Leukaemia inhibitory factor and interleukin 11 levels in uterine flushings of infertile patients with endometriosis

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BACKGROUND: Exact aetiology of infertility in stage I/II endometriosis patients is not known. Interleukin 11 (IL-11) and leukaemia-inhibitory factor (LIF) are factors associated with implantation window in human eutopic endometrium. We decided to test whether there is an altered secretion of these factors, which could explain receptivity defect in patients with minimal endometriosis. METHODS: Uterine flushing and endometrial samples were collected 7–9 days after ovulation (implantation window) from infertile patients with stage I/II endometriosis (n = 14) and fertile, endometriosis-free controls (n = 21). IL-11 and LIF were assessed in uterine flushings in eutopic endometria in all patients by enzyme-linked immunosorbent assay (ELISA). In eutopic endometrium, semiquantitative RT–PCR was performed for LIF and IL-11 mRNA expressions. RESULTS: No statistically significant differences were found in uterine flushing in women with and without endometriosis with regard to IL-11 levels (0.0 pg/ml versus 0.0 pg/ml) and LIF (25.53 pg/ml versus 36.26 pg/ml). These results were confirmed by the results of RT–PCR, where there were also no differences between studied groups. CONCLUSIONS: There is no receptivity defect with regard to LIF and IL-11 secretions by eutopic endometrium in infertile women with endometriosis.

Key words: endometrial receptivity/endometriosis/IL-11/infertility/LIF

Introduction

The problem of infertility affects approximately 80 million people in the world (World Health Organization, 2002). With multiple aetiologies, infertility it is often difficult to diagnose and treat. One of the factors affecting human fecundity is endometriosis (Halis and Arici, 2004). Because endometriosis is encountered in about 30% of women who have problems conceiving, and the treatment options, especially for mild endometriosis, remain questionable at best, it might be prudent to elucidate all the possible mechanisms involved in the pathogenesis of infertility in endometriosis. Although the cause of infertility in stage III and stage IV endometriosis (with deep infiltration and adhesions) is quite obvious, there are also reports linking minimal endometriosis with infertility (Rier and Yeaman, 1997). Some attribute those effects to hormonal imbalances, some to immunological factors (Zeitoun and Bulun, 1999; Szczepańska et al., 2001). With disillusionments about the role of histological assessment of the endometrium and recent advances in genetic technology, more attention has been brought to inherent differences on molecular level encountered in eutopic endometrium of infertile women with endometriosis compared with endometria from healthy, fertile women (Giudice et al., 2002; Halis, 2004). Although few in numbers, these studies might serve as a road map for further research. Because even in IVF–embryo transfer cycles (in a way bypassing problems with ovulation, patency of Fallopian tubes and embryo formation) the implantation rates of patients with endometriosis are very low (Barnhart et al., 2002), it is plausible to assume that the eutopic endometrium of patients with endometriosis might exhibit some molecular defects. Finding differences in concentrations of factors deemed to play a role in endometrial receptivity, within the uterine cavity in infertile patients with minimal endometriosis compared with fertile controls, might explain whether the changes in endometrial cells are responsible for infertility in patients with endometriosis.

