Analysis of intra-uterine cytokine concentration and matrix-metalloproteinase activity in women with recurrent failed embryo transfer

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BACKGROUND: In all IVF programmes, some patients fail to achieve an ongoing pregnancy, even after numerous embryo transfer procedures. An unfavourable environment within the uterus might be a contributory factor to such recurrent implantation failure. This question was addressed by measuring cytokine concentrations and matrix metalloproteinase activities in fluid derived from uterine irrigation of such patients. METHODS AND RESULTS: The uterine cavities of 22 patients who had previously undergone embryo transfer of at least 10 embryos without ongoing pregnancy were irrigated during the luteal phase. The resultant fluid was assayed for the concentration of interleukin (IL)-1beta, tumour necrosis factor (TNF)-alpha, interferon (IFN)-gamma, leukaemia inhibitory factor (LIF), IL-10 and matrix metalloproteinase (MMP) -2 and -9 activity. The results were compared with those of a control population of women known to be previously fertile (n = 16) and also with women with recurrent spontaneous abortion (n = 13). In the recurrent implantation failure group, the MMP score and IL-1beta concentration were significantly higher than those in the control group, whereas concentrations of IFN-gamma and IL-10 were significantly lower. CONCLUSIONS: In IVF patients with recurrent implantation failure, an altered pattern of intra-uterine cytokine concentration and MMP activity was observed.

Key words: implantation failure/interleukin-1beta/interleukin-10/matrix metalloproteinase/uterine flushing

Introduction

Despite continuing advances in assisted reproductive technology (ART), all IVF programmes include patients who fail to become pregnant despite numerous embryo transfer procedures. Whilst it is well known that much of the failure of embryo implantation may be explained by chromosomal abnormalities, it has been postulated that in some patients there may be a problem with uterine receptivity. This, hypothetically, could be at the level of an unfavourable intra-uterine milieu or an endometrial lining that is hostile to embryonic implantation.

In natural pregnancies, the developing embryo usually enters the uterine cavity at the morula stage and spends 2-3 days within the cavity prior to implantation at the blastocyst stage. Successful implantation therefore depends upon appropriate intra-uterine conditions as well as a receptive endometrium. In the case of embryo transfer following IVF or ICSI, embryos are commonly transferred into the uterine cavity at day 2 or 3, and spend 4 or 3 days respectively in the uterine cavity prior to implantation. The difficulties encountered by many IVF units in establishing efficient extended in-vitro blastocyst culture systems reinforces the importance of the extra-embryonic environment during this pre-implantation phase.

Substantial published data exist relating to possible factors involved in successful embryo implantation, both in animal models and in humans (Edwards, 1995; Bischof, 2000). Such studies have implicated a variety of cytokines (Lopata, 1996; Rice and Chard, 1998; Sharkey, 1998; Simon et al., 1998), prostaglandin-associated enzymes (Bonventre et al., 1997; Lim et al., 1997), matrix metalloproteinases (MMP) (Bischof et al., 1994), adhesion molecules (e.g. integrin beta3; Lessey et al., 1994, 1995; Lessey, 1997, 2000, 2002; Illera et al., 2000; Damario et al., 2001), cell surface mucins (e.g. MUC-1), altered cellular populations and pinopode formation (Martel et al., 1991; Bentin-Ley, 2000; Nikas, 2000; Nardo et al., 2002) in the implantation process. Despite this wealth of information, the relative contribution of these various factors to successful implantation remains unresolved. What can be stated is that the process requires a number of facilitatory events which include
maternal immunological tolerance of the semi-allogenic embryo.

A number of reports have implicated altered ratios of T-helper (Th) 1-type lymphocytes and changes in cytokine concentration with unexplained recurrent miscarriage (Hill et al., 1995; Makhseed et al., 1999; Raghupathy et al., 1999, 2000; Jenkins et al., 2000; Lim et al., 2000). However, these studies have generally focused on cytokine mRNA levels rather than the actual protein concentrations within the uterine cavity.

