Differential regulatory effect of progesterone on the proliferation and apoptosis of uterine leiomyoma tissue explants and primary leiomyoma cell cultures

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ABSTRACT

Objective: The growth of uterine leiomyomas is regulated by progesterone, although the underlying molecular mechanisms are not fully understood.

Methods: Primary leiomyoma cells were isolated by standard method from 16 samples of uterine leiomyoma tissue. Uterine leiomyoma explants and primary leiomyoma cell cultures were exposed to progesterone in concentrations of 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml for 24 h. Cell apoptosis was assessed with Annexin V assays performed in cell cultures by flow cytometry. The expression of PR-A, PR-B, Ki67, Akt, ERK, PTEN and PPARγ mRNAs was estimated in cultured leiomyoma cells and tissue explants by real time RT-PCR.

Results: Treatment with progesterone promoted viability and proliferation of cultured leiomyoma cells in a dose-dependent manner. Low and high doses of progesterone decreased early apoptosis of leiomyoma cells. High concentrations of progesterone increased the number of living cells in Annexin V assays. High doses of progesterone increased the expression of Ki67 mRNA, while low doses increased the expression of PR-A mRNA in cultured leiomyoma cells and tissue explants. In cell cultures, low doses of progesterone increased the expression of PR-B mRNA and the expression of PTEN and PPARγ mRNAs in a dose-dependent manner. Exposure of leiomyoma tissue explants to progesterone led to increased expression of PR-B and ERK mRNAs in a dose-dependent manner.

Conclusions: The effects of progesterone on the apoptosis and proliferation of leiomyoma cells was dose-dependent and different in cell cultures and leiomyoma explants, possibly as a result of impacts derived from the tumor microenvironment.

Keywords: leiomyoma, progesterone, cell cultures, proliferation, apoptosis

INTRODUCTION

Uterine leiomyoma (or fibroid) is a benign myometrial smooth muscle cell tumor, which consists of transformed smooth muscle cells, connective tissue fibroblasts, and a large amount of extracellular matrix (Chegini, 2010). Leiomyoma is the most common tumor of the female genital tract with an incidence of 40-70% in women of reproductive age (Commandeur *et al.*, 2015). This tumor has been associated with severe clinical symptoms such as excessive uterine bleeding, pelvic pain and pressure, dysmenorrhea, sexual dysfunction, infertility, recurrent pregnancy loss, and compression of adjacent pelvic organs by large uterine fibroids (Commandeur *et al.*, 2015). There is no effective medical treatment for uterine leiomyomas, with surgery standing as the treatment of choice (Havryliuk *et al.*, 2017). However, hysterectomy carries an increased

risk of postoperative complications and leads to partial or complete loss of reproductive function, a most undesired outcome for young women in particular. Therefore, searching for drug candidates to regulate uterine leiomyoma growth is of great importance for the development of effective therapies for leiomyomas.

Estrogen and progesterone are believed to regulate leiomyoma growth (Reis *et al.*, 2016). The development of uterine leiomyomas during one's active reproductive age and the significant decrease in the number of cases following menopause support this proposition (Reis *et al.*, 2016). Estrogen had been considered as the main hormone involved in leiomyoma development (Kasai *et al.*, 2004). However, recent studies showed that progesterone also plays an important role in leiomyoma growth (Kim *et al.*, 2009). The expression of progesterone receptors (PR) is significantly increased in uterine leiomyoma tissue when compared to the adjacent normal myometrium (Kim *et al.*, 2009). Acting via PR, progesterone interacts with the Bcl-2 promoter to induce the expression of Bcl-2 expression in leiomyoma tissue, thus suppressing the apoptosis of tumor cells (Yin *et al.*, 2007). The addition of progesterone to uterine leiomyoma cell cultures resulted in increased proliferation and cell nuclear antigen (PCNA) expression (Maruo *et al.*, 2000). Using an experimental leiomyoma xenograft model, Ishikawa *et al.* (2010) demonstrated that progesterone and its receptors directly stimulated tumor growth, while estrogen and its receptor only sustained PR expression in leiomyoma tissue. The stimulatory effects of progesterone in leiomyoma cell proliferation and growth have been clearly demonstrated, but the molecular mechanisms underlying this phenomenon have not been fully investigated.

