Deficient expression of monoamine oxidase A in the endometrium is associated with implantation failure in women participating as recipients in oocyte donation

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Successful implantation depends both on the quality of the embryo and on the endometrial receptivity. The latter depends on progesterone-induced changes in gene expression, a process that has been characterized by microarray analysis. One of the genes whose transcription appears to be enhanced during the receptive period is monoamine oxidase A (MAO-A). Our first objective was to confirm the increased expression of MAO-A in the endometrium during the receptive phase of spontaneous normal cycles using real time PCR and immunofluorescence. The second objective was to examine the endometrial expression of MAO-A during the receptive phase induced by exogenous estradiol (E2) and progesterone in patients whose endometrium was shown to have been either receptive or non-receptive to embryo implantation in repeated cycles of oocyte donation. Results showed that MAO-A transcript levels increased between the pre-receptive (LH+3) and receptive phase (LH+7) in all spontaneous cycles examined, with a median increase of 25-fold. Immunofluorescent labelling demonstrated MAO-A localization to the glandular and luminal epithelium with an increasing positive score between LH+3 and LH+7. Conversely, prior failure of embryo implantation was associated with a 29-fold decrease in MAO-A mRNA levels and a substantial reduction in MAO-A protein immunofluorescent label score. These results show a strong association between endometrial receptivity and MAO-A expression in the endometrial epithelium, suggesting an important role for this enzyme in normal implantation.

Key words: monoamine oxidase A/endometrium/implantation failure/implantation window/oocyte donation

Introduction

Implantation failure may result from a variety of abnormalities affecting the gametes, the embryo, the genital tract or the hormonal milieu and is one of the leading causes of ‘unexplained infertility’ estimated to occur in 10–15% of couples seeking fertility treatment (Crosignani et al., 1993). Implantation depends both on the blastocyst and on the endometrium. Although defects in uterine receptivity are viewed with scepticism by some, growing evidence suggests that for successful implantation the quality of the endometrium is no less important than the quality of the blastocyst (Valbuena et al., 1999).

Current data indicate that the expression of some genes at the onset of receptivity is temporarily turned on or increased whereas others are temporarily turned off or decreased (Lessey, 2000; Sunder and Lenton, 2000; Carson et al., 2002; Kao et al., 2002; Martin et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Mirkin et al., 2005). Many of these changes are likely to be essential for implantation and maintenance of pregnancy; however, distinguishing a mere association from a cause–effect relationship has proven difficult in the human female.

A gene whose expression increases during the receptive phase is the one coding for monoamine oxidase A (MAO-A), which is in keeping with previous findings of a marked increase in MAO-A activity in the human endometrium during the mid-luteal phase (Cohen et al., 1965; Southgate et al., 1968; Ryder et al., 1980). These data suggest that this enzyme may play an important role in receptivity to embryo implantation.

MAO is localized in the outer membrane of mitochondria in neuronal and glial cells among others (Cohen et al., 1965; Southgate et al., 1968; Mazumder et al., 1980; Ryder et al., 1980) and catalyses the oxidative deamination of a variety of monoamines using flavin adenosine dinucleotide as cofactor. Two forms of MAO, designated MAO-A and MAO-B, have been identified on the basis of biochemical properties (Johnston, 1968) and nucleic acid sequence (Bach et al., 1988). MAO-A oxidizes preferentially biogenic amines such as serotonin, norepinephrine (NE) and epinephrine although dopamine is a substrate for both (Johnston, 1968). Both isoenzymes are co-expressed in the majority of human tissues except placenta, which predominantly expresses MAO-A, whereas platelets and lymphocytes predominantly express MAO-B (Shih and Thompson, 1999).

Infertile women who have been submitted repeatedly and unsuccessfully to embryo transfer, using good quality embryos, represent a convenient model for examining possible defects in gene expression that render the endometrium non-receptive. The model is more robust when these women have been the recipients of embryos originated...
from oocyte pools that generated embryos that implanted successfully in the oocyte donors. The probability that implantation failure in these cases is due to an intrinsic endometrial defect is further enhanced by absence of discernible male factors. Additional strength of this model is attained when embryo transfer is performed in endometrial cycles induced with exogenous estradiol (E\(_2\)) and progesterone as adequate hormonal stimulation is insured.

On the basis of this reasoning, we hypothesized that implantation failure in infertile women with the features outlined above, may be due to an intrinsic defect in the expression of genes whose transcript level normally increases during the receptive period.

