ORIGINAL STUDY

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**VIA MEDICA**

# Immunohistochemical localization of selected pro-inflammatory factors in uterine myomas and myometrium in women of various ages

**Andrzej Plewka[1], Pawel Madej[2], Danuta Plewka[3], Anna Kowalczyk[4], Adam Miskiewicz[1], Piotr Wittek[1], Tomasz Leks[2], Rafal Bilski[2]**

[1]Department of Proteomics, Medical University of Silesia, Sosnowiec, Poland

[2]Chair and Department of Gynecological Endocrinology, Medical University of Silesia, Katowice, Poland

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

[4]Department of Human Histology and Embryology, University of Warmia and Masuria, Olsztyn, Poland

**Abstract:** Uterine myomas represent one of the most frequently manifested benign tumors in women. They originate from smooth muscle cells of myometrium or its blood vessels. Many studies suggest that inflammation and pro-inflammatory factors may play a role in the carcinogenesis with an involvement of the transcription factor NF-$\kappa$B which activity can be controlled by various environmental factors, including many cytokines. The aim of the study was to investigate the expression of NF-B, interleukin-1$\beta$ (IL-1$\beta$), tumor necrosis factor a (TNF-$\alpha$), cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in myometrium and uterine myomas of women of various age. The expression of NF-$\kappa$B, selected cytokines and enzymes was estimated in women of reproductive or perimenopausal age by semiquantitative immunohistochemistry. The expression of the examined proteins was higher in myomas than in control myometrium and was dependent on the size of myomas and the age of women. However, the expression of the cytoplasmic NF-$\kappa$B observed in uterine myomas was independent on the size of myomas and no significant differences were observed in the number of stained nuclei between control and myoma groups. Thus, the expression of proinflammatory factors in myomas was not accompanied by the nuclear activation of NF-$\kappa$B p65. The results of our study indicate that the examined factors may be involved in the pathogenesis of benign tumors and not only malignant diseases. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 1, 73–83)*

**Key words:** women, uterine myomas, myometrium, NF-$\kappa$B, IL-1$\beta$, TNF-$\alpha$, COX-2, iNOS, immunohistochemistry, age

## Introduction

Uterine myomas are the most common benign tumors in women, but in spite of this, the mechanisms and factors that control their formation and development are poorly understood. Many studies showed the par-

ticipation of pro-inflammatory factors in the pathogenesis of various types of cancer [1–4] through their effects on cell growth and differentiation, apoptosis, angiogenesis, and tissue remodeling [1, 2, 4, 5], i.e. events that are central to tumor growth.

Nuclear factor $\kappa$B (NF-$\kappa$B) represents an important transcription factor which participates in several cellular reactions to environmental changes. The activity of NF-$\kappa$B is an important element in the specific and non-specific mechanism of immune control over inflammatory processes and is tightly controlled by several regulatory proteins (e.g. tumor necrosis

**Correspondence address:** A. Plewka
Department of Proteomics, Medical University of Silesia,
Ostrogórska St. 30, 41–200 Sosnowiec, Poland;
tel.: + 48 32 364 14 30; fax.: + 48 32 364 14 40;
e-mail: aplewka@sum.edu.pl

factor $\alpha$ [TNF-$\alpha$] or interleukin-1). The genes regulated by NF-$\kappa$B involve those coding for cytokines, including interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6) or TNF-$\alpha$, and genes coding for anti-apoptotic proteins or proteins which control cell proliferation [4, 6–8]. They also include genes coding for cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) [9–12]. Cyclooxygenases form a group of enzymes involved in many inflammatory processes. Two forms of the enzyme include the constitutive form, COX-1 and the inducible form, COX-2. Normal uterine smooth muscle cells contain both forms of the enzyme. COX-2, which converts arachidonic acid into prostaglandin $H_2$ (precursor of all prostaglandins [PGs] and thromboxanes), represents a mitogen-induced form of cyclooxygenase, manifesting expression in response to various inflammatory stimuli including IL-1$\beta$ and TNF-$\alpha$. In myometrium and endometrium estrogens stimulate COX-2 to synthesize $PGE_2$ [13]. The $PGE_2$ is associated with proinflammatory mechanisms and could enhance the function of NFkB, whereas the $PGA_1$, $PGA_2$ and $PGJ_2$ are associated with suppression of inflammation and are able to inhibit NF-kB activation [14–16]. Inducible NOS was identified in several cell types, including macrophages and smooth muscle cells [17]. Nitric oxide, synthesized by iNOS, is a mediator involved in various cellular processes. Expression of iNOS was found also in tumors of female reproductive tract [18–20].

IL-1$\beta$ and TNF-$\alpha$ stimulate TRAF6 (tumor necrosis factor receptor associated factor 6) [21], the effect of which directly stimulates the expression of NF-$\kappa$B [22]. IL-1$\beta$ and TNF-$\alpha$ increase the level of iNOS mRNA by transcriptional activation [23]. These cytokines also increase the expression of COX-2, the activity of which is controlled by NOS, particularly by its inducible form [24]. Moreover, high level of estrogens activates iNOS [25, 26], which may significantly affect physiology of myometrium. An increased release of estrogens as a result of induction of aromatase [27] results in increased risk of myoma development due to a direct stimulation of proliferation [28] and increased activity of cyclooxygenase-2 [29].

