Expression of interleukin-6 and interleukin-6 receptors in human granulosa lutein cells

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Cytokines are important regulators of reproductive functions. Significant amounts of interleukin-6 (IL-6) have been detected in the serum and ascites of patients with ovarian hyperstimulation syndrome (OHSS). These findings suggest the involvement of IL-6 as a mediator in the pathogenesis of OHSS. This study was performed to analyse IL-6 and IL-6 receptor (IL-6-R) expression in human granulosa lutein cells (GC). GC were cultured after isolation from follicular fluid. IL-6 concentrations in follicular fluid and serum from individual patients and GC supernatants were measured by enzyme-linked immunosorbent assay. We found detectable concentrations of IL-6 in serum and follicular fluid of all patients. Expression of IL-6 in GC was shown immunocytochemically. IL-6 mRNA was detected in GC by in-situ hybridization. Gene expression for IL-6 and IL-6-R in GC was demonstrated using reverse transcription–polymerase chain reaction. IL-6 significantly inhibited human chorionic gonadotrophin (HCG)-induced progesterone secretion of GC. The results of our study suggest that IL-6 is expressed in HGC and that this cytokine is able to modulate GC function via its specific receptor. This is the first report that describes the presence of IL-6-R in human granulosa lutein cells.

Key words: angiogenesis/granulosa cells/interleukin-6/interleukin-6 receptor

Introduction

Cytokines have been shown to be important modulators of intraovarian processes (Adashi, 1990). Interleukin-6 (IL-6), a T-cell-derived factor, specifically induces differentiation of B lymphocytes into active antibody-producing plasma cells (Hirano et al., 1985). In addition, it can influence folliculogenesis and granulosa cell steroidogenesis (Adashi, 1990). IL-6 is produced by rat and bovine granulosa cells in vitro (Gorospe et al., 1992; Alpizar and Spicer, 1993; Gorospe and Spangelo, 1993). The IL-6 expression of GC is stimulated by follicle stimulating hormone (FSH), IL-1 and lipopolysaccharide (LPS) but not by tumour necrosis factor (TNF)-α (Gorospe and Spangelo, 1993). Immunohistochemical studies on human ovaries show IL-6 secretion in corpora lutea and in theca cells. IL-6 bioactivity has been detected in follicular fluid from in-vitro fertilization (IVF) patients. In patients with ovarian hyperstimulation syndrome (OHSS) IL-6 concentrations are significantly elevated in serum and ascites (Loret de Mola et al., 1996a; Agarwal et al., 1997). This suggests a gonadotrophin-dependent secretion of IL-6 by GC. The results of our study show the expression of IL-6 and IL-6-receptor (IL-6-R) in human granulosa cells in vitro. Furthermore IL-6 inhibits progesterone secretion by GC. The detection of specific IL-6-R on GC implies that IL-6 can modulate GC function, especially GC steroidogenesis via autocrine mechanisms.

Materials and methods

Luteinized GC were obtained from women undergoing oocyte retrieval for IVF. Experiments described below were performed from December 1996 to December 1997. During this time a total of 320 follicle punctures for IVF or intracytoplasmic sperm injection (ICSI) was performed. The mean age of the patients was 32.4 years (range 20–38 years). Reasons for infertility were tubal occlusion (42%), male factor infertility (54%) and infertility due to other reasons (4%). Written consent was obtained from all patients. Multiple follicular development was stimulated with pure FSH (225 IU/day; Gonal F; Serono, Munich, Germany) until follicular maturity. The mean total FSH dose administered per patient was 2175 IU (range 1200–3150 IU). Gonadotrophin therapy was preceded by complete desensitization of the pituitary gland with 400 mg nafarelin/day (Heumann AG, Nuremberg, Germany) according to the long protocol, starting on day 10 of the cycle preceding the stimulation cycle. Ovulation was induced by 10 000 IU HCG (Choragon; Ferring, Kiel, Germany). At the day of HCG administration blood samples were taken from the patients. Oestradiol, luteinizing hormone (LH), progesterone and IL-6 serum concentrations were determined and a vaginal ultrasound examination was performed. An average of eight follicles >14 mm was detected per patient and mean oestradiol concentrations were 2400 pg/ml (range 1200–3400 pg/ml). Patients with >15 follicles at ultrasound and/or oestradiol concentrations >3500 pg/ml were excluded from the study.

