Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene up-regulation

Meng-Hsing Wu¹, Pei-Ching Chuang², Hsiu-Mei Chen², Chen-Chung Lin² and Shaw-Jenq Tsai²,³

¹Institute of Clinical Medicine and Department of Obstetrics and Gynecology and ²Department of Physiology, College of Medicine, National Cheng-Kung University, Tainan 70101, Taiwan, R.O.C.

³To whom correspondence should be addressed. E-mail: seantsai@mail.ncku.edu.tw

Endometriosis is a polygenic disease with complex, multifactorial aetiologies affecting ~10% of women of reproductive age. Leptin is the product of the ob gene, which is related to reproductive function and immunological alteration. The angiogenic and mitogenic action of leptin may influence the formation of endometriosis. This study was aimed at determining whether leptin and leptin receptor expression differs in eutopic and ectopic endometria collected from laparoscopy and at investigating the pathophysiological role of leptin in the development of endometriosis. Leptin mRNA was undetectable in seven out of 14 eutopic endometria and only a minute amount was detected in the remaining samples. In contrast, there was a marked increase in leptin mRNA and protein expression in ectopic endometriotic lesions of patients with endometriosis (P < 0.05). Receptors for leptin were immunologically stained in eutopic endometrium as well as in ectopic endometriotic implants. However, the levels of mRNA for the long and total forms of leptin receptors were suppressed in association with the severity of endometriosis (P < 0.05). Administration of leptin stimulated its own mRNA expression in ectopic endometriotic stromal cells but decreased steady-state concentrations of mRNA encoding for leptin receptor (n = 6). In addition, leptin significantly enhanced both eutopic and ectopic endometrial stromal cell proliferation (P < 0.05). In conclusion, the differential distribution of mRNA for leptin and its receptor suggests an important autocrine and paracrine role for leptin in human endometriosis. The mitogenic and auto-augmentation effects of leptin may further contribute to the pathogenesis of endometriosis.

Key words: endometriosis/leptin/leptin receptor/quantitative RT–PCR/[³H]thymidine incorporation

Introduction

Endometriosis is a common disease that occurs primarily in women of reproductive age and is considered to be a disease with complex, multifactorial aetiologies. Several hypotheses have been proposed for the development of endometriosis. However, the exact mechanisms responsible for the development of this disorder are still unclear. Pelvic endometriosis is associated with overexpression of numerous factors such as steroids, prostaglandins, angiogenic factors and cytokines (Gonzalez et al., 2000b). Elevated 17β-estradiol concentrations in peritoneal fluid of patients with endometriosis has been documented (Lesorgen et al., 1984). This may be due to aberrant expression of steroidogenic acute regulatory protein (StAR) and aromatase in endometriotic lesions (Bulun et al., 1997a; Tsai et al., 2001a). In addition, defects in both cellular and humoral immunity may allow ectopic endometrium to implant and grow in the peritoneal cavity. For example, increased proinflammatory cytokines, such as interleukin (IL)-1, IL-8 and tumour necrosis factor-α in peritoneal fluid have been reported in women with endometriosis (Koninckx et al., 1998). Peritoneal macrophages isolated from women with endometriosis have been found to have relatively lower cytotoxic capacity and higher cytokine- and prostaglandin-secreting ability (Dunseldan et al., 1988; Braun et al., 1992; Karck et al., 1996), which may further stimulate the progression of the disease.

Leptin, the obese (ob) gene product, is a small peptide molecule synthesized and secreted mainly by adipocytes (Zhang et al., 1994). Serum leptin levels are dynamically regulated mainly through the brain leptin receptor (OB-R) (Tartaglia et al., 1995). Although there are at least four alternative mRNA splicing variants of OB-R, OB-R₂ is the primarily active signal-transducing isoform and is capable of activating the JAK-STAT signalling pathway (Cioffi et al.,...
Leptin and leptin receptor in endometriosis

