Expression of mRNA for Chemokines and Chemokine Receptors in Tissues of the Myometrium and Uterine Leiomyoma

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Tissues samples of leiomyoma and myometrium obtained intraoperatively were analyzed. For evaluation of the synthesis of MIP-1α, MIP-1β, RANTES, eotaxin, eotaxin-2, interleukin-8, CCR1, CCR3, CCR5, CXCR1, and CXCR2, mRNA isolated from tissues samples of leiomyoma and myometrium was subjected to reverse transcription-PCR and assayed by a semiquantitative method (relative to β-actin). The content of eotaxin, MIP-1α, MIP-1β, and CCR5 mRNA in leiomyoma tissue was lower than in the myometrium. The concentration of MIP-1β, CCR5, and eotaxin mRNA in common leiomyoma was much lower than in the myometrium. Eotaxin mRNA expression in myometrial tissue of patients with single nodes was much higher than in those with multiple nodes. Moreover, expression of eotaxin mRNA in common leiomyoma was higher than in proliferating leiomyoma. The concentration of mRNA for interleukin-8 in leiomyoma tissue, as well as the content of mRNA for MIP-1α and CCR3 in myometrial tissue increased in patients with submucosal nodes (as distinct from nodes of another location). A direct correlation was revealed between the size of the uterus and concentration of mRNA for interleukin-8 and MIP-1β in myometrial tissue. The concentration of mRNA for MIP-1α and MIP-1β in leiomyoma tissue negatively correlated with the size of the uterus (maximum size of the node) and duration of leiomyoma, respectively. Our results indicate that chemokines play an important role in the pathogenesis of uterine leiomyoma.

Key Words: *chemokines; chemokine receptors; mRNA; uterine leiomyoma; myometrium*

Little is known about the pathogenesis of uterine leiomyoma (UL). The main clinical sign of UL is overgrowth of myomatous nodes accompanied by pain syndrome and hemorrhage. Proliferation of smooth muscle cells and abnormal extracellular matrix (collagen) determines clinical signs of UL. The development and progression of UL are primarily associated with abnormal production of female sex hormones. This conclusion was derived from tumor regression in the majority of postmenopausal patients. However, the pathogenesis of UL is poorly understood. Hence, it is important to develop new markers for the state of the myometrium and UL tissue.

Chemokines are peptides producing a pleiotropic effect on cells and organism. Biological activity of chemokines is realized via the interaction with specific receptors belonging to a family of G protein-coupled receptors (GPCR).

Eotaxins are responsible for eosinophil recruitment. CCR3 is a specific receptor for these com-

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pounds [4]. Interleukin-8 (IL-8) binds to CXCR1 and CXCR2, which contributes to chemotaxis and activation of neutrophilic granulocytes [4]. Monocytes and T lymphocytes expressing CCR1 and CCR5 are the targets for MIP-1α, MIP-1β, and RANTES [4]. Little is known about chemokine RNA expression. The majority of data were obtained in studies of the endometrium. For example, MIP-1β RNA expression was identified in endometrial tissues after hysterectomy [9]. Constitutive secretion of MIP-1β and IL-8 is typical of endometrial cells [7]. Moreover, endometrial cells express RANTES and MIP-1α [5,8]. Previous experiments showed that eotaxin and CCR3 receptor are expressed in the endometrium [15]. Several studies were devoted to the effect of hormones on activity of chemokines and receptors. It was hypothesized that T lymphocytes expressing estrogen receptors produce a modulatory effect on autoimmune processes [13]. Expression of mRNA for CCR receptors increases after addition of estrogens to the culture of T lymphocytes [11]. The concentration of mRNA for CXCR1 and CCR5 in the endometrium depends on the phase of the menstrual cycle. mRNA production increases by hundreds of times during the luteal phase [6]. Expression of mRNA for IL-8 and CXCR1 in myometrial tissue around UL is 2-fold higher than in tumor tissue [12].

Hormonal disturbances in UL probably modulate local expression of mRNA for chemokines and receptors, which is followed by changes in proliferative function of myometrial cells.

Here we studied the expression of mRNA for chemokines and specific receptors in tissues of UL and myometrium. We evaluated the role of various chemokines in the pathogenesis of this disease.

