Lack of expression of endometrial prolactin in early implantation failure: a pilot study

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BACKGROUND: Animal models and experimental studies suggest a role for paracrine prolactin (PRL) signalling in decidualization and embryo implantation. We investigated the expression of endometrial prolactin (e-PRL) and prolactin receptor (PRL-R) in the endometrium of women affected by unexplained infertility (UI) and repeated miscarriages (RM). METHODS: Patients (n = 24) were divided into three groups: RM, n = 5; UI, n = 11; controls, n = 8. Endometrial samples were collected at the time of hysteroscopy in the late luteal phase. The presence of transcripts of e-PRL and PRL-R was investigated by qualitative RT–PCR. Pattern and site of expression of e-PRL were studied by immunohistochemistry. RESULTS: PRL-R mRNA was detected in all endometrial samples of the three groups. PRL gene expression was detected in all control samples, only in three of five samples of the RM group and in four of 11 samples of the UI group. RT–PCR results were largely confirmed by immunohistochemistry, study groups showing a defect of expression of e-PRL. CONCLUSIONS: In this pilot study we report a lack of expression of endometrial prolactin during the ‘implantation window’ in some patients affected by unexplained infertility and repeated miscarriages. These data, in association with those obtained in experimental animals, suggest that the lack of endometrial PRL expression is involved in reproduction failure.

Key words: endometrial prolactin/endometrium/embryo implantation/infertility/repeated miscarriages

Introduction

Embryo implantation and successful establishment of pregnancy require delicate and highly co-ordinated interactions that involve both embryonic and endometrial participation. Adequacy of the endometrium for fertility has been conventionally investigated by histological evaluation of a mid-luteal phase biopsy, and normal histology has been considered as a demonstration of receptive endometrium. As more is known about the gene products of the endometrium, it appears that many of the secreted products function to support the embryo and initiate the early communication that continues into the pregnancy (Lessey, 2000). Various growth factors and cytokines reveal a dynamic expression pattern through the menstrual cycle and have been implicated in endometrial growth, differentiation and receptivity (Lessey et al., 1996; Giudice, 1999). Some of these molecules could serve as markers of uterine receptivity and have been proposed to identify women at risk for implantation failure (Stewart et al., 1992; Lessey et al., 1995; Hambartsoumian, 1998; Dimitriadis et al., 2000).

Prolactin (PRL) is a peptide hormone essentially secreted by the anterior pituitary and, to a lesser extent, by other extrapituitary tissues (Ben-Jonathan et al., 1996). The endometrium is one of the first extrapituitary sites that has been reported to synthesize and secrete PRL (Brosens et al., 1999; Tseng and Mazella, 1999). It is synthesized by decidualized endometrial cells in the late secretory phase in a non-conception cycle and throughout pregnancy. Immunohistochemical techniques have demonstrated PRL expression in the stromal compartment and on most glandular epithelial cells of the secretory endometrium (Wang et al., 1994; Reis et al., 1999). PRL synthesis increases if pregnancy occurs and the production is maintained until the final stage of pregnancy (Wu et al., 1995). Endometrial PRL (e-PRL) is similar to hypophyseal PRL in chemical, biological and immunological properties. The messenger (m) RNA encoding e-PRL is structurally different from its pituitary counterpart due to the use of a different transcription start site (Gellersen et al., 1994). The use of a different promoter region probably accounts for the striking differences in regulation of PRL gene between the two tissues. It has been hypothesized that progesterone is essential for the initiation of e-PRL synthesis and the maintenance of its production (Tseng and Mazella, 1999). The weak or absent expression of progesterone receptors in decidualized stromal cells of human endometrium suggests, however, that progesterone does not induce e-PRL.
gene expression directly (Gellersen et al., 1994; Telgmann et al., 1997).

The effects of PRL are mediated by a membrane-bound receptor (PRL-R) which is a member of the superfamily of cytokine receptors. Through its receptor, e-PRL stimulates the tyrosine phosphorylation of Janus kinase 2 and STAT 1 and 5 (Jabbour et al., 1998, 1999). Recently Gubbay et al. demonstrated, within the secretory phase of the human endometrium, that e-PRL induces also the ERK/MAPK pathway (Pellegrini et al., 1992; Gubbay et al., 2002). In the non-pregnant human uterus, PRL-R has been localized in the stromal and glandular compartments of the mid- to late secretory phase endometrium (Jones et al., 1998). If pregnancy occurs, PRL-R expression is maintained and localized to the decidua, chorionic cytotrophoblast, placental trophoblast and amniotic epithelium (Jones et al., 1998).

