Halofuginone inhibits proliferation and collagen production by leiomyoma smooth muscle cells.

Romana Nowak*, Philip Low, Jen Wubben, and Robert Belton Jr.
Department of Animal Sciences, University of Illinois, Urbana, Illinois

ABSTRACT

One of the hallmarks of uterine leiomyomas is the excessive collagen that is laid down by the smooth muscle cells (SMCs) of these tumors. We are interested in the potential use of anti-fibrotic drugs as therapeutic agents for treatment of leiomyomas. Halofuginone is a drug that has been reported to inhibit collagen production and proliferation by renal mesangial cells and vascular SMCs.

Objectives: To determine 1) whether halofuginone inhibits proliferation of leiomyoma SMCs through effects on DNA synthesis and/or apoptosis; 2) halofuginone inhibits collagen type I and type III synthesis; and 3) whether these inhibitory effects are due to inhibition of TGFβ production.

Methods: Myometrial and leiomyoma SMCs from 5 patients were used to assess the effects of halofuginone on serum-stimulated DNA synthesis. Effects on apoptosis were measured using a caspase 3/7 assay. Cells from patients were treated with 0, 25, 100 or 200 ng/ml halofuginone for 24 hrs. The effects of halofuginone on levels of collagen type I, collagen type III, TGFβ1, and TGFβ3 mRNAs after 24 hrs treatment were determined in myometrial and leiomyoma SMCs by northern blot analysis.

Results: Halofuginone inhibited DNA synthesis in a dose dependent manner in both myometrial and leiomyoma SMCs. Caspase 3/7 levels were not increased over the untreated control for any of the concentrations tested. Halofuginone did not inhibit mRNA levels of collagen type I, collagen type II, or the TGFβs in myometrial SMCs. In contrast, halofuginone caused a dose dependent suppression of both collagen type I and type III mRNA levels in leioyoma SMCs. In addition, halofuginone inhibited both TGFβ1 and TGFβ3 mRNA levels in leiomyoma SMCs.

Conclusion: These results suggest that halofuginone may be a useful therapeutic agent for treatment of leiomyomas.

INTRODUCTION

Uterine leiomyomas or fibroids are common, benign tumors found in women that cause abnormal uterine bleeding. There are currently several surgical treatments available for women with these tumors including hysterectomy, myomectomy, focused ultrasound, endometrial ablation, and uterine artery embolization. However, few nonsurgical medical treatments for fibroids are available. Halofuginone is an anti-fibrotic compound that inhibits cell proliferation and collagen production. This compound has been shown to reduce the size of several types of tumors in animal models. Halofuginone is currently in phase III clinical trials for treatment of bladder cancer under the name of Tempstatin (Collgard Pharmaceuticals). We hypothesize that halofuginone may be a good therapeutic agent for treatment of fibroids and investigated its effects on fibroid and myometrial SMCs in vitro. Our previous studies showed that halofuginone treatment reduces cell number in vitro. Our first goal in the present study was to determine whether this decrease in cell number was due to an increase in apoptosis, a decrease in cell proliferation or a combination of the two. Our second goal was to determine whether halofuginone inhibits collagen production by leiomyoma and myometrial SMCs and whether this antifibrotic effect occurs as a result of inhibition of TGFβ production.

MATERIALS AND METHODS

Primary cultures of fibroid and myometrial SMCs were established from surgical biopsies obtained from hysterectomy cases. Myometrial and fibroid cells from five different patients were used in the various experiments.

Cell proliferation was assessed by measuring changes in DNA synthesis using [3H]thymidine uptake assays. For each DNA synthesis assay, myometrial and fibroid cells were grown in 96 well plates to approximately 75-80% confluence. Cells were given treatment medium consisting of DMEM + 10% FBS in the absence or presence of halofuginone for a period of 24 hours. [3H]thymidine was added during the last 6 hours of treatment. At the end of the treatment period cells were harvested onto filter mats and read on a Wallac beta-counter. Doses of halofuginone tested were 0.1, 1, 5, 10, 25, 50, 100 and 200 ng/mL. Five experiments were carried out for each cell type.

Effects of halofuginone on apoptosis were measured using the Caspase-Glo 3/7 assay from Promega. This is a luminescent assay that measures caspase activities in cultures of adherent cells. Cells were treated for 24 hr, lysed, and a proluminescent caspase 3/7 substrate was added which when cleaved by either caspase 3 or 7 generates a luminescent signal produced by luciferase. Cells were treated with staurosporine as a positive control and interferon-beta as a negative control.

Effects of halofuginone on collagen type I, collagen type III, TGFβ1, and TGFβ3 mRNAs were assessed using northern blot analysis. Cells were treated with either 0, 25, 100 or 200 ng/mL doses of halofuginone for 24 hours. 10 micrograms of total RNA from each treatment group was resolved on a 1% Glyoxal RNA gel (Ambion) and transferred to positively charged nylon. Hybridization was performed using random-primed DNA probes labeled with 32P alpha dATP at 45 °C for 14 hours in Ultrahyb solution (Ambion). Blots were washed at high stringency (0.1X SSC, 0.1% SDS, 45°C) and exposed to film with screens.

