Mechanism of collagen biosynthesis up-regulation in cultured leiomyoma cells

Monika Zbucka[1], Wojciech Miltyk[2], Tomasz Bielawski[1], Arkadiusz Surazynski[2], Jerzy Palka[2], Sławomir Wolczynski[1]

Departments of: [1]Reproduction and Gynecological Endocrinology, [2]Medicinal Chemistry,
Medical University in Białystok, Białystok, Poland

Abstract: Uterine leiomyoma is the most common tumour in women with a reported incidence of 25-30%. The tumors are benign, composed of smooth muscle cells with variable amount of collagen - rich fibrous tissue. It is well established that accumulation of extracellular matrix in leiomyoma is key feature of tissue fibrosis. However, the pathogenesis of leiomyoma is still unclear. The aim of this study was to evaluate the metabolism of collagen in cultured leiomyoma cells and in control myometrium cells. The effect of estradiol, selective modulators of estrogen receptors (raloxifene, tamoxifen) and estrogen receptor down regulator (ICI 182.780) on collagen biosynthesis (measured by 5-[^{3}H]-proline incorporation assay and measurement of prolidase activity) and collagen degradation (measured by metalloproteinase activity assay) was studied. It was found that collagen biosynthesis is strongly stimulated by low doses of estradiol (5 nM) in leiomyoma cells while it is not changed in control myometrium cells. An increase in estradiol concentration to 10 nM results in drastic decrease of this process both in leiomyoma as well as control cells. Although raloxifene and tamoxifen only slightly affected collagen biosynthesis in control myometrium cells, they significantly inhibited the process in leiomyoma cells. There was no coordinate correlation between collagen biosynthesis and prolidase activity suggesting that regulation of this process may take place at transcriptional level. Both estrogen and SERMs were found to inhibit MMP-2 in leiomyoma as well as in control myometrium cells. The data suggest that stimulatory action of estrogen on collagen biosynthesis and inhibitory effect on MMP-2 activity in uterine leiomyoma may contribute to accumulation of this protein in ECM of this tissue.

Key words: Leiomyoma - Myometrium - Collagen - Prolidase - MMP - β_1-integrin receptor

Introduction

Uterine leiomyoma is the most common benign tumor in women of reproductive age. It is characterized by excessive accumulation of extracellular matrix (ECM) in the tissue. Although it is not malignant disease, for one-third of these women, the tissue fibrosis may cause several reproductive and gynecological problems, such as menorrhagia, dysmenorrhoea, chronic pelvic pain, infertility, recurrent miscarriage, preterm delivery and postpartum haemorrhage [4]. Since there is no effective pharmacological treatment of the leiomyomas usually they are subject for hysterectomy. Growth of these tumors is dependent on ovarian hormones, estrogens and progesterone as evidenced by their remarkable shrinkage both in menopause and in the pseudomenopause state created by the use of GnRH analogue (GnRH-a) treatment. Leiomyomata rarely develop before puberty, increase in size during pregnancy, and markedly regress after menopause. Thus far, it has not been possible to demonstrate in vitro evidence of direct growth stimulatory action of steroid hormones on isolated myometrium and leiomyoma cells. This finding indicates that intermediary factors, such as growth factors or cytokines, most likely play pivotal roles in this process. Most of the available information on leiomyoma growth suggest a vital role of estrogen and progesterone in this process [7]. Actually, Brandon *et al.* [3] demonstrated increased expression of estrogen receptor and progesteron receptor in uterine leiomyomas compared to those in the adjacent normal myometrium.

During the past two decades, some progress has been made towards the understanding of the pathogenesis of the disease from the clinical, genetic, epidemi-

Correspondence: M. Zbucka, Dept. of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Marii Sklodowskiej-Curie Str. 24, 15-249 Białystok, Poland; tel.: (+4885) 7468818, e-mail: monikazbucka@wp.pl

ological and molecular perspectives [7]. However, the cause of the disease remain largely unknown.

Since ECM constitutes a significant proportion of leiomyoma mass the tissue is called fibroid. It has been reported [8] that leiomyomas contains 50% more ECM than the corresponding myometrium. The ECM of leiomyomas consists primarily of collagen, fibronectin, and proteoglycans. Several other studies reported relative overexpression of collagen by leiomyomas as compared with corresponding myometrium [17,19,20].

The present study was undertaken to evaluate the collagen biosynthesis, prolidase activity, expressions of integrin receptor and MMP in cultured *in vitro* human myometrium and leiomyoma cells treated with estradiol, selective estrogen receptors modulators and estrogen receptor down regulator, ICI 182.780.