To our knowledge, the localization and cellular expression of NF-$\kappa$B and iNOS in uterine myomas has not yet been examined, thus the aim of the presented study was to investigate by immunocytochemistry the expression of NF-$\kappa$B, IL-1$\beta$, TNF-$\alpha$, COX-2, iNOS in uterine myomas in women of reproductive or perimenopausal age.

## Material and methods

**Human material.** Recruitment of patients, clinical studies and hormonal tests were conducted in the Chair and Department of Gynecological Endocrinology, Medical University of Silesia in Katowice, Poland, while enzymatic and protein studies were executed in the Department of Proteomics.

The studies were conducted on 40 patients with myomas at the reproductive age (below 45th year of age, FSH < 30 mIU/mL; samples collected during the follicular phase of menstrual cycle) and 40 patients with myomas at the perimenopausal age (45–55 years, FSH >30 mIU/mL). Inclusion criteria involved myoma detected by USG, qualification of the patient to hysterectomy, informed consent to the planned studies. The exclusion criteria included: therapy with any drugs, including hormonal drugs for at least 3 months before inclusion to the studies, neoplastic disease, endometrial hypertrophy, metabolic and systemic disturbances, and nicotinism. In these studies we used only material from uteruses with one large myoma or one large and few small myomas.

Myometrial samples (control groups) were taken from 10 women (< 40 years old) undergoing hysterectomies for ovary tumors and 10 older women (> 52 years old) undergoing hysterectomies for uterine prolapse.

The investigative procedures were approved by the local Medical Bioethical Commission.

**Characteristics of studied groups**. Group 1 denoted as "control group of reproductive age women": myometrium of young women, in whom hysterectomy was performed for reasons other than uterine leiomyomas (n = 10). Group 2 denoted as "small myomas of reproductive age women": leiomyomas of < 3 cm in diameter (n = 20). Group 3 denoted as "large myomas of reproductive age women": leiomyomas of > 5 cm in diameter (n = 20). Group 4 denoted as "control group of perimenopausal age women: myometrium of perimenopausal age women, in whom hysterectomy was performed for reasons different than uterine leiomyomas (n = 10). Group 5 denoted as "small myomas of perimenopausal age women": leiomyomas of < 3 cm in diameter (n = 20). Group 6 denoted as "large myomas of perimenopausal age women": leiomyomas of > 5 cm in diameter (n = 20).

**Immunohistochemical studies.** Tissue samples were fixed in 10% (v/v) solution of buffered formalin for 24 h at 4°C, and then dehydrated, cleared in xylenes and embedded in paraffin. Paraffin sections (5 $\mu$m) were mounted on silane-coated slides, de-waxed, and rehydrated. The sections were treated with 10 mM citrate buffer, pH 6.0 in water bath (30 min at 95°C) for antigen retrieval, then treated with 1.5% (v/v) $H_2O_2$ in methanol for 10 min for quenching of endogenous peroxidase activity, and equilibrated in 10 mM PBS-0.05% v/v Tween 20 pH 7.5. Nonspecific binding was reduced by incubation in 1% BSA for 60 min. After this the slides were incubated with rabbit anti-NF-$\kappa$B p65 (sc-109, Santa Cruz Biotech, Santa Cruz, CA USA), anti-IL-1$\beta$ (ab2105, Abcam, Cambridge, MA, USA), anti-iNOS

**Table 1.** Quantitative evaluation of protein expression by immunohistochemical staining in myometrium and myomas of reproductive and perimenopausal age women

| Protein | Reproductive age | | | Perimenopausal age | | |
|---|---|---|---|---|---|---|
| | Control group | Small myomas | Large myomas | Control group | Small myomas | Large myomas |
| NF-$\kappa$B | 57.1 ± 10.7 | 120.6 ± 13.9 [a] | 119.5 ± 8.8 [b] | 59.0 ± 9.0 | 87.0 ± 10.2 [a,e] | 82.0 ± 11.3 [b,f] |
| IL-1 | 33.2 ± 3.9 | 183.4 ± 9.1 [a] | 210.6 ± 12.2 [b,c] | 38.4 ± 4.7 | 195.3 ± 12.4 [a] | 175.1 ± 11.1 [b,d,f] |
| TNF-$\alpha$ | 67.3 ± 7.9 | 121.5 ± 8.3 [a] | 133.3 ± 10.2 [b] | 72.2 ± 7.0 | 140.9 ± 9.0 [a,e] | 110.2 ± 8.9 [b,d,f] |
| COX-2 | 51.8 ± 11.7 | 110.4 ± 13.1 [a] | 116.5 ± 11.5 [b] | 56.9 ± 9.5 | 122.9 ± 14.1 [a] | 85.5 ± 10.5 [b,d,f] |
| iNOS | 63.4 ± 9.4 | 115.7 ± 6.2 [a] | 135.3 ± 8.0 [b,c] | 67.7 ± 10.6 | 130.7 ± 9.2 [a,e] | 124.6 ± 6.3 [b] |

The staining intensity was measured as described in methods. Data show an average optical density ± SD (see material and methods). Statistical significance was defined as a p < 0.05. [a]small myomas vs. healthy myometrium; [b]large myomas v. healthy myometrium; [c]small v. large myomas in reproductive age women; [d]small v. large myomas in perimenopausal age women; [e]small myomas in reproductive age women v. small myomas in perimenopausal age women; [f]large myomas in reproductive age women v. large myomas in perimenopausal age women