1996). Expression of leptin and OB-R is not restricted to adipocytes but is also detected in other tissues including those of the peripheral reproductive system, such as placenta and ovary (Cioffi et al., 1997). In the uterus, leptin and its receptor have also been detected in endometrium with levels of the functional OB-Rβ fluctuating during the menstrual cycle (Alfer et al., 2000; Gonzalez et al., 2000a; Kitawaki et al., 2000). Functionally, leptin has been found to enhance the estrogen-producing capability of luteinized human granulosal cells (Kitawaki et al., 1999) and elevated leptin levels have been shown to be associated with an earlier onset of menarche (Matkovic et al., 1997). These findings suggest that leptin may play important roles in the endocrine, paracrine and autocrine regulation of reproduction. Recently, the association of leptin and endometriosis has been investigated. A significant increase in leptin levels in peritoneal fluid of patients with endometriosis was observed (Matarese et al., 2000), but serum leptin levels are either elevated or unchanged (Matalliotakis et al., 2000; Matarese et al., 2000). In addition, the peritoneal leptin concentration was found to increase in women with peritoneal but not ovarian endometriosis, suggesting that different pathogenic mechanisms are involved in the development of these two forms of the disease (De Placido et al., 2001).

Although there is a potential novel role for leptin in the aetiopathology of endometriosis, it is not clear whether the peritoneal leptin is synthesized by ectopic endometriotic tissues or by other cell types and whether there is a functional role for peritoneal leptin in the development of ectopic endometriosis. To investigate the importance of leptin in the pathogenesis of endometriosis, we examined the leptin and OB-R expression profile in ectopic and eutopic endometria. In addition, we further explored the biological function of leptin with a cell proliferation assay of stromal cells to characterize the relationship between leptin expression and the development of endometriosis.

**Subjects and methods**

**Patient subjects**

Ectopic endometriotic samples of patients with endometriosis (n = 25) and eutopic endometrial tissues from disease-free patients of reproductive age undergoing hysterectomy for leiomyoma or ovarian pathology (n = 14) were collected at the time of laparoscopy or laparotomy at the Department of Obstetrics and Gynecology, National Cheng-Kung University Hospital. On four occasions, eutopic endometrium, peritoneal endometriotic lesions, and ovarian endometrioma were collected from the same patient. Endometriosis was classified according to a published classification (American Society of Reproductive Medicine, 1997) and was histologically confirmed. Stages I and II were combined as early endometriosis and stages III and IV were combined as severe endometriosis. None of the patients was receiving any hormone therapy, such as GnRH analogue, danazol, or pseudopregnancy therapy. There was no significant difference in the mean body mass index (BMI) among endometriosis and the otherwise disease-free (control) groups (22.76 ± 0.86, 21.48 ± 0.56 and 20.6 ± 0.51 for control, early endometriosis, and severe endometriosis respectively). The following cases were pre-excluded from the study: malignant neoplasms other than cervical carcinoma in situ, ovarian neoplasms, pelvic inflammation, and pregnancy. The tissues were immersed in Hanks’ solution supplemented with HEPES and antibiotics for further treatment. Informed consent was obtained from each woman and the protocol was approved by the Clinical Research Ethics Committee at the National Cheng-Kung University Medical Center.

**Isolation of endometrial stromal cells from eutopic and endometriotic tissues**

Isolation of endometrial stromal cells from uterine endometrium was achieved by using a published protocol (Ryan et al., 1994) with modifications. In brief, eutopic endometrium was obtained from the biopsies of hysterectomy specimens. The tissue samples were rinsed with phosphate-buffered saline (PBS), minced, and digested with type IV collagenase (2 mg/ml) at 37°C for 60 min with agitation. Stromal cells were separated from epithelial glands by filtration through narrow gauge sieves of 70 and 45 mesh. Filtrated cells were plated and allowed to adhere on plastic dishes for ~30 min, after which blood cells and debris were washed off by rinsing with PBS. The purity of the cells was determined by immunostaining with vimentin (stromal cell specific) and cytokeratin (epithelial cell specific). Stromal cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C until further assays.

