Endometrial leptin and leptin receptor expression in women with severe/moderate endometriosis

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The leptin system has been implicated in reproductive function, acting at endocrine and paracrine levels. Recently, deregulation of this gene family has been linked to endometrial changes caused by endometriosis. In the present study, we compare the expression of leptin receptor mRNA during the pre-receptive (LH+2) and receptive (LH+9) phases in the eutopic endometrium from patients with severe/moderate endometriosis (n=30) versus fertile controls (n=12). In each patient, two endometrial samples were obtained at LH+2 and LH+9 in their natural cycles. When real-time quantitative fluorescent PCR was performed, an up-regulation of OB-R L and all the isoforms investigated was observed at LH+9 versus LH+2 in patients with and without endometriosis. However, no difference was found in the expression pattern of the total leptin receptor OB-R, or in its long OB-R L and soluble HuB219.3 forms when the eutopic endometria of patients with severe/moderate endometriosis and fertile controls were compared. By means of in situ hybridization, total leptin receptor mRNA was localized in the luminal epithelium and the glands of the endometrium. The immunohistochemical analysis of the long form of leptin receptor was also performed in order to confirm these findings at the protein level. Finally, we have also shown similar leptin mRNA expression in both the control group and patients with endometriosis. In conclusion, we have not identified differences in the endometrial expression and localization of leptin and the leptin receptor when comparing the eutopic endometrium of women with severe/moderate endometriosis and fertile controls.

Key words: endometriosis/implantation/leptin/leptin receptor

Introduction

Embryonic implantation is a crucial event in reproductive success and is dependent on the interaction between the embryo and the receptive endometrium. Leptin, the product of the obese gene (ob), is a small pleiotropic peptide (16 kDa) composed of 146 amino acids that was initially related with homeostasis of energy and food consumption and fertility (González et al., 2000a). In ob/ob knockout mice, it was demonstrated that the deficiency in leptin synthesis is related to obesity and sterility (Zhang et al., 1994). The fertility of these animals is recovered with exogenous leptin treatment and not by food restriction, suggesting that leptin is necessary for normal reproductive function (Chehab et al., 1996). Such data are corroborated by other studies which have shown that leptin is crucial for the implantation process in the mouse (Malik et al., 2001).

There exist considerable data with respect to the leptin system’s probable involvement in the human embryonic implantation process. The expression of the leptin receptor in the human endometrium has been described (Alfer et al., 2000; González et al., 2000b; Kitawaki et al., 2000). Moreover, higher leptin concentrations are secreted by competent blastocysts than by arrested embryos (González et al., 2000b). Recently, our group confirmed that the endometrial OB-R increases in the late luteal phase and that human embryos possess OB-R mRNA throughout their preimplantation development, whereas leptin mRNA is expressed exclusively at the blastocyst stage (Cervero et al., 2004).

The leptin receptor is a class I membrane single-pass receptor of the cytokine receptor family, which presents several isoforms (Tartaglia et al., 1995). Their extracellular domains are identical, while their intracellular domains can be long (around 300 amino acids) or short (around 30 amino acids). The long form of the receptor, after binding leptin, activates transcription factors such as STATs, whereas the short forms are apparently incapable of triggering this pathway (Cunningham et al., 1999).

Endometriosis is an estrogen-dependent, benign gynaecologic and polygenetic disease with complex and multifactorial aetiologies, affecting 10–15% of women of reproductive age. Some researchers have reported that uterine receptivity can be adversely affected by this pathological situation (Yovich et al., 1988; Lessey et al., 1994; Arici et al., 1996). A previous study suggests an alteration of the endometrial leptin system in subfertile patients (Alfer et al., 2000), but the wider data on this matter are contradictory. Kitawaki et al. (2000) showed no difference in the leptin receptor mRNA expression when they compared endometriosis and control patients, whereas Kao et al. (2003) analysed eutopic endometrium in women with and without endometriosis and found that the leptin receptor was down-regulated in the former group. Regarding leptin, an increase in its expression has been reported in ectopic when compared to eutopic endometrium (Wu et al., 2002) and this molecule is significantly increased in the peritoneal fluid of patients with
Materials and methods

Patient subjects

The study group consisted of 15 patients who fulfilled the following inclusion criteria: at least 2 years’ primary infertility, stage III/IV endometriosis, regular cycles and normal male factor. Endometriosis was diagnosed by laparoscopy and histologically confirmed and classified according to the American Society of Reproductive Medicine classification (ASRM, 1997). The control group was composed of women with proven fertility and without endometriosis (n = 6). All patients were of reproductive age (23–37), with normal regular cycles, without other pathologies associated. BMI was comparable in the endometriosis and control groups (22.7 ± 3.6 and 21.3 ± 2.6, respectively). None of the subjects was receiving any form of hormone therapy. Informed consent was obtained from each woman and the project was approved by the Ethical Committee and Institutional Review Board on the use of human subjects in research at the University of Valencia and the Instituto Valenciano de Infertilidad.