Progesterone was shown to activate several intracellular signaling pathways, which effects are mediated by progesterone receptors (Lange, 2008). Ligand-activated PRs can activate the MAPK/ERK signaling pathway (Hennessy *et al.*, 2005). Increased MAPK activity may strongly increase the survival and proliferation of tumor cells in general and breast cancer cells in particular (Lange, 2008). Progesterone may also act via the PI3K/Akt pathway, which controls cells proliferation, survival, differentiation and apoptosis (Kim *et al.*, 2009). Hyperactivation of the PI3K/Akt pathway is common in breast, endometrial, prostate, lung, and ovarian cancer (Hennessy *et al.*, 2005). Recently, it was shown that progesterone may rapidly phosphorylate Akt in leiomyoma cells (Hoekstra *et al.*, 2009), with higher levels of Akt and the phosphorylated form of Akt reportedly occurring in uterine leiomyoma cells than in the myometrium (Kim *et al.*, 2009). A known important negative regulator of Akt, tumor suppressor PTEN (phosphatase and tensin homolog), a non-redundant, evolutionarily conserved phosphatase, affects cell cycle progression, proliferation, chemotaxis and apoptosis (Keniry & Parsons, 2008). Expression of PTEN mRNA and protein levels was increased in uterine leiomyomas when com-

pared to normal myometrium (Makker *et al.*, 2016). Recent reports indicated that transcription factor PPAR γ (peroxisome proliferator-activated receptor γ) transcriptionally up-regulates PTEN expression in tumor cells, with higher levels of expression of PPAR γ mRNA seen in uterine leiomyomas when compared to adjacent normal myometrial tissue (Jeong *et al.*, 2010). The relationship between PTEN, PPAR γ expression and progesterone action in uterine leiomyomas is still unclear.

Several hormone therapies have been proposed to treat uterine leiomyomas, including gonadotropin-releasing hormone (GnRH) agonists, selective estrogen receptor modulators (SERMs), and selective progesterone receptor modulators (SPRMs). All such therapies were designed to abolish steroid action, suppress leiomyoma growth, and diminish symptom severity (Chegini, 2010). However, these agents often provide limited clinical effect on the size of uterine leiomyomas. Significant side effects such as hot flashes and bone loss (Commandeur *et al.*, 2015) have been described. The limitations associated with medical treatment have been driven mostly by lack of complete information on how hormone therapies work at a molecular level. Almost every effect of progesterone has been studied using leiomyoma cell cultures, although the data gathered on the cell- and tissue-specific effects of ovarian steroids is rather compelling (Chegini, 2010). Special interest resides in comparing the impact of progesterone on leiomyoma tissue and leiomyoma cell cultures, describing the impact the tumor microenvironment may have on how progesterone regulates leiomyoma growth, and clarifying the relevance of cell culture models in the elucidation of the mechanisms by which progesterone operates in *in vivo* conditions.

The following were investigated in this study: i) the dose-dependent action of progesterone on the apoptosis and growth of leiomyoma cells; ii) the dose-dependent and tissue-specific action of progesterone on leiomyoma cell proliferative activity and expression of progesterone receptors PR-A and PR-B; iii) the dose-dependent tissue-specific action of progesterone on the activation of MAPK/ERK and Akt signaling pathways and on the expression of PTEN and PPAR γ to compare the effects of the hormone in cell cultures and whole tissue.

MATERIALS AND METHODS

Tissue collection and sample preparation

Samples of uterine leiomyoma tissue were collected from 16 women undergoing myomectomy for symptomatic uterine leiomyoma at the Gynecological Clinic of the Ivanovo State Research Institute of Maternity and Childhood (Ivanovo, Russia). Patients had been off hormone treatment for at least three months prior to surgery. The women included in the study were of reproductive age (32-42 years). All samples were collected in the proliferative phase of the cycle from relatively large myomas (6.23 ± 0.63 cm in diameter) of intramural localization. The collected samples did not have signs of hemorrhage or necrosis. The local Ethics Committee of the State Research Institute of Maternity and Childhood (Ivanovo, Russia) approved the use of these tissue samples. The patients gave written consent before joining the study.