In the present study the intra-uterine concentrations of a number of cytokines thought to be important for embryo implantation were analysed. In addition, MMP activity was assayed, as these proteases are thought to be intimately involved in the implantation process. The decision was made to analyse women on the authors’ IVF programme who had failed to achieve an ongoing pregnancy despite the transfer of at least 10 embryos. It was reasoned that in some of these women, there might be factors other than intrinsic problems with embryo quality that were contributing to their failure to achieve an ongoing pregnancy. The method used to assess cytokine and MMP concentrations within the uterine cavity was irrigation (flushing) followed by analysis of the resultant fluid. This method has been shown previously to provide a reproducible means of assaying both MMPs (Laird et al., 1999) and cytokines (Licht et al., 1998). Similar analyses were performed on two other groups of patients as controls. The first control group included multiparous women undergoing surgical reversal of sterilization; the reproductive history of this group suggested that previously they had not experienced any significant problem with embryonic implantation. The second control group included women with a history of recurrent spontaneous abortion; for these patients it was reasonable to suggest that the conditions within their uterus were facilitatory for early implantation events, with problems arising later in pregnancy.

The cytokines monitored in this preliminary study were interleukin (IL)-1beta, tumour necrosis factor (TNF)-alpha, interferon (IFN)-gamma, leukaemia inhibitory factor (LIF) and IL-10 because of their proposed roles in embryonic implantation. The IL-1 receptor is expressed on the endometrial surface epithelium throughout the menstrual cycle (Simon et al., 1993), and human pre-implantation embryos have been shown to express mRNA for IL-1beta (Krussel et al., 1998). Embryonic secretion of IL-1beta is thought to be important to the implantation process. However, high levels of this cytokine have also been observed in association with intra-uterine devices (IUD) and inflammatory responses (Dechaud et al., 1998); therefore, it is possible that high levels of this cytokine could be detrimental to successful implantation. In conclusion, the precise role of IL-1beta in implantation remains uncertain as gene knockout mice not expressing the IL-1beta gene are fertile (Abbondanzo et al., 1996).

TNF-alpha is produced by a number of cell types (including macrophages) and is associated with inflammatory responses. IFN-gamma produced largely by T lymphocytes and natural killer (NK) cells is a marker for activation of these cell types. IL-10 may play a role in the suppression of immune responses and therefore could potentially be important in maternal immunological tolerance of the embryo (Trautman et al., 1997; Piccinni et al., 2000; Clark and Croitoru, 2001). In addition, LIF was assayed as gene knockout studies have demonstrated a pivotal role for this cytokine in murine embryonic implantation (Stewart et al., 1992).

Materials and methods

Patients

Patients were recruited from the outpatient department. Those with recurrent implantation failure were categorized as patients who did not have an ongoing pregnancy after 10 or more embryos had been transferred, without possible aetiological factors such as parental chromosomal abnormalities, antiphospholipid antibodies and intra-cavitary uterine leiomyomata. The primary indication for IVF/ICSI in these women included unexplained infertility, endometriosis and male factor infertility. The aim was to obtain these samples during the putative window of receptivity for implantation (i.e. mid-luteal phase). Although 26 samples were collected for this study, four were eliminated for inappropriate dating by endometrial biopsy, as described below.

As controls, samples were taken from multiparous women with a history of tubal sterilization who were undergoing surgical tubal anastomosis. A number of these women became pregnant while the study was in progress. In the control group, 16 samples were obtained in the luteal phase and nine in the proliferative phase for comparative purposes. In addition, a group of women with a history of unexplained recurrent spontaneous abortion was studied; these included 13 patients categorized as having three or more consecutive spontaneous abortions without a live birth and who had been screened for thyroid function, parental chromosomal abnormalities, antiphospholipid antibodies, hereditary thrombophilias and infectious diseases. For patients with recurrent miscarriage, the level of serum progesterone was used to confirm that samples had been collected in the luteal phase.

