Endometrial LGR7 expression and implantation failure

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Abstract

Implantation failure is considered as a major cause of infertility in women with recurrent pregnancy loss (RPL) and in otherwise healthy women with unexplained infertility. Preliminary data in primates suggested that relaxin (RLX) is involved in endometrial preparation for implantation. In a prospective observational study, the endometrial RLX receptor (LGR7) expression was assessed in three groups of patients with regular ovulatory cycle and normal uterine cavity: 23 with RPL (Group A), 23 with unexplained infertility undergone at least three cycles of failed in vitro fertilization (IVF) reporting good oocyte and embryo quality (Group B), 23 with proven fertility (Group C). Assessment of LGR7 expression was performed with both polymerase chain reaction (PCR) analysis and immunohistochemistry on endometrial samples obtained with hysteroscopic biopsy performed in the secretory phase of the menstrual cycle. Endometrial LGR7 was less expressed in group A and B versus C, both by PCR analysis (p = 0.024) and immunohistochemistry. The decreased expression of the endometrial RLX receptor in women with implantation failures, both in vitro fertilization failure and recurrent pregnancy loss, suggests that RLX may play a crucial role in the structural and functional changes of the endometrium during the window of implantation.

Introduction

Implantation failure is considered as a major cause of infertility in women with recurrent pregnancy loss (RPL), otherwise in healthy women with unexplained infertility, and in several repeated unsuccessful IVF cycles. The human endometrium undergoes complex proliferative and secretory changes in each menstrual cycle and exhibits only a short period of receptivity known as the “window of implantation” [1], depending on paracrine signals from stromal cells underlying the luminal epithelium [2]. The timing and duration of the “window of implantation” are major endometrial determinants of the likelihood of reproductive success. A large variety of molecules, including adhesion molecules, cytokines, growth factors, lipids and others, are detected at high levels during this “window” and it has been postulated that they may be involved in the implantation process [3]. Indeed, the clinical use of these markers of endometrial receptivity has been hypothesized to improve the management of recurrent abortion and the implantation rate in women with previous failed IVF cycles [4].

Relaxin (RLX) is a peptide hormone produced by the corpus luteum during the luteal phase and in the first trimester of pregnancy. It belongs to the family of insulin-like growth factor and is composed of two peptide chains, A and B, of 24 and 29 amino acids respectively, linked by disulfide bridges. RLX LGR-7, the classical RLX receptor, is expressed in many tissues, including human endometrium [5–7]. Preliminary data in primates suggested that RLX is involved in endometrial preparation for implantation [8]. In a previous study, we showed increased LGR7 expression in the early secretory phase confirming the involvement of RLX in the physiology of human endometrium and suggesting a role for RLX in implantation [9]. Other studies showed that RLX levels are impaired in women with early pregnancy loss [10] and that granulosa cell production of RLX is predictive of pregnancy outcome in IVF cycles [11]. In this view, the aim of our study was to assess the RLX receptor expression in the endometrium of women with implantation failure.

Materials and methods

Patients

Two groups of patients referring to the outpatient infertility Clinic of the Second University of Naples were enrolled: 23 patients with three or more consecutive pregnancy losses before 12 weeks gestation (Group A) and 23 patients with unexplained infertility after having undergone at least three cycles of failed IVF and with reported fresh transfer of good quality embryos based on a morphological analysis [3] (Group B). All the women were aged >18 years and ≤35 years, had regular ovulatory cycle, normal pelvic examinations; hormone determinations were normal on day 3 of the menstrual cycle at the time the study was performed (FSH ≤10 mIU/ml, LH ≤10 mIU/ml, E2 >50 pg/mL), transvaginal ultrasound showed normal ovaries, hysteroscopy excluded endometrial pathologies, karyotype was normal, and a laparoscopy excluded endometriosis and confirmed a normal...
pelvis; semen analysis was normal. A homogeneous comparable group of 23 women was enrolled with proven fertility (defined as at least 2 spontaneous successfully delivered term pregnancies) and normal uterine cavity found at hysteroscopy performed because of ultrasound findings suspicious for endometrial pathology (Group C: control group). All patient presented similar demographic and baseline characteristics.

