Published in final edited form as:

Fertil Steril. 2014 July ; 102(1): 272–281.e2. doi:10.1016/j.fertnstert.2014.03.042.

Liarozole inhibits transforming growth factor- β –mediated extracellular matrix formation in human three-dimensional leiomyoma cultures

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Abstract

Objective—To investigate the impact of liarozole on transforming growth factor- β (TGF- β) expression, TGF- β controlled profibrotic cytokines, and extracellular matrix formation in a three-dimensional (3D) leiomyoma model system.

Design—Molecular and immunohistochemical analysis in a cell line evaluated in a three-dimensional culture.

Setting—Laboratory study.

Patient(s)—None.

Intervention(s)—Treatment of leiomyoma and myometrial cells with liarozole and TGF- β in a three-dimensional culture system.

Main Outcome Measure(s)—Quantitative real-time reverse-transcriptase polymerase chain reaction and Western blotting to assess fold gene and protein expression of TGF- β and TGF- β regulated fibrotic cytokines: collagen 1A1 (COL1A1), fibronectin, and versican before and after treatment with liarozole, and confirmatory immunohistochemical stains of treated three-dimensional cultures.

Result(s)—Both TGF- β gene and protein expression were elevated in leiomyoma cells compared with myometrium in two-dimensional and 3D cultures. Treatment with liarozole decreased TGF- β gene and protein expression. Extracellular matrix components versican, COL1A1, and fibronectin were also decreased by liarozole treatment in 3D cultures. Treatment of 3D cultures with TGF- β increased gene expression and protein production of COL1A1, fibronectin, and versican.

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G.L. has nothing to disclose. M.M. has nothing to disclose. J.B. has nothing to disclose. M.G. has nothing to disclose. J.S. has nothing to disclose. W.H.C. reports a contract with Bayer for use of cells and his spouse is employed by EMD Serono.

Conclusion(s)—Liarozole decreased TGF- β and TGF- β -mediated extracellular matrix expression in a 3D uterine leiomyoma culture system.

Keywords

Extracellular matrix; liarozole; leiomyoma; three-dimensional model; transforming growth factor-beta

Uterine leiomyoma are the most common benign tumors in women of reproductive age (1, 2) and account for over 200,000 hysterectomies annually in the United States (3). They are a significant cause of morbidity ranging from profuse vaginal bleeding to genitourinary and bulk symptoms. However, despite their widespread prevalence and morbidity, the pathogenesis of leiomyoma remains incompletely understood (4, 5), and as hysterectomy prevents future childbirth, novel medical therapies are warranted to treat this disease.

Uterine leiomyomata is a three-dimensional (3D) disease, and by growing cells in 3D, in vivo conditions are better simulated compared to two-dimensional (2D) cultures (6, 7). The superiority of the 3D system in uterine leiomyoma lies in its ability to resemble actual tissue by exposing cells to extracellular matrix (ECM) and its proteins and cytokines normally lost in standard 2D cultures. This provides cells with a natural foundation of differentiation and migration. Another advantage of the 3D system is its ability to maintain intercellular communication induced by mechanical stimulation (8). We have previously developed a 3D model for leiomyoma and demonstrated that both myometrial and leiomyoma cells grown in 3D culture retain their morphologic and molecular phenotype (8). The 3D cultures also responded to treatment and demonstrated an increased amount of ECM proteins compared with 2D culture. The introduction of these validated 3D leiomyoma cultures that mimic in vivo conditions present a novel opportunity to examine the impact of potential pharmacologic agents on ECM, which plays a central role in the bulk symptoms associated with uterine leiomyoma (8, 9).

The bulk symptoms caused by leiomyoma result from the overproduction of disordered extracellular matrix (3, 9, 10). This aberrant ECM production is likely the result of elevated transforming growth factor β (TGF- β) expression and signaling, which has been demonstrated in other fibrotic diseases such as glomerulonephritis (11), pulmonary fibrosis (12), liver cirrhosis (13), and keloids (14). The characteristic of elevated TGF- β expression seen in fibrotic disease has also been identified in uterine leiomyomas (15–17). TGF- β increased production of proteoglycans and collagens, which leads to the fibrotic phenotype of leiomyomata (15, 16, 18, 19). TGF- β exerts its effect through the mothers against decapentaplegic (Smad) signaling cascade that transfers signal from the membrane to the nucleus and effects gene expression (20). Mice devoid of Smads were resistant to TGF- β -mediated fibrosis (21).

