Leiomyoma Cells in 3-Dimensional Cultures Demonstrate an Attenuated Response to Fasudil, a Rho-Kinase Inhibitor, When Compared to 2-Dimensional Cultures

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Abstract

Uterine leiomyomata are common benign tumors in women of reproductive age and demonstrate an attenuated response to mechanical signaling that involves Rho and integrins. To further characterize the impairment in Rho signaling, we studied the effect of Rho-kinase inhibitor, fasudil, on extracellular matrix production, in 2-dimensional (2D) and 3-dimensional (3D) cultures of leiomyoma and myometrial cells. Leiomyoma 2D cultures demonstrated a rapid decrease in gene transcripts and protein for fibronectin, procollagen 1A, and versican. In 3D cultures, fibronectin and procollagen 1A proteins demonstrated increased levels at lower concentrations of fasudil, followed by a concentration-dependent decrease. Versican protein increased up to 3-fold, whereas fibromodulin demonstrated a significant decrease of 1.92-fold. Myometrial 2D or 3D cultures demonstrated a decrease in all proteins after 72 hours of treatment. The 3D leiomyoma cultures demonstrated a significant increase in active RhoA, followed by a concentration-dependent decrease at higher concentrations. A concentration-dependent increase in phospho-extracellular regulated signal kinase and proapoptotic protein Bax was observed in 3D leiomyoma cultures. Fasudil relaxed the contraction of the 3D collagen gels caused by myometrium and leiomyoma cell growth. These findings indicate that the altered state of Rho signaling in leiomyoma was more clearly observed in 3D cultures. The results also suggest that fasudil may have clinical applicability for treatment of uterine leiomyoma.

Keywords

uterine leiomyoma, 2D and 3D cultures, extracellular matrix, RhoA, fasudil, gel contraction

Introduction

Uterine smooth muscle leiomyomas are common benign fibrotic pelvic tumors, affecting about 40% reproductive-age women, increasing to 70% to 80% cumulative incidence by the age of 50.¹⁻⁴ Further, they represent a considerable economic burden for society and patients alike.^{5,6} Despite their widespread prevalence, the pathogenesis of leiomyomas is not completely understood.^{4,7,8} It is an accepted fact that leiomyomas are characterized by excessive and disordered extracellular matrix (ECM) which contributes to the total bulk of the tumor.^{9,10} Furthermore, the tumor is characterized by increased stiffness as studied in vitro^{11,12} or in vivo by magnetic resonance elastography.^{13,14}

Cells sense the changes in the surrounding stiffness as mechanical cues and translate them into biochemical signals.¹⁵ Norian and coworkers¹² detected an attenuated response of the leiomyoma cells in culture to mechanical cues. They further hypothesized that leiomyoma cells in vivo have adapted to their stiff environment and thus were insensitive to subtle mechanical cues in vitro. Increased stress in leiomyomas in vivo is

supported by increased levels of stress protein, ATF3.¹⁶ Notably, levels of active RhoA were higher in leiomyoma cells when compared to myometrium cells in vitro.^{12,17} The increased active RhoA and integrins may play a role in the attenuated response of leiomyoma cells.¹⁷ Integrins are transmembrane adhesion receptors that couple ECM to the intracellular cytoskeleton to influence cell proliferation and differentiation. Increased expression of integrins has been demonstrated in leiomyomas,^{17,18} and inhibition of integrin $\beta 1$ results in

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decreased RhoA activity.¹⁷ The small GTPase RhoA belongs to the Rho subfamily and has been implicated in many cellular functions, such as cell adhesion, growth control, and cell contraction.^{19,20} Overexpression of RhoA has also been demonstrated in breast cancer cells.²¹⁻²³ One of the main effectors of Rho is the Rho-associated protein kinase (ROCK), a serine/threonine kinase, which regulates smooth muscle contraction and cytoskeletal reorganization.²⁴

Fasudil, a highly selective inhibitor of ROCK, has been demonstrated to have antifibrotic effects in models of liver, renal, and cardiac fibrosis.²⁵⁻³⁰ Fasudil has been approved in Japan for the treatment of cerebral vasospasm following surgery for subarachnoid hemorrhage and associated cerebral ischemic symptoms.^{31,32} Inhibition of Rho/ROCK pathway has been shown to play an important role in collagen production in hepatic stellate cells.³³ The authors observed a decrease in collagen 1 transcript levels, together with an increase in matrix metalloproteinase 1 levels after fasudil treatment of hepatic stellate cells. The authors concluded that an increase in collagenase activity consequently led to degradation of the collagen matrix.

Uterine leiomyomata are a 3D disease, and in vivo conditions are better simulated by growing cells in a 3D cultural format, compared to 2-dimensional (2D) cultures.³⁴ The 2D leiomyoma cultures do not develop a 3D structure necessary to evaluate the ECM, which constitutes the majority of these uterine tumors. Furthermore, functional studies evaluating genetic manipulations or therapeutic strategies are limited by an inability to determine the impact of such treatments on the matrix development and dissolution of the in situ ECM. We have developed a 3D model for leiomyoma wherein the cells maintain the genomic changes relative to ECM genes, found in the surgical specimens when compared to patient-matched myometrium.³⁴ Further, we demonstrated that cells in this model system responded to transforming growth factor β 3 (TGF- β 3) and integrin β 1 inhibition,³⁴ estrogen and progesterone,³⁵ and drugs such as the cytochrome P26A1 inhibitor liarozole³⁶ and gonadotropin-releasing hormone antagonist, cetrorelix.³⁷ Support for this model system comes from recent work by Levy and coworkers³⁶ who demonstrated a decrease in TGF- β 3 protein amount in the ECM of 3D leiomyoma culture treated with liarazole. For the present study, we used both the 2D and 3D in vitro model of leiomyoma cells to assess the effects of fasudil hydrochloride on Rho/ROCK signaling and ECM proteins to further characterize the altered state of mechanical signaling in leiomyoma cells. Since leiomyoma tumors are mainly composed of collagen matrix and demonstrate an increased expression of ECM proteins fibronectin (FN1) and versican (V0), and leiomyoma cells show increased levels of RhoA, we hypothesized that inhibition of ROCK might inhibit ECM production in leiomyoma and/or myometrium cells.