(ab15323, Abcam) and anti-TNF-$\alpha$ (ab6671, Abcam) polyclonal antibodies or mouse anti-COX-2 monoclonal antibody (sc-58344, Santa Cruz) in a humidified chamber for 22 h at 4°C. After washing in PBS-Tween 20 the sections were incubated with biotinylated goat anti-rabbit or horse anti-mouse immunoglobulins (Vector Laboratories, Burlingame, VA, USA) for 30 min, and next with avidin-biotinylated peroxidase complex (Vector) for 30 min. The bound antibodies were visualised with diaminobenzidine (DAB) and $H_2O_2$ in PBS, pH 7.5 according to supplier's instructions (Vector). Finally, the tissues were stained with Gill's hematoxilin, dehydrated, and cover-slipped. Negative controls were performed by substituting the primary antibodies with rabbit IgG or mouse IgG respectively.

**Archives.** Photographic documentation was prepared using a light photomicroscope. In order to accentuate the immune reactions, every reaction was documented by 10 photographs under 200 × magnification (20 × objective and 10 × ocular) using a digital camera-equipped Nikon microscope.

**Optical density measurements.** In each positively stained cell, the intensity of staining was measured as the optical density of the reaction product, with the program KS 300 VIDAS video image analyzer served by IBAS 2.5 system and a digital camera. For each analyzed area, 173 × 130 $\mu$m average optical density was calculated [30]. Three sections for every studied protein and every patient were analyzed. In each section ten fields were examined. Finally, the arithmetic mean and standard deviation were calculated.

**Statistical analysis.** Normal distribution of the data was confirmed by the Kolmogorov-Smirnov test. Results are presented as a mean ± standard deviation. The Student's *t*-test was performed. A P value < 0.05 was considered to be statistically significant.

## Results

### *Expression of the nuclear transcription factor $\kappa$B (NF-$\kappa$B)*

NF-$\kappa$B showed both nuclear and cytoplasmic staining. No significant differences were observed in the number of stained nuclei between control groups and myoma groups (small and large myomas) in women of different age (data not shown).

### *Cytoplasmic localization and its analysis*

*Reproductive age.* Cytoplasmic expression of NF-$\kappa$B was about 100% higher in small and large myomas of reproductive age women than in the control (Table 1, Figure 1).
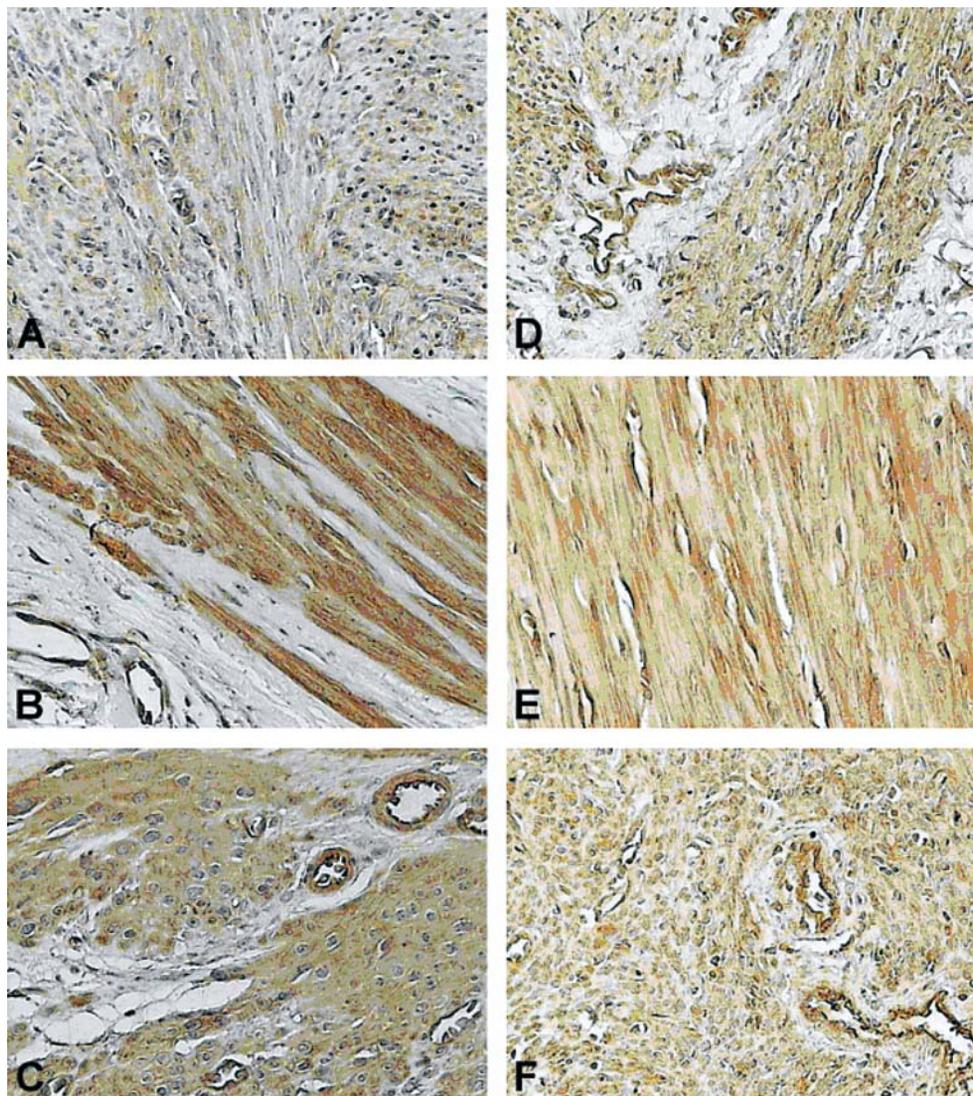
*Perimenopausal age.* Cytoplasmic NF-$\kappa$B expression in small myomas was increased to almost 150% of the control level, and in large myomas to 140% of the control level (Table 1, Figure 1).

