Interaction of interleukin-6 on human granulosa cell steroid secretion

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Abstract

Numerous studies have shown that a variety of cytokines are involved in the regulation of ovarian function. Interleukin-6 (IL-6), has been found in follicular fluid. In this report we show by immunocytochemical methods the localization of the extracellular domain of the IL-6-receptor and its associated signal transducer glycoprotein gp 130 on the surface of granulosa cells (GCs). The possibility that IL-6 may also influence the basal and FSH-stimulated production of estradiol (E2) and progesterone (Prog) by GCs *in vitro* was also investigated.

GCs were obtained from infertile patients undergoing *in vitro* fertilization and embryo transfer treatment and cultured for 72 h or were given increasing concentrations of human recombinant IL-6 (8–128 pg/ml) in the absence or presence of FSH (96 U/ml). For the time-course studies, FSH-stimulated GCs were treated in the absence or presence of IL-6 (128 pg/ml) and supernatants were assayed at 24 h intervals (24–96 h) for E2 and Prog

productions. The results show that increasing amounts of IL-6 significantly inhibit E2 production in the absence or presence of FSH vs untreated controls ($P=0.025$ at IL-6=128 pg/ml and $P=0.016$ at IL-6=16 pg/ml respectively). IL-6 also inhibited FSH-stimulated but not unstimulated Prog release ($P=0.038$ at IL-6=8 pg/ml). These findings suggest that IL-6 may be one of the factors that plays a local regulatory role in the course of FSH-stimulated E2 and Prog release. The time-course studies of the effect of the absence or presence of IL-6 demonstrated a significant inhibitory effect on both E2 and Prog secretion ($P<0.001$) by FSH-stimulated GCs. As infections of the female reproductive tract are often accompanied by elevated local IL-6 levels, this factor may be one of the links leading to endocrine reproductive dysfunction during genital infections.

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Introduction

Current views suggest that ovarian macrophages play a paracrine role in the regulation of ovarian function through the local secretion of regulatory molecules. Numerous studies have shown that a variety of cytokines such as interleukin-6 (IL-6) can exert profound effects on ovarian function and probably also on reproductive processes such as gonadal steroid secretion, corpus luteum function, embryo development and implantation (Adashi 1990, Spangelo *et al.* 1995). IL-6 was initially identified as a T-cell-derived cytokine that specifically promotes B-cell differentiation and antibody production (Hirano *et al.* 1985, Van Snick 1990). However, additional studies have shown that IL-6 is also produced by a variety of cell types that play important roles in both immunity and inflammation, including the activity of fibroblasts, mast cells,

endothelial cells, monocytes and keratinocytes (Akira *et al.* 1990, Van Snick 1990).

IL-6, a pleiotropic cytokine, acts on different cells, exerting growth promotion, growth inhibition and specific gene expression sometimes accompanied by cellular differentiation. In accordance with such a multifunctional property of IL-6, the involvement of IL-6 in several diseases, including inflammation, autoimmunities and lymphoid malignancies, has been well documented. (Kishimoto *et al.* 1992). IL-6 exerts multiple functions through a specific receptor system. This consists of two membrane proteins, a ligand-binding receptor (IL-6 receptor (IL-6R), 80 kDa) and a non-binding signal transducer glycoprotein (gp 130, 130 kDa), both of which belong to the cytokine receptor family. Binding of IL-6 to IL-6R triggers the association of IL-6R and gp 130, forming a high-affinity IL-6-binding site with the gp 130,

which transduces the signal (Taga *et al.* 1989, Hibi *et al.* 1990). Soluble forms of IL-6 receptor (sIL-6R) and gp 130 (sgp 130) have also been reported to occur in serum and in urine (Novick *et al.* 1989, Fernandez-Botran 1991, Narazaki *et al.* 1993, Kishimoto *et al.* 1994, Muller-Newen *et al.* 1998). Furthermore, recently evidence has accumulated suggesting that IL-6 is capable of influencing numerous physiological and pathophysiological processes, such as ovarian function, and may be an important regulator in reproductive function (Adashi 1990, Spangelo *et al.* 1995).

