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Differential expression of Akt/protein kinase B, Bcl-2 and Bax proteins in human leiomyoma and myometrium

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Abstract

The expression and activation of serine/threonine protein kinase, Akt, in leiomyoma and in adjacent myometrium of human uteri was studied parallel with the changes of Bcl-2, Bax proteins, estrogen and progesterone receptors during menstrual cycle and early stage of the menopause. Abundant expression of Akt protein was detected in the studied tissues during menstrual cycle, the rate of increase was higher in leiomyoma than in corresponding myometrium. The expression of estrogen receptor alpha, progesterone receptor and of Bcl-2 protein changed parallel with that of Akt protein. The level of phosphorylated Akt ($pAkt^{473}$) was seen only in leiomyoma samples from the growing period of tumors. At early stage of menopause levels of all studied proteins were lower than that in the menstrual cycle with the exception of Bax protein expression, which was high in leiomyoma. Our data suggest the involvement of phosphatidylinositol 3-kinase/Akt signaling in the pathomechanism of leiomyoma.

Keywords: Human myometrium; Leiomyoma; Akt/PKB; Bcl-2; Bax; Estrogen receptor

1. Introduction

Steroids control diverse biological processes in cells. They interact with intracellular receptors and regulate as ligand dependent transcription factors the expression of different target genes [1,2]. This cellular response is known as genomic action of steroid hormones.

Since late 1970s there is relevant evidence indicating the existence of cell membrane estradiol binding in endometrial cells [3]. Several reports on these binding sites suggest that these molecules mediate important cellular actions, known as non-genomic action, by recruitment of different signaling pathways such as MAPK cascades, several tyrosine kinases and lipid sensitive kinases [4]. Among them, the serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt) has emerged as a crucial regulator of widely divergent cellular processes including apoptosis [5,6], proliferation, differentiation [7] and metabolism [8].

Uterine leiomyoma is the most common tumor in women during fertile age. The tumors derive from myometrial cells, their hormonal responsiveness far exceeds that of mature, normal myometrium. Estrogen receptors (ER) are overexpressed [9,10], local formation of estrogen (E) in the tumor cells creates a local hyperestrogenic environment in the tumor [11]. Growth response of leiomyoma to estrogen has commonly been attributed to a hypersensitive state of tumor cells to this hormone. Progesterone (P) is also thought to play a role in the tumor growth [12]. More recently, local growth factors have been shown to mediate the action of estrogen and to play important roles in the development of leiomyoma [13].

Growth of tumors depends on the balance between cell proliferation and cell death, too much growth may be results of decreased cell death as well as of increased proliferation.

The Bcl-2 family of proteins comprises both cell death inhibiting and cell death promoting members. The possible role of changed apoptotic mechanism is widely investigated in different gonadal organs, endometrium, ovaries, breast and in human myometrium and leiomyoma. Recently

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published data demonstrates changed levels of antiapoptotic Bcl-2 and proapoptotic Bax protein in leiomyoma cells relative to myometrium [9,13–15]. The results are varied, might be due to methodological differences. According Wu et al. [15] only minor influence of the endocrine environment could be detected on the expression of the different members of Bcl-2 family in human uterine myometrium and leiomyoma, while in an in vitro study the Bcl-2 was shown to be upregulated by progesterone [14].

Recent data demonstrate that ERα in several types of cells interacts with lipid kinase phosphatidylinositol 3-kinase (PI3K)/Akt pathway [16] regulating the cell survival and apoptosis [17]. To date, there is no data concerning phosphatidylinositol 3-kinase/Akt pathway and its role in steroid response of human uterus myometrium and leiomyoma.

The aim of present investigation was to study the Akt/protein kinase B signaling in tissue samples from leiomyoma and adjacent myometrium of human uteri at different hormonal environment, parallel with the changes in the expression of antiapoptotic Bcl-2 and proapoptotic Bax proteins. Furthermore, the expression of ERα and PR was also determined in each tissue extracts to get parallel results for better evaluation of studied molecular events.