Tissue specimens

Two endometrial samples were obtained from each patient at LH + 2 and LH + 9 during the natural menstrual cycle. The timing of the LH surge was detected using a urinary kit (Felcontrol, Efik SA, Madrid). A total of 42 biopsies were obtained from patients with (n = 30) and without (n = 12) endometriosis and dated according to Noyes et al. (1950). Human adipose tissue and placenta were used as positive controls, after being obtained from patients undergoing gynaecological surgery or following routine delivery. None of the subjects was receiving any form of hormone therapy. Informed consent was obtained from each woman and the project was approved by the Ethical Committee and Institutional Review Board on the use of human subjects in research at the University of Valencia and the Instituto Valenciano de Infertilidad.

Nested PCR of leptin in human endometrium

Total RNA was extracted from endometrial tissues and placenta using Trizol reagent (GibcoBRL, Life Technologies, Paisley, UK). First strand cDNA was reverse-transcribed from 1 μg RNA using a Moloney murine leukaemia virus reverse transcriptase and Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). The first PCR amplification was carried out with the following primers: 5'-CCAAAACCTTCATAGAAGC-3' (forward) and 5'-CACTCTC-TGGGAGTAGT-3' (reverse) for leptin and 5'-ATCTGGACCAACACCTCTC-TACAATAGCTTGCG-3' (forward) and 5'-CGTGAGGATCCTTATGAG-G TAGA-3' (reverse) for β-actin. PCR amplification was performed in a final reaction volume of 25 μL containing 2 μL of cDNA. Annealing temperature was 59°C for both β-actin and leptin, 30 cycles for the former and 40 for the latter. The second PCR for leptin detection was performed with 3 μL of the first PCR for 20 cycles at 59°C. For this round, the forward primer was as mentioned above and the reverse primer was 5'-CTGTAGAGAAGGCACGC-3'. PCR products were analysed by gel electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide. The PCR assay was repeated at least three times for each cDNA sample.

Table I. Oligonucleotide primers for real-time QF-PCR and their respective product size

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward GAAGGTGAGATGGTCAGTCT</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Reverse GAAGATGGTGATGATGTTCC</td>
<td></td>
</tr>
<tr>
<td>OB-R</td>
<td>Forward TGGAAAGGGTGAAAGGAAAACCA</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>Reverse TTAAGTCCTTGTCGCCAGGAA</td>
<td></td>
</tr>
<tr>
<td>HuB219.3</td>
<td>Forward CAAGAATTTGTCTTGGCACAA</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Reverse ACTGTGGAGAAGTGGGCACA</td>
<td></td>
</tr>
</tbody>
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Real-time, quantitative fluorescent PCR (real-time QF-PCR)

The experiments for real-time QF-PCR were performed with the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). In short, primers were designed using the Primer Express™ software provided with the aforementioned system to accomplish the manufacturer’s universal conditions. SYBR Green I double-stranded DNA binding dye was used as the assay chemistry. Total RNA (1 μg) was reverse transcribed using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). We employed 100 ng of cDNAs for each sample analysed. cDNA from placenta was used to obtain the standard curve. All real-time QF-PCR assays were carried out according to the manufacturer’s universal thermal cycling parameters and the final products were analysed with the provided software (sequence detector v 1.7). The primer-cDNA sequences and the sizes of the amplified fragments are listed in Table I. Each assay was performed in duplicate. Data were presented as the relative average value of the investigated gene and normalized with the average value of the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH). Melting curves were analysed to confirm amplification specificity.

