Further studies on leptin and leptin receptor expression in myometrium and uterine myomas

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Summary

Aim: Examination of the potential role of leptin in the development of uterine myomas. Expression of the leptin gene and leptin receptor gene was tested in the myometrium of healthy women, and in myomas and the surrounding myometrium of women with benign tumors.

Methods: Using RT-PCR, expression of the leptin gene and leptin receptor gene were studied in myomas and in the surrounding myometrium in 30 women with uterine myomas at various phases of the menstrual cycle, and in the myometrium of ten women in a control group. Presence of leptin gene proteins and leptin receptor gene proteins in the women was also examined by Western blotting.

Results: Using RT-PCR, expression of the leptin gene was demonstrated both in myomas and in the surrounding myometrium. In contrast, expression of the gene could not be detected in the myometrium of healthy women. The results were confirmed by Western blotting, which documented the identical distribution of leptin proteins and leptin receptor proteins in studied tissues.

Conclusion: Demonstration of the expression of leptin genes and leptin proteins in uterine myomas and in the surrounding myometrium, and their absence in the myometrium of healthy women suggests the involvement of leptin in the development of uterine myomas.

Key words: Leptin; Leptin receptor; Uterine myoma.

Introduction

Leptin (LEP), a product of the *ob* gene, is mainly produced by adipocytes, and is secreted from these into the circulation where it may be found as a bioactive free form or as a conjugate with the short form of the leptin receptor, OB-R and with clusterin (apolipoprotein J). This anti-obesity cytokine inhibits the biosynthesis and activity of neuropeptide Y (a potent orexigenic neuropeptide) in the hypothalamus where it acts as a signal, switching from feelings of starvation to satiation [1-3].

LEP acts via its receptor which, along with the receptors for interleukin 6 (IL-6) and for granulocyte colony stimulating factor (GCSF), belongs to the family of class 1 cytokine receptors. To date, six variants of leptin receptors have been described and are formed by alternate splicing. They include one long form, OB-R_L (ObRb), and four short forms, OB-R_S (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf), comprised of a common extracellular ligand-binding domain and differing cytoplasmic domains which results in each having a different function. A soluble isoform, Ob-Re, has also been described which incorporates neither the trans-membranous nor the cytoplasmic domain [4-8]. The long isoform is mainly expressed in the hypothalamus while expression of the short isoforms has been detected in several tissues, including the endometrium, ovaries, mammary glands and placenta [7, 9].

Binding to its specific receptors, LEP mainly induces activation of the JAK-STAT (just another kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) systems which are linked to activation of the kinases and their phosphorylating activity, stimulation of receptors for various cytokines, control of gene expression and the initiation of various signaling pathways [10-13].

Obese individuals might be expected to manifest low LEP levels but, in fact, their plasma LEP levels are rather elevated. According to several investigators, this reflects resistance to endogenous LEP. Similarly, in obese patients with type II diabetes, the pathomechanism involves resistance against hyperinsulinemia, primarily the result of resistance to LEP at the level of the ß cells of the pancreatic islets, leading to derangement of the adipocyte-insulin axis [14-17]. Despite several attempts to do so, the mechanisms behind resistance to LEP have not been fully explained.

Serum LEP concentrations are related to several variables, including the proportion of adipose tissue, sex, age, levels of other hormones (for example insulin and steroids), pro-inflammatory cytokines, stress and diseases (elevated levels may be detected in diseases of the liver, kidneys and autoimmune system) [18-22]. Individuals of low weight and low body mass index (BMI) show low plasma levels of LEP while cytokine concentrations are higher in obese persons [14, 23, 24].

Conflicting opinions have been expressed regarding the correlation between serum LEP levels and those of estradiol and progesterone. In most studies, a positive relationship has been detected between LEP concentration and increased levels of progesterone in the luteal phase of

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the menstrual cycle [25-30]. Positive correlations have also been described between serum levels of insulin and LEP, and an interrelationship has been noted between the proportion of adipose tissue in the body and LEP levels [17, 31].

The studies we have undertaken on the potential relationship between LEP and the development of myomas have been prompted by, among others, the increased frequency of tumor development in obese relative to lean women [29, 32-34]. Moreover, in various organs, LEP stimulates angiogenesis, proliferation and migration, including in smooth muscle cells which are found in myomata [12, 35-37].

An earlier report, on the potential relationship between LEP and uterine myomas, indicates that, in certain patients, serum LEP levels are lower than in healthy women [38]. In our earlier studies, performed using RT-PCR, we demonstrated the expression of the leptin gene in uterine myomas and in the surrounding myometrium, while no such expression has been detected in myometrial fragments taken from a prevailing number of healthy women [39]. These results have been corroborated by the demonstration of LEP proteins in the studied tissue fragments using Western blotting and immunohistochemistry. Furthermore, in healthy myometrium and in uterine myomas, as well as in the surrounding unaltered myometrium, expression of the long receptor isoform, OB-R_L, has been detected. These data suggest that LEP expression in the myometrium may be linked to the development of myomas.