Some authors demonstrated a role for e-PRL signalling in reproduction in PRL-R-deficient mice (Bole-Feyset al., 1998). Female mice with a homozygous null mutation of the PRL receptor were infertile (Ormandy et al., 1997). Other studies are in progress to examine, in rodents, whether the absence of e-PRL leads to the expression of genes that are detrimental for normal decidualization and fetal survival (Bao et al., 2003).

The coordinated temporal pattern of expression of both e-PRL and PRL-R in non-pregnant and pregnant endometrium suggests that e-PRL may have an important role in the process of implantation and maintenance of pregnancy.

To verify the role of endometrial PRL in the early pregnancy processes, we investigated a group of patients whose reproductive failures were consistent with an implantation failure. The pattern and site of expression of e-PRL and PRL-R were therefore studied in endometrial samples collected in the late secretory phase from women selected for unexplained infertility and recurrent early miscarriages.

Patients

Sixty-five women were recruited from the patients referred to the San Paolo Hospital Sterility Unit, Milan. Of these, only 24 patients were admitted to the study. They were divided into three groups: repeated miscarriages (RM, n = 5 patients); unexplained infertility (UI, n = 11 patients); controls (C, n = 8 patients). The age of the patients of the three groups was not significantly different (Table I).

RM was defined by a history of at least two spontaneous miscarriages. Inclusion criteria were age between 18 and 40 years; normal anatomy of the genital tract determined by ultrasonography, hysterosalpingography and hysteroscopy; a history of regular menstrual cycles ranging from 25 to 32 days; ovulatory cycles determined by serum progesterone in late secretory phase; normal hormonal screen in days 3–5 of the cycle for FSH (<10 mIU/ml), LH, 17β-estradiol, thyroid-stimulating hormone (TSH), serum PRL and progesterone. Exclusion criteria were represented by known causes of miscarriages such as uterine pathologies or chromosomal anomalies. Only one patient of this group had previously delivered

UI was defined as women with failure of conception for 2 years having regular intercourse without contraception. Inclusion criteria were similar to the RM group. Exclusion criteria, to correctly classify these patients as UI, were known causes of infertility such as tubal disease or male factors evaluated by partners’ semen examinations. None of these women had previously given birth.

The C group consisted of women with at least one successful pregnancy with a previous partner, who underwent hysteroscopy as part of our routine protocol for assisted reproduction. Male factor infertility had been previously proven by the partners’ semen analysis, diagnosed as azoospermic or severe oligoteratozoospermic. Inclusion criteria were similar to the UI group. None of these patients had a previous history of miscarriages, infertility problems or any kind of gynaecological symptom or disorder.

The protocol was approved by the San Paolo Institute Board. Informed consent was obtained from all women.

Tissues and hormonal evaluation

Endometrial tissues were obtained by curettage during hysteroscopies performed to evaluate the uterine cavity morphology, according to our clinical protocol. Biopsies were harvested from the patient’s last menstrual period in late secretory phase (between days 21 and 27). All specimens were histologically examined by an experienced gynaecological pathologist according to Noyes et al.’s (1950) criteria and the data of Mutter and Ferenczy (2002) and only tissues in agreement with the date of the last menstrual period, corresponding to +7 to +13 days post-ovulation, were selected for the study. In each group, samples were collected in both the first (I) and the second (II) half of the late luteal phase (C: I, n = 5, II, n = 3; UI: I, n = 7, II, n = 4; RM: I, n = 3, II, n = 2).

All women underwent evaluation of progesterone and PRL serum levels the same day endometrial biopsy was performed (day 9 ± 2 post ovulation). Mean serum levels of progesterone and PRL showed no significant differences between the three groups (Table I). Progesterone level was, in all patients, suggestive of ovulation (>3 ng/ml).