This study was approved by the Research and Ethics committees at the Royal Women’s Hospital. Samples were collected only from patients who gave informed consent.

Uterine cavity irrigation

A bivalve speculum was inserted into the vagina and a flexible infant feeding tube (2.7 mm diameter) was introduced into the uterine cavity through the cervical canal. A syringe was then used to instil 5 ml of sterile saline into the uterine cavity. The uterine contents were aspirated quickly and collected without contamination by vaginal fluid. Samples were centrifuged at 1000 °C. Following uterine irrigation, all patients in the recurrent failed embryo transfer group underwent hysteroscopic examination of their uterine cavity; endometrial biopsies were performed to confirm secretory-phase endometrium.

Enzyme-linked immunosorbent assay (ELISA) of cytokines

A quantitative sandwich enzyme immunoassay technique was used in accordance with the manufacturer’s protocol. Quantitative ELISA kits for detecting IL-1beta, TNF-alpha, IFN-gamma, and IL-10 were obtained from R&D systems (Minneapolis, MN, USA). The limits of detection for these cytokines by ELISA were <1, 4.4, 8, 8 and 3.9 pg/ml respectively. None of the samples examined had a cytokine level >1000 pg/ml. Unfortunately, not all cytokines were measurable in all
samples due either to insufficient volume or to a limited number of ELISA assays being performed.

**Gelatin zymography measurement of MMP-2 and MMP-9**

Gelatin zymography was used to monitor picogram levels of MMP-2 and MMP-9, according to a published method (Fridman et al., 1992), albeit slightly modified (Kleiner and Stetler-Stevenson, 1994). Briefly, gelatin at a final concentration of 1 mg/ml was incorporated into the running polyacrylamide gel containing 10% acrylamide (Bio-Rad, CA, USA), 25% Tris buffer (1.5 mol/l, pH 8.8), 0.4% sodium dodecyl sulphate (SDS; ICN, Ohio, USA), 0.3% ammonium peroxodisulphate (APS; ICN) and 0.1% N,N,N,N-tetramethylethylenediamine (TEMED; ICN). The stacking gel containing 2% acrylamide, 25% Tris buffer solution containing 5.8% Tris±HCl, 1.7% Tris base, 0.1% NaN3, 0.7% sulphate (SDS; ICN, Ohio, USA), 0.3% ammonium peroxodisulphate (APS; ICN) and 0.1% TEMED was placed on top of the running gel. The same volume of sample buffer (Tris 0.5 mol/l, pH 6.5), 0.4% SDS, 0.4% APS and 0.1% TEMED was loaded into wells and the proteins were electrophoresed for ~1 h at 200 V. After electrophoresis, the gels were washed five times for 5 min in a Tris-based solution consisting of 3% Triton X-100 (ICN). Gels were then washed three times and incubated for 48 h at 37°C in a solution containing 5.8% Tris±HCl, 1.7% Tris base, 0.1% NaN3, 0.7% CaCl2·2H2O and 5% 100 μmol/l ZnCl2. After incubation, the gels were stained for 6 h with 0.1% Coomassie brilliant blue.

The presence of gelatinases was confirmed by their inhibition using EDTA and p-phenanthroline (data not shown). Other MMPs (e.g. MMP-1, -3 and -7), which can be detected using casein zymography including 1 mg/ml casein were not normally detected in the uterine irrigation fluid.

**Quantitation of MMPs on zymograms**

Although zymograms can be semi-quantitated using densitometric analysis (Kleiner and Stetler-Stevenson, 1994; Salamonsen et al., 1997), this is accurate only at picogram levels of MMPs when the bands on the zymograms are faint. Analysis of bands results in underestimation of levels. Because of the very high amount of gelatinase activity in many of the samples analysed, an alternative semi-quantitative technique was used. The neat samples of flushing fluid were diluted 40-fold before running gels for scoring.