2. Materials and methods

2.1. Tissues

Normal and pathological (leiomyoma) myometrial specimens were obtained from cyclic (*n*: 16) and menopausal (*n*: 5) women (aged 38–50 year) undergoing hysterectomy for benign indications with no history of hormonal treatment for at least 3 month before hospitalization. The last menstrual bleeding of menopausal women was at least 6 but less than 12 month. The Institutional Human Studies Committee approved the use of the tissues and informed consent was obtained from the patients.

We dissected one tumor from each uterus. The number of nodules ranged between one and five per patient, their sizes were 10–50 mm in average diameter. Nodules for study were 35–40 mm in diameter, and situated within the myometrial wall of the uterus. Leiomyoma specimens were obtained from the leiomyoma tissue just beneath the capsule of the tumor. Myometrial samples, for use as paired controls, were obtained from surrounding normal myometrium situated more than 10 mm away from the fibroid capsule. Part of selected nodules was examined by pathologist. All tissue samples used for this study were confirmed as histologically ordinary leiomyoma without any sign of degenerative changes. The pathologist to assess the stage of menstrual cycle and to exclude adenomyosis or malignant changes also examined the uterine samples. The tissue samples after dissection were immediately frozen in liquid nitrogen and were stored at −80 °C until use.

2.1.1. Hormone analysis

FSH, estradiol and progesterone concentrations in the serum of patients on the morning of hysterectomy were determined by two sites immunoluminometric assay (Byk-Sagtec Diagnostika, Germany) for exact diagnosis of the cycle phases and the early stage of menopause.

2.2. Chemicals

The following antibodies were used in Western blotting: Akt, pAkt (pSer^{473}) rabbit polyclonal antihuman (Sigma, St. Louis, Missouri. USA), ERα, Bcl-2, Bax and Actin rabbit polyclonal antihuman antibodies (Santa Cruz Biotechnology, CA, USA), and monoclonal antibody against progesterone receptor (clone: PRI0A9, Immunotech, Marseilles, France). All other chemicals unless stated otherwise were purchased from Sigma (St. Louis, MO, USA).

2.3. Western blot analysis

Methods used for experiments were carried out as reported earlier [9]. Briefly, the examined tissue samples were homogenized by Polytron homogenizer at 4 °C in 2% sodium dodecyl sulfate (SDS), 10 mM Tris, 1 mM sodium vanadate, and a mixture of protease inhibitors (2.5 μg/ml aprotinin plus 0.3 mM phenylmethylsulphonyl-fluorid) at a buffer/tissue ratio of 1 ml/100 mg tissue. Samples were boiled for 5 min and cleared by centrifugation at 10,000 × *g* for 10 min. Aliquots of samples were taken for protein determination (Bio-Rad protein assay, Bio Rad Labs. LA, CA, USA). The remaining samples were prepared for electrophoresis.

Proteins (10 μg/samples) from the same sample of tissue extracts were separated on 10% SDS polyacrylamide mini-gel, and electrophoretically transferred to a nitrocellulose membrane. Membranes were incubated for 1 h in blocking solution (PBS containing 5% dried milk, 0.1% Tween 20) and then transferred to primary antibody in PBS containing 2.5% dried milk to incubate overnight. To ascertain the specificity of the stained protein band, the first antibody was preincubated with blocking peptide overnight before being exposed to the membrane. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The signals were visualized by an ECL system (Amersham, IL, USA). The density of reacted bands was measured by densitometric scanning.

Eight patients were in the each of different phases of the menstrual cycle and five in menopause. In all 42 samples were analyzed, 21 samples were obtained from the myometrium and 21 from leiomyoma tissue. The Western blot analyses were conducted at three times in three independent tissue preparations from the same nodules with comparable results. To compare the rate of expression of receptor proteins in different blots, we applied arbitrary units. We extract proteins from myometrium of one menopausal uteri (myometrium from uteri of menopausal women, 6 month after the last bleeding), which was used as standard in all

blots. Densities of the examined bands in different blots after normalization were compared to the corresponding density of the "standard" myometrium (StM). In each series of experiments, the level of expression in the StM was arbitrarily set to 10 and the results from other examined samples on the same blot were expressed relative to these values. The level of actin expression was used as a loading control.