The samples were collected from the peripheral areas of the uterine leiomyomas, since these areas exhibited robust growth (Mas *et al.*, 2015). Fresh leiomyoma tissue samples were rinsed in RPMI 1640 culture medium with 1% antibiotic solution, containing penicillin 100 IU/ml and streptomycin 100 mg/ml; after washing, the samples were sliced manually into small pieces measuring approximately 2-3 mm³. Each sample was divided into two parts: one used as a tissue explant in the experiments with progesterone; and

the other was digested using an enzyme solution to receive the primary leiomyoma cell culture.

Primary cell isolation and cell culture

Primary leiomyoma cells were isolated as described in the literature (Koohestani *et al.*, 2013). In summary, minced leiomyoma tissue samples were digested by incubation in DMEM solution (Sigma Aldrich) containing Collagenase (Sigma Aldrich) 0.1 mg/ml and DNase IV 0.05 mg/ml (Sigma Aldrich) for 1-2 h at 37°C and 5% CO₂ with constant agitation. Ten milliliters of DMEM solution with enzymes was used to digest 1 g of leiomyoma tissue. Then the cells were incubated overnight in the same enzyme solution without agitation, and were subsequently incubated for 10 min under constant agitation after the addition of trypsin to a final concentration of 10 µg/ml. Cell suspensions were filtered to remove undigested tissues. The cells were resuspended in DMEM solution containing 1% antibiotic mixture and 10% heat-inactivated fetal calf serum; 1×10^6 cells were seeded in 24-well cultures plates and incubated overnight. Then, non-adherent cells were removed by washing with phosphate buffer solution (PBS) and the cell cultures were continued after the addition of fresh DMEM medium.

Progesterone treatment

To estimate the cell- and tissue-specific effects of progesterone on the proliferation and apoptosis of uterine leiomyomas, leiomyoma explants and primary leiomyoma cell cultures were exposed to progesterone (Sigma) at concentrations of 0.01 µg/ml, 0.1 µg/ml, and 1 µg/ml for 24 h. Control tissue explants and cell cultures were cultivated in DMEM solution alone. After incubation, tissue explants and culture cells were fixed for RNA isolation and RT-PCR testing. Additionally apoptosis of progesterone-stimulated primary culture cells was estimated by flow cytometry. To estimate the prolonged effect of progesterone on leiomyoma cells, primary cell cultures, after exposure to progesterone, were kept in fresh DMEM medium without progesterone for 72 h; the estimation of cell viability and morphology in the cultivation process was performed using a light microscope. Morphology analysis was performed in an Axiovert 200 inverted transmitted light microscope at 50x magnification (Carl Zeiss, Germany). Images were captured using an AxioCam digital camera from Zeiss.

Flow cytometry for apoptosis detection

The phenotype and apoptosis of leiomyoma culture cells were studied by flow cytometry. To this end, uterine leiomyoma primary culture cells exposed to progesterone for 24 hours were gently detached with a cell scraper, pipetted into single cells, and suspended at 1×10^6 /ml. The cells were washed twice with PBS, stained with desmin (a smooth muscle cell differentiation marker), and analyzed for apoptosis. Intracellular staining with anti-desmin monoclonal antibodies (eBioscience™, USA) was performed. Before antibody staining, a cell membrane fixation and permeabilization procedure was performed using Fixative Solution IOTest®3 10x Concentrate kit (Beckman Coulter, USA). Apoptosis of leiomyoma cells was measured using an Annexin V-FITC apoptosis detection kit (Invitrogen, USA) according to the manufacturer's protocol. Annexin V/Propidium iodide (PI) double staining made it possible to distinguish between viable (Annexin V-PI-), early apoptotic (Annexin V+PI-), late apoptotic (Annexin V+PI+), and dead cells (Annexin V-PI+). The analysis was performed in a BD FACSCanto II flow cytometer (Becton Dickinson, USA). At least 50,000 cells were counted in each analyzed sample. Mouse IgG (IgG1-FITC + IgG2a-PE) antibodies (Becton Dickinson, San Jose, CA, USA) were

used to estimate unspecific cell staining. The criteria for positive staining were set at a fluorescent intensity displayed by <0.5% of the cells stained with the appropriate fluorochrome-conjugated isotype control monoclonal antibodies (mAb). Data were analyzed using the Diva software (Becton Dickinson, USA). Gating strategies are shown in the figure legends.