Materials and methods

Cell culture. Human myometrium and leiomyoma tissue were obtained from six women (mean age 47 years, range 45-53) undergoing hysterectomy either for fibroids or for mennorhagia or benign ovarian tumors. Leiomyoma and myometrium cells were obtained from the patients operated in the Department of Gynecology and Department of Reproduction and Gynecological Endocrinology Medical University of Bialystok Poland. Tissues were etching in colagenase, after 5 hours rubbed by cell scraper. Cells were maintained in 75 cm^2 tissue culture flask (Sarsted, USA), under standard culture conditions; at 37°C, 5% CO_2 humidified incubator in Dulbeco's modified Eagle's medium (Sigma-Aldrich), were renewal every 2 days. Culture media contained 10% of adult bovine serum, and enriched with 50 U/ml antibiotic, antimycotic solution (penicillin, streptomycin, amphotericin B (Sigma-Aldrich Poland).

When cells reached 80% confluence, they were collected and seeded in 6 well culture plates (Sarsted, USA) at a density of 0.25 × 10^6 cells per well. The cells were incubated for 24 h prior to treatment and allowed to proliferate to 70% - 80% confluence before commencement of each experiment, without serum in D-MEM/F12 medium without phenol red.

The control was media with PBS only, which was the solvent for E2, ICI, R, T.

Determination of prolidase activity. The activity of prolidase was determined according to the method of Myara [14] which is based on measurement of proline by Chinard`s reagent [5]. Briefly, the monolayer was washed three times with 0.15 M of NaCl. Cells were harvested by scraping and suspended in 0.15 M NaCl, centrifuged at low speed (200 × g) and the supernatant was discarded. The cell pellet was suspended in 0.3 ml of 0.05 M Tris-HCl, pH 7.8, and sonicated 3 times for 10 seconds at 0°C. Samples were then centrifuged (16.000 × g, 30 min.) at 4°C. Supernatant was used for protein determination and then prolidase activity assay. Activation of prolidase required incubation with manganese; 100 μl of supernatant incubated with 100 μl of 0.05 M Tris-HCl, pH=7.8 containing 2 mM MnCl (II) for 2 hours at 37°C. The prolidase reaction was initiated by adding 100 μl of the incubated mixture to 100 μl of 94 mM glycyl-proline (Gly-Pro) for a final concentration of 47 mM Gly-Pro. After additional incubation for 1 hour at 37°C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes reaction was terminated at time "zero" (without incubation). The released proline was determined by adding of 0.5 ml of the trichloroacetic acid supernatant
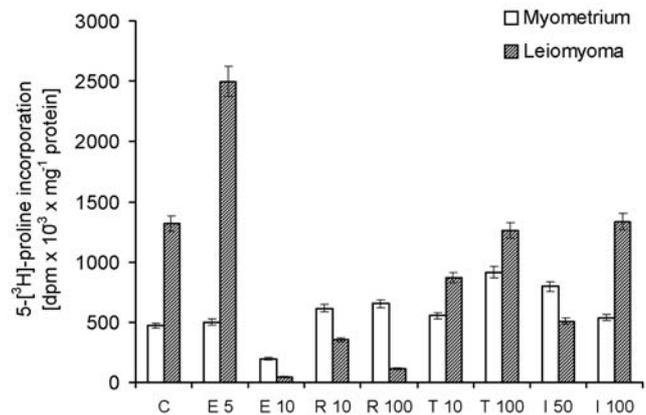
Fig. 1. Collagen biosynthesis in myometrium and leiomyoma cells treated for 24 h with various concentrations of estradiol (E), raloxifene (R), tamoxifen (T) and ICI 182.780.
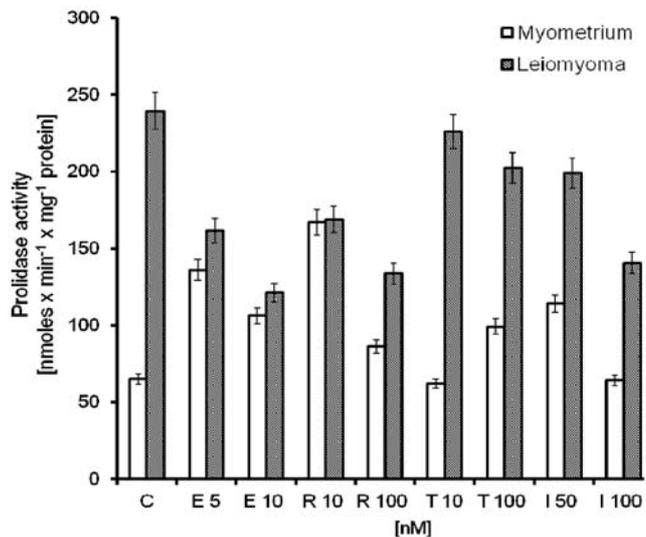
Fig. 2. Prolidase activity in myometrium and leiomyoma cells treated for 24 h with various concentrations of estradiol (E), raloxifene (R) and tamoxifen (T).

