Elevated expression of tumour necrosis factor α in cultured granulosa cells from women with endometriosis

Magdalena Carlberg1,3, Jelve Nejaty1, Berit Fröysa2, Yongmei Guan1, Olle Söder2 and Agneta Bergqvist1

1Department of Clinical Sciences, Unit for Obstetrics and Gynaecology, Huddinge University Hospital, SE-141 86 Huddinge, Sweden
2Department of Woman and Child Health, Astrid Lindgren Children’s Hospital, Karolinska Hospital, SE-171 76 Stockholm, Sweden
3To whom correspondence should be addressed at: Department of Clinical Sciences, Unit of Obstetrics and Gynaecology, Huddinge University Hospital, SE-141 86 Huddinge, Sweden. E-mail: magdalena.carlberg@kfcmail.hs.sll.se

Fertilization and oocyte cleavage rates have previously been demonstrated to be lower for women with endometriosis undergoing IVF compared with controls. This might be related to impaired oocyte function, possibly due to an inflammatory milieu in the pelvis of these women, where an elevated concentration of many cytokines is documented. The aim of this study was to examine whether granulosa cells from women with endometriosis deviated with respect to production of the inflammatory cytokines interleukin-1β, interleukin-6, interleukin-8 and tumour necrosis factor α (TNFα) compared with granulosa cells from healthy women, undergoing IVF for male infertility. The effect of human chorionic gonadotrophin on cytokine production was also investigated. Granulosa cells in follicular fluid were obtained at oocyte retrieval for IVF. Incubated cell culture media were analysed by enzyme-linked immuno-sorbent assay. The basal production of all four cytokines was higher in cells from women with endometriosis when compared to controls, although the increase was only significant for TNFα. Chorionic gonadotrophin had no significant effect, although it had a tendency to suppress cytokine release in both patient categories. Whether aberrant cytokine production in granulosa cells from women with endometriosis may disturb fertilizing capacity of oocytes requires study.

Key words: endometriosis/granulosa cell/TNFα

Introduction

The fertilization and oocyte cleavage rates are significantly lower in women suffering from endometriosis in comparison to women with tubal factor infertility and couples with male factor associated fertility undergoing IVF (Gutiérrez et al., 1994; Simón et al., 1994; Tanbo et al., 1995; Bergendal et al., 1998; Hull et al., 1998; Pal et al., 1998). In addition, it has previously been shown that in comparison to healthy controls, the follicular growth rate is slower in patients with endometriosis, which may disturb the synchronization of oocyte maturation and ovulation in these women (Doody et al., 1988).

Cytokines are proteins with pleiotrophic regulatory effects on many cell types and can be produced by practically every nucleated cell in the body. Unlike hormones, cytokines only occasionally reach the circulation as endocrine mediators, and mainly act locally as paracrine and/or autocrine signals (Simón et al., 1995). The interleukins (IL) belong to a cytokine family modulating cellular proliferation and have the capacity to induce other cytokines, for instance the cascade-like cytokine release accompanying acute inflammation (Fay and Grudzinskas, 1991). It is well established that inflammatory cytokines, for instance IL-1β, IL-6, IL-8 and tumour necrosis factor α (TNFα), are elevated in the peritoneal fluid in women with endometriosis (Rier et al., 1994, 1995). An increased concentration of many compounds, for instance the cytokines IL-1, IL-8 and TNF, has been suggested to affect fertility adversely (Ramey and Archer, 1993; Arici et al., 1996). For instance, peritoneal fluid from patients with endometriosis has been shown to have a toxic effect on ovum retrieval, sperm mobility/survival, sperm–oocyte interaction, and embryonic development (Taketani et al., 1992; Ramey and Archer, 1993). Cytokines have also been suggested to influence oocyte fecundity (Zolti et al., 1992). Endometriotic tissue has been shown to, at least in tissue culture, produce different cytokines as IL-1β, IL-6 and TNFα and may contribute to elevated cytokine concentrations in the peritoneal fluid of women with endometriosis (Akoum et al., 1996; Bergqvist et al., 2000). Hypothetically, the inflammatory components in peritoneal fluid in women with endometriosis might diffuse into the ovarian follicles, or by paracrine mechanisms impair the oocyte maturation. Peritoneal fluid constituents might also interfere with the function of granulosa cells, or granulosa cells might by another disease-related mechanism have an altered expression of, e.g. cytokines.