Real-time PCR

Quantitative real-time PCR was performed to examine Ki67, PR-A and PR-B, ERK, Akt, PTEN, PPAR γ , mRNAs expression in leiomyoma tissue explants and primary culture cells after incubation with progesterone. Total RNA was extracted from samples using the standard acid guanidinium thiocyanate-phenol-chloroform method. The amount of RNA in each sample was estimated with a Nano Drop 2000C (Thermo Scientific, USA) spectrophotometer. Five micrograms of RNA was reverse-transcribed into cDNA using a commercial kit (Fractal Bio, St. Petersburg, Russia), according to the manufacturer's protocol. Real-time PCR was performed with iCycler IQ software (BIO-RAD, CA, USA). Commercial kits for quantitative estimation of specific gene mRNAs expression (Fractal Bio, St. Petersburg, Russia) were used. Quantification was performed for the expression of β -actin, a compound expressed consistently in uterine leiomyoma tissue and leiomyoma cells used as housekeeping gene. A series of control DNA dilutions were used to generate standard curves and the number of copies of cDNA of specific genes was defined and normalized to β -actin expression. Results for mRNAs expression of the studied genes are presented as a normalized number of copies $\times 10^3$ per μ l.

Statistical analysis

Data were treated using the STATISTICA 6.0 software program. The results were presented as mean $\pm$ standard error for variables with a normal distribution, and as median, first quartiles, third quartiles for variables with non-normal distributions. Initial statistical analysis was made using ANOVA. Statistical analysis was performed with the Bonferroni correction on two-tailed Student's t-test for variables with a normal distribution and the Mann-Whitney U-test for variables following a non-normal distribution. *P* values <0.05 were considered significant.

RESULTS

Morphology of the uterine leiomyoma cell cultures after treatment with different concentrations of progesterone

After 72 hours of incubation, control leiomyoma cells had comparatively small round shapes with oval nuclei (Fig. 1a). The cells treated with progesterone 0.01 μ g/ml and 0.1 μ g/ml were more elongated and had intact cigar shaped nuclei (Fig. 1b and 1c). The number of leiomyoma cells treated with progesterone 0.1 μ g/ml grew by 40% and some cellular crowding, suggestive of proliferation, was observed (Fig. 1c) (*p*=0.025). The cells treated with progesterone 1 μ g/ml increased 3-fold (*p*=0.011). These cells appeared to be robust, were spindle-shaped and had elongated nuclei; they showed maximal cellular crowding and proliferation (Fig. 1d).

Effect of different concentrations of progesterone on leiomyoma cell apoptosis

Annexin V-FITC/PI assays were performed to investigate whether the stimulatory effect of progesterone on the proliferation of leiomyoma cell cultures was attributed to the apoptotic mechanism. The analyzed leiomyoma cells had predominantly the phenotype of muscle cells, which was confirmed by staining with desmin (Fig. 2c).

Approximately 70-80% of the analyzed cells were positive for intracellular desmin expression. A statistically significant decrease was observed in the number of early apoptotic Annexin V+PI- cells in cultures after treatment with progesterone 0.01 μ g/ml and 1 μ g/ml (Fig. 2e). High doses of progesterone (1 μ g/ml) significantly increased the number of living Annexin V-PI- cells (Fig. 2e). The number of late apoptotic and dead cells remained unchanged after incubation with progesterone.