The present study aimed at: (i) characterization of leptin receptor isoforms present in normal myometrium, and in myomata and their unaltered surrounding myometrial tissue; (ii) detection of the incidence of leptin gene expression and the manifestation of its receptor isoforms in normal myometrium relative to phases of the menstrual cycle, serum levels of LEP and insulin, BMI scores and to the proportion of adipose tissue in the body; (iii) determination of the frequency with which the leptin gene is expressed and leptin receptor isoforms are present in uterine myomas and unaltered surrounding myometrial tissue, relative to phases of the menstrual cycle, serum levels of LEP and insulin, BMI scores and to the proportion of adipose tissue in the body.

Material and Methods

Both the myoma and the surrounding myometrium were obtained from 30 women who underwent surgery for uterine myomas. The ages of the patients ranged from 30 to 79 years and none of the women had been treated with hormones. In all cases the histopathological diagnosis showed *leiomyomata uteri*. Among the studied patients, ten women were in the proliferative phase of their menstrual cycle while ten were in the secretory phase. Ten patients were postmenopausal. The control group consisted of 30 women aged between 25 and 64 years who had undergone surgery owing to the presence of benign ovarian cysts, uterine prolapse or postmenopausal bleeding. In this control group, ten women were in the proliferative phase of their menstrual cycle, ten were in the secretory phase and ten women were postmenopausal. Phases of the cycle were defined by menstrual anamnesis and by histopathological appraisal of endometrial material obtained during surgery.

In the morning, prior to surgery, fasting blood was sampled in order to estimate serum levels of LEP, estradiol, progesterone and insulin. Also, body weight, BMI and waist/hip circumference ratio (WHR) were estimated in a routine manner and a detailed history was collected to exclude patients burdened with metabolic diseases such as diabetes and hyper or hypothyroidism from the studies.

The percentage of body fat was calculated according to Deurenberg [40]: % body fat = 1.2 x (BMI) + 0.23 x age – 10.8 x sex (males: 1, females: 0) – 5.4.

The Bioethical Committee of the University of Medical Sciences in Poznań approved the study protocol.

Tissue material

The tissues (samples of unaltered myometrium, myomas and in some cases white adipose issue) were placed in saline, at 4°C immediately after excision. Within 10 to 20 minutes, fragments for RNA isolation were frozen at –80°C and stored at that temperature. The remaining tissue fragments were used for histopathological diagnosis. Subcutaneous adipose tissue served as a control.

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

RT-PCR studies were performed in all studied patients. RNA extraction and RT-PCR were performed using reagents from Qiagen (USA) according to the manufacturer's manuals (RNA extraction using reagents from companies other than Qiagen has been described separately).

Total RNA was extracted by a coupled method using TRI REAGENT (Sigma) and purification in mini columns (RNeasy Mini Kit, Qiagen), 30-60 mg of tissue were homogenized in TRI REAGENT, chloroform and isopropanol were added as specified by Sigma. The sample was pipetted onto RNeasy mini columns and the Qiagen protocol was applied [41]. Contaminating DNA was eliminated by DNase I treatment (RNase-Free DNase Set); 10-20 μg RNA were obtained in a single procedure.

Reverse transcription was performed using an Omniscript RT Kit with random hexamers as primers (Gibco-BRL, USA). PCR was performed using the HotStarTaq PCR Master Mix Kit. PCR primers were purchased from Invitrogen (USA). Primer sequences are shown in Table 1. PCR reactions were performed under the following conditions:

For GAPDH amplification: hot start Taq polymerase activation –95°C, 15 min.; (denaturation: 94°C, 30 sec.; annealing: 59°C, 30 sec.; extension: 72°C, 1 min.) – 30 cycles; final extension 72°C, 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, 60 sec.; annealing: 60°C, 60 sec.; extension: 72°C, 2 min.) – 40 cycles; 72°C, 10 min.

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

Two RT reactions were performed for each RNA prep after DNase I treatment: (1) without reverse transcriptase (RT negative control) and (2) with reverse transcriptase (RT positive control), followed by PCR with GAPDH primers. Only those RNA preps which produced no band for the RT negative control (confirmation of lack of contaminating DNA) and gave a strong GAPDH band for the RT positive control (confirmation of good