Some of the key players in the receptivity of the endometrium are cytokines belonging to interleukin 6 (IL-6) superfamily, namely leukaemia-inhibitory factor (LIF) and IL-11. LIF has been linked with infertility in mouse (Stewart et al., 1992). It is one of few factors that affect the fecundity in all-or-nothing way. When mice were devoid of LIF, thanks to gene knockout techniques, it resulted in complete implantation block. The effect was attributable to endometrium, because blastocysts from LIF-null mice could implant in wild-type mice. Also, in humans, it has been shown that infertile women exhibited low levels of LIF in endometrial flushing and immunohistochemical staining (Kojima et al., 1994; Laird et al., 1997; Mikolajczyk et al., 2003). However, whether or not LIF secretion within the uterine cavity plays a role in decreasing the receptivity of eutopic endometrium in women with endometriosis has not been studied so far.
Another cytokine that has not been studied extensively in women with endometriosis is IL-11. IL-11 is a cytokine with pleiotropic actions on multiple cell lineages (Heinrich et al., 1998). It is involved in modulating the reactions of inflammation and injury, and also it has been linked to decidual reaction in endometrium (Leng and Elias, 1997; Dimitriadi et al., 2002). Also, IL-11 has a potential to inhibit apoptosis, so it might explain the longer life of endometrial cells within the peritoneal cavity in patients with endometriosis (Orazi et al., 1996). There are reports linking IL-11 to increased expression of aromatase which is necessary for estrogen formation, which again favours the development and survival of endometriotic implants (Zhao et al., 1995). Because endometriosis development is attributable to inflammatory reactions, the involvement of IL-11 might be substantial. The only report so far linking IL-11 with endometriosis suggested no association between peritoneal fluid IL-11 levels and endometriosis presence (Gazvani et al., 2000).

The aim of this study was to assess the concentrations of IL-11 and LIF secreted into the uterine cavity in the period of maximal receptivity, i.e. ‘implantation window’, to determine whether eutopic endometrium of infertile women with endometriosis exhibits different characteristics compared with endometria from fertile women, which might in some way explain the pathogenesis of infertility in minimal endometriosis.

Materials and methods
A total of 84 consecutive patients were recruited to this study. Patients were admitted to Division of Reproduction in Poznan during routine infertility workup. The mean duration of infertility was 3.5 years (2–8 years). The mean age of the patients was 26 years (22–34 years). During the routine workup, patients underwent laparoscopy 7–9 days after ovulation, assessed by serial ultrasound follicular tracking. The diagnosis of endometriosis was based on visualization of endometrial lesions found during laparoscopy. In 70% of patients, the diagnosis was also confirmed by histopathologist. Only patients with stage I and stage II endometriosis according to the revised American Fertility Society endometriosis staging (Canis et al., 1996) were considered for further studies, as with higher stages of endometriosis the cause of infertility appears obvious. Also, we wanted to see whether the suspected changes in endometrium are present from the earliest forms of endometriosis. The patients also had ovulation tracking, semen analysis and tubal patency tests. Any anomaly found within those tests excluded the patient from the study group. Finally, a total of 14 women with stage I (n = 10) and stage II (n = 4) endometriosis were analysed. All laparoscopies were performed by a skilled surgeon with over 15 years of experience in detection and treatment of endometriosis.

On the day of operation, before laparoscopy, a uterine flushing was performed according to a protocol described elsewhere (Mikolajczyk et al., 2003). It involves placing a sterile speculum in the vagina, visualizing the cervical os and positioning insemination catheter into the uterine lumen. The catheter is connected to 10-ml syringe filled with 3.5 ml of sterile normal saline. The saline is slowly infused into the uterine cavity and aspirated, and the procedure is repeated a few times to achieve turbulent flow and homogenic distribution of substances within the fluid. The fluid was centrifugated at 3 g for 3 min, immediately frozen and kept until further research. Also, an endometrial biopsy was performed with Pipelle or during hysteroscopy for LIF and IL-11 mRNA assessments.

Twenty-one healthy women aged 24–35 years (mean 24 years) constituted the control group. All women from this group had children; none of them experienced a miscarriage. The indication for diagnostics was suspicion of endometriosis or pelvic pain of unknown origin. Only those without endometriosis and inflammation of the pelvis at the time of diagnostic laparoscopy (sterile fluid samples from cul-de-sac) were considered as controls. None of the women (both in control and endometriosis groups) have used any form of hormonal treatment or ovulation-inducing drugs for at least 3 months before study. All patients signed an informed consent, and the design of the study was approved by local ethical committee.