Exclusion criteria were as follows: PCOS, hyperprolactinemia, thrombophilic conditions, ovarian or adrenal disease, previous uterine surgery within 3 months, abnormal uterine bleeding, leiomyoma in or near the uterine cavity, known or suspected endometriosis, chronic diseases (e.g. systemic lupus erythematosus and diabetes mellitus), abnormal semen analysis of male partner; in addition, poor ovarian response (<3 oocytes previous pick up) in previous ART cycles for group B. All patients did not receive hormones for 6 months before sample collection. Institutional review approval was obtained for the study. Written consent was obtained from the patients for the collection and analysis of data.

Endometrium collections

An endometrial biopsy was obtained from all the patients in the secretory phases [12–14] of the menstrual cycle confirmed by serum progesterone assay (>5 mU/ml). All the patients were requested to abstain from unprotected intercourse in the cycle the hysteroscopy was planned. The endometrial biopsy was performed during the hysteroscopic procedure on both anterior and posterior aspect of the uterine cavity with a 5 Fr grasping forcep. Two endometrial specimens were obtained from all the biopsies: one was fixed in formalin and subsequently paraffin embedded for immunohistochemistry analysis and one was immediately immersed in RNA later and stored at −20°C for PCR analysis.

Histological analysis

The specimens were considered adequate according to the routine criteria for adequacy of endometrial biopsies applied by the consultant pathologists in the routine Biopsy Service. Histological examination was performed on sections stained with hematoxylin and eosin [15]. Immunohistochemistry was carried out as previously described [16]. Slides were then incubated at 4°C overnight with antibodies raised against human LGR7 (sc-50328, rabbit polyclonal IgG, Santa Cruz Biotechnology) at a 1:100 dilution. Negative controls were prepared by substituting the primary antiserum with nonimmune IgG, and positive controls by using the antibody on a tissue section where the protein had already been demonstrated. Three observers evaluated the staining pattern separately, then scored each specimen for staining intensity signal: 0 (absent immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity). An average of 22 fields was observed for each tissue.

Detailed protocols for RNA extraction, cDNA amplification with PCR method using specific oligonucleotides raised against the human LGR7 sequence, electrophoresis of PCR products and densitometry of each sample are reported in detail in Supplemental Digital Content (S1).

Statistical analysis

All analyses were performed with R software, version 2.9.1 (Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2009). For both PCR analysis and immunohistochemistry, the data reported represent the mean and median values of each group. A Kruskal–Wallis test for multiple comparisons was performed to compare LGR7 within each groups and between different groups.

Results

For PCR analysis, a remarkable difference between groups was found (p = 0.02422). Mean values and medians were 1.10 and 1.12 for group A, 0.94 and 0.95 for group B, 1.70 and 1.68 for group C (detailed data of LGR7 expression value are reported in Supplemental Digital Content S2). Multiple comparison tests show a significant difference between group B versus C and A versus C (Figure 1). Regarding immunohistochemistry, in groups A and B RLX LGR7 was expressed at a low level, whereas a significantly higher immunopositivity (moderate/intense) was present in samples of group C (p = 0.05) (Figures 2 and 3).

Discussion

Our study shows a decreased expression of the endometrial receptor LGR7 in women with implantation failure, suggesting that RLX plays a critical role in the complex mechanisms of endometrial preparation to embryo implantation. Such endometrial development resulting in endometrial receptivity during the window of implantation requires subtle collaboration of an extremely large number of different factors. Many of them have been described and are highly detected [3]. It has been suggested that these factors could be used as markers of endometrial receptivity: this could lead to their potential usefulness in the treatment of female infertility secondary to implantation failure [17].