Materials and Methods

Two-Dimensional RNA and Protein Protocol

Immortalized myometrial and leiomyoma cells³⁸ were plated in 6-well plates at concentration of 2×10^4 cells/well and

maintained in complete media (CM; 10%; Dulbecco Modified Eagle Medium [DMEM]-F12 [with phenol red; Invitrogen] supplemented with 10% fetal bovine serum [FBS; HyClone], $1 \times$ glutamax, $1 \times$ penicillin and streptomycin (Pen Strep) antibiotics and 0.25 μ g/mL amphotericin B [all from Invitrogen]) at 37°C and 5% CO₂. Once the monolayer cultures reached approximately 40% confluence, the media was changed to CH 10% (DMEM-F12 [without phenol red, Invitrogen] supplemented with 10% charcoal-treated FBS [Hyclone], 1X Glutamax, 1X Pen Strep antibiotics, and 0.25 μ g/mL amphotericin B) for 48 hours. This was followed by treatment with fasudil hydrochloride (Tocris Bioscience, Bristol, United Kingdom) at concentrations ranging from 10^{-7} mol/L to 10^{-5} mol/L in CH 10% for 24 hours and 72 hours. Fresh media containing treatment concentrations of fasudil were replaced after 48 hours. At specified time points, cells were either collected for RNA or protein for further analysis. Experiments were repeated 3 times with a minimum of 2 replicates for each experiment.

The RNA was purified using the Direct-zol RNA miniprep (Zymo Research) method according to manufacturers' protocol. Briefly, samples in trizol were mixed with ethanol at 1:1 (v: v) ratio and mixed by vortexing. The mixture was loaded on to the Zymo-spin column provided and centrifuged at 12 000g for 1 minute. After DNase treatment and 2 washes with the wash solution provided, the RNA was eluted using the DNase/RNase-free water and measured spectrophotometrically. The RNA was diluted and stored at -80°C .

Three-Dimensional RNA and Protein Protocol

Cell growth in 3D culture. Three-dimensional collagen gel was prepared as described previously³⁴ with some modifications. Briefly, rat tail collagen 1 was used at a final concentration of 3 mg/mL (60%, Cultrex) with 10% phosphate-buffered saline (PBS, 10 $\times$), 28% CM 10%, and 1 N NaOH (1.5%-2%). The gels were chilled on ice at all times, and all steps were carried out in safety hood. Immortalized myometrium and leiomyoma cells grown in CM 10% at 37°C in the presence of 5% CO₂ were trypsinized, counted, and resuspended in CM 5% media (DMEM/F12 containing 5% FBS). From this stock, cells were mixed with collagen 1 solution to give a final concentration of 0.5×10^4 cells/mL such that the volume of cell suspension was less than 10% of final solution. For RNA and protein collection, the cells were plated at a concentration of 1×10^5 cells/well in 6-well plates. Fresh 5% media was replaced every other day until gels were visually 40% confluent (8-10 days). The CM 5% media was replaced by CH 10% for 48 hours. This was followed by treatment with fasudil hydrochloride in CH 10% for 72 hours at concentrations as described for 2D cultures. Fresh media containing treatment concentrations of fasudil were replaced after 48 hours.

RNA and protein collection. After specified time points, the gels in each of the 6 wells were divided into 2, one for RNA and other for protein. The experiment was repeated twice, 2 replicates for each experiment. RNA and protein collection has been described before.³⁴ Briefly, RNA was isolated using Trizol

method. The gels, still in plates, were washed once with ice-cold $1 \times$ PBS before being put into 5-mL tubes and centrifuged at 5000 rpm/4°C for 6 minutes. The solution was decanted and Trizol (0.7 mL) was added and sample rested on ice for 10 minutes or frozen at -80°C for storage prior to analysis. The gels in Trizol were sonicated 2×30 seconds each with 10-minute rest on ice in between until the gels dispersed. Further steps were according to the manufacturers' protocol (Invitrogen). RNA was purified using Turbo DNase (Ambion) and measured and stored at -80°C .

For Western blot analysis, the 3D gels were transferred into Eppendorf tubes on ice and washed 2 more times with ice-cold $1 \times$ PBS, and each wash was followed by centrifugation at 5000 rpm/6 min/4°C. To each tube, 0.4 mL of radioimmunoprecipitation assay (RIPA) extraction buffer containing $1 \times$ of Halt protease inhibitor, phosphatase inhibitor, and EDTA (Pierce Biotech, Rockford, Illinois) was added. The samples were sonicated till the gels were completely dispersed. The tubes were centrifuged at 13 000 rpm for 20 minutes at 4°C. A clear solution was seen which was aliquoted and stored at -80°C . Protein concentrations were determined using bicinchoninic acid (BCA) assay (Pierce Biotech).

Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) method was used to evaluate expression of ECM genes; procollagen 1A, V0, and FN1 as described previously.³⁴ The 18S ribosomal RNA gene was used as an internal control, and each sample was analyzed in triplicate. Bio-Rad iCycler software, version 3.1, was used for data analysis.