At the time of oocyte retrieval, follicular fluids from each patient were collected. Serum samples, follicular fluid and GC supernatants were stored at –80°C after centrifugation for 10 min at 300 g before assaying IL-6 concentration.

Interleukin-6 enzyme-linked immunosorbent assay (ELISA)

IL-6 concentrations were determined in patients’ serum, pooled follicular fluid and in supernatants of GC cultures by ELISA using a commercially available kit (Quantikine; R&D Systems GmbH, Wiesbaden, Germany).
Granulosa cell preparation

For cell preparation follicular aspirates from individual patients were pooled. Cell preparation was performed as described elsewhere (Neuten et al., 1995). To eliminate blood contamination, salt treatment (Beckmann et al., 1991) and immunobead treatment (Best et al., 1995) were performed. Magnetic beads (Coulter-Immunotech, Hamburg, Germany) were coupled with specific mouse CD68-antibodies (Dako, Hamburg, Germany) and the cell suspension was incubated with immunobeads for 10 min at room temperature. Then the suspension was exposed to a cobalt-samarium magnet for removal of anti-CD68 bead-bound macrophages. The isolated cells were plated in medium 199 supplemented with 1% fetal calf serum (FCS) (Biochrom, Berlin, Germany) and 100 μg/ml gentamycin and incubated at 37°C in 5% CO2. Cells were cultured for up to 14 days and culture media were changed every second day. Granulosa cell viability was assessed by 1% Trypan Blue exclusion. The day on which cells were collected and seeded into wells was designated day 0.

Flow cytometry

Flow cytometry was performed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using the Lysis II software for data processing. GC were removed from plastic dishes with trypsin (Biochrom) containing 0.5 mM EDTA. All subsequent steps were performed at 4°C with labelling buffer [phosphate-buffered saline (PBS) containing 1.1 mM CaCl2, 0.4 mM MgCl2] and 3% FCS). The primary cell suspension (before immunobead treatment) and GC (after immunobead treatment, free of macrophages) were incubated as follows: 25 μl of CD68 monoclonal antibody (10–50 μg/ml) was added to the cells for 20 min. After washing with ice-cold labelling buffer, 25 μl biotinylated anti-mouse IgG antibodies (Sigma, Deisenhofen, Germany) were added to the cells for another 20 min followed by washing. For the last step, the cells were incubated with 25 μl phycoerythrin-linked streptavidin (1:100) (Southern Biotechnology Associates, Birmingham, AL, USA) for 20 min, washed and resuspended in 300 μl labelling buffer for FACS analysis.

Immunocytochemical techniques

Macrophages were localized using the monoclonal antibody CD68 and cellular IL-6 production in GC was localized using the monoclonal antibody anti-IL-6 (Pharmingen GmbH, Freiburg, Germany). Before staining, cells were fixed with a methanol–acetone solution (1:1). Endogenous peroxidase activity was blocked by incubation in cold labelling buffer, 25 μl biotinylated anti-mouse IgG antibodies (Sigma, Deisenhofen, Germany) were added to the cells for another 20 min followed by washing. For the last step, the cells were incubated with 25 μl phycoerythrin-linked streptavidin (1:100) (Southern Biotechnology Associates, Birmingham, AL, USA) for 20 min, washed and resuspended in 300 μl labelling buffer for FACS analysis.

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RNA extraction

Total cellular RNA was isolated from cell pellets by the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987) with all solutions made in diethylpyrocarbomate-treated water (DEPC water). Extracted RNA was dissolved in 50 μl 0.25 mol/l Na acetate (pH 6.0), precipitated with 2.5 volumes of absolute ethanol at –80°C washed several times with cold 80% ethanol and resuspended in 20 μl DEPC water.

