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Antitumor Effects of Flavopiridol on Human Uterine Leiomyoma In Vitro and in a Xenograft Model

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Abstract

Dysregulated cyclin-dependent kinases (CDKs) are considered a potential target for cancer therapy. Flavopiridol is a potent CDK inhibitor. In this study, the antiproliferative effect of the flavonoid compound flavopiridol and its mechanism in human uterine leiomyoma cells were investigated. The present study focused on the effect of flavopiridol in cell proliferation and cell cycle progression in primary cultured human uterine leiomyoma cells. Cell viability and cell proliferation assays were conducted. Flow cytometry was performed to determine the effect of flavopiridol on cell cycle. The expression of cell cycle regulatory-related proteins was evaluated by Western blotting. Cell viability and proliferation of uterine leiomyoma cells were significantly reduced by flavopiridol treatment in a dose-dependent manner. Flow cytometry results showed that flavopiridol induced G1 phase arrest. Flavopiridol-induced growth inhibition in uterine leiomyoma cells was associated with increased expression of $p21^{cip/wafl}$ and $p27^{kip1}$ in a dose-dependent manner. Downregulation of CDK2/4 and Cyclin A with a concomitant increase in dephosphorylation of retinoblastoma was observed. This study demonstrates that flavopiridol inhibits cell proliferation by initiating G1 cell cycle arrest in human uterine leiomyoma. We also found that flavopiridol is effective in inhibiting xenografted human uterine leiomyoma growth. These results indicate that flavopiridol could prove to be a promising chemopreventive and therapeutic agent for human uterine leiomyoma.

Keywords

uterus, leiomyoma, flavopiridol, $p27^{kip1}$, xenograft

Introduction

Uterine leiomyomas, commonly referred to as myomas, fibromyomas, or fibroids, are benign smooth muscle tumors that originate from the myometrium. Uterine leiomyomas are a major public health concern, occurring in more than 40% of reproductive-aged women, and the incidence rises to 70% to 80% by 50 years of age.[1] Patients with uterine leiomyomas may experience symptoms requiring medical intervention, such as anemia, menorrhagia, pelvic pain, infertility, spontaneous abortion, abnormal uterine bleeding, and recurrent pregnancy loss.

Treatment of leiomyoma has mainly been surgical although some medical therapies are available for women who wish to preserve their uterus. Currently, leiomyoma management relies on reducing the circulating levels of ovarian hormones using gonadotropin-releasing hormone (GnRH) agonists[2]; however, GnRH agonists frequently cause side effects such as hot flushes, and the use of these drugs is approved only for short-term therapy because of safety concerns (loss of bone mass and cardiovascular changes).[3,4] Therefore, there is a dire need to develop safe and effective nonsurgical treatments for women having uterine leiomyoma.

Flavopiridol, a synthetic flavone derivative isolated initially from the stem bark of *Dysoxylum binectariferum*, is a pan inhibitor of cyclin-dependent kinases (CDKs), currently in clinical development. Flavopiridol directly competes with adenosine triphosphate and is capable of inducing either cell cycle arrest or apoptosis through the inhibition of CDK2, CDK4, and CDK6 but without a direct effect on protein stability.[5,6] Inhibition of CDKs by flavopiridol blocks cell cycle progression and induces G1 phase arrest through negative regulation of the

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phosphoinositide-3-kinase/protein kinase B signaling pathway.[7] The primary response of tumor cells to flavopiridol is cytostatic growth arrest with delayed cytotoxicity although cell arrest and apoptosis may occur concomitantly with cell death the preferential response.[8] Flavopiridol was the first CDK inhibitor to enter clinical trials.[9] However, the feasibility of flavopiridol administration in the treatment of uterine leiomyoma remains to be assessed.

Expression of the CDK inhibitor $p27^{kip1}$ is a powerful prognostic and diagnostic marker in several tumor models, including breast, colorectal, gastric, thyroid, and lung carcinomas.[10-12] Our previous studies demonstrated that $p27^{kip1}$ controls growth and cell cycle progression in human uterine leiomyoma.[13] Furthermore, upregulation of $p27^{kip1}$ by progestin can suppress growth of human endometrial glandular cells.[14] These data suggest a role for $p27^{kip1}$ as a possible tumor suppressor in the tumorigenesis of the female reproductive system.

In human germ cell tumor-derived cell lines, flavopiridol induces apoptosis,[15] whereas in human breast carcinoma cell lines, cell cycle progression is blocked in G1 phase because of elevation in $p27^{kip1}$.[16] Upregulation of $p27^{kip1}$ induced by flavopiridol has been reported in various cell lines, suggesting that $p27^{kip1}$ may be a target of the flavopiridol cell cycle arrest pathway.[17] However, it remains to be established whether alterations in flavopiridol affect the expression of $p27^{kip1}$ in a xenograft model.

