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Novel Effects of Simvastatin on Uterine Fibroids: In vitro and Patient-Derived Xenograft Mouse Model Study

Mostafa A. BORAHAY, MD, PhD^{1,2,*}, Kathleen VINCENT, MD^{1,3}, Massoud MOTAMEDI, PhD³, Elena SBRANA, PhD⁴, Gokhan S. KILIC, MD¹, Ayman AL-HENDY, MD, PhD⁵, and Darren BOEHNING, PhD^{2,*}

¹Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX, 77555

²Department of Biochemistry and Molecular Biology, University of Texas Health Sciences Center at Houston, Houston, TX, 77030

³Biomedical Engineering Center, University of Texas Medical Branch, Galveston, TX, 77555

⁴Department of Pathology, University of Texas Medical Branch, Galveston, TX, 77555

⁵Department of Obstetrics and Gynecology, Georgia Regents University, Augusta, GA, 30912

Abstract

Objective—Uterine leiomyomas represent a common gynecologic problem with no satisfactory long-term medical treatment. The purpose of this study is to examine the effects of simvastatin on uterine leiomyoma, both in vitro and in vivo.

Study Design—This is a laboratory-based experimental study. For in vitro studies, we used human and rat leiomyoma cells. For in vivo studies, we used immunodeficient mice supplemented with estrogen/progesterone pellets xenografted with human leiomyoma tissue explant.

Results—For in vitro studies, cells were treated with different concentrations of simvastatin for 48 hours. Simvastatin induced dose-dependent apoptosis in leiomyoma cells as measured by a fluorometric caspase-3 activity assay, and inhibited proliferation as demonstrated by an MTT assay (both were significant at 5 and 10 μ M). In addition, simvastatin decreased Akt signaling pathway phosphorylation as examined using Western blot analysis. For in vivo studies, animals were treated for 28 days with simvastatin (20 μ g/ gm body weight/ day) vs vehicle control. The treatment inhibited tumor growth as measured weekly using calipers and/ or ultrasound ($P < .01$).

*Corresponding authors: Mostafa BORAHAY, 301 University Blvd., Galveston, TX 77555-0587, maboraha@utmb.edu, Phone: (409) 772-1574. Darren BOEHNING, 6431 Fannin St., Houston, TX 77030, Darren.F.Boehning@uth.tmc.edu, Phone: (713) 500-6167.

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Finally, simvastatin decreased expression of the proliferation marker Ki67 in xenograft tumor tissue as examined by immunohistochemistry ($P=.02$).

Conclusion—Simvastatin can be a promising treatment for uterine leiomyoma. Further studies, including pharmacokinetic and drug delivery studies, are required.

Keywords

leiomyoma; simvastatin; tumor; treatment; patient-derived xenograft model

INTRODUCTION

Uterine leiomyomas are the most common tumors of the female genital tract¹, with 50%–70% of women developing uterine leiomyomas during their lifetime.² They cause several gynecologic symptoms, including heavy bleeding and pelvic pain. Unfortunately, no satisfactory long-term medical treatment is currently available and a hysterectomy is ultimately needed in many cases. In fact, about 600,000 hysterectomies are performed annually in the US, and about half of these are related to leiomyomas.³

Simvastatin is a semisynthetic member of the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor family. Statins have been used to treat hyperlipidemia for over 20 years, and therefore they have a well-known safety profile.⁴ Remarkably, they were also found to have profound and broad-reaching effects on various types of tissues.⁵ Furthermore, statins have cell-specific effects on cellular proliferation. For example, they stimulate proliferation of endothelial progenitor cells,⁶ while inhibiting it in vascular smooth muscles, hepatocytes, and certain neoplastic cell lines.^{7, 8}