**RNA isolation and construction of native and competitor plasmids for quantitative (QC)-RT–PCR**

Total RNA was extracted from eutopic and ectopic endometrial samples using an RNeasy mini kit according to the manufacturer’s protocol (Qiagen, http://www.qiagen.com) and the first strand complementary DNA (cDNA) was synthesized from total RNA using standard procedures. The resulting first strand cDNA was used for PCR amplification with the primers listed in Table I. The primer sequences and PCR product size for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported previously (Tsai et al., 2001b).

In order to evaluate mRNA quantitatively, native and competitive plasmids for in-vitro transcription of native and competitive RNA were prepared as described previously (Tsai et al., 2001b). We had cloned partial human leptin, OB-Rβ (total forms) and OB-Rβ cDNA by RT–PCR. The PCR products were then subcloned into PCR II vector (Invitrogen) and the sequences were determined. From these plasmids (which contained the native sequences), we deleted a portion of the internal region by a PCR method as previously described (Tsai et al., 2001a) and subcloned these shorter cDNA (the competitors) into PCR II vector. Previously constructed human GAPDH native and competitor plasmids were used for internal control of the QC–RT–PCR assay (Tsai et al., 2001b).

**Evaluation of standard curve QC–RT–PCR**

The procedure of standard curve QC–RT–PCR for measuring mRNA from small samples has been described previously (Tsai and Wiltbank, 1996, 1998b). Samples of test RNA or native RNA were combined with competitor RNA and reverse-transcribed. The resulting cDNA samples were then amplified by PCR with appropriate programmes using primers for human leptin, OB-Rβ and OB-Rβ genes, followed by a final 5 min of extension step at 72°C. The PCR products were separated on a 5% acrylamide gel with electrophoresis and visualized by ethidium bromide staining to verify the PCR products. The expected 473, 478 and 463 bp PCR products were detected for the leptin, OB-Rβ and OB-Rβ respectively. The lengths of competitors were 368, 329 and 346 bp for leptin, OB-Rβ and OB-Rβ respectively. The resulting images were analysed using AlphaImager™ software (Alpha Innotech Corp., San Leandro, CA, USA). A standard curve...
was produced by the logarithmic ratio of native to competitor bands against the initial amounts of native RNA used in the RT–PCR. The concentrations of specific mRNA transcripts were obtained by comparison of ratio of band intensity with the standard curve (Figure 1).

**Cell culture and treatment**

After subcultured stromal cells (2×10⁴ cells/well) from eutopic or ectopic endometrium reached 70% confluency in 24-well plates, the cells were incubated under serum-free medium for 12 h. Cells were then treated with various doses of leptin (from 0.1 ng/ml to 10 ng/ml) for 24 h in the presence of 10% FBS. The cells were then lysed in the well using lysis buffer and subjected to the isolation of mRNA by the procedure described previously (Tsai and Wiltbank, 1996, 1998b). Steady-state concentrations of mRNA encoding for leptin, total form leptin receptor (OB-RT), OB-R L and GAPDH were quantified using standard curve QC-RT–PCR (Tsai and Wiltbank, 1996, 1998b).

**Cell treatment and [³H]thymidine incorporation assay**

Subcultured stromal cells were deprived of serum for 12 h and then treated with different doses of leptin (0.1–10 ng/ml) for 48 h in the presence of 10% FBS. Cells were then incubated with [³H]thymidine (0.5 mCi/ml) for 24 h and then washed twice with PBS. After the addition of ice-cold 10% trichloracetic acid (TCA) for 20 min, the TCA was removed and cells were washed with PBS. The acid-insoluble fractions were dissolved by the addition of 1 mol/l NaOH. The contents were then neutralized with an equal volume of 1 mol/l HCl to a final concentration of 0.5 mol/l. Aliquots of 500 µl were transferred to a scintillation vial containing 3.5 ml counting fluid (Ready safe; Beckman). The radioactivity was measured in a liquid scintillation counter.