MATERIALS AND METHODS

We examined 22 women of 34-54 years (average age 46.0±4.9 years, Table 1). Myometrial samples were obtained during biopsy. UL tissue was isolated during laparotomy or laparoscopy for hysterectomy or myomectomy. UL nodes were removed from the abdominal cavity, washed with cold physiological saline, and cut with a scalpel. Samples of myomatous tissue were excised from the peripheral (subcapsular) area of this node. In patients with multiple UL, biopsy specimens were obtained from the largest node. All samples were randomly encoded by the digital code, immediately frozen at -20°C, and stored until the study.

Tissue fragments of UL or myometrium (~0.005 g) were put in a plastic Eppendorf tube (1.5 ml) with 300 μl lysing solution D. The tube was maintained in a FinnSonic ultrasound washer at 65°C for 30 min. Isopropanol (400 μl) was added by the end of lysis. The sample was agitated on a Vortex for 3-5 sec. The tubes were centrifuged in an Eppendorf centrifuge (5415R) at 14,000 rpm and 4°C for 5 min. The supernatant was removed. Ethanol (500 μl, 70% solution) was added to the pellet. The tube was carefully turned over (3-5 times) and centrifuged at 14,000 rpm and 4°C for 5 min. The supernatant was removed. Acetone (500 μl) was added to the pellet. The tube was carefully turned over (3-5 times) and centrifuged at 14,000 rpm for 5 min. The supernatant was removed. The pellet was dried at 65°C for 5 min and dissolved in 50 μl DEPC H_2O at 65°C for 10 min. Reverse transcription was performed with Reverta kit (AmpliSens) according to manufacturer's recommendations.

The polymerase chain reaction (PCR) with specific primers was conducted as follows: denaturation of DNA at 95°C for 4 min; 30 cycles of amplification (95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec); and elongation at 72°C for 7 min.

We studied the expression of mRNA for chemokines (MIP-1α, MIP-1β, RANTES, eotaxin, eotaxin-2, and IL-8). Besides this, we measured the concentration of mRNA for specific chemokine receptors (CCR1, CCR3, CCR5, CXCR1, and CXCR2). These chemokines were selected to perform complex study of the chemokine system.

β-Actin served as the reference gene. PCR products were visualized in 1.5% agarose gel stained with ethidium bromide. The study was performed in UV light on a Volna transilluminator. Semiquantitative study involved Gel-Pro software. Fluorescence of β-actin was taken as 100%. To evaluate the role of chemokines in clinical signs of UL, we studied the dependence of mRNA concentration for chemokines and receptors on the type of tumor (common or proliferating tumor), number and location of myomatous nodes, growth rate, duration of UL, size of the uterus, and presence or absence of adenomyosis.

The results were analyzed by means of SPSS 13.0 software. Statistical treatment involved the nonparametric test with 2 variables (Mann—Wilcoxon—Whitney test), correlation study (Spearman test and Pearson test), and graphic presentation of data (histogram). Intergroup differences were significant at $p \leq 0.05$.

RESULTS

Expression of genes for eotaxin (p=0.005), MIP-1α (p=0.029), MIP-1β (p=0.003), and CCR5 (p=0.034) in UL tissue was much lower than in the myomet-

TABLE 1. Clinical Characteristics of Patients with UL (n=22)

Characteristic	Number of patients	Percentage of the total number
Duration of disease		
less than 5 years	11	50
more than 5 years	11	50
Myoma growth rate		
no data	5	23
slow	7	32
rapid	8	36
Type of uterine myoma		
common	16	73
proliferating	6	27
Number of nodes		
single (≤3)	8	36
multiple	16	64
Location		
submucosal	13	59
intramural and subserous	8	36
Adenomyosis		
with	7	32
without	15	68

rium (Table 2). Expression of mRNA for eotaxin, MIP-1β, and CCR5 in tissues of common UL was significantly lower than in the myometrium. These differences were not found in patients with proliferating UL (Table 3). We studied the dependence of mRNA expression in myometrial tissue on the number of myomatous nodes. Eotaxin expression in the myometrium of patients with single nodes was much higher than in those with multiple nodes (p=0.02, Fig. 1). We studied the dependence of mRNA expression in tissues of UL and myometrium on the number of nodes. Eotaxin mRNA expression in the myometrium of patients with single nodes was significantly higher than in UL (p=0.01). However, no differences were found in eotaxin mRNA expression in the myometrium and UL of patients with multiple nodes. We studied the dependence of mRNA concentration in myometrial tissue on the type of UL. Eotaxin gene expression in patients with common UL was much higher than in those with proliferating tumor (p=0.004, Fig. 2).