Statins have been shown to have general anti-tumor properties^{9, 10}, particularly against breast cancer^{11, 12} and gynecologic malignancies.^{13–16} In addition, recent studies showed that certain statins, specifically simvastatin and atorvastatin, have beneficial effects on benign steroid-dependent gynecologic conditions, such as endometriosis.^{17–20} Therefore, it seems intriguing to examine the effects of statins on uterine fibroids. We previously demonstrated that simvastatin inhibits proliferation, interrupts cell cycle progression, and induces apoptosis through a calcium-dependent mechanism in human leiomyoma cells in vitro.²¹ In this study, we proceeded to investigate in vivo effects of simvastatin on a patient-derived xenograft leiomyoma murine model. In addition, we investigated the effects of treatment on a human as well as a rat leiomyoma cell line. We report, for the first time to the best of our knowledge, that simvastatin treatment is associated with tumor growth inhibition in leiomyoma xenograft animal models and thus possibly has therapeutic potential for treating uterine leiomyoma.

MATERIALS AND METHODS

Cells

The Eker rat leiomyoma cell line (ELT-3) was a kind gift from Dr. Cheryl Walker, professor and director at the Texas A&M Health Science Center Institute of Biosciences and Technology in Houston, TX. These cells were established and have been fully

characterized.²² The immortalized human leiomyoma cell line (huLM) was derived from a patient with a uterine leiomyoma after hysterectomy. They were immortalized by the Dr. Darlene Dixon group using telomerase induction and have been previously characterized.²³

HuLM cells were cultured and maintained in a Smooth Muscle Growth Medium-2 (SmGMTM-2) containing 5% fetal bovine serum (FBS), 0.1% insulin, 0.2% basic human fibroblast growth factor, 0.1% gentamicin/amphotericin B, and 0.1% human epidermal growth factor; all of which were purchased from Lonza (Walkersville, MD). ELT-3 leiomyoma cells were cultured and maintained in a DF8 medium as previously described.²² Cells were incubated in a 5% CO₂ atmosphere under 37 °C and split once 70%–80% confluent.

Materials

The simvastatin was purchased from Cayman Chemical (Ann Arbor, MI). A complete protease inhibitor cocktail without EGTA was purchased from Roche Applied Science (Indianapolis, IN). Z-DEVD-R110 used for the caspase-3 assay was purchased from the American Peptide Company (Sunnyvale, CA). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was purchased from Calbiochem (EMD Millipore, Merck KGaA, Darmstadt, Germany). Monoclonal anti-phospho Akt and anti-total Akt antibodies, used for Western blotting, were purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal anti-Ki67 antibody, used for immunohistochemistry, was purchased from Abcam (Cambridge, MA).

A MatrigelTM basement membrane matrix was purchased from BD Biosciences (San Jose, CA). Sixty-day release pellets with 17 β -Estradiol (0.05 mg) + Progesterone (50 mg) were purchased from Innovative Research of America (Sarasota, FL).

Simvastatin activation

The simvastatin was activated prior to use to convert the prodrug (lactone) to an active (beta-hydroxyacid) form. Activation was done as previously described.²⁴ In brief, 25 mg of simvastatin was dissolved in 625 μ L of absolute ethanol and then added to 935 μ L of 0.1 NaOH. After mixing, the solution was placed in a water bath at 50°C for 2 hours and then diluted with water to the required concentration. Finally, the solution was sterile filtered and kept at 4°C until use.

Proliferation assay

An MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to monitor cellular proliferation. ELT-3 cells were seeded into 96-well plates. After 24 hours, the medium was replaced with a medium containing 0, 0.1, 0.5, 1, 5, and 10 μ M of simvastatin. 48 hours later, the MTT assay was performed as previously described.²⁵

Caspase-3 assay

Caspase-3 activity was measured using a quantitative fluorometric assay as previously described.²⁶ In brief, cells were seeded in 10 cm dishes. After 24 hours, the medium was replaced with a medium containing 0, 0.1, 0.5, 1, 5, and 10 μ M of simvastatin. After 48

hours, cells were harvested and cell lysates were obtained. Cell lysates containing equal amounts of proteins were loaded in a 96-well plate in addition to a reaction mixture containing Z-DEVD-R110 (caspase-3 substrate). Caspase-3 activity was measured fluorometrically over a period of 60 minutes.