To date, there are no studies evaluating the growth inhibition effect of flavopiridol on human uterine leiomyoma. We hypothesized that flavopiridol could be useful in the treatment of human uterine leiomyoma and investigated its influence on cell cycle progression and cell cycle-associated gene expression. Our results provide the first evidence that flavopiridol strongly inhibits the growth of uterine leiomyoma cells in vitro and xenografted uterine leiomyoma tumors in vivo and that its effects correlate with upregulation of $p27^{kip1}$.

Materials and Methods

Tissue Collection

Uterine leiomyoma and the adjacent normal myometrial tissues were obtained from hysterectomies that were conducted on benign diseases at the Keimyung University Dongsan Medical Center, Korea. Informed written consent was obtained from patients, and sample collection and handling were performed based on approval and guidance of the Ethical Committee of the Keimyung University Dongsan Medical Center. Uterine leiomyoma tissues were obtained from central to peripheral parts of the biggest fibroid from women undergoing elective hysterectomy for uterine leiomyoma. The central part refers to the volume limited to 5 mm from the center of the tumor, and the peripheral part refers to the outer 5 mm under the capsule. Histological diagnosis of each uterine specimen was determined by a pathologist. All intramural leiomyomas were included in this study and calcified; hydropic degenerative leiomyomas were excluded. The patients ranged in age from 40 to 48 years, with a mean age of 45 years and had received no hormonal therapy for at least 6 months before surgery. In all, 3 samples were collected from the proliferative phase of the menstrual cycle and 3 samples were obtained from the secretory phase of the menstrual cycle. Fibroid tissues were harvested aseptically, immediately submerged in phosphate-buffered saline (PBS) containing 100 units/mL antibiotics on ice under sterile conditions for transportation from the operating room to the laboratory.

Preparation of Primary Uterine Leiomyoma Cell Cultures

Tissue was washed twice in cold PBS before being minced into 5-mm pieces in a sterile culture dish. The minced pieces were transferred into 50-mL conical tubes containing Hanks' balanced salts (HBSS; Sigma-Aldrich, St Louis, Missouri), supplemented with 25 mmol/L 2-(4-[2-hydroxyethyl]piperazin-1-yl)ethanesulfonic acid, 100 units/mL antibiotics, 1.5 mg/mL collagenase IV (Sigma-Aldrich), and 0.2 mg/mL of DNase I (Roche Diagnostics, Mannheim, Germany). All the tubes were kept at 37°C in a water bath with gentle agitation for 3 hours. Undigested tissue was filtered and the cells were centrifuged at 1000 rpm for 5 minutes. The pellet was rinsed once with HBSS and dispersed in complete medium composed of Dulbecco Modified Eagle Medium: Nutrient Mixture F-12 with 10% fetal bovine serum, 100 units/mL antibiotics. After the cell isolation protocol, characterization of uterine leiomyoma cells was carried out. We used antibodies to vimentin (1:50, Dako, Carpinteria, California), a class of intermediate filament protein present in fibroblasts to confirm the purity of the cells. In addition, desmin (1:100, Dako) staining was used to identify myometrial smooth muscle cells by immunohistochemical staining.

Cell Viability Assay

The number of viable cells was evaluated by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Initially, cells were seeded at a density of 1×10^5 cells/mL in 24-well plates and then cultured to allow their adhesion to the plates. After this preincubation, the culture medium was changed to experimental medium supplemented with flavopiridol 50, 100, 150, and 200 nmol/L or dimethyl sulfoxide (DMSO; control) for 24 hours. The MTT reagent was added and incubated for an additional 3 hours at 37°C. The intensity of the purple color formed in this assay is proportional to the number of viable cells. Optical density (OD) was measured at 495 nm. Cell survival was calculated by subtracting the background OD of media alone and then normalized by dividing the OD of test wells by the OD of control (untreated) wells. The mean value and their standard deviation were calculated from triplicate experiments.

Cell Proliferation Assay

The ability of flavopiridol to inhibit DNA synthesis was determined by estimating the amount of 5-bromo-2′-deoxyuridine

(BrDU) incorporation into DNA by colorimetric immunoassay, using cell proliferation enzyme-linked immunosorbent assay with BrDU-colorimetric kit (Roche Diagnostics, Germany). Uterine leiomyoma cells were cultured in 96-well plates (1×10^4 cells/well). After 24 hours of postseeding, the cells were treated with flavopiridol at the required concentrations and the assay was carried out according to the manufacturer's instructions. The developed assay color was measured at 490 nm. The color intensity and the absorbance values directly correlated with the amount of BrDU incorporated into the DNA. The results were expressed as percentage inhibition of BrDU incorporation over the control.