**Western blotting**

Tissues were homogenized under Tris–sucrose–EDTA buffer (10 mmol/l Tris, 250 mmol/l sucrose, and 0.1 mmol/l EDTA, pH 7.4) and centrifuged at 600 g for 30 min at 4°C to remove debris. Protein concentrations were determined by a published method (Lowry et al., 1951). Samples of 50 µg of protein were loaded into each lane, separated on a 14% sodium dodecyl sulphate (SDS)–polyacrylamide electrophoretic gel, and transferred onto a PVDF membrane (Millipore Co., Bedford, MA, USA). Non-specific binding was blocked by immersing the membrane in 5% skimmed milk at 4°C overnight. The membrane was then incubated with goat anti-leptin polyclonal

---

**Table I. Sequences of primers used and sizes of PCR product of native and competitor sequences for leptin, total form leptin receptor (OB-RT), and long form leptin receptor (OB-RL)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>PCR length (bp)</th>
<th>GenBank no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>hOB-F</td>
<td>5'-GAACCCCTGTGGGATTC-3' (50–68)</td>
<td>473</td>
<td>XM_004625</td>
</tr>
<tr>
<td>Native</td>
<td>hOB-R</td>
<td>5'-CAGCTGACAGACATGCAGTCTC3' (522–505)</td>
<td>478</td>
<td>U66497</td>
</tr>
<tr>
<td>Competitor</td>
<td>hOB-IR</td>
<td>5'-CAGCTGACAGACATGCAGTCTC3' (522–505)</td>
<td>368</td>
<td></td>
</tr>
<tr>
<td>OB-RT</td>
<td>OB-RT-F</td>
<td>5'-CCACCAATTGGTACCCATTCC-3' (904–923)</td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>OB-RT-R</td>
<td>5'-GCATTGACATGTCAGCAG-3' (1381–1362)</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td>Competitor</td>
<td>OB-RT-IR</td>
<td>5'-GGCATTCATGTTCATTGCAG-3' (2771–2790)</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td>OB-RL</td>
<td>OB-RL-F</td>
<td>5'-GGCATTCATGTTCATTGCAG-3' (2771–2790)</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>OB-RL-R</td>
<td>5'-AATGCCTGGCGCTTCATCTC3' (3233–3214)</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td>Competitor</td>
<td>OB-RL-IR</td>
<td>5'-AATGCCTGGCGCTTCATCTC3' (3233–3214)</td>
<td>463</td>
<td></td>
</tr>
</tbody>
</table>

**Table II. Days of menstrual cycle at the time when tissues were collected**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases in phases of menstrual cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early follicular (days 1–7)</td>
</tr>
<tr>
<td>Eutopic</td>
<td>5</td>
</tr>
<tr>
<td>Early (stages I and II)</td>
<td>3</td>
</tr>
<tr>
<td>Severe (stages III and IV)</td>
<td>4</td>
</tr>
</tbody>
</table>

---

**Figure 1. Standard curve QC-RT–PCR using long form leptin receptor as an example. (A) Ethidium bromide-stained PCR products for the long form leptin receptor (OB-RT); (B) standard curve produced from analysing the intensity of bands shown in (A). In the presence of 0.5 amol of competitor RNA, 2-fold serial dilutions of native RNA (OB-RL, 6.4–0.05 amol) was reverse-transcribed and PCR-amplified. The band intensity was quantified by Alphalmager computer software and used to construct the standard curve shown in (B). The inset in (B) shows two samples that were RT–PCR-amplified in the presence of the same amount of competitor. The ratios of band intensity in lanes 1 and 2 were logarithmically transformed and compared to the standard curve (solid line for lane 1 and dashed line for lane 2) to calculate the concentration of the transcripts (vertical lines cross x-axis). M = DNA molecular weight marker.
antibody (R&D Systems, Minneapolis, MN, USA) at a 1:1000 dilution for 1 h at 37°C. After washing with TBST (10 mmol/l Tris, pH 8.0, 150 mmol/l NaCl, 0.05% Tween-20) for 3×10 min, the membrane was further incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma, St Louis, MO, USA) at a 1:25 000 dilution for 1 h at room temperature. The membrane was washed for 1 h with TBST and detected by ECL (Amersham, Life Science, Little Chalfont, Bucks, UK). The blots were then stripped with stripping buffer (100 mmol/l 2-mercaptoethanol, 2% SDS, and 62.5 mmol/l Tris–HCl, pH 6.7) and re-detected as described above, except that mouse anti-β-actin monoclonal antibody (Amersham) and horse-radish peroxidase-conjugated goat anti-mouse IgG (Sigma) were used.