Measurement of RhoA Activity

Absorbance-based G-Lisa RhoA activation assay and total RhoA enzyme-linked immunosorbent assay (Cytoskeleton, Inc) were used according to manufacturers' protocol. Briefly, protein was collected from treated myometrial and leiomyoma cells grown in 6-well plates for 2D experiments, centrifuged, aliquoted, snap frozen in liquid nitrogen, and stored at -80°C . For 3D cultures grown in 24 wells, the gels were washed once with ice-cold PBS before 0.3 mL of lysis buffer provided by the manufacturer was added. Samples were sonicated twice at 4°C before centrifugation, were snap frozen, and stored at -80°C . Protein amount was measured using the Precision Red provided in the kit. Absorbance was measured at 490 nm using spectrophotometric plate reader. The absorbance was normalized to untreated control samples, and all samples are reported as fold change. Data are representative replicates of 3 separate experiments.

Western Blot Analysis

Protein was isolated using RIPA lysis and extraction buffer containing $1 \times$ Halt protease and phosphatase inhibitor (all

from Pierce Biotech). Briefly, aliquots of the proteins extracted from cultured cells treated with different concentrations of fasudil for different time points underwent electrophoresis on a sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. For the detection of proteins, blots were incubated overnight at 4°C, with primary antibody against collagen 1A (COL-1A; sc-59772; 1:200), Bax (sc-7480; 1:200), V0 (ab286711:1000), FN1 (ab6584, 1:5000), fibromodulin (FMOD; sc-166498, 1:250), and total RhoA (Cell signaling #2117). Horseradish peroxidase (HRP)-conjugated secondary antibody (ImmunoPure, Pierce Biotech) in combination with the SuperSignal West Pico (Pierce Biotech) was used for detection of the proteins. As an internal standard between the samples, HRP-labeled antihuman β -actin (Sigma; 1:50 000) was used.

Gel Contraction Assay

Three-dimensional collagen gel was prepared as described earlier. Briefly, cells at a concentration of $1 \times 10^5/\text{mL}$, plated in a 24-well culture dish, were allowed to grow for 2 weeks reaching a visual confirmation of 30% to 40% cell confluence. The media was changed to CH 10% (DMEM/F12 media with 10% charcoal-treated FBS) for 24 hours. The cells in 3D culture were treated with different concentrations of fasudil for 72 hours. At the end of the treatment, for comparison and photographs, the gels were moved to 6-well culture plate. The experiment was repeated 3 times.

Statistical Analysis

For real-time RT-PCR data, the results are reported as mean $\pm$ standard error of the mean. For each result, the average expression of 3 replicates was calculated before relative quantification using normalization against housekeeping gene (18S) was done. Relative expression was calculated based on Pfaffl method.³⁹ Wilcoxon signed-rank test was used for nonparametric statistical evaluation. Values below $P < .05$ were considered significant. For Western blot analysis, calculations were done using QualityOne software 4.1 from Bio-Rad. Data are presented as fold difference between relative density units of treated and untreated samples and were corrected for internal control, β -actin.

Results

Leiomyoma Response in 2D Cultures

As demonstrated in Table 1, fasudil treatment of leiomyoma cells grown on polystyrene culture plates (2D format) in 24 hours resulted in a decrease in ECM gene transcripts. Fibronectin transcripts were increased at low fasudil concentrations (1.34 ± 0.27 -fold), followed by a decrease at 10^{-6} mol/L (0.84 ± 0.02 -fold) and 10^{-5} mol/L (0.76 ± 0.05 fold) compared to untreated controls. Pro-COL-1A transcripts demonstrated a similar 1.2- to 1.4-fold downregulation at higher concentrations of fasudil. By end of 72 hours of

Table 1. Expression of Extracellular Genes in 2-Dimensional Leiomyoma Cultures.^a

Genes	10 ⁻⁷ mol/L Fasudil		10 ⁻⁶ mol/L Fasudil		10 ⁻⁵ mol/L Fasudil	
	24 h	72 h	24 h	72 h	24 h	72 h
Fibronectin (FNI)	1.34 ± 0.27 ^b	1.05 ± 0.22	0.84 ± 0.02 ^b	0.88 ± 0.12	0.76 ± 0.05 ^b	0.92 ± 0.06
Collagen IA (COL-1A)	1.11 ± 0.07	0.97 ± 0.05	0.83 ± 0.03 ^b	1.03 ± 0.06	0.71 ± 0.02 ^b	1.21 ± 0.04
Versican (V0)	0.41 ± 0.03 ^b	1.21 ± 0.11	0.65 ± 0.08 ^b	0.53 ± 0.10 ^b	0.61 ± 0.19 ^b	0.38 ± 0.07 ^b
Fibromodulin (FMOD)	1.61 ± 0.08 ^b	0.86 ± 0.15	1.28 ± 0.23	1.04 ± 0.05	1.09 ± 0.09	0.64 ± 0.09 ^b

^aData shown represent mean ± standard error of the mean (SEM) fold difference in expression in treated leiomyoma cells relative to leiomyoma cells at baseline.
^bP < .05.

treatment, expression of both genes resembled untreated control samples. Transcripts of the V0 isoform of V0 were significantly decreased at 24 hours and 72 hours of treatment compared to the untreated control. The reduction ranged from 2-fold at the lower concentration of fasudil (0.41 ± 0.03 ; 10^{-7} mol/L) to 1.54-fold (0.65 ± 0.08) at 10^{-6} mol/L and 1.64-fold (0.61 ± 0.19) at 10^{-5} mol/L concentrations. At higher concentrations, after the end of 72 hours of treatment, V0 transcripts were significantly reduced to 1.9-fold at 10^{-6} mol/L and 2.6-fold at 10^{-5} mol/L, whereas at 10^{-7} mol/L, gene expression resembled the untreated control. Compared to untreated controls, FMOD transcripts demonstrated significant increase after 24-hour treatment (1.61 ± 0.08 -fold) at 10^{-7} mol/L and no significant change at end of 72-hour treatment. At 10^{-6} mol/L and 24-hour treatment, the observed increase (1.28 ± 0.23 -fold) was not significant, and no changes were observed at 72-hour treatment. At the highest concentration (10^{-5} mol/L) and at 72-hour treatment, leiomyoma cells demonstrated a decrease in FMOD transcripts by 0.64 ± 0.09 -fold compared to untreated control.