Table 1. — *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	[64, 65]
	GAPDH-2(E5/E6)	atgagccccagccttctccat		J04038
Leptin	OB-1(E2/E3)	cacacacgcagtcagtctcc	298	[66]
	OB-2(E3)	accacctctgtggagtagcc		D63708, D63709, D63710,
Leptin receptor (extracellular domain)	OBRu-1(E4/E5)	gtgaagcctgatccaccatt	340	[67, 68]
	OBRu-2(E7)	cattagaccccacacttgtcaga		U66497
Leptin receptor (leptin binding domain[3])	OBR_{LBD}-1(E7)	tgcagtgtactgcaatg	414	[67]
	OBRLBD-2(E10)	tctgctttcacactggatgg		U66497
Long isoform of receptor (L)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	439	[67, 53]
	OBRL-2(E18)	ctgatcagcgtggcgtattt		U43168
Short isoform of receptor (s1)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	227	[67, 53, 5]
	OBRs1-2(E18)	ctgtggccttccgcagtg		U52912
Short isoform of receptor (s2)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	176	[67, 53, 5]
	OBRs2-2(E18)	acctccacccagtagttcctt		U52913
Short isoform of receptor (s3)	OBRc-1(E16)	ttgtgccagtaattatttcctctt	200	[67, 53, 5]
	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.*, [65] and Mix *et al.*, [53] (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.

quality of RNA) were used for the examination of LEP and leptin receptor isoforms.

Moreover, in each set of assays the following control reactions were performed:

(1) negative control: without RNA – to exclude contamination of the reagents used for RNA isolation and RT-PCR reactions. mRNAs coding for leptin and isoforms of its receptor are widespread in the human body, making it difficult to find a reliably negative control. Because of this, for each set of RNA preparations from tissues, one blind control was prepared – isolation without the addition of any human material. This "RNA free" prep was then used as a negative control in each RT-PCR test round.

(2) positive control: using RNA extracted from adipose tissue – to confirm the appropriate reaction conditions of RT-PCR for GAPDH, OB, OBRu, OBRLBD; OBRL, OBRs1 and OBRs3 [26].

Western blotting

In some randomly chosen cases Western blotting was used to identify leptin and leptin receptor proteins. 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 on 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 membranes were halved. One part contained proteins with a molecular weight similar to that of the long form of the leptin receptor (116.5 kDa) and in the other part were proteins of a molecular weight resembling that of leptin (16 kDa). Non-specific binding was blocked by immersing the membrane in 5% BSA at 4°C overnight. Leptin-containing membranes were then incubated with primary antibodies from Santa Cruz Biotechnology, Inc (USA), at a 1:1,000 dilution for one hour at 37°C. Ob (A-20) (sc-842) is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at 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 at the carboxy terminus of Ob-R of mouse origin. After washing with TBST (10 mmol/l Tris, pH 8.0, 150 mmol/l NaCl, 0.05% Tween-20) for 3 x 10 min, leptin-containing membranes were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO, USA) at a 1: 20,000 dilution for 1 hour at room temperature. For detection of Ob-R, horseradish peroxidase-conjugated anti-goat IgG (Sigma, St. Louis, MO, USA, at a 1: 20,000 dilution for 1 hour at room temperature) was used. The membrane was washed for one hour with TBST and the target protein was detected by means of CL-X Posure Film from Pierce Biotechnology.

Estimation of hormone concentrations in serum

Serum levels of estradiol and progesterone were estimated by electrochemoluminescence (ECL) in an ELECSYS analyzer (Roche), using commercial reagent kits. Insulin concentration was estimated by the immunoenzymatic MEIA technique in an Axsym analyzer (Abott), using a reagent kit supplied by the producer. The concentration of LEP in serum was estimated by an ELISA technique using the Leptin (human) ELISA Kit, AK-153 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA).

Statistical analysis of results

Quantitative traits were characterized by their mean values and respective standard deviations ($x \pm SD$). 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 $p < 0.05$. Inter-relationships between selected variables were defined using Spearman's rank correlation index. Statistical analyses were performed using Statistica 5.0 software.

Results

The ages of the examined women, their body weights and heights are presented in Table 2. As is evident from the data, women with uterine myomas were older and had higher body weight as compared to women without myomas. Moreover, postmenopausal women in the control group proved to be older compared to premenopausal women.

Table 2. — *General characteristics of the control group and women with myomas. Each of the groups included ten women. The results are given as x ± SD.*

Group	Age [years]	Body weight [kg]	Height [cm]
	Control		
Proliferative phase	36.5 ± 6.8	58.6 ± 6.4	163 ± 4
Secretory phase	35.0 ± 7.6	58.3 ± 8.0	162 ± 7
Postmenopausal	53.3 ± 4.6 *A, B*	70.4 ± 11.0 *a, b*	163 ± 3
	Myomas		
Proliferative phase	45.9 ± 4.6 **	67.6 ± 6.6 **	163 ± 4
Secretory phase	42.8 ± 6.6 *	66.5 ± 9.5 *	166 ± 7
Postmenopausal	54.5 ± 10.1 *a, B*	66.2 ± 12.3	163 ± 4

Statistical evaluation of inter-group differences (Mann-Whitney's test): significantly different within the control group or myoma group respectively, at $p < 0.05$ (lower case letter) or at $p < 0.01$ (upper case letter); Significantly different from respective control group at $p < 0.05$ * or at $p < 0.01$ **.

