Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation

N.Lédée-Bataille1,4, G.Lapréé-Delage1, J.-L.Taupin2, S.Dubanchet1, R.Frydman3 and G.Chaouat1

1INSERM U131, Clamart, 2CNRS, UMR 5540, Bordeaux and 3Service de Gynécologie-Obstétrique et Médecine de la Reproduction, Hôpital Antoine Béclère, Clamart, France

4To whom correspondence should be addressed at: Service de Gynécologie-Obstétrique, Hôpital Antoine Béclère, 157, Rue de la Porte de Trivaux, 92141 Clamart cedex, France. E-mail: Ledeenathalie@aol.com

BACKGROUND: There is strong evidence that locally secreted cytokines control the implantation process and can cause implantation failure. Uterine flushing fluids were analysed to determine their concentrations of leukaemia inhibitory factor (LIF) and tumour necrosis factor (TNF).

METHODS AND RESULTS: We began by flushing the uterine cavities of 33 infertile patients on day 26 of two consecutive cycles. The concentrations of LIF (by enzyme-linked immunosorbent assay) and TNF (by bioassay) were significantly correlated during these cycles ($r = 0.762$, $P = 0.0001$ and $r = 0.822$, $P = 0.001$ respectively) and hence reliable. Then, after a reference flushing of 30 infertile patients, we followed the outcome of their first consecutive cycle of ovarian stimulation, which preceded either IVF or intrauterine insemination. A total of 10 patients became pregnant. The median concentration of LIF was 0 pg/ml (range: 0–177) and of TNF was 0 U/ml (range: 0–6.17) among those who became pregnant, and 203 pg/ml (range: 0–1620) and 2.14 U/ml (range: 0–16) respectively among those who did not. The LIF concentration was significantly lower in the pregnant group ($P = 0.0013$).

CONCLUSION: A low concentration of LIF in the uterine flushing fluid at day 26 was predictive of subsequent implantation. Use of this procedure should increase the number of IVF attempts yielding successful pregnancies and also lead to corrective therapies.

Key words: implantation/leukaemia inhibitory factor/tumour necrosis factor/uterine flushing

Introduction

Implantation remains an unsolved problem in reproductive medicine and is the major factor restricting the success of assisted conception in humans. Only 10–15% of the embryos transferred after IVF lead to the birth of a healthy baby (de Mouzon, 1998). It is generally assumed that approximately two-thirds of implantation failures are imputable to inadequate uterine receptivity or to defects in the essential embryo–endometrium dialogue. The embryo in and of itself is thus probably directly responsible for only one-third of IVF failures (Edwards, 1994; Simon et al., 1998). We have, however, no reliable tools for the routine exploration of uterine receptivity. Every parameter explored in ultrasound assessment has been notable for its poor positive predictive value, and no histological indicators can predict adequate uterine receptivity.

There is now strong evidence that implantation is controlled primarily by locally acting growth factors and cytokines, some regulated by steroids (Giudice, 1994; Chaouat et al., 1995; Chard, 1995; Lessey and Castelbaum, 1995; Tabibzadeh et al., 1995a,b). These steroid hormones may initiate a downstream cascade of molecular events through local paracrine/autocrine molecules, events including the intricately entwined mechanisms of uterine receptivity and preimplantation embryo development.

In 1992 Stewart and colleagues demonstrated the absolute necessity of leukaemia inhibitory factor (LIF) for murine implantation by monitoring the reproductive performance of LIF-knockout mice (Stewart et al., 1992). LIF–/– transgenic mice are fertile (the number of LIF–/– embryos is normal) but the embryos do not implant in the mother; similarly, LIF+/– embryos do not implant in a LIF–/– mother. Both types of embryos do implant in a LIF+ foster mother, and the implantation blockade observed in LIF– mice can be partly corrected by an infusion of recombinant murine LIF in the peritoneal cavity. The role of LIF in implantation has been documented to be as important in the monkey as in mice (Yue et al., 2000).