IL-1 exists in two forms, IL-1α and IL-1β, which, although encoded by distinct genes, share the same receptors and biological properties (Simón et al., 1993; Robertson and Seamark, 1994). It is expressed in both acute and chronic inflammation, mediating a plethora of immunological and haematological effects, e.g. activation of T- and B-lymphocytes, macrophages, neutrophils, natural killer cells and endothelial cells (Robertson and Seamark, 1994). IL-1 is secreted in response to TNFα, but the transcription of IL-1 is suppressed by IL-6 as well as by IL-1 itself (Robertson and Seamark, 1994). The IL-1β protein has been found in total follicular fluid cells as well as in both cultured granulosa cells and macrophages (Baranao et al., 1995; Machelon et al., 1995).
The latter study also demonstrated expression of IL-1β mRNA in total follicular fluid cells as well as in cultured granulosa cells (Machelon et al., 1995).

IL-6 is a multifunctional cytokine involved in immunological, proliferative, and neoplastic processes (Akoum et al., 1996). IL-6 is critical for the differentiation and immunoglobulin production of B-cells (Akoum et al., 1996). It also promotes T-cell activation, proliferation and differentiation (Lotz, 1993). Furthermore, IL-6 has a prominent role in the co-ordinated systemic host defence response to injury (Lotz, 1993). IL-6 is not constitutively expressed but is transiently induced by various stimuli, for example IL-1 and TNF-α (Lotz, 1993; Machelon et al., 1994). Interestingly, IL-6 has the capacity to down-regulate its own inducers, demonstrating the complexity of the cytokine network regulation; IL-6 namely increases IL-1 transcription (Robertson and Seamark, 1994) and under certain circumstances suppresses the release of TNF-α (Aderka et al., 1989). The production of IL-6 has in primates been shown to increase at ovulation (Machelon et al., 1997), and IL-6 has been suggested to participate in ovulatory rupture of the follicular wall as well as in follicular angiogenesis (Motro et al., 1990; Machelon et al., 1997). IL-6 mRNA, as well as the IL-6 protein and IL-6 activity, has been demonstrated to be present in follicular fluid and granulosa cell cultures (Machelon et al., 1994).

IL-8 is a potent chemotactic and angiogenic factor (Hébert and Baker, 1993; Arici et al., 1996). Various cell types have been reported to produce IL-8, for instance in response to IL-1α, IL-1β and TNFα (Hébert and Baker, 1993; Arici et al., 1996; Laham et al., 1997). Both IL-8 mRNA and the IL-8 protein have also been detected in follicular fluid and granulosa cell cultures (Runesson et al., 1996).

TNFα is an important regulator in inflammation with anti-tumorigenic effects; it is also involved in metabolic and apoptotic pathways (Zolli et al., 1990; Machelon et al., 1997). Furthermore, TNFα has many roles in ovarian regulation including ovulation (Machelon et al., 1997). Production of TNFα has earlier been demonstrated in human granulosa cells (Zolli et al., 1990). TNFα increases IL-6 production in various cell types, including granulosa cells (Machelon et al., 1997).

To test the hypothesis that granulosa cells in women with endometriosis have a deviating cytokine production, the production of IL-1β, IL-6, IL-8 and TNFα was compared in cultured granulosa cells from patients with endometriosis and healthy controls undergoing IVF. The effect of human chorionic gonadotrophin (HCG) on the production of these cytokines was also investigated.

**Materials and methods**

**Patients**

Ten women with laparoscopically verified endometriosis and 10 women without any known endometriosis, but with a male factor related infertility, were included in the study. All women had at least 2 years of infertility but were otherwise healthy and were not receiving any pharmacological treatment; their age range was 28–40 years. The stages of endometriosis according to the revised American Fertility Society classification were four in stage I, two in stage II, three in stage III and one in stage IV. The study was approved by the local ethics committee at Huddinge University Hospital and all patients had given their informed consent before being included in the study. Granulosa cells were obtained at oocyte retrieval at the time of IVF treatment at Huddinge University Hospital, Huddinge, Sweden.