Non-radioactive in situ hybridization

Total RNA from endometrium (1 μg) was reverse transcribed using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) and PCR amplified with specific primers in order to detect all isoforms of human OB-R mRNA (forward 5'-GGCCCAACCCCAAACTCA-3' and reverse 5'-GGTAAGACACAGGAGGA-3'). The PCR product (394 bp) was purified and inserted into the SrfI site of the pPCR-Script™ Amp SK (+) cloning vector using a commercial kit (Stratagene, La Jolla, CA, USA). Cloning DNA sequence and orientation were determined by sequencing using T3 and T7 primers. Digoxigenin cRNA sense and antisense probes were generated by either T3 (antisense) or T7 (sense) RNA polymerase-mediated transcription of linearized plasmids with HindIII and NotI. Paraffin-embedded sections of endometrial tissue were baked for 2 h at 60°C, dewaxed by means of two xylene baths and rehydrated in a series of alcohol solutions. A further bath with 0.2 M HCl and DEPC-treated water was then performed. Proteinase K digestion (10 mg/ml) was carried out at 37°C for 30 min and a further wash with 0.1 M triethanolamine and acetic anhydride (0.25% v/v) was subsequently performed. Sections were prehybridized at 42°C for 3 h with hybridization buffer containing 60% deionized formamide, 25 mM Tris–HCl pH 7.4, 1 mM EDTA pH 8.0, 0.4 mM NaCl, dextran sulphate (12% w/v), and Denhardt’s solution (1×). Following this, 100 μg/ml tRNA and 200 μg/ml salmon sperm DNA were added to the buffer. Hybridization was performed overnight in a hybridization buffer at 42°C with 0.1% (v/v) of 100 mM dithiothreitol stock solution, 1% (v/v) of a 10% sodium thiosulphate stock and 1% (v/v) of a 10% sodium dodecyl sulphate stock solution with 500 ng/ml sense and antisense probes. Slides were consecutively washed in 2× saline sodium citrate (SSC) at room temperature, 2× SSC at 42°C, 1× SSC, and finally 0.1× SSC. RNase A (20 μg/ml) digestion was carried out by shaking for 1 h at 37°C. Afterwards, 1× blocking solution (Roche Diagnostics, GmbH Mannheim, Germany) was added to buffer 1 pH 7.5 containing 100 mM maleic acid and 150 mM NaCl. Sections were incubated at room temperature for 2 h with alkaline phosphatase anti-DIG antibody (Roche Diagnostics, GmbH Mannheim, Germany, diluted 1:500) in 1× blocking solution with buffer 1. Colour development took place at room temperature for 3 h in buffer 3 (100 mM NaCl, 50 mM MgCl2, 100 mM NADH and 5 mg/ml nitroblue tetrazolium chloride) and 100 mM Tris–HCl pH 9.5.
Tris–HCl pH 9.5) containing 1% NBT/S-bromo-Δ-chloro-3-indolyl phosphate (Roche Diagnostics, GmbH Mannheim, Germany) (v/v) and 0.1% (v/v) of a 1 mM levamisole stock solution. Counterstaining with 0.1% methyl green was performed for 30 s. Sections were mounted using Kaiser’s glycerol gelatine (Sigma, Madrid, Spain). Photomicrographs were obtained using a digital camera (Coolpix 995; Nikon, Tokyo, Japan).

Immunohistochemistry
Endometrial tissues were formalin-fixed and paraffin-embedded and then sectioned (5 μm) and mounted on glass coated with Vectabond™ (Vector Laboratories, Burlingame, CA, USA). Following deparaffinization and rehydration with a graded series of ethanol, immunohistochemistry was performed using an LSAB peroxidase kit (DAKO Corp., Barcelona, Spain). In short, sections were incubated with 3% hydrogen peroxide at room temperature for 5 min in order to suppress endogenous peroxidase activity. After 20 min incubation with the linker, streptavidin-peroxidase was added for the following 20 min and the substrate–chromogen solution was used for 5 min to stain the slides. Subsequent to each incubation step, the tissues were washed three times with PBS 50 mM Tris–HCl buffer. Counterstaining was carried out with Mayer’s Hematoxylin and the slides were mounted with entellan (Merck, Darmstadt, Germany).

Statistical analysis
Data were expressed as mean ± SEM. Wilcoxon test—a non-parametrical, paired test—was used based on the number of subjects and the non-homogeneous feature of each group. The Mann–Whitney U-test was used for pair-wise comparisons of the control and study groups. Significance was defined in all cases as P < 0.05. Statistical analysis was performed using Graph-Pad Prism software version 3.0 (San Diego, CA, USA).

Results
Leptin receptor isoforms expression in eutopic endometrium at LH + 2 and LH + 9 in women with and without endometriosis using real-time QF-PCR
We have analysed the mRNA expression of the total leptin receptor (OB-R T), its long form (OB-R L) and the short isoform HuB219.3. Data are represented as a relative average value for each isoform studied and have been normalized according to the average value of the housekeeping gene (Figure 1).