LIF protein and mRNA are present in the human endometrium only during the luteal phase of the cycle (Charnock-Jones et al., 1994; Arici et al., 1995) and then mainly in the glandular epithelium (Chen et al., 1995; Vogiagis et al., 1996). The epithelial cells express mRNA for LIF receptor components gp190 and gp130 and LIF binding is confined
to the luminal epithelium (Cullinan et al., 1996). Cultured endometrial explants obtained in the luteal phase from infertile women produce less LIF than those from fertile ones, suggesting that, as in mice, reduced maternal LIF concentrations at the time of implantation are associated with a defect in uterine receptivity (Delage et al., 1995). Recently, discrete mutations of the LIF gene have been described in a population of infertile women (Giess et al., 1999). Finally, it has been found that the blastocyst itself expresses gp190 and gp130, which suggests that LIF plays a role in the embryo–endometrium dialogue (Chen et al., 1999).

Tumour necrosis factor (TNF-α) has been detected in the ovariies (Roby et al., 1990), oviduct (Hunt, 1993), preimplantation embryo (Zolti et al., 1991; Sharkey et al., 1995) and endometrium (Hunt et al., 1992; Philippeaux and Piguet, 1993; Tabibzadeh et al., 1995a). All types of endometrial cells (fibroblast, immunocompetent, glandular epithelial and vascular) express TNF-α. This expression is cycle-dependent and occurs mainly in the endometrial gland (Hunt et al., 1992; Philippeaux and Piguet, 1993; Tabibzadeh et al., 1995a; Von Wolff et al., 1999). Increased TNF-α expression has been associated with implantation failure (Hazout, 1995), immunologically-mediated abortion (Giacomucci et al., 1994) and endometriosis (Zhang et al., 1993).

In the present study, we analysed LIF and TNF production in uterine flushing and by endometrial biopsies. The concentrations of LIF and TNF were measured in the flushing liquid by enzyme-linked immunosorbent assay (ELISA) and bioassay respectively. Explant cultures from the endometrial biopsy tissue made it possible to calculate the LIF production index (LPI). Infertile patients (n = 33) underwent uterine flushing on day 26, in the late luteal phase, of two consecutive cycles, to confirm the reproducibility and hence the reliability of the data. Then, after a reference uterine flushing and biopsy with measurements of the LIF and TNF concentrations and determination of the LPI for 30 patients, we followed the outcome of their first consecutive cycle of either IVF or intra-uterine insemination (IUI).

**Materials and methods**

**Patients**

The first part of the study analysed endometrial tissue samples and uterine flushing fluids from 15 women with ovarian failure enrolled in an in-vitro oocyte donation program and from 18 women with idiopathic sterility enrolled in an IVF program. The mean age for the overall group was 33 years (range 25–40). The inclusion criteria for the ovum donation group were the absence of ovarian function, high serum gonadotrophin (FSH >15 IU/l) and low oestradiol levels (<90 pmol/l). The women in the idiopathic sterility group were required to have ovarian functioning, low serum gonadotrophins (FSH <8 IU/l), low oestradiol levels (<50 pmol/l), a normal laparoscopy and a partner whose sperm parameters were normal.

The second part of the study included 30 patients who were evaluated under hormone replacement treatment two months after their laparoscopic exploration for infertility and then followed prospectively. We excluded from the study patients older than 42 years and those patients with a poor ovarian reserve, measured by FSH >8 mIU/ml on day 3 of the cycle. The aetiology of sterility was distributed as follows: 15 idiopathic, seven related to a tubal factor, seven endometriosis, one related to male factors. The 15 women with idiopathic sterility were previously involved in the first part of the study; the second uterine flushing was then retained as the reference one. All the patients subsequently underwent one cycle of ovarian stimulation, completed for 17 patients by IVF and for 13 patients by IUI. We then correlated the immunological data with the reproductive outcome after the first cycle of ovarian stimulation. All patients provided informed consent, and this investigation received the approval of our Institutional Review Board.

**Hormone administration**

All patients received oestradiol–progestin replacement treatment to standardize the data of the endometrial evaluation, performed on day 26, in the late luteal phase of the cycle. They received micronized oestradiol (Provames; Cassenne, Paris, France) (4 mg daily on days 1–26). Micronized progesterone (Utrogestan; Besins-Isovesco Pharmaceuticals, Paris, France) (300 mg daily) was administered vaginally on days 14–26 (Ludwig and Driedrich, 2001). This protocol is applied in our centre in the placement of frozen embryos (de Ziegler et al., 1994).