2.4. Statistics

The data are presented as mean ± S.D. from at least three experiments giving similar results. Group differences were analyzed by ANOVA followed by Student-Newman-Keul's multiple range tests. Differences between tissues for the individual groups of patients were calculated by paired t tests. Differences were considered to be statistically significant at $P < 0.05$ levels.

3. Results

3.1. Hormone analyses

In women having menstrual cycles, the steroid concentrations in serum were as expected for the corresponding cycle phase, oestradiol 285 ± 37 pmol/l (mean ± S.D.) and progesterone 1.32 ± 0.7 nmol/l in patients from proliferative phase, and 349 ± 115 pmol/l of estradiol and 22.7 ± 6.9 nmol/l of progesterone in patients from secretory phase. Serum concentrations of FSH on the morning of hysterectomy were 38 ± 7.9 IU/l and 10 ± 98 IU/l in our patients having menopause and menstrual cycle, respectively.

3.2. Sex steroid receptor analysis

Fig. 1 shows the variation of ERα in the examined samples. In agreement with our earlier results [9], the expression of ERα in all leiomyoma samples was increased. This increase was at least two folds, in comparison with corresponding myometria during menstrual cycle. No cyclic differences in ERα were seen in the examined tissues. No differences were seen in ERα expression between leiomyoma and adjacent myometrium in samples from menopausal uteri.

Fig. 2 shows the changes in the levels of PR in the examined tissues. In all samples only one molecular species of PR (~116 kDa) reacts specifically with the applied antibody. The PR proteins in leiomyoma during menstrual cycle were slightly elevated compared to that of adjacent myometrium. PR is more abundantly expressed in the secretory, than in proliferative phase. The pattern of changes in PR protein expression in the myometrium is similar to that of leiomyoma. No difference was found in the examined tissues from menopausal uteri.

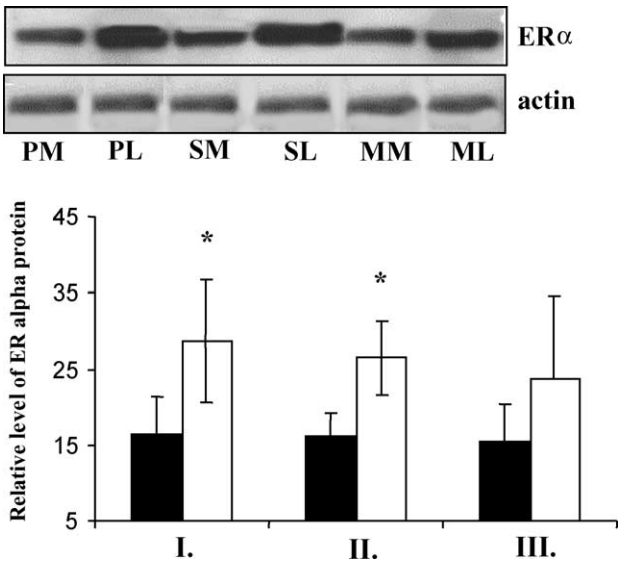

Fig. 1. Changes of ERα protein level in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause, see Section 2). Inset (representative blots): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Statistically significant differences ($P < 0.05$) are indicated by asterisks between leiomyoma and corresponding myometrium.