For each patient, gelatinolytic activity was estimated when seen as clear bands against a blue background. The individual bands revealed the presence of various molecular weight enzymes and bands identified as proMMP-2, active MMP-2, proMMP-9, active MMP-9 and dimeric MMP-9, the intensities of which were individually graded by visual inspection on a scale of 0 (no band) to 5 (very strong) and summarized for the total score. All analyses were performed by two independent observers who were blinded to the sample type. A total MMP score for each sample was achieved by adding the scores for each band in individual lanes. Inter-assay variability was assessed as 10.3%. Any gel on which the intensity of the bands in the standard sample was not consistent was repeated.

**Protein assay**

Protein concentrations in the irrigation fluid were monitored using the Bio-Rad Detergent Compatible Protein Assay (Bio-Rad) according to the product manual.

**Statistical analysis**

Data were analysed using the Wilcoxon–Mann–Whitney U-test, Welch’s unpaired t-test and Student’s t-test where appropriate. For the statistical analysis, P < 0.05 was considered significant. Pearson’s correlation tests were performed using Microsoft EXCEL.

**Results**

When samples were obtained during the luteal phase, the aim was to achieve this during the putative window of uterine receptivity for implantation. The mean (±SD) for day of sampling was day 21.1 ± 2.2 (n = 16) for the control samples, day 22.0 ± 2.7 (n = 22) for the patients with implantation failure, and day 22.2 ± 3.7 (n = 12) for the patients with recurrent miscarriage (Table I).

The control population of multiparous women undergoing surgical reversal of sterilization showed a low MMP score, and low levels of IL-1beta, TNF-alpha and IFN-gamma in the luteal phase as well as in the proliferative phase (Table I). No sample in the luteal phase contained >10 pg/ml IL-1beta. LIF

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**Table I.** Matrix metalloproteinase (MMP) score and cytokine concentrations in irrigation fluid of patients with recurrent implantation failure on unexplained recurrent miscarriage, and in control patients. Values are mean ± SD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Day in menstrual cycle</th>
<th>MMP score</th>
<th>IL-1beta (pg/ml)</th>
<th>TNF-alpha (pg/ml)</th>
<th>IFN-gamma (pg/ml)</th>
<th>LIF (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
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<tbody>
<tr>
<td>Control group</td>
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<tr>
<td>Luteal phase</td>
<td>33.6 ± 3.9</td>
<td>21.1 ± 3.2</td>
<td>0.5 ± 1.3</td>
<td>2.15 ± 1.82</td>
<td>9.31 ± 6.73</td>
<td>16.28 ± 12.69</td>
<td>15.32 ± 26.54</td>
<td>7.24 ± 3.95</td>
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<tr>
<td>(n = 16)</td>
<td>16</td>
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<td>(n = 16)</td>
<td>(n = 13)</td>
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<tr>
<td>Proliferative phase</td>
<td>35.9 ± 5.6</td>
<td>10.3 ± 3.6</td>
<td>1.4 ± 1.6</td>
<td>6.21 ± 10.35</td>
<td>7.96 ± 5.82</td>
<td>13.73 ± 8.98</td>
<td>4.50 ± 2.65</td>
<td>5.27 ± 2.36</td>
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<td>(n = 9)</td>
<td>9</td>
<td>(n = 9)</td>
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<td>(n = 9)</td>
<td>(n = 7)</td>
<td>(n = 6)</td>
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<tr>
<td>Recurrent implantation failure group</td>
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<tr>
<td>Luteal phase</td>
<td>35.0 ± 3.6</td>
<td>22.0 ± 2.7</td>
<td>5.0 ± 3.1</td>
<td>80.57 ± 160.21</td>
<td>21.69 ± 35.16</td>
<td>1.65 ± 2.38</td>
<td>5.86 ± 4.25</td>
<td>1.27 ± 2.63</td>
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<tr>
<td>(n = 22)</td>
<td>22</td>
<td>(n = 22)</td>
<td>(n = 22)</td>
<td>(n = 22)</td>
<td>(n = 22)</td>
<td>(n = 22)</td>
<td>(n = 15)</td>
<td>(n = 15)</td>
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<tr>
<td>Unexplained recurrent miscarriage group</td>
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<tr>
<td>Luteal phase</td>
<td>31.8 ± 5.0</td>
<td>22.2 ± 3.7</td>
<td>0.0 ± 0.0</td>
<td>2.66 ± 2.22</td>
<td>18.57 ± 25.73</td>
<td>15.32 ± 20.56</td>
<td>5.51 ± 5.19</td>
<td>5.75 ± 2.70</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>13</td>
<td>(n = 12)</td>
<td>(n = 7)</td>
<td>(n = 9)</td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
</tr>
</tbody>
</table>

