Follicular fluid concentration of leukaemia inhibitory factor is decreased among women with polycystic ovarian syndrome during assisted reproduction cycles

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BACKGROUND: The possibility that a specific cytokine profile could be detected in the ovaries of patients with polycystic ovarian syndrome (PCOS) was investigated. METHOD: Enzyme-linked immunosorbent assay (ELISA) or bioassays were used to assess the concentrations of leukaemia inhibitory factor (LIF), tumour necrosis factor, interleukin 11, gamma interferon, progesterone and oestradiol in follicular fluids from preovulatory follicles collected after ovarian stimulation from 15 PCOS patients, 15 infertile control patients with regular cycles, and 8 oocyte donors. RESULTS: LIF and progesterone concentrations were significantly lower in the follicular fluid of PCOS patients (LIF median: 265 pg/ml) compared with controls (LIF median: 816 pg/ml); LIF and progesterone follicular fluid concentrations were correlated (r = 0.720, P = 0.0001). The LH/FSH ratio was negatively correlated with LIF concentrations (r = –0.714, P = 0.0075). Although the PCOS and control patients did not differ significantly in age, ovarian reserve or IVF indication, the implantation rate was significantly lower among the women with PCOS (IR = 9 versus 21%, P = <0.01). CONCLUSION: The specific cytokine profile of the PCOS patients is probably related to the lower implantation rate since follicular fluid LIF appears to function as an embryotrophic agent.

Key words: cytokines/ follicular fluid/ leukaemia inhibitory factor/ polycystic ovarian syndrome

Introduction

Follicular growth and maturation is a complex process regulated by autocrine and paracrine factors. Follicular fluids provide the environment in which oocyte maturation occurs. Follicular fluids may therefore affect fertilization and early embryonic development (Brannström and Norman, 1993, Vinatier and Monnier, 1993; Adashi, 1994). For this reason, several authors have postulated that cytokines are involved in ovarian function (Deshpande, 2000). Some cytokines have been detected in human follicular fluids and even in human embryo culture medium (probably because it contains residual follicular fluids) (Austgulen, 1995).

Four cytokines were the focus of this study: leukaemia inhibitory factor (LIF), interleukin-11 (IL-11), tumour necrosis factor (TNF), and gamma interferon (IFNγ). The first two are already known to be key determinants of successful implantation and the latter two are responsible at high doses for implantation failure and early pregnancy loss in mice and humans.

LIF is the first cytokine found to be mandatory for implantation in mice (Stewart et al., 1992). Indeed, LIF–/– embryos obtained by gene knockout are fertile (the number of LIF–/– embryos is normal), but neither LIF–/– nor LIF+/+ embryos implant in LIF–/– foster mothers. Both types of embryo do implant in LIF+ foster mothers, and the implantation blockade observed in LIF– mice can be partly corrected by an infusion of recombinant LIF in the peritoneal cavity. The presence of LIF in the follicular environment has been described by several authors (Arici et al., 1997; Coskun et al., 1998; Jean et al., 1999; Ozornek et al., 1999), but its role is not yet clear: it cannot be essential because LIF-deficient mice usually ovulate. Nonetheless, the concentration of LIF in human follicular fluid rises around the time of ovulation (Arici et al., 1997). In-vitro LIF improves the development of murine blastocysts (Mitchell et al., 1994; Kauma and Matt, 1995) and increases the rate of hatching in both murine and ovine species (Lavranos et al., 1995; Tsai et al., 1999). LIF also decreases the rate of embryo degeneration and increases the pregnancy rate in ovine experiments (Fry et al., 1992). In humans, follicular fluid LIF has been described as a marker of oocyte quality (Jean et al., 1997) and correlated with embryo quality (Arici et al., 1997). Follicular fluid LIF in humans has also been reported to enhance the rate of blastocyst formation (Dunglison et al., 1996), although this...
finding has not been confirmed (Jurisicova et al., 1995) and thus remains controversial. Ovarian granulosa, stromal cells and macrophages all express LIF mRNA and actively secrete the protein (Loukides et al., 1990).

IL-11, reported to be present in preovulatory follicular fluids, belongs to a cytokine subfamily that shares a common signal transducing receptor, gp130, with LIF. Although its paracrine and/or autocrine role in human ovarian follicular function is not yet known, IL-11 is strongly suspected to be an immunomodulator that may down-regulate the expression of various pro-inflammatory cytokines. Reports of high IL-11 concentrations in the follicular fluids of atretic follicles raise the issue of this protein’s involvement in the process of atresia (Branisteau et al., 1997).

