Inverse correlation between peritoneal fluid leptin concentrations and the extent of endometriosis

Neal G.Mahutte1, Ioannis M.Matalliotakis1,2, Anastasia G.Goumenou2, Simon Vassiliadis3, Georgios E.Koumantakis2 and Aydin Arici1,4

1Yale University School of Medicine, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, New Haven, CT 06520-8063, USA and 2Departments of Obstetrics and Gynecology and 3Biology, University of Crete, Greece
4To whom correspondence should be addressed. E-mail: aydin.arici@yale.edu

BACKGROUND: The role of leptin in reproductive processes has received increasing attention. Because leptin has intrinsic angiogenic properties, may be induced by inflammatory cytokines and induces matrix metalloproteinases, we examined peritoneal fluid (PF) leptin concentrations in women with endometriosis. METHODS: PF samples were collected from 60 women undergoing laparoscopy for endometriosis, and 18 controls undergoing tubal sterilization. Fifty of the women with endometriosis had received no prior hormonal treatment, while 10 with moderate–severe endometriosis were using GnRH agonists. RESULTS: Women with untreated endometriosis had significantly higher (mean ± SD) PF leptin levels (34.9 ± 7.9 ng/ml) than controls (17.9 ± 4.1 ng/ml; P < 0.001). However, PF leptin levels were inversely correlated with the stage of disease (r = −0.62; P < 0.001). Nevertheless, women with stage III–IV endometriosis maintained significantly higher PF leptin levels (26.3 ± 4.8 ng/ml; P < 0.001) than controls. Although PF leptin levels were significantly higher in the secretory versus proliferative phase of the menstrual cycle, they remained higher in both phases in women with untreated endometriosis. PF leptin levels in women on GnRH agonists were similar to controls. CONCLUSIONS: PF leptin levels are elevated in women with endometriosis, but inversely correlated with extent of disease. These findings suggest a potential role for leptin in the pathogenesis of peritoneal endometriosis.

Key words: angiogenesis/cytokines/endometriosis/leptin/peritoneal fluid

Introduction

Endometriosis is defined as the presence of endometrial tissue outside of the uterus. Although the pathogenesis of endometriosis is unknown, the theory of retrograde menstruation with attachment of endometrial fragments in conjunction with immunological and peritoneal factors that stimulate angiogenesis and cell growth is currently the most widely accepted explanation (Senturk and Arici, 1999; Starzinski-Powitz et al., 2001; Gazvani and Templeton, 2002; Witz, 2002).

Growth factors and inflammatory mediators produced by peritoneal leukocytes are postulated to participate in the pathogenesis of endometriosis (Harada et al., 2001). These include interleukins (IL), tumour necrosis factor (TNF)-α, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) (Eisermann et al., 1988; Khorram et al., 1993; Keenan et al., 1994; Ryan et al., 1995; Akoun et al., 1996; Arici et al., 1996; Ho et al., 1996; McLaren et al., 1996; Punnonen et al., 1996; Arici et al., 1997; Harada et al., 1997; Gazvani et al., 1998; Zeyneloglu et al., 1998; Mahnke et al., 2000). All of these cytokines are increased in the peritoneal fluid (PF) of women with endometriosis. In most cases (RANTES, MCP-1, IL-6, IL-8) levels correlate with the severity of the disease. However, in some cases (IL-15) there is an inverse correlation between cytokine levels and the stage of endometriosis (Arici et al., 2003).

Angiogenic factors are involved in the pathogenesis of endometriosis (Arici et al., 1997; Harada et al., 2001; Arici, 2002). VEGF, also known as vascular permeability factor, is one of the most potent and specific. PF-activated macrophages are a major source of VEGF in endometriosis, and VEGF expression is directly regulated by estradiol and progesterone (McLaren et al., 1996). IL-8, another potent angiogenic agent, is also postulated to play a role in endometriosis. IL-8 is a chemoattractant for neutrophils, is produced in human endometrial tissues (Arici et al., 1998a) and induces proliferation of endometrial stromal cells thereby acting as an autocrine growth factor (Arici et al., 1998b).

Leptin, an adipocyte hormone and recently described type-1 cytokine, has angiogenic properties and appears to have a relationship with some reproductive processes
The menstrual cycle was determined by the patient’s last menstrual period and was confirmed by histological dating of the endometrium in 14/18 controls and 38/50 untreated endometriosis cases. Controls were equally divided between the proliferative and secretory phase of the menstrual cycle. Twenty-one endometriosis cases were in the proliferative phase and 17 in the secretory phase of the menstrual cycle. Twenty-one endometriosis cases. Controls were equally divided between the proliferative and secretory phases of the menstrual cycle. However, in a study of 20 women with endometriosis we were unable to demonstrate significant differences in serum leptin between women with and without endometriosis (Matalliotakis et al., 2000). Moreover, in that study serum leptin levels actually increased during medical therapy with either danazol or depot leuprolide, and returned to previous levels after cessation of treatment.