Propidium Iodide Flow Cytometry

To determine cell cycle distribution, uterine leiomyoma cells were treated with flavopiridol. After 24 hours, the cells were harvested, washed with PBS, and fixed in ice-cold 70% ethanol. The fixed cells were suspended in 0.1% RNase A and propidium iodine (50 μg/mL in PBS) to determine cell cycle dynamics. DNA fluorescence was measured by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, New Jersey). The percentage of cell cycle phase was determined using ModFit LT software (Becton Dickinson) based on the DNA histogram.

Protein Extraction and Western Blot Analysis

Flavopiridol-treated cells were harvested in 1 × radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, Illinois) containing protease and phosphatase inhibitors with EDTA (Thermo Scientific). Protein concentrations were measured using protein assay reagent (Bio-Rad, Hercules, California) following the manufacturer's protocol. Aliquots of protein were separated by 8% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by semi-dry transfer cell (Bio-Rad). The membrane was blocked with Tris-buffered saline containing 5% skim milk. After washing, the membranes were incubated with primary antibodies to CDK2/4 (1:1000, Santa Cruz Biotechnology, Santa Cruz, California) and retinoblastoma (Rb; 1:1000, Santa Cruz Biotechnology), $p21^{cip/wafl}$ (1:1000, BD Pharmingen, Franklin Lakes, New Jersey) and $p27^{kip1}$ (1:1000, BD Pharmingen), Cyclin A (1:500, Abcam, Cambridge, Massachusetts), and β-actin (1:1000, Santa Cruz Biotechnology). After reaction with horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology), bands on the membranes were visualized by an enhanced chemiluminescence (ECL) system (Thermo Scientific). The density of respective bands was analyzed by the Chemi-doc XRS imaging system (Bio-Rad). The data were presented as percentage of controls.

Animals and Animal Care

Eight-week-old female nonobese diabetic-severe combined immunodeficiency interleukin-2 receptor-γ^{null} (NSG) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animals were kept in microisolator cages and housed separately in a barrier facility with a well-controlled, pathogen-free environment. The animals were cared in monitored ambient temperature at 22°C with 12-hour light–dark cycle. All housing materials were autoclaved before use. Mice were fed a laboratory diet and water ad libitum.

Uterine Leiomyoma Tumor Xenografts in Mice

For transplantation, NSG mice were anesthetized using 30 μL of a mixture of ketamine and xylazine (80 and 20 mg/mL, respectively) injected intraperitoneally (ip). Serum hormone levels were maintained at high levels by subcutaneous implantation of sustained-release 17β-estradiol (0.05 mg/90 d) and progesterone pellets (25 mg/60 d; Innovative Research of America, Sarasota, Florida) as described previously.[18] Tissues were minced with scalpel to fragments of no more than 2 to 3 mm^3 and dipped into Matrigel (Becton Dickinson), and the wounds were then closed with suturing. Primary uterine leiomyoma cells (1×10^7) were mixed with 0.5 mL Matrigel and allowed to warm at room temperature for 5 minutes with gentle mixing. The cells were injected under the skin using a 1- cm^3 syringe. The entire transplantation was completed within 30 minutes. Mice and developed lesions were closely monitored once per week.

Pathological Evaluation at 8 Weeks After Grafting

Tissues were harvested and examined for gross evidence of tissue morphology. There were no anesthesia-related or perioperative complications in this implantation model. The tissues were formalin fixed, paraffin embedded, and subjected to hematoxylin and eosin (H&E) histological evaluation. We also used immunohistochemistry to evaluate typical histological features of leiomyoma, including anti-α smooth muscle actin (αSMA; 1:200, Dako) and desmin (1:100, Dako). Expression of estrogen receptor (ER) and progesterone receptor (PR) was evaluated with anti-ER α antibody (1:100, Dako) and anti-PR antibody (1:75, Dako, USA).

Testing the Effect of Flavopiridol on Xenografted Uterine Leiomyoma Tumors

Tumor volume was measured once per week manually, using a digital vernier caliper and tumor volume was calculated using the formula (smallest diameter^2 × largest diameter)/2, for the assessment of flavopiridol's effect. Eight weeks after tumor cell implantation in mice, tumor growth was examined. Volume assessment commenced once the tumors reached a palpable size (approximately 35-45 mm^3), and mice were randomized into control and treatment groups ($n = 5$ per group). Control mice received vehicle (0.1% DMSO in saline) and treated mice received 5.0 or 7.5 mg/kg of flavopiridol ip, 5 days a week for a total of 3 weeks. This was followed by an observation period of 2 weeks without drug treatment. Tumor growth was monitored as before using a digital vernier caliper. The percentage of tumor volume was calculated by considering the starting tumor volume of the day of drug injection as 100%.