**Uterine flushing and endometrial biopsy**

Both the biopsy and uterine flushing took place on day 26. The in vivo method we used for uterine flushing is neither painful nor invasive. The absence of pain is an essential feature of this exploration since pain may enhance cytokine production and thereby produce inconsistent results. We therefore adopted the standard method previously described, which involves use of an 8 Fr paediatric Foley to instill 10 ml of saline water. Instead, we gently inserted into the uterus an embryo transfer catheter (Frydman catheter; CDD Laboratories, Paris, France) connected to a 5 ml syringe. We then instilled, twice, 1 ml of saline water, which was immediately and gently aspirated. The low quantity and lack of viscosity of the saline water instilled minimized leakage.

The volume of fluid retrieved from the uterus varied, as it did in studies for pp14 assays (Li et al., 1993; Mackenna et al., 1993). Therefore, like those authors, we considered only the cytokine concentration, but not the amount of cytokine harvested. We did assess the volume of fluid after centrifugation; the liquid was stored at 4°C until the assays in mini-vials containing 0.05% Tween-20.

The endometrial biopsy was performed with the standard technique and a standard Cornier pipelle (CCD Laboratories). All procedures were performed by a single operator and the samples were immediately processed in the laboratory. One sample of endometrium was embedded in paraffin for histological dating according to the Noyes criteria (Noyes et al., 1950). Another endometrial sample was cultured as described below.

**Culture of tissue samples**

The explant culture protocol has previously been described in detail (Delage et al., 1995). Briefly, endometrial samples were cultured in 20 ml culture vials (Polylabo, Strasbourg, France) containing Roswell Park Memorial Institute 1640 culture medium (Gibco BRL; Life Technologies, Paris, France) supplemented with 10% heat-inactivated fetal calf serum (Dominique Sutschler SA, France), 1% antibiotics (Peni Strep; Gibco) and non-essential amino acids (Gibco) and then incubated in a 5% CO₂ humid incubator at 37°C. LIF accumulation in the culture medium was determined on days 1 and 5 after the culture began: 0.5 ml was collected at the same time and transferred to mini-vials containing 0.05% Tween-20. The aliquots of culture supernatants were kept at 4°C until analysis. The LPI was calculated as the ratio of the concentration of LIF on day 5 over the concentration
of LIF on day 1, determined by ELISA in the aliquots of culture supernatants.

Cytokine assays
The ELISA used for LIF quantification has been described previously (Taupin et al., 1997). The two monoclonal antibodies used in the system are known not to interfere with ligand-binding to the receptor; therefore, the test is not affected by the presence of soluble receptors in the samples. The threshold of detection was calculated by adding 2 SD to the mean of six blank wells and never exceeded 25 pg/ml. Results are expressed as a concentration (pg/ml). Intra-assay coefficients of variation were 4.45, 3.55 and 4.59 respectively, for LIF concentrations of 80, 400 and 1000 pg/ml and inter-assay coefficients of 4.26, 3.42 and 1.02. For the purpose of calculation, samples containing undetectable levels of LIF (i.e. <25 pg/ml) were assumed to contain 0 pg/ml LIF.

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 does not distinguish between the α and β forms of TNF, but measures all, and only, the bioactive TNF present in the samples (Wang et al., 1985). Results are expressed in units (U), one unit corresponding to the amount of TNF required for half maximal cell lysis.

Statistical analysis
Because we could not confirm the normal distribution of the data, results are expressed as their median values with their range. The statistical analysis used a non-parametric method, the Mann–Whitney U-test, to compare pregnant and non-pregnant patients and the Spearman correlation test for the correlation between cytokines (LIF and TNF) at the same point for two consecutive cycles and for distinct cytokines. A P value of < 0.05 was considered significant.

Results
Correlations between LIF and TNF concentration during two consecutive cycles
The correlation between the LIF concentrations in fluid flushed from the uterine cavity on day 26 of two consecutive cycles for 33 patients was statistically significant ($r = 0.762, P = 0.0001$) (Figure 1). This correlation was determined for 15

patients without ovarian activity in our ovum donation program ($r = 0.882, P = 0.001$) and for 18 patients with idiopathic sterility ($r = 0.616, P = 0.0073$). We also assessed the correlation between the TNF concentrations in the same fluid samples for 27 patients: it was also statistically significant ($r = 0.822, P = 0.001$) (Figure 2). This correlation involved 13 patients without ovarian activity ($r = 0.909, P = 0.016$) and 14 with idiopathic sterility ($r = 0.743, P = 0.074$).