The location of myomatous nodes determines the clinical course and outcome of UL. The incidence of pain syndrome and hemorrhage in submucosal nodes is much lower compared to that in intramural and subserous nodes. The concentration of mRNA for IL-8 in UL (p=0.013) and expression of mRNA for MIP-1α (p=0.003) and CCR3 in the myometrium (p=0.046) increased in patients with submucosal node/nodes (as compared to nodes of another location).

We studied the dependence of chemokine mRNA expression on the growth rate of UL. CCR5 mRNA concentration in the myometrium of patients with slow growing or non-growing UL was much higher compared to rapidly growing tumors (p=0.003, Fig. 3). Besides this, CCR5 gene expression in the myometrium was higher than in UL tissue of patients with slow growing tumors. These differences were not found in patients with rapidly growing UL.

The concentration of mRNA for eotaxin-2 and CXCR2 in UL tissue and the content of MIP-1β mRNA in the myometrium of UL patients with adenomyosis were significantly lower compared to those with no adenomyosis (p<0.05).

Correlation analysis was performed with clinical signs and chemokine mRNA concentration. A positive correlation was revealed between the size

TABLE 2. Expression of mRNA for Chemokines and Receptors in Tissues of UL and Myometrium ($M\pm\sigma$, n=22)

Chemokine/chemokine receptor	Myometrium	UL	p
CCL3/MIP-1α	10.7±9.0	6.5±7.0	0.029
CCL4/MIP-1β	6.5±4.7	1.7±1.5	0.003
CCL5/RANTES	3.6±6.5	3.0±6.8	0.385
CCL11/eotaxin	4.2±5.9	1.5±1.9	0.005
CCL24/eotaxin-2	5.1±5.5	4.5±5.7	0.367
CXCL/8/IL-8	5.9±7.3	3.4±4.5	0.182
CCR1	55.4±21.5	47.9±20.8	0.333
CCR3	48.3±23.6	40.7±19.3	0.230
CCR5	58.9±23.0	44.7±23.1	0.034
CXCR1	45.1±24.6	17.1±20.1	0.173
CXCR2	30.9±20.8	24.7±14.6	0.857

TABLE 3. Dependence of Expression of mRNA for Chemokines and Receptors in Tissues of UL and Myometrium on the Type of UL ($M \pm \sigma$)

Chemokine/ chemokine receptor	Type of UL				p 1-2	p 2-4
	common (n=16)		proliferating (n=6)			
	myometrium 1	UL 2	myometrium 3	UL 4		
Eotaxin	3.9±2.9	1.5±1.9	4.7±10.6	1.5±2.0	0.004	0.007
MIP-1β	7.8±6.1	3.6±4.5	11.3±10.4	3.3±5.6	0.002	0.687
CCR5	61.9±21.6	43.1±21.8	51.9±26.9	49.1±27.8	0.009	0.500

of the uterus and concentration of mRNA for IL-8 (r=0.62, p=0.004) and MIP-1β (r=0.5, p=0.02) in myometrial tissue. However, MIP-1α mRNA concentration in UL negatively correlated with the size of the uterus (r=-0.49, p=0.02) and maximum size of the node (r=-0.43, p=0.047). A negative correlation was found between MIP-1β mRNA concentration in UL and duration of the disease. Hence, long-term disease is manifested in the reduced production of MIP-1β by abnormal tissue.