In order to confirm that transcript changes translated to changes in protein expression, we measured the total amount of extracellular proteins in leiomyoma cells at the end of 72 hours of treatment (Figure 1). Protein levels of FN1 and COL-1A demonstrated a concentration-dependent decrease at all concentrations studied. Fibronectin demonstrated a significant decrease of 1.20-fold (0.83 ± 0.008) at 10^{-7} mol/L, 1.43-fold (0.71 ± 0.007) at 10^{-6} mol/L, and 1.69-fold (0.59 ± 0.006) at 10^{-5} mol/L. The COL-1A protein similarly demonstrated a significant decrease of 1.54-fold (0.65 ± 0.04) at 10^{-7} mol/L, 1.72-fold (0.58 ± 0.03) at 10^{-6} mol/L, and 2.43-fold (0.41 ± 0.02) at 10^{-5} mol/L. Versican demonstrated a significant 1.6 ± 0.1-fold increased level of protein at the lowest concentration of fasudil (10^{-7} mol/L) followed by a decrease of 1.5-fold at 10^{-6} mol/L (0.67 ± 0.08) and 2-fold at 10^{-5} mol/L (0.49 ± 0.06). Significant decreases in FMOD protein were observed at 10^{-6} mol/L (0.74 ± 0.1) and 10^{-5} mol/L (0.29 ± 0.03) concentrations of fasudil.

Leiomyoma Response in 3D Cultures

Response of both transcript levels and protein concentration in leiomyoma cells grown in 3D cultures was examined after 72 hours of fasudil treatment. No significant changes were

Figure 1. Western analysis of extracellular matrix proteins in leiomyoma cells grown in 2-dimensional (2D) culture after 72 hours of fasudil treatment. Significant (*P ≤ .05) changes in fibronectin (FN1), collagen IA (COL-1A), versican (V0), and fibromodulin (FMOD) are calculated to untreated controls (0 mol/L). Data shown represent mean ± standard error of mean (SEM) fold. Results were normalized to β-actin.

observed in gene expression levels of transcript encoding FN1, pro-COL-1A, and FMOD. Versican demonstrated a significant increase of 1.56 ± 0.02 -fold at 10^{-5} mol/L (data not shown). Most ECM proteins increased in response to fasudil treatment at the end of 72-hour treatment at low concentrations. As depicted in Figure 2, FN1 and COL-1A demonstrated a significant increase at lower concentrations of fasudil (10^{-7} mol/L and 10^{-6} mol/L) followed by levels equivalent to the untreated controls at 10^{-5} mol/L. Fibronectin 1 protein increased significantly to 1.74 ± 0.3 -fold at 10^{-7} mol/L when compared to untreated controls. The protein levels fell to 1.40 ± 0.21 -fold at 10^{-6} mol/L and reached control levels at 10^{-5} mol/L (1.19 ± 0.36 -fold). A similar pattern was observed for levels of COL-1A protein (Figure 2), which demonstrated a significant (2.17 ± 0.42 -fold) increased amount at 10^{-7} mol/L followed by a decrease to 1.87 ± 0.25 -fold at 10^{-6} mol/L that reached untreated control levels of 0.96 ± 0.21 -fold at 10^{-5} mol/L. Versican protein demonstrated a significant increase at all concentrations that was concentration dependent. Versican protein was 1.58 ± 0.24 -fold higher at 10^{-7} mol/L, increased to 1.98 ± 0.06 at 10^{-6} mol/L and 3.00 ± 0.21 at 10^{-5} mol/L compared to untreated control. Fibromodulin did not demonstrate any significant change at lower concentrations, but protein levels fell to almost 2-fold (0.52 ± 0.19) at

Figure 2. Western analysis of extracellular matrix proteins in leiomyoma cells grown in 3-dimensional (3D) culture after 72 hours of treatment with fasudil. Significant ($*P \leq .05$) changes are calculated to untreated controls (0 mol/L). Data shown represent mean $\pm$ standard error of the mean (SEM) fold difference in protein amount in treated leiomyoma cells relative to untreated control. Results were normalized to β -actin. Data presented are representative of 3 experiments.

10^{-5} mol/L compared to the untreated leiomyoma control. Thus, the response of leiomyoma cells in 3D culture was biphasic, with an increase in ECM protein at low concentrations, but reduced secretion at high concentrations, with the notable exception of versican V0 transcript.

Myometrium Response in 2D and 3D Cultures

Changes in expression of genes in response to fasudil were also examined in myometrial cells grown in 2D or 3D culture (Figure 3A-D). In the 2D culture system, FN1 demonstrated a significant reduction in transcript levels within 24 hours of treatment at 10^{-6} mol/L (0.77 ± 0.041) and 10^{-5} mol/L (0.66 ± 0.036) fasudil compared to untreated controls, which increased, although not significantly, at the end of 72-hour exposure (Figure 3A1). Fibronectin protein demonstrated a significant lower expression at 10^{-6} mol/L (0.45 ± 0.12) and 10^{-5} mol/L (0.68 ± 0.23) treatment at the end of 72-hour exposure (Figure 3A2). In 3D format and at the end of 72-hour treatment, the myometrial cells demonstrated no statistically significant effect at transcript (Figure 3A3) or protein (Figure 3A4) level for FN1 gene in response to fasudil. Pro-COL-1A gene expression demonstrated statistically significant reduction only at the protein level in myometrial cells grown in both 2D and 3D (Figure 3B). As depicted in Figure 3B2, in 2D system, COL-1A protein was significantly reduced at 10^{-6} mol/L (0.56 ± 0.18) and 10^{-5} mol/L (0.48 ± 0.14). In 3D culture, reduction in protein amounts were observed at all concentrations tested, compared to the untreated control (Figure 3B4). Versican isoform V0 demonstrated a significant reduction in transcripts at the end of 72-hour treatment in both 2D and 3D cultures of myometrium cells (Figure 3C). In the 2D cultures, a concentration-dependent reduction in V0 transcripts ranged from 2-fold at 10^{-7} mol/L, 2.9-fold at 10^{-6} mol/L to