Statistical Analysis

The data are presented as the mean ± standard deviation. Statistical analysis was conducted using 1-way analysis of variance followed by Duncan multiple range test for post hoc comparison by SPSS 17.0 (SPSS Inc., Chicago, Illinois). Statistical significance was set at $P < .05$.

Results

Decreased Cell Viability After Flavopiridol Treatment

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to analyze the effect of flavopiridol on uterine leiomyoma cell growth. As shown in Figure 1, flavopiridol-treated cells showed decreased cell growth in a dose-dependent manner. Treatment with 200 nmol/L flavopiridol blocked more than 50% of cell growth during the tested time period. The viability of normal myometrium cells was not affected by flavopiridol treatment and did not cause any significant reduction in cell growth.

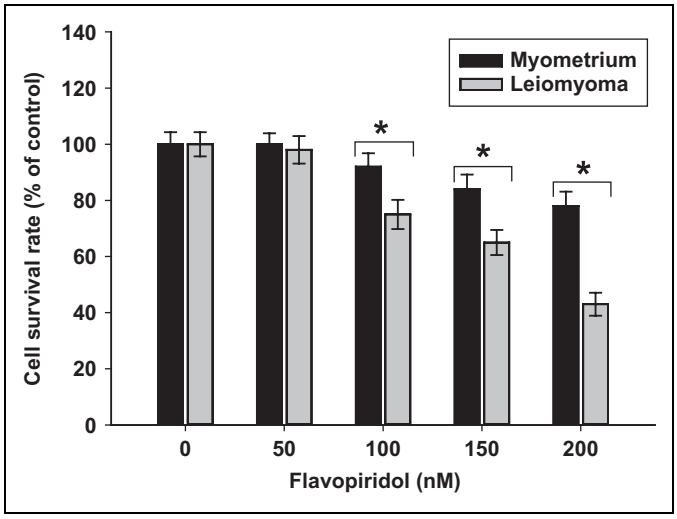

Figure 1. Antiproliferative effect of flavopiridol in uterine leiomyoma cells. Growth inhibition in uterine leiomyoma cells treated with the indicated dose for 24 hours. Cell viability was measured by MTT assay and the results are expressed as a percentage of viable cells. Values are ± SD of 3 measurements, *$P < .05$. MTT indicates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation.

Inhibition of Cell Proliferation After Flavopiridol Treatment

To determine whether the decrease in the number of viable cells was due to decreased proliferation, incorporation of BrDU into DNA was tested in the presence and absence of flavopiridol in leiomyoma cells. As shown in Figure 2, analysis of BrDU incorporation revealed that treatment with flavopiridol resulted in decreased DNA synthesis, leading to decreased cell proliferation. Flavopiridol treatment at 200 nmol/L caused significant reduction in DNA synthesis, correlating with viability and decreased cell growth.

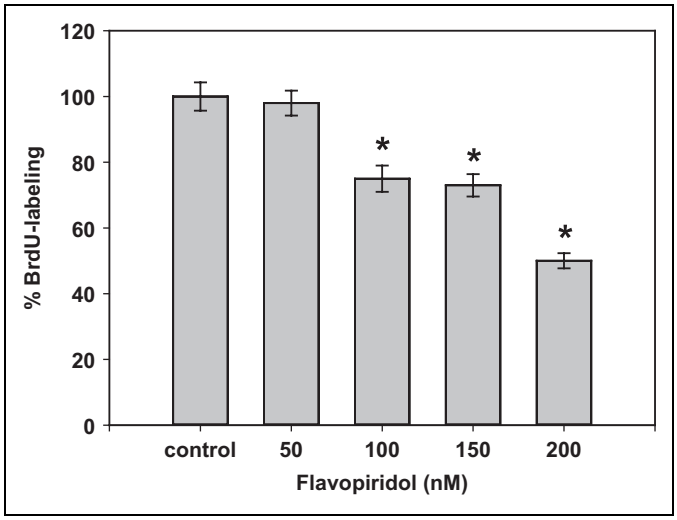

Figure 2. Inhibition of DNA synthesis in uterine leiomyoma cells by flavopiridol. Antiproliferative effects were determined after treating uterine leiomyoma cells for 24 hours with 100 and 200 nmol/L flavopiridol and BrDU labeling (10 μmol/L) for 24 hours. Results are expressed as a percentage of BrDU incorporation relative to control cells. Values are ± SD of 3 measurements, *$P < .05$. BrDU indicates 5-bromo-2′-deoxyuridine; SD, standard deviation.