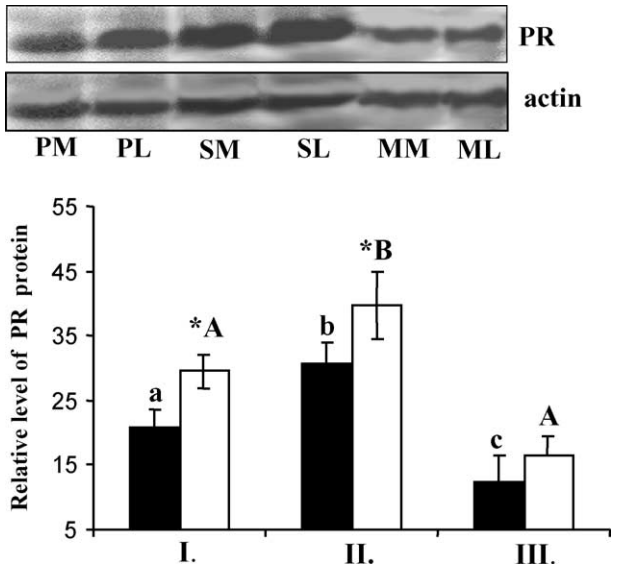

Fig. 2. Changes of PR protein level in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause). Inset (representative blot): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Myometrial values marked by different lower case letters (a–c) and leiomyoma values marked by different capitals (A, B) are significantly different from each other at $P < 0.05$ level. Asterisks (*) indicate statistically significant differences between leiomyoma and corresponding myometrium. For further details see legend of Fig. 1.

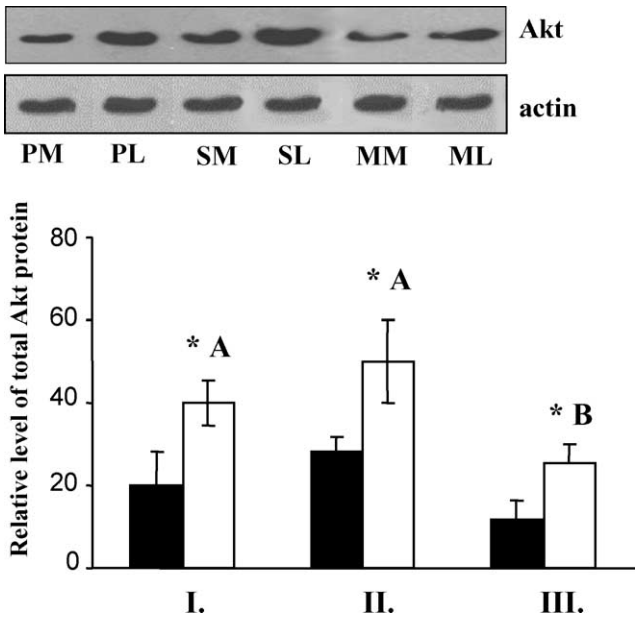

Fig. 3. Changes of Akt protein level in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause). Inset: (representative blots): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Values marked by different letters are significantly different from each other at $P < 0.05$ level. Asterisks (*) indicate statistically significant differences between leiomyoma and corresponding myometrium. For further details see legend of Fig. 1.

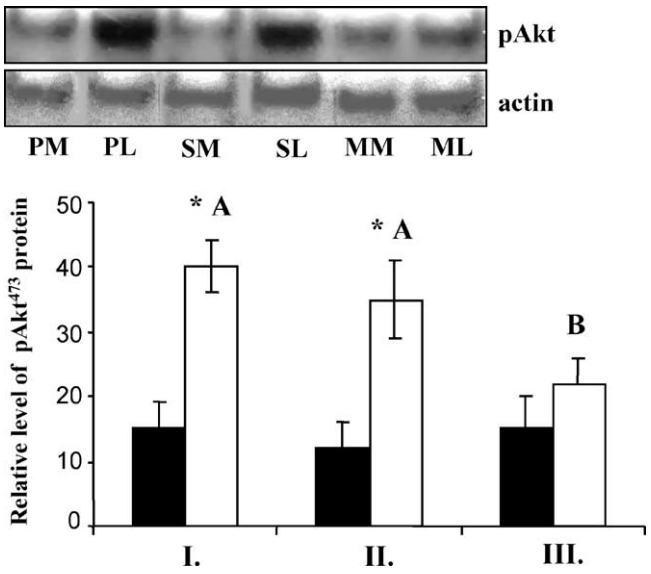

Fig. 4. Changes of phospho-Akt (pSer^{473}) protein level in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause). Inset (representative blots): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Values marked by different letters are significantly different from each other at $P < 0.05$ level. Asterisks (*) indicate statistically significant differences between leiomyoma and corresponding myometrium. For further details see legend of Fig. 1.