*P < 0.05 versus control group (luteal phase and proliferative phase) and the unexplained recurrent miscarriage group.

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and IL-10 concentrations in the control group were 15.32 ± 26.54 and 7.24 ± 3.95 pg/ml in the luteal phase. The mean age of the control patients was 33.6 ± 3.9 years in the luteal phase, and 35.9 ± 5.6 years in the proliferative phase.

The patients in the unexplained recurrent miscarriage group also showed low levels of MMP score, IL-1beta, TNF-alpha and IFN-gamma (Table I).

Patients in the recurrent implantation failure group showed significantly higher levels of MMPs and IL-1beta than those in the control group (Table I). When the irrigation fluid was diluted 40-fold for scoring, some 54.5% of women (12/22) showed MMP scores >5 in the recurrent implantation failure group, compared with 6% (1/16) in the control luteal group. A detailed analysis of each MMP band showed that the total score of MMP-9 was significantly higher than the total score of MMP-2 (Table II). For samples with a total MMP score >5, the individual scores of active MMP-9 and total MMP-9 were significantly higher than those of active MMP-2 and total MMP-2 (Table II). The score of proMMP-9 also tended to be higher than that of proMMP-2, although the difference was not significant.

In the recurrent implantation failure group, 14 of 22 samples showed an IL-1beta level >10 pg/ml, and nine samples showed a level >30 pg/ml, whereas in the control group (luteal phase) no sample showed a level >10 pg/ml. Some of the patients in the recurrent implantation failure group showed very high level of IL-1beta (Figure 2b). The MMP score was significantly correlated (r = 0.595; P = 0.0033) with IL-1beta in the recurrent implantation failure group (Figure 3a), but no correlations were detected between MMP score and protein concentration, nor between IL-1beta and protein concentration (Figure 3b and c). This implies that, in the recurrent implantation and MMP-9 (92 kDa)
active MMP-9 (87 kDa)
proMMP-9 (180 kDa)
dimeric MMP-9 (180 kDa)
active MMP-2 (66 kDa)
proMMP-2 (72 kDa)

Table I. Matrix-metalloproteinase (MMP) scores in patients with recurrent implantation failure following embryo transfer. Lane 1: Standard from the supernatant of BHK9 breast carcinoma cell culture. Lanes 2, 3, 4, 5, 6, 7 and 8: Samples that were scored 1, 12, 0, 3, 7, 4 and 0 respectively. Lane 2: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0. Lane 3: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 5, activeMMP-9: 2, dimericMMP-9: 4. Lane 4: proMMP-2: 0, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0. Lane 5: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 2, activeMMP-9: 0, dimericMMP-9: 0. Lane 6: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 3, activeMMP-9: 1, dimericMMP-9: 2. Lane 7: proMMP-2: 1, activeMMP-2: 0, proMMP-9: 2, activeMMP-9: 2, activeMMP-9: 0, dimericMMP-9: 1. Lane 8: proMMP-2: 0, activeMMP-2: 0, proMMP-9: 0, activeMMP-9: 0, dimericMMP-9: 0. The details of scoring are described in the Materials and methods.