The assessment of LIF concentration was performed with Bender MedSystems GmbH kit (Vienna, Austria). The detection of LIF was made according to manufacturer’s instructions. Concentration assessment of all samples and controls were performed in duplicate. Hundred microlitres of standard dilutions were added in wells covered with anti-LIF antibody ranging from 3.13 to 200 pg/ml (including negative control) and 100 μl of uterine fluid diluted at 1:1. To each well, we added 50 μl of biotinylated secondary antibody (anti-LIF). This mix was incubated for 2 h in room temperature. After incubation, the wells were washed three times in buffer supplied by Bender MedSystems. Finally, the wells were filled with 100 μl of streptavidin–peroxidase conjugate (DAKO) and incubated for 1 h. The plates were then washed and 100 μl of tetramethylbenzidine (TMB) staining substance was added. After 10 min, the stop solution was added, and the wells were read at 450 nm wavelength in BioTek Instruments spectrophotometer (Winsko, VT, USA). The negative controls were fluids without the primary antibody.

The assessment of IL-11 concentration was performed with Quantikine R&D Systems kit (Minneapolis, MN, USA). The detection of IL-11 was performed according to manufacturer’s instructions. Hundred microlitres of standard dilutions were added in wells covered with anti-IL-11 antibody ranging from 0 to 1000 pg/ml (including negative control) and 100 μl of uterine fluid diluted at 1:1. To each well, we added 200 μl of biotinylated secondary antibody (anti-IL-11). This mix was incubated for 2 h at room temperature. After incubation, the wells were washed three times in 400 μl of buffer supplied by R&D Systems. Finally, the wells were filled with 200 μl of streptavidin–peroxidase conjugate (DAKO) and incubated for 1 h. The plates were then washed and 100 μl of TMB staining substance was added. After 10 min, the stop solution was added, and the wells were read at 450 nm and 540 nm wavelengths in BioTek Instruments spectrophotometer according to manufacturer’s instructions. The negative controls were fluids without the primary antibody.

Concentration assessment of all samples and controls were performed in duplicate.

The semiquantitative RT–PCR assay of LIF and IL-11 mRNA
The endometrial sample (<0.5 cm³) was immediately placed after collection in adequate amount (10 μl/1 mg tissue) of RNAlater Stabilization Reagent from Qiagen (Australia). The tissue samples were kept in –20°C till the extraction of RNA. RNA was extracted from endometrial cells with RNAeasy Protect Mini Kit (Qiagen, Australia) according to manufacturer’s instructions. Reverse transcription reaction was performed with Qiagen OneStep RT–PCR kit. The following oligonucleotide primers were designed from published nucleotide sequences: for LIF (forward: GATGAGTGGAAGATAGAGAGG, reverse: CGTCTTGAATCCCGTCC) and for IL-11 (forward:
GATGAGTGGAGATAGAGAGG, reverse: CGTCTTGATCCCA GTCC). The amplification protocol was as follows: 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (extension). This protocol was repeated in 35 cycles. Each investigation was performed in duplicate. PCR products were visualized by ethidium bromide and a 1.5% agarose gel. Quantification of PCR products was performed with ImageQuant TL software from Amersham (USA) with corrections for background staining. The house-keeping gene was glyceraldehyde-3-phosphatedehydrogenase (GAPDH). Results are presented as LIF/GAPDH and IL-11/GAPDH expression ratios.

**Statistical analysis**

The normality of distribution was assessed using Shapiro-Wilk’s test. Because results for both IL-11 and LIF levels in uterine flushing did not conform to normal distribution, the differences between the groups were assessed using non-parametric Mann–Whitney test, and the analysis of correlations was performed with the Spearman test. Statistical analysis was performed using SigmaStat 3.1 software (Systat Software, Inc., USA). P < 0.05 was considered statistically significant.

**Results**

Table I presents the values of LIF in uterine flushings in infertile women with endometriosis and in fertile controls. There was no statistically significant difference between the levels of LIF in uterine flushing between infertile women with endometriosis compared with controls. In 14.3% of women with endometriosis, we have found undetectable LIF levels in uterine flushing compared with 4.8% in control group.