TNF is a cytokine with an essential role in folliculogenesis and ovarian maturation (Wang et al., 1992). It is a potent modulator of ovarian function, affecting steroidogenesis of both granulosa and interstitial thecal cells (Spaczynski et al., 1999). TNF has been shown to play an important role in oestradiol secretion (Wang et al., 1992, Cianci et al., 1996) as well as in the induction and demise of the corpus luteum (Wang et al., 1992). The administration of dexamethasone to patients with polycystic ovarian disease has been shown to decrease follicular fluid TNFα concentrations (Zolti et al., 1992).

The pro-inflammatory cytokine IFNγ has been found in follicular fluids, while other pro-inflammatory cytokines are either not detectable or detected in insignificant amounts (Srivastava et al., 1996). This suggests that it has a specific role, perhaps the prevention of infection. In mice, some gene knockout experiments have shown that neither IFNγ nor its receptor is essential for fertility and maintenance of pregnancy (Dalton et al., 1993; Huang et al., 1993), but other studies report the contrary (Ashkar and Croy, 1999; Croy et al., 2000). The reason for such discrepancies between various strains of knockout mice is still unresolved.

We postulated that the cytokine profiles of women who spontaneously ovulate (control and donor patients) should differ from those of women with polycystic ovarian syndrome (PCOS) who have difficulty ovulating without stimulation. Nonetheless, ovarian stimulation of these patients usually leads to abnormal results, such as a premature ovarian response to LH by granulosa cells, albeit healthy (Almahbobi et al., 1996; Willis et al., 1998), and a tendency to ovarian hyperstimulation. Accordingly, patterns of cytokine expression in follicular fluids were investigated that might be related specifically to PCOS and that might lead to a better understanding of this syndrome, which has not yet been completely characterized.

Diagnosis of PCOS (n = 15) was based on oligo- or anovulatory cycles associated with oligo-amenorrhoea or amenorrhoea, LH/FSH >2, or on ovarian ultrasonography findings of increased stromal and ovarian volume and many (>10) tiny (<3 mm) cysts studded along the periphery of the ovary (Dewailly et al., 1992; Dewailly, 1997). Patients were selected if they exhibited all the criteria for the diagnosis of PCOS. Eight patients had hyperandrogenism and seven did not. IVF indications in the PCOS group were male factors (n = 8), tubal disease (n = 3) or idiopathic infertility (n = 4). The women in the control group (n = 15) and the oocyte donors (n = 8) had regular cycles, an LH/FSH ratio <1, and <10 follicles at the beginning of the cycle, with normal stromal volume. The patients in the control group were selected according to the inclusion criteria during the same period. In the control group, the IVF indications were male factors (n = 7), tubal disease (n = 3), or idiopathic infertility (n = 5).

Protocol for controlled ovarian stimulation

All patients received a standard gonadotrophin-releasing hormone (GnRH) agonist regimen that began on day 21 of a spontaneous menstrual cycle. Leuprolide acetate (1 mg per day s.c. Lutrin; Takeda Pharmaceuticals, Paris, France) was administered for 10–14 days until complete pituitary desensitization was documented. Stimulation was initiated once sonographic evidence indicated no ovarian follicular activity and an endometrial lining <5 mm thick and serum oestradiol measured <50 pg/ml. Recombinant gonadotrophin (rFSH) (Purogen; Organon Pharmaceuticals, Saint Denis, France) therapy then began at 225 IU/day for control and oocyte-donor groups and 150 IU/day for the PCOS group for the first five days of controlled ovarian stimulation. Further rFSH doses were determined according to the standard criterion of follicular maturation, assessed by ultrasound and serum oestradiol measurements. Similarly, HCG was administered (10 000 IU i.m. ‘endo’ chionic gonadotrophin; Organon Pharmaceuticals) when at least three follicles exceeded 17 mm in diameter and when the oestrogen concentration per mature follicle (diameter >17 mm) was >300 pg/ml. 36 h after HCG administration, oocytes were retrieved by needle aspiration, with transvaginal ultrasound guidance and routine intravenous sedation. After the cumulus–oocyte complexes were removed, the preovulatory follicular fluid were pooled for each patient and then centrifuged at 600 g for 10 min. The cell-free supernatants were then divided into aliquots and stored at −80°C until assay.

Cytokine assays

The enzyme-linked immunosorbent assay (ELISA) used to quantify LIF has been described previously (Taupin et al., 1997). The two monoclonal antibodies it uses are known not to interfere with the ligand receptor binding; therefore the test is not affected by the presence of soluble receptors in the samples. The detection threshold was calculated by adding 2 standard deviations to the mean of 6 blank wells and never exceeded 25 pg/ml. Results are expressed in pg/ml. Intra-assay coefficients of variation were respectively 4.45, 3.55 and 4.59 for LIF concentration of 80, 400 and 1000 pg/ml. Inter-assay coefficients of variation were respectively 4.26, 3.42 and 1.02 for LIF concentration of 80, 400 and 1000 pg/ml.