3.4-fold at 10^{-5} mol/L (Figure 3C1). The reduction in V0 gene transcripts was statistically significant, 1.5-fold in 3D culture at 10^{-6} mol/L (0.68 ± 0.04) and 10^{-5} mol/L (0.65 ± 0.07 ; Figure 3C3). At end of 72-hour treatment, compared to untreated controls, in 2D cultures, lower amounts of the V0 protein were observed at 10^{-6} mol/L (0.51 ± 0.09) and 10^{-5} mol/L (0.72 ± 0.16 ; Figure 3C2). In 3D cultures, a concentration-dependent decrease in V0 protein was observed ranging from 2.07-fold (0.48 ± 0.13) at 10^{-7} mol/L to 4.17-fold (0.24 ± 0.06) at 10^{-5} mol/L (Figure 3C4). As depicted in Figure 3D1, FMOD gene transcripts demonstrated a significant increase within 24 hours treatment of 2D cultures; 10^{-6} mol/L (1.46 ± 0.06) and 10^{-5} mol/L (1.31 ± 0.002), an effect that was reversed at end of 72-hour treatment with significant reduction of 0.79 ± 0.02 -fold at 10^{-6} mol/L and 0.64 ± 0.07 -fold at 10^{-5} mol/L. The reduction observed at transcript level translated to decreased protein at all concentrations (Figure 3D2), 10^{-7} mol/L (0.88 ± 0.30), 10^{-6} mol/L (0.42 ± 0.06), and 10^{-5} mol/L (0.24 ± 0.03). In 3D cultures, at the end of 72-hour treatment, a significant reduction in FMOD transcripts (Figure 3D3) was observed only at 10^{-5} mol/L (0.62 ± 0.02) although the protein (Figure 3D4) demonstrated a lower amount at 10^{-6} mol/L (0.68 ± 0.02) and 10^{-5} mol/L (0.39 ± 0.14). In sum, we noted that the ECM proteins in myometrial cells responded similarly to fasudil treatment irrespective of growth in 2D or 3D culture system.

Active RhoA Analysis in 2D and 3D Cultures

We previously observed that when cyclic strain was applied to leiomyoma cells, inhibition of ROCK by LY-27632 did not facilitate cell reorientation.¹² To further characterize the altered state of Rho signaling, we measured active and total RhoA in fasudil-treated myometrial and leiomyoma cells grown in 2D or 3D cultures. Leiomyoma cells demonstrated a higher amount of active RhoA in 2D (1.53 ± 0.06 -fold) and 3D (2.1 ± 0.11 -fold) when compared to myometrial cells (Figure 4A). No significant changes were observed in total RhoA with fasudil treatment of leiomyoma or myometrium cells in 2D and 3D cultures (data not shown). In 2D cultures, no significant effect on active RhoA was observed in myometrial cells after fasudil treatment; whereas leiomyoma cells demonstrated slight decrease in active RhoA at 10^{-6} mol/L (0.89 ± 0.008 -fold) and 10^{-5} mol/L (0.86 ± 0.01 -fold) compared to untreated control. Active RhoA expression was increased in both myometrial and leiomyoma cells in 3D cultures after 72 hours of treatment. As shown in Figure 4B, the lower concentrations of fasudil at 10^{-7} mol/L increased active RhoA by 1.19-fold (myometrium) and 1.32-fold (leiomyoma) when compared to untreated controls. At higher concentrations, in myometrial cells, active RhoA reached the untreated control, whereas in leiomyoma cells, the activity of RhoA was significantly lowered compared to control. We interpret the findings to indicate an altered state of Rho signaling in leiomyoma cells, which was responsive to fasudil at higher concentrations.

Figure 3. Transcript and protein analysis of extracellular matrix (ECM) genes, fibronectin (A), procollagen IA (B), versican (C), and fibro-modulin (D) in myometrial cells after fasudil treatment. Under each gene (A-D): (1) Represents the gene expression at transcript level in 2-dimensional (2D) cultures at the end of 24 and 72 hours of treatment; (2) protein amount in 2D cultures at end of 72 hours of treatment; (3) expression of gene transcript in 3-dimensional (3D) cultures at the end of 72-hour treatment; (4) protein amount in 3D cultures at end of 72 hours of treatment. Significant (* $P \leq .05$) changes are calculated to untreated controls (0 mol/L). Figures represent data from 3 experiments.