The experimental strategy chosen to test this hypothesis was to compare endometrial gene expression profiles during an artificially induced implantation window in two groups of women, one having a past history of successful implantation and the other the antecedent of failed implantation in repeated cycles in an oocyte donation program. Here, we describe our findings in relation to MAO-A expression, whereas general results of the microarray profiling in the two groups will be reported elsewhere.

We first confirmed the increased expression of MAO-A in the endometrium during the receptive phase of spontaneous menstrual cycles of fertile women, using real time PCR and immunofluorescence. Subsequently, we compared the endometrial expression of MAO-A during the implantation window of artificially induced endometrial cycles in the two group of patients. Herein, we show for the first time that the deficient expression of a gene in the endometrium during the receptive phase is consistently associated with refractoriness to implantation in infertile women.

Materials and methods

Approval and informed consent

This study was approved independently by the Ethics committee of the Chilenian Institute for Reproductive Medicine, University of Santiago de Chile and that of Clinica Las Condes. All women participating in this study were informed of the procedures and purpose of the study and signed a consent form.

Endometrial biopsies

Spontaneous cycles of normal fertile women

Sixteen endometrial biopsies were obtained from eight healthy fertile women aged between 28 and 39 years, who had regular menstrual cycles (26–35 days), had been surgically sterilized, had no history of endometriosis or pelvic inflammatory disease and were not using drugs or taking hormones. Two samples were obtained within the same cycle from each volunteer, one on day 3 (LH+3, pre-receptive phase) and the other on day 7 (LH+7, receptive phase) after the LH peak (LH peak = day 0). Endometrial biopsies were taken from the uterine fundus using Pipelle catheters (CCD Laboratories, Paris) under sterile conditions. These biopsies were used to confirm the differential gene expression of MAO-A in the endometrium during the pre-receptive and receptive phase of the luteal phase.

Occurrence of ovulation was confirmed by monitoring follicular growth and rupture with daily transvaginal ultrasound (TVU). A blood sample of 10 ml was obtained daily from the antecubital vein, when the leading follicle reached 12 mm until ovulation and every other day thereafter until the first biopsy was obtained. E\(_2\), progesterone, LH and FSH were measured in these samples to pin-point the LH peak and confirm retrospectively that the biopsies had been preceded by a normal mid-cycle endocrine profile.

Induced cycles in infertile and fertile women

Seventeen endometrial biopsies were obtained during the receptive period from three groups of volunteer women: Group A, women who had previously participated as recipients in oocyte donation cycles and who repeatedly failed to have implantation of the transferred embryos (n = 5, one of these women had no ovarian function); group B, women who had succeeded in having implantation as recipients in the same oocyte donation program (n = 6, three of these women had no ovarian function) and group C, fertile women who conceived spontaneously in natural cycles and had given birth to three or more children (n = 6). In groups A and B, the same oocyte pool that provided their embryos also provided embryos that implanted successfully in the uteri of the donors. Women having spontaneous menstrual cycles received first appropriate treatment to suppress spontaneous cyclicity as illustrated in Figure 1. This treatment started on the first day of menstrual bleeding or at any convenient time in amenorrheic women. Women with ovarian function received contraceptive pills containing levonorgestrel 0.25 mg and ethinyl estradiol 0.05 mg for 10–21 days, according to the convenience of the biopsy schedule. Down-regulation with the GnRH agonist leuprolide acetate (Lupron; TAP Pharmaceuticals, Deerfield, IL, USA) was initiated on the last day of contraceptive administration at a dose of 0.5 mg s.c. daily for 7 days. Women with no ovarian function did not receive GnRH agonist therapy. In the case of cycling women, treatment with leuprolide acetate started on day 22 of the previous cycle. Ten to twelve days later, the ovaries and uterus were scanned by TVU to exclude women with any abnormal structure in these organs. On the last day of the GnRH agonist treatment, a blood sample was taken to confirm that E\(_2\) serum levels were <100 pmol/l and LH serum levels were ≤ 3 IU/l. Endometrial proliferation was then induced by the oral administration of micronized E\(_2\): 4 mg/day on days 1–7 and 6 mg/day on days 8–20. The endometrium was checked by TVU on day 14 to confirm adequate growth. Micronized progesterone, 600 mg/day, was administered from day 14 to 20 as follows: 400 mg/day orally and 200 mg/day vaginally. On day 20 (day 7 of progesterone administration), additional TVU was performed for endometrial assessment, and an endometrial biopsy was taken. These biopsies were performed as previously described for spontaneous cycles.