Pregnancy outcome and LIF and TNF concentrations in uterine flushing fluids
Ten of the 30 patients became pregnant during the first cycle of stimulation after the uterine flushing (Table I). The women who became pregnant and those who did not were comparable in age, FSH level on day 3 of the cycle and duration of infertility. In the pregnant group, seven underwent IUI (five for idiopathic sterility, one tubal and one endometriosis-related) and three IVF (one tubal, one idiopathic and one for endometriosis-related). In the other group, six underwent IUI (five for idiopathic sterility and one tubal) and 14 IVF (four idiopathic, four tubal, five endometriosis-related and one male-factor). The failures in this group could not be explained by a lack of oocytes or by poor embryo quality, since nine oocytes, on average, were retrieved for each patient and a mean of six good quality embryos obtained after IVF. The characteristics of the two groups are summarized in Table I.

The median LIF concentration measured in the uterine flushing fluid of the women in the pregnant group was 0 pg/ml (range: 0–177). The median LIF concentration in the other group was 203 pg/ml (range: 0–1620). This difference was statistically significant ($P = 0.0013$). LIF was not detectable in the fluid of six patients who became pregnant (60%), compared with three in the group that did not (15%).

The median TNF concentration in the uterine flushing fluid of the pregnant group was 0 U/ml (range: 0–6), and only one of 10 patients had detectable TNF. The median TNF concentration measured in the other group was 2.1 U/ml.

Figure 1. Concentration of LIF (determined by ELISA) in two separate flushings of the uterine cavity performed on day 26 of two consecutive cycles among 33 patients. Eleven patients had no detectable LIF in either flushing. The two LIF measurements were significantly correlated ($r = 0.762, P = 0.0001$).

Figure 2. Concentration of TNF (measured by bioassay) in two separate uterine flushings performed on day 26 of two consecutive cycles among 27 patients. Eighteen patients had no detectable TNF in either flushing. The two TNF measures were significantly correlated ($r = 0.822, P = 0.001$).
Discussion

A non-invasive technique enabled us to determine that a low LIF concentration in the fluid from uterine flushing in the late luteal phase is predictive of implantation.

We began by confirming that the uterine flushing technique is reproducible and that the results are therefore reliable. The difference in the correlation between women receiving oocyte donations and those with idiopathic sterility may suggest greater variability among women with ovarian activity than among those without, despite the standardizing conditions induced by the oestrogen–progestin replacement therapy.

Laird et al. previously used uterine flushing to detect endoluminal LIF secretion during the late luteal phase (Laird et al., 1997). They used a different method to flush the uterine cavity, but that alone is unlikely to account for the substantial differences in the amount of LIF detected and in the interpretation of the results. They reported a mean LIF concentration of 8.1 ± 3.3 pg/ml in the late luteal phase among fertile patients and undetectable LIF in the flushing fluid of infertile patients. The limit of detection reported for the LIF ELISA was 2 pg/ml. They considered the presence of LIF in flushing fluid to be a good result. Our results are dramatically different. We have found LIF in uterine flushing fluid to be associated with a poor prognosis. This discrepancy may be partially explained by the different ELISA assays used in their study (ELISA from Amersham Canada) and ours. Human LIF is known to be highly glycosylated, and sugar moieties represent as much as 50% of the total weight of the naturally produced protein. Several peptide epitopes may therefore be masked, and the protein may not be detectable with certain antibodies. We have previously reported that commercially available ELISA kits have found LIF in uterine flushing fluids (P = 0.0013). They did not differ for the TNF concentration or the in-vitro LPI.

Comparison of the two groups used the non-parametric Mann–Whitney U-test. The two groups were comparable in age, basal FSH level and duration of infertility. The groups differed significantly in the LIF concentration (pg/ml) found in uterine flushing fluids (P = 0.0013). They did not differ for the TNF concentration or the in-vitro LPI.

Nonetheless, the difference between the groups in TNF concentration was not statistically significant (P = 0.142).

**LPI and histological dating results**

No difference was observed in the LPI between the pregnant (5.4) and the non-pregnant (4.6) patients. The mean LPI was >3 in both groups, so it can be considered normal. The LIF concentration in uterine flushing fluid (a direct ex-vivo test) was not correlated with the LPI (an in-vitro method) (r = −0.436, P = 0.1).

According to the Noyes criteria (Noyes et al., 1950), all the endometrial samples were in phase with the theoretical timing of the uterine assessment. There was no histological difference between those who became pregnant and those who failed to conceive.