*Effect of age.* Cytoplasmic NF-$\kappa$B expression was similar in both control groups (Table 1). In perimenopausal age women the expression of NF-$\kappa$B was lower in both studied myoma groups as compared to myomas of young women.

## Interleukin-1$\beta$ (IL-1$\beta$)

### Reproductive age

Expression of IL-1$\beta$ in young women with small myomas was clearly higher than in the control and reached 550% of the control level (Table 1, Figure 2). IL-1$\beta$ expression in large myomas amounted to 635% of the control level. Expression of the interleu-

**Figure 1.** NF-$\kappa$B presence in uteri from reproductive (**A–C**) and perimenopausal age women (**D–F**). A and D — myometrium of control groups, B and E — small myomas, C and F — large myomas. NF-$\kappa$B was detected by IHC as described in Methods. Total magnification 200 ×

kin in large myomas in women of reproductive age was higher than in small myomas.

### Perimenopausal age

In women of perimenopausal age IL-1$\beta$ expression in small myomas increased to 510%, and in large myomas to 455% of the control level (Table 1, Figure 2). In large myomas expression of IL-1$\beta$ was lower than that in small myomas.

### Effect of age

Comparison of IL-1$\beta$ expression in control groups demonstrated that it was comparable in young and perimenopausal women (Table 1, Figure 2). Similarly, comparison of small myomas in women of the two studied age groups revealed no differences. In large myomas the expression of IL-1$\beta$ at the perimenopausal age was lower and amounted to 80% of the expression seen in young women.

### Tumor necrosis factor-$\alpha$ (TNF-$\alpha$)

Reproductive age
In young women the expression of TNF-$\alpha$ in small myomas was higher than in control myometrium and amounted to about 180%. TNF-$\alpha$ expression in large myomas reached 200% of the control level (Table 1, Figure 3).
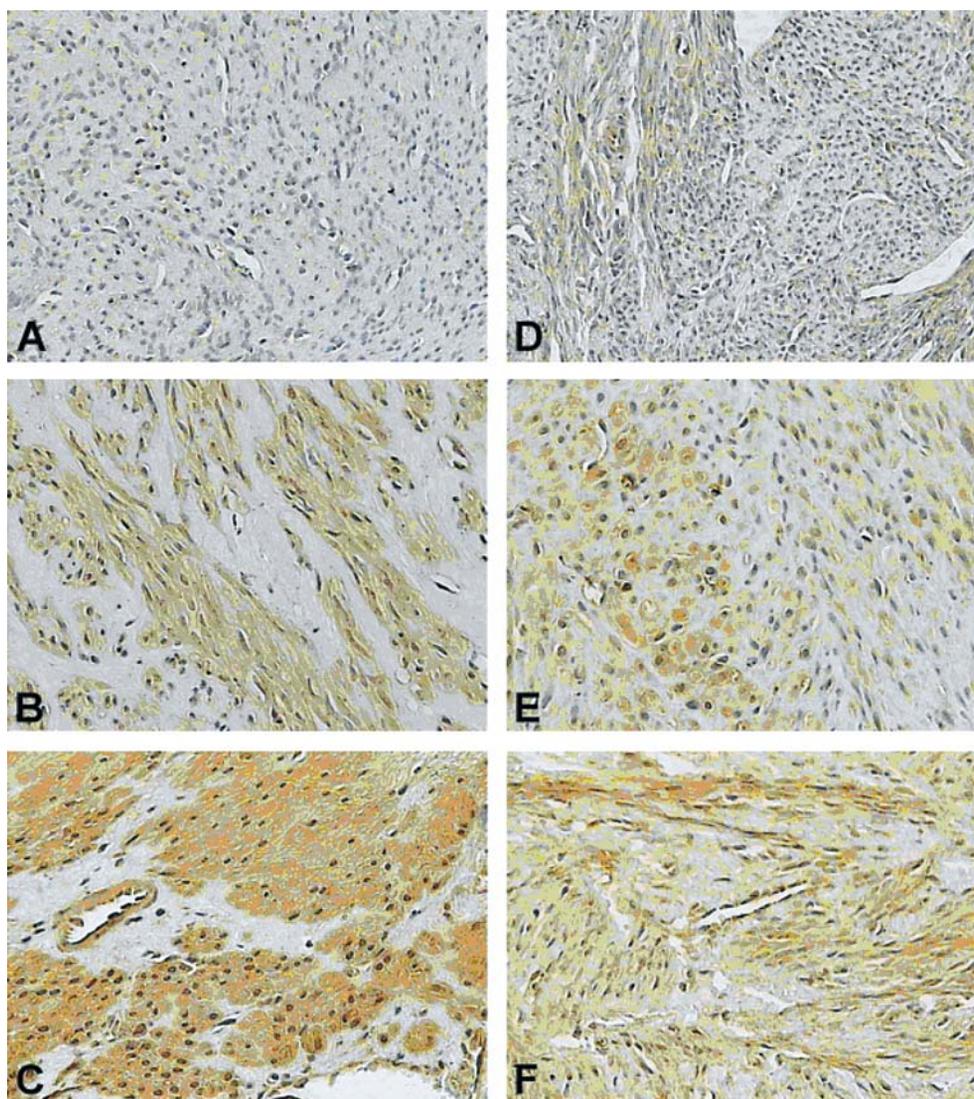
**Figure 2.** IL-1b presence in uteri from reproductive (**A–C**) and perimenopausal age women (**D–F**). A and D — myometrium of control groups, B and E — small myomas, C and F — large myomas. IL-1b was detected by IHC as described in Methods. 200 ×