Reverse transcription–polymerase chain reaction (RT–PCR) analysis

One μg of total cellular RNA isolated from GC-enriched preparations (three to five patients) was converted into cDNA by incubating overnight with 50 μl of Ready to Go-T primed First Strand Kit (Pharmacia, Freiburg, Germany). One-half of each RT reaction mixture was used in 38 cycles of PCR amplification in a Perkin Elmer Cetus Thermal Cycler using 2.5 U Taq polymerase and the oligonucleotides 5′ AGTAACCTCTTTCCCAAAACGCG 3′ (sense strand primer) and 5′ GAAGAGCCCTCAGGCTGACTG 3′ (anti-sense strand primer) for IL-6 (Ehlers and Smith, 1991) as well as 5′ CATTGCATTTGGCCTGACTGTC 3′ (sense primer) and 5′ ATGATGCTGGATCTGACTGTC 3′ (antisense primer) for IL-6-R (Stark et al., 1993). PCR amplification was performed in 38 cycles, each cycle consisting of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C. After amplification, 10 μl of the PCR reaction mix were electrophoresed in a 2% agarose gel. Hae III restriction endonuclease-digested Phi-x 174 replicative from DNA was used as a size marker.

In-situ hybridization

In-situ hybridization was performed as previously described (Fischer et al., 1991). As a source of IL-6-specific sequences, we used a complementary DNA (cDNA) clone of human IL-6. The hybridization probe was labelled with digoxigenin-uridine triphosphate (Dig-UTP) (Boehringer Mannheim, Mannheim, Germany). GC were cytopsin, air dried, and fixed with 4% paraformaldehyde. Cell spots were hybridized with a Dig-UTP-labelled antisense RNA probe, that was complementary to nucleotides of the coding sequence of human IL-6 in a solution containing 50% (v/v) deionized formamide, 0.3 mol/l NaCl and 10 mmol/l Tris. Hybridization solution was placed over each cellular spot and covered with coverslips. Cells were hybridized by incubation overnight at 45°C. Sense RNA probes were used as controls.

Statistical analysis

Since GC were used from different patients the effects of rhIL-6 on HCG- and cAMP-stimulated progesterone production inevitably showed some variation between experiments. However, the same trend was always seen in each set of experiments. One representative set of data is shown. Experiments were repeated four times with different original suspensions. Triplicate measurements were performed and variations were expressed as the mean ± SE. Statistical significance was determined using the SPSS-PC, using one-way analysis of variance, followed by Scheffé’s F-test. Student’s t-test was used for unpaired data.
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Table I. Interleukin-6 (IL-6) concentrations in serum, follicular fluid and culture medium of granulosa lutein cells (GC) from patients undergoing in-vitro fertilization (mean ± SE)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>IL-6</th>
</tr>
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<tbody>
<tr>
<td>Serum (pg/ml)</td>
<td>3.34 ± 0.8a</td>
</tr>
<tr>
<td>Follicular fluid (pg/ml)</td>
<td>32.28 ± 4.5b</td>
</tr>
<tr>
<td>GC supernatant with macrophages (ng/ml)</td>
<td>8.5 ± 1.5c</td>
</tr>
<tr>
<td>GC supernatant without macrophages (ng/ml)</td>
<td>8.1 ± 1.0c</td>
</tr>
</tbody>
</table>

IL-6 concentrations were significantly different in serum, follicular fluid and GC supernatants (a/b P < 0.01; a/c P < 0.05; b/c P < 0.01). There was no difference between IL-6 concentrations in GC supernatants with and without macrophages.

Results

Immunoreactive IL-6 concentrations were detected in serum and pooled follicular fluid from each individual patient and in GC supernatants. The mean concentrations in follicular fluid were significantly higher (32.28 ± 4.5 pg/ml) than serum concentrations (3.34 ± 0.8 pg/ml). There were no differences in the mean concentrations of IL-6 in GC cultures with (8.5 ± 1.5 pg/ml) or without macrophages (8.1 ± 1.0 pg/ml) (mean ± SEM) (Table I).

Treatment with anti-CD68 immunobeads significantly reduced the amount of contaminating macrophages in the cell suspension to <1% of the total cell population. The purity of our GC preparation (absence of macrophages) could be demonstrated by flow cytometry.

Immunocytochemical detection of IL-6 revealed intense staining of GC (Figure 1a). No staining was detected in the absence of the primary antibody (Figure 1b). NIH:OVCAR-3 cells served as positive control.

The effects of rhIL-6 on GC progesterone production were tested in serum-free cultures. HCG and cAMP increased progesterone secretion in GC. The addition of 100 U/ml rhIL-6 to GC without macrophages inhibited cAMP- and HCG-stimulated progesterone production significantly, whereas inhibition of basal progesterone production was not significant (Figure 2).