**Table I. Patients’ characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Pregnant group (n = 10)</th>
<th>Non-pregnant group (n = 20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.5 (28–41)</td>
<td>32 (27–41)</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>5.7 (3.9–8)</td>
<td>5.4 (3–8)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>3.5 (2–8)</td>
<td>4 (2–7)</td>
<td>NS</td>
</tr>
<tr>
<td>LIF concentration in flushings (pg/ml)</td>
<td>0 (0–177)</td>
<td>203 (0–1620)</td>
<td>0.0013</td>
</tr>
<tr>
<td>TNF concentration in flushings (U/ml)</td>
<td>0 (0–6.1)</td>
<td>2.1 (0–16.1)</td>
<td>NS</td>
</tr>
<tr>
<td>LPI</td>
<td>5.4 (1.6–12.8)</td>
<td>4.6 (2–10)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are medians with the range in parentheses.

LPI = LIF production index; NS = not significant.

Thirty patients were followed prospectively after an endometrial biopsy and a reference flushing during the first ovarian stimulation cycle. Ten became pregnant. A P value < 0.05 is considered significant.

In a previous report by our group (Delage et al., 1995) the LPI was interpreted as an indicator of the potential for implantation and was assessed by an in-vitro method. The LIF concentration in uterine flushing fluid, on the other hand, reflects in vivo the inflammatory state of the uterine cavity during the implantation window. We previously reported that the LPI was lower in infertile than fertile women: LPI <3 appears to signal a poor prognosis for conception. In our study, the prognosis was good for both groups, with a median LPI in the group that became pregnant of 5.4 (1.6–12.8) and in the group that did not of 4.6 (2–10).

LIF is a key cytokine in the implantation process. Maternal expression of LIF is essential for implantation in the mouse (Stewart et al., 1992) and monkey (Yue et al., 2000) and most authors consider that it may be almost equally important in humans. These aspects might be more than simply quantitative, since the complex organization of the LIF gene results in the expression of three independently regulated LIF transcripts, LIF-D, LIF-M and LIF-T. Haines and colleagues have demonstrated that intracellular and extracellular LIF proteins can have distinct cellular activities that are mediated by different signalling pathways (Haines et al., 2000). Intracellular LIF activity, unlike extracellular LIF protein, is independent of receptor-mediated signalling.

Theoretically, a protein translated by LIF-T encoding mRNA
is intracellular and is thus not secreted and not detectable, while protein translated by LIF-M encoding mRNA remains attached to the matrix and also should not be detectable. We therefore assume that the protein translated by the LIF-D transcript is what we detected in the uterine flushing fluid. However, ELISA does not allow us to determine which isoform is present in flushin fluid. Three heterozygous mutations of the LIF gene have been reported in infertile women (Giance et al., 1995). The consequences of these mutations on the activity of the protein are still unknown, however two of the three mutations are silent. Expression of LIF-D and -M transcripts is up-regulated in response to the pro-inflammatory cytokines IL1b and TNF. We can therefore assume that the excess LIF detected in the lumina signals inflammation in the uterine cavity. It is now known that the beginning of the implantation process can be compared with an inflammation-like reaction that is rapidly down-regulated. An excess of pro-inflammatory cytokines at this stage could have the same deleterious effects on embryo survival as it does on fetal survival in an established pregnancy. This phenomenon has been shown in mice.

Another point that remains unclear is the direction of the LIF secretions produced from the polarized uterine epithelium. Our results suggest that secretion in the apical direction towards the uterine lumen is harmful for implantation. Secretion towards the basal cells, on the other hand, seems necessary for implantation: LIF action on the uterine stroma, particularly in the basal cells, on the other hand, seems necessary for implantation. Hogan, M.M. and Vogel, S.N. (1988) Production of tumor necrosis factor by LIF gene have been reported in infertile women (Giess et al., 1995; Sharkey, 1995; Nachtigall et al., 1996).

This study suggests that uterine flushing may detect the unresponsive uterus before assisted reproduction treatments and, in the future, help to verify the normalization of cytokine concentrations after specific therapy to improve uterine receptivity before ovarian stimulation. A better comprehension of cytokine profiles before and during implantation should help us to define strategies that enhance implantation rates after embryo transfer and therefore increase the number of IVF attempts that lead to successful pregnancies.

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References


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