**Immunohistochemical staining**

Paraffin-embedded tissues were sectioned at 5 µm thickness and mounted onto poly-lysine-coated slides, deparaffinized and re-hydrated. Tissue sections from eutopic endometrium, early lesions and severe lesions were stained simultaneously throughout all immunostaining procedures. Tissue sections were rinsed three times (5 min each) in PBS containing 0.4% Triton X-100 (PBST) prior to blocking with 10% normal bovine serum for 15 min at room temperature. The sections were again rinsed in PBST solution and incubated with primary antibody (goat anti-human leptin R; R&D Systems, Cat. no. AF389) at a concentration of 5 ng/µl (overnight at 4°C). A negative control was included by omission of the primary antibody or by using an equal concentration of rabbit immunoglobulin as primary antibody. Following incubation with the primary antibody, the tissue sections were rinsed three times (5 min each) in PBST and then incubated with a biotinylated rabbit anti-goat immunoglobulin (1:500) for 60 min at room temperature. The sections were then quenched of endogenous peroxidase activity (3% H2O2 in PBST at 1:500) for 60 min at room temperature. The sections were then incubated with a biotinylated rabbit anti-goat immunoglobulin (1:500) for 60 min at room temperature. The colour reaction was precipitated using 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA, USA) for 60 min at room temperature. The tissue sections were again rinsed in PBST solution and counterstained with haematoxylin.

**Statistical analysis**

The data were expressed as mean ± SEM. Concentration differences in a given mRNA transcript between groups were analysed with one-way analysis of variance (ANOVA). Multiple comparisons were performed using Tukey’s multiple comparison procedure, once significance was found by the F-test.

**Results**

**Expression of leptin in eutopic endometrium and ectopic endometriotic lesion**

The basal concentrations of mRNA encoding for leptin were very low or even undetectable in seven out of 14 samples of eutopic endometrium. There was a significant increase in leptin mRNA expression in ectopic lesions of patients with endometriosis, both in the early and severe stages, as compared with the eutopic endometrium (Figure 2A). There was also no detectable amount of leptin transcripts in four eutopic endometria collected from women with endometriosis (Figure 2B). However, the peritoneal implants and ovarian endometrioma collected from the same individuals expressed high levels of leptin transcripts (Figure 2B). Western blot analysis detected very little or no leptin in eutopic endometrium and marked increases in the leptin protein in ectopic endometriotic implants (n = 4, Figure 2C).

**Expression of OB-R in eutopic endometrium and ectopic endometriotic lesion**

Immunohistostaining clearly identified expression of OB-R in both epithelial cells and stromal cells in eutopic endometrium (Figure 3A). Expression of OB-R was also seen in ectopic endometriotic stromal cells of early stage (Figure 3C) and severe stage endometriosis (Figure 3E). Negative stains with normal bovine serum or rabbit immunoglobulin instead of first antibody show no non-specific staining (Figure 3B, D, F). Since the antibody used cannot distinguish different splicing variants of OB-R, we used QC-RT–PCR to determine amounts of transcripts encoding for the long form and total form of OB-R. Steady-state concentrations of mRNA encoding for
OB-R<sub>T</sub> were significantly suppressed in ectopic endometriotic stromal cells compared with eutopic endometrium (Figure 4A). The decrease was associated with the severity of endometriosis. The expression of OB-R<sub>L</sub> transcripts was also decreased in ectopic endometriotic cells in a pattern similar to that in OB-R<sub>T</sub> (Figure 4B). There was no significant difference in OB-R<sub>T</sub> expression between stromal and epithelial cells in eutopic endometria (Figure 5). However, steady-state concentration of mRNA encoding for OB-R<sub>L</sub> was greater in stromal cells than in epithelial cells obtained from eutopic endometria (Figure 5).
Leptin and leptin receptor in endometriosis