Cells in G1 Phase Increased by Flavopiridol Treatment

The effect of flavopiridol on the cell cycle is illustrated in Figure 3. Flow cytometry showed that flavopiridol induced uterine leiomyoma cell cycle arrest in the G1 phase in a dose-dependent manner. After 24 hours exposure to 100 and 200 nmol/L flavopiridol, the fraction of uterine leiomyoma cells in the G1 phase increased from 43% to 54% while the fraction of cells in the S phase decreased from 19% to 14%, and cells in the G2 phase increased from 18% to 22% (Figure 3).

Inhibition of Cell Cycle Regulatory Proteins in Uterine Leiomyoma

Since the cell cycle effects may, in part, be responsible for the antiproliferative response to flavopiridol, we evaluated the expression of CDK2 and CDK4 in uterine leiomyoma cells. Cells were treated with flavopiridol for 24 hours and protein expression analyzed by Western blotting. Levels of $p21^{cip/wafl}$ and $p27^{kip1}$ were significantly increased while CDK2, CDK4, cyclin A, and Rb were downregulated. Therefore, downregulation of cyclin A and CDKs and upregulation of $p21^{cip/wafl}$ and $p27^{kip1}$ in uterine leiomyoma cells contributed to the G1 cell cycle arrest induced by flavopiridol (Figure 4).

Histological Assessment of Human Uterine Leiomyoma Xenografts

Anatomical, physiological, and histopathological changes in tissue were compared with cell graft model. The H&E staining

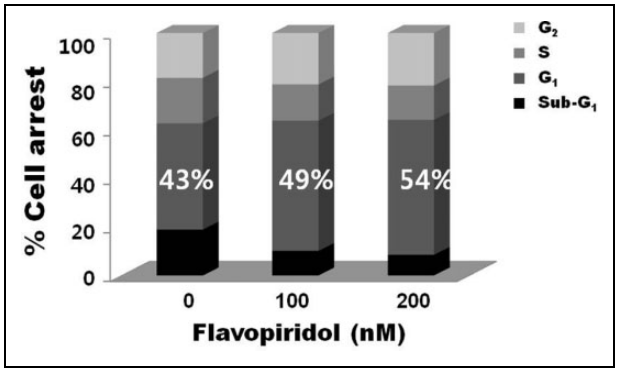

Figure 3. Effect of flavopiridol treatment on the cell cycle profile. After treatment with the indicated dose of flavopiridol for 24 hours, uterine leiomyoma cells were collected, fixed, stained with PI, and analyzed by flow cytometry. Values shown represent the number of cells in each phase as a percentage of the total cells. PI indicates propidium iodide.

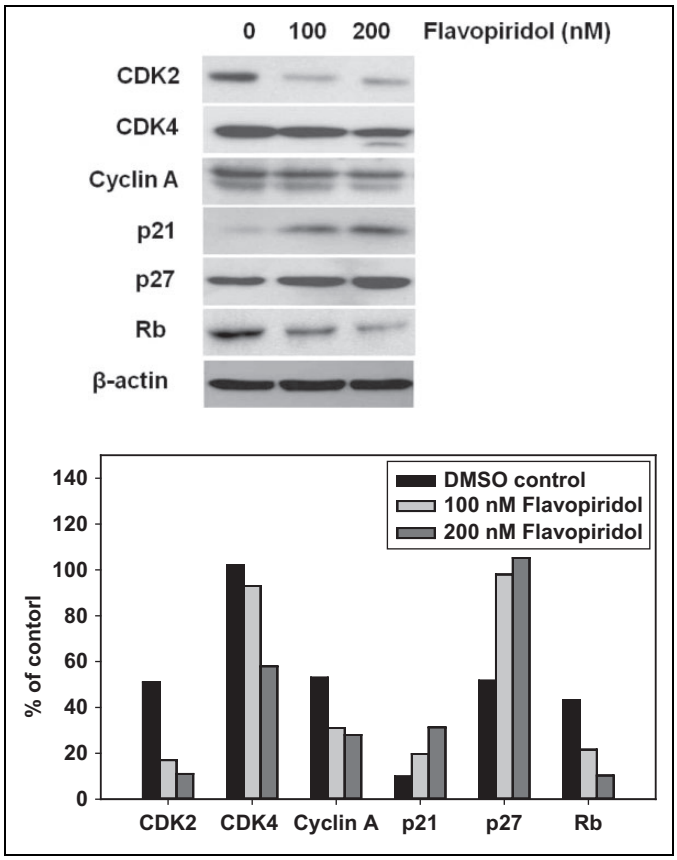

Figure 4. Effect of flavopiridol on cell cycle-related protein expression in uterine leiomyoma cells. Twenty-four hours after treatment, cell extracts were prepared and subjected to immunoblotting analysis. β-Actin was used as an internal loading control. Each band was quantified by densitometric analysis and presented in a bar graph.