Table II presents the values of IL-11 in uterine flushings in women with endometriosis and in fertile controls. There was no statistically significant difference in the IL-11 level between those groups. The range for IL-11 levels in uterine fluid was between 0 and 11.21 pg/ml and 0–17.4 pg/ml in infertile women with endometriosis and in controls, respectively. Only in 21% of patients in the endometriosis group and 14% of patients in the control group, we noticed an IL-11 level above 0 pg/ml.

Table III and IV summarize the eutopic endometrial mRNA expression results for LIF/GAPDH and IL-11/GAPDH, respectively. Again, there was no difference in the expression of either cytokine in women with and without endometriosis.

There was also a statistically significant correlation between levels of LIF measured in uterine flushing and the expression of LIF mRNA in endometrium ($r_s = 0.46; P < 0.00005$). No correlation was found between IL-11 levels in uterine flushing and respective mRNA expression results ($r_s = 0.13; P < 0.5$).

**Discussion**

The aetiology of infertility in women with low-stage endometriosis (I/II) remains an enigma. Whether this effect is due to factors found in peritoneal cavity or is due to intrinsic defects of eutopic endometrium found in patients with endometriosis is still unknown (Szczepańska et al., 2001; Szczepanska et al., 2003). However, recent studies using gene-matrix technology have revealed some differences in the expression of many molecules, cytokines and other factors in eutopic endometrium of women with endometriosis compared with fertile women (Giudice et al., 2002). These differences might contribute to aberrations in a fine-tuned programme of endometrial receptivity, which results in infertility. A confirmation of these molecular studies and the assumptions based on them comes from meta-analysis of IVF trials. It has been confirmed that women with endometriosis have similar ovulation and embryo formation rates compared with patients scheduled for IVF treatment due to different reasons (e.g. blocked tubes). However, implantation rates of women with endometriosis are only half of those achieved for other causes of infertility (Barnhart et al., 2002). These results indicate that there might be a receptivity defect within the eutopic endometrium in women with endometriosis that affects fertility regardless of other causes of infertility in endometriosis (adhesions, etc.).

Some of the factors associated with endometrial receptivity and implantation window are IL-11 and LIF. Both belong to

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<th>Table I. Median, 25–75% range and statistical significance for LIF levels in uterine flushing in all groups (results in pg/ml)</th>
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| **Number** | Median | Range (25–75%) | *P*
| Infertile women with endometriosis | 14 | 25.53 | 12.63–43.32 | NS |
| Control | 21 | 36.26 | 14.45–59.32 | – |

LIF, leukaemia-inhibitory factor; NS, non-significant.

*Compared with fertile control.

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<th>Table II. Median, 25–75% range and statistical significance for interleukin 11 levels in uterine flushing in all groups (results in pg/ml)</th>
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| **Number** | Median | Range (25–75%) | *P*
| Infertile women with endometriosis | 14 | 0.48 | 0.39–0.59 | NS |
| Control | 21 | 0.52 | 0.42–0.61 | – |

GAPDH, glyceraldehyde-3-phosphatedehydrogenase; IL, interleukin; NS, non-significant.

*Compared with fertile control.
IL-6 family of cytokines, share the same receptor (gp-130) and therefore might have overlapping functions (Heinrich et al., 1998).