A portion of each biopsy was immediately frozen in liquid nitrogen for subsequent RNA isolation. The remainder was transported in cold phosphate-buffered saline (PBS), placed in 30% sucrose in PBS overnight and subsequently embedded in OCT compound for immunohistochemistry, histological examination and dating by a pathologist according to Noyes et al. (1950).

RNA isolation

RNA extraction was performed according to a modification of the method of Chomczynski and Sacchi (1987), using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One millilitre of Trizol reagent was added for every 100 mg of tissue. Total RNA was separated from DNA and protein by adding chloroform and was precipitated with isopropanol. The precipitate was washed with ethanol 75%, air-dried and resuspended in RNase–DNase free water. RNA was quantified by spectrophotometry on a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA). The 260/280 ratio varied between 1.8 and 1.9, and integrity was determined by the visual inspection of RNA fractionated by agarose gel electrophoresis. Each RNA sample was treated with DNase-I, (amplification grade, Invitrogen) to remove contaminating genomic DNA.

RT

All reagents used for RT were purchased from Invitrogen. RT was carried out at 46°C for 50 min in a total volume of 20 µl. Each reaction mixture contained
1 μg of total RNA from endometrial tissue, 4 μl of buffer 5x RT (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 μl of dithiothreitol (DTT) 0.1 M, 10 mM of mix dNTPs, 0.5 μg of oligo (dT) primer and 200 IU of SuperScript II reverse transcriptase. Reaction tubes were incubated at 46°C for 50 min at the end of which reactions were stopped by heating at 70°C for 15 min. Finally, RT products were treated with Ribonuclease H to remove mRNA for the second-strand cDNA synthesis.

**Real time PCR**
The relative expression of mRNA of MAO-A was determined using TaqMan® probes (Applied Biosystems, Foster City, CA, USA). The ABI PRISM 7900HT detection system (Applied Biosystems) and probes labelled with 5’ FAM and 3’ TAMRA (Molecular probes, Eugene, OR, USA) were used to determine the relative expression of MAO-A gene in all endometrial samples. Pre-synthesized primers and probe sets were used to determine the relative level of mRNA encoding human MAO-A (assay ID Hs00165140_m1, Assays-on-demand, Applied Biosystems). The endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was assayed with reagents from the same manufacturer, and was used as a control to normalize for differing amounts of starting material. Real time PCR assays were run using TaqMan Master Mix and the Applied Biosystems 7900HT Sequence Detection System. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Quantitative analysis was based on the relative quantification of each gene of interest in the samples of each group using the ‘Delta-delta method’ developed by PE Applied Biosystems; [\(\Delta \Delta^\text{Ct} \) (gene X in reference – gene X in endometrial samples)]

\[\Delta \Delta^\text{Ct} = \text{Ct (GAPDH in reference – GAPDH in endometrial samples)} – \text{Ct (GAPDH in reference – GAPDH in endometrial samples)}] (Soong et al. 2001). Where Ct (Cycle thresholds) is the cycle in the amplification reaction in which the fluorescence begins to be exponential above the background base line and gene X corresponds to each gene of interest analysed.

**Immunofluorescence**
This technique was applied to endometrial samples obtained from four fertile women with spontaneous cycles, four subjects of group A and four of group B. After transportation in cold PBS, we transferred tissues to 10% w/v sucrose in PBS for 60 min at 4°C followed by 30% w/v sucrose in PBS at 4°C for at least 1 h. Frozen cryostat sections (4–6 μm thick) were placed onto gelatin-coated slides. Sections were incubated with ammonium chloride 100 mM 45 min to quench autofluorescence, followed by PBS washing and permeation in cold acetone for 10 min. After rinsing in PBS three times, we blocked the sections with 10% rabbit normal serum in PBS for 90 min. Sections were then washed three times with PBS and incubated overnight at 4°C with the primary polyclonal antibody against MAO-A from Santa Cruz Biotechnology (Santa Cruz, CA, USA), at a working dilution 1:25. Three PBS rinses were followed by incubation for 60 min at room temperature with the secondary antibody rabbit anti-goat IgG conjugated with Alexa® Fluor 488 (Molecular Probes). After three PBS rinses, we counterstained the samples with 1 μg/ml propidium iodide and mounted in 1,4-diazobicyclo-(2,2,2)-octane (DABCO) (Sigma, St Louis, MO, USA). The primary antibody was omitted as negative control, and sections of human placenta were used as positive control. The resulting staining was evaluated on a Carl Zeiss confocal laser scanning microscope. The label intensity was classified independently by two observers blinded to the slide identification as absent (−), weak (+), moderate (++), or strong (+++). Discrepancies were resolved by seeking consensus.