of cell graft showed a well-demarcated nodule with marked proliferation of variable-sized blood vessels similar to tissue graft. Expression of αSMA was analyzed as a specific marker for smooth muscle. The level of αSMA and desmin was compared among the 2 groups. The cell graft was strongly positive for both proteins. The tissue and cell graft were composed of whorled, anastomosing fascicles of leiomyocytes while the leiomyoma cells had an eosinophilic cytoplasm with elongated or oval nuclei. Abundant extracellular matrix (ECM) was noted along with extensive cellular vascularity. Immunohistological analysis of ER and PR antibodies showed similar protein levels and proliferating cells between tissue and cell xenograft (Figure 5).

Antitumor Effects of Flavopiridol in a Cell-Based Model System of Human Uterine Leiomyoma

Three weeks after administration of flavopiridol, mean tumor growth in the treated group decreased significantly compared with the control group (Figure 6A). Analysis of tumor weights at the end of the treatment period indicated a significant decrease in average tumor volume (Figure 6C). These results indicate that flavopiridol is effective in inhibiting xenografted human uterine leiomyoma growth.

Discussion

Uterine leiomyoma is the most common benign tumor in women and the leading worldwide cause of hysterectomy. Although the nature of the initial event is unknown, the role of ovarian hormones in the pathogenesis of uterine leiomyoma is well established as leiomyomas grow during reproductive years and regress after menopause.[19] The environment within and around leiomyomas is hyperestrogenic; therefore, estrogen is thought to modulate tumor growth.[20] However, estrogen is not mitogenic for mature normal myometrial cells, suggesting that estrogen responsiveness is altered in fibroids.[21,22] Thus, the cell cycle regulatory properties of flavopiridol may become a new target for the control of abnormal uterine leiomyoma growth.

It is well known that eukaryotic cell proliferation is a tightly regulated system controlled by a network of cyclin–CDK complexes.[23,24] Cyclin-dependent kinases and their associated pathways represent attractive targets for the development of anticancer therapeutics. Anticancer effects of flavopiridol have been established in variety of preclinical studies, using numerous cancer cell lines including the lymphoma,[8] cervix,[25] glioma,[26] and breast.[16] To our knowledge, we show here for the first time the antiproliferative effects of flavopiridol on uterine leiomyoma cells.

In the present study, flavopiridol inhibited cell proliferation of human uterine leiomyoma cells. We found that flavopiridol causes G1 phase arrest in uterine leiomyoma cells by inhibiting the expression of CDKs, cyclin A, and Rb accompanied by upregulation of $p21^{cip/wafl}$ and $p27^{kip1}$. Expression and interaction of CDKs, cyclin A, and their inhibitors account to a great extent for cell cycle timing. The major regulatory checkpoint control is the transition from G1 phase to S phase, which is characterized by inactivation and phosphorylation of Rb.[27] Retinoblastoma has a critical role in the proliferative response of cells to mitogenic signals, and the phosphorylation of Rb is

