Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction?

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We determined the concentrations of tumour necrosis factor (TNF-α), interleukins (IL)-1β, -6, -8 and -1-receptor antagonist (IL-1-ra) and of oestradiol and progesterone in the follicular fluid of 111 women undergoing in-vitro fertilization (IVF) and of six women with ovarian cysts in order to elucidate mid-cycle mechanisms causing dissociation of the follicle wall and local rupture of the ovarian tissue complex. Four stimulation protocols were administered: gonadotrophin releasing hormone agonist/human menopausal gonadotrophin (GnRHa/HMG), clomiphene citrate/HMG (CC/HMG), HMG and follicle-stimulating hormone (FSH). Concentrations of TNFα and IL-1β were below 15 and 3 pg/ml respectively. IL-6 (median 4.1, 3.5–4.4 pg/ml, 95% CI) was higher after stimulation with FSH (5.6 pg/ml) than with HMG (3.2 pg/ml, P < 0.05) or GnRHa/HMG (3.7 pg/ml, P < 0.05), and after stimulation with CC/HMG (5.5 pg/ml) than with HMG (P < 0.01) or GnRHa/HMG (P < 0.001). IL-8 ranged from 32 to 1241 pg/ml (147, 117–178 pg/ml) and IL-1-ra from <31 to >10 000 pg/ml (156, 109–192 pg/ml). Cytokine levels did not correlate to oestradiol or progesterone concentrations. The ovarian cysts contained similar IL-8 (14–540 pg/ml) and IL-1β (<30 pg/ml), but higher IL-6 (13.6–>500 pg/ml) and lower IL-1-ra concentrations. We assume that IL-6, IL-8 and IL-1-ra are involved in peri-ovulatory cellular interactions. Thus, ovulation appears to be a cytokine-regulated process of an ‘inflammation’ (IL-6 and IL-8) followed by ‘anti-inflammatory’ reactions (IL-1-ra).

Key words: follicular fluid/interleukin-6/interleukin-8/interleukin-1 receptor antagonist/ovulation physiology

Introduction

Inter-relationships between the endocrine and the cytokine system have been known for a long time. Cytokines exert direct effects on active endocrine tissues and on the other hand are produced by them in noteworthy concentrations (Mandrup-Poulsen et al., 1995; Vinatier et al., 1995). Oestrogens inhibit interleukins (IL)-1, IL-6 and tumour necrosis factor (TNF)-α production and progestrones and androgens inhibit IL-1 production by mononuclear cells (Hu et al., 1988; Pacifici et al., 1989; Polan et al., 1989; Pottratz et al., 1994). For TNF-α and IL-1, interactions with the reproductive system in the ovary have been proven in vitro (Zolli et al., 1990; Best and Hill, 1995). In vivo, TNF-α, IL-1 and IL-6 were found in the follicular fluid of women undergoing in-vitro fertilization (IVF) (Buyalos et al., 1992; Wang and Norman, 1992; Wang et al., 1992). These and other cytokines like GM-CSF (granulocyte-macrophage-colony-stimulating factor) seem to play a pivotal role in the regulation of the development and atresia of follicles in the ovary (Jasper et al., 1996; Kaipia and Hsueh, 1997).

The ovary itself is capable of initiating inflammatory reactions. A total of 5–15% of the cellular pool of the follicular fluid consist of macrophages (Loukides et al., 1990). At mid-cycle, ovarian IL-1 synchronously rises with the IL-1-mRNA in peripheral blood monocytes in women undergoing IVF (Polan et al., 1994). Intra-ovarian macrophages are moreover involved in the production of IL-1β (Machelon et al., 1995).

Furthermore, autochthonous functional ovarian cells like thecal and granulosa cells are involved in the precise regulation of the immuno-endoctrine system (Adashi et al., 1989; Kokia et al., 1993). Meanwhile for IL-1 the existence of a highly compartmentalized and hormonally dependent intra-ovarian IL-1-system has been demonstrated with ligands, receptors and receptor antagonists (Hurwitz et al., 1992; Wang et al., 1997).

An oestradiol pulse of sufficient potency and adequately long duration (Goh and Ratnam, 1990) causes ovulation in the first half of the female menstrual cycle by its effect on the hypothalamus and pituitary. The peri-ovulatory luteinizing hormone (LH) surge activates enzymes in the ovary that weaken the follicle wall to facilitate extrusion of the oocyte. Ovulation takes place 1–1.5 days after the LH peak. Oestrogens also increase the responsiveness of granulosa cells to follicle-stimulating hormone (FSH) and LH in the ovary by inducing receptor formation in these cells. There is evidence that cytokines are involved in both the inhibition and stimulation of follicular responsiveness to gonadotrophins (Gougeon, 1994). Paracrine interrelationships that cause follicular growth and tissue damage, however, have not been elucidated. Therefore it was our objective to investigate the role of cytokines in ovulation as mediators of follicular rupture, oocyte expulsion from the ovary and ensuing reparative reactions.