Tumor samples

Approval from the Institutional Review Board (IRB) at the University of Texas Medical Branch (UTMB) was obtained, and verbal consents were obtained from patients. Leiomyoma samples (otherwise discarded) from patients undergoing hysterectomies were obtained and immediately transported to the laboratory under sterile conditions. For this study, independent samples were obtained and processed from two separate patients at two different time points.

Animals

Approval from the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch (UTMB) was obtained. All animal handling was performed in accordance with IACUC and other appropriate guidelines. We modified a previously described leiomyoma xenograft animal model.²⁷ Six-week-old female immunodeficient NOG (NOD/Shi-*scid*/IL-2R γ^{null}) mice were purchased from Taconic (Hudson, NY). These mice have multiple immunodeficiencies and were originally developed by the Central Institute for Experimental Animals (CIEA) in Japan in 2000 from 3 mice strains: NOD/Shi, SCID, and IL-2R γ^{null} . These combined defects rendered the mice more appropriate as human xenograft models.²⁸ All procedures were performed under sterile precautions and, the mice were kept in an appropriately isolated environment.

Estrogen-progesterone pellet insertion

Mice were anesthetized by isoflurane (1%–2%), administered by mask. At least 5 days before leiomyoma xenograft placement, 1 17 β -estradiol (0.05 mg)/ progesterone (50 mg) 60-day pellet was placed subcutaneously (SQ) in each animal. For pain control, buprenorphine (0.05–0.1 mg/kg SQ twice daily, then as needed) was administered.

Tissue processing and implantation

Leiomyoma tumors obtained from patients were immediately processed under sterile conditions. Uniform 2 $\times$ 2 $\times$ 3 mm cylinders were obtained from the tumor using a 2 mm Keyes' biopsy punch and then dipped in the MatrigelTM basement membrane matrix. The tumors were then inserted subcutaneously into 20 mice through a small skin incision in their flanks, which were then closed with sterile surgical staples (2 tumors per mouse). The animals were closely observed for pain and signs of infection after procedures. One week after xenograft placement, the staples were removed and treatment was initiated. The twenty animals were randomly assigned to treatment ($n=10$) or control ($n=10$) groups.

Animal treatment and sacrifice

Animals were treated by a daily subcutaneous injection of simvastatin (20 μ g/ gm body weight/ day) or vehicle control for 28 days. Animals were closely observed on a daily basis,

and tumor sizes were measured weekly by caliper. After 28 days, tumor sizes were measured by calipers for the last time.

High-resolution ultrasound system was used to measure tumor sizes in three dimensions using a 18–38MHz probe (Vevo 2100, Visualsonics, Toronto, Canada). This measurement was done at the biomedical engineering center at the University of Texas Medical Branch. Tumor volumes were then calculated using formula $\text{volume} = 0.52 * \text{length} * \text{width} * \text{height}$ (this is a simplification of tumor volume = $4/3 * \pi * \text{length}/2 * \text{width}/2 * \text{height}/2$).

After ultrasound, animal euthanasia was performed using an isoflurane overdose followed by cervical dislocation. Skin incisions were made and the tumors were removed. Representative images of the tumors by ultrasound and at sacrifice are shown in Figure 1. Tumor sizes were measured again by calipers and wet tumor weights were obtained. Finally, tumors were placed in a 10% buffered formalin solution and kept at 4°C until immunohistochemistry (IHC). The research personnel administering treatment and measuring tumor size were blinded in regard to treatment vs control groups.