Figure 5. Expression of total form leptin receptor (OB-RT) and long form leptin receptor (OB-RL) in stromal (S) and epithelial (E) cells obtained from eutopic endometrium (n = 8 batches of cells). (A) Representative photograph from two sets of samples collected from the same individual. (B) Mean concentrations of mRNA transcripts. The asterisk indicates a significant difference between stromal cells and epithelial cells for the long form leptin receptor (P < 0.05).

Effect of leptin on OB-R and leptin mRNA expression
The elevation of leptin and suppression of OB-R transcripts in ectopic endometriotic cells prompted us to evaluate the effect of leptin on its own receptor. Treatment of stromal cells isolated from eutopic endometria with different doses of leptin resulted in decreases in OB-RT transcripts (Figure 6A). The inhibition was >50% at the concentration of 10 ng/ml. The OB-RL splicing variant was also inhibited by treatment with leptin in a pattern identical to that of OB-RT (Figure 6B). Similar inhibition pattern of OB-RT and OB-RL by leptin was also seen in ectopic endometriotic stromal cells (n = 3, data not shown).

We further evaluated the effect of leptin on its own mRNA transcripts. As has been shown previously, eutopic endometrial stromal cells expressed barely detectable amounts of the leptin transcript. Treatment of eutopic endometrial stromal cells with leptin failed to stimulate mRNA expression (data not shown).

In contrast, cultured endometriotic stromal cells expressed high levels of leptin (6.8 ± 0.5 amol/µg mRNA). Addition of leptin caused a significant increase in leptin mRNA in stromal cells isolated from ectopic endometriosis in a dose-dependent manner (Figure 7). The mRNA encoding for GAPDH, a housekeeping gene, was not affected by treatment with leptin (data not shown).

Effect of leptin on stromal cell proliferation
The biological role of leptin was evaluated by measuring its mitogenic capability in cultured eutopic and ectopic endometrial stromal cells using [3H]thymidine incorporation. Figure 8 shows that leptin stimulated a significant increase in eutopic as well as ectopic endometrial stromal cell proliferation. However, this mitogenic effect of leptin was somewhat different in eutopic endometrial stromal cells compared with ectopic endometriotic stromal cells. In eutopic endometrial stromal cells, leptin caused a greater extent of cell proliferation and at much lower doses (Figure 8A). In stromal cells obtained from ectopic endometriotic implants, only high doses of leptin (3 and 10 ng/ml) induced cell proliferation and the induction was less pronounced (Figure 8B).
Leptin is known as the protein primarily released by fat cells and it acts in an endocrine fashion via the leptin receptor in the hypothalamus to regulate satiety status. Recently, the role of leptin has become more and more versatile after the discovery of leptin and its receptor in peripheral tissues including ovary, uterus and placenta (Cioffi et al., 1997; Karlsson et al., 1997; Masuzaki et al., 1997; Alfer et al., 2000; Gonzalez et al., 2000a; Kitawaki et al., 2000). Leptin has been reported to exert immunoregulatory, proinflammatory, mitogenic and angiogenic effects in several tissues (Gainsford et al., 1996; Wolf et al., 1999; Caprio et al., 2001). This makes it a potential candidate for contributing to the progress of endometriosis. A recent report even demonstrated that leptin levels in peritoneal fluid and serum of patients with pelvic endometriosis are increased (Matarese et al., 2000). However, the cellular origin and mechanism by which leptin modulates the formation of endometriosis is not clear. We herein present evidence showing that leptin and its receptor are differentially expressed in endometriosis and are involved in the proliferation of endometrial stromal cells.