Perimenopausal age
The expression of TNF-$\alpha$ increased in women of perimenopausal age, in small myomas, reaching the level corresponding to 195% of the control value (Table 1, Figure 3). The expression of TNF-$\alpha$ in large myomas was higher than in the control, reaching 155%. In uterine myomas of women at perimenopausal age the expression of the cytokine was slightly lower in large than in small myomas, reaching 80% of the level in the latter.

Effect of age
The expression of TNF-$\alpha$ was found to be similar in young women and in women at perimenopausal age (Table 1, Figure 3). In small myomas, in young women the expression of TNF-$\alpha$ reached 90% of the value disclosed in the older women. The expression of TNF-$\alpha$ in large myomas of women at perimenopausal age was noted to be lower, amounting to 75% of the value detected in young women.

*Cyclooxygenase-2 (COX-2)*

Reproductive age
COX-2 expression in small myomas of young women was increased to 215% of the control value (Table 1, Figure 4), and in large myomas to 225% of the control value. In large myomas COX-2 expression was similar to the one found in small myomas.
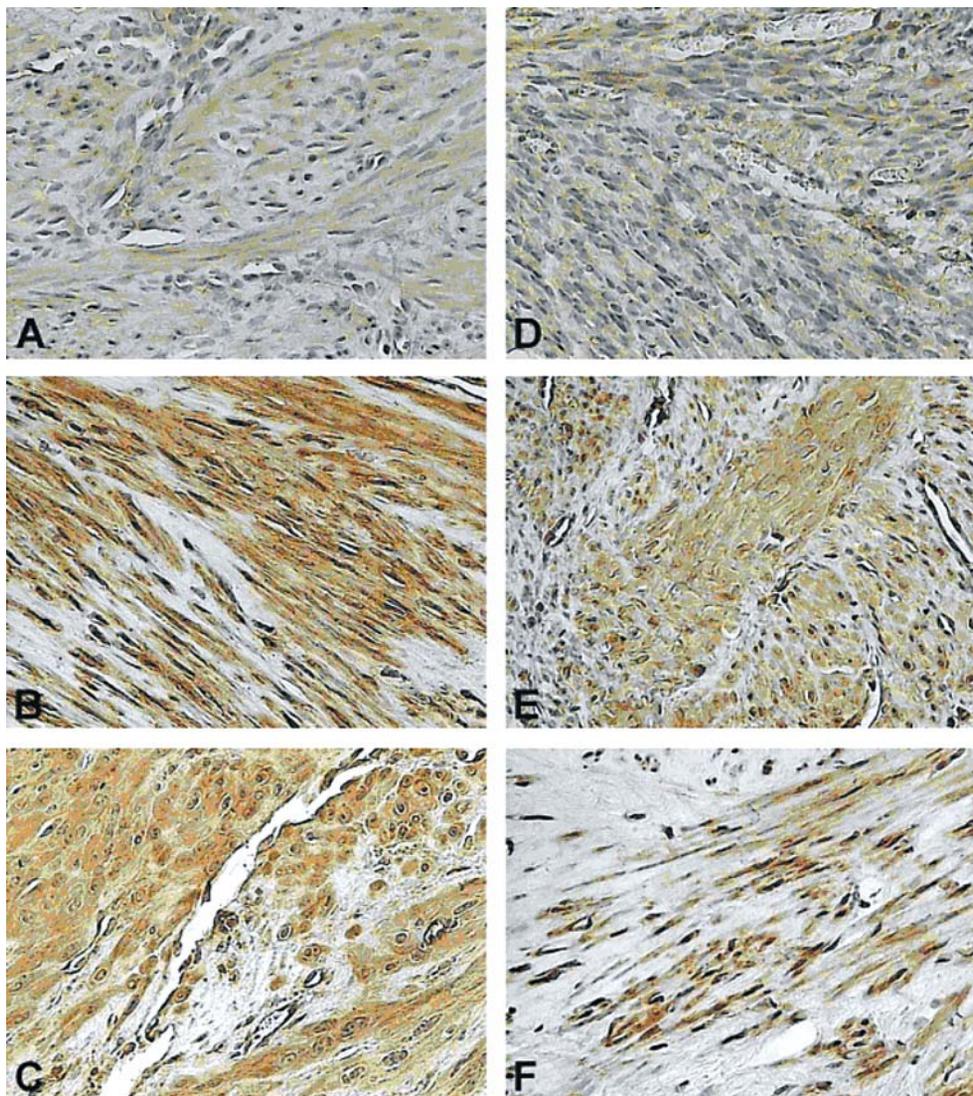
**Figure 3.** TNF-$\alpha$ presence in uteri from reproductive (**A–C**) and perimenopausal age women (**D–F**). A and D — myometrium of control groups, B and E — small myomas, C and F — large myomas. TNF-$\alpha$ was detected by IHC as described in Methods. 200 ×