These studies, with their limited sample sizes and somewhat contradictory findings motivated us to investigate PF leptin levels in women with and without endometriosis. We also sought to examine the suggested relationships between leptin, the stage of endometriosis and the effects of medical treatment. Finally, we documented variations in PF leptin levels during the menstrual cycle.

Materials and methods
Peritoneal fluid samples were obtained from 60 women with endometriosis undergoing laparoscopy at Yale-New Haven Hospital for evaluation of infertility or pelvic pain, and from 18 controls undergoing tubal sterilization. The Human Investigation Committee at Yale University approved the study, and informed consent was obtained from each woman prior to surgery. In 10 patients, eight of whom had stage III–IV endometriosis, a GnRH agonist (Depot Lupron; TAP Pharmaceuticals, USA) had been used during the 6 months prior to the surgery. None of these 10 women had undergone pelvic surgery in the 6 months prior to initiating treatment with the GnRH agonist.

Participants with previously untreated endometriosis were classified into three groups according to the operative findings: women with superficial peritoneal endometriosis (n = 24), women with deep endometriotic implants (n = 18), and women with endometriomas (n = 8). The depth of invasion of peritoneal implants was based on the visual impression of the excised lesion(s) at the time of surgery. All surgeries were performed by reproductive endocrinologists with extensive experience in endometriosis surgery, and a threshold of 5 mm was used to distinguish superficial from deep. The extent of endometriosis was also scored according to the revised classification of the American Fertility Society (1985), and biopsy specimens were taken to confirm the diagnosis histologically. Endometriosis implants included both typical and atypical lesions. All patients underwent laparoscopy under general anaesthesia the morning after fasting for ≥12 h.

The phase of the menstrual cycle was determined by the patient’s last menstrual period and was confirmed by histological dating of the endometrium in 14/18 controls and 38/50 untreated endometriosis cases. Controls were equally divided between the proliferative and secretory phase of the menstrual cycle. Twenty-one endometriosis cases were in the proliferative phase and 17 in the secretory phase of the menstrual cycle.

The PF was collected by aspiration from the posterior cul-de-sac at the beginning of laparoscopy. Samples were not used if bleeding into the pelvic cavity from the abdominal stab punctures was observed. The PF sample was placed in a sterile tube and centrifuged at 600 g for 10 min. The supernatant was collected and stored at −80°C until assayed.

Determination of leptin
The leptin levels in the PF of women with and without endometriosis were detected by enzyme-linked immunosorbent assay (ELISA). The inter- and intra-assay coefficients of variation were <5%. The primary antibody used in this work was purchased from Santa Cruz Biotechnology, Inc. (USA). This affinity-purified rabbit polyclonal antibody (sc-843; IgG) reacts with leptin of mouse, rat and human origin. The secondary antibody was goat anti-mouse IgG, (Fab2)2 fragment specific, horse-radish peroxidase-conjugated (Pierce, USA).

The ELISA experiments were performed as follows. Briefly, PF samples at a concentration of 1:100 in carbonate buffer pH 9.6 were coated in 96-well flat bottom plates (Sarstedt, Germany), incubated overnight at 4°C and washed four times in 5% Tween-20 (Sigma, USA). The remaining protein-free sites in the plate were blocked by 2% phosphate-buffered saline–bovine serum albumin (PBS–BSA) solution after an incubation of 2 h at room temperature. After washing four times, 100 µl of primary antibody diluted in 0.1% PBS–BSA were added and incubated for 1 h at room temperature. Extensive washing of the plates was followed by addition of 100 µl of secondary antibodies coupled to horse-radish peroxidase (1:1000 dilution; Sigma) and incubated for 1 h at room temperature, in the dark. Finally, the reaction was developed by adding 100 µl/well of tetramethyl benzidine–H2O2 (Sigma) for 20 min. The enzymatic reaction was stopped with 50 µl H2SO4 (4 N). Optical density was measured at 450 nm using a Titertec ELISA photometer (Digiscan; ASYS Hitech GmbH, Austria). The results are expressed as factor concentrations calculated from the corresponding standard curves.