No patient in the three groups had taken any hormonal medication during the 6 months before entry into the study.

Immunohistochemistry

Immunohistochemistry was conducted on endometrial samples collected during hysteroscopy at the same time of the biopsy for PCR. The pathologist (G.B.) was blinded with regard to the groups from which the samples were derived.

Paraffin-embedded sections (3 μm) were cut for immunohistochemistry detection, mounted on Super-frost slides (Bio-Optica, Italia) and dried at room temperature. After de-waxing and rehydrating, the sections were submitted to the immunohistochemical reaction in the Genomix-i 6000 autostainer (BioGenex Laboratories).

The endogenous peroxidase was inhibited with 3% hydrogen peroxide in water for 10 min. Primary antibody used was a rabbit anti-human prolactin (1:200 dilution; 60 min; DakoCytomation, USA). The detection system used was the DakoEnvision™+ System (30 min; DakoCytomation) labelled polymer, horseradish peroxidase.

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Table I. Serum prolactin and progesterone levels

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Serum prolactin (ng/ml)</th>
<th>Progesterone (ng/ml)</th>
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<tbody>
<tr>
<td>Unexplained infertility</td>
<td>34.5 ± 3.8 (29–40)</td>
<td>16.0 ± 8.6 (7.8–30.2)</td>
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<tr>
<td>Repeated miscarriages</td>
<td>36.8 ± 4 (31–40)</td>
<td>19.0 ± 10.2 (6.5–27.7)</td>
</tr>
<tr>
<td>Controls</td>
<td>37.1 ± 2.6 (33–40)</td>
<td>20.2 ± 5.8 (12–28.9)</td>
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Values are mean ± SD.
(HGPRT). The staining was developed with 3,3′-diaminobenzidine (5 min; DakoCytomation) and counterstained with Mayer’s haematoxylin (2 min; Bio-Optica). Immunohistochemical observations and their negative controls were performed using two consecutive histological sections. The section for control was treated with the same study protocol without incubation with the anti-PRL antibody.

**Ribonucleic acid (RNA) extraction and RT–PCR**

Endometrial samples stored at −80°C were homogenized in phenol–guanidine isothiocyanate (Trizol®; Gibco BRL, USA). Total RNA was extracted in chloroform and precipitated by centrifugation in isopropanol (12 000 g for 10 min, at 4°C). The RNA pellet was washed with 75% ethanol, resuspended in diethylpyrocarbonate-treated water and quantified by absorbance at 260/280 nm.

For detection of PRL and PRL-R mRNA, 3 μg of total RNA, 2 μl of dNTP and 1.5 μl of random hexamer primers (Promega) were denatured at 65°C for 10 min and RT reaction was performed at 37°C for 60 min in the presence of M-MLV RT (Moloney Murine Leukemia Virus reverse transcriptase; Promega) and recombinant ribonuclease inhibitor (Promega). Complementary DNA fragments were amplified by using intron spanning primers designed to prevent the amplification of contaminating genomic DNA. Primers for PRL–R RT–PCR amplification (expected fragment length: 650 bp) were previously reported (Jones et al., 1998). The following primers were newly designed for PRL RT–PCR amplification: PRL sense, 5′-GCCCTCTGATCATCCTGTCACG-3′; PRL antisense, 5′-TGCGTAGGAGTGAG-3′ (expected fragment length: 251 bp). The amplification of the ubiquitously expressed dehydropyrimidine guanine phosphoribosyl transferase (HGPRT) gene was used as RT–PCR control, as previously described (Fugazzola et al., 2002).

The amplification product of a first strand reaction performed without reverse transcriptase was used as a negative control, whereas RNA extracted from hypophyseal tissue was used as positive control.

Qualitative PCR protocol comprised 30 amplification cycles in all cases (annealing temperature: 56°C for PRL and 62°C for PRL-R). The PCR products were run on a 3% polyacrylamide gel stained with ethidium bromide and visualized under UV light.

**Statistical analyses**

Between-group differences in PRL gene expression were tested by Fisher’s exact test.