Perimenopausal age
COX-2 expression increased in small myomas to the level of almost 215% of the control value (Table 1, Figure 4). In large myomas the expression of COX-2 was higher than in the control, reaching 150% of the control value.

Effect of age
Expression of the enzyme in control samples was similar in both studied age groups (Table 1, Figure 4). A similar situation was detected upon quantitative evaluation of the enzyme expression in small myomas in women of the two age groups. In large myomas the

level of COX-2 expression in women of perimenopausal age was lower and corresponded to 65% of the level detected in the younger women.

### Inducible nitric oxide synthase (iNOS)

*Reproductive age.* In young women iNOS expression increased in both small and large uterine myomas, reaching 185% and 215% of the control level, respectively (Table 1, Figure 5). In large myomas the expression of iNOS was higher than in small myomas and it reached almost 120% of the enzyme expression detected in small myomas.
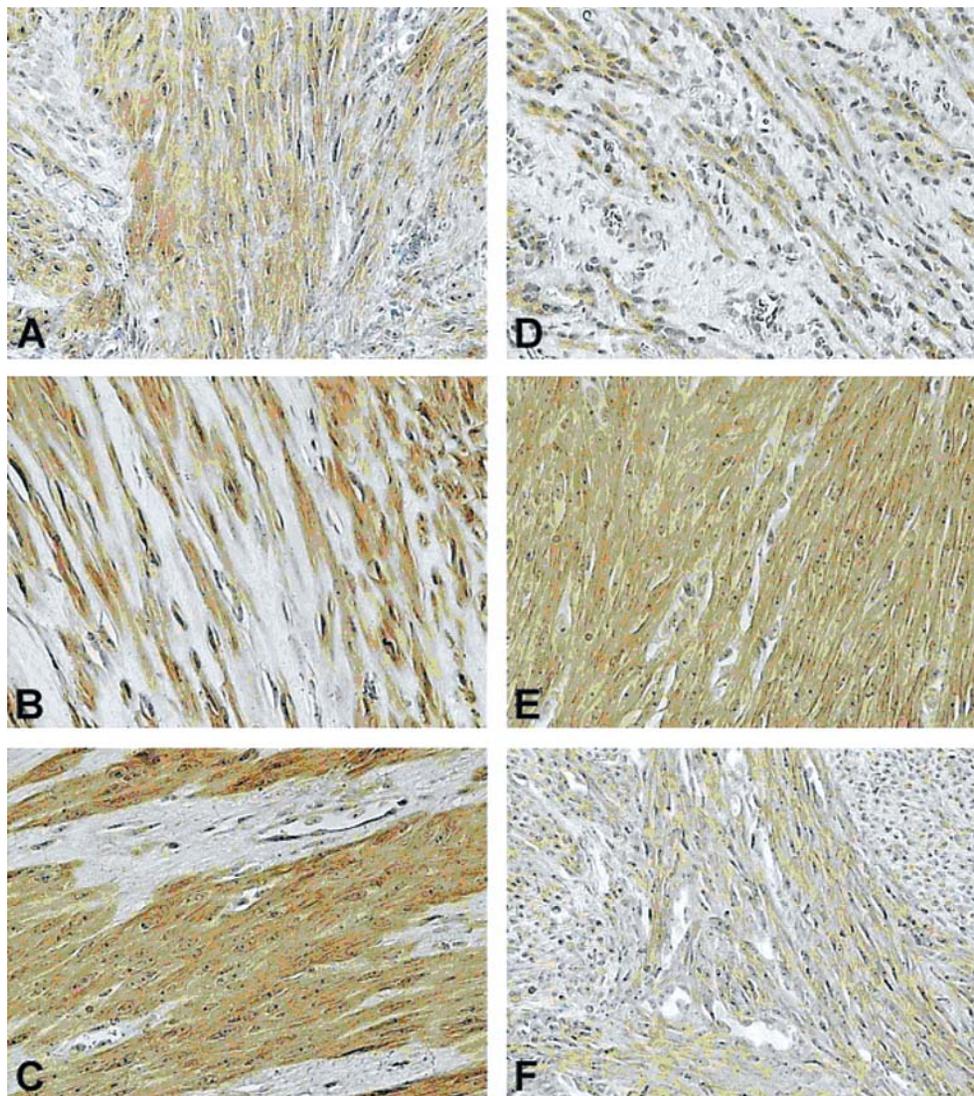
**Figure 4.** COX-2 presence in uteri from reproductive (**A–C**) and perimenopausal age women (**D–F**). A and D — myometrium of control groups, B and E — small myomas, C and F — large myomas. COX-2 was detected by IHC as described in Methods. 200 ×
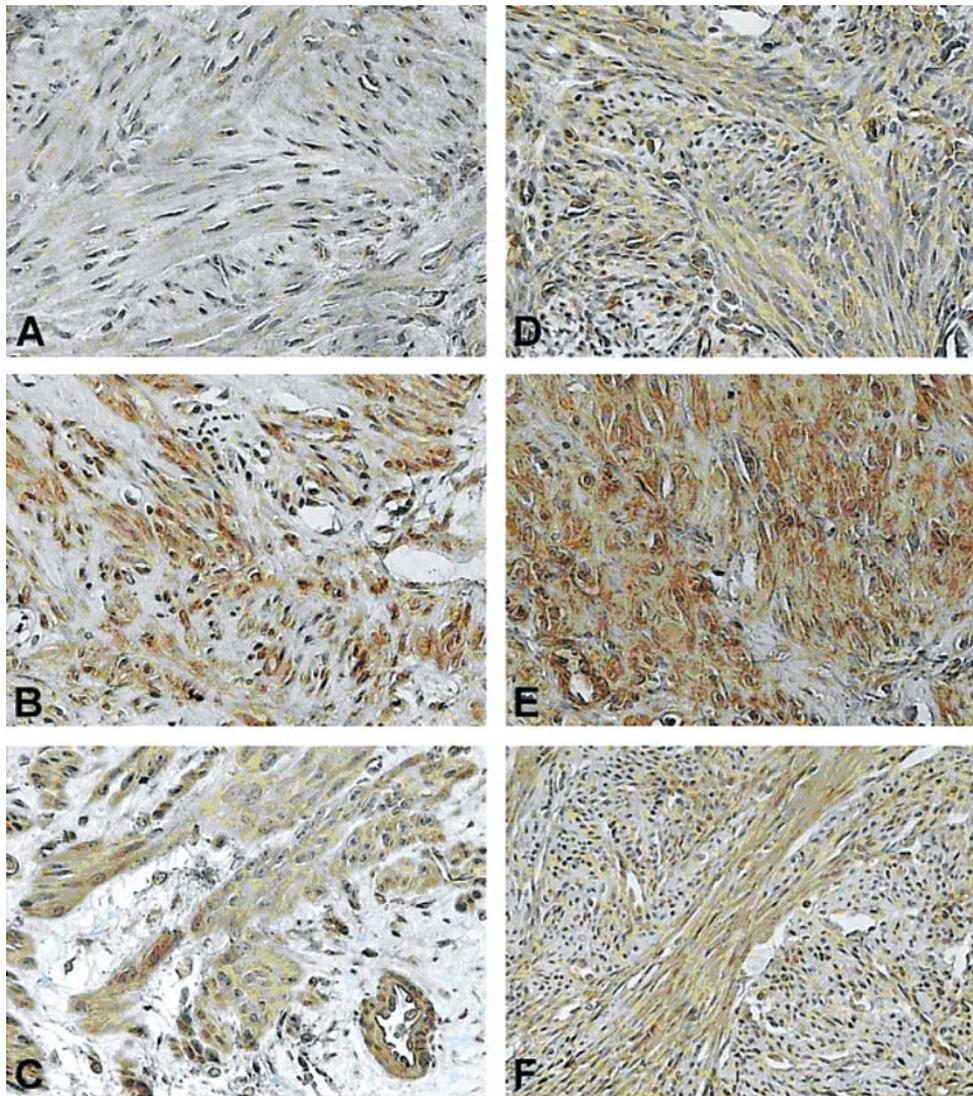
Perimenopausal age
iNOS expression in perimenopausal women increased in small myomas to the level corresponding to 190% of the control level (Table 1, Figure 5). In large myomas, as compared to the control, the expression was higher, amounting to 185% of the control.