By using quantitative, competitive RT–PCR and Western blot analysis, we showed that both leptin transcripts and protein are highly expressed in ectopic endometriotic lesions. In eutopic endometrium, leptin was not detected in a half of the samples and only extremely low amounts of leptin were detected in the other half of the endometria. In concordance with our finding, contradictory reports have shown either positive or negative leptin expression in normal human endometrium (Alfer et al., 2000; Gonzalez et al., 2000a; Kitawaki et al., 2000). The reasons for differences in leptin transcript expression in eutopic endometrium are not known. Nevertheless, leptin was highly expressed in ectopic endometriotic lesions. The elevation of leptin in ectopic endometriosis was not due to differences in the stages of menstrual cycles or body mass as evidenced by marked increase of leptin in ectopic endometriotic tissues as compared to the eutopic endometrium collected from the same patients (n = 4). In addition, the mean BMI was not different between eutopic and endometriosis groups. Thus, elevated expression of leptin in ectopic endometriotic tissues may reflect the distinct biochemical nature of endometriotic lesions. Whether this is causal or consequent to the pathogenesis of pelvic endometriosis remains to be determined.

Our result showing that leptin is markedly expressed in ectopic endometriotic lesions supports previous reports that the peritoneal fluid concentration of leptin was increased in women with endometriosis (Matarese et al., 2000; De Placido et al., 2001). Furthermore, peritoneal macrophages purified from patients with peritoneal endometriosis, ovarian endometrioma, or without any pelvic implants did not express leptin as analysed by RT–PCR (n = 6 per group, data not shown), indicating that the ectopic endometriotic lesion may have an important contribution to the elevated leptin concentration in peritoneal fluid. However, the contribution made by peritoneal fat cells should not be excluded and warrants further investigation. One report (De Placido et al., 2001), showing that peritoneal fluid concentrations of leptin are elevated in patients with peritoneal endometriosis but not ovarian endometriosis, has raised questions regarding which determinants cause the differences. It is hypothesized that these two kinds of endometrial lesions may have distinct leptin biosynthesizing capacity. In that particular report, the cellular origin of leptin contributing to the peritoneal fluid was not examined. In the present report, we have detected the leptin transcript and protein in both peritoneal implants and ovarian endometrioma and the quantity of leptin transcripts was not different between these two groups. Thus, it appears that a distinct leptin biosynthesis capacity may not be the determinant leading to differential leptin concentrations in peritoneal fluid with these two kinds of endometriosis. Alternatively, the authors suggested that a possible explanation is that leptin may be sequestered into the cystic fluid of the endometrioma instead of being diffused into peritoneal fluid. This seems likely given our current result showing that similar amounts of leptin were produced. However, more direct evidence is needed before any conclusion can be drawn.

To address whether the elevation of leptin expression in endometriotic implants would be involved in development of endometriosis via autocrine/paracrine mechanisms, we next characterized the expression profile of the leptin receptor in eutopic as well as ectopic endometriotic implants. Immunohistostaining showed that OB-R is expressed in both stromal...
and epithelial cells in eutopic endometrium. In ectopic endometriotic implants, OB-R was also positively stained. Using quantitative assays, we found that both OB-R_1 and the functional OB-R_1 transcripts were detected in the eutopic as well as ectopic endometria and in both stromal and epithelial cells throughout the entire menstrual cycle. Our results are mostly consistent with a previous report by Alfer et al., but slightly different in that they found that OB-R_1 was strongly stained in stromal cells of mid–late luteal phase while the staining was weak during follicular phase (Alfer et al., 2000). Using semi quantitative RT–PCR, another study reported a fluctuation of OB-R in endometrium during the menstrual cycle, but the peak concentration was found to be in the early luteal phase (Kitawaki et al., 2000). Furthermore, a recent work showed that progesterone but not estradiol reduced OB-R_1 expression in endometrium (Koshib et al., 2001), indicating that high concentrations of progesterone in the luteal phase may suppress the expression of OB-R_1. The reasons for the discrepancies between these studies are not clear since different kinds of antibodies and/or quantification methods were used. Thus, the expression pattern of OB-R during the menstrual cycle cannot be definitely concluded at this point.