Effect of different concentrations of progesterone on Ki67, PR-A and PR-B mRNAs expression by uterine leiomyoma explants and primary leiomyoma cells

We studied proliferation marker Ki67 mRNA expression in leiomyoma explants and primary leiomyoma cells after treatment with different concentrations of progesterone for 24 h to confirm the stimulatory action of progesterone. We found that high doses of progesterone (1 μ g/ml) significantly increased Ki67 mRNA expression both in tissue explants and cell cultures (Fig. 3a). It is well known that progesterone action in the uterus is mediated through specific nuclear progesterone receptors – PR-A and PR-B isoforms (Kim *et al.*, 2009). We found that in leiomyoma tissue explants the expression of PR-A mRNA significantly increased after the explants were treated with progesterone at a low concentration (0.01 μ g/ml) (Fig. 3b). PR-B mRNA expression in tissue explants was elevated after tissue exposure to progesterone at concentrations of 0.1 μ g/ml and 1 μ g/ml (Fig. 3c). Leiomyoma cell cultures showed increased PR-A and PR-B mRNAs expression after treatment with progesterone 0.01 μ g/ml (Fig. 3b, 3c). PR-A mRNA expression was greater in myoma explants than cell cultures after treatment with progesterone 0.1 μ g/ml and 1 μ g/ml (Fig. 3b).

Effect of different concentrations of progesterone on ERK, AKT, PTEN and PPAR γ mRNAs expression in uterine leiomyoma explants and leiomyoma cells

To estimate the stimulatory action of progesterone on leiomyoma cells cultures and leiomyoma tissue, we looked into the effects of progesterone on the activation of two intracellular pathways associated with cell activation and proliferation, MAPK/ERK and Akt (Fig. 4). We found that progesterone 0.01 μ g/ml and 1 μ g/ml significantly increased ERK mRNA expression in uterine leiomyoma tissue explants (Fig. 4a). Progesterone did not affect ERK and Akt mRNAs expression in uterine leiomyoma cell cultures. It must be noted, however, that uterine leiomyoma cell cultures expressed Akt mRNA more intensively than tissue explants. Significantly higher expression of Akt mRNA was noted in cell cultures incubated with DMEM medium only (control) and with progesterone 1 μ g/ml (Fig. 4b). Differently from tissue explants, leiomyoma cell cultures showed decreases in PTEN and PPAR γ mRNAs expression after incubation with progesterone 0.1 μ g/ml and 1 μ g/ml (Fig. 4c, d). The level of PPAR γ mRNA expression by control leiomyoma cells was significantly higher than that of tissue explants (Fig. 4d).

DISCUSSION

Our results have demonstrated the stimulatory effect of progesterone on uterine leiomyoma cell growth. We found that progesterone acts on primary cultures of uterine leiomyoma cells in a dose-dependent manner, increasing the proliferation and inhibiting the apoptosis of leiomyoma cells. Increased Ki67 mRNA expression and greater number of living cells in Annexin V tests after exposure of leiomyoma cells to progesterone confirmed this statement. Our results presented good agreement with the literature, demonstrating the growth-promoting action of

Figure 1. Primary cultures of leiomyoma cells after incubation with different concentrations of progesterone. Photographs showing the effect of progesterone 0.01 µg/ml (b), 0.1 µg/ml (c) and 1 µg/ml (d) on leiomyoma cell cultures after 72 h of treatment. Cells incubated only in culture medium were used as controls (a). Photographs were taken with a phase-contrast microscope (50x magnification).

progesterone on leiomyoma cells (Reis *et al.*, 2016; Kim *et al.*, 2009). The stimulatory action of progesterone on leiomyoma cells in vitro is mediated via the elevation of activity of anti-apoptotic factor Bcl-2 (Yin *et al.*, 2007; Maruo *et al.*, 2000) and increased expression of proliferating cell nuclear antigen (PCNA) (Maruo *et al.*, 2000). Using an in vivo mouse model of leiomyoma, it was shown that progesterone stimulates tumor cell proliferation, accumulation of extracellular matrix, and cell hypertrophy (Ishikawa *et al.*, 2010). However, the precise molecular mechanisms tied to progesterone have not been elucidated in full. According to our data, progesterone significantly decreases PTEN and PPAR γ mRNAs expression in uterine leiomyoma cell cultures in a dose-dependent fashion. Additionally, we identified a stimulatory effect of progesterone in low concentrations on PR-A and PR-B mRNAs expression by primary cell cultures.