**Isolation of granulosa cells from follicular fluid and cell culture**

After the oocytes had been identified under the microscope and removed from the follicle samples, the follicular fluid was pooled from all follicles from each woman and centrifuged at 52 g for 2 min. To remove erythrocytes, the cell preparation was washed at least twice in cold phosphate buffered saline (PBS) (without calcium and magnesium; SBL Vaccine, Stockholm, Sweden). After the second wash, the cells were resuspended in 5 ml cell culture medium (Dulbecco’s Modified Eagle’s Medium:nutrient mixture F12 (Ham’s) (DMEM:F12; 1:1), supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin). All cell culture reagents were from Life Technologies, Gibco BRL, Paisley, UK. The cell suspension then consisted of partly granulosa cell colonies (GCC), and partly of singular cells (SC). As SC might include contaminating theca cells or fibroblasts, the cell suspension was passed through a cell strainer (size 40 µm; Becton Dickinson, Labora, Sweden) in order to isolate GCC. The retained GCC were obtained by back-washing, after which GCC were divided into equal volumes and seed in DMEM:F12 supplemented as above in 24-well flat bottom culture plates (Becton Dickinson) in a humidified 5% CO₂/95% air atmosphere at 37°C. The total volume of medium in each well was 500 µl. With the intention of removing macrophages, which very readily adhere to plastics (Machelon et al., 1996), from granulosa cells, the cells were transferred to new wells in the same plate after 1 h incubation. Next day, cells were rinsed twice in culture medium to remove any remaining red blood cells or leukocytes, which do not adhere to plastic (Machelon et al., 1995), and given fresh medium.

Following transfer and an adhesion period of 24–72 h, contamination of leukocytes was checked and found to be 0–1% (data not shown) by immunostaining using a monoclonal antibody against the leukocyte antigen CD45 (Dakopatts, Glostrup, Denmark), which is found on macrophages and all other leukocytes.

**Experimental procedure**

As GCC appeared more viable in culture than SC over time, it was decided that the study should be focused on these cells. After an initial overnight incubation, new medium was added and the granulosa cells were cultured for an additional 48 h. At this time, 500 µl of serum-free medium, with or without HCG (Serono Nordic AB, Solna, Sweden) in two concentrations (10 and 100 IU/ml respectively) was added. Following another 48 h, incubated cell culture media were collected and the cells were given new serum-free medium with or without HCG. The incubated media were centrifuged at 1500 g for 5 min in order to clear the supernatant from dead cells. After an additional 48 h the second change of incubated media was collected and centrifuged as before. All media were thereafter stored in −20°C until cytokine assay.

**Cell counting**

After the last medium collection, 200 µl trypsin (5×concentrated; Gibco) was added to the GCC samples which were then incubated for 30–40 min at 37°C in an atmosphere of 5% CO₂/95% air until cells loosened from the bottom of the plates and from each other. The cell suspension was mixed by pipetting several times to disperse the GCC. The cells were then counted in a Burkner chamber. In total,
Basal production of IL-1β of the GCC-containing fractions (data not shown).

Evaluation of samples with detectable cytokine concentrations using Spearman's rank correlation test. The absence of cytokines in any of the groups according to median values. Although the concentrations of IL-6 were about 10 times higher in the endometriosis group, this was not enough to reach significance.

**Effect of HCG**

HCG did not have any significant effect on cytokine release in any of the patient groups. It had, however, a tendency to suppress the cytokine production in both controls and women with endometriosis (Table I). This tendency appeared more pronounced in the control group, especially in the case of 10 IU/ml HCG. Using absolute figures, this dose induced a 31% inhibition of IL-1β release in the control group and 12% in the endometriosis group (data not shown). For IL-6, the inhibitory effect was 89 and 53% respectively, for IL-8 82 and 26%, and for TNFα 65 and 23% (data not shown). HCG 100 IU/ml did not further suppress cytokine production in any of the patient groups.

**Production of IL-1β, IL-6, IL-8 and TNFα in control and endometriosis groups**

Since no significant differences were observed in cytokine production with respect to HCG treatment, control and endometriosis groups including both HCG-treated and untreated GCC were compared regarding release of IL-1β, IL-6, IL-8 and TNFα. The endometriosis group had significantly elevated concentrations of TNFα (P = 0.01) in the GCC cultures compared to controls (Table II). IL-1β was only slightly higher in the endometriosis group, and IL-8 concentrations were not detectable in any of the groups according to median values. Although the concentrations of IL-6 were about 10 times higher in the endometriosis group, this was not enough to reach significance.

**Evaluation of samples with detectable cytokine concentrations**

Nearly all of the samples had detectable concentrations of IL-1β in both patient groups (Table II). When excluding the non-detectable samples, the median concentration was about two times higher in the endometriosis group compared to the control group.

For IL-6, very few samples were positive in both the control and the endometriosis group (Table II). However, also when comparing these samples, the median concentration was about five times higher in the endometriosis group.

Only one-third of the samples in the control group, but four-fifths of the samples in the endometriosis group, had detectable TNFα concentrations (Table II). The median concentration of this cytokine was about three times higher in the endometriosis group.

**Correlation coefficients of cytokine concentrations**

The mutual concentration dependency of the different cytokines was investigated by correlation analysis and the correlation coefficients were as shown in Table III.