Altered retinol metabolism, resulting in decreased intracellular all-trans retinoic acid (ATRA) is another characteristic of uterine leiomyoma (22). Furthermore, retinoids have also been shown to influence ECM production in other tissues such as the vascular smooth muscle (23, 24), and TGF- β interacts with the retinoic acid (RA) pathway in various tissues including leiomyoma (22, 25, 26). Treatment of leiomyoma cells with ATRA results in a

reduction of ECM formation to levels comparable with myometrium (22). In addition, ATRA treatment of leiomyoma cells decreased TGF- β expression and TGF- β -regulated gene expression signals responsible for overproduction of ECM (22). Because the ECM is decreased by ATRA treatment in vitro, ATRA is an attractive candidate for pharmacologic therapy of uterine leiomyoma. Unfortunately, ATRA is highly toxic, so alternative medical therapies that target the RA and TGF- β pathways to modulate leiomyoma ECM production are warranted.

Liarozole, a RA metabolizing blocking agent (RAMBA) inhibits RA metabolism and thereby increases intracellular RA levels in cells that actively produce RA (27–30). Liarozole has been used in the treatment of a variety of conditions characterized by ECM overproduction such as ichthyosis (28, 30–33) and also has a mild side-effect profile, making it a candidate for medical treatment of leiomyoma. Previously, we found that liarozole inhibits ECM production in leiomyoma cells in 2D cultures (34). However, because 2D cultures do not accumulate ECM, the impact of liarozole on ECM formation, maintenance, and dissolution is unknown. We therefore hypothesized that treatment of 3D leiomyoma cultures with liarozole would inhibit TGF- β gene and protein expression. Furthermore, we hypothesized that decreased activity of the TGF- β pathway will decrease leiomyoma fibrosis in 3D leiomyoma cultures.

MATERIALS AND METHODS

This study was conducted under institutional review board approval from the human use committee of the Uniformed Services University and Walter Reed National Military Medical Center. Institutional review board approval was obtained before tissue procurement.

Tissue Collection

Tissue was collected after institutional review board approval and consent from patients undergoing hysterectomy for leiomyoma at Walter Reed National Military Medical Center, Bethesda, had been obtained. Generation of 2D primary cultures was previously described elsewhere (22, 35).

Cell Growth in 3D Cultures

Generation of 3D cultures has been described elsewhere (8) but will be briefly reviewed here. Rat-tail collagen 1 was prepared by mixing it (50%, 4 mg/mL) with 25% complete medium (Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% fetal bovine serum, 1X penicillin/streptomycin, and 0.25 μ g/mL amphotericin B), 5X Dulbecco's modified Eagle's medium with phenol red or phosphate-buffered saline (PBS) with phenol red at 15%, and 0.1 M sodium hydroxide (NaOH) (10%) to give a final concentration of 2 mg/mL collagen-1 gels. The gels were kept on ice at all times. Immortalized cells of patient-matched myometrium and leiomyoma were grown in complete medium at 37°C in the presence of 5% CO₂ until they reached 80% confluence. For 3D cultures, the cells were resuspended at final concentration of 6.25×10^4 cells/mL in 5% medium. From this stock, cells were mixed with collagen-1 solution to give final concentration of 2.5×10^3 cells/0.4 mL, ensuring the volume of the cell suspension was less than 10% of the final solution.

Treatment of the 3D Cultures

Leiomyoma 3D cultures were grown to 80% confluency before being exposed to graded liarozole concentrations from 1×10^{-8} M to 1×10^{-9} M. These concentrations were chosen based on our liarozole studies in 2D cultures that identified liarozole concentrations that impact ECM formation but do not effect cellular proliferation (34). The 3D culture was allowed to grow for 3 days with liarozole in 5% Dulbecco's modified Eagle's medium/Ham's F-12 containing 5% fetal bovine serum, 1X penicillin/streptomycin, and 0.25 $\mu\text{g/mL}$ amphotericin B, and was replaced every other day. Patient-matched myometrial controls were grown similarly.

For cultures treated with TGF- β (Sigma-Aldrich), it was added to leiomyoma 3D cultures for 24 hours at concentrations of 1.0 $\mu\text{g/mL}$ in serum-free medium to examine the effect of TGF- β on collagen 1A1 (COL1A1), fibronectin, versican, and TGF- β . These ECM genes were chosen because they are established and validated as overexpressed ECM genes in uterine leiomyoma (1, 10, 35, 36) and are known to be regulated by TGF- β (15, 16).

