Studies on leptin and leptin receptor gene expression in myometrium and uterine myomas of GnRH analogue-treated women

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Summary

Aim: To test if treatment with GnRH analogue, which leads to a significant reduction in myoma volume, changes expression of leptin genes and gene coding leptin receptor isoforms in uterine myomas and in the surrounding unaltered myometrium.

Methods: Using RT-PCR, expression of leptin genes and leptin receptor genes was studied in myomas and in the surrounding myometrium in women with uterine myomas, untreated or treated with GnRH analogue. In the randomly selected cases presence of leptin protein and of leptin receptor proteins was examined also by Western blotting.

Results: Expression of leptin genes was demonstrated both in myomas and in the surrounding myometrium, and a similar pattern of expression was found for leptin receptor isoforms. The results of RT-PCR were confirmed by Western blotting, which documented the identical distribution of leptin proteins and leptin receptor proteins in studied tissues. Treatment with GnRH analogue had no effect on the expression pattern of studied genes.

Conclusion: The results of the present study on the administration of GnRH analogue to females with myomas suggest that no direct or immediate inter-relationship exists between expression of leptin genes in uterine myomas on one hand and estrogen, progesterone and leptin levels in the blood on the other. Expression seems to be of a more durable nature but factors that induce such expression remain unknown.

Key words: Leptin; Leptin receptor; Uterine myoma; GnRH analogue.

Introduction

Uterine myomas represent benign monoclonal uterine tumors that arise from smooth muscle cells. They are estimated to affect around 20-30% of women older than 30 years of age [1, 2]. Detailed histopathological studies demonstrated their presence in as many as 70-77% females [3, 4]. Such differences in the frequency of manifestation reflect their detection in females with complaints: depending on the localization they may induce extensive hemorrhage, pain and a feeling of compression in the small pelvis as well as disturbances in reproductive function. Occasionally, myomas remain undetected since in the absence of complaints gynecological check-ups may not be undertaken.

Causes of myoma development have not been unequivocally determined, although they have been considered to be linked to steroid hormones - estrogen and progesterone. This hypothesis is formed by the absence of myomas in girls before menarche and in women after menopause, as well as by a decrease in their size following pharmacological gonadectomy with GnRH analogues or following administration of drugs which modulate estrogen receptors, such as raloxifen, or anti-progesterones, such as RU 486 [5-10].

Studies pointing to differences in plasma levels of estrogen and progesterone as well as to differences in the density of their receptors in uterine myomas has yielded, however, contradictory results [11-14]. Similarly, results on genetic alterations in uterine myomas have proved to be equivocal [4, 15, 16]. In recent years differences have been demonstrated in the expression of Il-8 and its receptor in myomas and in the surrounding myometrium; expression of the cytokine has been suggested to represent one of the causes of myoma growth [2].

Our earlier studies have shown [17, 18] that leptin genes are expressed in uterine myomas in contrast to the surrounding unaltered myometrium. In constrast to leptin, expression of various isoforms of its receptor takes place both in myomas and in the unaltered myometrium. Moreover, we have shown that in myoma cell cultures supplementation with leptin stimulates their proliferation. Observations suggest that expression of leptin genes may be linked to development of uterine myomas.

Taking into account the known effect of the decreasing size of myomas following pharmacological gonadectomy with GnRH analogues, the present study was aimed at examining whether this type of treatment alters expression of leptin and its receptor isoforms in uterine myomas.

Material and Methods

The studies were performed on material obtained from 14 females, aged 24 to 49, who underwent surgery for uterine myomas and who were treated earlier with GnRH analogues. In

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the course of the earlier treatment, the patients received a luteinizing hormone-releasing hormone analogue, Decapeptyl depot (Triptorelin), at an IM dose of 3.75 mg every 28 days. One to three courses of the treatment were applied, depending on their effect on the menstrual cycle. Four females received the analogue for one month, seven for two months and three women received the treatment for three months (Table 1).