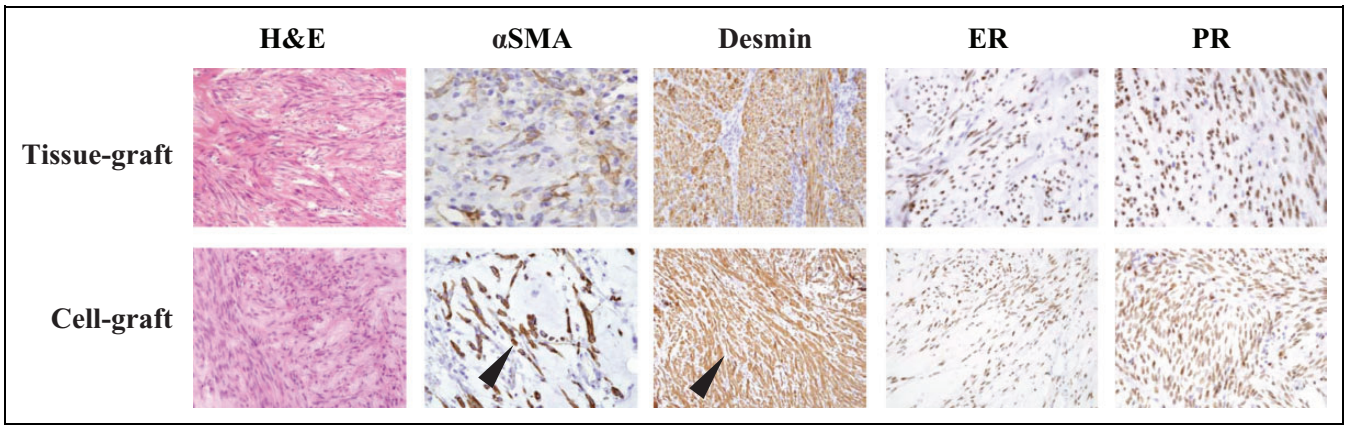

Figure 5. Histological evaluation of human uterine leiomyoma xenografts. Representative histological sections stained with hematoxylin and eosin (H&E). Immunohistochemistry using antibodies against α-smooth muscle actin (αSMA), desmin, estrogen receptor (ER) α, and progesterone receptor (PR) from matched tissue and cell graft. Original magnification was ×200.

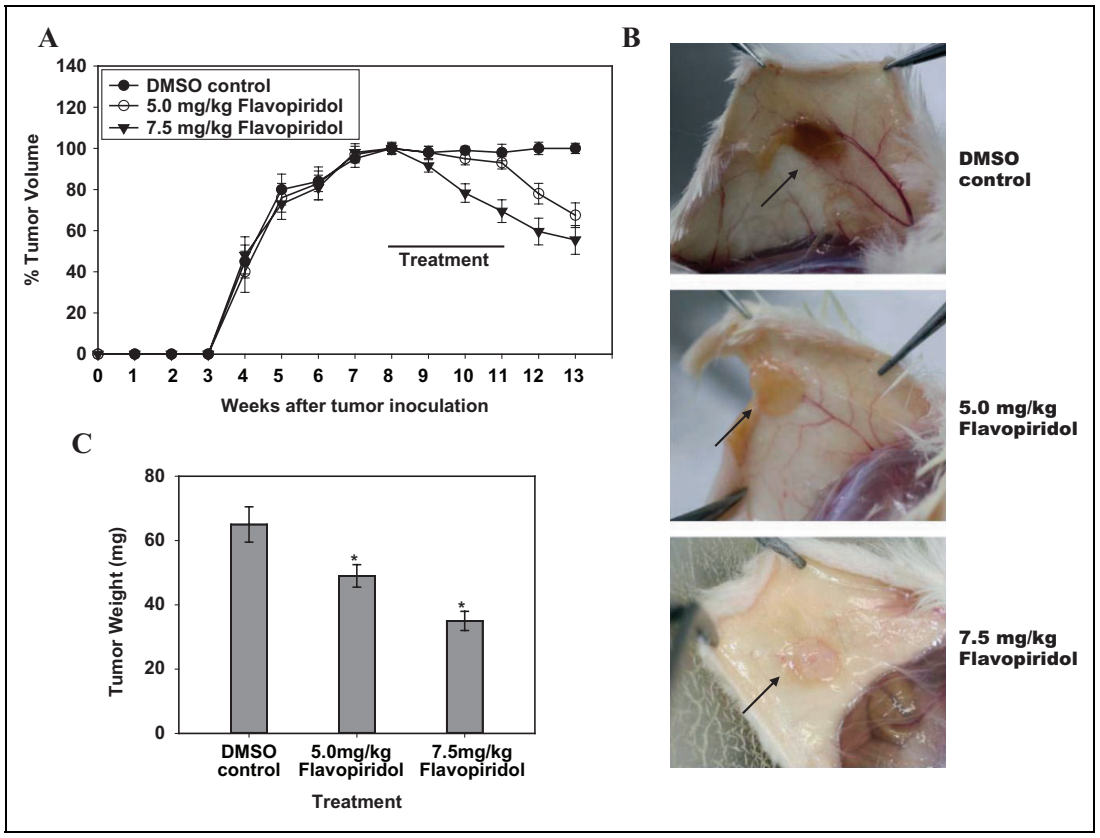

Figure 6. Effect of flavopiridol on xenografted tumors. A, Mice bearing established cell grafts were treated with flavopiridol at 5.0 or 7.5 mg/kg or DMSO control for 3 weeks starting on week 8. The graphs indicate the percentage of tumor growth in control and flavopiridol-treated groups. B, Representative image of control mice and mice treated with flavopiridol. C, Average tumor weights at the termination of the protocol in the control, 5.0, and 7.5 mg/kg of flavopiridol-treated groups. Values are ±SD of 3 measurements, *$P < .05$. DMSO indicates dimethyl sulfoxide; SD, standard deviation.

regulated in a cell cycle-dependent manner. In association with the cyclin A, D, E, and CDK2, CDK4 governs the progression of G1 and S phases through sequential phosphorylation of Rb and $p27^{kip1}$.[28] The effects of flavopiridol on cancer cells are varied and dependent on cell type. A previous study reported that flavopiridol leads to sub-G1 phase arrest accompanied by downregulation of cyclin D1 in head and neck squamous cell carcinomas and immortalized nontumorigenic cells.[29] Reduced expression of cyclins has also been documented in a mouse model,[30] and Sedlacek et al[31] documented that

flavopiridol induces G2 phase arrest concomitant with a marked inhibition of cyclin B1 and induction of the CDK inhibitor $p21^{cip/wafl}$ in cancer cells.