RNA Isolation and Quantitative Real-time Reverse-transcriptase Polymerase Chain Reaction

Once the 3D cultures of immortalized myometrial and leiomyoma cells reached 70% to 80% confluence, the RNA was isolated by the use of the TriZol method (Invitrogen) (22). For 3D cultures, gels were washed with cold 1X PBS before placement into 5-mL tubes and centrifugation at 5,000 rpm at 4°C for 6 minutes. After another wash, TriZol (0.7 mL) was added to the tube, and the sample was left on ice for 10 minutes, after which the gels were sonicated twice for 30 seconds, each with a 10-minute pause on ice in between each sonication. Further steps were according to the manufacturer's protocol.

Any residual DNA was removed using Turbo DNase (Ambion), and the purified RNA was measured and stored at -80°C. The real-time reverse-transcriptase-polymerase chain reaction (RT-PCR) method was used to evaluate the expression of TGF- β , COL1A1, fibronectin, and versican. We used 18S ribosomal RNA gene as an internal control, and each sample was analyzed in triplicate. Bio-Rad thermal cycler software, version 3.1, using the Pfaffl method was used for data analysis (37).

Protein Collection and Western Blot Analysis

The 3D gel in each well of a 24-well plate was washed with ice-cold 1X PBS. Each gel was moved into an Eppendorf tube on ice and was washed with ice-cold 1X PBS and centrifuged at 5,000 rpm for 6 minutes at 4°C. To each tube, 0.4 mL of radioimmunoprecipitation (RIPA) buffer containing 1X Halt protease inhibitor with phosphatase inhibitor (Pierce Biotechnology) was added. The samples were sonicated for 30 seconds three times, followed by a 10-minute rest on ice. The tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C. The aqueous layer was aliquoted and stored at -80°C. Protein concentrations were determined using Precision Red Assay (Cytoskeleton Inc.). For Western blot analysis, equal amounts of protein were loaded into Tris-glycine or Bis-Tris 4% to 20% gels and were transferred to nitrocellulose membranes (Invitrogen). After they were incubated in blocking solution (5% nonfat milk in 0.1% Tween 20 in 1X TBS [TBST]), the

membranes were washed and exposed to primary antibody against collagen-1 (sc-59772, 1:100; versican V0/V2, ab8671, 1:200; fibronectin ab6584, 1:500; phosphorylated SMAD 2/3, sc-133098, 1:200; TGF- β 3, sc-82, 1:200) overnight at 4°C. For detection of proteins, blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibody 1:5000–1:15,000 (ImmunoPure; Pierce Biotechnology) for 1 hour at room temperature. SuperSignal West Pico (Pierce Biotechnology) was used for detection of the proteins. As an internal standard between samples, HRP-labeled anti-human β -actin (sc-1616, 1:10,000) was used.

Immunohistochemistry

For immunohistochemistry, tissue samples and 3D cultures were stored in 4% paraformaldehyde or 10% formalin. Slides were deparafinized and hydrated in xylene and graded ethanol followed by distilled water. Slides were then treated with 3% hydrogen peroxidase solution to block endogenous peroxidases and treated with chondroitin ABC lyase (0.15 U/mL). Blocking was performed with normal goat serum. Slides were treated with primary antibody at 1:500 dilution. Secondary antibody was used at 1:100–1:200 dilution followed by DAB solution. Finally, slides were dehydrated and mounted with Hypermount (Thermo Shandon). No staining was observed on control slides of tissue or 3D cultures that were not exposed to either the primary or secondary antibodies for TGF- β 3 (data not shown).

Statistical Analysis

For qRT-PCR data, the results are reported as mean $\pm$ standard error of the mean (SEM). For each result, the average expression of four replicates was calculated before the relative quantification using normalization against the housekeeping gene (18S) was performed. Relative expression was calculated based on the Pfaffl method (37). The Wilcoxon signed rank test was used for nonparametric statistical evaluation, and $P < .05$ was considered statistically significant. For the Western blot analysis, calculations were performed with QualityOne software from Bio-Rad Laboratories. Data are presented as the fold difference between relative density units of treated and untreated samples, and are corrected for the internal control β -actin.