Immunohistochemical studies

Tumor tissues obtained from the animals were fixed using 10% buffered formalin, and blocks were used to prepare tissue sections. These sections were next stained with hematoxylin and eosin (H&E) to examine the tissue morphology. Further sections were used for immunostaining for the proliferation marker Ki67, using the immunoperoxidase method with DAB as the chromogen. Immunostaining was then quantitated using the Image-Pro Plus software (Media Cybernetics, Rockville, MD). This analysis software generates arbitrary numbers using a computer grading algorithm that takes into account the tissue area, the percentage of positively-stained cells to the total number of cells, and the intensity of the staining. This was performed in 10 separate high-power fields (20x) per slide. The pathologist who read the slides was blinded in regard to treatment vs control groups.

Statistical analysis

In vitro experiments were performed in triplicates and independently repeated at least 3 times. Data were checked for normality using the Shapiro–Wilk test. Whenever applicable, data were presented as mean $\pm$ SEM (standard error of the mean). For in vitro experiments where several concentrations of simvastatin were used, one-way analysis of variance (ANOVA) was used with Bonferroni post-hoc analysis to analyze each treatment concentration vs control. Different statistical methods were used for the in vivo experiments. To evaluate within-animal concordance of the volumes of the 2 xenografts in each animal, we used intraclass correlation coefficient (ICC). This was calculated using SPSS for Windows, version 20 (IBM Corporation, Armonk, NY). We used the Student's t-test to compare tumor size measurements and IHC results between the treatment and control groups. A 2-sided alternative to the null hypothesis of no difference was used. *P* values less than .05 were considered statistically significant. We used SigmaPlot software (Systat Software Inc, San Jose, CA) for the statistical analysis.

RESULTS

Induction of apoptosis in leiomyoma cells in vitro

To investigate the effects of simvastatin on apoptosis, we treated ELT-3 cells with different concentrations of simvastatin for 48 hours, followed by a fluorometric caspase-3 activity assay using cell lysates. ANOVA analysis demonstrated significant difference among groups ($P=.004$). These results are similar to our previous findings using HuLM cells.²¹ Bonferroni post-hoc analysis showing significant differences at 5 and 10 μM compared to controls ($P=.005$ and $P=.025$, respectively) (Figure 2).

Inhibition of proliferation of leiomyoma cells in vitro

Next, we proceeded to determine if simvastatin affects proliferation of leiomyoma cells. After treating ELT-3 cells for 48 hours, we performed an MTT assay. ANOVA analysis demonstrated significant difference among groups ($P=.019$). These results are similar to our previous findings using HuLM cells.²¹ Bonferroni post-hoc analysis showed that simvastatin significantly inhibits proliferation of cells at 10 μM ($P=.021$) (figure 3).

Inhibition of Akt signaling pathway in leiomyoma cells in vitro

We next sought to examine the effects of simvastatin on proliferation signaling pathways. To this end, we used Western blotting to examine the phosphorylation of Akt protein in HuLM cells. Phosphorylation was calculated by dividing the expression of phosphorylated fraction by the total Akt protein. Analysis of variance demonstrated a statistically significant decrease in Akt phosphorylation ($P=.002$), with post-hoc analysis showing the 2, 5, and 10 μM concentrations statistically significant ($P=.049$, .017, and .014, respectively) (figure 4).

Inhibition of leiomyoma xenograft growth in the animal model

To determine the antitumor efficacy of simvastatin in a patient-derived xenograft leiomyoma model, mice were treated with a daily dose of 20 $\mu\text{g/kg}$ simvastatin (treatment group, $n=10$) or vehicle control (control group, $n=10$). Animals in both groups were regularly checked for any signs of poor general health, and both groups stayed healthy till sacrifice. Since each animal received 2 tumor xenografts, we had 20 tumors in each group (total of 40 tumors). The sizes of tumors were measured weekly by calipers. At the time of sacrifice, the tumor sizes were measured by both calipers and ultrasound. The intraclass correlation coefficient (ICC), a concordance measure, of the volumes of the 2 xenografts in each animal was 0.576. As graphically presented in Figure 5(A and B), tumor sizes were 37.9% and 49.1% smaller in the treatment group compared to controls at weeks 3 and 4 ($P=.003$ and .002, respectively). In addition, as measured by ultrasound prior to sacrifice, the mean tumor size was 43.1% smaller in the treated group compared to the control group ($P=.043$) (Figure 5C). Finally, the mean wet tumor weight was 28.5% smaller in treated group compared to the control; however, this difference did not reach significance (Figure 5D; $P=.17$).