In the control group, the BMI of postmenopausal women was significantly higher than in women in the proliferative and secretory phases but no significant differences in this respect were observed among women with myomas (Table 3). Among women of the control group, mean WHR index values were higher in postmenopausal women than in women examined at the proliferative phase. In all women with myomas this index was higher than in the respective subgroups of women who were free of myomas. Body adipose tissue content was higher in the control post-menopausal women than in either subgroup of premenopausal women. On the other hand, no differences in body adipose tissue content were observed in individual subgroups of women with myomas although the values were lower in the menstruating control women than in the corresponding group of women with myomas.

Table 3. — *Body mass index (BMI) [kg/m^2], ratio of waist/hip circumference (WHR) and percentage of adipose tissue in the control group and in women with myomas. In each group of 10 women the results are given as x ± SD.*

Group	BMI	WHR	% adipose tissue
	Control		
Proliferative phase	21.9 ± 2.2	0.728 ± 0.050	29.28 ± 3.27
Secretory phase	22.3 ± 2.8	0.747 ± 0.053	29.42 ± 4.52
Postmenopausal	26.6± 3.6 *A,B*	0.790 ± 0.059 *a*	38.14 ± 5.39 *A,B*
	Myomas		
Proliferative phase	25.6 ± 2.5**	0.832 ± 0.089**	35.86 ± 3.46**
Secretory phase	24.1 ± 2.6	0.812 ± 0.067*	33.68 ± 3.80*
Postmenopausal	24.7 ± 3.9	0.853 ± 0.057*	37.12 ± 4.97

Statistical evaluation of inter-group differences (Mann-Whitney's test): significantly different within the control group or myoma group respectively, at $p < 0.05$ (lower case letter) or at $p < 0.01$ (upper case letter); Significantly different from respective control group at $p < 0.05$ * or at $p < 0.01$ **.

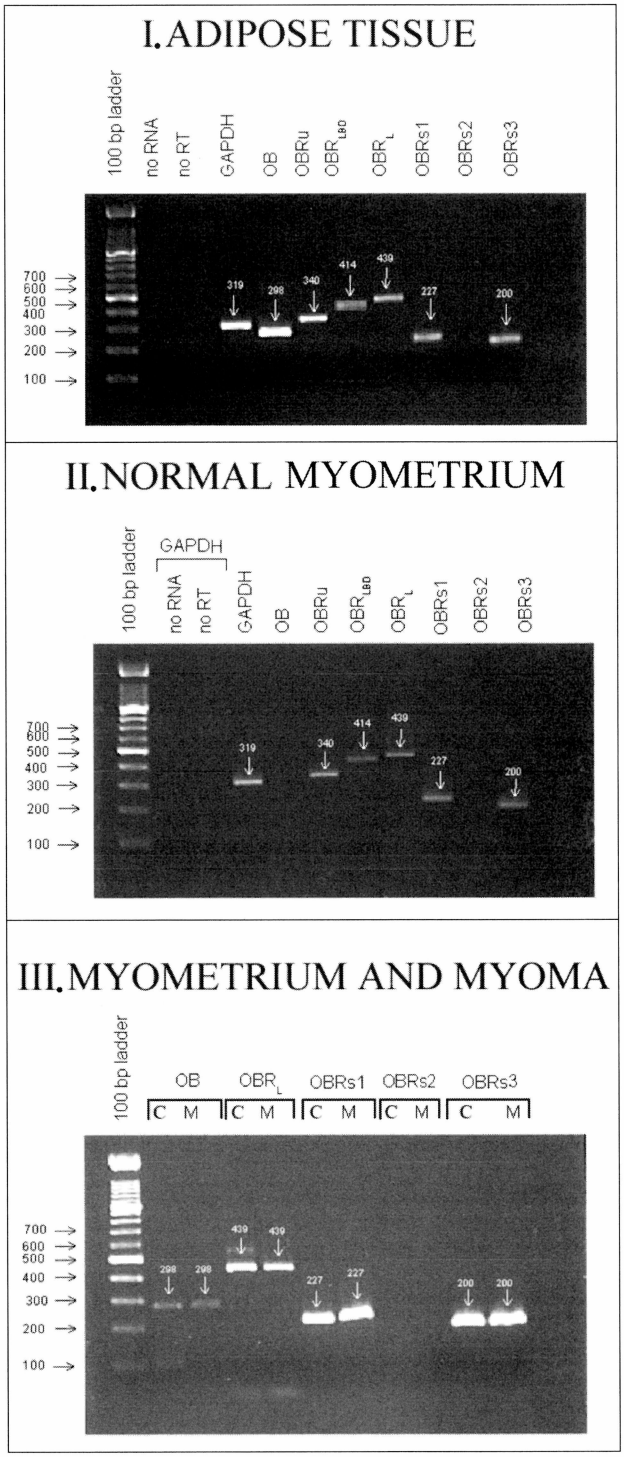

Figure 1. — Examples of typical results of RT-PCR studies on expression of leptin and its receptor mRNAs in: I - human adipose tissue, II - in normal myometrium and III - in myometrium and uterine myomas of patient with myomatous myometrium. RT – reverse transcriptase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; OB - leptin; OBRu – extracellular domain of leptin receptor; OBRLBD – leptin binding sequence of the receptor; OBRL - long isoform of the receptor; OBRs1, OBRs2 and OBRs3 – short isoforms of the receptor ; C – myometrium; M - uterine myoma.