TNF was evaluated by the standard cytotoxicity bioassay, with the L929 murine fibroblast cell line (Wang et al., 1985; Hogan and Vogel, 1988). This assay, while it does not distinguish between the alpha and beta isoforms, measures all—and only—the bioactive TNF present in the samples (Wang et al., 1985). Murine and human TNF lyse equally well with the L929 murine fibroblast cell. Results are expressed in units, each corresponding to the TNF concentration required for half maximal lysis.

Materials and methods

Patient characteristics

This study enrolled 38 patients between November 1998 and November 1999. All patients provided an informed consent, and this investigation was approved by our Institutional Review Board. Human follicular fluids were collected from preovulatory follicles of patients (aged 26–41 years) undergoing IVF and embryo transfer at the Antoine Béclère Hospital, Clamart, France.
IL-11 concentrations were measured with the Quantikine kit (R&D, Abingdon, UK); its sensitivity, calculated as the 95% coefficient of variation of repeated blank measurements, was 8 pg/ml. Intra- and inter-assay coefficient of variation values were 2.4 and 1.8% respectively. For IFNγ we used the commercial ELISA kit produced by Coulter-Immunotech, Marseille, France. Its sensitivity was 0.08 IU/ml. Intra- and inter-assay coefficient of variation values were 4.7 and 7.8% respectively.

**Oestradiol and progesterone assays**

Oestradiol and progesterone concentrations in serum were determined on the day of triggering, and in follicular fluid, on the day of oocyte collection. Hormone measurements were performed with an automated chemiluminescence system (ACS-180; Bayer Diagnosis, USA). Sensitivities were 30 pg/ml (conversion factor to SI units, 3.67) for oestradiol and 0.1 ng/ml (conversion factor to SI units, 3.18) for progesterone. Intra- and inter-assay coefficient of variation values were respectively, 5 and 7% for oestradiol and 3.5 and 7% for progesterone. Before testing, follicular fluid was diluted with a specific diluent at 1/100 for oestradiol and 1/10 for progesterone.

**Evaluation of embryo quality**

The quality of embryos was evaluated by their morphological aspect, the regularity of the embryo cells and the percentage of fragmentation.

**Statistical analysis**

The normal distribution of the follicular fluid cytokine concentrations could not be confirmed. Therefore, results were expressed as median values. The nonparametric Mann–Whitney U-test was used to compare groups. We used the Spearman’s rank test for correlation analysis of the control group, the PCOS group and the pooled group of all infertile patients (control and PCOS). Embryo quality was compared with the $\chi^2$ test. The statistical assessment used StatView software (Abacus Concepts, Inc., Berkeley, CA, USA). A $P$ value < 0.05 was considered significant.

**Results**

**Patients, controlled ovarian stimulation and embryology data**

Table I summarizes the patients’ clinical features. The PCOS and control groups did not differ significantly as to mean (± SEM) age, duration of infertility, indication for IVF, serum FSH and oestradiol on the third day of the menstrual cycle, or number of oocytes and embryos collected. Stimulation of PCOS patients used significantly fewer units of gonadotrophin ($P < 0.0001$), in accordance with their ovarian profiles.

**Cytokine concentrations in the follicular fluid**

All follicular fluid samples contained LIF. In the control group, the LIF concentrations ranged from 403 to 2894 pg/ml with a median value of 816 pg/ml, not significantly different from the donor group (range: 553–1230 ; median: 749 pg/ml). These values were significantly higher than those in the PCOS group ($P < 0.0001$), where LIF ranged from 78 to 594 pg/ml, with a median at 265 pg/ml (Table II). In the PCOS group, median LIF values did not differ significantly according to hyperandrogenism.

TNF was detected in all samples of follicular fluid except for a single case in the PCOS group (Table II). The PCOS and control groups did not differ significantly for TNF concentrations, but significant differences were observed for the donors, compared with both the control ($P = 0.0225$) and PCOS patients ($P = 0.0340$). IL-11 and IFNγ, on the other hand, were less commonly found in follicular fluid samples. IL-11 was found in 50% of donor follicular fluid samples, 29% of control samples and only 21% of PCOS patients. Although the donor median differed, there were no significant statistical differences between the 3 groups (Table II).

IFNγ was detected in 50% of control follicular fluid samples, 66% of PCOS patients, and 75% of donors. Again, the differences were not significant (Table II).

**Oestradiol and progesterone in follicular fluids**

The follicular fluid oestradiol concentrations did not differ significantly, with medians of 655×10³ pg/ml for controls, 753×10³ pg/ml for donors, and 526×10³ pg/ml for PCOS patients (Table II). Follicular fluid progesterone concentrations were significantly lower among the PCOS group than among the control ($P = 0.0046$) or donor ($P = 0.0072$) groups.