Some authors found that IL-6 levels in follicular fluid (FF) were significantly higher in an ovarian hyperstimulation syndrome (OHSS) group than in controls (Buyalos *et al.* 1992, Loret de Mola *et al.* 1996, Geva *et al.* 1997) and claim that elevated levels of IL-6 in FF at the time of oocyte retrieval for *in vitro* fertilization (IVF) may predict the development of early-form OHSS.

The presence of lymphocytes, monocyte subpopulations and significant amounts of IL-6 has been demonstrated in the FF of women undergoing follicular aspiration for IVF (Droesch *et al.* 1988, Loukides *et al.* 1990, Machelon *et al.* 1994). This has been assumed to indicate local production of these substances. In bovines, rabbits and rats, IL-6 has been found to be produced by cultured granulosa cells (GCs), influencing folliculogenesis and GC steroidogenesis (Adashi 1990, Gorospe *et al.* 1992, Gorospe & Spangelo 1993, Alpizar & Spicer 1994, Breard *et al.* 1998). The majority of such studies have been carried out in animals. Only limited data are available about the effects of IL-6 on GC function in the human system. By means of immunohistochemistry it was possible to visualize the IL-6 content of human corpus luteum theca cells (Machelon *et al.* 1994, Loret *et al.* 1996). The potential role of immune cell secretory products, such as cytokines, in ovarian function has meanwhile become a field of intensive investigation. The aim of the present study was to determine the potential role of IL-6 on the basal production and follicle-stimulating hormone (FSH)-induced secretion of estradiol (E2) and progesterone (Prog) by cultured human GCs. Furthermore, we report the occurrence of IL-6R and its associated gp 130 on GCs, implying that IL-6 can modulate GC function, especially GC steroidogenesis via autocrine or paracrine mechanisms.

Materials and Methods

Cell collection

GCs were obtained from 15 infertile patients undergoing IVF, intra-cytoplasmic sperm injection, and IVF and embryo transfer (IVF-ET) treatment. The patients undergoing IVF-ET were superovulated with human menopausal gonadotropin (Menogon; Ferring, Kiel, Germany), administered from the 3rd, 4th or 5th cycle day onwards.

Daily monitoring of follicle development by real-time ultrasonic scans and serum E2 levels was performed from the 14th to 16th day of the cycle. Once the leading follicle measured >1.8 cm in diameter and the 17- β E2 level was adequately increased, but still less than 3000 pg/ml in serum, 10 000 IU of human chorionic gonadotropin (hCG) (Choragon; Ferring) were administered i.m. Follicles were aspirated 36 h after administration of hCG and GCs were harvested from the FF after oocyte collection for IVF.

FF was centrifuged at 350 *g* for 5 min and the supernatant was removed. The cells from all follicles aspirated from 15 (3 $\times$ 5) patients (3–14 oocytes) were pooled and resuspended in 5–10 ml Ham's F-10 medium (Sigma Chemical Co., Deisenhofen, Germany). The medium was supplemented with glutamine (2 mM; Sigma), androstenedione (40 ng/ml; Sigma), penicillin (100 IU/ml; Sigma) and streptomycin (100 μ g/ml; Sigma). The cells were separated over a gradients containing 2 ml 64% and 2 ml 36% Percoll solution (Sigma). After centrifugation at 450 *g* for 15 min at room temperature the interphase cells were pooled and washed twice with Hank's balanced salt solution (HBSS). The cells were then treated with collagenase (0.1% w/v; Sigma) for 30 min, washed twice with HBSS and resuspended in 3 ml HBSS. To remove the CD45-positive blood cells (such as lymphocytes), 50 μ l magnetic M-450 CD45 Dynabeads (DYNAL Inc., Sigma) were added. The supernatant of the cell suspension was collected in magnetic rack. The cells were washed three times with HBSS and suspended in the serum-free Ham's F-10 culture medium supplemented with 1 U/ml penicillin/streptomycin (CM).