Table 4. — *Serum concentrations of estradiol and progesterone in the control group and in women with uterine myomas. In each group of ten women the results are given as x ± SD.*

Group	Estradiol [pg/ml]	Laboratory norm [pg/ml]	Progesterone [ng/ml]	Laboratory norm [ng/ml]
		Control		
Proliferative phase	84.24 ± 25.96	12.5-166.0 (62.2)	0.47 ± 0.27	0.2-1.5 (0.7)
Secretory phase	158.92 ± 123.07	43.8-211.0 (106.0)	7.72 ± 4.63 *A*	1.7-27.0 (11.0)
Postmenopausal	18.20 ± 3.47 *A,B*	5.0-54.7 (12.0)	0.32 ± 0.19 *B*	0.1-0.8 (0.3)
		Myomas		
Proliferative phase	132.56 ± 77.98		0.56 ± 0.26	
Secretory phase	154.99 ± 77.22		6.75 ± 7.23 *A*	
Postmenopausal	45.83 ± 43.94 A,B		0.80 ± 0.62 *B*	

Statistical evaluation of inter-group differences (Mann-Whitney's test): significantly different within the control group or myoma group respectively, at $p < 0.05$ (lower case letter) or at $p < 0.01$ (upper case letter); Significantly different from respective control group at $p < 0.05$ * or at $p < 0.01$ **.

Table 5. — *Serum concentrations of leptin and insulin in the control group and in women with uterine myomas. In each group of 10 women the results are given as means ± SD.*

Group	Leptin [ng/ml]	Insulin [μU/ml]
	Control	
Proliferative phase	4.30 ± 3.29	11.13 ± 9.34
Secretory phase	4.22 ± 2.17	12.10 ± 9.65
Postmenopausal	3.80 ± 2.71	9.50 ± 5.89
	Myomas	
Proliferative phase	8.50 ± 3.45*	5.51 ± 1.68
Secretory phase	5.25 ± 1.95 *a*	11.10 ± 7.07 *a*
Postmenopausal	5.26 ± 3.15 *a*	10.95 ± 11.52

Statistical evaluation of inter-group differences (Mann-Whitney's test): significantly different within the control group or myoma group respectively, at $p < 0.05$ (lower case letter) or at $p < 0.01$ (upper case letter); Significantly different from respective control group at $p < 0.05$ * or at $p < 0.01$ **. Normal values for insulin concentration: 0 – 17 [μU/ml].

Both in the group of women free of myomas and among women with myomas serum estradiol levels were higher in menstruating women than in postmenopausal women (Table 4). Progesterone levels in all the examined women were higher at the secretory phase than in the proliferative one or following menopause.

Serum LEP and insulin levels in women with no myomas were similar at the proliferative, secretory and postmenopausal phases (Table 5). In contrast, LEP levels in women with myomas were higher at the proliferative phase than at the secretory or postmenopausal phases and also higher than in myoma-free women at this phase. Conversely, in women with myomas, insulin concentration at the secretory phase was higher than in women at the proliferative phase.

In the white adipose tissue RT-PCR studies revealed expression of both the leptin gene and the gene for the isoforms of its receptor. The isoforms were identified jointly by demonstration of expression of the common extracellular domain (OBRu) and of the leptin-binding extracellular domain (OBRLBD). Expression of the OBR_L, $OBRS_1$ and $OBRS_3$ leptin receptor isoforms were also selectively demonstrated (Figure 1). No expression of the OB gene could be shown in the myometrium of women in the control group although the tissue exhibited expression of the short receptor isoforms, $OBRS_1$ and $OBRS_3$. In contrast to the control group, the myomas and myometrium of women in the study group showed expression of the OB gene as well as genes for the OBR_L, $OBRS_1$ and $OBRS_3$ receptor isoforms. Expression of $OBRS_2$ was not detected in any of the examined cases. Western blotting failed to demonstrate the presence of the leptin protein in the myometrium of the control group women but its presence was demonstrated in both the myometrium and in the myomas of myomatous women (Figure 2). The presence of the protein for the long leptin receptor isoform was documented in both the myometrium and the myomas of all the randomly chosen examined cases.

The cumulative results of the RT-PCR studies are presented in Table 6. As is evident from data presented in the table, women with myomas show more frequent expression of the OB gene, both in their myomas and in their myometrium, than may be seen in the myometrium of women with no myomas. It should be noted that in some women with myomas, expression of the gene was noted only in the myometrium (n = 6) or only in the tumor (n = 4).