Table II. Analysis of the individual matrix metalloproteinase (MMP) bands in patients with recurrent implantation failure. Values are mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Pro MMP-2</th>
<th>Active MMP-2</th>
<th>Total MMP-2</th>
<th>Pro MMP-9</th>
<th>Active MMP-9</th>
<th>Dimeric MMP-9</th>
<th>Total MMP-9</th>
<th>Total MMP score</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients with recurrent implantation failure (n = 22)</td>
<td>1.4 ± 1.2</td>
<td>0.1 ± 0.3</td>
<td>1.5 ± 1.3</td>
<td>1.9 ± 1.4</td>
<td>0.4 ± 0.7</td>
<td>1.2 ± 1.1</td>
<td>3.5 ± 2.9</td>
<td>5.0 ± 3.1</td>
</tr>
<tr>
<td>Patients with a total MMP score &gt;4 (n = 12)</td>
<td>1.8 ± 1.3</td>
<td>0.1 ± 0.3</td>
<td>1.9 ± 1.5</td>
<td>2.7 ± 0.8</td>
<td>0.8 ± 0.8b</td>
<td>1.9 ± 1.0</td>
<td>5.3 ± 2.6c</td>
<td>7.3 ± 2.0</td>
</tr>
</tbody>
</table>

Total MMP-2 = pro MMP-2 + active MMP-2.
Total MMP-9 = pro MMP-9 + active MMP-9 + dimeric MMP-9.
\( ^a P < 0.05 \) versus total MMP-2.
\( ^b P < 0.05 \) versus active MMP-2.
\( ^c P < 0.05 \) versus total MMP-2.
(Willcoxon--Mann--Whitney U-test, Welch’s unpaired t-test and Student’s t-test)
ation failure group, the high levels of MMP score and IL-1beta were independent of protein concentration in the irrigation fluid.

There was a tendency for the concentration of TNF-alpha in the recurrent implantation failure group to be higher than that in the control group, but this was not statistically significant (Table I). The concentration of IFN-gamma in the recurrent implantation failure group was significantly lower than that in the control group (Table I; Figure 2c).

There was no statistically significant difference in the concentration of LIF between the implantation failure and control groups (Table I; Figure 2d).

The concentration of IL-10 in the recurrent implantation failure group was significantly lower than that in control group (Table I; Figure 2e); however, the absolute values were very low and approaching the lower level of sensitivity of the assay.

Discussion
The relatively low rate of successful implantation following embryo transfer after IVF and ICSI can be partly explained by intrinsic problems within the embryos transferred. This has been estimated to be as high as 60–80% (Schelesseman, 1979; Rudak et al., 1985; Papadopoulos et al., 1989; Edirisinghe...
et al., 1992; Jamieson et al., 1994). However, even when the embryos are screened for chromosomal abnormalities using pre-implantation genetic diagnosis (PGD) prior to transfer, the resulting implantation rate is generally <50% (Gianaroli et al., 1997; Kahraman et al., 2001). This observation raises the possibility that additional factors might contribute towards failed implantation, particularly for younger women whose embryos would be expected to have a lower incidence of karyotypic abnormality.

A number of studies have investigated the role of endometrial factors in implantation. For example, a correlation has been noted between decreased expression of the vitronectin receptor α,β3 (Lessey et al., 2000) and decreased uterine receptivity (Lessey et al., 1994, 1995; Lessey, 1997, 1998; Illera et al., 2000; Damario et al., 2001). MUC-1 expression has also been implicated in uterine receptivity (Braga and Gendler, 1993; Hey et al., 1994; Croy et al., 1997; Aplin, 1999; Horne et al., 2001). Similarly, pinopode formation is thought to be important for embryonic implantation (Martel et al., 1991; Nikas and Psychos, 1997; Nikas, 1999, 2000; Stavreus-Evers et al., 2001; Nardo et al., 2002). In contrast, there is a relative paucity of information regarding the in-vivo conditions within the uterine cavity around the time of implantation. For this reason, cytokine concentrations and MMP activities were analysed in women who had undergone multiple unsuccessful embryo transfers. These women (and partners) had been screened for other possible causes of implantation failure such as karyotypic abnormalities, antiphospholipid antibodies, thyroid function and inherited thrombophilias.

