Profile of Selected Cytokines and Growth Factors in Uterine Myomas in Females of Various Age

P. Madej[1], D. Plewka[2], D. Pluta[*,1], R. Bilski[1], G. Franik[1], A. Madej-Czech[3], M. Maksym[1], M. Nylec[1] and S. Czech[4]

[1]*Department of Endocrinological Gynecology, Medical University of Silesia, Katowice, Poland*

[2]*Department of Histology and Embryology, Medical University of Silesia, Katowice, Poland*

[3]*Department of Intensive Care and Neonatal Pathology Medical University of Silesia, Zabrze, Poland*

[4]*District Hospital of Orthopedics and Trauma Surgery, Piekary Śląskie, Poland*

Abstract: *Objective*: Uterine myomas are the most common non-malignant tumours in adult females. Causes of development and progression of the tumour remain unclear but, apart from steroid hormones, factors which stimulate growth of uterine myomas include cytokines and growth factors. In this study an analysis was conducted of cytokine environment in myomas of various size and in healthy uterine tissue in women of various age. *Methods*: Concentrations of selected cytokines and growth factors were evaluated using immunohistochemical techniques in small (<3cm) and large (>5cm) uterine myomas, in women of generative age or perimenopausal age. Immunohistochemical studies were performed on properly prepared paraffin sections, to which the primary antibodies was applied. The obtained immunohistochemical reactions were evaluated under a light microscope. The results were subjected to statistical analysis. Photographic documentation was prepared with photographic attachment. *Results*: Interleukin-1 level in young women in small myomas was higher than in the control, amounting to 345%. In large myomas - it was 415%. In women of perimenopausal age in small myomas was augmented to 320% and in large myomas - 240%. Interleukin-6 level in young women in small myomas was higher than in the control, reaching 255%. In large myomas – it was 285%. In women of perimenopausal age in small myomas was augmented to 240% and in large myomas - 235%. TNF-α level in young women in small myomas was higher than that in the control, amounting to 265%. In large myomas – it was 230%. In women of perimenopausal age in small myomas was augmented to 270% and in large myomas - 140%. TGF-β1, TGF-β2, TGF-β3 levels in the young women in small myomas amounting to 310%, 285%, 335% of the control levels for, respectively, TGF-β1, TGF-β2 and TGF-β3. In large myomas – it was respectively 390%, 375%, 320%. In women of perimenopausal age in small myomas was augmented, respectively, to 260%, 270%, 385% and in large myomas - 295%, 295%, 355%. IGF-1 and IGF-2 levels in young women in small myomas was higher than in the control, amounting to respectively 210% and 250%. In large myomas – it was respectively 290% and 200%. In women of perimenopausal age in small myomas was augmented, respectively, to 245% and 195% and in large myomas - 265% and 215%. *Conclusions*: in myomatous uterine cells of young women an increase was noted in expression of IL-1 and IL-6 both in small and in large myomas. In women of perimenopausal age, the increase in expression of TNF-α took place only in small myomas with lower expression in large myomas. In women of generative age subjected to evaluation of transforming growth factors, high expression of the peptide was detected in all groups of leiomyomas. In both types of leiomyomas a comparable expression of insulin-like growth factors was detected, irrespectively of women's age.

Keywords: Uterine myomas, interleukins, tumour necrosis factor, transforming growth factors, insulin-like growth factors, age.

1. INTRODUCTION

Uterine myomas pose a significant health problem. They are frequently accompanied by signs/symptoms such as menorrhagia, which may lead to anaemia, infertility, unsuccessful pregnancies [1]. They represent the most frequent indication for hysterectomy [2,3].

Transformation of normal uterine muscle into a myoma represents a complex and a step-wise process. It is assumed that the primary factor which induces pathological growth of myometrium involves alterations at the level of cellular genome [1]. A cell stripped of the potential of entering the process of programmed death begins to divide in an uncontrolled manner, providing grounds for tumour development. Estrogens are thought to provide the principal growth controller of leiomyomas [1,4,5]. Nevertheless, studies *in vitro* on human tissues aimed to provide proof for direct action of the hormones in stimulation of myoma growth provided equivocal results [1,6,7]. This suggested existence of intermediate elements, such as cytokines or growth factors, through which ovarian hormones may exert their stimulatory effect on cell growth of uterine leiomyomas [1,7]. Estrogens and progesteron may control expression of genes coding for the cytokines and growth factors, which, in turn, modifies transcription of other genes. The modified production and release of cytokines and growth factors may result in a stimulated cellular proliferation and pronounced accumulation of extracellular matrix (ECM) [7].

*Address correspondence to this author at the Department of Endocrinological Gynecology, Medical University of Silesia, ul. Medyków 14, 40-752 Katowice, Poland; Tel: +32 789 43 51; Fax: +32 252 59 55;
E-mail: dagmara.pluta@med.sum.edu.pl

E-ISSN: 2309-4400/14

Myomas are composed of smooth muscle cells of myometrium, vast amounts of ECM, which may be more abundant by as much as 50% than in corresponding healthy myometrium [7]. The leiomyoma structure is dominated by collagen types I and III but the fibres demonstrate disturbances in arrangement similar to those observed in keloids [3].

Among cytokines and growth factors analyzed from the perspective of their potential role in cellular proliferation and growth of uterine myomas, our particular attention was focused on isoforms of transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF 1); insulin-like growth factor 2 (IGF 2), tumour necrosis factor α(TNF-α), interleukins 1 and 6 (IL-1; IL-6).

TGF-β seems to play a significant role in accumulation of extracellular matrix, particularly though an increased expression of fibronectin and collagen [8]. Arici *et al.* demonstrated a higher level of TGFβ3 expression in myoma than in myometrium [9]. In several studies leiomyomas were found to contain higher levels of IGF than normal myometrium [10-13]. TNF–α represents a promoter of neoplastic lesions in multiple animal models, pointing to possible role of the factor in pathogenesis of uterine myomas. Interleukines 1, 6, 8 and tumour necrosis factor (TNF-α) are elevated in peritoneal fluid of women with pathological lesions in uterus [14].

This study aimed at analysis of cytokine environment in uterine myomas of various size and in healthy uterine tissues looking for an appropriate approach of a potential conservative treatment.

2. MATERIAL AND METHODS

The studies took advantage of ready paraffin blocks, which served for immunohistochemical stainings using antibodies specific for defined epitopes.

The studies included altogether 67 women of generative or perimenopausal age with diagnosis of small uterine myomas (below 3 cm) or large myomas (above 5 cm), which required surgery. In the group of young women 9 preparations represented the control group, 14 preparations contained small myomas and 12 represented large myomas. In the group of perimenopausal women the respective numbers amounted to, respectively, 11, 16 and 15 cases. From every paraffin block three microscopic preparations were made, selecting every fifth section. Upon sectioning, the test was performed in order to find whether the consecutive sections included the uterine lesion.

The control groups included young women in the follicular phase of menstrual cycle, whose uteri were removed due to cystic, relapsing, benign lesions in ovaries while in the group of older women the control included uteri removed due to prolapse of the uterus. Patients with postoperatively histologically diagnosed malignant uterine and/or ovarian tumours, with myomas who received hormonal therapy or oral contraceptives, with autoimmune diseases and smoking patients were eliminated from the investigated material.

Protocol of the studies received consent of Bioethical Commission, Silesian Medical University.

The sampled tissue fragments were fixed in 4% formalin (v/v), dehydrated in a row of alcohols of growing concentrations, made translucent in xylene and infiltrated with paraffin, to be finally embedded in paraffin blocks.

The paraffin blocks were cut in a microtome to 5 μm-thick sections which were placed on silanized microscope glasses. The preparations were deparafinized and rehydrated in a row of alcohols from absolute alcohol to 30% alcohol. At the end, the preparations were rinsed with distiled water, with PBS, pH 7.5.