PPAR γ or peroxisome proliferator-activated receptor γ belongs to the nuclear hormone receptor super family and is involved in the regulation of lipid metabolism, cell proliferation, tumor growth and inflammation (Yun

et al., 2018). It was shown that PPAR γ is expressed in different malignant tissues such as bladder, colon, prostate and breast cancer (Yun *et al.*, 2018). Once activated by its natural (polyunsaturated fatty acids, oxidized low density lipoproteins, eicosanoids and prostaglandin metabolites) and synthetic ligands (members of antidiabetic thiazolidinedione class of drugs, including rosiglitazone, pioglitazone, ciglitazone and troglitazone), PPAR γ inhibits cell proliferation and induces apoptosis by up-regulating PTEN, Bax, inhibitors of cyclin-dependent kinase expression and down-regulating X-linked inhibitor of apoptosis, Bcl-2, COX-2 and NF- κ B (Yun *et al.*, 2018). The literature shows that different synthetic PPAR γ agonists act as tumor suppressors in many types of malignant tissues (Yun *et al.*, 2018). The role of PPAR γ in the pathogenesis of uterine leiomyomas is not fully understood. Jeong *et al.* (2010) reported higher PPAR γ expression in uterine leiomyoma tissue compared to the myometrium. The PPAR γ synthetic ligand ciglitazone reduced the survival of leiomyoma cells due to the activation of the Fas-mediated apoptosis signaling cascade (Nam *et al.*, 2007). The molecular mecha-

Figure 2. Effect of progesterone treatment on the apoptosis of primary leiomyoma cells. Dot plot pictures of flow cytometry assessment of leiomyoma cell apoptosis (a-d). Gate was built on the FSC (forward light scatter) vs. SSC (side light scatter) dot plot (a) to analyze cells with predominantly smooth muscle features, confirmed with desmin staining (c). The cells were stained with anti-mouse IgG1-FITC and IgG2a-PE antibodies (negative control) to discern positive from negative cells (b). Representative dot plot picture of flow cytometry data from Annexin test is shown at various progesterone (P4) concentrations (0.01 µg/ml, 0.1 µg/ml and 1 µg/ml) (d). Effect of different concentrations of progesterone (0.01 µg/ml, 0.1 µg/ml and 1 µg/ml) on the relative content of early apoptotic (Annexin+PI-), late apoptotic (AnnexinV+PI+) and viable (Annexin V-PI-) leiomyoma cells (e). Error bars represent means $\pm$ SEM. * $p < 0.05$, two-tailed Student's t test with controls.

nisms of PPAR γ action in uterine leiomyoma tissue remains elusive, but some authors suggested that the inhibitory effects of PPAR γ were mediated by the interaction between estrogen receptors and PPAR γ signaling pathways (Houston *et al.*, 2003). This study demonstrated that uterine leiomyoma cells have decreased PPAR γ mRNA expression, thus revealing a potential inhibitory effect tied to progesterone. The dose-dependent diminishment of PPAR γ mRNA expression after exposure of uterine leiomyoma cells to progesterone occurred concomitantly with increased cell proliferation and growth. The initially very high level of PPAR γ synthesis in uterine leiomyoma cells incubated only in culture medium confirms the role of this factor in the pathogenesis of leiomyoma.