Table 6. — *Leptin and isoforms of its receptor in the uterine myometrium of control group women and in the myometrium and myomata of women with myomas. In every group the studies were conducted on 10 individuals. Gene expression was examined by RT-PCR. In the table the number of positive results per 10 tests is given in every group.*

Tissue	Leptin	OBRL	OBRs1	OBRs2	OBRs3
		Control			
Myometrium	0/10	9/10	10/10	0/10	10/10
		Myomas			
Proliferative phase - myometrium	8/10***	10/10	10/10	0/10	10/10
Proliferative phase - myoma	6/10*	10/10	10/10	0/10	10/10
Secretory phase - myometrium	7/10**	10/10	10/10	0/10	10/10
Secretory phase - myoma	7/10**	9/10	10/10	0/10	10/10
Postmenopausal - myometrium	7/10**	10/10	10/10	0/10	10/10
Postmenopausal - myoma	6/10*	7/10	10/10	0/10	10/10

Statistical evaluation of differences according to Fishers' test. Differences as compared to the control group are significant at $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. from respective control group at $p < 0.05$ * or at $p < 0.01$ **. Normal values for insulin concentration: 0 – 17 [μU/ml].

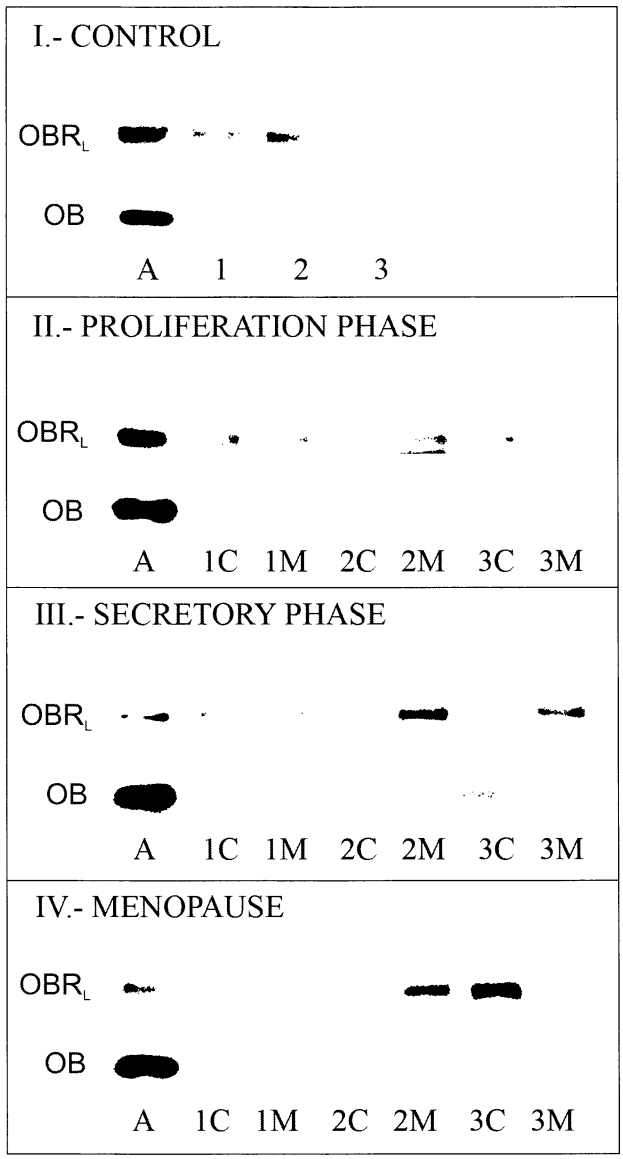

Figure 2. — Western blotting of leptin (OB) and long isoform of leptin receptor (OBRL) in myometrium of women of control group (without uterine myomas; I – control) and in women with uterine myomas (II – proliferation phase, III – secretory phase, and IV - menopause). A – positive control (white adipose tissue); C – myometrium; M – uterine myoma. Numbers – number of studied patient.

Expression of the long form of leptin receptor (OBR_L) was demonstrated in nine out of ten studied myometrium fragments from the control group. In the group with myomas, the expression was shown in all women at the proliferative phase (both in myomas and in the surrounding myometrium), in all women at the secretory phase in the myometrium and in nine out of ten women in myomas, in all postmenopausal women in the myometrium and in seven out of ten women of the group in myomas. Expression of the short leptin receptor isoforms $OBRS_1$ and $OBRS_3$ was demonstrated in all the examined tissues while expression of the short $OBRS_2$ form could not be documented in any case (Table 6).

No correlation could be detected in either the control group or in the myoma patient group between concentrations of LEP and insulin on the one hand, and body weight, BMI, WHR and adipose tissue content on the other (Table 7). Also, no correlation was found in women with uterine myomas between serum LEP concentration and the expression of the OB gene or the gene for its long receptor isoform (OBR_L) (Table 8).

No correlation could be detected between expressions of the OB gene in the myometrium or myomas of myomatous women and the BMI or body adipose tissue content (Table 9). No inter-relationships could be detected between concentrations of LEP and insulin in the sera of the studied groups of women (Table 10).