2.2. Immunohistochemical Studies

Characteristics of antibodies is presented in Table **1**. In order to unmask antigens, the preparations were incubated in a water bath at the temperature of 95^{o}C in Tris EDTA solution, pH 9 or in a citrate buffer, pH 6 for 30 minutes and, then, cooled for around 20 minutes. After cooling, the preparations were rinsed in PBS. Sites of non-specific antibody bonding were blocked with 1% solution of BSA in PBS, for 30 minutes at room temperature. After removing BSA solution, the section was overlaid with respective primary antibodies. The incubation was conducted for 22 hours at the temperature of 40^{o}C. Activity of endogenous peroxidase was blocked by incubation in 0.3% (v/v) hydrogen peroxide in 0.1% solution of NaN_3 in PBS for 10 minutes. Binding of complexed antibodies was visualized using ABC technique: (Vectastain Elite ABC Kit, Vector Laboratories).

Table 1: Characteristics of Employed Antibodies and Conditions of their Application

Antibody	Type	Host	Producer	Antibody concentration	Conditions of antigen unmasking
Anti-IL-1	Monoclonal	Mouse	Santa Cruz Biotechnol. Inc.	1 μg/ml	pH = 6
Anti-IL-6	Polyclonal	Rabbit	Abcam	1 μg/ml	pH = 9
Anti-TNF-α	Polyclonal	Rabbit	AbD Serotoc	10 μg/ml	pH = 9
Anti-TGF-β1	Polyclonal	Rabbit	Abcam	2.5 μg/ml	pH = 6
Anti-TGF-β2	Polyclonal	Rabbit	Abcam	5 μg/ml	pH = 9
Anti-TGF-β3	Polyclonal	Rabbit	Santa Cruz Biotechnol. Inc.	2 μg/ml	pH = 6
Anti-IGF-1	Polyclonal	Rabbit	Abcam	2 μg/ml	pH = 6
Anti-IGF-2	Polyclonal	Rabbit	Abcam	1 μg/ml	pH = 6

The ABC complex was visualized following the manufacturer's protocol (Vector Laboratories). The preparations were counterstained with Gill'es hematoxylin. The negative control involved sections in which primary antibody was substituted by IgG of rabbit, mouse or goat, respectively. The control reaction was performed in parallel on every slide in order to detect nonspecific binding of primary antibody.

The obtained immunohistochemical reactions were evaluated under a light microscope. The evaluation included both cellular localization of selected proteins and, due to computer-assisted image analysis, their amount. The results were subjected to statistical analysis.

Photographic documentation was prepared with photographic attachment. Intensity of every immuno-logical reaction was evaluated using magnification of x200 and x400 and a Nikon Eclipse E200 microscope with Nikon DS-Fi1 digital camera.

In studied sections quantitative analysis was conducted of the studied protein content. Employing NIS-AR 3.0 computer software optical density of microscopic preparations was evaluated in fields with immunohistochemical colour reaction for a given protein. Absorption of the employed wavelength pointed to optical density of cell cytoplasm containing antigen-antibody complexes or to its content of the reaction product.

2.3. Statistical Analysis

Characteristics of the examined parameters was presented in the form of an arithmetic mean, as a measure of central tendency, and a standard deviation as a measure of variability. Normal distribution was verified using tests of Kolmogorov-Smirnov and of Shapiro-Wilk and visually, inspecting the histograms. The distributions were thought to resemble normal one when the significance level in both tests exceeded 0.05 and the histogram shape was symmetrical. Distributions of all studied parameters were found to be normal.

Effects of studied variables, myoma size and patient's age were appraised using ANOVA analysis of variance. The assumption of a uniform variance was verified using Levene's test. In the ANOVA analysis, as a post-hoc test for comparisons of individual means with the control values, an advantage was taken of the multiple comparison test of Dunnett. The results were considered significant at $p<0.05$. The statistical

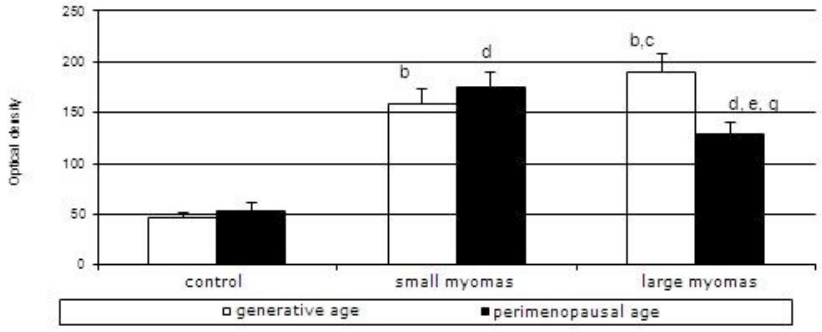

Figure 1: Optical density of reaction product for interleukin 1 (IL-1) in control and in myomas.

analyses were conducted using the professional kit of statistical procedures STATISTICA PL version 8.0.

3. RESULTS

3.1. Interleukin 1

3.1.1. Women of Generative Age

Interleukin-1 level in young women optical density of imunohistochemical reaction product in small myomas was clearly higher than in the control, amounting to 345% of the latter (Figure **1**).

In large myomas, the former was found to amount to 415% of the control level.

3.1.2. Women of Perimenopausal Age

IL-1 level in women of perimenopausal age optical density of the reaction product in small myomas was augmented to 320% of the control level (Figure **1**).

In large myomas, the expression was high, amounting to 240% of the level found in the control group (Figure **2**).

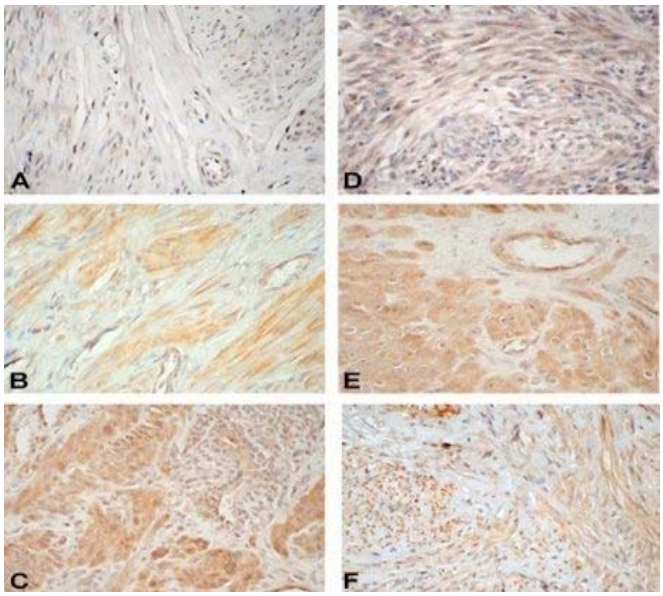

Figure 2: Immunohistochemical expression of interleukin 1 (IL-1) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

3.2. Interleukin 6

3.2.1. Women of Generative Age

Interleukin-6 level in young women optical density of the immunohistochemical reaction product in small myomas was evidently higher than in the control, reaching 255% of the latter level (Figure **3**).

In large myomas, the expression amounted to 285% of the control level.

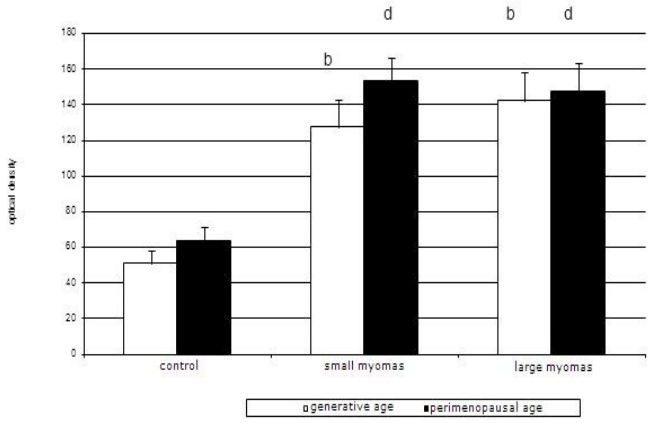

Figure 3: Optical density of reaction product for interleukin 6 (IL-6) in the control and in myomas.