Synthesis and degradation of the cell cycle inhibitor $p27^{kip1}$ are key events in the regulation of cell cycle progression. It is generally accepted that $p27^{kip1}$ and the related $p21^{cip/wafl}$ inhibitor block the cell cycle by inhibiting the kinase activity of CDK2-cyclin A complexes, binding and inhibiting the activity of CDK complexes and then functioning as a regulator of cell cycle progression in G1 or late G1 phase. In this study, we demonstrated that flavopiridol potentially downregulates CDK2/4. Moreover, $p21^{cip/wafl}$ and $p27^{kip1}$ were also found to be upregulated in a dose-dependent manner after treatment with flavopiridol. Dysregulated CDK activity is a hallmark of human cancer and a variety of genetic and epigenetic events.[32,33] Inhibition of CDK1/2 and CDK4/6 largely accounts for growth arrest or delayed cell cycle progression.[34] This in vitro study demonstrates that the drug has the potential to overcome abnormal multiplication of fibroid tissue. Additionally, our study will add support to verify the underlying mechanism responsible for human uterine leiomyoma.

In this study, tumors were found in 9 of 10 inoculated mice. We have developed a procedure for successful cell inoculation of primary human uterine leiomyomas in NSG mice. Using this method, we have consistently achieved high tumor take rates (>90%). This was due to a number of factors, including the utilization of NSG mouse hosts, adjustment of hormonal status, convenient site, and easy techniques. Strong staining of H&E and αSMA expression on the tissue grafted and cell-grafted tissue proved tumor establishment. Moreover, the absence of inflammatory cells in cell grafted model when compared to tissue grafted model indicates advancements in our model compared to the tissue grafted model.

Uterine leiomyomas are characterized by a large amount of ECM proteins that lead to fibrosis.[35] Previous studies have demonstrated that the ECM-associated protein type I collagen was found to be overexpressed and to play an important role in the pathogenesis of uterine leiomyomas.[36] Flavopiridol treatment reduced the expression of type I collagen in uterine leiomyoma cells (data not shown) suggesting that flavopiridol may reduce ECM production, possibly leading to decreased tumor volume. The dosing schedule of flavopiridol administrated in this study is based on previous preclinical reports.[37] It was reported that after daily ip injections of 7.5 mg/kg of flavopiridol for 5 days, 11 of 12 advanced stage subcutaneous human promyelocytic leukemia cell xenografts underwent complete regression. In phase I clinical trials, flavopiridol was given as a continuous 72 hours infusion every 2 weeks. The maximum tolerated dose (MTD) of infused flavopiridol was 50 mg/m^2 daily for 3 days, with dose-limiting secretory diarrhea at 62.5 mg/m^2 daily for 3 days. The MTD was 78 mg/m^2 daily for 3 days in combination with antidiarrheal prophylaxis, with dose-limiting hypotension at 98 mg/m^2 daily for 3 days. The plasma levels observed were in the range of 200 to 400 nmol/L.[38] Although flavopiridol may induce apoptotic cell death at high concentrations, the lack of cell death at concentrations with profound inhibitory effects on cell proliferation and G1 phase suggests that cytostasis and not cytotoxicity is the most important biological effect of this compound.

In conclusion, this study demonstrated that the pan-CDK inhibitor flavopiridol is effective in inhibiting uterine leiomyoma tumor growth in a xenograft model, correlating with in vitro data. These findings strengthen our hypothesis that flavopiridol possesses potent antitumor properties in uterine leiomyoma cells and may be associated with G1 phase arrest and a reduction in the levels of cyclin A and Rb. This compound inhibits proliferation of human uterine leiomyoma without inducing cell death and thus may be advantageous in treating human tumors by restricting proliferation in a manner unlikely to induce myometrium cytotoxicity and death. These data may provide a basis for flavopiridol treatment of patients with uterine leiomyoma.

Authors' Note

Hyun-Gyo Lee, and Jong-Woo Baek contributed equally to this article.

Declaration of Conflicting Interests

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