Activation of Extracellular Regulated Signal Kinase

To further explore the mechanisms responsible for the increase in ECM proteins after fasudil treatment in 3D leiomyoma cultures, we examined activation of MAPK/extracellular regulated signal kinase (ERK) pathway proteins. As depicted in Figure 5, in 2D and 3D cultures of myometrial cells, phospho-ERK (pERK) was significantly reduced in response to fasudil treatment. Compared to untreated controls, 2D cultures of myometrium cells demonstrated a 1.25-fold reduction at 10^{-7} mol/L (0.79 ± 0.08) followed by a 1.5-fold reduction at concentrations of 10^{-6} mol/L (0.66 ± 0.1) and 10^{-5} mol/L (0.68 ± 0.006). A concentration-dependent fold decrease was observed in 3D cultures ranging from 0.79 ± 0.15 (10^{-7} mol/L), $0.51 \pm$

0.06 (10^{-6} mol/L), and to 0.39 ± 0.02 (10^{-5} mol/L) compared to untreated control. In response to fasudil treatment, 2D cultures of leiomyoma cells also demonstrated a reduction in pERK ranging from 1.48-fold at 10^{-6} mol/L (0.67 ± 0.08) to 2.42-fold at 10^{-5} mol/L (0.41 ± 0.1). In 3D leiomyoma cultures, a significant increase in pERK was observed at all concentrations of fasudil compared to untreated controls. At 10^{-7} mol/L fasudil 1.69 ± 0.09 -fold increase was followed by slight but not significant decrease at 10^{-6} mol/L (1.47 ± 0.07) and 10^{-5} mol/L (1.51 ± 0.11 ; Figure 5). Thus, culture of leiomyoma cells in the 3D format revealed a differential response to inhibition of ROCK, which was associated with increased levels of pERK.

Figure 3. (Continued)

Apoptosis in 2D Leiomyoma Culture

The biphasic response of leiomyoma cells in 3D culture to fasudil as shown by ECM secretion increased levels of active RhoA and increased pERK suggested that fasudil might be acting to reverse, in part, the attenuated mechanical signaling of leiomyoma cells. In addition to the increased secretion of ECM, cells within a leiomyoma have continued growth and reduced apoptosis. We suspect that if the mechanical signaling was altered by fasudil, apoptosis might be increased in leiomyoma cells. Leiomyoma cells in 2D cultures demonstrated an increase in proapoptotic protein Bax, a 22- to 23-KDa protein that is involved in induction of apoptosis (Figure 6). The fold increase ranged between 1.18 ± 0.2 (10^{-7} mol/L), 1.23 ± 0.09 (10^{-6} mol/L), and 1.27 ± 0.02 (10^{-5} mol/L) to 1.37 ± 0.13 -fold (10^{-4} mol/L) when compared to untreated control (0 mol/L). As shown in Figure 6, at higher concentrations of fasudil (10^{-5} mol/L and 10^{-4} mol/L), a band

was observed at ~ 18 KDa that may represent the cleaved Bax. In 3D leiomyoma cultures, an increase in the Bax protein was also observed although not statistically significant.

Collagen Gel Contraction

In prior experiments using the ROCK inhibitor, Y27632, we observed little response of leiomyoma cells to ROCK inhibition in an assay of applied cyclic strain.¹² Since we had observed leiomyoma and myometrium cells cultured for 14 days in 3D cultures led to collagen gel contraction, we were interested to test for an effect of fasudil in this system. The Rho/ROCK pathway plays an important role in cytoskeletal rearrangement and contraction of actin fibers.^{24,40} As illustrated in Figure 7, untreated leiomyoma 3D cultures contracted after the end of 3-week growth. Fasudil treatment at 10^{-7} mol/L (Figure 7B) and

Figure 4. Quantitation of Rho activity in myometrial and leiomyoma cells in 2-dimensional (2D) and 3-dimensional (3D) cultures. A, Levels of active RhoA in untreated leiomyoma cells grown in 2D (L-2D) or 3D (L-3D) were higher when compared to untreated myometrial cells (MY; patterned bar) grown in corresponding culture format. B, Levels of active RhoA in 3D cultures after fasudil treatment for 72 hours. Myometrial cells (MY; patterned bars) grown in 3D cultures demonstrated significant increase at 10⁻⁷ mol/L fasudil. Leiomyoma (L; gray bars) demonstrated a biphasic response with an increase in active RhoA at 10⁻⁷ mol/L fasudil, which was significantly decreased at higher concentrations. The significant (**P* < .05) changes are calculated to untreated myometrium or leiomyoma controls (0 mol/L). Data shown represent mean $\pm$ standard error of the mean (SEM) fold.

10⁻⁶ mol/L (Figure 7C) relaxed the gel slightly compared to controls. At higher concentrations of 10⁻⁵ mol/L (Figure 7D), maximum relaxation of the gel was observed which was equivalent to the control collagen gel containing no cells (Figure 7E). Similar effects were observed in 3D cultures of myometrial cells (not shown). These results further demonstrate that fasudil inhibited the contractile activity of leiomyoma and myometrial cell lines and is consistent with the conclusion that fasudil might reverse the attenuated response of leiomyoma cells.

Discussion

The excessive ECM production is responsible for the bulk of the leiomyoma tumor and increased bulk may be related to

Figure 5. Western blot of phosphorylated extracellular regulated signal kinase (pERK) in myometrium and leiomyoma 2-dimensional (2D) and 3-dimensional (3D) cultures treated with fasudil for 72 hours. * indicates statistically significant difference (*P* $\leq$.05) when compared to untreated controls (0 mol/L). Data shown represent mean $\pm$ standard error of the mean (SEM) fold. Results were normalized to β -actin.