3.2.2. Women of Perimenopausal Age

IL-6 level in women of perimenopausal age optical density of the reaction product increased in small myomas to 240% of the control level (Figure **3**).

In large myomas, the expression was high, reaching 235% of the control level (Figure **4**).

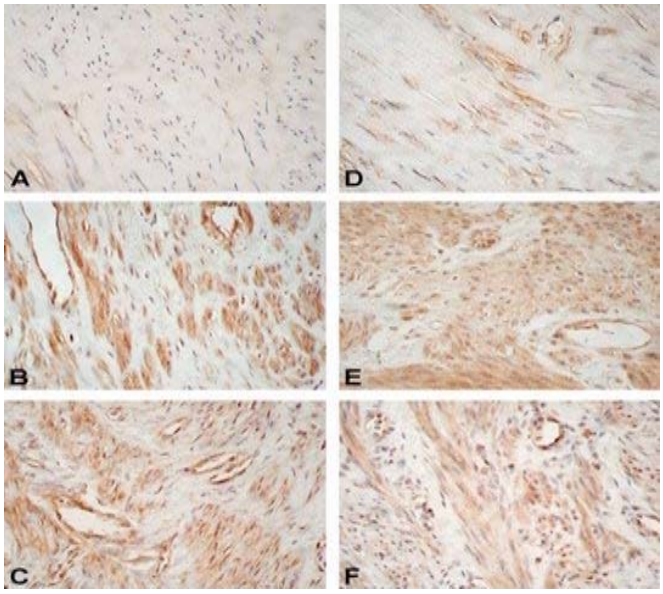

Figure 4: Immunohistochemical expression of interleukin 6 (IL-6) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

3.3. Tumour Necrosis Factor-α (TNF-α)

3.3.1. Women of Generative Age

TNF-α level in women of generative age was found to manifest higher optical density of the immunohistochemical reaction in small myomas than that in the control, amounting to 265% of the latter (Figure **5**).

In large myomas, the former was found to amount to 230% of the control level.

3.3.2. Women of Perimenopausal Age

TNF-α level in women of perimenopausal age optical density of the reaction product was found to increase in small myomas to 270% of the control level (Figure **5**).

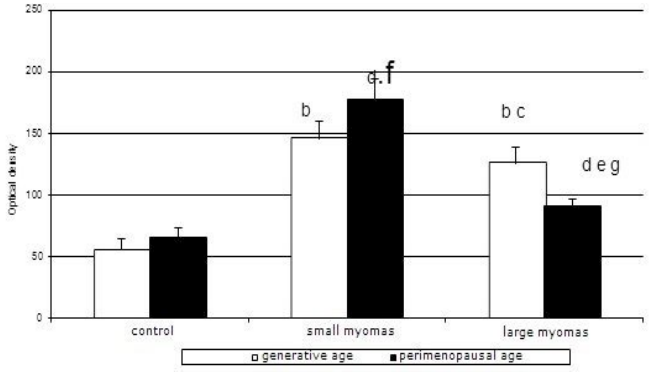

Figure 5: Optical density of reaction product for TNF-α in the control and in myomas.

In large myomas, the expression was found to reach around 140% of the control level (Figure **6**).

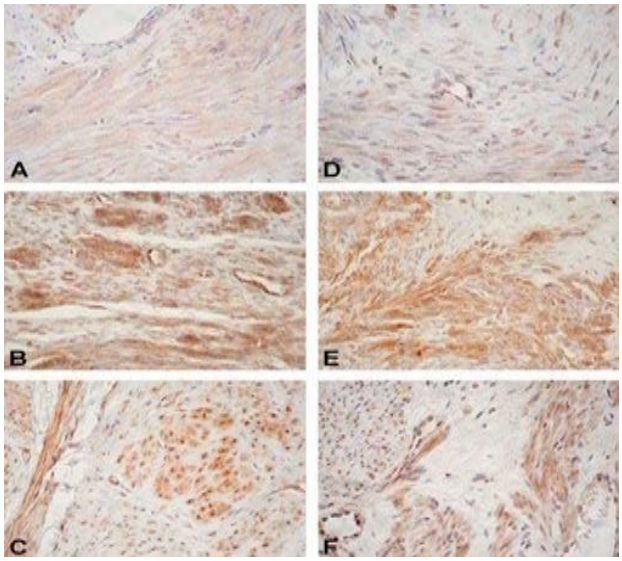

Figure 6: Immunohistochemical expression of tumour necrosis factor-α (TNF-α) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

3.4. Transforming Growth Factor-β (TGF-β)

3.4.1. Women of Generative Age

TGF-β1, TGF-β2, TGF-β3 levels in the young women manifested optical density of immunohistochemical reaction clearly higher in small myomas, which amounted to 310%, 285% and 335% of the control levels for, respectively, TGF-β1, TGF-β2 and TGF-β3 (Figures **7**, **9**, **11**).

In large myomas, expressions were found to amount to 390% of the control level for TGF-β1, 375% for TGF-β2 and 320 % for TGF-β3.

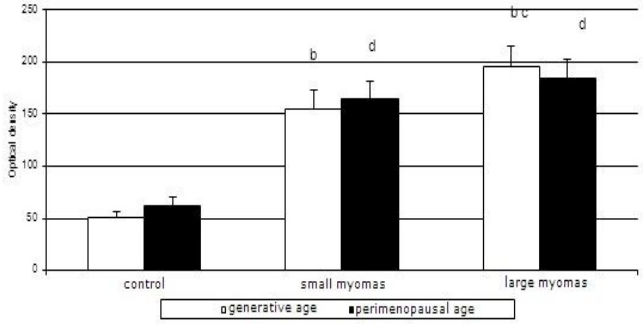

Figure 7: Optical density of reaction product for TGF-β1 in the control and in myomas.

3.4.2. Women of Perimenopausal Age

TGF-β1, TGF-β2, TGF-β3 levels in women of perimenopausal age optical density of immunohistochemical reaction product for TGF-β1, TGF-β2, TGF-β3 was found to increase in small myomas to, respectively, 260%, 270%, 385% of control levels (Figures **7**, **9**, **11**).

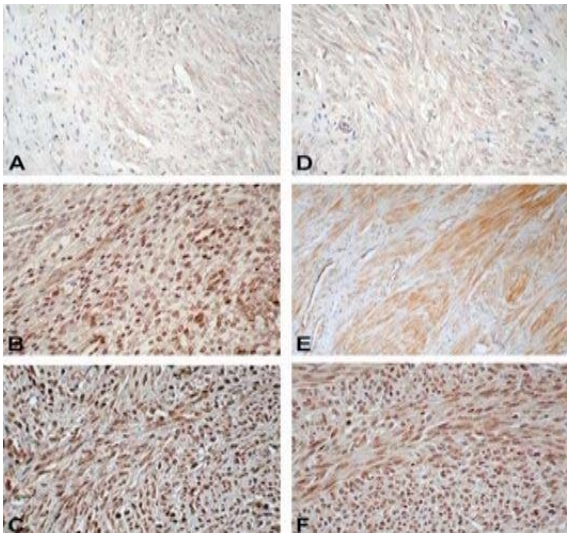

Figure 8: Immunohistochemical expression of transforming growth factor β1 (TGF-β1) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age, and D, E, F to women of perimenopausal age. Magnification: 400x.