Effect of age
iNOS expression in young and older women was similar (Table 1, Figure 5). In small myomas of women at perimenopausal age the expression of iNOS was higher (12%) than in small myomas of young women. iNOS expression in large myomas of perimenopausal women was the same as that in young women.

## Discussion

Pro-inflammatory factors and their regulators are multifunctional agents and it has been shown that they can control various processes associated with tumorigenesis [1–4]. IL-1$\beta$ mRNA expression was identified for example in cells of melanoma, colon adenocarcinoma or non-small-cell lung cancer. It was found that this pluripotent cytokine promotes angiogenesis, tumor growth and metastasis [2]. Our studies revealed the expression of IL-1$\beta$ in myometrium and uterine myomas of women of various age, whereas several times higher expression was observed in myomas. IL-1 may affect the growth of myomas cells, as

**Figure 5.** iNOS presence in uteri from reproductive (**A–C**) and perimenopausal age women (**D–F**). A and D — myometrium of control groups, B and E — small myomas, C and F — large myomas. iNOS was detected by IHC as described in Methods. 200 ×

it was demonstrated in the case of vascular smooth muscle cells [31] or airway smooth muscle cells [32]. TNF-$\alpha$ is another cytokine with dual role in cancer biology. This multifunctional cytokine plays a key role in apoptosis and cell survival as well as inflammation and immunity [33]. It can destroy blood vessels, but also induce several angiogenic factors [1]. TNF-$\alpha$ is mainly produced by activated macrophages, T lymphocytes, and natural killer cells, but lower expression was also observed in other cell types, including fibroblasts, smooth muscle cells, and tumor cells. TNF-$\alpha$ mRNA and protein were detected in malignant and/or stromal cells in human ovarian, breast, prostate, bladder and colorectal cancer, lymphomas and leukemia's, often in association with IL-1 [1, 33].