**Results**

Table II presents results for e-PRL and PRL-R investigated by RT–PCR in the endometrial samples. Expression of PRL-R mRNA was detected in all samples of the three groups, whereas PRL gene showed a discrepant expression pattern between the three groups. PRL mRNA was detected in all control samples (8/8); only in three out of five samples of the RM group (P = 0.128 versus controls) and in four out of 11 samples of the UI group (P = 0.013) (Figure 1). Expression of e-PRL in study groups was not related to the timing of endometrial biopsy (first or second half of the late luteal phase). No significant association was observed between expression of e-PRL and previous history of deliveries or serum levels of progesterone and PRL.

Immunohistochemistry conducted on endometrial samples to verify the pattern of expression, and to localize the site of production of e-PRL, revealed immunoreactive signal in all control samples (Figure 2) according to the RT–PCR results. We also recognized a variable pattern of expression throughout the late luteal phase. The early immunoexpression of e-PRL appeared in endometrial surface epithelial cells and in the strictly connected stromal layer (Figure 2A). A weaker e-PRL immunostaining was detected in glandular epithelial cells and perivascular endometrial stromal cells (Figure 2C). In the last days of the late luteal phase (11th–13th day post-ovulation) e-PRL expression was strong in the glandular epithelial cells, as well as in the endometrial stromal compartment, but weaker in epithelial surface cells (Figure 2B).

Negative controls were performed for all samples and showed no staining.

In study groups, immunohistochemistry confirmed the expression or the defect of expression of e-PRL consistently with RT–PCR results. Only two of the immunohistochemistry results, both regarding samples of the UI group, were not consistent with PCR. In particular, one of the PCR positive samples did not reveal immunoreactive e-PRL and, on the other hand, one PCR negative sample was positive when investigated for e-PRL immunostaining.

In both study groups, positive samples showed a pattern of e-PRL expression similar to the controls. Otherwise, studying in a semi-quantitative way the degree of e-PRL expression, marked differences were detected. In fact, variable degrees of immunoexpression (Figures 3B and C; 4B–D) were

| Table II. Prolactin (PRL) and PRL-R mRNA expression in endometrial samples |
|-----------------|-----------------|
|                | PRL             | PRL-R            |
| Unexplained infertility | 4/11* (36)      | 11/11 (100)      |
| Repeated miscarriages     | 3/5* (60)       | 5/5 (100)        |
| Controls                  | 8/8 (100)       | 8/8 (100)        |

Values refer to number of patients showing expression/total number of patients (%).

*P = 0.013 versus controls.

**p = 0.128 versus controls.**
observed, ranging between the samples with null e-PRL expression (Figures 3A and 4A) to the few samples (one by RM group and one by UI group) that presented a strong expression comparable to the control samples (Figure 3D).

In all IHC positive samples of the three groups, immunoreactive e-PRL was detected in the epithelial cells as strongly as in the stromal fibroblasts. Immunostained e-PRL was strictly confined to the cytoplasm.

Discussion

This is, to our knowledge, the first study investigating endometrial PRL and PRL-R expression in patients with reproductive failure. We report an alteration of PRL expression in patients affected by unexplained infertility and repeated miscarriages, characterized by a defect of PRL in secretory endometrium.

It has become increasingly evident that endometrial-derived cytokines and growth factors play an essential role in pregnancy recognition, early embryonic development, implantation and, ultimately, in the establishment of a successful pregnancy (Hunt, 1989; Giudice, 1999). Possible defects in these mostly non-characterized biological processes at the maternal–fetal interface has been implicated in the failure of implantation (Hunt, 1989; Hambartsoumian, 1998).

In our study, RT–PCR analyses revealed the presence of PRL-R transcripts in all investigated samples. Moreover, e-PRL mRNA expression was found in all endometrial samples of the control group and detected with large individual variations in the UI and RM groups. With the exception of two biopsies, immunohistochemistry confirmed the RT–PCR results. Differences in e-PRL expression cannot be explained by differences in the timing of endometrial biopsies since all samples in the three groups were in the late secretory phase, as confirmed by histology.