Statistical analysis
Non-parametric data were described as median and parametric data as mean ± SD. An unpaired t-test was used for comparison of means and Mann–Whitney U-test for medians. Differences between groups were analysed using Fisher’s exact test for non-parametric data. Correlation analysis was performed by Spearman’s rank test. Note that in the correlation analysis, both stage of disease and depth of invasion (superficial = 1, deep = 2, endometrioma = 3) were treated as ordinal variables. P < 0.05 was accepted as statistically significant.

Results
There was no significant difference in mean age between controls (31.2 ± 6.9 years), women with untreated endometriosis (32.5 ± 6) and women undergoing GnRH agonist treatment for endometriosis (32.7 ± 6). Moreover, no significant difference was seen in body mass index among the above groups (mean 22.1–22.9 kg/m²).

The mean concentration of PF leptin was significantly higher in women with untreated endometriosis compared with controls (Table I). This finding was consistent throughout the different phases of the menstrual cycle. However, PF leptin levels were significantly higher in the secretory versus the proliferative phase of the menstrual cycle.

In women with endometriosis who were not on GnRH agonist therapy, mean leptin levels were significantly higher...
with superficial peritoneal disease (38.2 ± 5.1 ng/ml) than with deep peritoneal implants (32.0 ± 8.1 ng/ml; *P < 0.001) or endometriotic cysts (27.4 ± 8.2 ng/ml; *P = 0.004). A negative correlation was found between PF leptin levels and the depth of invasion (superficial, deep, endometrioma) of endometriosis (*r = -0.50, *P < 0.001). Similarly, the stage of endometriosis was inversely correlated with PF leptin levels (*r = -0.62, *P < 0.001).

Significantly higher concentrations of PF leptin were found in stage I–II (combined mean 38.3 ± 6.1 ng/ml) than stages III–IV endometriosis (combined mean 26.3 ± 4.8 ng/ml; *P < 0.001; Figure 1). The discriminating point occurred between stage II and stage III (37.3 ± 7.5 versus 26.1 ± 3.2 ng/ml; *P < 0.001). Nevertheless, even women with stage III–IV endometriosis had significantly higher PF leptin levels than controls (*P < 0.01). Women with stage III–IV endometriosis treated with a GnRH agonist (Lupron) prior to surgery (n = 8) demonstrated significantly lower PF leptin levels (20.3 ± 4.8 ng/ml) than untreated women with stage III–IV endometriosis. There was no significant difference in PF leptin levels between controls and women treated with a GnRH agonist. Finally, among women with untreated endometriosis, no significant difference in PF leptin levels was found between women with infertility (36 ng/ml) and women with pelvic pain (34.5 ng/ml).

Discussion

We find that leptin is significantly increased in the PF of women with endometriosis. This observation is in accordance with previous smaller studies demonstrating that PF leptin levels were significantly higher in women with endometriosis than controls (Matarese et al., 2000; De Placido et al., 2001). We have also shown that PF leptin levels are higher in women with superficial, minimal–mild stage endometriosis than those with more invasive, advanced stage disease. Indeed, we note a significant decline in PF leptin levels with advancing depth of invasion and stage of disease. Again, this confirms earlier, small studies suggesting that PF leptin may be more elevated in women with early than advanced stage endometriosis (Matarese et al., 2000; De Placido et al., 2001).

There is evidence that estradiol and progesterone mediate serum leptin levels (Messinis et al., 2001). A rise in serum leptin has consistently been documented in the luteal and late follicular phase of both natural and gonadotrophin-stimulated cycles (Mannucci et al., 1998; Messinis et al., 1998; Yamada et al., 2000). Our PF data complement the observation that serum leptin concentrations are higher in the secretory phase than in the proliferative phase of the cycle (Messinis and Milingsos, 1999; Messinis et al., 2001). However, we find that despite variation during the menstrual cycle, PF leptin levels are significantly higher in women with endometriosis than controls in both phases of the cycle.

An interesting finding in this study is that women with stage III–IV endometriosis undergoing medical treatment with GnRH agonist had lower PF leptin levels than women with similar stage, previously untreated disease. In fact, PF leptin levels in women on GnRH agonist were similar to controls undergoing spontaneous menstrual cycles. In pre-menopausal women who undergo bilateral oophorectomy a significant drop in serum leptin levels has been documented (Messinis et al., 1999). Interestingly, this decline may be prevented by the exogenous administration of estradiol plus progesterone (Messinis et al., 2000). If changes in serum leptin levels closely correlate with changes in PF leptin levels, one might predict a decline in PF leptin after GnRH agonist treatment to levels below those of normally cycling women.