RESULTS

TGF- β 3 Expression and Signaling in Myometrial and Leiomyoma Cells in 3D Culture

We previously demonstrated that TGF- β 3 was expressed at a higher concentration in leiomyoma tissue than myometrium and in leiomyoma cells when compared with myometrial cells in 2D cell cultures (15, 16, 36). However, it was not clear whether TGF- β 3 would remain elevated in the presence of ECM. As a result, we evaluated TGF- β 3 expression in the 3D leiomyoma and patient-matched myometrial model systems. Using qRT-PCR, we demonstrated that untreated leiomyoma 3D cell cultures demonstrated a 1.86 ± 0.49 -fold increase in TGF- β 3 compared with patient-matched myometrial cells ($P < .05$). Leiomyoma cells treated with liarozole at concentrations ranging from 1×10^{-8} to 1×10^{-9} for 72 hours demonstrated a concentration-dependent decrease in the amount of TGF- β 3 gene expression compared with untreated leiomyoma cells (Table 1).

After confirming the elevated TGF- β gene expression in 3D leiomyoma cultures and inhibition by liarozole, we evaluated whether this finding would translate to protein. Untreated leiomyoma cells previously demonstrated an elevation in TGF- β protein production in 2D culture (15). In 3D culture, untreated leiomyoma cells demonstrated a 4.36 ± 1.92 increase of TGF- β protein compared with untreated myometrial cells. After treatment with liarozole at concentrations of 1×10^{-9} for 72 hours, leiomyoma cells demonstrated a dose-dependent decrease in TGF- β protein (Fig. 1A). In tissue and in 3D culture immunohistochemistry, TGF- β was elevated in leiomyoma compared to myometrium (see Fig. 1B–1E), and decreased with liarozole treatment (Fig. 1F).

TGF- β –mediated Signaling Pathway

The TGF- β signaling cascade requires TGF- β interaction with the TGF- β receptor, followed by receptor phosphorylation, then interaction of the phosphorylated receptor complex with the Smad pathway by phosphorylation of the Smad 2/3 complex. Prior studies in 2D cultures demonstrated an elevated level of phosphorylated Smad 2/3 in leiomyoma cells (15), but it is unknown whether similar regulation occurs in 3D culture. Our evaluation of downstream TGF- β signaling in 3D cultures showed that untreated leiomyoma cells have a 5.18 ± 0.085 ($P < .05$) increased expression of phosphorylated Smad 2/3 protein compared with myometrial cells, indicating a greater activation of the TGF- β pathway in leiomyoma cells in 3D cultures. After treatment with liarozole at concentrations of 1×10^{-8} M to 1×10^{-9} M for 72 hours, liarozole-treated leiomyoma cells demonstrated a decrease in phosphorylated Smad 2/3 protein to 4.32 ± 0.72 -fold; $P < .05$ (Supplemental Fig. 1, available online) indicating a decrease in Smad protein production. Collectively these results demonstrate that in 3D cultures, leiomyoma samples contain elevated levels of TGF- β transcript and protein expression, and a greater activation of TGF- β signaling with a greater amount of intracellular phosphorylated-Smad 2/3 protein. Treatment with liarozole decreases TGF- β gene and protein expression and reduces TGF- β signaling via Smad pathway of leiomyoma cells in our 3D culture model.

TGF- β Regulated Extracellular Matrix Genes

We (16) and others (17) have demonstrated elevated expression of fibronectin in leiomyoma compared with myometrium, and have demonstrated that TGF- β stimulates fibronectin expression in myometrial and leiomyoma cells. In prior studies, treatment of myometrial cells with ATRA decreased fibronectin expression in a dose-dependent matter in 2D cell culture (22). In our present study, we evaluated whether leiomyoma TGF- β –mediated ECM would decrease with liarozole treatment. In leiomyoma cells, fibronectin mRNA demonstrated 8.53 ± 0.60 -fold greater expression in untreated leiomyoma cells compared with patient-matched myometrium (Table 1). Treatment of 3D leiomyoma cultures with TGF- β increased fibronectin gene expression fourfold (Fig. 2B). With the addition of 10^{-9} M liarozole, leiomyoma cells showed decreased fibronectin mRNA expression. Higher liarozole concentrations decreased fibronectin mRNA expression below the untreated myometrial expression in 3D cultures (see Table 1).