The present study was designed to analyse the humoral contents of follicular fluid at the time of ovulation. Based on the hypothesis that ovulation has to be regarded as a local inflammatory event that is followed by anti-inflammatory reactions, we selected TNF-α, IL-1β, IL-6 and IL-8 as medi-
ators of cytotoxicity and inflammation and IL-1-ra as an immunosuppressive agent for examination and determined their levels in the follicular fluid of 111 women undergoing IVF. We assumed that the synchronous determination of several cytokines might clarify the physiology of ovulation in IVF-stimulated ovarian follicles.

In order to compare these follicles with the situation in physiological ovarian follicles, we analysed follicular fluid from punctures of ovarian cysts.

Materials and methods

Patients

A total of 111 samples of follicular fluid were obtained from women who underwent IVF at the Universitätsfrauenklinik Virchow-Klinikum, Humboldt-Universität zu Berlin. The women’s age ranged from 20–46 years [median 33, 31–34, 95% confidence interval (CI)]. Infertility was due to tubal sterility in 51.8% (58 cases). In 16.1% (18) it was due to male infertility. In 2.7% (3) it was due to hormonal sterility. In 25.9% (29) the cause of infertility was due to both male and female factors. In 3.6% (4) no aetiology could be found or another cause was underlying.

Six samples of cystic fluid were obtained from premenopausal women with ovarian cysts who underwent diagnostic and/or therapeutic treatment for conditions that required no preceding hormonal treatment. These women’s ages were 21, 34, 38, 38, 47 and 51 years. We included only those cysts in our study that were histologically proven to be follicular cysts. The small quantities of cystic fluid in these specimens precluded concurrent measurement of TNF-α.

IVF stimulation protocols

Four different stimulation protocols were administered, two protocols using either a series of follicle stimulating hormone (FSH, Serono, Unterschleisheim, Germany) or human menopausal gonadotrophin (HMG; Organon, Oberschleißheim, Germany) and two protocols, both with human menopausal gonadotrophin in the follicular phase, either with the gonadotrophin-releasing hormone agonist Decapeptyl (GnRHa/HMG; Ferring AB, Kiel, Germany) or with clomiphene citrate (CC/HMG; Serono) in the mid-luteal phase for luteal phase down-regulation.

Aspiration of follicular fluid

Transvaginal follicular aspiration was performed under vaginal sonographic guidance 36 h following the administration of human chorionic gonadotrophin (HCG). Each ovary was aspirated with separate needles. We sampled the fluid from the leading follicle on each side only if oocyte aspiration was successful at the first attempt. If flushing of the follicle with medium was necessary for retrieving the oocyte, the content was not used for measurement.

The number of oocytes retrieved ranged from 2–28. The number of oocytes after stimulation with GnRHa/HMG was significantly higher than after stimulation with CC/HMG (median of 9 versus 6, P < 0.0002).

Cytokine measurement by ELISA (enzyme-linked immunosorbent assay)

To select any cytokine alterations only blood free samples were selected. Aliquots were put into pyrogen-free tubes and immediately stored at –80°C until assay.

IL-6 was measured by an ultrasensitive enzyme-linked immunosorbent assay (ELISA; Cytoscreen US™ ultrasensitive, human IL-6 ultrasensitive, Immunoassay Kit, ASY-03S, Standard Lot no. S 112294; BioSource International, Camarillo, CA, USA). The assay was sensitive down to 104 pg/ml, with intra-assay and interassay variabilities of 4.71–8.33% and 6.7–10.0% respectively.

Tumour necrosis factor-α was measured by an ELISA (Quantikine™ Human TNF-α Immunoassay, DTA50, Standard Lot no. 941115 D; R&D Systems, Minneapolis, MN, USA). The assay was sensitive down to 4.4 pg/ml with intra-assay and interassay variabilities of 4.2–5.2% and 4.6–7.4% respectively.

IL-1 receptor antagonist was measured by an ELISA (Quantikine™ Human IL-1β Immunoassay, DRA00, Lot no. 9419128; R&D Systems). The assay was sensitive down to 6.5 pg/ml with intra-assay and interassay variabilities of 2.6–8.4% and 4.8–5.9% respectively.