GC culture

Serum-precoated 24-well plastic dishes were inoculated with triplicate portions of GC suspensions (2 $\times$ 10⁴ viable cells per well) in CM and preincubated for 36 h. Supernatant was removed and GCs were treated with FSH and IL-6.

In this set of experiments the cells were either cultured in CM alone (control) or were treated with increasing concentrations of IL-6 (Sigma), which were added in the absence or presence of recombinant human FSH (Serono, Munich, Germany) to give a final volume of 500 μ l. The final concentration of the IL-6 was adjusted to 8, 16, 32, 64 and 128 pg/ml in the CM and the incubation time was 72 h. The cells were then incubated at 37 °C in a humidified tissue culture incubator gassed with 95% air and 5% CO₂. For the time-course studies, FSH-stimulated GCs were treated in the absence or presence of IL-6 (128 pg/ml). The supernatants were collected for the E2 and Prog assays after 24, 48, 72 or 96 h.

GC viability was assessed by 1% (w/v) Trypan Blue exclusion. The presence of monocytes/macrophages in

separated GCs was tested immunocytochemically by the monoclonal antibody Ki-M₁P, as reported by Radzun *et al.* (1991).

E2 and Prog assay

E2 and Prog levels were measured by an enzyme immunoassay with the SR1 auto system (Serono) within the range 5–3000 pg/ml for E2 (sensitivity 5 pg/ml) and 0.05–40 ng/ml for Prog (sensitivity 0.2 ng/ml). For E2, 250 µl (1:2 diluted) and for Prog 100 µl of the culture supernatant were taken. Briefly, an enzyme-linked polyclonal antibody specific for the analyte (hormones), fluorescein isothiocyanate (FITC)-labeled analyte and sample are added to make an immunoreactions system. Analyte in the sample and FITC-labeled analyte are competitive for binding with the same enzyme-labeled antibody. The absorption of light by the reactive system is measured at a wavelength of 554 nm. The results are calculated and printed automatically by the SR1 auto system.

IL-6, sIL-6R and sgp 130 assay in serum and FF

Blood (serum) and FF were taken from 95 IVF-ET patients on the day of follicle puncture, centrifuged for 10 min at 350 g and 5 °C, snap frozen and kept at –80 °C. After pick-up of the oocytes the FF underwent the same treatment as the blood. IL-6 levels in FF ranged between 15.6 and 500 pg/ml with a sensitivity of <2 pg/ml. In serum the IL-6 level ranged between 0.15 and 10 pg/ml with a sensitivity of 0.104 pg/ml. Precision was <8% for inter-assays and <7% for intra-assays. The sIL-6R levels ranged between 6.25 and 2000 pg/ml with a sensitivity of <5 pg/ml. Precision was <7% for inter-assays and <7% for intra-assays. The sgp 130 levels ranged between 40 and 4500 pg/ml with a sensitivity of 18 pg/ml. Precision was <8% for inter-assays and <4% for intra-assays. All measurements were performed with an ELISA method (Biosource, Ratingen, Germany).

Immunocytochemical detection of IL-6R and gp 130 on human GCs

The GCs were separated from 15 patients as described above, suspended in PBS with the addition of 1% (w/v) human serum albumin, and cytopins prepared. After air drying (24 h) the cytopins were fixed in acetone for 10 min. The IL-6R on the GC surface was detected by the immunocytochemical method with a monoclonal antibody (CD126; Coulter Immunotech, Krefeld, Germany) and the avidin-biotin complex-labeled alkaline phosphatase (DAKO, Hamburg, Germany).

Table 1 Concentration (means ± S.D.) of IL-6, sIL-6R and sgp 130 in serum and FF of IVF-ET patients (n=95)

	IL-6 (pg/ml)	sIL-6R (ng/ml)	sgp 130 (ng/ml)
Serum	7.84 ± 1.06	67.8 ± 10.2	724.3 ± 49.2
FF	25.37 ± 5.23	22.5 ± 2.01	729.9 ± 56.6

Applying the same method, gp 130 on the surface of GCs was detected by its corresponding primary monoclonal antibody (mouse IgG against human CD130; Biologo, Kiel, Germany). Up to eight cytopins were prepared from each patient.