The expression of the OB-R transcript in this study was inversely related to the expression pattern of leptin and the severity of endometriosis. We believe that this is due, at least in part, to the down-regulation effect of leptin on its own receptor. Indeed, the in-vitro study using eutopic endometrial stromal cells demonstrated that leptin dose-dependently inhibited the mRNA for its receptor (both the long form and total forms of leptin receptor). The physiological significance of receptor down-regulation by its homologous ligand is a safeguarding system that prevents overstimulation by the ligand, as has been reported for prostaglandin F_2α and its receptor (Tsai and Wiltbank, 1998a; Tsai et al., 1998). Whether this down-regulation of OB-R by leptin plays any significant role in disease progression remains an open question and requires further investigation.

The addition of leptin to the culture medium stimulated growth of endometrial stromal cells, suggesting that leptin may be involved in the development of endometriosis by inducing stromal cell proliferation. This may increase the chance of survival in the peritoneum, and, to some degree, contribute to the formation of endometriosis. The finding that low concentrations of leptin (0.1–1 ng/ml) failed to stimulate ectopic endometriotic stromal cell proliferation may be due to one (or both) of the following two reasons. The first explanation could be that the low levels of leptin receptor in ectopic endometriotic lesion provide fewer binding sites for leptin and thus limit the mitogenic effect of leptin. Hence, only a higher dose of leptin can have enough binding to its receptor to exert its action. Alternatively, endogenous production of leptin may already act on these cells even without the addition of exogenous leptin. Thus, one would anticipate that the basal level of [H]thymidine incorporation is greater and the mitogenic effect of leptin would not be apparent unless the leptin-induced cell proliferation rate is much higher.

One of the most intriguing findings of our current study is that leptin stimulates its own mRNA expression in ectopic endometriotic stromal cells. To our knowledge, there has been no previous report showing that leptin can stimulate its own mRNA expression. This autoamplification effect of leptin was only seen in ectopic endometriotic stromal cells but not in eutopic endometrial stromal cells, indicating again that the biochemical natures of eutopic endometria and ectopic endometriotic implants are different. As we have learned from the study of endometriosis, the microenvironment of the peritoneal cavity plays an important role in the process of establishment of ectopic endometrium, especially during early stages of endometriosis. Retrograde endometrial cells, after exposure to potent stimulators in the peritoneal fluid such as insulin-like growth factor-1 or leptin or adipocyte origin, appear to undergo differentiation and acquire the capacity to produce leptin. This provides a self-supporting system for growth of pelvic endometrial cells. It is generally believed that ectopic endometriotic tissues are capable of evolving similar self-supporting systems in order to survive under hostile environments such as pelvic peritoneum. One of the examples is the acquisition of estrogen-producing ability in ectopic endometriotic implants (Noble et al., 1996; Bulun et al., 1999, 2000). Our recent data have also indicated that ectopic endometriotic cells of early endometriosis express high quantities of steroidogenic acute regulatory protein and produce high levels of progesterone (Tsai et al., 2001c). As a consequence, the ectopic endometriotic tissues become independent of the survival factors generated from gonads, and proliferate continuously throughout the cycle.

In summary, differential expression of leptin and its receptor in eutopic and ectopic endometrium suggests that leptin may have a critical role in endometriosis development. Elevated leptin expression by endometriosis lesions appears to enhance the proliferation of ectopic endometriotic stromal cells. Our findings may open a new field of investigation into the actions of leptin in the pathogenesis of endometriosis and provide a reasonable rationale for developing a therapeutic regime for endometriosis by targeting leptin and its action.

Acknowledgements
The authors are very grateful to Ms Mei-Fang Huang for technical assistance. This work was financially supported by the grants from National Science Council of Republic of China (NSC89-2320-B-006-119 to S.J.T.) and National Cheng-Kung University Hospital (NCKUH-90-044 to M.H.W.).

References


Submitted on August 20, 2001; resubmitted on November 8, 2001; accepted on February 20, 2002