Figure 6. Quantification of proapoptotic protein Bax (22-23 KDa) in leiomyoma cells grown in 2-dimensional culture. An increase in expression of Bax was observed after fasudil hydrochloride treatment. Inset: The 2 bands represent the Bax- α and Bax- β forms of the Bax protein. The significant (**P* < .05) changes are calculated to untreated controls (0 mol/L). Results were normalized to β -actin. Experiments were repeated twice.

worsening of symptoms. Thus, evaluating the steps that are critical in either the production of the altered ECM or the breakdown of the ECM might help clarify the pathogenesis of these uterine tumors. The large deposit of the ECM creates stiffness which is a measure of the ability of a material to resist deformation measured as Young modulus (YM).⁴¹ Rogers and coworkers¹¹ demonstrated that in unconfined compression, leiomyoma tissue exhibited an increased YM compared to surrounding myometrium. Further, the pseudo-dynamic modulus was significantly increased in leiomyoma tissues (207.7 $\pm$ 27.8 megapascals [MPa] per mm²) versus myometrium (48.1 $\pm$ 25.6 MPa per mm²).¹² Both measures indicated that the tumors are stiffer than the surrounding myometrium.

Figure 7. Collagen I gel contraction in response to fasudil treatment. Leiomyoma cells seeded in collagen I gels and grown for 2 weeks before treatment with fasudil at 0 mol/L (A1 and A2), 10^{-7} mol/L (B), 10^{-6} mol/L (C), and 10^{-5} mol/L (D) for 72 hours. A concentration-dependent relaxation of the gel was observed with little contraction observed at 10^{-5} mol/L equivalent to no-cell control matrix (E). Results are representative of 3 experiments.

Cells possess the ability to sense the stiffness in the surrounding ECM and respond accordingly by converting the mechanical cues into complex intracellular signaling pathways that regulate cellular responses.⁴¹ These signaling pathways include transmembrane receptors, integrins, and Rho GTPases. The 3 GPa YM of the polystyrene plastic culture plates, in which the cells grow in 2-dimension culture system, is greater than 5 orders of magnitude stiffer than skeletal muscle.⁴²⁻⁴⁴ The YM of most human tissues is below 100 kPa⁴⁵; therefore, the in vivo environment is considerably more elastic than polystyrene substrate. Furthermore, when grown in various matrices in a 3D culture, cells from different tissues, where the YM may differ from Pa to kPa, tend to retain more of their in vivo characteristics.^{43,45} Thus, the 3D system more closely resembles the in vivo state.

Leiomyoma is a fibrotic disease, and Rho/ROCK signaling is involved in initial steps of the fibrotic response. For instance, ROCK inhibitors have been shown to be effective to mitigate a large number of fibrotic diseases⁴⁶ including reperfusion injury in the liver,⁴⁷ cardiovascular diseases,^{48,49} tissue fibrosis,⁵⁰ cerebral ischemia,⁵¹ peritoneal fibrosis,²⁸ amyotrophic lateral sclerosis,⁵² and pulmonary hypertension.^{53,54} Fasudil is a specific ROCK inhibitor⁵⁵ and is the first kinase inhibitor drug used in a clinical setting in Japan.⁵⁶⁻⁵⁸ In this study, we have demonstrated that targeting the mechanosensing/mechanore-sponse Rho/ROCK pathway^{59,60} by inhibiting the action of the ROCK in leiomyoma cells regulates the response of the ECM genes in leiomyoma cells. Importantly, we have shown that the leiomyoma cells respond to manipulation of the Rho pathway differently when grown in 3D culture compared to 2D culture.

Our 3D culture system permits characterization of the growth of leiomyoma and myometrial cells in collagen I matrix, an important attribute because collagen I is a main component of the abnormally formed leiomyoma matrix.⁶¹ We have further demonstrated that at the end of 2 weeks, using IHC methodology, control leiomyoma and myometrium 3D

cultures demonstrate a difference in ECM production (unpublished results). Furthermore, 3D leiomyoma cultures respond with a decrease in TGF β 3 protein production on treatment with liarazole.³⁶ The 3D system thus provides both the ability to assess the development of the ECM as well as the impact of therapeutic manipulations on leiomyoma ECM in vitro.^{34,36} Collagen 1, fibronectin, and versican are ECM proteins that are known to be increased in the leiomyoma tumor, as compared to normal myometrium, and therefore dysregulation of these genes is a crucial component of tumor formation.^{3,9,10,62,63} In present study, we examined the changes in expression of these genes in response to fasudil treatment of leiomyoma and myometrial cell lines in 2D and 3D culture systems. In 2D cultures of both myometrial and leiomyoma cells, downregulation of transcripts resulted in reduced amount of ECM proteins in response to fasudil treatment. An early response by ECM gene transcripts, to higher concentrations of fasudil, was observed in leiomyoma cells when compared to myometrial cells in 2D culture. Similar to the response in 2D cultures, the myometrial cells in 3D cultures had decreased ECM protein production at 72 hours of treatment. Leiomyoma cells in 3D cultures responded differently, compared to 2D cultures, and demonstrated an increase in most ECM proteins at lower concentrations of fasudil as compared to untreated controls, followed by decreasing expression reaching the control levels. These findings support our earlier results of an attenuated response of leiomyoma to mechanical cues¹² and support the use of 3D culture system to understand the impact of biological compounds on ECM.

Myometrial cells in 2D and 3D culture system demonstrated a concentration-dependent reduction of ECM proteins in response to fasudil hydrochloride. Similarly, leiomyoma cells in a 2D culture system responded with decreased ECM protein production. Interestingly, leiomyoma cells grown in collagen I matrix in 3D culture did not respond in a manner similar to 2D cultures. Instead of a concentration-dependent decrease, an increase in ECM proteins collagen 1 and fibronectin at lower concentration of fasudil (10^{-7} mol/L) was observed, followed by concentration-dependent decrease. Thus, a biphasic response to ROCK inhibition was observed in the 3D leiomyoma cell cultures, with an initial increase, followed by a decrease in ECM proteins; notably a differential effect of inhibition compared to myometrial cells. Further, versican, a sulfated glycosaminoglycan, demonstrated a concentration-dependent increase in treated leiomyoma cells. Norian and coworkers⁶⁴ had demonstrated increased amount of versican variants in leiomyoma tissue as compared to myometrium. Fibromodulin was the only ECM protein to be decreased below control levels in 3D cultures of leiomyoma cells in response to fasudil treatment.