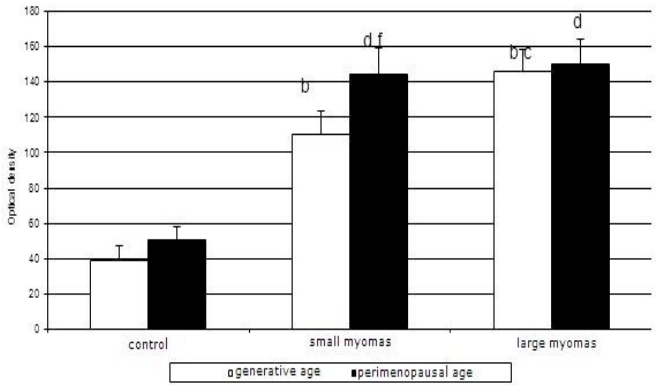

Figure 9: Optical density of the reaction product for TGF-β2 in the control and in myomas.

In large myomas, expressions proved to be also high, amounting to, respectively, 295%, 295%, 355% of the control levels (Figures **8**, **10**, **12**).

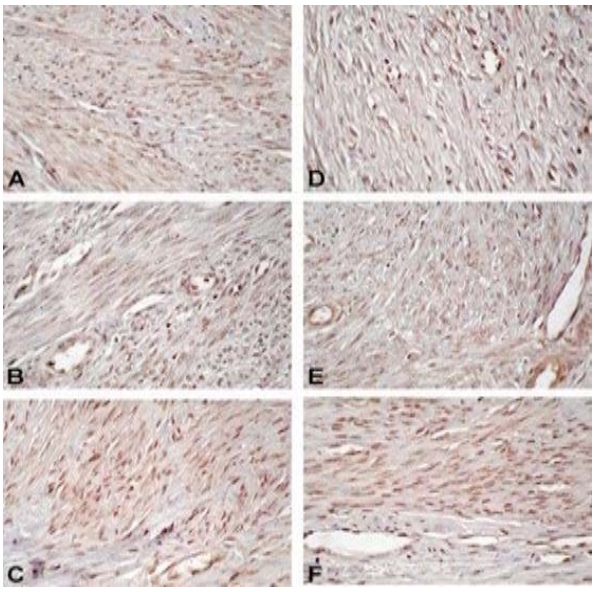

Figure 10: Immunohistochemical expression of transforming growth factor-β2 (TGF-β2) in the control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

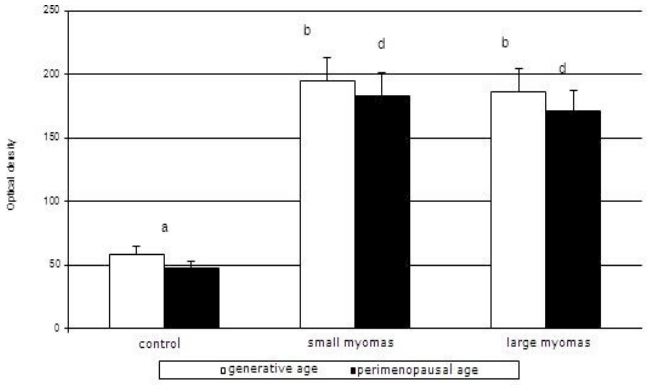

Figure 11: Optical density of reaction product for TGF-β3 in control and in myomas.

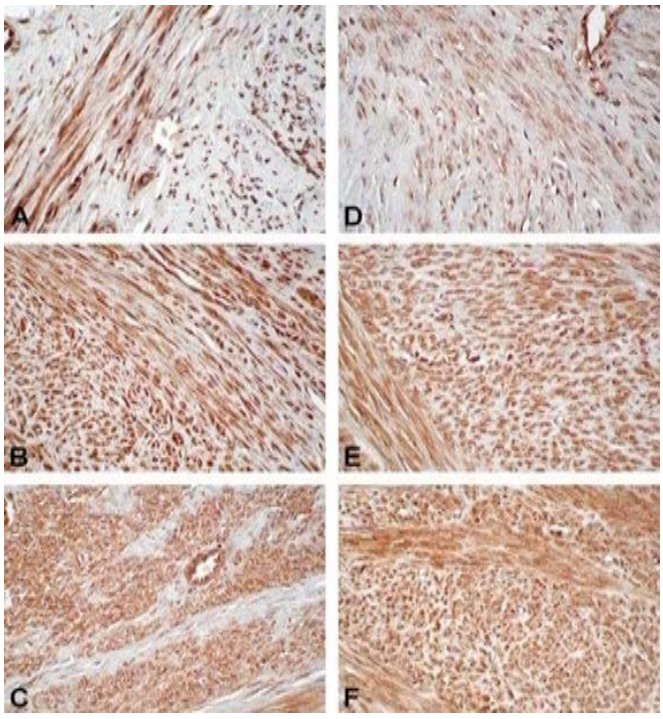

Figure 12: Immunohistochemical expression of transforming growth factor β3 (TGF-β3) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

3.5. Insulin-Like Growth Factors

3.5.1. Women of Generative Age

IGF-1 and IGF-2 levels in women of generative age optical density of immunohistochemical reaction product in small myomas was found to be higher than in the control, amounting to 210% of the latter for IGF-1 and 250% for IGF-2 (Figures **13**, **15**).

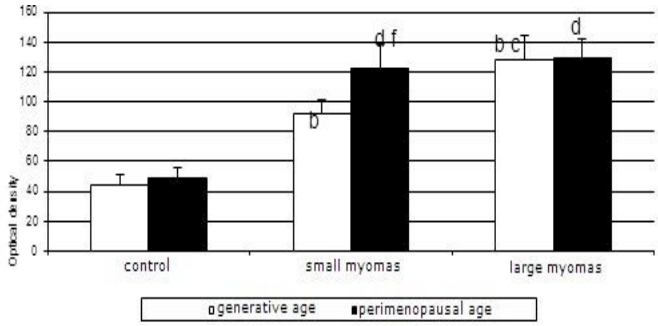

Figure 13: Optical density for the reaction product for insulin-like growth factor 1 (IGF-1) in the control and in myomas.

Comparing expressions in large myomas with those in the control group, the former were found to amount to, respectively, 290% of the control for IGF-1 and 200% for IGF-2.

3.5.2. Women of Perimenopausal Age

IGF-1, IGF-2 levels in women of perimenopausal age optical densities of the reaction products in small myomas were augmented to 245% of the control level for IGF-1 and 195% for IGF-2 (Figures **13**, **15**).

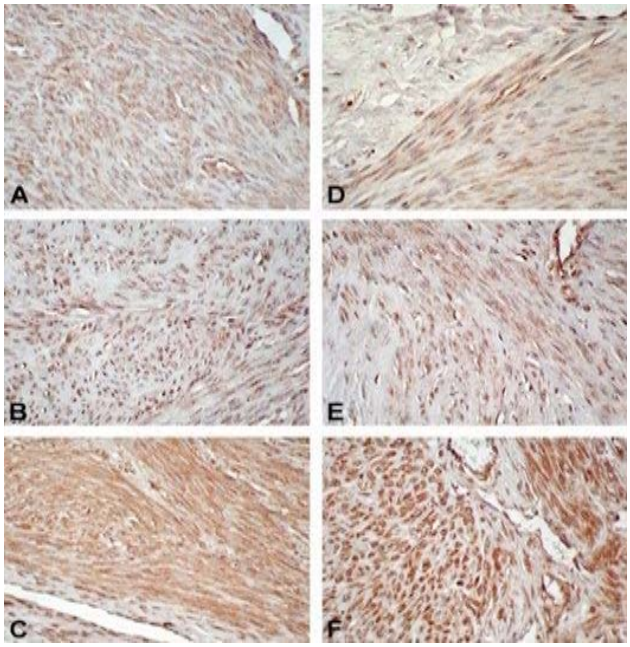

Figure 14: Immunohistochemical expression of insulin-like growth factor 1 (IGF-1) in the control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimanopausal age. Magnification: 400x.