When different doses of IL-6 (10 IU, 50 IU and 100 IU) were added to GC stimulated with a fixed dose of 100 ng/ml HCG, the progesterone production was dose-dependently inhibited (Figure 3).

Total RNA was isolated from GC. By RT–PCR using oligonucleotide primers corresponding to known cDNA sequences for IL-6, a 628 base pair band characterizing the amplification products of IL-6 transcripts could be detected for up to 9 days of culture (Figure 4). In addition, a 240 bp band characterizing amplification products of IL-6-R transcripts could frequently be detected in GC for up to 13 days of culture (Figure 5). At day 13 another distinct band at around 580 base pairs was evident.

Cellular localization of IL-6 transcripts was identified by in-situ hybridization (Figure 6).

Discussion

Growth factors and cytokines are important regulators of ovarian function. IL-6, a 26 kDa glycoprotein, is produced and secreted by various haematopoietic as well as non-haematopoietic cell types, either constitutively or in response to specific stimuli (Akira et al., 1990; Van Snick, 1990). In addition, this cytokine seems to play a pivotal role in the regulation of female reproductive functions (Adashi, 1990). This prompts the questions as to what are the factors regulating IL-6 production and secretion in the ovary and what are the effects of IL-6 on intragonadal processes?

Gorospe et al. (1992) and Gorospe and Spangelo (1993) studied the production and function of IL-6 in rat granulosa cells and showed that IL-6 release was significantly enhanced by FSH, IL-1α, IL-1β and LPS, but not TNF-α. Basal and FSH-stimulated IL-6 secretion was inhibited by INFγ. Incubation of rat GC with cAMP alone, or in combination with forskolin and/or 3-isobutyl-1-methyl-xanthine (IBMX) significantly stimulated IL-6 secretion, indicating that cAMP serves as a second messenger.

Motro et al. (1990) showed that in pregnant mare serum gonadotrophin-primed mice a transient expression of IL-6 mRNA is clearly associated with angiogenesis in the ovary, suggesting that IL-6 may play a role in the regulation of angiogenesis-dependent processes such as follicular maturation, selection and corpus luteum formation.

Figure 1. Immunocytochemical detection of interleukin (IL)-6 in granulosa lutein cells (GC). (a) Positive immunostaining for IL-6 in GC. Bar = 25 µm. (b) No signal was seen in an adjacent sections processed without primary antibody. Bar = 50 µm.
Figure 2. Effects of recombinant human interleukin-6 (IL-6) on human chorionic gonadotrophin (HCG)- and cAMP-stimulated progesterone (P) production of GC. Stimulation was performed with rhIL-6 at doses of 100 IU/ml for 24 h in serum-free medium. Data are expressed as mean (M) values ± SE (triplet measurements). *P < 0.05; NS = P > 0.05.

Figure 3. Effects of increasing recombinant human interleukin-6 (IL-6) doses added to culture medium on HCG-stimulated progesterone (P) production. Data are the mean ± SE (triplet measurements). Effects of different IL-6 concentrations were significant (P < 0.05).

The peri-ovulatory period is characterized by a moderate rise in progesterone secretion followed by a marked increase due to corpus luteum formation. Numerous cytokines have been shown to regulate progesterone secretion by GC. Investigating the effects of IL-6 on porcine GC, Pitzel et al. (1993) did not see an effect on progesterone secretion. However, in rat granulosa cells, Gorospe et al. (1992) and Gorospe and Spangelo (1993) showed that IL-6 inhibits FSH-stimulated progesterone production while not appreciably affecting basal progesterone steroidogenesis. Using an in-vitro ovarian perfusion system, Mikuni (1995) showed that 100 ng/ml IL-6 suppressed LH-induced oestradiol secretion but did not affect progesterone secretion. At the same dose IL-6 inhibited ovulation. In cultured granulosa cells, IL-6 inhibited FSH-induced progesterone secretion (Mikuni, 1995). Alpizar and Spicer (1993) demonstrated that IL-6 inhibits FSH-induced oestradiol production of bovine granulosa cells in a dose-dependent manner. However, there was a significant difference between granulosa cells from large and small follicles. GC from small follicles were much more sensitive to IL-6 effects compared with GC from large follicles. This also applied to FSH-induced oestradiol production and to cell proliferation. These findings suggest that the effects of IL-6 are dependent on cell differentiation.