to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard`s reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min. at 90°C. The amount of proline released was determined colorimetrically by absorbance at 515 nm and calculated by using proline standards. Protein concentration measured by the method of Lowry. Enzyme activity was reported as nanomoles per minute per milligram of supernatant protein.

Collagen production. Incorporation of radioactive precursor into extracellular matrix components was measured after labeling confluent cells in serum-free medium for 24 h with the 5-[^{3}H] proline (5 μCi/ml, 28 Ci/mmol). Incorporation of label into collagen was determined by digesting proteins with purified C. histolyticum collagenase according to the method of Peterkofsky *et al.* (16).

SDS-PAGE. Slab SDS/PAGE was used, according to the method of Laemmli [11]. Samples of cell supernatants (25-50 μg of protein) were incubated for 10 min. at 100°C in 62.5 mmol/l Tris-HCl,
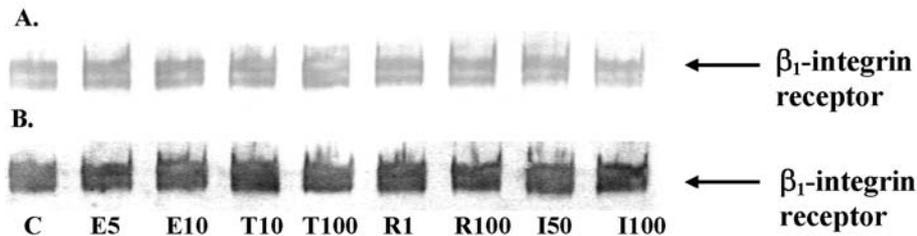
Fig. 3. Western immunoblot analysys of β_1-integrin receptor in myometrium (A) and leiomyoma (B) cells treated for 24 h with various concentrations estradiol (E), raloxifene (R), tamoxifen (T) and ICI 182.780.
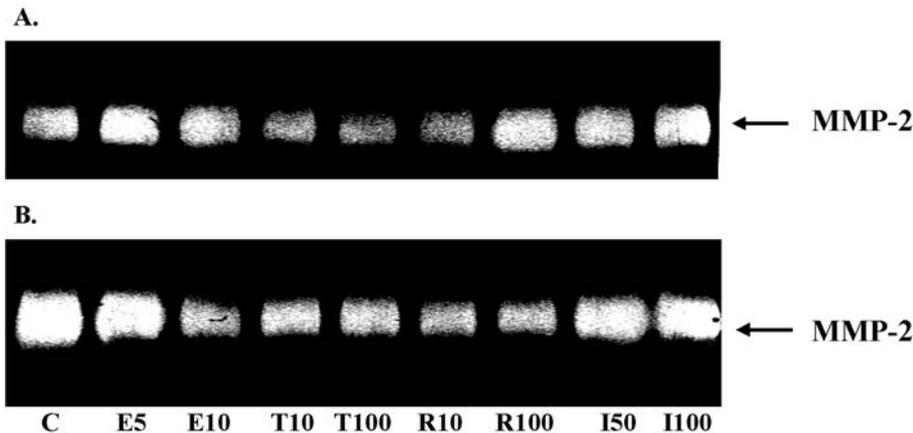
Fig. 4. MMP-2 activity in cell culture medium of myometrium (A) and leiomyoma (B) treated for 24 h with various concentrations estradiol (E), raloxifene (R), tamoxifen (T) and ICI 182.780.

pH=6.8, containing 2.0% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples were electrophoresed on a Tris-Glycine gels at 100V per gel for 30 minutes at room temperature.