Fibronectin protein production was also decreased in liarozole-treated leiomyoma cells. Untreated leiomyoma controls showed a twofold increase in fibronectin protein

concentration compared with untreated myometrial cells (Fig. 3). At 1×10^{-9} M liarozole treatment, the fibronectin protein expression increased 2.56-fold. Continued exposure to liarozole at 1×10^{-8} M decreased the fibronectin protein expression to 0.83-fold of myometrial cells (Fig. 3), indicating a decrease in fibronectin protein production with exposure to increasing concentrations of liarozole. Immunohistochemical evaluation of fibronectin in 3D cultures demonstrated an increased staining for fibronectin in leiomyoma cultures compared with myometrium ($P < .05$). Treatment of 3D cultures with liarozole decreased staining for fibronectin in 3D cultures (Supplemental Fig. 2A, available online).

Expression of type I collagen in leiomyoma specimens is known to be greater than in myometrium (16, 38, 39). Furthermore, TGF- β exerts a dose-dependent stimulatory effect in myometrial and leiomyoma cells on COL1A1 mRNA and protein production (16). We also showed that COL1A1 expression was inhibited by ATRA treatment (22). We therefore hypothesized that leiomyoma cells would respond similarly when in the presence of ECM and that treatment with liarozole would decrease COL1A1 gene and protein expression.

Untreated leiomyoma cells demonstrated a 1.99 ± 0.44 -fold ($P < .05$) increase in COL1A1 mRNA expression compared with untreated myometrial controls in 3D cultures. Treatment of leiomyoma cells with liarozole at concentrations of 1×10^{-9} M resulted in COL1A1 mRNA expression similar to myometrial cells (see Table 1).

Prior work demonstrated that COL1A1 protein was also overproduced in leiomyoma cells when compared with myometrium (16, 22). In 2D cultures, myometrial and leiomyoma cells produced COL1A1 protein in response to varying concentrations of TGF- β (16) and responded with decreased protein production when treated with ATRA (22). We first treated 3D cultures with TGF- β and stimulated a 3.1-fold increase in COL1A1 expression in treated leiomyoma cells compared with untreated myometrial cells (Fig. 2C). Consequently, we evaluated whether leiomyoma cells in 3D cultures treated with liarozole decreased COL1A1 protein production. Untreated leiomyoma cells demonstrated a 1.33 ± 0.11 increase in COL1A1 protein expression compared with patient-matched myometrial cells. At increasing concentrations of liarozole, leiomyoma cells demonstrated decreased production of COL1A1 protein at levels below those of myometrial cells (Fig. 3B). Immunohistochemical staining of 3D myometrial and leiomyoma untreated and liarozole-treated cultures demonstrated increased expression of COL1A1 in leiomyoma cultures and a decrease with liarozole exposure (see Supplemental Fig. 2B).

Versican is an overproduced glycoprotein in the ECM of uterine leiomyoma (15). We previously demonstrated that versican production was induced in leiomyoma and myometrial cells with TGF- β and inhibited with anti-TGF- β antibodies in 2D cultures (15). Furthermore, versican was down-regulated with ATRA treatment (22). Based on the ATRA effect on versican in 2D cultures, we hypothesized that treatment with liarozole would result in the down-regulation of versican in 3D cultures. In 3D cultures, untreated leiomyoma cells demonstrated a 3.87 ± 0.79 -fold increase in versican mRNA expression compared with myometrial cells, and treatment of leiomyoma cells with increasing concentrations of liarozole in 3D cultures demonstrated a decrease in versican gene expression (see Table 1).

After we had demonstrated a down-regulatory effect on versican mRNA production, we evaluated protein production. Untreated leiomyoma cells demonstrated a 1.99 ± 0.85 -fold increase in versican protein production compared with patient-matched myometrial cells. Treatment of leiomyoma cells with liarozole demonstrated a decrease in versican protein production that resembled myometrial cells (Fig. 2C). The immunohistochemical evaluation demonstrated increased staining for versican in 3D leiomyoma cultures compared with myometrium and a decrease in staining in liarozole-treated cultures (see Supplemental Fig. 2C).

Leiomyoma and Myometrial Cells Treated with Liarozole after TGF- β Exposure

Because TGF- β induces fibroid-like changes in myometrial cells and promotes extra deposition of ECM in leiomyoma in a 2D system (16), we hypothesized that ECM expression would be decreased with liarozole treatment in TGF- β -exposed leiomyoma cells in 3D cultures.