Table 1. — *Approximate changes in volume of uterine myomas in females treated with GnRH analogue.*

No.	Age (years)	Myoma: single (S) or multiple (M)	Number of courses of analogue application	Diameter (cm)		Decrease in volume (%)
				Before treatment	After GnRH analogue treatment	
1	48	S	3	7.7	5.5	63.6
2	49	M	1	3.0	1.5	87.5
3	41	S	2	6.5	4.3	71.1
4	45	S	1	4.5	4.0	29.8
5	48	S	2	2.5	1.5	78.4
6	47	M	1	5.5	5.0	24.9
7	46	M	3	2.8	2.0	63.6
8	24	S	2	4.5	4.0	29.8
9	36	S	3	5.0	4.0	48.8
10	44	S	2	5.5	4.7	37.6
11	46	S	2	5.5	3.5	74.2
12	44	S	1	4.4	3.4	53.9
13	25	S	2	5.0	3.5	65.7
14	45	S	2	3.5	3.0	37.0

Diameters of myomas were measured by ultrasonography. The diameter permitted the approximate volume of myomas to be calculated and in cases of multiple myomas the volume of the largest myoma was considered. The data permitted an approximate decrease in myoma volume to be measured.

The sampled study material included both myomas and surrounding unaltered myometrium. The results were compared to those obtained in a group of myomatous women who had been pharmacologically untreated and who were described earlier [18]. The control group (myomatous females not subjected to pharmacotherapy) included 20 women, 30 to 50 years of age, who underwent surgery for uterine myomas. In all the females samples of the myometrium and the myoma were taken and processed with RT-PCR; in randomly chosen cases Western blotting was also employed. In all cases the histopathological diagnosis involved *leiomyomata uteri.*

In females with myomas the size of the tumor was established by ultrasonography, measuring the diameters. The measurements were conducted before and after treatment with the analogue. The diameter of the myoma permitted us to calculate the approximate volume of the myoma and in cases of multiple myomas the volume of the largest tumor was calculated. The data permitted us to define an approximate decrease in tumor volume.

Before surgery, fasting blood was sampled in all the females to establish serum levels of leptin, estradiol, progesterone and insulin. Body weight body mass index (BMI), and waist to hip circumference ratio (WHR) were established routinely and accurate anamnesis was taken, excluding women suffering from other metabolic diseases (diabetes, hyper- or hypothyroidism, etc.) from the study.

The content of adipose tissue in the body was calculated according to Deurenberg *et al's* [19] formula: (BF) % body fat = 1.2 x (BMI) + 0.23 x (age) – 10.8 x (sex) – 5.4, the sex coefficient amounting to 1 for males and 0 for females.

The Bioethical Committee of Poznań Medical University gave consent for the study protocol.

Tissue material

The tissues (samples of unchanged myometrium, myomas and white adipose tissue) were placed in saline at 4°C immediately after surgical excision. Within 10 to 20 min fragments devoted to RNA or protein isolations were frozen at -80°C and stored at this temperature. The remaining tissue fragments were used for histopathological diagnosis. Subcutaneous adipose tissue served as a respective control.

RNA extraction and RT/PCR (reverse transcription-polymerase chain reaction)

Methods used for RNA extraction and RT-PCR have been described earlier [18]. Briefly, total RNA was extracted by a coupled method using TRI REAGENT (Sigma) and purification in mini columns (RNeasy Mini Kit, Qiagen). Thirty to 60 mg of tissue were homogenized in TRI REAGENT and chloroform and isopropanol were added, as specified by Sigma. Then, the sample was pipetted onto RNeasy mini columns and, next, the Qiagen protocol was used. Contaminated DNA was eliminated by DNase I treatment (RNase-Free DNase Set). Ten to 20 μg RNA were obtained in a single procedure.

Reverse transcription was performed using Omniscript RT Kit with random hexamers as primers (Gibco-BRL, USA). PCR was performed using HotStarTaq PCR Master Mix Kit. PCR primers were purchased from Invitrogen (USA). Primer sequences are shown in Table 2. PCR reactions were performed under the following conditions:

for GAPDH amplification: hot start Taq polymerase activation – 95°C for 15 min.; (denaturation: 94°C for 30 sec.; annealing: 59°C for 30 sec.; extension: 72°C for 1 min.) – 30 cycles; final extension 72°C for 10 min.

for OB, OBRu; OBR_{LBD}; OBR_L; OBRs1; OBRs2 and OBRs3 amplifications: hot start Taq polymerase activation: 95°C 15 min.; (denaturation: 94°C for 60 sec.; annealing: 60°C for 60 sec.; extension: 72°C for 2 min.) – 40 cycles; 72°C for 10 min.