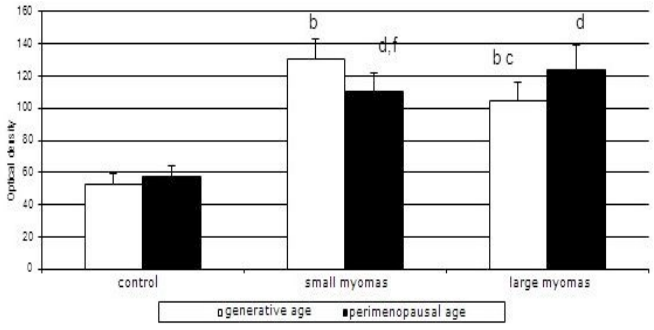

Figure 15: IGF-2 Optical density of reaction product for insulin-like growth factor 2 (IGF-2) in the control and in myomas.

In large myomas, the expressions were found to be higher, amounting to 265% of the control level for IGF-1 and 215% for IGF-2 (Figures **14**, **16**).

4. DISCUSSION

Cytokines comprise a large family of protein intercellular mediators, which control cell growth processes, their differentiation, migration and apoptosis. Data on cytokines and their receptors point to their significant role in development of tumours due to their effects in reciprocal interaction of various components in tumour microenvironment. They influence growth and survival of neoplastic cells, controlling, i.a., infiltration of neoplastic tumours by leukocytes, stimulation of new blood vessel formation and involvement in shaping of extracellular matrix.

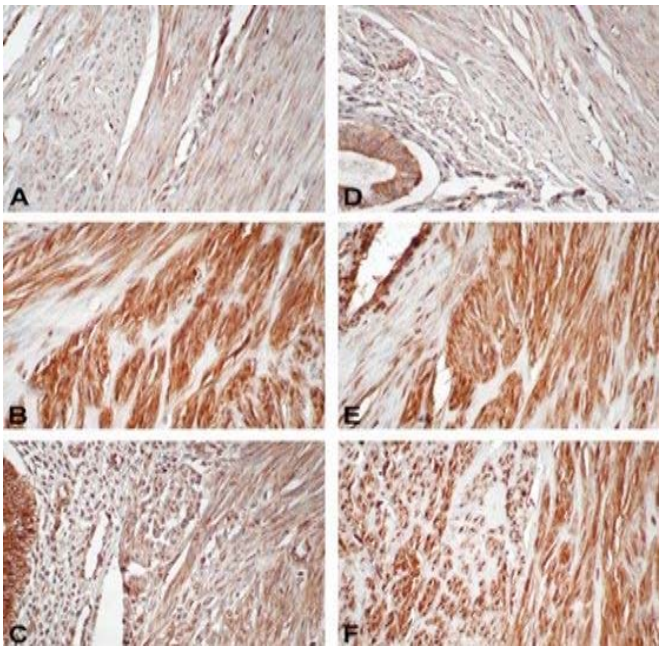

Figure 16: Immunohistochemical expression of insulin-like growth factor 2 (IGF-2) in control (A, D), small myomas (B, E) and large myomas (C, F). Figures A, B, C correspond to women of generative age while D, E, F to women of perimenopausal age. Magnification: 400x.

Significance of proinflammatory cytokines in pathogenesis of tumours was relatively well documented, but not completely understood. Proinflammatory cytokines, such as TNF-α or IL-1β, are capable of activating signalling cascades, including induction of various cytokines.

As a result, levels of, e.g., pro-inflammatory cytokine of IL-6 were markedly higher in sick women [15,16]. Even if it is known that macrophages provide the main source of cytokines, exogenous TNF-α is known to up-regulate expression of IL-6 through activation of NF-κB or MAPK pathway in cells of uterine stroma [17,18].

Release of IL-6 was observed in cells of several tumours, such as carcinoma of uterine cervix or urinary bladder. The interleukin inhibits proliferation of certain

neoplastic cells. Nevertheles, the role of IL6 in a neoplastic disease has not been unequivocally determined [19,20]. On one hand it stimulates cytotoxicity of lymphocytes T, on the other, also due to neoplastic cachexia, it stimulates angiogenesis in developing tumours and growth of numerous types of neoplastic cells [21]. High concentration of IL-6 in a neoplastic disease argues for existence of, a based on the cytokine, an autocrine or paracrine mechanism which promotes tumour expansion, which has been confirmed in our studies.

The obtained results are interesting since they may indicate that IL-6 may represent a significant marker, which might be used to evaluate progression of human myometrium.

Following radical surgery before implementation of chemotherapy in patients who manifested a progression at the later stage of the disease, IL-6 concentration proved to be twofold higher than in persons manifesting a remission. This may indicate prognostic value of IL-6 estimations.

Results related to estimations of IL-1, which I obtained in my study, are in a sense unexpected. I expected that alterations in expression of the cytokine will at least in part resemble those obtained for IL-6. However, the similarity involved only myomas in women of generative age. I was also surprised to see that the results obtained for IL-1 resembled those obtained for TNF-α. Explanation of the similarity remains difficult since such a coincidence of cytokines remains rare in literature of the subject. If this will be confirmed, it will be rather IL-1 and not as expected IL-6, which will constitute an element of kits used to prognose development of myomas [22].

TNF-α was demonstrated to markedly increase expression of IL-6 genes and proteins in stroma cells [23,24], and the mechanism was shown to be definitely dependent on activation of NF-κB pathway.

The discoveries suggest that uterine stroma cells may manifest characters which need not to be controlled for the purpose of maintaining a balance between pro- and anti-inflammatory cytokines [24]. TNF-α is known to be capable of inducing an inhibition of cancer cell growth *in vitro*, since proofs are available that, e.g. women suffering from ovarian cancer manifest elevated serum levels of TNF-α and, thus, also TNF-α may be involved in control of *in vivo* growth of cancer cells.

The ranges of concentrations manifested by TNF-α and its receptors noted in the serum of affected patients manifest a correlation with the tumour type, degree of its histological differentiation and with its clinical advancement. A high serum concentration of the cytokine and its tissue expression suggest a rather unfavourable course of the disease [25,26].

Evaluating expression of TNF-α, I have noticed that in evaluated uterine myomas the cytokine level increased manifesting no relationship with myoma size and age of the patients.

Janes *et al.* [27] suggested that cells may respond to TNF-α by a sequential release of a number of TNF-α-stimulated cytokines and by some growth factors. Even if the authors examined effects of exogenously added cytokines, it seems rather sure that expression of TNF-α may also develop such an endocrine cascade and that upon an absence of a strong apoptotic signal such a cascade may be favourable for tumour cells. The results obtained up to now suggest that a direct therapeutic targeting of TNF-α itself in tumour cells may provide a strategy capable of controlling progression ability of neoplastic cells. The till now published results indicate that both antibodies and soluble receptors are well tolerated by patients and that they may exert stabilizing and tumour growth inhibiting effect [28]. A direct inhibition of TNF-α using RNAi technology or blocking of signalling pathways for molecular inhibitors of TNFα, which would down-regulate the network of inflammatory cytokines, may provide an interesting intervention, even if it would be conducted in parallel with the other, conventional therapies.

In most of the tissues physiological role played by TGF-β has not been fully clarified. TGF-β inhibits cell proliferation, induces apoptosis and influences morphogenesis through its effect on extracellular matrix [29, 30]. TGF-β controls its own expression, expression of ECM, ECM metalloproteinases and tissue inhibitor of metalloproteinases, of growth of leiomyoma and uterine leiomyoma [9]. TGF-β induces a much more pronounced thymidine incorporation to leiomyoma cells than to normal myometrium smooth muscle cells [31].