IL-1β was measured by an ELISA (Quantikine™ Human IL-1β Immunoassay, DLB50, Standard Lot no. 9349083; R&D Systems). The assay was sensitive down to 0.3 pg/ml, with intra-assay and interassay variabilities of 2.3–3.1% and 3.4–4.1% respectively.

IL-8 was measured by an ELISA (Quantikine™ Human IL-8 Immunoassay, D800, Lot no. 9449079; R&D Systems). The assay was sensitive down to 3 pg/ml, with intra-assay and interassay variabilities of 2.4–3.9% and 7.3–12.2% respectively.

All samples were tested in duplicate.

Oestradiol and progesterone measurement by EIA (enzyme immunoassay)

Concentrations of oestradiol and progesterone were determined using a fully automatic EIA-analysing system (SR 1®; Serono Diagnostics, Freiburg, Germany). The oestradiol assay was sensitive down to 0.1 ng/ml, with intra-assay and interassay variabilities of 4.0–26.2% and 9.1–22.2% respectively. The progesterone assay was sensitive down to 0.2 ng/ml, with intra-assay and interassay variabilities of 5.4–16.9% and 7.4–20.4% respectively.

The concentrations of oestradiol ranged from 13.2–1500 ng/ml (median 306 ng/ml, 264–476 ng/ml, 95% CI, mean 524 ± 55 ng/ml) and those of progesterone from 0.1–165 µg/ml (median 9.6 µg/ml, 8.4–12.3 µg/ml, 95% CI, mean 13.4 ± 2.6 µg/ml).

Statistical analysis

For statistical analysis we used the statistics programme SPSS for Windows. We decided to choose medians with a 95% CI because our patients did not represent a Gaussian distribution. We used the Wilcoxon–Mann–Whitney test to test differences of the medians. No sample had concentrations above 15 pg/ml.

Results

Cytokine concentrations

TNF-α

No sample had concentrations above 15 pg/ml.

Interleukin-1β

47 (81%) out of 58 samples were under the lowest standard level (<3.9 pg/ml). No sample had concentrations higher than 20 pg/ml.

The concentrations did not differ by the stimulation protocol.

Interleukin-6

The samples contained IL-6 in concentrations from <0.3 pg/ml to over 20 pg/ml with a median of 4.1 pg/ml (3.5–4.4 pg/ml, 95% CI) and a mean of 5.2 (±0.4 SEM) pg/ml.

Stimulation protocol had a significant influence. The IL-6 concentrations were significantly higher following stimulation.
Table I. Concentrations of progesterone, oestradiol, interleukin (IL)-8, IL-6, IL-1β and IL-1-ra in follicular fluid of six women with ovarian cysts

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Progesterone µg/ml</th>
<th>Oestradiol ng/ml</th>
<th>IL-8 pg/ml</th>
<th>IL-6 pg/ml</th>
<th>IL-1β pg/ml</th>
<th>IL-1-ra pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>1905</td>
<td>&gt;300 000</td>
<td>14.0</td>
<td>13.64</td>
<td>&lt;0.3</td>
<td>&lt;31</td>
</tr>
<tr>
<td>47</td>
<td>54</td>
<td>13 730</td>
<td>266</td>
<td>&gt;500</td>
<td>&lt;0.3</td>
<td>&lt;31</td>
</tr>
<tr>
<td>38</td>
<td>79</td>
<td>248 300</td>
<td>38.0</td>
<td>241</td>
<td>&lt;0.3</td>
<td>207</td>
</tr>
<tr>
<td>51</td>
<td>&lt;0.2</td>
<td>1770</td>
<td>26.0</td>
<td>48.4</td>
<td>&lt;0.3</td>
<td>&lt;31</td>
</tr>
<tr>
<td>21</td>
<td>&lt;0.2</td>
<td>62</td>
<td>540</td>
<td>&gt;500</td>
<td>&lt;0.3</td>
<td>&lt;31</td>
</tr>
<tr>
<td>38</td>
<td>&gt;4000</td>
<td>&gt;300 000</td>
<td>488</td>
<td>186</td>
<td>27.8</td>
<td>&lt;31</td>
</tr>
</tbody>
</table>

Figure 1. Concentrations of interleukin (IL)-6 in follicular fluid dependent on the stimulation protocol administered.

with FSH (5.6 pg/ml median) than with HMG (3.2 pg/ml, \( P < 0.05 \)) or with GnRHα/HMG (3.7 pg/ml, \( P < 0.05 \)). The IL-6 concentrations were higher following stimulation with CC/HMG (5.5 pg/ml) than only with HMG (\( P < 0.01 \)) or with GnRHα/HMG (\( P < 0.001 \)) (Figure 1).