Inhibition of leiomyoma cell proliferation in xenografted tumors

Finally, we wanted to examine the effect of simvastatin treatment on proliferation markers in vivo. Xenograft tumors were removed and fixed in formalin after animal sacrifice.

Thereafter, immunohistochemistry was done to examine the expression of the proliferation marker Ki67. Using computer-aided quantitation, Ki67 expression was measured and statistically analyzed. As graphically presented in figure 6, simvastatin treatment was associated with a significant reduction of the expression of the proliferation marker Ki67 by 43.7% ($P=.015$).

COMMENT

The results of this study demonstrated that simvastatin induces apoptosis and inhibits proliferation of leiomyoma cells in vitro. In addition, it inhibits leiomyoma tumor growth in a patient-derived xenograft animal model.

This study has several strong points. First, using simvastatin in the treatment of uterine leiomyoma is innovative as simvastatin is a commonly used, FDA-approved drug with well-known pharmacokinetics and safety profile.⁴ Second, we used an innovative animal model in which we modified a previously described protocol.²⁷ Instead of using estrogen-only pellets, as previously described²⁷, we used estrogen-progesterone pellets, which is important as progesterone has been demonstrated to be critical for maintenance and growth of uterine leiomyoma.²⁹ A third advantage of this model is the use of human leiomyoma tissue since the extrapolation of findings will be more reasonable. Also, the subcutaneous location of tumors allows more accurate and relatively easier measurement. Finally, the tumor growth rate in our model is closer to a clinical pattern than NSG mice models in which tumors grow 6–7 folds.^{27, 29} The average tumor volume at 4 weeks was 142% of initial volume. This is close to what we expected as Tsuigi and colleagues demonstrated almost no tumor growth at 8 weeks with estrogen only supplementation and 1.3 times growth on estrogen and progesterone supplementation.²⁷

Animals in our study were not ovariectomized and, therefore, had endogenous sex steroids. It was previously demonstrated that endogenous steroids are insufficient to sustain leiomyoma xenografts. Tsuiji and colleagues found that mice leiomyoma xenografts in animals without steroid supplementation undergo complete necrosis.²⁷ In addition, since this is the first study with such a design, we do not have information about the therapeutic impact of simvastatin when only estrogen is used versus estrogen and progesterone. Future studies are warranted to further address this question.

On the other hand, the study has some weaknesses. First, we have not used a myometrial control to determine differential effects of simvastatin on leiomyomas vs normal myometrium or to detect any myometrial toxicity. Second, we have not done a drug pharmacokinetics study to evaluate plasma and tumor drug levels. However, we plan to use myometrial control and study pharmacokinetic in future studies. Finally, the tumor microenvironment within the steroid sensitive uterine wall, including growth factors, inflammatory factors and immune cells, is likely different from subcutaneous site used in this study. Therefore, development of an orthotopic xenograft leiomyoma animal model can be a powerful tool in leiomyoma research.