Statistical evaluation

As GCs were pooled from five different patients and measurements were performed three times. The effect of IL-6 on basal and FSH-stimulated E2 and Prog production inevitably showed some variation between the three experiments; however, the same trend was evident in each experiment. Triplicate measurements were performed and variations were expressed as the mean ± S.D.

The statistical analysis was performed by SPSS ANOVA at $P < 0.05$. Differences between the groups were analyzed by multiple comparison using the Dunnet (post hoc) method. This test treats one group as the control and compares all other groups against it.

The inhibitory effects (%) for E2 and Prog release at concentrations of IL-6 of 8, 16, 32, 64 and 128 pg/ml in the presence of FSH were calculated by formula I (inhibition (%)) = $((C_{\text{with FSH}} - C_{\text{with FSH+IL-6}}) / C_{\text{with FSH}}) \times 100$, and in the absence of FSH by formula II (inhibition (%)) = $((C_{\text{without IL-6}} - C_{\text{with IL-6}}) / C_{\text{without IL-6}}) \times 100$, where C represents E2 or Prog mean concentration in pg/ml and ng/ml.

The inhibitory effects (%) for E2 and Prog release at 24, 48, 72 and 96 h were also calculated by formula II.

Results

Measurement of IL-6, sIL-6R and sgp 130 in serum and in FF

The levels of IL-6, sIL-6R and sgp 130 in serum and FF from infertile patients undergoing IVF-ET treatment are summarized in Table 1.

IL-6 levels in FF were significantly higher than those in serum ($P < 0.005$) whereas sIL-6R levels in FF were significantly lower ($P < 0.001$) than those in serum. In contrast to sIL-6R levels, no significant difference in sgp 130 levels in the serum or FF of IVF-ET patients was found.

Immunocytochemical detection of IL-6R and gp 130 on human GCs

Figure 1a shows the localization of the extracellular domain of human IL-6R and Fig. 1b the associated signal transducer gp 130 on the surface of GCs with reference to a negative control (Fig. 1c). Fig. 1d shows as positive control the localization of human IL-6R on the surface of monocytes isolated from blood.

The purity of our GC preparations (absence of macrophages) could be demonstrated by the immunocytochemical test with the monoclonal antibody Ki-M₁P, which recognizes CD68, to be over 99% of cells in all samples.

Effect of IL-6 on human GCs in E2 and Prog production in vitro

To determine whether IL-6 can directly influence GC steroidogenesis (production of E2 and Prog), GCs were treated for 72 h with increasing concentrations of IL-6 (8–128 pg/ml) in the presence or absence of FSH (96 U/ml). The differences between the groups, as analyzed by ANOVA, were significant at $P=0.05$ in the absence and $P<0.001$ in the presence of FSH.

As shown in Fig. 2, increasing amounts of IL-6 alone had a significant inhibitory effect on E2 production compared with untreated controls in both the presence and absence of FSH. The suppressing levels ranged from 8.9 to 41.6% in the absence of FSH and from 22.2 to 62.0% in the FSH-stimulated controls as calculated by formula I and II.

The significant IL-6 inhibition effect on E2 production analyzed by multiple comparisons in the absence of FSH was determined at a concentration of 128 pg/ml ($P=0.025$) and in the presence of FSH at a concentration higher than 16 pg/ml ($P=0.016$) in comparison with the corresponding untreated controls.

As shown in Fig. 3, increasing amounts of IL-6 in the absence of FSH had no effect on Prog production compared with untreated controls, but caused a significant dose-dependent inhibition of FSH-stimulated Prog production. The levels of suppression ranged from 26 to 64% in the presence of FSH as calculated by formula II.

The differences between the groups were not significant ($P=0.81$) in the absence and highly significant ($P<0.001$) in the presence of FSH. The inhibition effect in the presence of FSH was also determined by the multiple comparison at a concentration of 8 pg/ml ($P=0.038$) and higher ($P<0.001$) as compared with IL-6. Higher concentrations of IL-6 (256 pg/ml) had no additional inhibitory effect and therefore no additional doses were included in Figs 2 and 3.