UL is a long-term disease with various clinical manifestations. Tumor regression in the postmenopause period reflects the major role of hormones in the development and progression of this disease. However, local regulatory factors (*e.g.*, chemokines) are also of considerable clinical and diagnostic significance. MIP-1β mRNA concentration progressively increases from the early proliferative phase (minimum level) to the late secretory phase of the menstrual cycle. Addition of progesterone to the culture of endometrial stromal cells was followed by an increase in MIP-1β expression in the supernatant [9]. Expression of CCR1 and CCR3 in estrogen-receiving gonadectomized mice was much higher than in the placebo group [11]. Estrogens and progesterone can modulate the growth of UL, which is mediated by activation of growth factors and inhibition of apoptosis [10]. The development of UL is probably associated with reduced expression of chemokines and receptors in myometrial tissue. These changes are followed by uncontrolled proliferation of myocytes and collagen during hormonal disturbances.

We showed that expression of genes for eotaxin, MIP-1α, MIP-1β, and CCR5 in UL tissue is lower than in the myometrium. This fact is probably related to impaired production of regulatory factors (chemokines and receptors) in abnormal tissue, which results from the loss of regular cellular structure. As distinct from proliferating UL, the content of MIP-1β, CCR5, and eotaxin in tumor tissue of common UL was significantly lower than in the myometrium. Previous studies showed that the number of natural killer cells in the peripheral blood from patients with proliferating UL is higher compared to those with common tumors [1]. MIP-1β contributes to migration of natural killer cells in uterine tissue [9]. Low expression of mRNA for MIP-1β and CCR5 in myometrial tissue of patients with proliferating UL probably promotes a compensatory increase in the synthesis of this chemokine and specific receptor in the peripheral blood. These changes are followed by an increase in the number of natural killer cells. Eosinophilic infiltration was observed in 3 patients with UL. However, the genesis of this symptom remains unclear [14]. Our study showed that eotaxin mRNA expression in the myometrium of patients with common UL is higher compared to those with proliferating tumor. Besides this, eotaxin mRNA expression in the myometrium of UL patients with single nodes was much higher than in those with multiple nodes. Our previous studies showed that the concentration of IL-5 (eosinophil growth factor) in the peritoneal fluid of UL patients with single nodes is higher than in those

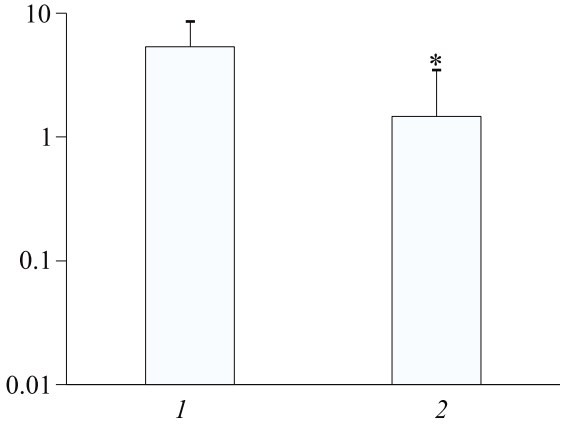

Fig. 1. Eotaxin mRNA concentration in myometrial tissue during single (*1*) and multiple nodes (*2*). *p=0.02 compared to single nodes.

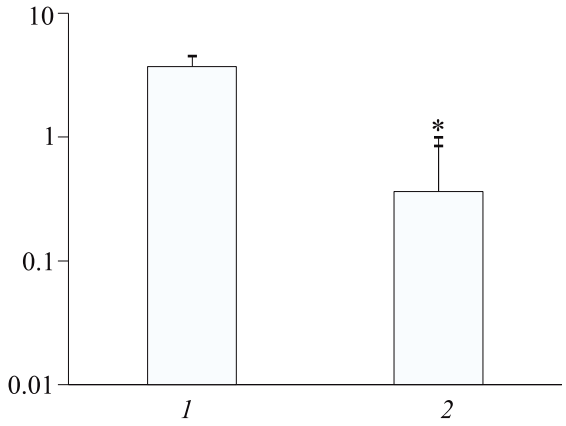

Fig. 2. Eotaxin mRNA concentration in myometrial tissue during common (*1*) and proliferating UL (*2*). *p=0.004 compared to common UL.