A statistically significant increased expression was observed in receptive (LH + 9) versus pre-receptive (LH + 2) endometrium both in patients with and without endometriosis (P < 0.05) (Figure 1). This pattern was repeated when an individual analysis of each patient was carried out (data not shown). However, no statistical differences in leptin receptor expression was found between patients with and without endometriosis in the receptive endometrium (LH + 9), although a lower quantitative expression was noted during the pre-receptive phase (P < 0.05) (Figure 1).

Leptin receptor localization at the mRNA and immunoreactive levels at LH + 2 and LH + 9 in the eutopic endometrium of women with and without endometriosis
We performed a non-radioactive in situ hybridization on paraffin sections in order to corroborate and localize the presence of OB-R T mRNA in the endometrium. A clear positive signal was observed in the luminal and glandular epithelium of the endometrium at

Figure 1. Quantitative mRNA analysis of different isoforms of leptin receptor in human endometrium at LH + 2 and LH + 9 in patients with and without endometriosis. (A) OB-R T, (B) OB-R L, (C) HuB219.3 mRNA expression. Data were normalized with the GAPDH gene and are represented as relative average value. The experiment was performed in 15 endometrial samples for the endometriosis group and six for the control group in order to obtain the mean presented in the graphs. All isoforms showed the same expression pattern, with a significant increase (*P < 0.05) at LH + 9 compared to LH + 2 in both groups, with and without endometriosis. No differences exist in the expression of any isoform at LH + 9 between the endometriosis and control group. A lower expression is observed at LH + 2 in the endometriosis group compared with the control group (**P < 0.05).
The stromal cells are also stained but slightly less so. Magnification, £
the endometrial tissue. They are the expression controls when the endometriosis is both present (B sense probe, negative control of patients, with and without endometriosis, respectively. (Figure 2E,G,I,K). Sense and anti-sense β-actin probes were employed as controls of expression (Figure 2A–D).

We have also carried out immunostaining in order to localize the long form of the leptin receptor (OB-R_L) in the endometrium and to verify the immunoreactive pattern in patients with and without endometriosis. A similar expression pattern was found in both groups, with a strong signal in the luminal epithelium and a weaker signal in the stromal cells during LH + 2 (Figure 3d,h). A similar staining was also present during LH + 9, though slightly stronger (Figure 3f,j). There were no obvious morphological differences in the location of the expression between the groups. Adipose tissue was used as positive control for the expression of the leptin receptor (Figure 3b). Deletion of the first antibody was used as negative control (Figure 3a,c,e,g,i).

### Nested PCR to analyse the expression of leptin mRNA in the human endometrium

Due to the low abundance of this transcript, nested PCR was carried out to detect the leptin expression in the endometrium of patients with and without endometriosis. We found a clear band in all the samples analysed when the second PCR was performed (Figure 4). Placental mRNA was used as positive control (+). Water, rather than cDNA, was the negative control (−). The mRNA quality was verified based on the β-actin expression and no obvious differences were observed.

**Figure 2. In situ hybridization of OB-R_E in the eutopic endometrium of patients with and without endometriosis at LH + 2 and LH + 9. (A and C): β-actin sense probe, negative control of patients, with and without endometriosis, respectively. (B and D): β-actin antisense probe, and showing a signal present in all the endometrial tissue. They are the expression controls when the endometriosis is both present (B) or absent (D). Pictures E, G, I, K correspond to the OB-R_E sense probe in patients with (E–I) and without (G–K) endometriosis at LH + 2 (E,G) and LH + 9 (I,K). OB-R_E antisense probe at LH + 2 (F,H) and LH + 9 (J,L) in endometria with (F,J) and without (H,L) endometriosis. A clear positive signal can be observed in the luminal and glandular epithelium (see arrows). The stromal cells are also stained but slightly less so. Magnification, ×200.**

**Discussion**

In this study, we have meticulously compared the mRNA expression and localization of leptin and its receptors in pre-receptive and receptive endometrium in infertile patients with moderate/severe endometriosis (III/IV) with fertile women. We provide evidence that leptin is expressed at significantly low levels, while its receptors OB-R_F, OB-R_L and OB-R_S are considerably expressed and up-regulated in receptive versus pre-receptive endometrium. However, no differences were observed in the leptin system in the eutopic endometrium during the window of implantation in moderate/severe endometriosis versus that of disease-free, fertile patients.