PCR reactions were performed using the Peltier Thermal Cycler PTC 200 (MJ Research). PCR products were analyzed by electrophoresis in 2% agarose gels and staining with ethidium bromide.

Two RT reactions were performed for each RNA prep after DNaseI treatment: (1) without reverse transcriptase (RT negative control) and (2) with reverse transcriptase (RT positive control), followed by PCR with GAPDH primers. Only these RNA preps, which gave no band after RT negative control (confirmation of lack of contaminating DNA) and gave a strong GAPDH band after RT positive control (confirmation of good quality of RNA), were used for examination of leptin and leptin receptor isoform expression.

Western blotting

In some randomly chosen cases Western blotting was used to identify leptin and leptin receptor proteins, as described previously [17, 18]. In these cases myometrium and uterine myoma samples were homogenized in 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 the method of Bradford. Samples of 20 μg of protein were loaded into each lane, separated in a 15% sodium dodecyl sulphate (SDS)-polyacrylamide electrophoretic gel, and transferred onto a PVDF membrane (Millipore Co., Bedford, MA, USA). Transferred proteins were stained with Ponceau S. Subsequently, the mem-

Table 2. — *PCR primers used in the study.*

Gene	Primers[1]	Primer sequence 5' □ 3'	Product size (bp)	References[2]
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH-1(E2/E3)	ggtcggagtcaacggatttg	319	[36, 37]
	GAPDH-2(E5/E6)	atgagccccagccttctccat		J04038
Leptin	OB-1(E2/E3)	cacacacgcagtcagtctcc	298	[38]
	OB-2(E3)	accacctctgtggagtagcc		D63708, D63709, D63710
Leptin receptor (extracellular domain)	OBRu-1(E4/E5)	gtgaagcctgatccaccatt	340	[39, 40]
	OBRu-2(E7)	cattagaccccacacttgtcaga		U66497
Leptin receptor (leptin binding domain[3])	OBR_{LBD}-1(E7)	tgcagtgtactgcaatg	414	[39]
	OBR_{LBD}-2(E10)	tctgctttcacactggatgg		U66497
Long isoform of receptor (L)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	439	[39]
	OBRL-2(E18)	ctgatcagcgtggcgtattt		U43168
Short isoform of receptor (s1)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	227	[32, 39, 41]
	OBRs1-2(E18)	ctgtggccttccgcagtg		U52912
Short isoform of receptor (s2)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	176	[32, 39, 41]
	OBRs2-2(E18)	acctccacccagtagttcctt		U52913
Short isoform of receptor (s3)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	200	[32, 39, 41]
	OBRs3-2(E18)	agttggcacattgggttcat		U52914

[1]- starter localization with regard to exons is shown in parentheses (i.e. E7 – starter anneals to a sequence in exon 7; E4/E5 – starter spans exon 4/ exon 5 boundaries)

[2]- gene sequence references and GeneBank accession numbers. Primers for GAPDH, OBRL, OBRs1, OBRs2 and OBRs3 were according to Brossart *et al.* [37] and Mix *et al.* [32], respectively; primers for OB; OBRu and OBRLBD were designed using the software Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3).

[3]- part of extracellular domain

branes were halved, with one part containing proteins of molecular weight similar to that of the long form of the leptin receptor (116.5 kDa), and the other part carrying proteins of molecular weight resembling that of leptin (16 kDa). Leptin-containing membranes were then incubated with primary antibodies from Santa Cruz Biotechnology, Inc. (USA), at a 1:1000 dilution for 1 h at 37°C. OB (A-20) (sc-842) is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping to the carboxy terminus of the Ob gene product of human origin. Leptin-receptor containing membranes were also incubated with primary antibodies from Santa Cruz Biotechnology, Inc. (USA). Ob-R (M-18) (sc-1834) is an affinity-purified goat polyclonal antibody raised against a peptide mapping to the carboxy terminus of Ob-R of mouse origin.

Estimation of serum hormone levels

Serum estradiol and progesterone concentrations were quantitated by electrochemiluminescence (ECL) using the ELECSYS analyzer (Roche) and reagent kit supplied by the manufacturer. Insulin concentration was determined by an immunoenzymatic technique, MEIA, in the Axsym analyzer (Abbott) and using the manufacturer's kit of reagents. Serum concentration of leptin was estimated by the ELISA technique, employing the Leptin (human) ELISA Kit, AK-153 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA).