Moreover, expression of TGF-β in uterine myometrium cells adhering to leiomyoma cells was significantly augmented. Expression of TGF-β was found to increase twice as fast in rapidly growing leiomyomas in patients as compared to the control [32].

ECM molecules, such as collagen, fibronectin and proteoglycans are used to localize cytokines or growth factors close to neoplastic cells by strict binding to them and anchorage to the sites [7]. However, metalloproteinases play a mediatory role in dissociation of cytokines, such as TGF-β, from proteoglycans by proteolysis of ECM [33]. Proteoglycans interactions with other proteins modulate arrangement if the matrix, promote tumour growth and cellular proliferation. Existence of a TGF-β3-dependent pathway was also demonstrated as well as the fact that the cytokine significantly controls expression of ECM proteins.

The augmented expression of certain proteoglycans demonstrated in uterine myomas may lead to disarrangement of ECM and an increased compactness of the tumours, linked to an increased contents of total glycosaminoglycan sulphates and of collagen [34]. This was demonstrated in several studies which documented elevated levels of total glycosaminoglycans in leiomyomas as compared to myometrium [35,36].

The altered expression of TGF-β3 leads to several disturbences, including tissue fibrosis [37,38]. Multiple investigators showed that TGF-β3 functions through signalling pathways in various tissue types [39-41] including tissue and cells of leiomyoma. Treatment using TGF-β was found to stimulate various ECM gene transcripts in cells of smooth cell myomas, including collagen type I, fibronectin and connective tissue growth factor (CTGF) in cells of primary leiomyoma and in myometrium [42]. The increasing concentration of TGF-β was paralelled by a concentration-dependent growth in contents of collagen, fibronectin and CTGF in cell lines of uterine myometrium and uterine myoma, with expression in myometrial cells of all studied ECM genes resembling expression in leiomyomas. The obtained results support the hypothesis that upon increased stimulation with TGF-β, myometrium cells manifest characteristic traits common to molecular phenotype of myoma cells and they support the hypothesis that myometrial cells may manifest molecular alterations under effect of TGF-β, which resemble fibrotic phenotype of uterine myoma cells.

Earlier studies conducted in tissue cultures and cultured cells proved that TGF-β manifests high expression in myomas [43], in which in synergy with other growth factors [44,45] it can stimulate development of various ECM proteins, such as various proteoglycans [46], collagens and fibronectin [47]. Also expression of TGFβ was shown to be stably elevated in myoma cells in the patients, as compared to normal myometrial cells [48]. Considering that proteins of intercellular matrix and synergistic growth factors remain under effect of modulation, both in myometrium and in myoma taking part along the TGF-β3-dependent pathway, it is logical to assume that a myoma develops from a myometrium due to an initiating event, which leads to overexpression of TGF-β3, resulting in overexpression of ECM genes and secretion of matrix proteins. Ding *et al.* [49] showed that also the TGF-β1 isoform directly stimulates expression of signalling pathways linked to synthesis of ECM components.

In parallel to the increase of TGF-β3 concentrations to supraphysiological levels, expression of mRNA in myoma cells demonstrated a significant reduction as compared to the expression at lower TGF-β3 concentrations. High therapeutic concentrations of TGF-β3 statistically failed to alter MMP-2 expression, while expression of MMP-11 continued to be reduced.

Certain studies demonstrated that MMP-2 is significantly higher in human leiomyomas than in myometrium tissue [50-54].

TGF-β3 represents the dominating isoform in smooth muscle cells. As compared to the surrounding myometrium not affected by the lesions, leiomyomas manifest high expression of TGF-β3 [55]. A lowered expression of TGF-β in smooth muscle cells of myometrium and leiomyomas was described to reflect an *in vitro* effect of GnRH-a [56]. Regression of myomas induced by GnRH-a is linked *in vivo* to down-regulation of expressions manifested by TGF-β and its receptors [57]. For example, in patients treated with GnRH-a a decreased size of uterus was accompanied by an unaltered expression of TGF-β3 in myometrium and a lower one in leiomyomas, as compared to cases of untreated patients [58]. The observations confirm role of TGF-β3 in progression of myomas. In uterine myomas, up-regulation of TGF-β3 by progesterone is manifested in a lowered activity of matrix metalloproteoinases (MMP), the key enzymes in ECM degradation, with a subsequent increase in ECM production [9].

Moreover, progression of the neoplastic process is accompanied by an autocrine stimulation of TGF-β secretion, in increasing amounts of its active form and by release of proteases which activate the latent TGFβ form, linked to extracellular matrix [59].

Evaluating expression of TGF-β1 its level in leiomyomas may be noted to increase with develop-

ment of the tumour, it is at least two-fold higher in the leiomyoma as compared to myometrium. The results confirm suggestions presented in the world literature that TGF-β1 in high concentrations becomes a pro-proliferative factor [60].

In this study we have demonstrated that IGF-I is clearly exposed on human myoma cells. This suggests that IGF-I may participate in control of various metabolic and cellular responses in this type of cells in an autocrine or paracrine manner.

It is suggested that IGF-I may stimulate growth of myoma cells by increasing their proliferative potential [55,61], and that IGF-I may also inhibit apoptosis of myoma cells [62]. In addition the data are available, showing that mRNA for IGF-I receptor [63-65] manifests high expression in uterine leiomyomas, as compared to the surrounding healthy myometrium. Such data suggest that growth potential of myoma cells may be elevated as compared to healthy myometrium cells.

However, few data are available on effects of pro-gesterone antagonists on expression of growth factors and their receptors in cultured leiomyoma cells and healthy cells of myometrium [55].

IGF-I was demonstrated to markedly increase cell proliferation and to inhibit apoptosis in cultured cells of leiomyomas as compared to control cultures [62]. In view of these discoveries, down-regulation of the IGF-I/IGF-IR system may reduce the cell proliferative and anti-apoptotic effect of IGF-I in cultured myoma cells. TGF-β3 was demonstrated to inhibit synthesis of DNA in cells of myometrium but to stimulate it in myoma cells [55]. A disturbance in the TGF-β3/TGF-β RII system may result in inhibition of the downstream signalling, such as cell proliferation in cultured myoma cells. Altogether, the IGF-I/IGF-IR system and TGF-β3/TGF-β RII system may provide targets for asoprisnil (a selective modulator of progesterone receptor) in cultured leiomyoma cells, leading to inhibition of myoma cell proliferation [66].

REFERENCES

[1] Flake GP, Andersen J, Dixon D. Etiology and pathogenesis of uterine leyomyomas: a review. Environ Helth Persp 2003; 111: 1037-54. http://dx.doi.org/10.1289/ehp.5787

[2] Robak-Chołubek D, Jakiel G. Uterine myomas (in Polish). Przegl Menopauzalny 2006; 6: 409 12.

[3] Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil Steril 2007; 87: 725-36. http://dx.doi.org/10.1016/j.fertnstert.2007.01.093

[4] Andersen J, Barbieri RL. Abnormal gene expression in uterine leiomyomas. J Soc Gynecol Invest 1995; 2: 663-72. http://dx.doi.org/10.1016/1071-5576(95)00021-6

[5] Walker CL, Stewart EA. Uterine fibroids: the elephant in the room. Science 2005; 308: 1589-92. http://dx.doi.org/10.1126/science.1112063

[6] Otubu JA, Buttram VC, Besch NF, Besch PK. Unconjugated steroids in leiomyomas and tumor-bearing myometrium. Am J Obstet Gynecol 1982; 143: 130-133.