Impaired decidualization of the endometrium prior to conception is a relevant mechanism underlying RPL, either by prolonging the implantation window, thereby disabling natural embryo selection or by disrupting the maternal responses to embryonic signals [18]. Endometrial failure to express a receptive phenotype is thought to be a major cause of infertility in IVF treatment failure [19]. The average implantation rate in IVF is around 25% [20] and two-thirds of implantation failures recognized as a trigger inadequate uterine receptivity, whereas the embryo itself is responsible for only one-third of these failures [12,21]. In addition, prolonged endometrial receptivity facilitates implantation of delayed or compromised embryos and has a strong association with early pregnancy loss [13,18]. To increase implantation rates, an increased knowledge of the factors involved in embryo implantation is required. A better understanding of the mechanisms regulating embryo implantation may improve the ability of clinicians to treat infertility, to prevent early pregnancy loss and improve IVF outcome. Evaluation of the implantation markers with endometrial biopsy samples may help to detect occult implantation deficiency and predict pregnancy outcome [14]. In the future, optimizing endometrial receptivity in fertility treatment and manipulating the expression of key endometrial genes with medical, surgical or future cell and gene-based therapies may improve implantation rates.

Although many molecules enhance progesterone-induced decidualization, only cAMP and RLX are known to stimulate human endometrial stromal cells (HESC) decidualization in vitro, independent of progesterone [22]. A large body of data that support a role for RLX in human endometrial decidualization has been generated in in vitro models. RLX is an extremely potent stimulator of the secretion of various hormones and growth factors, including insulin-like growth factor-binding protein and prolactin, hallmarks of decidualization. The effects of RLX on implantation is a relevant mechanism underlying RPL, either by prolonging the implantation window, thereby disabling natural embryo selection or by disrupting the maternal responses to embryonic signals [3]. It has been suggested that these factors could be used as markers of endometrial receptivity: this could lead to their potential usefulness in the treatment of female infertility secondary to implantation failure [17].

Statistical computing. R Foundation for Statistical Computing. (Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2009). For both PCR analysis and immunohistochemistry, the data reported represent the mean and median values of each group. A Kruskal–Wallis test for multiple comparisons was performed to compare LGR7 within each groups and between different groups.
endometrium in all the four phases of menstrual cycle (early and late proliferative phase, early and late secretory phase) and that LGR7 expression shows a striking significant increase in the early secretory phase, suggesting a role for RLX in implantation. To date, data on correlation between impaired function of endometrial RLX and defective implantation are lacking.

The current study describes for the first time the expression of LGR7 in the biopsies of human endometrium obtained from women with different reproductive history: women with proven fertility, with RPL and with repeated IVF failures. In the latter group, only those patients who reported a good oocyte and embryo quality in the previous failed IVF cycles were considered to avoid including failures of IVF due to gametes alterations. The decreased expression of the endometrial receptor LGR7 in women with implantation failures, both IVF failure and RPL, suggests which RLX may play a crucial role in the structural and functional changes of the endometrium during the “window of implantation”. In addition, the reduced expression of LGR7 in women affected by RPL leads to hypothesize a role of RLX in the timing of the “window of implantation”; assuming that a deficient action of this hormone can contribute to a desynchronization of events that lead to endometrial receptivity, with the result of an increased abortion rate [13,18]. Since an increase in mRNA expression does not mean that this is associated with the presence of a functional protein, we also performed an assessment of the tissue expression of the protein by immunohistochemistry. Results confirmed a lower presence of LGR7 in endometrial samples of patients with implantation failure. Indeed, our study has some limitations: in
References


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Declaration of interest

The authors report no declarations of interest.

The control group the time elapsed between the last pregnancy and the sample collection and the possible occurrence of confounding factors were not considered. Moreover, like all in vivo studies, our study was performed in nonconception cycles, and cyclic expression of endometrial factors is thus unrelated to a subsequently ensuing pregnancy.

Establishing an etiology for preimplantation and preclinical losses is not easy, but the one proven explanation is morphologic abnormalities in the early embryo. It is presumed that most are due to chromosomal abnormalities, but such chromosomal abnormalities have not been evaluated in the study because not allowed by Italian law at the time of the study. In conclusion, our work opens new possibilities to study and understand the mechanisms underlying embryo implantation, underscoring the importance that RLX through the action of its receptor LGR7 plays in endometrium in order to ensure a successful implantation.

Figure 3. Immunostaining localization of LGR7 in endometrium samples. Low immunopositivity of LGR7 in glandular and stroma of endometrium samples of group A and B, elevated immunopositivity of LGR7 in glandular compartment and moderate immunopositivity in stroma compartment of endometrium samples of group C. Magnifications: \( \times 10 \) (A), \( \times 20 \) (B), \( \times 40 \) (C).