Statistical analysis of results

Quantitative data were characterized by their mean values and respective standard deviations (x ± SD). Inter-group differences in the distribution of quantitative trait values were verified using the non-parametric Mann-Whitney test. Differences in the frequencies of positive results were verified using Fisher's exact test. Detected differences were considered statistically significant at a credibility level of $p < 0.05$. Statistical analyses were performed using Statistica 5.0 software.

Results

Age, body weight and height of examined myomatous females, pharmacologically untreated or receiving GnRH analogue, were similar (Table 3). No significant differences between the two groups were detected in BMI, WHR or percent of body fat (Table 4). In myomatous women treated with GnRH analogue serum estradiol, progesterone and leptin concentrations were lower than those in women with uterine myomas but receiving no pharmacological treatment (Table 5). Insulin concentra-

Table 3. — *General characteristics of studied females with uterine myomas at the proliferative and secretory stage (n = 20) not subjected to pharmacotherapy and of females with uterine myomas following therapy with GnRH analogue (n = 14). Results are given as x ± SD.*

Group	Age (years)	Body weight (kg)	Stature (cm)
Not subjected to pharmacotherapy	44.2 ± 5.7	66.5 ± 8.2	163.6 ± 5.2
Treated with GnRH analogue	42.0 ± 8.1	64.6 ± 7.7	163.6 ± 5.7

Table 4. — *Body mass index (BMI) [kg/m^2], waist to hip circumference ratio (WHR) and % body fat in myomatous females at the proliferative or the secretory stage (n = 20), not subjected to pharmacotherapy and in myomatous females following therapy with GnRH analogue (n = 14). The data are given as x ± SD.*

Group	BMI	WHR	% body fat
Not subjected to pharmacotherapy	25.0 ± 2.5	0.82 ± 0.08	34.8 ± 3.68
Treated with GnRH analogue	24.3 ± 4.3	0.81 ± 0.06	33.5 ± 5.71

Table 5. — *Concentrations of estradiol, progesterone, insulin and leptin in serum of myomatous females at the proliferative or secretory stage (n = 20) not subjected to pharmacotherapy and in myomatous females following treatment with GnRH analogue (n = 14). Results are given as x ± SD.*

Group	Estradiol (pg/ml)	Progesterone (ng/ml)	Insulin (μM/ml)	Leptin (ng/ml)
Not subjected to pharmacotherapy	139.4 ± 77.0	3.25 ± 4.51	8.95 ± 6.19	6.88±3.21
Treated with GnRH analogue	16.7 ± 5.0**	0.40 ± 0.27**	9.53 ± 10.42	4.63±1.75*

Differences are significant at: * $p < 0.05$; ** $p < 0.001$.

Table 6. — *Leptin and isoforms of its receptor in the myometrium and in myomas in females not subjected to pharmacotherapy (n = 20) or in females following treatment with GnRH analogue (n = 14). Gene expression was studied by RT-PCR. The results are given as the number of positive results per total number of studied cases in each group.*

Material	Leptin	OBRL	OBRs1	OBRs2	OBRs3
Females not subjected to pharmacotherapy					
Myometrium	15/20	20/20	20/20	0/20	20/20
Myomas	13/20	19/20	20/20	0/20	20/20
Females treated with GnRH analogue					
Myometrium	9/14	14/14	14/14	0/14	14/14
Myomas	9/14	13/14	14/14	0/14	14/14

All the differences proved to be insignificant.

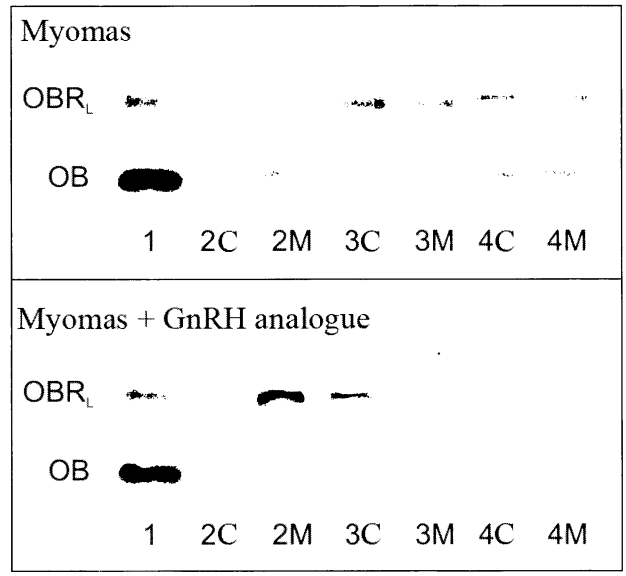

Figure 1. — Western blotting of leptin (OB) and long isoform of leptin receptor (OBRL) in myometrium (C) and myomas (M) of untreated women (myomas) or of GnRH analogue treated women (myomas + GnRH analogue). 1 – white adipose tissue as a control; 2, 3 and 4 – studied cases.