Western immunoblot analysis. After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2 μm pore-sized nitrocellulose at 100 mA for 90 minutes using a BioRad electrophoresis unit according to the method described in the manual accompanying the unit. The membrane was blocked with 5% dried milk in TBS-T for 1 hour in room temperature, slowly shaking. Then the nitrocellulose was incubated with monoclonal antibody against β_1-integrin at concentration 1:5,000 or polyclonal antibodies against prolidase or β-actin at concentration 1:1,000 overnight. After the incubation, nitrocellulose was washed with TBS-T (1 x 15 min and 2 × 10 min) intensively shaking. In order to analyze the proteins second alkaline phosphatase labeled antibodies against mouse`s Fc IgG or against rabbit's IgG were added at concentration 1:2,500 in TBS-T and incubated for 1 hour slowly shaking. Then the membrane was extensively washed with TBS-T (5 × 10 min) and submitted to BCIP/NBT western blotting detection system.

Zymography. Gelatinolytic activity was determined according to the method of Unemori and Werb (23). Cell culture medium was concentrated 10 times using 3 kDa cut off centricons, mixed with Laemmli sample buffer [11], containing 2,5% SDS (without reducing agent). An equal amounts (about 20 μg) of protein was electrophoresed under non-reducing conditions on 10% polyacrylamide gels impregnated with 1 mg/ml gelatin. After electrophoresis, the gels were incubated in 2% Triton X-100 for 30 min at 37°C to remove SDS and incubated for 18-24 h at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH=8.0, containing 5 mM CaCl$_2$). After staining with Coomassie briliant blue R250, gelatin-degrading enzymes present in tissue extract were identified as clear zones in a blue background.

Statistical analysis. In all experiments, the mean values for six independent experiments ± standard deviation were calculated. The results were submitted to statistical analysis using Student's t-test, accepting $p<0.05$ as significant.

Results

It is well known that estrogens participate in collagen metabolism [1,18]. We used estradiol, SERMs (tamoxifen and raloxifene) and ICI 182.780 to test their effect on collagen biosynthesis in cultured *in vitro* human myometrium and leiomyoma cells. Collagen biosynthesis in the cells was measured by 5[^{3}H] - proline incorporation into proteins susceptible to the action of bacterial collagenase. As can be seen from Fig. 1, leiomyoma cells produce higher amount of collagen compared to myometrium cells. Estradiol at 5 nM concentration is a potent stimulator of collagen biosynthesis in leiomyoma cells while it has no effect on protein synthesis in myometrium cells. Increase in estradiol concentration to 10 nM contributed to decrease in collagen biosynthesis to 40% of control value in myometrium cells and almost completely inhibited the process in leiomyoma cells. The addition of raloxifene into the culture medium contributed to decrease in collagen biosynthesis in leiomyoma cells while it had no significant effect in myometrium cells. On the other hand tamoxifen slightly induced collagen biosynthesis in myometrium cells and slightly inhibited in leiomyoma cells.

We considered whether increase in collagen biosynthesis is a result of increase of prolidase activi-

ty. Intracellular prolidase activity was measured in the cells incubated for 24 hours with estradiol, raloksifen or tamoxifen as well as with estrogen receptor down regulator, ICI 182.780. The results are shown in Fig. 2. Leiomyoma cells showed about four fold increase in prolidase activity in comparison to normal myometrium cells. Estradiol and raloxifen however inhibited collagen biosynthesis in leiomyoma in dose dependent manner, but stimulated collagen biosynthesis in normal myometrium cells. In respect to tamoxifen it was found to slightly decrease of collagen biosynthesis in leiomyoma cells and slightly induce this process in myometrium cells. As described previously, prolidase activity and collagen biosynthesis are stimulated through the β_1-integrin subunit, presumably a component of the $\alpha_2 \beta_1$ integrin receptor (15). Our results show that β_1-integrin receptor expression is increased in leiomyoma cells comparing to normal cells (Fig. 3). We observed no effect of estradiol, tamoxifen, raloxifene and ICI on the receptor expression in normal and leiomyoma cells. Since disturbances in collagen metabolism are usually accompanied by deregulation of collagen degradation we determined the gelatinolytic activity in the studied cells. As can be seen from Figure 4, normal myometrium and leiomyoma cells contain gelatinase of 72 kDa (MMP2) however, its activity is much higher in leiomyoma than in control cells. It is interesting that estradiol slightly increased the activity of the collagenase in myometrium cells while in leiomyoma cells it inhibited the enzyme activity. Both, tamoxifen and raloxifen inhibited the activity of MMP2, compared to control.