Although we know of no data specifically addressing PF leptin levels in post-menopausal women, we found the similarity between controls and women treated with GnRH agonist to be somewhat surprising. One possible explanation is that factors related to the presence of endometriosis sustain leptin production in the absence of estrogen/progesterone. Both the leptin receptor and, to a lesser extent, leptin itself are expressed in human endometrium (Gonzalez et al., 2000; Kitawaki et al., 2000; Gonzalez and Leavis, 2001). Moreover,

Table I. Leptin concentrations in peritoneal fluid of women with and without endometriosis, and in relation to the phase of the menstrual cycle

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 18)</th>
<th>Endometriosis (n = 50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>17.9 ± 4.1</td>
<td>34.9 ± 7.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(10–27.2)</td>
<td>(20.8–48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>14.1 ± 2.4</td>
<td>30.1 ± 6.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>phase</td>
<td>(10–17.5)</td>
<td>(20.8–35)</td>
<td></td>
</tr>
<tr>
<td>Secretory phase</td>
<td>21.7 ± 3.4a</td>
<td>40.3 ± 3.4a</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(17.5–27.2)</td>
<td>(35–46)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed in ng/ml as mean ± SD (range).

*Proliferative versus secretory phase leptin in the control group: *P < 0.001.

**Proliferative versus secretory phase leptin in women with endometriosis: **P < 0.001.

Figure 1. Peritoneal fluid (PF) leptin levels in women with endometriosis divided according to the stage of disease. Women with stage I–II endometriosis had significantly (*P < 0.001) higher PF leptin levels than women with stage III–IV endometriosis, women with stage III–IV endometriosis previously treated with Lupron and controls. Additionally, women with stage III–IV had significantly higher PF leptin levels (**P < 0.01) than women with stage III–IV endometriosis previously treated with Lupron or controls. Data are presented as mean ± SD.
the expression of leptin mRNA and protein are significantly increased in endometriosis cells compared with eutopic endometrium (Wu et al., 2002). Finally, although endometriosis cells express the leptin receptor, mRNA levels for the receptor decrease in association with increasing stages of the disease (Wu et al., 2002). Thus, endometriosis implants are both a potential source of leptin production and a potential target for its action, particularly in stages I–II of the disease.

On the basis of these findings, it is tempting to hypothesize that leptin may be an active participant in the pathogenesis of peritoneal endometriosis. Leptin is known to stimulate angiogenesis (Bouloumie et al., 1998; Sierra-Honigmann et al., 1998; Cao et al., 2001; Park et al., 2001). Angiogenesis is believed to be a necessary requirement for the sustenance of endometriosis implants. Leptin also induces vascular permeability, and synergistically stimulates angiogenesis with VEGF and fibroblast growth factor-2 (Cao et al., 2001). Moreover, TNF and IL-1 are known to increase leptin in vivo. In fact, leptin may induce expression of matrix metalloproteinases both in vitro (Park et al., 2001). Matrix metalloproteinases are believed to play a critical role in the initial invasion of endometrial cells into the mesothelium (Spuijbroek et al., 1992; Arici, 2002). In addition, high doses of leptin have been shown to significantly enhance mitogenic activity in cultured eutopic and ectopic endometrial stromal cells (Wu et al., 2002). Thus, leptin may provide a link between acute inflammation, angiogenesis, invasion of the mesothelium and endometrial stromal proliferation.

In conclusion, leptin has intrinsic angiogenic and mitogenic properties, may be induced by inflammatory cytokines, and induces matrix metalloproteinases. PF leptin levels are elevated in women with endometriosis, particularly in minimal–mild stage, peritoneal disease. Moreover, despite fluctuations during the menstrual cycle, PF leptin levels remain in both phases significantly higher in women with endometriosis than controls. Finally, prolonged exposure to a GnRH agonist suppresses endometriotic implants and lowers PF leptin to levels comparable with control populations. All of these characteristics strongly suggest a role for leptin in the pathogenesis of stage I–II, peritoneal endometriosis.

Acknowledgements

We would like to thank I.Athanassakis, the Chief of the Department of Biology, University of Crete, for her invaluable contribution to this work. In addition, we would like to thank Mrs E.Dionysopoulou and L.Papadimitriou from the Department of Obstetrics and Gynecology, University of Crete for their technical assistance. The co-operation of Nacile Mulayim, MD and Begin Selam, MD from the Department of Obstetrics and Gynecology, Yale University School of Medicine was appreciated.

References


Keenan, J.A., Chen, T.T., Chadwell, N.L., Torry, D.S. and Caudle, M.R. (1994) Interferon-gamma (IFN-gamma) and interleukin-6 (IL-6) in


Submitted on December 5, 2002; resubmitted on January 22, 2003; accepted on February 10, 2003.