**Analyses of data**
Real time PCR data were analysed with the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney test for pair-wise comparison when overall significance was detected. The immunofluorescence data were analysed using Fisher’s exact probability test. Statistical significance was set at \(P < 0.05\).

**Results**

**MAO-A mRNA levels in the endometrium during pre-receptive and receptive phase of normal spontaneous cycles**
All cycles were ovulatory and exhibited normal endocrine profile, and all endometrial biopsy specimens taken from groups A and B were in phase according to Noyes criteria (Noyes et al., 1950) (not shown).

MAO-A mRNA levels on days LH+3 and LH+7 of the same cycle \((n = 8)\). Women having induced cycles had a single sample taken on day 7 of progesterone treatment (P+7). These were women who had previously participated as recipients in oocyte donation cycles, who repeatedly failed to have implantation of the transferred embryos (group A; \(n = 5\); women who had succeeded in having implantation as recipients in the same oocyte donation program (group B; \(n = 6\)) and fertile women who conceived spontaneously in natural cycles (group C; \(n = 6\)). Values shown by box plots represent the median plus 75% and 25% quartiles and the range.

**Figure 2.** Relative level of monoamine oxidase A (MAO-A) mRNA, standardized against level of glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA, in endometrial samples taken from various groups of women. Normal fertile women having spontaneous cycles had samples taken on days LH+3 and LH+7 of the same cycle \((n = 8)\). Women having induced cycles had a single sample taken on day 7 of progesterone treatment (P+7). These were women who had previously participated as recipients in oocyte donation cycles, who repeatedly failed to have implantation of the transferred embryos (group A; \(n = 5\); women who had succeeded in having implantation as recipients in the same oocyte donation program (group B; \(n = 6\) and fertile women who conceived spontaneously in natural cycles (group C; \(n = 6\)). Values shown by box plots represent the median plus 75% and 25% quartiles and the range.

**Deficient endometrial MAO-A expression in implantation failure**

MAO-A mRNA levels in the endometrium during the luteal phase of induced cycles
Real time PCR was used to document MAO-A transcript levels in women who had been recipients in an oocyte donation program and normal fertile women. As shown on the right hand side of Figure 2, women in group A (no implantation) had endometrial MAO-A mRNA levels that were 29- and 6.8-fold lower during the receptive phase as compared to normal fertile women having spontaneous cycles and LH+3 to LH+7 of the same cycle \((n = 8)\). Women having induced cycles had a single sample taken on day 7 of progesterone treatment (P+7). These were women who had previously participated as recipients in oocyte donation cycles, who repeatedly failed to have implantation of the transferred embryos (group A; \(n = 5\); women who had succeeded in having implantation as recipients in the same oocyte donation program (group B; \(n = 6\)) and fertile women who conceived spontaneously in natural cycles (group C; \(n = 6\)). Values shown by box plots represent the median plus 75% and 25% quartiles and the range.

**Expression and localization of MAO-A in the endometrium**
MAO-A protein expression levels were determined by immunofluorescence in four samples taken during the luteal phase of spontaneous cycles. MAO-A green fluorescent staining was localized mainly to the luminal and glandular epithelium and scattered in the stromal compartment both on LH+3 and on LH+7 (Figure 3). Staining intensity increased significantly during the receptive phase (LH+7) in comparison with the pre-receptive period (LH+3) (Table I). The results of positive and negative controls (PC and NC, respectively in Figure 3) were as expected.