**Interleukin-8**

Interleukin-8 was found in all samples that were analysed. The concentrations ranged from 32 pg/ml to 1241 pg/ml with a median of 147 pg/ml (117–178 pg/ml, 95% CI) and a mean of 196 (±16 SEM) pg/ml. Concentrations were unaffected by the stimulation protocol.

**Interleukin-1 receptor antagonist**

The concentrations of this cytokine had the widest range of all determined cytokines. Ten patients had concentrations below 31 pg/ml. Five values were higher than 10 000 pg/ml with one of them being even higher than 20 000 pg/ml. So concentrations differed by more than 1000% and left room for speculation about the underlying conditions.

The median was at 156 pg/ml (109–192 pg/ml, 95% CI). The mean was at 867 (±258 SEM) pg/ml. The concentrations did not differ by the stimulation protocol.

**Number of oocytes**

According to our experience with the GnRHα/HMG protocol, the number of oocytes retrieved was significantly higher than after stimulation with CC/HMG. This is probably due to the lack of physiologic LH so that aromatase activity is reduced and many follicles develop at the same time. Despite the reciprocal relationship of the concentration of IL-6 to the number of oocytes retrieved in these two subgroups, no correlation between the number of oocytes and cytokine concentrations in follicular fluid in general could be found.

**Cytokines in follicular ovarian cysts**

IL-6 concentrations were remarkably higher than in IVF follicles. Five out of six samples contained concentrations over 20 pg/ml while less than 10% of the IVF follicles did so. And IL-1-ra could only be detected in one out of six cysts, but in more than 90% of the IVF follicles. IL-8 occurred in all cysts, as well as in all IVF follicles, and as in IVF follicles IL-1β was not found at concentrations above 30 pg/ml (Table I).

**Discussion**

Numerous studies have shown evidence that cytokines not only occur during immunopathological reactions, but also regulate physiological processes. Thus cytokines play an important role in the endocrine, paracrine or autocrine mediated interactions of cells and tissues.

Because of their short half-life, rapid changes in concentrations and low systemic concentrations (\( 10^{-12} – 10^{-15} \) mol/l), cytokines have highly specific effects depending on the target cell and local interactions with other cytokines.

**Cytokines in IVF follicles**

We examined aspirates of follicular fluid that were taken from IVF patients during oocyte retrieval procedures. Our objective was to depict the immuno-endocrine situation in the stimulated preovulatory and peri-ovulatory ovarian follicle. A small compartment and a defined time made peri-ovulatory determination of cytokine concentrations useful.

In keeping with the results of previous studies (Wang and Norman, 1992; Barak et al., 1992; Huyser et al., 1994), we found no correlation between cytokine concentrations and oestradiol or progesterone concentrations in follicular fluid.

Yet the idea of interlocking relationships between the endocrine and the cytokine system was supported by our observation that the stimulation protocol for IVF influenced cytokine concentrations in follicular fluid.

Different stimulation protocols resulted in significant differences in IL-6 concentrations (\( P < 0.05 \)). Both hormone and cytokine production in the ‘stimulated ovarian follicle’ compartment were influenced by the stimulation protocol for IVF and therefore by the systemic hormonal constellation.
Cytokines and ovulation

Still little is known about the intra-ovarian paracrine system that allows Graafian follicles to break through the ovarian cortex and to empty their contents into the abdominal cavity. Based on the presence of prostaglandins and leukotriens (Lipner, 1988) it has been suggested that a modified inflammatory process was involved in these events. Nitric oxide has been proposed to induce ovulation by exerting an IL-1 induced cytotoxic effect on the granulosa cell complex with succeeding hyperaemia and rupture of the follicle (Mandrup-Poulsen et al., 1995). For a short period of time the ovarian stroma also has to tolerate a local dissolution of its tissue complex.

The occurrence of IL-8 in all samples indicates a kind of inflammatory event followed by an increased immigration of neutrophils. A preventive secretion accompanied by a chain of physiological reactions could account for these reactions, all of them aiming at the prevention of potential infectious or damaging processes after ovarian rupture. There is evidence of a peri-ovulatory surge in neutrophils in the theca of the leading follicle contributing to follicular rupture and dissociation of the ovarian wall (Chun et al., 1993; Bonello et al., 1996; Oral et al., 1997).