Previous reports immunolocalized e-PRL protein only in stromal cells, in contrast to PRL-R whose immunostaining was detected in stromal and in epithelial cells (Wang et al., 1994; Jones et al., 1998). By contrast, we detected immunostaining for e-PRL in stromal fibroblast cells as strong as in luminal and glandular epithelial cells. In agreement with the present study, immunostaining in most glandular epithelial cells has been previously reported (Reis et al., 1999) and a possible explanation has been raised that stromal e-PRL might reach the glands by paracrine diffusion. However, this hypothesis can only partially explain the strong e-PRL expression of luminal epithelial cells observed in the present study.

Cases with negative e-PRL but positive PRL-R expression argue against the hypothesis put forward by other authors that PRL could directly induce the expression of its own receptor (Jabbour et al., 1998).

RT–PCR results were not confirmed in two of the samples examined by immunohistochemistry. One of these was PCR positive and immunohistochemically negative; this sample was histologically consistent with the 21st day of the menstrual cycle. In this case, the superior sensitivity of qualitative PCR protocol may be a suitable explanation. For the second sample, showing negativity in PCR and positivity in IHC, we cannot exclude a technical problem in biopsy execution or laboratory manipulation.

The role of endometrial PRL has been demonstrated in PRL and PRL-receptor knockout mice (Bao et al., 2003;
Ormandy et al., 1997), with progesterone treatment unable to avoid fetal death and premature abortion. In these studies, a strong expression of interleukin (IL-6), hydroxysteroid dehydrogenase (20α-HSD) and activin A, detrimental for normal decidualization and fetal survival, was found in the null mice decidua whereas these genes were silenced in the wild type mice. Preliminary results showed that PRL treatment was able to restore the normal pattern of decidual expression and fertility in the PRL null mice (Bao et al., 2003).

Decidual changes are necessary for correct implantation, and the products of decidual cells, such as e-PRL, could be key to the normal or abnormal tissue communication between blastocyst and decidua.

Some authors have suggested a role for e-PRL in the process of implantation, by modification of the immune environment of the endometrium or by regulation of the expression of factors within the glandular secretions that may control trophoblast proliferation and invasion of the endometrium (Jabbour et al., 1998; Jones et al., 1998). PRL-R gene expression has been detected in CD56+ natural killer (NK) cells (Pellegrini et al., 1992; Gubbay et al., 2002), i.e. large granular lymphocytes, the predominant leukocyte population in non-pregnant endometrium and decidua (King et al., 1989). Their interaction with the invading trophoblast cells, limiting the invasion, has been suggested to be necessary for successful implantation (Klentzeris et al., 1994). Moreover, a number of studies have described the effects of cytokines released by decidual macrophages and other bone marrow-derived cells on endometrial PRL release. The demonstration, in primary decidual cell cultures, of a dose-dependent inhibition of e-PRL synthesis and release by the exposure to tumour necrosis factor (TNFα), IL-1α, IL-1β, tumour growth factor (TGF-β) and IL-8 (Jikihara and Handwerger, 1994), to IL-2 (Kanda et al., 1999), to interferon (IFN-γ) (Christian et al., 2001) as well as the induction of e-PRL release by PRL-releasing peptide (Reis et al., 2002) suggests a pivotal role, for e-PRL, in the decidual cytokine network.

One potential limitation to the study is that controls were women who had previously given birth. Although a group of nulligravidae would not represent controls with proven fertility, they are indispensable as an additional control group since it is not known how previous pregnancy will affect endometrial prolactin expression. Larger, well-controlled studies are needed before definitive conclusions can be reached. Our pilot study shows that a number of patients, suffering from unexplained infertility and repeated miscarriages, present a defect in endometrial PRL production. These data, in association with those accumulated in experimental animals, may indicate that lack of expression of endometrial PRL is involved in reproduction failure and strongly suggest that e-PRL may be more than a simple marker of decidualization,
but rather an active cytokine. It is possible that the endometrial PRL defect, probably mediated by other endometrial cytokines, may be associated with an impairment in CD56+ NK cell endometrial recruitment, ultimately resulting in unsuccessful embryo implantation and inadequate placenta
c tion.

Recent knowledge about feto-maternal immune regulation may open new and promising methods for the management of patients affected by infertility and repeated miscarriages. Further larger studies are needed, including a nulligravi-
dae control group, to test whether the evaluation of e-PRL expression may constitute a useful clinical parameter in the diagnosis of implantation defect.

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