**Correlation between cytokines and hormones**

Folicular fluid concentrations of LIF and progesterone were correlated in the control group ($r = 0.550, P = 0.0396$) and

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Values are reported as medians with the range in parentheses. The fertilization and implantation rates are percentages. The LH/FSH ratio, the number of gonadotrophin units administered for ovarian stimulation and the implantation rate were significantly different in the PCOS and control groups ($P < 0.05$).

PCOS = polycystic ovarian syndrome.
A growing body of evidence points to an interaction between the immune and reproductive systems. Cytokines and growth factors are the candidates most likely to participate in such an interaction. Nevertheless, whether high follicular fluid cytokine concentrations have any significance for ovarian function must be assessed.

In this study the highest cytokine concentrations were found in the donor follicular fluid and the lowest in the PCOS follicular fluid. TNF has usually been described as deleterious in follicular fluid (Yan et al., 1993), but in studies that used an ELISA assay specific for TNFα which is unable to measure bioactive TNF. The current investigation used a bioassay that is unable to distinguish between the α and β forms of TNF but measures all the bioactive TNF (Wang et al., 1985; Hogan and Vogel, 1988).

LIF concentrations <15 pg/ml have usually been reported in pooled follicular fluids isolated from preovulatory follicles and collected in conditions like ours (Arici et al., 1997; Coskun et al., 1998). The results of the current study differ dramatically from those reported previously. This discrepancy may be explained by the fact that the ELISA assay used measured all LIF fractions, whether or not they were bound to soluble receptors. The specificity of our LIF ELISA assay made possible a 100-fold increase in follicular fluid LIF detection. Such differences in the specificity of LIF ELISA tests have been reported previously (Taupin et al.; 1997; Jean et al., 1999).

A previous report found follicular fluid LIF to be correlated with follicular fluid oestradiol (Arici et al., 1997). The strong correlation between follicular fluid LIF and progesterone found in this study was more in agreement with that reported by Ozornek et al. (Ozornek et al., 1999).

Except in cases of severe inflammation, LIF is reported to be absent from circulating blood due to its short half-life in the vascular compartment (Hilton et al., 1991). Therefore, blood contamination is very unlikely to be responsible for the high follicular fluid LIF concentrations. The precise sites that produce and secrete LIF and TNF have not yet been determined. Candidate sources include granulosa cells, resident ovarian cells or leukocytes present in follicular fluid. Resident macrophages and monocytes make up 5–15% of human follicular tissue cells. The lymphocyte concentration and T-cell subpopulations in follicular fluid change significantly during oocyte maturation (Loukides et al., 1990). Although all these cells may contribute to the regulation of the cytokines tested here, the best candidate is the granulosa cell, which expresses LIF.
mRNA at the preovulatory follicle stage (Arici et al., 1997). Indirect support for this proposition may be found in the difference in the LIF detected in control and PCOS follicular fluid and the correlation between LIF and progesterone. Indeed, PCO granulosa cells have an abnormal capacity to synthesize progesterone in vivo and in vitro (Doldi et al., 1998). Recent data suggest that follicular fluids may play an important role in the endocrine balance of PCOS by its effect on the relation between theca and granulosa cells. An abnormal interaction between theca and granulosa cells. An abnormal interaction between the LH/FSH ratio, one of the criteria by which PCOS is defined (Tarlatzis et al., 1995), and the follicular fluid LIF concentration may thus be a key factor in this abnormal interaction.

Recombinant human LIF has already been tested in murine IVF. Its addition to mouse embryo culture medium enhances blastocyst development (Kauma and Matt, 1995; Tsai et al., 1999), significantly increases the number of embryos that hatch and improves embryo survival in vivo (Fry et al., 1992; Lavranos et al., 1995).

The effects of adding recombinant human LIF to culture medium for human blastocysts is still a matter of debate. It has been found that adding LIF to the medium enhances in-vitro human blastocyst formation (Dunglison et al., 1996), while others have not found such an effect (Jurisicova et al., 1995). Nonetheless, follicular fluid LIF concentration has been found to be correlated with embryo quality in an oocyte donor group; and a similar correlation has been reported between LIF concentration and oocyte quality (Arici et al., 1997). LIF receptors have been found in human embryos (Charnock-Jones et al., 1994; Sharkey et al., 1995; Van Eijk et al., 1996; Chen et al., 1999): this suggests that LIF may affect embryo development by acting as an 'embryotrophin' before implantation and as a factor required for embryo implantation.

In this study lower concentrations of LIF follicular fluid were found in the PCOS group. We therefore speculate that the lower implantation rate in this group might be due to the LIF concentrations.

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References

LIF in preovulatory follicles of women with PCOS


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