In the course of the design of this study, efforts have been undertaken to avoid any methodological bias. The BMI was normal in all patients and comparable in both endometriosis and fertile groups. Endometriosis was accurately diagnosed via laparoscopy and histological examination, and all other known causes of infertility, such as endocrine or male factor, were ruled out. All endometrial samples were obtained from ovulatory cycles, confirmed by urinary LH measurement, ultrasonographic confirmation and histological dating. Furthermore, each patient constituted her own control, since endometrial biopsies were obtained at LH + 2 and LH + 9 of the same cycle.

Three different groups (Alfer et al., 2000; Gonzalez et al., 2000a,b; Kitawaki et al., 2000) detected endometrial leptin receptor at the mRNA and protein level. Nevertheless, results regarding mRNA leptin expression were discordant. In 2002, it was reported that leptin expression increased in ectopic endometriotic lesions, whereas, in eutopic endometrium, the signal was not always detected (Wu et al., 2002). In the present study, we have found that leptin mRNA is clearly expressed in the eutopic endometrium of patients both with and without endometriosis, but only when nested
PCR is employed. Therefore, leptin is expressed in the human endometrium at very low levels, a finding that confirms our previous results (González et al., 2000b; Cervero et al., 2004). Considering the negligible presence of endometrial leptin, other sources of leptin, such as endocrine or embryonic leptin, may also drive the endometrial receptor activation.

We observed, through real-time QF-PCR, that the total leptin receptor, and its long form and a short isoform, are expressed in a similar pattern in both infertile patients with moderate/severe endometriosis and fertile women. A statistically significant up-regulation was observed in the expression of all leptin receptor isoforms studied in receptive (LH + 9) when compared to pre-receptive endometrium (LH + 2). These results suggest that, in this pathological situation, the expression of the leptin receptor is not modified in the endometrium during the endometrial receptive phase (at least at LH + 9).

It was possible to validate real-time QF-PCR data for the total leptin receptor (OB-R_T) and locate its expression morphologically using non-radioactive in situ hybridization. We identified maximum expression in the luminal and glandular epithelium and a minor signal in stromal cells in both groups. Immunohistochemistry analysis also showed an OB-R_L protein pattern similar to that of its mRNA, with a more intense signal in the luminal and glandular epithelium and a weaker signal in the stroma. Additionally, we noted a higher

**Figure 3.** Immunohistochemical localization of OB-R_L in human endometrium, with and without endometriosis. Adipose tissue, as positive control (a) and without primary antibody (b). OB-R_L immunostaining is predominant in the luminal epithelial cells (see arrows) when endometriosis is both present (d,f) and absent (h,j). The positive signal is slightly higher at LH + 9 (f,j) than at LH + 2 (d,h) in both groups. (c,e,g,i) Corresponding negative controls with deletion of the primary antibody. Magnification, ×200.
expression during the receptive phase (LH + 9) in both groups, although this measurement was limited due to the semi-quantitative value of this technique. These studies reveal that there are no differences in the morphological localization of the leptin receptor in the fertile and endometriosis group.

Using microarray DNA technology, Kao et al. (2003) compared endometriosis patients with fertile subjects and reported that certain genes were up- or down-regulated during the implantation window in the former group. The leptin receptor was among those genes that were down-regulated in endometriosis. The discrepancies of these results with our data may be due to the difference in the stage of endometriosis studied; while patients with mild/moderate endometriosis were used in the Kao et al. study, we analysed women with moderate/severe endometriosis. In fact, it has been reported that leptin levels in peritoneal fluid are higher in women with minimal endometriosis than in those at a more advanced stage of the disease (Mahutte et al., 2003). Therefore, leptin receptor expression would seem to be affected by the progression of the disease. Moreover, we have only studied infertile patients, whereas Kao et al. did not mention whether the patients investigated were fertile or infertile. The different methodological approaches could be another reason.

To summarize, there is an increase in the leptin receptor expression at both protein and mRNA levels in receptive versus pre-receptive endometrium in infertile women with a moderate/severe endometriosis, and also in fertile controls. Moreover, leptin mRNA is expressed in the endometrium of both subject types. All these findings lead to the conviction that the leptin system remains unaffected during the implantation window in moderate/severe endometriosis, at least with respect to mRNA and protein expression. Further studies are necessary to demonstrate whether the functionality of the receptor is affected in these conditions.

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References

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