[7] Sozen I, Arici A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. Fertil Steril 2002; 78: 1-12. http://dx.doi.org/10.1016/S0015-0282(02)03154-0

[8] Joseph DS, Malik M, Nurudeen S, Catherino WH. Myometrial cells undergo fibrotic transformation under the influence of transforming growth factor beta-3. Fertil Steril 2010; 93: 1500-8. http://dx.doi.org/10.1016/j.fertnstert.2009.01.081

[9] Arici A, Sozen I. Transforming growth factor-b3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. Fertil Steril 2000; 73: 1006-11. http://dx.doi.org/10.1016/S0015-0282(00)00418-0

[10] Manni A, Wei L, Badger B, *et al.* Expression of messenger RNA for insulin-like growth factors and insulin-like growth factor binding proteins by experimental breast cancer and normal breast tissue *in vivo*. Endocrinology 1992; 130: 1744-6.

[11] Chandrasekhar Y, Heiner J, Osuamkpe C, Nagamani M. Insulin-like growth factor I and II binding in human myometrium and leiomyomas. Am J Obstet Gynecol 1992; 166: 64-9. http://dx.doi.org/10.1016/0002-9378(92)91831-T

[12] Wolańska M, Bańkowski E. An accumulation of insulin-like growth factor I (IGF-I) in human myometrium and uterine leiomyomas in various stages of tumour growth. Eur Cytokine Net 2004; 15: 359-63.

[13] Burroughs KD, Howe SR, Okubo Y, Fuchs-Young R, LeRoith D, Walker CL. Dysregulation of IGF-I signaling in uterine leiomyoma. J Endocrinol 2002; 172: 83-93. http://dx.doi.org/10.1677/joe.0.1720083

[14] Iwabe T, Harada T, Tsudo T, Tanikawa M, Onohara Y, Terakawa N. Pathogenetic significance of increased levels of interleukin-8 in the peritoneal fluid of patients with endometriosis. Fertil Steril 1998; 69: 924-30. http://dx.doi.org/10.1016/S0015-0282(98)00049-1

[15] Rier SE, Zarmakoupis PN, Hu X, Becker JL. Dysregulation of interleukin-6 responses in ectopic endometrial stromal cells: correlation with decreased soluble receptor levels in peritoneal fluid of women with endometriosis. J Clin Endocrinol Metab 1995; 80: 1431-7.

[16] Tsudo T, Harada T, Iwabe T, *et al.* Altered gene expression and secretion of interleukin-6 in stromal cells derived from endometriotic tissues. Fertil Steril 2000; 73: 205-11. http://dx.doi.org/10.1016/S0015-0282(99)00496-3

[17] Sakamoto Y, Harada T, Horie S, Iba Y, Taniguchi F, Yoshida S. Tumor necrosis factor-α-induced interleukin-8 (IL-8) expression in endometriotic stromal cells, probably through nuclear factor-κB activation: gonadotropin-releasing hormone agonist treatment reduced IL-8 expression. J Clin Endocrinol Metab 2003; 88: 730-5. http://dx.doi.org/10.1210/jc.2002-020666

[18] Yamauchi N, Harada T, Taniguchi F, Yoshida S, Iwabe T, Terakawa N. Tumor necrosis factor-α induced the release of interleukin-6 from endometriotic stromal cells by the nuclear factor-κB and mitogen activated protein kinase pathways. Fertil Steril 2004; 82(Suppl 3): 1023-8. http://dx.doi.org/10.1016/j.fertnstert.2004.02.134

[19] Wang Y, Yang J, Gao Y, *et al.* Regulatory effect of E2, IL 6 and IL-8 on the growth of epithelial ovarian cancer cells. Cell Mol Immunol 2005; 2: 365-72.

[20] Palena C, Hamilton DH, Fernando RI. Influence of IL-8 on the epithelial-mesenchymal transition and the tumor microenvironment Future Oncol 2012; 8: 713-22. http://dx.doi.org/10.2217/fon.12.59

[21] Łukaszewicz M, Mroczko B, Szmitkowski M. Clinical significance of interleukin 6 (IL-6) as a prognostic factor in a neoplastic disease (in Polish). Pol Arch Med Wewn 2007; 117: 247-51.

[22] Roy D, Sarkar S, Felty Q. Levels of IL-1β control stimulatory/inhibitory growth of cancer cells. Front Biosci 2006; 11: 889-98. http://dx.doi.org/10.2741/1845

[23] Tsudo T, Harada T, Iwabe T, *et al.* Altered gene expression and secretion of interleukin-6 in stromal cells derived from endometriotic tissues. Fertil Steril 2000; 73: 205-11. http://dx.doi.org/10.1016/S0015-0282(99)00496-3

[24] Tagashira Y, Taniguchi F, Harada T, Ikeda A, Watanabe A, Terakawa N. Interleukin-10 attenuates TNF-alpha-induced interleukin-6 production in endometriotic stromal cells. Fertil Steril 2009; 91(5 Suppl): 2185-92. http://dx.doi.org/10.1016/j.fertnstert.2008.04.052

[25] Kulbe H, Thompson R, Wilson JL, *et al.* The inflammatory cytokine tumor necrosis factor-α generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. Cancer Res 2007; 67: 585-92. http://dx.doi.org/10.1158/0008-5472.CAN-06-2941

[26] Szlosarek PW, Grimshaw MJ, Kulbe H, *et al.* Expression and regulation of tumor necrosis factor alpha in normal and malignant ovarian epithelium. Mol Cancer Ther 2006; 5: 382-90. http://dx.doi.org/10.1158/1535-7163.MCT-05-0303

[27] Janes KA, Gaudet S, Albeck JG, Nielsen UB, Lauffenburger DA, Sorger PK. The response of human epithelial cells to TNF involves an inducible autocrine cascade. Cell 2006; 124: 1225-39. http://dx.doi.org/10.1016/j.cell.2006.01.041

[28] Madhusudan S, Foster M, Muthuramalingam SR, *et al.* A phase II study of ethanercept (ENBREL) a tumour necrosis factor-a inhibitor in recurrent ovarian cancer. J Clin Oncol 2005; 10: 6528–34.

[29] Zhu L, Skoultchi AI. Coordinating cell proliferation and differentiation. Curr Opin Genet Dev 2001; 11: 91-7. http://dx.doi.org/10.1016/S0959-437X(00)00162-3

[30] McClellan KA, Slack RS. Novel functions for cell cycle genes in nervous system development. Cell Cycle 2006; 5: 1506-13. http://dx.doi.org/10.4161/cc.5.14.2980

[31] Arici A, Sozen I. Expression, menstrual cycle-dependent activation, and bimodal mitogenic effect of transforming growth factor-beta1 in human myometrium and leiomyoma. Am J Obstet Gynecol 2003; 188: 76-83. http://dx.doi.org/10.1067/mob.2003.118

[32] Zeyneloglu HB, Esinler I, Ozdemir BH, Haydardedeoglu B, Oktem M, Batioglu S. Immunohistochemical characteristics of intramural leiomyomata that enlarge during controlled ovarian hyperstimulation for *in vitro* fertilization. Gynecol Obstet Invest 2008; 65: 252-7. http://dx.doi.org/10.1159/000113049

[33] Wight TN. Versican: a versatile extracellular matrix proteoglycan in cell biology. Curr Opin Cell Biol 2002; 14: 617-23. http://dx.doi.org/10.1016/S0955-0674(02)00375-7

[34] Rogers R, Norian J, Malik M, *et al.* Mechanical homeostasis is altered in uterine leiomyoma. Am J Obstet Gynecol 2008; 198: 474.e1-11.