IL-1-ra occurred at concentrations comparable to those in serum in most of the follicles, in 10% even at concentrations >1000 pg/ml. These observations may be evidence of an increased peri-ovulatory occurrence of macrophages in the ovary and in the ovarian follicle, respectively. One potential reason for the wide range of concentrations is the rapid changing of cytokine secretion with short peaks and quick decreases. These ups and downs suggest an important role of IL-1-ra in ovulation. An immunosuppressive effect has to be assumed antagonizing the deleterious effects of IL-1. Thereby, the receptor antagonist suppresses the IL-1α and -β mediated reactions of the immune system against ovulatory tissue damage. Thus, we consider ovulation as a course of inflammatory events and that the organism apparently attempts to limit the reaction cascade that follows.

Not surprisingly, we found low concentrations of IL-1β in our samples, for it is known that high oestradiol concentrations inhibit IL-1β production.

Nevertheless regulative interactions between IL-1 and its receptor antagonist are more complex. Previous publications indicate a common regulatory circle of IL-1 and IL-1-ra with increased concentrations of the latter expressing an enhanced activity of the former (Arend, 1990).

Furthermore, much higher concentrations of IL-1-ra are necessary to block the effects of IL-1, although IL-1-ra and IL-1 bind to the receptor with equimolar concentrations. In healthy controls, normal serum concentrations of the receptor antagonist are about 100-fold higher than those of IL-1β.

Despite its cytotoxic qualities we did not find TNF-α in concentrations above the detection limit (15 pg/ml) in any of the samples.

Concentrations of IL-6 (0–20 pg/ml), whose production is induced by TNF-α as well as by IL-1 (Dinarello and Thompson, 1991), differed significantly ($P < 0.05$) depending on the stimulation protocol used. Hormonal influences obviously affect ovarian cytokine formation. In addition, our results show that even very low concentrations seem to have specific effects.

Is it IL-1-ra antagonism of IL 1-mediated induction of TNF-α and IL-6-production in the ovary that keeps concentrations of these two cytokines low, while they are otherwise frequently involved in inflammatory conditions? Or is there a superior regulator at the time of ovulation for both cytokines and the endocrine system?

Finally, the occurrence of IL-8 in pre-ovulatory follicles with its chemotactic activity towards neutrophils signals that there is a kind of inflammatory reaction in progress. Our findings agree with a recent study which postulated a pre-ovulatory increase of IL-8 concentrations in the follicle (Arici et al., 1996) and with the results of Runesson et al. (1996), who found 30-fold higher values in follicular fluid than in plasma.

The non-physiological influence of IVF on the endocrine system must not be neglected. As differences in LIF (leukaemia inhibitory factor) and GM–CSF levels between stimulated and unstimulated follicles have already been described (Jasper et al., 1996; Arici et al., 1997), our interest focused on the situation in physiological non-stimulated ovarian follicles. Therefore we included in our study ovarian cysts that were histologically recognized as follicular cysts with an intact wall of epithelial cells. The small number and the uncertainty regarding the cyclic status at the time of puncture do not allow us to apply our results to non-stimulated ovarian follicles in general, let alone on peri-ovulatory follicles. But accidental puncture of the follicle made them likely to be non-periovulatory follicular cysts. As a matter of speculation, in these follicles ovulation may just be overdue so they developed into symptomatic cysts. At least they contribute to elucidate the role of cytokines in the reproductive system.

In the cystic fluid we found remarkably higher concentrations of IL-6 than in IVF follicles. And IL-1-ra could only be detected in one out of six ovarian cysts, but in more than 90% of the IVF follicles. IL-8 occurred in all cysts, as in all IVF follicles, and as in IVF follicles no cyst contained IL-1β at a level above 30 pg/ml.

The present study demonstrates that human peri-ovulatory follicular fluid contains substantial concentrations of IL-1-ra. IL-8 could be detected in all samples at concentrations above normal serum levels. IL-6 was measured at serum levels. We postulate that the ovary is a site of the production of IL-1-ra, IL-8 and IL-6 as well as a target of their action.

We demonstrated the influence of the stimulation protocol on IL-6 concentrations in follicular fluid. We conclude that IL-6, IL-8 and IL-1-ra are involved in cellular interactions in peri-ovulatory follicles and that they play an important role in the process of ovulation. In this sense ovulation may be regarded as a regulated process with an inflammatory reaction as a result of follicular rupture carried out by the inflammatory cytokines IL-6 and IL-8 and with reactions suppressing inflammation following ovulation. These reactions are mediated by the ‘anti-inflammatory’ cytokine IL-1-ra and possibly other cytokines which prevent the deleterious effects of the physiological process of ovulation.