3.3. Expression and activation of Akt protein

Western blot analyses revealed that Akt proteins were expressed in both leiomyoma and myometrium. The level of total Akt protein was more abundant in leiomyoma than in myometria, and no significant changes regarding phases of menstrual cycle were detected in the studied tissues. However, at the early phase of menopause the expression of Akt protein decreases in comparison with results from uteri with menstrual cycle. No significant difference between samples from leiomyoma and adjacent myometrium was found (Fig. 3).

Measurement of activation of Akt proteins, recognized by an antibody raised against phospho-Akt (pAKT^{473}), showed abundant level of pAKT^{473} in leiomyoma from uteri of fertile age. The pAKT^{473} was higher in leiomyoma than in myometrium. In postmenopausal patients, only weak bands of pAKT^{473} were measurable in extracts from both myometria and leiomyoma (Fig. 4).

3.4. Analysis of Bcl-2 proteins

Western immunoblot analysis of leiomyoma and adjacent myometrium revealed that antiapoptotic Bcl-2 protein with molecular weight of 26 kDa is abundantly expressed in leiomyoma during menstrual cycle. The rate of increase is higher in secretory than in proliferative phase, in menopause no change was detected in leiomyoma relative to myometrium (Fig. 5). Bcl-2 protein in myometrium was barely detected and no significant changes were found during menstrual cycle and menopause.

Fig. 6 shows the Western analysis of proapoptotic Bax protein. Expression of Bax protein with molecular weight 21.5 kDa is detectable in all studied tissues. Most abundant expression of Bax protein is observed in leiomyoma of menopausal uteri, when the tumor regression takes place. During menstrual cycle, Bax protein in leiomyoma tends to increase in secretory phase, its level during proliferative phase is low as it is in the myometria.

4. Discussion

The present study demonstrates the expression of PKB/Akt in human uterus leiomyoma and myometrium. As our data show the expression and activation of Akt in leiomyoma is higher during menstruation cycle, than in menopause. In the myometrium levels of both Akt and pAkt are at only detectable levels. Furthermore, our data show similarities in the expression of PKB/Akt, steroid response and expression of apoptotic markers in uterine smooth

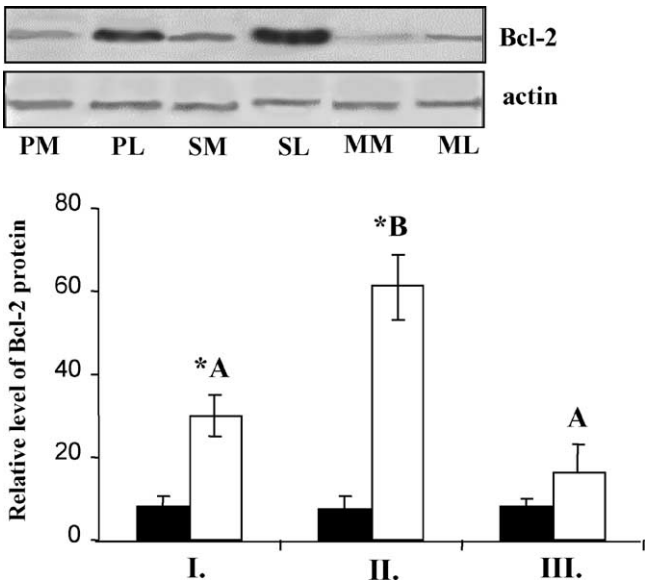

Fig. 5. Changes of Bcl-2 protein level in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent the mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause). Inset (representative blot): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Values marked by different letters are significantly different from each other at $P < 0.05$ level. Asterisks (*) indicate statistically significant differences between leiomyoma and. corresponding myometrium. For further details see legend of Fig. 1.