Time-course studies

Time-course studies of FSH-stimulated hormone release between 24 and 96 h after a single FSH pulse (96 U/ml)

showed the characteristic cumulative increase in E2 and Prog levels (Figs 4 and 5). Aliquots of the cultures additionally treated with IL-6 (128 pg/ml) revealed significantly lower values when compared with the results of the same time in the absence of IL-6. From 24 to 96 h the inhibitory effect on E2 and Prog secretion persisted. The inhibitory effect amounted to 26, 66, 65 and 70% for E2 release at 24, 48, 72 and 96 h respectively, as calculated by formula II. The corresponding inhibitory effect on Prog levels amounted to 38, 52, 66 and 75% as calculated by formula II (Figs 4 and 5 insets).

The analysis of time effects by ANOVA revealed $P=0.01$ in E2 groups and $P=0.008$ in Prog groups (in both the presence and absence of IL-6). The results with the multiple comparison indicated for E2 experiments $P=0.003$ after 96 h as compared with 24 h culture time and for Prog group $P=0.04$ after 72 h against 24 h culture time. The analysis of IL-6 effects by *t*-test (independent sample test) revealed $P<0.001$ for both E2 groups and for the Prog groups.

The levels of E2 and Prog under IL-6 influence did not change during the entire time-course. This indicate that IL-6 prevents hormone secretion, whereas in the absence of IL-6 the steroid secretion continues.

Discussion

In the present study we measured the concentration of IL-6, sIL-6R and sgp 130 in FF in comparison with serum. Our results show that although IL-6 concentration in FF is significantly higher than in serum, its soluble receptor is lower by a factor of 3 in FF compared with in serum. The signaling component of the IL-6 system, the gp 130, reveals a similar concentration in both media. The higher level of IL-6 in the FF in our results is well in line with the findings of other authors (Buyalos *et al.* 1992, Huyser *et al.* 1994, Buscher *et al.* 1999). This implies an intra-follicular production and a potential autocrine or paracrine role of IL-6 within the follicular microenvironment.

The concentration of this soluble variant of IL-6 receptor in the serum prevails where both splicing variants of this protein, the 80 and 55 kDa, occur. The soluble variants have been shown to bind IL-6 and the cellular gp 130 resulting in an effective signaling. In contrast, complexing of the receptor ligand with serum sgp 130 does not lead to an effective signaling and may act as a regulator of cytokine activities (Thibault *et al.* 1997). The signaling protein gp 130 is present in the FF as well as in the serum in a far higher concentration than IL-6 itself. sgp 130 has been shown to be involved not only in the IL-6-mediated signaling, but also in that of IL-11, leukemia inhibitory factor, oncostatin M and ciliary neurotrophic factor (Narazaki *et al.* 1993, Modrell *et al.* 1994). Our results confirm the findings of Keck *et al.* (1998) showing expression of membrane-bound IL-6R by GCs. Using a

Figure 1 Immunocytochemical demonstration of (a) the extracellular domain of IL-6R and (b) the signal transducer glycoprotein (gp 130) on human GCs. (c) Negative control, GCs without the primary antibody, and (d) positive control, localization of IL-6R on monocytes isolated from blood. Immunoalkaline phosphatase reaction using monoclonal antibodies to IL-6R and gp 130 ($\times 400$).

highly specific monoclonal antibody to gp 130, we could also show the expression of this protein by GCs, a finding that has not been described by other authors so far. As variously reported, monocytes and tissue macrophages may express IL-6R (Wognum *et al.* 1993). These cells were used as positive controls throughout this study.