Thus, the groups differed in that no negative correlation coefficients were obtained in the endometriosis group. The
Table I. Effect of human chorionic gonadotrophin (HCG) on cytokine release from granulosa cell colonies. Cytokine concentrations (fg/1000 cells) assayed in incubated media from granulosa cell colonies unexposed or exposed to HCG (10 or 100 IU/ml respectively), presented as median values and range (within brackets)

<table>
<thead>
<tr>
<th>HCG (IU/ml)</th>
<th>0</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Controls 281 (0–2928)</td>
<td>502 (10–1028)</td>
<td>165 (58–2571)</td>
</tr>
<tr>
<td></td>
<td>Endometriosis 525 (0–3003)</td>
<td>142 (0–4004)</td>
<td>452 (0–3303)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Controls 0 (0–4012)</td>
<td>57 (0–257)</td>
<td>0 (0–288)</td>
</tr>
<tr>
<td></td>
<td>Endometriosis 252 (0–4084)</td>
<td>113 (0–2927)</td>
<td>106 (0–4144)</td>
</tr>
<tr>
<td>IL-8</td>
<td>Controls 0 (0–1499)</td>
<td>0 (0–321)</td>
<td>0 (0–84)</td>
</tr>
<tr>
<td></td>
<td>Endometriosis 0 (0–67568)</td>
<td>0 (0–52478)</td>
<td>0 (0–43243)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Controls 843 (0–2196)</td>
<td>0 (0–771)</td>
<td>0 (0–3446)</td>
</tr>
<tr>
<td></td>
<td>Endometriosis 1062 (0–2808)</td>
<td>602 (0–3540)</td>
<td></td>
</tr>
</tbody>
</table>

IL = interleukin, TNFα = tumour necrosis factor α.

Table II. Cytokine concentrations (fg/1000 cells) assayed in incubated media from granulosa cell colonies from the controls and endometriosis groups respectively. Median values and range (within brackets) are shown. d/t indicates number of detectable samples/total number of samples

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Controls</th>
<th>Endometriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All samples</td>
<td>Detectable samples</td>
</tr>
<tr>
<td>IL-1β</td>
<td>281 (0–2928)</td>
<td>283 (10–2928)</td>
</tr>
<tr>
<td></td>
<td>d/t 23/24</td>
<td>234 (21–4012)</td>
</tr>
<tr>
<td>IL-6</td>
<td>10 (0–4012)</td>
<td>11/23</td>
</tr>
<tr>
<td></td>
<td>d/t 4/29</td>
<td>871 (84–1499)</td>
</tr>
<tr>
<td>IL-8</td>
<td>0 (0–1499)</td>
<td>4/29</td>
</tr>
<tr>
<td></td>
<td>d/t 9/24</td>
<td>415 (50–3446)</td>
</tr>
<tr>
<td>TNFα</td>
<td>0 (0–3446)</td>
<td>415 (50–3446)</td>
</tr>
</tbody>
</table>

Table III. Co-correlation of cytokine concentrations

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Endometriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β versus IL-6</td>
<td>-0.44*</td>
<td>0.24 (ns)</td>
</tr>
<tr>
<td>IL-1β versus IL-8</td>
<td>-0.15 (ns)</td>
<td>0.08 (ns)</td>
</tr>
<tr>
<td>IL-1β versus TNFα</td>
<td>-0.76***</td>
<td>0.42*</td>
</tr>
<tr>
<td>IL-6 versus IL-8</td>
<td>0.22 (ns)</td>
<td>0.69***</td>
</tr>
<tr>
<td>IL-6 versus TNFα</td>
<td>-0.56*</td>
<td>0.19 (ns)</td>
</tr>
<tr>
<td>IL-8 versus TNFα</td>
<td>0.11 (ns)</td>
<td>0.08 (ns)</td>
</tr>
</tbody>
</table>

*p = 0.05, Spearman’s correlation test.
**p = 0.01, Spearman’s correlation test.
***p = 0.001, Spearman’s correlation test.
ns = not significant.

Discussion

In both groups, a positive correlation coefficient was obtained between IL-1β and TNFα, although this was of weaker significance in the endometriosis group. Also, in both groups there was no significant correlation between IL-8 and TNFα.