In the present study, levels of cytokines and MMPs were measured in the uterine flushings of women with recurrent failed embryo transfer, and also in two control groups. Following uterine irrigation, a hysteroscopy was performed to exclude any intra-uterine abnormality and an endometrial biopsy was taken to confirm secretory-phase endometrium. In the recurrent implantation failure group, statistically significant elevated levels of IL-1beta and reduced levels of IFN-gamma and IL-10 were found. Although not statistically significant, there was also a trend towards TNF-alpha being higher and LIF lower in this group. The MMP score was significantly higher in the recurrent implantation failure group, and also correlated with the level of IL-1beta. Differentially increased MMP-9 activity made the major contribution to the increased total MMP score in these patients. These observations could not be explained by differences in the protein concentration of the uterine flushings from the different patients.

The pattern of cytokine expression in the recurrent implantation failure group was intriguing. Raised IL-1beta levels may occur as a result of chronic inflammation, for example chronic endometritis, but there was no histopathological evidence of endometritis in any of the endometrial biopsy specimens. There also appears to be an association between IL-1beta levels and MMP score. This observation is biologically plausible as MMP-2 and MMP-9 production by endometrial stromal cells can be induced by IL-1beta (Huang et al., 1998; Zhang et al., 1998). It was also confirmed that IL-1beta induced the expression of proMMP-2, active MMP-2, proMMP-9 and active MMP9 bands by cultured endometrial stromal cells (data not shown).

The low level of IL-10 in the implantation failure group is an intriguing observation, as it might be hypothesized that underexpression of this immunosuppressive cytokine could be detrimental to the immunological tolerance of the embryo. However, the low measurable concentrations of IL-10 in all the groups suggests that caution be taken against overinterpretation.

In the present study, cytokine concentrations and MMP activities were measured during ‘natural’ menstrual cycles. It is possible that the high estrogen and progesterone levels found in the setting of an IVF-stimulated cycle might influence the results. However, it should also be noted that in the present group of patients with recurrent implantation failure a significant number of thawed embryos had been replaced during ‘natural’ cycles without success. It is believed therefore that the natural cycle results obtained are relevant to the implantation failure in this group.
A recent report has shown that increased intra-uterine concentrations of LIF and TNF-alpha was a negative prognostic factor for subsequent pregnancy (Ledee-Bataille et al., 2002). These results are only partially consistent with those of the present study, but it should be noted that the former study included patients undergoing intra-uterine insemination as well as IVF-embryo transfer. Moreover, another report described a significantly lower expression of LIF in patients with unexplained infertility due to uterine flushing (Lass et al., 2001).

In any biological study, a distinction must be drawn between correlation and cause. In the present preliminary study, the initial aim was to determine if there was any detectable difference in the intra-uterine expression of MMPs and cytokines in patients with recurrent implantation failure compared with normally fertile women. With this in mind, a number of cytokines were selected, the known roles of which in implantation and immunology raised the possibility that their differential expression could have an impact upon embryonic implantation. Having observed some differences in the pattern of cytokine expression, the next challenge would be to elucidate the biological basis for these observations. With the expansion of PGD, it would be interesting to see if these findings are borne out for women who continue to have failed implantation following embryo transfer despite the demonstration of chromosomally normal embryos. Additionally, it would be interesting to determine, in a prospective trial, whether there is any correlation between intra-uterine cytokine/MMP expression and reproductive outcome. Ultimately, of greatest interest would be to identify whether modulation of the pattern of cytokine expression could improve implantation rates in women with recurrent implantation failure. These studies will be the focus of ongoing research.

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