PPAR γ up-regulates the expression of PTEN (Jeong *et al.*, 2010). PTEN is a dual-specificity phosphatase,

which is capable of removing phosphates from PI3K (phosphatidylinositol 3-kinase), thereby inhibiting the activation of the PI3K/Akt (protein kinase B) signaling pathway. This pathway plays an important role in the regulation of cell metabolism, survival, proliferation, apoptosis, growth and migration. Thus, PTEN exerts tumor suppressive functions (Keniry & Parsons, 2008). It has been reported that PTEN mRNA expression was significantly increased in uterine leiomyoma tissue comparing to adjacent normal myometrium (Makker *et al.*, 2016). Increased PTEN activity in uterine leiomyomas might prevent the hyperactivation of the Akt pathway and produce benign tumor growth. We found a significant dose-dependent inhibition of PTEN mRNA expression in uterine leiomyoma culture cells after exposure to progesterone. Apparently, progesterone-induced decreases in PTEN activity may be

Figure 3. Comparative characteristic of PR-A, PR-B and Ki67 mRNAs expression in leiomyoma tissue explants and leiomyoma primary cultures cells, incubated at different progesterone concentrations (P4). Real-time PCR shows the effect of progesterone on Ki67 (a), PR-A (b) PR-B (c) mRNAs expression in leiomyoma tissue explants and leiomyoma primary cultures cells. Results are presented as median, first quartiles, third quartiles. * - $p < 0.05$, Mann-Whitney U-test with controls, x - $p < 0.05$, Mann-Whitney U-test with explants.

associated with uterine leiomyoma cells proliferation and growth. However, we did not observe significant changes in Akt mRNA expression by leiomyoma cells treated with progesterone. It is well known that PTEN activity is mediated through interactions with components of the Akt-signaling pathway; one might suppose, therefore, that decreased PTEN synthesis might lead to Akt up-regulation. On the one hand, the level of Akt mRNA expression in leiomyoma cells in our experiments was consistently high and significantly exceeded that of tissue explants. Thus, decreased PTEN activity in uterine leiomyoma cultures after treatment with progesterone and stable levels of Akt activation may shift the balance of cell apoptosis and proliferation towards increased cell survival and growth. On the other hand, the absence of changes in Akt mRNA expression in

leiomyoma culture cells after incubation with progesterone may be explained by specific cell culture conditions.

Primary cultures of uterine leiomyoma cells have been widely used in the investigation of fundamental biological mechanisms tied to the mediation of leiomyoma development and growth, and many authors have described the differences between cell cultures and leiomyoma tissue (Serna *et al.*, 2018). The relevance of the in vitro leiomyoma model to native tumor tissue was studied by Bonazza *et al.* (2016). The authors described a classical, well-established procedure for the isolation and cultivation of uterine leiomyoma cells and reported on the expression of smooth muscle and myoblast markers and response to steroid hormones. But this in vitro leiomyoma model had several limitations such as having a limited life span, slow proliferation, and genetic diversities (Bonazza *et al.*,

Figure 4. Comparative characteristic of ERK, AKT, PTEN and PPAR γ mRNAs expression in leiomyoma tissue explants and leiomyoma primary cultures cells, incubated at different progesterone concentrations (P4). Real-time PCR shows the effect of progesterone on ERK (a), AKT (b) PTEN (c) and PPAR γ (d) mRNAs expression in leiomyoma tissue explants and leiomyoma primary cultures cells. Results are presented as median, first quartiles, third quartiles. *- $p < 0.05$, ** - $p < 0.01$, Mann-Whitney U-test with controls, x- $p < 0.05$, xx- $p < 0.01$, Mann-Whitney U-test with explants.

2016). In another study, significant alterations of gene expression profiles between native tumor tissue and isolated fibroid cells were also described (Zaitseva *et al.*, 2006). It was demonstrated that cultivation of uterine leiomyoma cells reduced the difference between model and normal myometrial cells and decreased the expression of specific estrogen and progesterone receptors mRNAs (Zaitseva *et al.*, 2006). Our results stressed the similarities and differences in the response of leiomyoma culture cells and tissue explants to progesterone stimulation. Similar to cell cultures, high doses of progesterone induced increased expression of Ki67 mRNA, while low doses of progesterone increased PR-A mRNA expression in leiomyoma tissue explants. Unlike cell cultures, the exposure of leiomyoma tissue explants to high concentrations of progesterone produced increased PR-B mRNA expression. PR-A synthesis in tissue explants was significantly higher in these conditions than in cell cultures. Our results were consistent with published data suggesting decreased progesterone receptor expression in uterine leiomyoma cell cultures (Zaitseva *et al.*, 2006). Progesterone stimulated cell proliferation in cell cultures and tissue explants alike, but in the case of

uterine leiomyoma explants this stimulatory effect was obviously mediated by PR-B.