Discussion

Since the tumors of uterus do not exist in animals model, cultures of human myometrium and leiomyoma cells are the only model for studying the regulation of cell proliferation and biosynthesis of extracellular matrix in the tissue. We examined the influence of the estrogen receptor activation on the metabolism of collagen in leiomyoma and myometrium cultured cells. It is known that collagen interaction with cell surface integrin receptor induces signaling that it is involved in regulation of cell proliferation and survival and also may play a pivotal role in the development and tumor growth.

The biosynthesis of extracellular matrix is dependent on estrogen receptor activation [13,21]. The basic massage of our study is that estradiol much stronger inducer of collagen biosynthesis in leiomyoma cells than in myometrium cells. In opposite raloxifene was found to be much stronger inhibitor of the protein synthesis in tumor cells than in control cells. It suggests that estrogen receptor activation may represent mechanism for up-regulation of collagen synthesis in

leiomyoma cells. It is supported by studies of Leppert *et al.* [12] showing that leiomyomas contain an abnormal collagen fibril structure and orientation, which suggests that the well-regulated fibril formation in myometrium is altered in leiomyomas. Alterations in collagen genes expression and collagenolytic activity may play a role in the pathogenesis of leiomyomas as well as some post-transcriptional regulations. One such a regulatory mechanism was found at the level of prolidase.

Prolidase plays an important role in collagen biosynthesis at post-transcriptional level, because provides proline for collagen biosynthesis and may be a rate-limiting factor in the regulation of the protein production. The leiomyoma cells evokes higher prolidase activity in comparison to myometrium cells. The mechanism of the up-regulation of prolidase activity is not known. It can not be due to estradiol since it induces inhibition of prolidase activity in leiomyoma cells while it stimulates in myometrium cells. Additionally, the effect of raloxifene or tamoxifen on prolidase activity has no or little correlation with collagen biosynthesis.

The activity of prolidase is known to be regulated by interaction of collagen with β_1-integrin receptor. In our study we observed increased β_1-integrin receptor expression in leiomyoma cells in comparison to normal cells.

Activation of signaling through β_1-integrin cascade represents important mechanism for stimulation of collagen production in collagen synthesizing cells [22]. Upregulation of the signaling cascade may contribute to stimulation of collagen synthesis which is the dominant process during the development of leiomyoma [9,10].

In our study we found increased MMP2 activity in leiomyoma cells comparing to myometrium cells. It is interesting that estradiol increased the activity of the gelatinase in myometrium cells while it inhibited the enzyme activity in leiomyoma cells. Both, tamoxifen and raloxifene were found to decrease the activity of MMP2, compared to control. Ubiquitination and degradation of estrogen receptor by ICI 182.780 does not lead to any changes in MMP activity.

MMP plays an important role in the balance between collagen biosynthesis and degradation and its activity reflects the rate of collagen turn-over [2]. Tumor remodelling plays a major role in both growth and regression of uterine tumors and it is regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) [6]. We observed up-regulation of MMP-2 in leiomyoma cells in comparison to myometrium cells.

Wolanska *et al.* [24] examined the activities of matrix metalloproteinases (MMPs) in human myometrium and uterine leiomyomas in various stages

of growth. It was found that both myometrium and the investigated tumors contain collagenolytic enzymes: MMP-1, MMP-2, MMP-3 and MMP-9. It seems that during leiomyoma remodeling the most important is MMP-2. In control myometrium only 10% of MMP-2 exists in an active form, whereas in tumors, especially in large ones, the values reach 30%. It is suggested that the high activity of MMP-2 is responsible for remodeling of extracellular matrix in the growing tumors.

Since raloxifene strongly inhibited the collagen biosynthesis, our results confirm the clinical observations of shrinkage of leiomyomata in women treated with raloxifene.

In conclusion, the data are consistent with a mechanism for up-regulation of extracellular matrix collagen, which is characterized by increase in collagen synthesis and decrease in collagen breakdown. The results suggest that altered regulation of extracellular matrix in leiomyoma may be due to integrin - dependent signaling and estrogen activity. We postulate that the both factors are likely candidates to promote collagen biosynthesis and enlarge the tumor volume of leiomyoma.

References

[1] Baldekas JC, Smith B, Gerstenfeld L, Sonenshein GE, Franzblau K. Effects of 17 estradiol on the biosynthesis of collagen in cultured bovine aortic smooth muscle cells. *Biochemistry.* 1981;20:2162-2163.