[35] Mitropoulou TN, Theocharis AD, Stagiannis KD, Karamanos NK. Identification, quantification and fine structural characterization of glycosaminoglycans from uterine leiomyoma and normal myometrium. Biochimie 2001; 83: 529-36. http://dx.doi.org/10.1016/S0300-9084(01)01281-0

[36] Berto AG, Sampaio LO, Franco CR, Cesar RM Jr, Michelacci YM. A comparativeanalysis of structure and spatial distribution of decorin in human leiomyoma and normal myometrium. Biochim Biophys Acta 2003; 1619: 98-112. http://dx.doi.org/10.1016/S0304-4165(02)00446-4

[37] Bierie B, Moses HL. Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer 2006; 6: 506-20. http://dx.doi.org/10.1038/nrc1926

[38] Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. N Engl J Med 2000; 342: 1350-8. http://dx.doi.org/10.1056/NEJM200005043421807

[39] He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massague J. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGF-β pathway. Cell 2006; 125: 929-41. http://dx.doi.org/10.1016/j.cell.2006.03.045

[40] Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-β signal transduction. J Cell Sci 2001; 114: 4359-69.

[41] Dijke P, Hill CS. New insights into TGF-β-Smad signaling. Trends Biochem Sci 2004; 29: 265-73. http://dx.doi.org/10.1016/j.tibs.2004.03.008

[42] Joseph DS, Malik M, Nurudeen S, Catherino WH. Myometrial cells undergo fibrotic transformation under the influence of transforming growth factor beta-3. Fertil Steril 2010; 93: 1500-8. http://dx.doi.org/10.1016/j.fertnstert.2009.01.081

[43] Leppert PC, Catherino WH, Segars JH. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. Am J Obstet Gynecol 2006; 195: 415-20. http://dx.doi.org/10.1016/j.ajog.2005.12.059

[44] Abreu JG, Ketpura NI, Reversade B, Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signaling by BMP and TGF-β. Nat Cell Biol 2002; 4: 599-604.

[45] Muroski ME, Roycik MD, Newcomer RG, Van den Steen PE, Opdenakker G, Monroe HR. Matrix metalloproteinase-9/gelatinase B is a putative therapeutic target of chronic obstructive pulmonary disease and multiple sclerosis. Curr Pharm Biotechnol 2008; 9: 34-46. http://dx.doi.org/10.2174/138920108783497631

[46] Norian JM, Malik M, Parker CY, *et al.* Transforming growth factor β3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. Reprod Sci 2009; 16: 1153-64. http://dx.doi.org/10.1177/1933719109343310

[47] Leppert PC, Baginski T, Prupas C, Catherino WH, Pletcher S, Segars JH. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. Fertil Steril 2004; 82: 1182-7. http://dx.doi.org/10.1016/j.fertnstert.2004.04.030

[48] Malik M, Catherino WH. Novel method to characterize primary cultures of leiomyoma and myometrium with the use of confirmatory biomarker gene arrays. Fertil Steril 2007; 87: 1166-72. http://dx.doi.org/10.1016/j.fertnstert.2006.08.111

[49] Ding L, Xu J, Luo X, Chegini N. Gonadotropin releasing hormone and transforming growth factor beta activate mitogen-activated protein kinase/extracellularly regulated kinase and differentially regulate fibronectin, type I collagen, and plasminogen activator inhibitor-1 expression in leiomyoma and myometrial smooth muscle cells. J Clin

Endocrinol Metab 2004; 89: 5549-57.
http://dx.doi.org/10.1210/jc.2004-0161

[50] Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JR, Sawicki G, Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. Circulation 2002; 106: 1543-9.
http://dx.doi.org/10.1161/01.CIR.0000028818.33488.7B

[51] Sawicki G, Leon H, Sawicka J, *et al.* Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. Circulation 2005; 112: 544-52.
http://dx.doi.org/10.1161/CIRCULATIONAHA.104.531616

[52] Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006; 69: 562-73.
http://dx.doi.org/10.1016/j.cardiores.2005.12.002

[53] Préaux AM, Mallat A, Nhieu JT, D'Ortho MP, Hembry RM, Mavier P. Matrix metalloproteinase-2 activation in human hepatic fibrosis regulation by cell-matrix interactions. Hepatology 1999; 30: 944-50.
http://dx.doi.org/10.1002/hep.510300432

[54] Bogusiewicz M, Stryjecka-Zimmer M, Postawski K, Jakimiuk AJ, Rechberger T. Activity of matrix metalloproteinase-2 and -9 and contents of their tissue inhibitors in uterine leiomyoma and corresponding myometrium. Gynecol Endocrinol 2007; 23: 541-6.
http://dx.doi.org/10.1080/09513590701557416

[55] Lee BS, Nowak RA. Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-beta 3 (TGF beta 3) and altered responses to the antiproliferative effects of TGF beta. J Clin Endocrinol Metab 2001; 86: 913-20.

[56] Chegini N, Ma C, Tang XM, Williams RS. Effects of GnRH analogues, “add-back” steroid therapy, antiestrogen and antiprogestins on leiomyoma and myometrial smooth muscle cell growth and transforming growth factor-beta expression. Mol Hum Reprod 2002; 8: 1071-8.
http://dx.doi.org/10.1093/molehr/8.12.1071

[57] Dou Q, Zhao Y, Tarnuzzer RW, *et al.* Suppression of transforming growth factor-β (TGF-β) and TGF-β receptor messenger ribonucleic acid and protein expression in leiomyomata in women receiving gonadotropin-releasing hormone agonist therapy. J Clin Endocrinol Metab 1996; 81: 3222-30.

[58] De Falco M, Staibano S, D'Armiento FP, *et al.* Preoperative treatment of uterine leiomyomas: clinical findings and expression of transforming growth factor-beta3 and connective tissue growth factor. J Soc Gynecol Investig 2006; 13: 297-303.
http://dx.doi.org/10.1016/j.jsgi.2006.02.008

[59] Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol 2002; 17: 599-621.

[60] Chechlińska M. Role of cytokines in neoplastic processes (in Polish). Nowotwory. Journal of Oncology 2003; 53: 648-59.

[61] Maruo T, Ohara N, Wang J, Matsuo H. Sex steroidal regulation of uterine leiomyoma growth and apoptosis. Hum Reprod Update 2004; 10: 207-220.
http://dx.doi.org/10.1093/humupd/dmh019

[62] Gao Z, Matsuo H, Wang Y, Nakago S, Maruo T. Up-regulation by IGF-I of proliferating cell nuclear antigen and Bcl-2 protein expression in human uterine leiomyoma cells. J Clin Endocrinol Metab 2001; 86: 5593-9.
http://dx.doi.org/10.1210/jcem.86.11.8008

[63] Van der Ven LTM, Roholl PJM, Gloudemans T, *et al.* Expression of insulin-like growth factors (IGFs), their receptors and IGF binding protein-3 in normal, benign and malignant smooth muscle tissues. Br J Cancer 1997; 75: 1631-40.
http://dx.doi.org/10.1038/bjc.1997.278

[64] Englund K, Lindblom B, Carlström K, Gustavsson I, Sjöblom P, Blanck A. Gene expression and tissue concentrations of IGF-I in human miometrium and fibroids under different hormonal conditions. Mol Hum Reprod 2000; 6: 915-20.
http://dx.doi.org/10.1093/molehr/6.10.915

[65] Dixon D, He H, Haseman JK. Immunohistological localization of growth factors and their receptors in uterine leiomyomas and matched myometrium. Environ Health Perspect 2000; 108 (Suppl. 5): 795-802.
http://dx.doi.org/10.1289/ehp.00108s5795

[66] Schubert G, Elger W, Kaufmann G, Schneider B, Reddersen G, Chwalisz K. Discovery, chemistry, and reproductive pharmacology of asoprisnil and related 11β-benzaldoxime substituted selective progesterone receptor modulators (SPRMs). Semin Reprod Med 2005; 23: 58-73.
http://dx.doi.org/10.1055/s-2005-864034

Received on 06-11-2014 Accepted on 18-11-2014 Published on 15-12-2014

DOI: http://dx.doi.org/10.14205/2309-4400.2014.02.02.2