Immunofluorescent labelling of MAO-A was detected only in a single sample from group A that displayed weak intensity (Table II). All samples from group B displayed strong fluorescence intensity comparable with that of samples from spontaneous cycles in LH+7 (Table II) (Figure 4).
Discussion

The results presented herein confirm that MAO-A transcript levels increase in the human endometrium from the pre-receptive to the receptive phase, as previously reported using microarray analyses (Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Tapia-Pizarro et al., unpublished data). A concomitant increase was also observed in the level of MAO-A immunoreactive protein. Previous reports had also shown that MAO-A activity increases markedly in human endometrium during the mid-secretory phase of the menstrual cycle, coinciding with the period of endometrial receptivity (Ryder et al., 1980). Progesterone increases MAO-A activity in the rat uterus (Mazumder et al., 1980), and therefore, it is likely that it also increases MAO-A protein expression in the human endometrium, through progesterone response elements.
Deficient endometrial MAO-A expression in implantation failure

When the pattern of MAO-A expression was compared between groups A, B and C, a strong association was found between embryo implantation failure and decreased MAO-A transcript levels in all five subjects examined. Decreased MAO-A protein immunofluorescence was also associated with prior implantation failure in the four cases examined in group A. We cannot assume that these differences will persist when a healthy embryo is present and begins to interact with the endometrium. However, a critical difference is revealed at the molecular level between those women who failed repeatedly to obtain implantation and those who succeeded under the same procedures and conditions. Our experimental design was developed to assess endometrial gene expression associated with implantation failure under conditions in which an intrinsic defect in receptivity was the most likely cause.

Both MAO isoforms are present in the endometrium; however, only MAO-A levels change during the endometrial cycle, suggesting that its substrates or products are important for implantation. However, the function of MAO-A in endometrial physiology is unknown at present. Several years ago, it was proposed that MAO-A can improve the rate of implantation by inactivating 5-hydroxytryptamine, a potentially toxic molecule (Poulson et al., 1960). Microarray experiments (Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Tapia-Pizarro et al., unpublished data) have identified several receptors for these biogenic amines whose role in endometrial physiology has not been established.

It is well known that monoamines are potent vasoactive agents and that the regulation of uterine blood flow is important throughout the endometrial cycle and during pregnancy when blood flow increases markedly. Reduced uterine blood flow due to sympathetic hyperactivity may be prevented by the partial inactivation of NE by MAO. The reported association between unexplained infertility and impairment of endometrial perfusion, assessed by Doppler ultrasound, is in keeping with this speculation (Chien et al., 2002; Edi-Osagie et al., 2004). However, MAO-A protein was found mainly in epithelial cells rather present in the promoter region of MAO-A gene (Borthwick et al., 2003).

Table II. Monoamine oxidase A (MAO-A) protein label intensity in groups A and B on day LH+7

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EC, epithelial cells; SC, stromal cells.
Grading of immunofluorescent staining of MAO-A in sections of human endometrium obtained from women who had previously participated as recipients in oocyte donation cycles, who repeatedly failed to have implantation of the transferred embryos (group A) or had succeeded in having implantation as recipients in the same oocyte donation program (group B). The samples were taken on day 7 of progesterone treatment during induction of an artificial endometrial cycle with exogenous estradiol and progesterone.

Figure 4. Monoamine oxidase A (MAO-A) green immunofluorescence labelling in sections of human endometrium obtained from women who had previously participated as recipients in oocyte donation cycles, who repeatedly failed to have implantation of the transferred embryos (group A) or had succeeded in having implantation as recipients in the same oocyte donation program (group B). The samples were taken on day 7 of progesterone treatment during the induction of an artificial endometrial cycle with exogenous estradiol (E2) and progesterone. Note the lower fluorescent staining intensity in group A in comparison with group B.
than capillaries and stromal cells, suggesting an alternate role for this enzyme. Beta adrenergic receptors are expressed in mouse preimplantation embryos, and the cell number in these embryos exposed to isoproterenol is lower than that in control embryos (Cikos et al., 2005), suggesting that low concentrations of beta adrenoreceptor agonists in uterine fluid may be required for normal embryo development. Because MAO-A is present in epithelial secretory cells, it may control beta adrenoreceptor agonist concentrations in endometrial fluid.

The implantation requires a complex network of interactions with redundant mechanisms to ensure reproductive success. This complexity makes it difficult to identify critical genes and pathways involved in receptivity. On the basis of the present results, we believe that MAO-A is one of these critical genes. Lack of endometrial receptivity among infertile women in our study group is likely to be due to the poor expression of MAO-A and/or compromised expression of other genes regulated by MAO-A activity.

If MAO-A is a pivotal gene in implantation, it is a potential target for new strategies aimed at diagnosing and improving defective endometrial receptivity in infertile women. Further studies in a larger number of subjects should be performed to validate deficient levels of MAO-A expression in endometrial refractoriness to embryo implantation.

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