[2] Bodner-Adler B, Bodner K, Kimberger O, Czerwenka K, Leodolter S, Mayerhofer K. Expression of matrix metalloproteinases in patients with uterine smooth muscle tumors: an immunohistochemical analysis of MMP-1 and MMP-2 protein expression in leiomyoma, uterine smooth muscle tumor of uncertain malignant potential, and leiomyosarcoma. *J Soc Gynecol Investig.* 2004;11(3):182-6.

[3] Brandon DD, Erickson TE, Keenan EJ, Strawn EY, Novy MJ, Burry KA, Warner C, Clinton GM. Estrogen receptor gene expression in human uterine leiomyomata. *J Clin Endocrinol Metab.* 1995;80:1876-81.

[4] Buttram VC Jr, Reiter RC. Uterine leiomyomata: etiology, symptomatology, and management. *Fertil Steril.* 1981;36: 433-45.

[5] Chinard FP. Photometric estimation of proline and ornithine. *J Biol Chem.* 1952;199:91-95.

[6] Dou Q, Tarnuzzer RW, Williams RS, Schultz GS, Chegini N.Differential expression of matrix metalloproteinases and their tissue inhibitors in leiomyomata: a mechanism for gonadotrophin releasing hormone agonist-induced tumour regression. *Mol Hum Reprod.* 1997;3(11):1005-14.

[7] Flake GP, Andersen J, Dixon D.Etiology and pathogenesis of uterine leiomyomas: a review. *Environ Health Perspect.* 2003;111(8):1037-54.

[8] Fujita M. Histological and biochemical studies on collagen in human uterine leiomyomas. *Hokkaido Igaku Zasshi.* 1985;60: 602-15.

[9] Hynes RO. Integrins: versitility, modulation and signaling in cell adhesion. *Cell.* 1992;69:11-25.

[10] Juliano RL. The role of B1 integrins in tumor. *Cancer Biology.* 1993;4:277-283.

[11] Laemmli UK. Cleavage of structural proteins during the assembly of the head of baceriophage T4. *Nature (London).* 1970;227:680-685.

[12] 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(supp l):1182-7.

[13] Miltyk W, Anchim T, Wolczynski S, Palka J. Estrogen-dependent regulation of prolidase activity in breast cancer MCF-7 cells. *Gynecol Endocrinol.* 1999;13(3):166-74.

[14] Myara I, Charpentier C, Lemonnier A. Optimal conditions for prolidase assay by proline colorimetric determination: application to imidodipeptiduria. *Clin Chim Acta.* 1982;125:193-205.

[15] Pałka JA, Phang J. Prolidase activity in fibroblasts is regulated by interaction of extracellular matrix with cell surface integrin receptors. *Journal of Cellular Biochemistry.* 1997;67: 166-175.

[16] Peterkofsky B, Chojkier M & Bateman J. Determination of collagen synthesis in tissue and cell culture system. In: Eds M Furthmay. CRC Press, Boca Raton, Fl. *Immunochemistry of the Extracellular Matrix.* 1982;19-47.

[17] Puistola U, Ristili L, Ristili J, Kauppila A. Collagen metabolism in gynecologic patients: changes in the concentration of the aminoterminal propeptide of type 3 procollagen in serum. *Am J Obstet Gynecol.* 1990;163:1276-81.

[18] Schmid MECh, Froesch ER. Enhanced osteoblast proliferation and collagen gene expression by estradiol. *Proc Nat Acadf Sci USA.* 1998;85:2307-2310.

[19] 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):1-12.

[20] Stewart EA, Friedman AJ, Peck K, Nowak RA. Relative over-expression of collagen type 1 and collagen type 3 messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J Clin Endocrinol Metab.* 1994;79:900-6.

[21] Surazynski A, Jarzabek K, Haczynski J, Laudanski P, Palka J, Wolczynski S. Differential effects of estradiol and raloxifene on collagen biosynthesis in cultured human skin fibroblasts. *Int J Mol Med.* 2003;12(5):803-9.

[22] Taylor CV, Letarte M, Lye SJ. The expression of integrins and cadherins in normal human uterus and uterine leiomyomas. *Am J Obstet Gynecol.* 1996;175(2):411-9.

[23] Unemori EN, Werb Z. Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. *J Cell Biol.* 1986;103:1021-1031.

[24] Wolańska M, Sobolewski K, Bańkowski E, Jaworski S. Matrix metalloproteinases of human leiomyoma in various stages of tumor growth. *Gynecol Obstet Invest.* 2004;58(1): 14-8.