The abundance of IL-6 in FF implies a physiological role for this IL. Numerous cytokines have been shown to be involved in Prog secretion by GCs. The majority of such studies have been on experimental animals. Investigating the effect of IL-6 on porcine GCs Pitzel *et al.* (1993) did not find an effect on Prog secretion. On the

Figure 2 Effect of increasing doses of recombinant human IL-6 on E2 secretion in GCs under basal and recombinant human FSH-stimulated (96 U/ml) conditions after 72 h culture. Results are the means $\pm$ S.D. of three experiments performed in triplicate. Number of cells 2×10^4 . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with corresponding control experiment with basal GCs (IL-6=0, FSH=0) and at FSH-stimulated GCs (IL-6=0, FSH=96 U/ml).

other hand, Gorospe & Spangelo (1993) showed that IL-6 inhibits the FSH-driven Prog production, while not appreciably affecting basal Prog steroidogenesis. In the human system, only a few sporadic references have been made to this context. Keck *et al.* (1998) studied hCG-driven Prog secretion by GCs, which was found to be inhibited by IL-6. This context was thoroughly analyzed by us on highly purified viable human GCs excluding contamination by monocytes and macrophages. This is an essential step, since monocyte and macrophages produce significant amounts of IL-6. We systematically examined the effect of IL-6 on the basal non-stimulated production of E2 as well as Prog by GCs and showed that increasing doses of IL-6 led to a significant reduction in the production of E2, but not of Prog. Using FSH-stimulated GCs in an identical experimental set-up, disclosed a massive inhibitory effect on E2 and Prog secretion. These results show the same trend as described for hCG-stimulation by Keck *et al.* (1998) in human and by Gorospe *et al.* (1992), Gorospe & Spangelo (1993) and Breard (1998) in rats applying FSH. IL-6 concentration proved critical in the inhibition of hormone synthesis and secretion in GCs.

Figure 3 Effect of increasing doses of recombinant human IL-6 on Prog secretion in GCs under basal and recombinant human FSH-stimulated (96 U/ml) conditions after 72 h culture time. Results are the means $\pm$ S.D. of three experiments performed in triplicate. Number of cells 2×10^4 . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with corresponding control experiment with basal GCs (IL-6=0, FSH=0) and with FSH-stimulated GCs (IL-6=0, FSH=96 U/ml).

Hormone level cumulatively increased with culturing period. Our results show that relatively low concentrations of IL-6 are capable of exerting a potent inhibitory effect on the E2 production of human GCs. The inhibitory effect of IL-6 on the FSH-driven E2 and Prog production may suggest that gonadotropin regulation of GC steroidogenesis can be modulated by non-steroidal factors, including cytokines. This track seems to be one of the mechanisms of the ovarian autoregulation. IL-6 production by GCs may well mean that the steroidogenesis of GCs is coupled to IL-6 production. The inhibitory effect of IL-6 could then readily be regarded as part of a rebound mechanism to downregulate hormone production. This effect is concentration-dependent. It has also been claimed that locally available IL-6 possibly originated from resident ovarian macrophages or ovarian stromal cells. The contribution of these cells to local IL-6 concentration has been shown to represent the centerpiece of an intra-ovarian regulatory loop promoting GC differentiation into lutein cells (Hurwitz *et al.* 1991, Schoester *et al.* 1994, Breard *et al.* 1998).

Significantly enhanced levels of circulating IL-6 have been correlated with inflammatory and neoplastic

Figure 4 Effect of increasing culture time on E2 secretion by FSH-stimulated (96 U/ml) GCs in the absence or presence of recombinant human IL-6 (128 pg/ml). Results are the means $\pm$ S.D. of three experiments performed in triplicate. Number of cells 2×10^4 . Inset shows inhibition as calculated by formula II. ** $P < 0.01$ compared with 24 h culture in the absence or presence of IL-6.

Figure 5 Effect of increasing culture time on Prog secretion by FSH-stimulated (96 U/ml) GCs in the absence or presence of recombinant human IL-6 (128 pg/ml). Results are the means $\pm$ S.D. of three experiments performed in triplicate. Number of cells 2×10^4 . Inset shows inhibition calculated by formula II. * $P < 0.05$, ** $P < 0.01$ compared with 24 h culture in the absence or presence of IL-6.

alteration in women with OHSS (Friedländer *et al.* 1993, Moradi *et al.* 1993). Considering the pleiotropic effect of IL-6 and its central role in the regulation of hormone production by ovarian follicle cells, pathological alterations of IL production may have a decisive bearing on endocrine reproductive dysfunction.

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