Discussion

LEP plays an important role in the female reproductive system, both within the gonads and in the genital tract. In recent years, RT-PCR studies have reproducibly demon-

Table 7. — *Spearman's rank correlation indices for serum concentrations of leptin or insulin vs body mass, BMI, WHR and adipose tissue content of the body in studied women.*

Concentration of leptin or insulin in serum	Leptin [ng/ml]		Insulin	
	Control	Myomas	Control	Myomas
Body weight	0.028	- 0.049	- 0.234	- 0.198
BMI	0.058	- 0.159	- 0.129	- 0.013
WHR	- 0.213	- 0.139	- 0.098	- 0.221
% adipose tissue	- 0.025	- 0.143	- 0.111	- 0.204

None of the indices reached values suggestive of even slight correlation between analysed variables.

Table 8. — *Serum leptin concentrations vs expression of the leptin gene (OB) and the gene for the long isoform of the leptin receptor (OBRL) in myometrium and myomas of myomatous women. Concentrations of leptin [ng/ml] are given as means ± SD; numbers with + or – signs correspond to the number of cases with, respectively, positive or negative results of the test for gene expression). Number of examined women: 30.*

	myometrium		myoma	
OB	8 -	5.39 ± 2.54	11 -	5.73 ± 3.77
	22+	6.52 ± 3.30	19 +	6.35 ± 2.75
OBRL	0 -	0	4 -	4.58 ± 1.42
	30+	6.24 ± 7.79	26 +	6.38 ± 3.20

Mann-Whitney's test was used for statistical evaluation of the results: differences in serum leptin levels were not related to expression of the OB gene; no relationships were detected between expression of the OB gene and blood leptin levels; owing to a low number of negative results, similar calculations for OBRL could not be performed.

Table 9. — *Expression of the leptin gene (OB) in the myometrium and in myomas of myomatous women as related to BMI [kg/m2] and percentage of body adipose tissue [%]. Results are given as means ± SD. numbers with + or – signs correspond to the number of cases with, respectively, positive or negative results of the test for gene expression. Number of examined women: 30.*

	OB expression (myometrium)		OB expression (myoma)	
BMI	8 -	26.19 ± 3.83	11 -	26.27 ± 2.72
	22 +	24.13 ± 2.41	19 +	24.20 ± 3.07
% adipose tissue	8 -	36.49 ± 6.42	11 -	37.11 ± 3.49
	22 +	34.94 ± 2.66	19 +	34.74 ± 4.49

Statistical evaluation of the results using Mann-Whitney's test: no differences between the individual groups; no relationships between expression of the OB gene in the myometrium or in myomata and BMI and content of adipose tissue.

Table 10. — *Spearman's rank correlation indices for insulin concentration vs leptin concentration in serum.*

Insulin concentration vs:	Spearman's rank correlation in the group of:	
	Control	*Myomas*
Leptin concentration	0.211	0.126

Conclusion: no relationships were detected between insulin concentration and leptin concentration in the serum of either group.

strated the expression of the leptin receptor in the endometrium (both in the lining, glandular epithelium and in its sublayer) and in the myometrium. In uterine epithelium the long receptor isoform is mainly expressed while in the sublayer and in the myometrium both the long and short isoforms are present [42-46]. In contrast to this receptor, no expression of the leptin gene has been noted in unaltered endometrium or myometrium [39, 42, 43, 46, 47]. Only Gonzales *et al.* [48] have been able to demonstrate the expression of the OB gene in the endometrium. Expression of the long isoform of the leptin receptor in the endometrium and myometrium suggests that LEP participates in the control of endometrial growth and function. The role of LEP has been shown to be linked to blastocyst implantation [48-52]. However, recent studies demonstrate that the leptin-leptin receptor system of the uterine wall may also be important for the development of pathology within the organ. Disturbed expression of the leptin receptor may be observed in, for example, certain forms of infertility [42].

The results of this study have confirmed our earlier observations regarding the lack of expression of the OB gene in the myometrium of control group women [39]. Our preliminary observations have now been extended by the discovery that the receptor's long isoform (OBR_L) and short isoforms ($OBRS_1$ and $OBRS_3$) are present in the myometrium of such women. Significantly more frequent expression of the OB gene has been observed in the myomas and the surrounding, macroscopically unchanged myometrium of affected patients than in the myometrium of myoma-free women. It should also be noted that in a proportion of the examined women with myomas, expression of the gene was detected only in the myometrium (n = 6) or only in myomas (n = 4). Since in practically all the studied cases (groups of women with or without myomas) and in all tissues (normal myometrium, myomas, myoma-surrounding myometrium) expression of the genes for the leptin receptor (its long form and its short forms, $OBRS_1$ and $OBRS_3$) was observed, it seems reasonable to suggest that LEP may be locally produced in myomas and in the surrounding myometrium and that it may be involved in the development of benign uterine tumors. The expression of the leptin gene and its receptor gene which we have observed suggests a potential paracrine action of the cytokine within pathologically altered myometrium. This is supported by the results of studies into LEP serum levels in examined women which showed no significant differences (except for in the proliferative phase of women with uterine myomas). It should be noted that Chan *et al.* [38] noted lower LEP levels in women with leiomyomas than in a control group; in the former group the levels failed to correlate with BMI. Possibly, the difference between those results and ours stems from use of different techniques for the assaying of serum LEP levels (we employed an ELISA approach while Chan *et al.* used a RIA technique). In any case, such discrepancies in the serum LEP levels of women with myomas (lowered levels or no change) would seem to lend support to our hypothesis regarding a local (most probably paracrine or autocrine) action of LEP in myoma development.