A decrease in collagen production and deposition accompanied with increase in collagenase activity by fasudil has been demonstrated in hepatic stellate cells³³ and cardiac remodeling.⁶⁵ In a rat model of peritoneal sclerosis, Washida and coworkers demonstrated that fasudil downregulated fibrosis markers such as TGF- β and fibronectin.²⁸ Rho-kinase inhibition

also suppressed glucose-induced fibrosis markers such as collagen in rat cardiac fibroblasts,²⁹ FN1 in human mesangial cells,⁶⁶ and TGF- β in human pleural mesothelial cell line.²⁸ We observed a concentration-dependent significant reduction in COL-1A, FN1, versican, and FMOD proteins in 2D cultures of leiomyoma and myometrial cells. Given the highly disorganized nature of the leiomyoma collagen,⁶¹ and the fact that mammalian collagenases degrade only specific bonds in the collagen molecule and do not degrade it completely,⁶⁷ it may be speculated that collagenases may not degrade all the collagen in a leiomyomata and a combination of different drugs may be required to treat uterine fibroids.⁶⁷ Collectively, our 2D data suggest that activation of the Rho/ROCK pathway is essential for ECM synthesis in uterine myometrium and leiomyoma cells and inhibition of the pathway might have therapeutic potential, as fasudil is associated with decreased matrix protein production as well as increase in signals for apoptosis.

To further evaluate whether fasudil at higher concentrations affected ECM synthesis, we observed that in 2D cultures, 10^{-4} mol/L concentration was toxic to leiomyoma cells, as demonstrated by a loss of cells in treated wells (data not shown). This was supported by increase in proapoptotic protein Bax. Interestingly, at the 2 higher concentrations of 10^{-5} mol/L and 10^{-4} mol/L, an 18-KDa band was observed that may be a cleaved product of Bax that is usually observed during stress-related apoptosis.^{68,69} The Bax is cleaved by cysteine protease calpain that increases the intrinsic cytotoxic property of the protein.^{69,70} This may explain that, despite a concentration-dependent increase in Bax protein in leiomyoma cells on fasudil treatment, it was at the higher concentrations that loss of cells was observed. The apoptosis in 3D culture, in response to higher concentrations of fasudil, was not significant, and further work is required to answer this disparity in cytotoxic response in 3D cultures when compared to 2D cultures of leiomyoma cells.

As an initial attempt to examine the mechanisms responsible for increased ECM production, we quantified levels of active RhoA. A significant increase in active RhoA was observed at lower concentrations of fasudil treatment in 3D cultures of leiomyoma cells, which decreased to below control levels at higher concentrations. Although fasudil is an inhibitor of ROCK, a downstream kinase, we had not expected an increase in active RhoA but importantly we observed a biphasic effect with a further increase at low concentrations of fasudil, which may relate to a reduction in substrate for the elevated levels of active RhoA observed. Also, others have observed an increase in RhoA with ROCK inhibition. Thumkeo and coworkers⁷¹ reported some functional redundancy between Rho isoforms and ROCK knockout mice models of different diseases, where an increase in different Rho isoforms may be found in response to ROCK knockout. We theorize that the altered state of mechanotransduction in leiomyoma cells in 3D cultures (altered solid state signaling) spatially coordinates upstream cell receptors and their downstream effectors leading to activation of the MAP kinase pathway and ERK activity either through increase in RhoA activity and/or independently. This

activation could then increase the ECM production. In support of this notion, we observed an increase in pERK activity in 3D cultures of leiomyoma cells.

Collectively, these results support the hypothesis that leiomyoma cells in vivo have adapted to high-stress environment and thus have an attenuated response to mechanical cues in vitro.¹² Norian and coworkers observed that leiomyoma cells did not respond to uniaxial straining when the Rho/ROCK was inhibited. This finding supported their earlier finding of an increased level of the Rho-GEF AKAP13 and differential response to AKAP13 knockdown in leiomyoma cells compared to myometrial cells.^{11,72} Our work further supports the observation that leiomyoma cells exposed to a 3D culture environment demonstrate an altered response to ECM cues, since there was a biphasic response to ROCK inhibition, and the responses differed from myometrial cells in 2D or 3D cultures. The results of fasudil hydrochloride on leiomyoma cells grown in 3D cultures closely resembled the in vivo conditions and demonstrated a differential response between leiomyoma and myometrial cells, in the direction consistent with a favorable clinical response.

The Rho/ROCK pathway is important in uterine contraction. Fitzgibbon and coworkers⁷³ demonstrated that collagen gel embedded with immortalized myometrial cells lost contractibility after inhibition of ROCK. To determine whether ROCK inhibition affected the cytoskeletal organization in our 3D cultures, we examined the collagen 1 gel contraction caused by both myometrial and leiomyoma cell growth. We observed an increasing relaxation of 3D cultures of myometrium and leiomyoma cells with increasing fasudil concentrations. We conclude that both myometrial and leiomyoma cells retained their contraction capability in 3D culture but, as established by Norian and coworkers,¹² the leiomyoma cells have an attenuated response to mechanical cues as well as a diminished ability to change morphology in response to substrate stiffness. As contractile activity is correlated with differentiation and with influence of TGF- β is known to induce fibrosis development, a more in-depth study is required to understand the contractibility of leiomyoma cells in vitro. We have also established that as the effects of ECM mechanics in 3D cultures are distinct from those in 2D, it may be prudent to study the mechanical role of cell matrix interactions in leiomyomas in a 3D environment that may lead to better understanding of therapeutic manipulations on ECM for clinical interventions.

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