In this study, we have found that infertile women with endometriosis have lower levels of LIF in uterine flushing compared with controls; however, the differences failed to reach statistical significance. These results were confirmed by the assessment of LIF mRNA in RT–PCR reactions, which also, despite a trend to lower expression observed in women with endometriosis, failed to show statistical significance. The statistically significant correlation between the results obtained in uterine flushing and the results of LIF mRNA expression in endometrium confirms the method used for flushing the uterine cavity. These results disagree with those observed by Dimitriadis et al. (2006). According to these authors, immunostaining intensities for LIF in infertile patients with endometriosis have been found to be statistically significantly lower compared with controls. There could be two possible explanations. In our study, we have used two methods that are unbiased by observer. The results of uterine flushing are given in pg/ml and the mRNA expression is depicted as a ratio of LIF/GAPDH. This allows for the precise comparison of two studied groups. Also, in our paper, the number of patients in each studied group was higher (almost 3-fold) compared with study of Dimitriadis. Nevertheless, our study is in general agreement with the paper by Dimitriadis. In some patients with endometriosis, there might be a decreased secretion of LIF, but this defect is not a common feature of patients with endometriosis. A study by Illera et al. (2000) in mouse endometrium found that injection of peritoneal fluid derived from patients with endometriosis to mice’s peritoneal cavity resulted in decreased expression of LIF mRNA in endometrium. One should be very careful about extrapolating the results of studies in mouse onto human subjects.

Dimitriadis et al. (2006) have also found significantly lower staining intensities for IL-11 and IL-11Ra in infertile women with endometriosis compared with fertile controls. There was no staining in glandular epithelium in the endometriosis group, whereas all patients from the control group exhibited positive staining for those factors. In this study, we have found no differences between IL-11 levels in uterine cavity between infertile women with endometriosis and control, because most patients exhibited levels of IL-11 that were below the detection threshold for the kit used. Confirmation of our results comes from studies of IL-11 in peritoneal fluid of women with endometriosis, which also failed to show any significant differences between fertile controls and patients with endometriosis, although the concentration in peritoneal fluid was much higher compared with that observed in uterine flushings (Gazvani et al., 2000). The results of IL-11 mRNA expression also showed no differences in IL-11 expression pattern in endometrium among studied groups. The difference in results of our study and the study of Dimitriadis et al. (2006) might be attributable to posttranslational differences in the expression of IL-11 molecules. It might be speculated that the actual expression of IL-11 molecule in glandular endometrium of infertile women with endometriosis might be suppressed by some unknown factors present in the uterine fluid. Although when one considers the results of experimental studies that showed up-regulation of IL-11 expression after E2 treatment, together with a fact that endometrium in endometriosis possesses aromatase that produces estrogens, lower expression of IL-11 in patients suffering from endometriosis seems a little surprising (Zeitoun and Bulun, 1999; von Rango et al., 2004). Alternatively, because the IL-11 is required for the transformation of the endometrium in the preimplantation phase and von Rango study points to maximal expression of IL-11 in the endometrium during the follicular and early luteal phases, it is possible that at the time of maximal endometrial receptivity (7–9 days after ovulation), there is no need for secretion of IL-11 to the uterine fluid (von Rango et al., 2004).

Recently, three papers have addressed the pregnancy outcome after IVF in patients with endometriosis (Kuivasaari et al., 2005; Suzuki et al., 2005; Witsenburg et al., 2005). They have found that implantation rates in women with stage I/II endometriosis are no different to those achieved in patients with tubal infertility. Dimitriadis compared patients with all stages of endometriosis (Dimitriadis et al., 2006). It is possible that higher stages of endometriosis might influence the hormonal and immunological environments in such way that they alter the receptivity of the eutopic endometrium.

In a review of possible causes of infertility in women with endometriosis, Garrido et al. (2002) have concluded that the infertility encountered in women with endometriosis is more because of follicular growth disturbances and higher rate of embryo development arrest rather than endometrial receptivity defect. Also, studies aimed at specific markers of endometrial receptivity such as integrins and pinopods also have not found any differences between those factors in endometria in women with and without minimal endometriosis (Ordi et al., 2003), although one cannot exclude that in some women with endometriosis, there might be decreased expression of integrins such as alphaVbeta3 (Lessey, 2002).

On the basis of our studies and the review of literature, we postulate that there is no general inherent receptivity defect (at least with respect to IL-11 and LIF) in eutopic endometrium of women with minimal endometriosis. Further studies are needed to elucidate the possible role of other factors found in uterine cavity and peritoneal fluid that could possibly explain decreased fecundity in patients with grade I/II endometriosis.

References


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