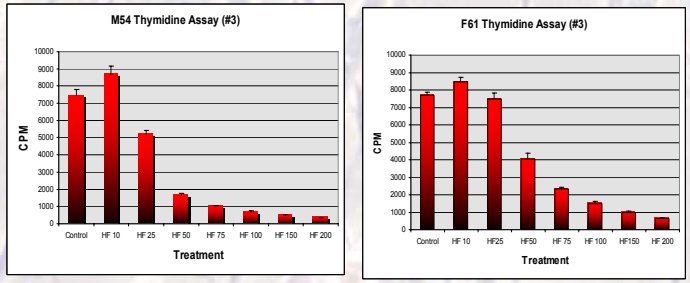

Figure 2. Effects of halofuginone on DNA synthesis by cultured myometrial and leiomyoma SMCs. Two representative experiments are shown of a total of 10 experiments.

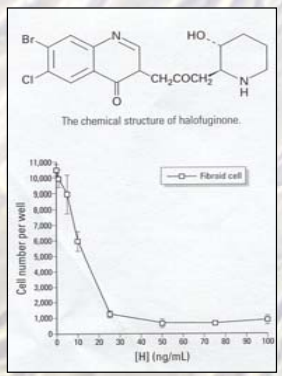

Figure 1

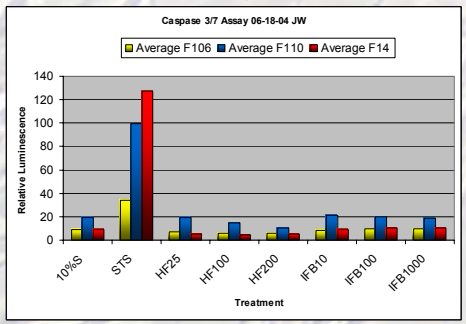

Figure 3. Effect of halofuginone on apoptosis of leiomyoma SMCs. Cells were treated with either staurosporine (STS, 0.25 uM), halofuginone (25, 100 or 200 ng/ml) or interferon-beta (10, 100, and 1000 U/ml) for 24 hr. Results are shown for three leiomyoma cell lines. Similar results were obtained for myometrial SMCs (data not shown).

RESULTS

Thymidine Assay
Halofuginone inhibited DNA synthesis in a dose dependent manner in every leiomyoma or myometrial cell line tested. Concentrations of halofuginone between 25-50 ng/ml resulted in 50% inhibition of DNA synthesis. The responses of leiomyoma and myometrial SMCs were quite similar.

Caspase 3/7 Assay
Halofuginone did not cause any increase in caspase 3/7 levels in any of the three leiomyoma or myometrial cell lines tested.

Northern Blot Analysis
Halofuginone caused a dose-dependent 30-65% decrease in the mRNA levels of collagen type I, collagen type III, TGFβ1 and TGFβ3 in leiomyoma SMCs. Halofuginone had little to no effect on myometrial SMCs.

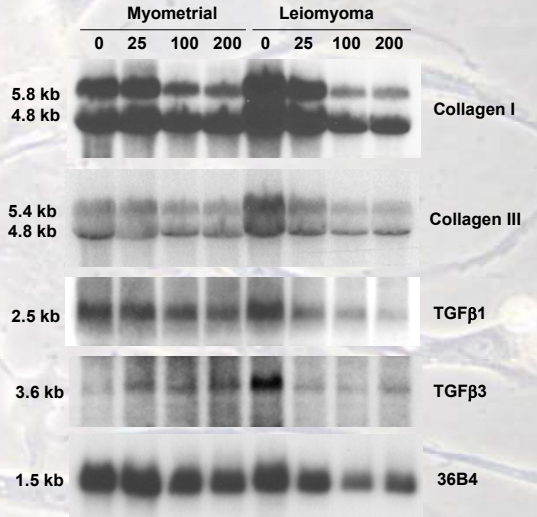

Figure 4. Effects of halofuginone on the expression of collagen and TGFβ. Cells were treated with halofuginone at 0, 25, 100, or 200 ng/ml. Three experiments using myometrial and leiomyoma SMCs from three patients were carried out. Cells were treated for 24 hours and cells were harvested for total RNA using Trizol reagent (Life Technologies). Collagen type I and type III mRNA levels were reduced by 30-60% in leiomyoma SMCs treated with halofuginone. TGFβ mRNA levels were also reduced by 30-65% in leiomyoma SMCs. Myometrial SMCs showed only a 5-10% decrease in collagen type I.

DISCUSSION

The results of our studies show that halofuginone is a potent inhibitor of DNA synthesis in both normal myometrial and leiomyoma SMCs. Halofuginone does not however, cause an increase in apoptosis in these cells. The antifibrotic effect of halofuginone was only evident in the leiomyoma SMCs where an inhibitory effect of halofuginone on collagen and TGFβ mRNA levels was observed. No similar inhibitory effect was observed for myometrial SMCs. The difference in response of the two cell types suggests that halofuginone is able to specifically target abnormal collagen production. These data support our hypothesis that halofuginone (Tempstatin) may be an effective medical therapy for treatment of leiomyomas.

ACKNOWLEDGEMENTS

This work was supported by NIH HD046227 to R.A..N. We also thank Eddie Greene of the Mayo Clinic for useful discussions.