tions were similar in the two groups of women. In all myomatous females treated with GnRH analogue decrease in tumor volume was noted, ranging from 29.8 to 87.5% (mean around 55%) (Table 1).

RT-PCR studies demonstrated expression of the OB gene in unaltered myometrium in 15 out of 20 cases (75%) of studied women who received no pharmacological treatment and in myomas in 13 women (65% cases) (Table 6). In females treated with GnRH analogue, expression of the OB gene was observed both in the unaltered myometrium and in myomas in nine out of 14 treated women (statistically insignificant). In almost all the cases of women treated or untreated with the analogue expression of OBRL, OBRs1 and OBRs3 receptor isoforms were observed both in the myometrium and myomas. In contrast, expression of OBRs2 receptor was not noted in any of the studied materials.

In randomly selected cases (3 females with myomas treated with GnRH analogue and 3 untreated females with myomas) Western blotting demonstrated the presence of proteins of leptin and the long leptin receptor isoform both in the unaltered myometrium and in the myomas (Figure 1).

Discussion

Although uterine myomas represent the most frequent benign tumors in women, their pathogenesis remains to be fully understood. Both estrogen and progesterone are thought to be involved in the development but results on the role of the hormones in the incidence of myomas and their growth are divergent [14, 20, 21]. Clinicians are aware that myomas do not develop in girls before menarche or in postmenopausal women, and that they increase in size during pregnancy [11, 14, 20]. Rein *et al.* [22] and Friedman *et al.* [23] advanced a hypothesis that concentration of estrogen receptors (ER) and progesterone receptors (PR) in myomas and in the myometrium may be important for the control of myoma growth.

Independent of the role of estrogen and progesterone, other factors also seem to be involved in myoma development. Among them, the most significant role is ascribed to IL-8 and its receptor [2]. Results of our earlier studies suggest that such factors include leptin, the hormone secreted mainly by adipocytes, which represents one of the most important peptides controlling energetic homeostasis in the body. The suggestion has been backed up by the following observations: (1) the *OB* gene manifests expression in uterine myomas and in unaltered myometrium while in women with no myomas the myometrium manifests no expression or only sporadic expression of the gene, (2) various isoforms of leptin receptors are manifested in myomas and in myometrium which may suggest a paracrine mechanism of the cytokine action, (3) leptin stimulates proliferation of myoma cells in *in vitro* culture. In the context of such results, observations of Wu *et al.* [24] on leptin expression in endometriosis cells are worth attention. In their study leptin mRNA was undetectable in seven out of 14 eutopic endometria while in ectopic endometriosis leptin mRNA and protein expression were found in all studied cases. Moreover, those authors demonstrated a significant stimulating effect of leptin on endometrial stromal cell proliferation.

Clinical observations, imaging and laboratory results prove that application of GnRH analogues results in decreased myoma size. Such treatment has been applied

mainly to prepare patients for planned surgery since it improves the biochemical variables in blood (increase in Hgb and RBC levels) which permits blood transfusion to be avoided, reduces myoma volume, reduces uterine blood supply and, thus, intraoperative blood loss, facilitates conservative treatment of myomas (myomectomy) and decreases duration of the operation [5, 22, 23, 25]. The mechanisms that lead to decreased myoma volume under the effect of GnRH analogues are variable. According to Colgan *et al.* [7], the reduced size of a myoma reflects anoxia and atrophy of its cells. In studies of Sreenan *et al.* [25] treated and untreated myomas did not differ in atrophy of cell nuclei, calcification, foci of necrosis, hemorrhage, vascular lesions, hyaline degeneration or mitotic activity. Rein *et al.* [22] and Friedman *et al.* [23] noted that in shrinkage of myomas following treatment with GnRH analogue, concentrations of estrogen and progesterone receptors were higher (concentration of ER was six-fold higher) than in the unaltered myometrium, and the effect might explain an abrupt growth of myomas after discontinuation of GnRH analogue administration.