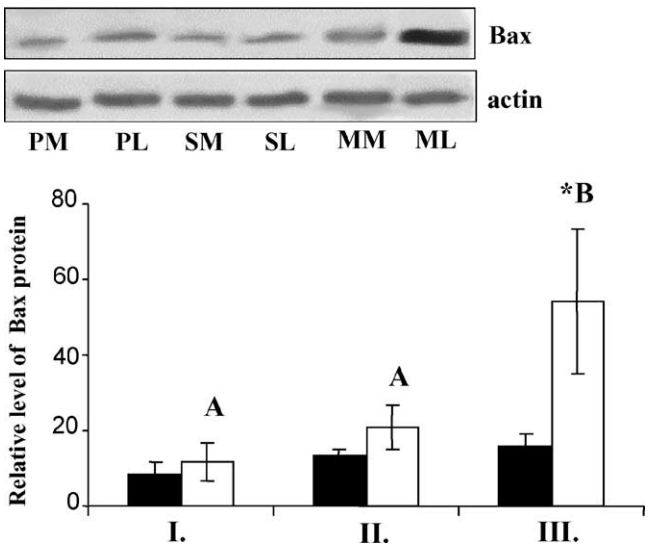

Fig. 6. Changes of Bax protein concentration in leiomyoma (open bars) and adjacent myometrium (black bars) of human uteri. I: Proliferative phase; II: secretory phase; III: menopause. Bars represent mean ± S.D. of data from the same phases (24–24 determinations from uteri with menstrual cycle phases, 15 from menopause). Inset (representative blot): PM, proliferative myometrium; PL, proliferative leiomyoma; SM, secretory myometrium; SL, secretory leiomyoma; MM, menopausal myometrium; ML, menopausal leiomyoma. Values marked by different letters are significantly different from each other at $P < 0.05$ level. Asterisks (*) indicate statistically significant differences between leiomyoma and corresponding myometrium. For further details see legend of Fig. 1.

muscle tissues suggesting the possibility of functional link between these responses.

Estrogen action and PKB/Akt pathway shows intimate relation. Estrogen activates Akt by triggering the binding of ERα to the p85 regulatory subunit of PI3-kinase [18,19]. Physically and functionally coupling of ERα, but no ER beta, to PI3K is responsible in cardiovascular system for the rapid E2 induced vascular effects, eNOS phosphorylation [16]. Enzymatic activation of Akt mediates estrogenic function of EGF and IGF in breast cancer cells [20]. The activation of Akt by E2 results in expression of different genes in vascular endothelial cells [21]. The PI3K/Akt2 pathway regulates ERα transcriptional activity by phosphorylation of serine^{167} of ERα, and in turn, ERα activates PI3K/Akt2 kinase [22]. PI3K/Akt pathway is involved also in the action of progesterone. Progesterone induces Akt phosphorylation, and this phosphorylation is associated with the inhibition of apoptosis in MCF-7 cells. These data suggest that progesterone may protect breast cancer cells from apoptosis by altering PI3K/Akt activity [23]. In addition, activation of Akt/PKB appears to be involved in the effects of E2 or P induced growth factors (EGF, IGF) in steroid responsive cells [14,24,25].

Expression of Akt was higher in leiomyoma than in corresponding myometria and in addition, the Akt activation, recognized by monitoring of pAkt^{473} protein level, was transiently higher in leiomyoma during the growing period of tumor than in the adjacent myometrium, where it was only barely detected. At the regression period of the tumor, in the early stage of menopause, no difference was seen between the two types of studied tissues. Akt is activated by lipid sensitive PI3K and is translocated to cell membrane. Akt is then further activated by membrane associated kinase which phosphorilates Akt at Ser^{473} or at Thr^{308} [26]. Disruption of normal PKB/Akt signaling has now been documented as a frequent occurrence in several human tumors [27–29]. Amplification of genes encoding Akt protein has been found in several types of human tumors [27]. In addition, mutation in the phosphatase and tensin homolog deleted chromosome ten (PTEN) gene has emerged as a primary cause in many of gynaecological tumors, such as endometrial carcinoma, endometriosis [30–32]. PTEN protein, product of one tumor suppressor gene, down regulates the activators of Akt. Expression of PTEN protein is suppressed in leiomyoma relative to myometrium [33]. Taken together these findings with our results, similar changes might be supposed in the pathogenesis of leiomyoma. Downstream targets of Akt in human uterus at present are not known.