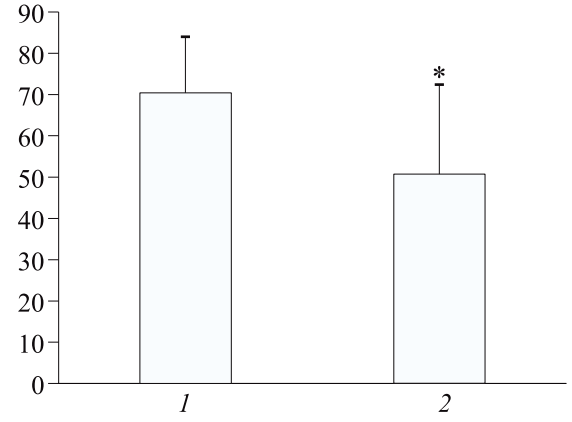

Fig. 3. CCR5 mRNA concentration in myometrial tissue during slow growing (*1*) and rapidly growing UL (*2*). *p=0.003 compared to slow growth.

with multiple nodes [3]. The data indicate that eosinophils play a protective role in the clinical course of this disease.

Measurement of IL-8 activity in tissues of UL and myometrium by means of an immunohistochemical study and enzyme immunoassay showed that expression of chemokine mRNA in UL tissue is lower than in the myometrium. Addition of monoclonal antibodies against IL-8 to the culture of native myometrial cells was followed by the decrease in myocyte proliferation [12]. The increase in IL-8 mRNA expression in UL tissue of patients with submucosal nodes probably reflects the involvement of neutrophil granulocytes in the pathological process. Cell influx contributes to the release of proteolytic enzymes (collagenase and myeloperoxidase) from granules of neutrophils. Our previous studies showed that allelic variants of collagenase genes (MMP-1) are associated with an unfavorable prognosis for the number of nodes and incidence of adenomyosis during UL [2]. The increase in CXCR1 mRNA concentration (IL-8 receptor) in the myometrium of patients with submucosal nodes reflects the relationship between expression of genes for the chemokine and receptor. It is manifested in increased synthesis of IL-8 in UL tissue and elevated production of CXCR1 in surrounding normal cells.

The increase in the concentration of mRNA for MIP-1α and MIP-1β in the myometrium of patients with submucosal nodes reflects the involvement of monocytes and T lymphocytes into the pathological process (as distinct from nodes of another location). CCR5 mRNA concentration in slow growing UL was higher compared to that in rapidly growing tumors. It can be hypothesized that the feedback between the synthesis of chemokines and receptors determines the course of UL. CCR5 is a specific receptor for MIP-1α, MIP-1β, and RANTES. This receptor probably serves as a factor inhibiting UL growth under conditions of increased expression of CCR5 in myometrial tissue.

The pathogenetic significance of MIP-1β expression in UL tissues was confirmed by comparing the expression of mRNA for this chemokine and duration of UL. A negative correlation was found between MIP-1β mRNA concentration and duration of UL. Hence, the ability of UL tissue to synthesize this chemokine decreases with progression of the disease.

The positive correlation between an increase in the size of the uterus and concentration of mRNA for IL-8 and MIP-1β probably reflects a compensatory increase in the synthesis of these chemokines during UL progression. The synthesis of MIP-1α in UL tissue negatively correlated with the size of the uterus and maximum size of the node. This chemokine probably serves as a factor inhibiting progression of the pathological process.

Eotaxin-2 synthesis in UL tissue of patients with adenomyosis was lower than in those without adenomyosis. These differences are probably associated with dysfunction of eosinophils during ectopia of the myometrium. CXCR2 mRNA concentration in UL tissue and MIP-1β mRNA content in the myometrium decreased in patients with adenomyosis, which reflects a deficiency of chemokine regulation.

Our results indicate that the abnormal tissue of UL is characterized by low synthesis of chemokines (eotaxin, MIP-1α, and MIP-1β) and chemokine receptors (CCR5). The concentration of mRNA for chemokines and specific receptors depends on the type of tumor, location and number of nodes, size of the uterus, duration of UL, and presence or absence of adenomyosis. These data illustrate the relationship between chemokine synthesis and chemo-

kine-receptor interaction. Therefore, molecules of this class play an important role in the development of UL and adenomyosis.

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