In view of our earlier observations [17, 18] on expression of leptin genes and manifestation of leptin proteins in uterine myomas, and in view of the known effect of decreasing myoma size under effect of pharmacological gonadectomy using GnRH analogues, in the present study we decided to examine whether this type of treatment alters the expression of leptin and its receptor isoforms in uterine myomas, as compared to females with myomas who had not been subjected to pharmacotherapy. The administered doses of the analogue have reflected its effect on menstrual blood loss, decrease in myoma size, and increase in hemoglobin level in anemic patients due to blood loss as well as the mental attitude of the patient. In accordance with the data of Nowak *et al.* [5], Friedman *et al.* [23] and Nakamura and Yoshimura [26], we detected lowered concentrations of both progesterone and estrogen after only four weeks of treatment which points to pharmacologically induced gonadectomy and which was accompanied by reduced myoma size, detected by ultrasonography. Therapy with the analogue did not alter blood insulin levels but markedly decreased blood leptin levels [from 6.88 ± 3.21 to 4.63 ± 1.75 ng/ml ($p < 0.025$)], even if no changes in BMI or percent of body fat were seen following treatment.

The results of other authors on blood leptin levels following treatment with GnRH analogues have been divergent. Matalliotakis *et al.* [27] employed GnRH analogues and obtained a significant increase in blood leptin levels in patients treated for endometriosis although the patients developed no changes in BMI during the treatment and, thus, the increase in leptin concentration was independent of changes in body fat. Males treated with GnRH analogues due to prostate cancer also developed augmented blood leptin levels only after four weeks of therapy but the increase was associated with a significant increase in body weight [28]. However, other authors have failed to note altered blood leptin levels following treatment with GnRH analogues (Krotkiewski *et al.* [29] – in females with PCOS; Witchel *et al.* [30] – in pubertas praecox; Tommaseli *et al.* [31] – in premenopause; Nowicki *et al.* [5] – in uterine myomas).

Moreover our results have shown that treatment with GnRH analogue does not affect the expression pattern of leptin receptors in unaltered myometrium or in myomas. In all the examined cases expression of OBRL, OBRs1 and OBRs3 receptor isoforms was noted both in treated and in untreated patients. On the other hand, in none of the cases could OBRs2 receptor expression be demonstrated, in line with our earlier observations [18]. The literature data indicate that expression of this isoform is very restricted: it has been detected only in human fetal livers and in the epithelial cells of the stomach [32]. In view of the present results, this isoform of the leptin receptor does not appear to be involved in the growth of uterine myomas.

In our studies on myomatous women treated with GnRH analogue we observed a significant decrease in serum leptin levels, as compared to females who had not been subjected to pharmacotherapy even if no changes developed in the two groups in body weight or percent of body fat. Nevertheless, the changes were not accompanied by alterations in expression of leptin genes or genes for leptin receptor isoforms in myomas or unaltered myometrium. The absence of the latter alterations seems to reflect the relatively short duration (up to three courses) of GnRH analogue administration. This observation also implies that no parallelism exists between blood leptin levels and expression of leptin or leptin receptor isoform genes in myomas, and we suggest that the role of leptin in the growth of uterine myomas is most probably linked to the paracrine or autocrine action of cytokines in the myometrium.

It should be stressed that similar results on the expression of leptin genes were noted by Wu *et al.* in endometriosis cases [24]. In their study, leptin mRNA was undetectable in seven out of 14 eutopic endometria while in ectopic endometriosis leptin mRNA and protein expression were found in all studied cases. Similar observations were published by Lima-Couy *et al.* [33]. Nevertheless, Vigano *et al.* [34] stressed a lack of inter-relationships between the development of endometriosis and serum leptin levels while Matarese *et al.* [35] expressed the opinion that blood leptin levels are higher at early stages of endometriosis, suggestive of the role of leptin in the pathogenesis of endometriosis.

Results of the presented studies on administration of GnRH analogues to women with uterine myomas suggest that no direct or immediate relationship exists between the expression of leptin genes in uterine myomas on one hand and blood concentrations of estrogen, progesterone and leptin on the other. The expression seems to be of a more durable nature but factors leading to such expression remain to be defined.

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