In agreement with findings by us [34] and by others [14,15] the data in this study demonstrate the expression of antiapoptotic Bcl-2 and proapoptotic Bax protein in leiomyoma and adjacent human myometrium. Bcl-2 protein abundantly expressed in pre-menopausal leiomyoma, similar to

that of changes in level pAkt^{473}. Expression of Bax protein was dominant in leiomyoma from post-menopausal uteri, when the Akt expression and activation was suppressed.

The Bcl-2 gene products have determining role in the regulation of apoptosis.

The expression of pro- and antiapoptotic members of Bcl-2 gene family is regulated by several ways. Several data suggest that sex steroids take part in the regulation of apoptotic markers. Estrogen has been shown to inhibit apoptosis in different tissues [35] by inducing Bcl-2 expression via two estrogen responsive elements located within the coding region [36]. Expression of Bcl-2 protein showed strong correlation to estrogen receptor in endometrium [37], in breast [38,39], in leiomyoma [15,34]. Contribution of progesterone in the regulation of Bcl-2 in leiomyoma was also reported [14].

PI3K/Akt pathway is also essential in cell survival as it is shown in many types of cells [17]. However, the cellular mechanism by which Akt inhibits cell death varies, Akt regulates apoptosis at a post- or premitochondrial level [17,40], up-regulates Bcl-2 expression through cAMP response element binding protein [6], or by inhibiting Bax conformational change [41]. Furthermore the activation of Akt/PKB phosphorilates BAD on Ser^{136} resulting in suppression of apoptosis [42]. No data are available concerning the cellular mechanism of apoptosis in uterine smooth muscle tissues.

Endometrial carcinoma, endometriosis and leiomyoma are common tumors of the female genital tract. All three diseases develop in estrogen dependent fashion and in the lesions increased intratumoral estrogen synthesis has also been demonstrated [43]. All three tumors contain ER and PR receptors, changes in their expression show tissue specificity and depend on the stages of diseases. In these disorders the control of cell cycle and cell death varies but the mechanism of this variation is not fully understood [44,45]. On the basis of our present data obtained on leiomyoma the implication of PI3K/Akt signaling may play some role in the pathomechanism of endometrial carcinoma and endometriosis. Expression and activation of Akt in leiomyoma (present data) and in endometrial carcinoma [46] were detected. No data are available about it in endometriosis, however, changes in PTEN protein expression were published [32]. This protein inhibits downstream functions mediated by the PI3K pathway, such as activation Akt/protein kinase B, cell survival and cell proliferation [47]. Negative correlation between loss of PTEN expression and consequent Akt phosphorylation found in endometriosis [32] and endometrial carcinoma [46] can be detected. Not too much informations are available about the hormonal control of PTEN protein. Mutter [30] found that PTEN expression increases in endometrium in estrogenic follicular phase assuming a direct upstream link between estrogen response pathway and PTEN gene. It seems to be that the PTEN/pAkt ratio one of the most important regulator in cell cycle and cell death. As it has been assumed [27] simultaneous regulation by other signaling pathways may act also on the different cellular processes thought to be modulated by Akt and, the response of cells to Akt activation may be dependent upon the levels of activity of these other signals.

In conclusion, the results of our present data suggest that Akt/PI3K signaling seems to be involved in the tightly regulated hormonal hierarchy in uterine smooth muscles. When the level of circulatory sex steroids and their receptors in the uterus elevate, the expression and activation of Akt are dominant, the apoptosis is prevented, the cyclin D1 expression is increased [9] and leiomyomas grow. In menopause, when the circulatory level of sex steroids is low, the expression of anti-proliferative ERbeta is dominant in the uterus [9], the Akt signaling is declined, the Bax protein is activated, the apoptosis is enhanced, and the tumor regresses.

At present stage of investigation the causative links between the mentioned events are not known. The are many questions: whether changes of E2- ERα signaling or the changes of PI3K/Akt signaling are initiative events in the leiomyoma pathogenesis; how are influenced the responses of tissue by different growth factors initiated signaling mechanisms; how does the PTEN expression link to the observed molecular changes; does the in situ estrogen production due to alteration in different enzyme activity contribute in the activation of mentioned signaling pathways? Answers for these questions and to elucidate the exact molecular events and the links between them further investigations are required.

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