Unlike cell cultures, leiomyoma tissue explants treated with progesterone did not show alterations in PPAR γ or PTEN mRNAs expression and had increased ERK mRNA expression after exposure to low and high concentrations of progesterone. It has been demonstrated that progesterone acting via its receptors may increase the activity of the MAPK/ERK signaling pathway in cells (Ishikawa *et al.*, 2010). The mitogen-activated protein kinase (MAPK) signaling pathway plays a key role in human cancer cell survival, migration and resistance to drug therapy (Burotto *et al.*, 2014). MAP kinases are serine/threonine-specific protein kinases that include four signaling families: MAPK/ERK classical pathway, Big MAP kinase-1, c-Jun N-terminal kinase and h38 signaling families (Burotto *et al.*, 2014). The MAPK/ERK (extracellular signal regulated kinases) pathway is activated by numerous signals, such as internal metabolic stress, DNA damage, altered protein concentrations, in addition to signaling from growth factors, cell-matrix and cell-cell interactions (Yang *et al.*, 2013). Engagement of the ERK pathway activates the AP-1 (activating

protein-1) family of transcription factors, which modulates the transcription of several target genes regulating proliferation and invasion (Borahay *et al.*, 2015). Recent investigations have demonstrated the complex and bidirectional interaction between steroid hormones and the ERK-signaling pathway (Borahay *et al.*, 2015). On the one hand, estrogens and progesterone may directly activate the ERK pathway; on the other hand, growth factors can modulate ERK response to steroids, thus targeting steroid receptors (Lopez *et al.*, 2001). We suppose that the increased ERK mRNA expression seen in uterine leiomyoma tissue explants after incubation with progesterone may be induced by the direct action of progesterone and by the specific tumor microenvironment. It is well known that numerous growth factors and proinflammatory cytokines, including TGF (transforming growth factor), EGF (epidermal growth factor), IL (interleukin) -1 and -8, TNF α (tumor necrosis factor α) and others, are synthesized in the uterine leiomyoma tissue and play an important role in the mechanisms of leiomyoma growth (Chegini, 2010). The stimulatory effect of IL-8, TNF α and TGF β 3 upon the ERK pathway has also been demonstrated (Chan *et al.*, 2017; Wolczyk *et al.*, 2016; Tao *et al.*, 2015). It might be inferred that the specific leiomyoma tissue microenvironment, characterized by increased expression of a variety of growth factors and proinflammatory cytokines, might interfere with progesterone and promote the proliferation of uterine leiomyoma cells by activating the MAPK/ERK pathway via PR-B receptors as well. Our results allow us to speculate about the differences in the stimulatory action of progesterone on leiomyoma cell cultures and whole tissue explants and their potential associations with the specific cytokine microenvironment in tissue explants.

CONCLUSION

This study described a direct dose-dependent inhibitory effect of progesterone on the apoptosis of primary uterine leiomyoma cell cultures. This finding was associated with elevated Ki67 mRNA expression in tumor cells and enhanced cell growth in response to high doses of progesterone. This effect may be mediated through the effects of progesterone on PTEN and PPAR γ synthesis in leiomyoma cells. Low doses of progesterone increased PR-A and PR-B mRNAs expression in tumor cells. High doses of progesterone increased Ki67 and PR-A mRNAs expression. In tissue explants, a dose-dependent effect of progesterone on PR-B mRNA expression and a stimulatory effect of low and high doses of progesterone on ERK activity were observed. Therefore, progesterone acts quite differently in uterine leiomyoma cell cultures and tissue explants, a factor that must be taken into the account in the development of pharmacological strategies to regulate progesterone activity in uterine leiomyomas.

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Author Contributions

Y.S. contributed in conception and design. D.V. contributed in all stages of experimental work, N.S. contributed in data analysis and interpretation. A.M. was responsible for overall supervision and consultation. S.N. contributed in data collection. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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