In the present study we have shown that IL-6 inhibits HCG- and cAMP-stimulated progesterone production by human granulosa cells. However, as granulosa cells and follicular fluid from each patient were pooled, we could not correlate the effects of IL-6 with follicular size. The inhibition of GC progesterone secretion was IL-6 specific and not due to cytotoxicity, as demonstrated by post-culture cell counts.
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between control and IL-6-treated granulosa cells after Trypan Blue staining. Our findings are in contrast to those published earlier on the effects of IL-6 on human granulosa cell steroidogenesis. Machelon et al. (1994) explored cellular sites of IL-6 biosynthesis in human follicular aspirates from patients undergoing IVF and studied the effects of IL-6 on GC steroidogenesis. Adding 10–200 IU/ml recombinant human IL-6 to human GC under experimental conditions of low endogenous IL-6 concentrations, the authors could not see any effect on GC progesterone production and/or on FSH-stimulated aromatase activity. The differences between our findings and those of Machelon et al. (1994) are difficult to explain as in both studies a similar experimental model was used. Trying to explain why, in GC cultures cleared of macrophages, IL-6 had no effect on basal and stimulated steroidogenic activities of GC, Machelon et al. (1994) suggested that the lack of IL-6-mediated effects might occur either with or without the loss of IL-6-R. However, in contrast to Machelon et al. (1994), who could not show the presence of IL-6-R on GC, we were able to detect the IL-6-R for up to 13 days of culture by means of RT–PCR. It may be possible that the different findings are due to methodological differences, differences in specific activity of IL-6 formulations used or variability of cellular response to cytokines in the different groups of patients studied. On day 13 of the experiments showing IL-6-R expression, an additional band of ~580 bp was detected. The physiological significance of this product is unclear.

Besides the potential role of IL-6 for regulation of intra-gonadal processes and its effects on GC steroidogenesis, the question as to whether or not this cytokine might play a role in the pathophysiology of OHSS has been intensively discussed. IL-6 has been detected in serum, follicular fluid, ascites and pleural effusions from patients undergoing ovarian stimulation for IVF treatment and its concentrations are significantly elevated in patients developing OHSS (Buyalos et al., 1992; Friedlander et al., 1993; Orvieto et al., 1995; Loret de Mola et al., 1996a, 1997). Abramov et al. (1996) followed IL-6 serum concentrations in seven patients with OHSS. Comparing these seven patients with two control groups, the authors showed that high concentrations of IL-6 were detected in all individuals upon admission which then fell to normal concentrations as the symptoms of OHSS disappeared. However, the question whether IL-6 may serve as predictor for the development of OHSS is controversial. Loret de Mola et al. (1996b) performed a prospective case-controlled study to assess if preovulatory cytokine serum concentrations can predict the occurrence of OHSS. The authors determined preovulatory IL-6, IL-1-R antagonist and TNF-α serum concentrations, claiming that there is no evidence for cytokine peaks or nadirs prior to ovulation, regardless of whether or not patients develop OHSS. It is unlikely that serum concentrations of these cytokines would predict the development of OHSS.

In contrast to Loret de Mola et al. (1996b), Geva et al. (1997) analysed follicular fluid from patients undergoing ovarian stimulation for IVF and determined concentrations for IL-6, IL-2 and TNF-α. Ten patients who developed severe
OIHSS were compared with controls. IL-6 concentrations in the follicular fluid were significantly higher in the OIHSS group than in control patients whereas no differences were found in IL-2 and TNF-α concentrations. The authors postulate that elevated concentrations of IL-6 in the preovulatory follicular fluid at the time of oocyte retrieval may predict the development of early-form OIHSS in high responders. The principal difference between the two studies mentioned is that IL-6 concentrations have been detected in different matrices. Whereas IL-6 concentrations in follicular fluid reflect intraovarian IL-6 concentrations, the cytokine is removed from the circulation with a half-life of ~3 min so that the diagnostic and/or predictive value of IL-6 measurements in serum can differ from those in follicular fluid significantly. In the present study we confirmed that in patients undergoing ovarian stimulation for IVF at the day of oocyte retrieval, IL-6 concentrations in follicular fluid are significantly higher compared with serum.

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References


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