In our investigations immunoreactivity of TNF-$\alpha$ was significantly higher in myoma cells than in cells of control myometrium. These results are consistent with studies of Kurachi et al. [34] who showed that the expression of TNF-$\alpha$ in leiomyoma cells was higher than that in the normal myometrial cells and could be down-regulated by progesterone. Activity of IL-1$\beta$ and TNF-$\alpha$ is particularly important since, on the one hand, they may directly affect the level of inducible cyclooxygenase [16,35] and, on the other, they may indirectly, through TRAF proteins, stimulate activation of the NF-$\kappa$B transcription factor [21]. We found that the expression profile of COX-2 was similar to the one of TNF-$\alpha$. Higher imunnoreactivity of COX-2 was observed in myoma tissue than the control myometri-

um. The overexpression of this enzyme may be related to its function in the process of tumorigenesis. It was found that COX-2 prevented apoptosis and was associated with inflammation, cell growth and differentiation [5]. Prostaglandins produced via COX-2 activation, including prostaglandin E2 (PGE2), are believed to be the major contributors to cell proliferation [36], and PGE2 has been regarded as a primary COX-2 product in smooth muscle cells [37]. Transfection of rat intestinal epithelial cells with COX-2 gene caused inhibition of apoptosis accompanied by increased spontaneous output of $PGE_2$ and increased levels of an anti-apoptotic protein Bcl-2 [38]. Furthermore, it was shown that overexpression of COX-2 promoted angiogenesis and tumor growth in endometrial carcinoma [39], and that COX-2 inhibitor in human prostate cancer cells inhibited their growth [40]. The probability of participation of COX-2 in the process of cell growth is supported by the fact that this enzyme is inducible by growth factors and mitogens [41]. Similarly to results of our investigations, up-regulation of COX-2 expression was observed e.g. in carcinomas of the lung, colon and breast [3], however, in uterine leiomyomas, Erol et al. [42] reported opposite observations.

IL-1$\beta$ and TNF-$\alpha$ are the most prominent cytokines involved in iNOS stimulation. iNOS is one of three key enzymes generating nitric oxide (NO) which plays an important role in numerous physiological and pathophysiological conditions. It is well known that NO affects the expression of VEGF, angiogenesis, apoptosis, and prostaglandin production. The role of iNOS during tumor development is highly complex and presumably depends on the local concentration of iNOS within the tumor microenvironment [43]. To our best knowledge, our study is the first report on the localization and the immunoreactivity of iNOS in human uterine myomas. We found significantly higher immunoreactivity of iNOS in myoma tissue than in the control myometrium, which suggests an involvement of this enzyme in the pathogenesis of benign tumors. It seems to be consistent with other investigations that which reported that iNOS expression correlated with progression of human astrocytoma [44], human melanoma [45] and prostate cancer [46].

Expression of many cytokines and enzymes, such as TNF-$\alpha$, COX-2 and iNOS, which may control cell proliferation, cell survival or tumor development, was found to be regulated by a transcription factor-NF-$\kappa$B [47, 48]. On the other hand NF-$\kappa$B activity may be reciprocally regulated by the cytokines TNF-$\alpha$ or IL-1. They lead to the phosphorylation and degradation of I$\kappa$B, which results in the nuclear transfer of NF-$\kappa$B. The classic form of NF-$\kappa$B is normally retained in cytoplasm through interactions with inhibitor proteins I$\kappa$B$\alpha$ and I$\kappa$B$\beta$ [4]. Such a condition probably occurred in our study, since in samples obtained from control uteri, which contained no myomas, and in samples with myomas the high immunoreactivity of NF-$\kappa$B was restricted exclusively to the cytoplasmic compartment. No significant differences were observed in the number of stained nuclei between control groups and myomas groups. It might have been caused by the fact that there are five members of the mammalian NF-$\kappa$B/Rel family and the heterodimer composed of the p50 and 65 subunits is the classic form, and we examined only this type of NF-$\kappa$B. However, studies of Cogswell et al. [49] indicated that only p50, p52, and c-Rel subunits were activated in human breast cancers without a corresponding consistent activation of the p65 subunit. The NF-$\kappa$B is generally considered active when it is in the nuclear localization rather than the cytoplasm. However, the overall expression level of this protein may also be related to its activity. Annunziata et al. [50] reported that an increase in cytoplasmic NF-$\kappa$B transcription factor p50 was significantly associated with poorer patient survival. In myomas, cytoplasmic expression of NF-$\kappa$B was highly elevated. Two observations have drawn our attention. For the first, independently whether data obtained from small or large myoma samples were compared, the levels of immunoreactivity in each age group of women were practically the same. However, samples obtained from women in reproductive age manifested higher cytoplasmic expression of NF-$\kappa$B. Differences in the expression levels of the examined factors in tissues of women of different age may be dependent on the hormonal status. Our studies, for the first time, indicated the localization and expression level of NF-$\kappa$B in uterine myomas.

In conclusion, higher expression of the examined factors in uterine myomas in comparison to the control myometrium suggests involvement of these agents in the pathogenesis of uterine myomas.

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