Among the available statins, we chose simvastatin for this study because of its favorable chemical and pharmacokinetic properties. Statins are classified as lipophilic (e.g.

simvastatin, lovastatin, atorvastatin) or hydrophilic (e.g. pravastatin, fluvastatin, rosuvastatin). This solubility profile significantly affects the hepatoselectivity of a certain statin. While lipophilic statins passively diffuse to hepatic and non-hepatic tissues, the hepatic uptake of hydrophilic statins is active and carrier-mediated through membrane transporters, including organic anion-transporting polypeptide (OATP). Therefore, hydrophilic statins are more hepatoselective (concentrated in the liver), while lipophilic statins (including simvastatin) can reach hepatic and non-hepatic tissues.^{30–32}

In this study, we based our formulation and administration of simvastatin on previous studies utilizing rodent models where simvastatin has been administered subcutaneously,^{33, 34} intra-peritoneally,³⁵ or by oral gavage.^{36–38} In published animal studies, oral dosages ranged from 5–140 µg/ gm body weight/ day^{36–38} while subcutaneous and intraperitoneal dosages ranged from 2–40 µg/ gm/ day.^{33–35}

In the in vitro part of this study, we used simvastatin concentrations of 0.1–10 µM. We extensively reviewed the literature regarding bioavailability, hepatic metabolism, serum levels (including peak plasma levels [C_{max}]), and maximum tolerated dosages of simvastatin. After an oral dosage of 80 mg/ day (used for hypercholesterolemia) in an average adult, simvastatin C_{max} is around 0.3 µM.^{39–41} In addition, the maximum tolerated dosage of simvastatin in humans was noted to be 15 mg/ kg/ day (15 µg/ gm/ day).⁴² Furthermore, in humans, bioavailability of simvastatin is about 5% of the orally administered dosage.^{40, 43} Therefore, we chose our concentrations by taking into account a future need to increase local tissue concentrations of simvastatin to reach higher doses using technologies discussed later.

Simvastatin pharmacokinetics and pharmacodynamics in mice and rats are different than in humans. Rodents require higher doses of simvastatin to achieve the same effects and plasma levels as humans. This is due to several factors, including the hepatic uptake and clearance of statins and feedback regulation of HMG-CoA reductase expression after statin administration.^{44, 45} In fact, it was demonstrated that mice treated with 25, 100, and 400 µg/ gm of body weight resulted in plasma levels 1, 4, and 8 times the mean human plasma level observed after 80 mg oral dosage, respectively.⁴⁶ Similarly, another study⁴⁷ showed that oral administration of lovastatin (very similar molecular weight, lipophilicity and pharmacokinetics to simvastatin) to mice at a dosage of 25 µg/ gm was associated with a peak lovastatin acid plasma level of 0.4 µM (similar to human levels at 80 mg/day). As simvastatin undergoes extensive uptake by the liver on the first pass (more than 95%), we decided to administer it parenterally. The dosage used in this study is somewhat in the middle compared to doses used in other simvastatin animal experiments (2–40 µg/ gm/ day using subcutaneous and intraperitoneal routes).^{33–35}

Simvastatin exists in a lactone or acid form. The lactone form is a prodrug and is converted to the active (beta-hydroxy acid) form by opening the lactone ring by hydrolysis. The lactone form is more readily extracted by the liver, and is metabolically cleared faster than the acid form.^{48, 49} Therefore, it is logical to use the lactone form when the liver is targeted, e.g. treating hypercholesterolemia. However, when extra-hepatic tissues are targeted, e.g.

treating extra-hepatic tumors, activation and conversion to an acid form is more appropriate. Therefore, we activated simvastatin prior to use in this study.

Several mechanisms have been proposed for the anti-tumor effects of statins in specific tumor types.^{7, 8, 12, 13} In this study, we demonstrated that simvastatin inhibits phosphorylation of the growth factor signaling pathway Akt with the resulting inhibition of proliferation. In addition, Akt phosphorylation has been demonstrated to phosphorylate the BH3-only pro-apoptotic Bcl-2 family member Bim which leads to its proteasomal degradation.⁵⁰ Therefore, inhibition of Akt phosphorylation by simvastatin leads to an increase of the Bim level and, therefore, activation of the apoptotic cascade. This links the anti-proliferative and pro-apoptotic effects of simvastatin through Akt pathway. In addition, our group recently described a novel calcium-dependent mechanism for simvastatin effects on leiomyoma cells.²¹ In this mechanism, in addition to the previously described Bim level elevation, simvastatin causes an elevated cytosolic calcium level through engagement of voltage-gated calcium channels. These combined lead to mitochondrial cytochrome C leak with subsequent activation of apoptotic caspase machinery. Because in vivo tumor microenvironment is more complex than cell culture environment, additional mechanisms can be operating. Therefore, further studies are warranted to examine the anti-tumor effects of simvastatin against leiomyoma in vivo.