It should be stressed that in all of the studies on myomas, the surrounding myometrium and the myometrium of healthy women, expression of the short isoform $OBRs_2$ could not be demonstrated. Literature data indicate that expression of that isoform is very restricted: it has been detected only in human fetal liver and in the epithelial cells of the stomach [53]. 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 recent years two reports have documented the expression of the leptin gene and the leptin receptor gene in cases of endometriosis [46, 47]. Wu *et al.* [47] expressed the opinion that the angiogenic and mitogenic activity of LEP might be linked to the development of endometriosis. In the eutopic endometrium of women with endometriosis, and in women in control groups, only the gene for the leptin receptor has been expressed while in ectopic endometrium (endometriotic implants) mRNAs for both leptin and for its receptor have been detected. According to the authors, the differential distribution of leptin and its receptor suggests an autocrine and/or paracrine role of LEP in the pathogenesis of endometriosis. Even if related to a distinct pathology – uterine myomas – our results suggest an analogous role for LEP.

Numerous studies into the epidemiology of uterine myomas indicate that such tumors develop more frequently in older and obese women than in young and lean women [14, 32, 33, 54, 55]. These observations have been corroborated in multiple reports and in our presented data, including those regarding the content of adipose tissue in the body.

Interesting observations arose from the WHR measurements of the studied women. In all groups of women with myomas, the index proved to be significantly higher than in women of the control group. As noted by Shimokata *et al.* [25], WHR represents one of anthropometric indices used for the classification of various groups of body structure. The results would seem to suggest that women with higher WHR values, usually belonging to the group of women with visceral obesity, are more susceptible to the development of uterine myomas, as they are similarly more susceptible to other diseases, including metabolic disturbances [56-58]. Thus, it seems that uterine myomas develop more frequently in women of such somatotype.

One of theories related to the development of myomas points to their relationship to the sex hormones, estrogen and progesterone [14, 25, 31]. Nevertheless, opinions regarding the effects of these hormones on uterine myomas and their development are divergent. The stimulatory role has been ascribed to both estrogen and to gestagens [33, 36, 59]. In our studies, serum estradiol and progesterone levels were the same in the group of women

with uterine myomas and in the control group. Divergent results have been reported on serum LEP levels at various phases of the menstrual cycle. According to some authors the levels are higher at the luteal phase [5, 26, 28] but results of other authors have failed to document effects of circulating sex hormones on LEP levels in blood [27, 29, 43, 61, 62]. Our results obtained in the control group have been consistent with the latter reports. Slightly different results have been observed in women with a myomatous uterus: LEP levels were significantly higher at the proliferative phase as compared to women in the control group at the same phase. LEP levels at the secretory phase and following menopause were significantly lower than in women with myomas at the proliferative phase. Such a result cannot be explained by a reciprocal relationship between circulating levels of LEP and sex hormones or between LEP levels and insulin levels in the blood, as postulated by Zimmet *et al.* (1996), Boden *et al.* (1997) and Seufert *et al.* (2004) [17, 31, 63]. In this study, no correlation between the two variables could be detected and insulin levels were lowest at the proliferative phase in myomatous women.

In our studies on the control group and on the myomatous patient group studied as a whole, no relationship between LEP and insulin or any relationships between serum LEP levels and BMI, WHR and body fat content were detected. Chen *et al.* [38] also stressed the lack of relationships between BMI and serum LEP levels in women with myomas (but not in healthy women). The authors interpreted the finding as a disturbance of a normally existing inter-relationship between LEP production and the amount of adipose tissue, which is absent in women with myomas.

No studies have appeared in the literature regarding the effect of LEP on the growth of uterine myomas. Among the potential influences cited are: genetic predisposition, exposure to environmental factors, genetic mutations and reactions of myometrial cells to steroid hormones. Cytokines and growth factors are mentioned most frequently [33, 36, 59, 60]. Only Chen *et al.* [38], suggested that lowered blood LEP levels are linked to myoma pathogenesis, based on lowered blood LEP levels in women with myomas. Our studies have failed to confirm these suggestions since they indicate that a local system of leptin/leptin receptor and not blood LEP levels per se, is most probably linked to the development of uterine myomas. Most likely, this involves autocrine and/or paracrine signaling and is observed more frequently in women with somatotypes associated with elevated WHR.

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