Outcome of IVF

In both groups, the pregnancy rate was 30% (three of 10 patients in each group). No particular differences could be seen in cytokine concentrations when comparing pregnant versus non-pregnant women within the control group (data not shown). Interestingly, within the endometriosis group, the women who became pregnant had about three times higher IL-1β concentrations and at least five times lower IL-6 concentrations than the ones who did not become pregnant (data not shown). However, since only three women in each group became pregnant, the numbers in this study are far too small to allow such comparisons, although these findings are worth studying further.
IL-1β was only slight, both when the median production from all samples was studied and when including only samples with detectable cytokine concentration. On the other hand, the production of IL-6 was about 10 times higher in granulosa cells from women with endometriosis when taking the whole material into account and about four times higher when considering only samples with detectable IL-6 concentrations. These results are in line with the findings in a recent study (Pellicer et al., 1998), which demonstrated higher IL-6 production in granulosa cells from women with endometriosis in comparison to healthy controls. They did not find any significant differences in granulosa cell IL-1β release between the two groups, either. Although very few of the samples in this study had detectable concentrations of IL-8, granulosa cells from women with endometriosis had about five times higher IL-8 concentration taking into account only the positive samples. The finding of significantly elevated concentrations of TNFα in granulosa cell cultures derived from women with endometriosis might be related to the fact that very few samples in the control group had detectable concentrations of this cytokine. Nevertheless, the TNFα concentration was about three times higher in the endometriosis group when considering only detectable samples. In accordance with this finding, increased concentrations of TNFα have earlier been correlated to infertility (Naz et al., 1995), in that significantly higher TNFα concentrations have been demonstrated in the cervical mucus of infertile and idiopathic infertile women compared to fertile controls.

The human oocyte and human cumulus cells have been demonstrated to express the TNFα type II receptor, but not the apoptosis-mediating TNFα type I receptor (Naz et al., 1997). Hence, the finding in this study of significantly increased TNFα production from granulosa cells from endometriosis patients might directly affect the oocyte. It is not known whether the oocyte has receptors for IL-1β, IL-6 and IL-8.

Thus, the increased TNFα production in granulosa cells from patients with endometriosis might be a result of the inflammatory pelvic environment associated with the disease, either directly by diffusion of the inflammatory reagents into the follicles, or, indirectly, by ovary-mediated communication. The reason for no measurable production of some cytokines from some GCC samples is not known, and will be the subject of future study.

Indications were found that GCC from women with endometriosis were less sensitive to HCG than control GCC. The tendency to suppression of cytokine release by 10 IU/ml HCG was stronger in the control group than in the endometriosis group for IL-1β, IL-6, IL-8 and TNFα. These findings are of interest, but need to be confirmed in an extended study. In any event, these results might be related to the decreased number of HCG receptors demonstrated in granulosa cells from women with endometriosis (Kauppila et al., 1982; Rönnberg et al., 1984).

The leukocyte antigen CD45 was present in <1% of the GCC-containing fractions; therefore any contribution to cytokine production by contaminating macrophages must be considered extremely small, using our cell isolation method. It should also be pointed out that very few of the cells in the potential macrophage-containing fractions stained positive for CD45 (data not shown), therefore these fractions cannot be considered to consist of macrophages only. Thus, cytokine production of macrophages is beyond the scope, and was not the aim, of this study.

In a pilot study including four control patients and four endometriosis patients, basal IL-6, IL-8 and TNFα production from SC and macrophage-containing fractions was also investigated. Some of the IL-6 and IL-8 concentration values were higher in the media from potential macrophage-containing wells, indicating that contaminating macrophages indeed were present in these wells and that the cell separation procedure was satisfactory. No obvious differences or trends were, however, observed in this pilot study when comparing patients with endometriosis and the control group, except that IL-8 concentrations in this study were also about five times higher in GCC cultures from the endometriosis group. The singular cell fractions and the macrophage-containing cell fractions had an apparently more heterogeneous morphology than the GCC and thus the cytokine production from these cell groups is probably of mixed origin, therefore they were not included in further studies. For instance, the fractions potentially containing macrophages sometimes also contained GCC and SC that had adhered very rapidly (data not shown), and the SC had a diverse morphology, indicating the presence of cell types other than just singular granulosa cells, possibly also fibroblasts obtained at follicle aspiration. Furthermore, GCC appeared more viable than SC in culture over time. Thus, in this study, investigations were limited to the cytokine production of GCC.

No specific differences in cytokine concentrations or HCG response were observed when comparing women who subsequently became pregnant from IVF versus women who did not within each group. It is recognized, however, that the material is too small for any statistical calculations.

The results of this study suggest that granulosa cells from women with endometriosis have an up-regulated production of all the cytokines IL-1β, IL-6, IL-8 and TNFα in comparison to healthy women. Furthermore, HCG appeared to suppress cytokine release from granulosa cells from both patient groups.

In conclusion, the aberrant TNFα expression in granulosa cells from women with endometriosis might disturb the synchronization of oocyte maturation, ovulation and uterine receptivity and might, at least partly, explain the reduced fertilization rate previously observed for endometriotic women undergoing IVF.

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