In conclusion, simvastatin seems to have a potential therapeutic utility in uterine leiomyoma. However, further studies are required. In future studies, we plan to use a myometrial control to examine for differential effect against leiomyoma and any myometrial toxicity. To achieve higher tumor drug concentrations while minimizing systemic side effects, localized/targeted delivery approaches can be explored, such as medicated intra-uterine devices or targeted simvastatin-loaded nanoparticles.^{51, 52} We plan to develop these targeted delivery systems in our future work. In addition, we plan to study pharmacokinetics of simvastatin in our animal model by measuring drug plasma and intra-tumor levels as previously described.⁵³

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Figure 1. Leiomyoma xenograft animal model. A: Ultrasound image of tumor. B: Macroscopic photo of tumor after animal sacrifice. Arrows demonstrate tumor.

Figure 2.

Effect of simvastatin treatment of leiomyoma cells on apoptosis. ELT-3 leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by fluorometric caspase-3 activity assay. The data were normalized to control and presented as means $\pm$ SEM of independently repeated 3 experiments. * $P < .05$; *** $P < .001$ vs control.

Figure 3.

Effect of simvastatin treatment of leiomyoma cells on cellular proliferation. ELT-3 leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by an MTT assay. The data were normalized to control and presented as means $\pm$ SEM of independently repeated 3 experiments. * $P < .05$; *** $P < .001$ vs control.

Figure 4.

Effect of simvastatin treatment of leiomyoma cells on activation of Akt cellular signaling pathway. HuLM leiomyoma cells were treated with the indicated doses of simvastatin (SIMV) for 48 hours, followed by western blotting of cell lysates for active (phosphorylated) Akt and total Akt protein. Protein levels were quantified and data presented as the ratio of phosphorylated to total Akt. The data were normalized to control and presented as means $\pm$ SEM of independently repeated 3 experiments. * $P < .05$; ** $P < .01$ vs control.

Figure 5.

Effect of simvastatin treatment of patient-derived leiomyoma xenograft animal model on tumor volume. Treated animals ($n=10$) received 20 $\mu\text{g}/\text{gm}$ body weight/ day subcutaneously for 28 days while controls ($n=10$) received vehicle. This figure shows tumor volumes in control and simvastatin groups during the study period. A: Tumor volume in mm^3 as measured weekly by calipers. B: Tumor volumes as percentage of week 0 as measured weekly by calipers. C: Tumor size at sacrifice as measured by ultrasound. D: Tumor weight immediately after sacrifice. Data are presented as means $\pm$ SEM. * $P < .05$; ** $P < .01$ vs control.

Figure 6.

Effect of simvastatin treatment of patient-derived leiomyoma xenograft animal model on expression of Ki67 by immunohistochemistry (original magnification 40x). Animals were treated as described in Figure 5. Tumor tissues obtained after sacrifice were placed in 10% buffered formalin and then processed to prepare tissue sections. These sections were next used for immunostaining with the proliferation marker Ki67, using DAB as a chromogen. Expression was then graded using an arbitrary grading system generated by the image analysis software, which correlates with the percentage of positively staining cells and the intensity of the staining in 10 separate high power field (20x) per slide. The expression is presented by arbitrary numbers. Data are presented as means $\pm$ SEM from 20 independent slides from the treatment group and 20 slides from control group.. * $P < .05$ vs control.