Treatment of myometrial cells with TGF- β in 3D cultures demonstrated a statistically significant 1.95 ± 0.08 -fold increase in TGF- β gene expression, comparable to the levels seen in leiomyoma cells (1.86 ± 0.51) ($P < .05$). When TGF- β -exposed leiomyoma cells were treated with liarozole in 3D cultures, they demonstrated a dose-dependent, statistically significant decrease in TGF- β gene expression (Fig. 2A).

Next, we evaluated TGF- β -controlled ECM gene expression in leiomyoma cells treated with TGF- β and subsequently liarozole. The TGF- β treatment induced a statistically significant increase in fibronectin expression in both myometrium and leiomyoma cells (18.22 ± 3.32 , 13.79 ± 1.89), respectively, when compared with untreated myometrial 3D cultures (Fig. 2B). Liarozole treatment of TGF- β -exposed leiomyoma cells decreased fibronectin gene expression to levels significantly below those induced by TGF- β exposure (Fig. 2B). When evaluating COL1A1, we found that treatment of leiomyoma TGF- β -exposed cells with liarozole decreased COL1A1 gene expression to levels seen in leiomyoma levels that are unexposed to TGF- β (Fig. 2C). Versican expression was increased with TGF- β exposure in both myometrial and leiomyoma cells in 3D cultures (5.98 ± 1.46 , 7.11 ± 1.28) ($P < .05$). When TGF- β -exposed leiomyoma cells were treated with liarozole, they demonstrated a statistically significant decrease to levels seen in untreated leiomyoma cells (Fig. 2D).

DISCUSSION

Our results demonstrated that exposure to the RA metabolic blocking agent liarozole decreased TGF- β expression and signaling and consequently lowered ECM production of leiomyoma 3D cultures. Liarozole at concentrations of 10^{-8} to 10^{-9} M inhibited TGF- β expression and regulated the production of ECM structural components and proteoglycans to levels that approximated myometrial cells. These results demonstrate that liarozole can modulate leiomyoma fibrosis by altering TGF- β expression.

Liarozole's mechanism of action is prevention of hydroxylation of ATRA via inhibition of CYP450. In clinical studies evaluating liarozole for the treatment of cutaneous fibrotic

pathology, liarozole was demonstrated to be effective and very well tolerated (40, 41). The minimal effective oral dose of liarozole implemented in clinical trials has been 50 mg (42), with an oral dose of 300 mg being equivalent to 1×10^{-5} molar in human blood (43). The effective concentrations used in our studies implemented to inhibit TGF- β formation and signaling were significantly lower than those previously tested in clinical trials. Based on liarozole's proven clinical effectiveness in the treatment of fibrotic disorders along with its tolerability, liarozole is a possible, attractive novel intervention for uterine leiomyoma. Further clinical studies are warranted to examine liarazole's impact on leiomyoma in vivo.

There are several limitations to our study. First, the results demonstrate an almost universal effect of liarozole in the decrease of the ECM. Rarely, such unequivocal results may be a result of the direct application of any intervention, not necessarily the agent of interest. We did not directly address this in our study with a negative control. However, we were able to counter the effect of TGF- β by the addition of liarozole, indicating that the primary mode of action was indeed the modulation of the ECM. Additionally, in clinical trials liarozole has demonstrated an effect at up to 12 weeks. Our studies examined protein translation up to 72 hours; it is difficult to ascertain whether those results may also be obtained with prolonged treatment, as the longevity of the 3D culture limits the opportunity to examine such a treatment duration.

The major strength of this study lies in it being the first study to evaluate the use of liarozole in a 3D culture system and its impact on TGF- β and leiomyoma ECM. Additional strengths lie in our methodology. We confirmed our qRT-PCR and Western blot findings with immunohistochemical analysis, and by addition of liarozole we countered the impact of TGF- β on the ECM, which further buttressed our findings.

Interaction between retinoids and TGF- β pathways is highly complex and is likely to be cell and tissue dependent (44). Retinoids can affect TGF- β signaling either positively or negatively, and TGF- β affects the retinoid pathway by suppressing CYP26, leading to decreased RA concentrations (44). For example, in human mesangial cells, RA suppresses TGF- β -mediated action of fibrosis by stimulation of hepatocyte growth factor (45) and in fibroblasts ATRA decreases p-Smad mediated transcription (46). Retinoids are required for normal development of essential embryonic structures. Vitamin A-deficient quail embryos that develop abnormal cardiovascular systems can be rescued by ATRA (44). In these retinoid-deficient embryos, the TGF- β gene, protein, and signaling were increased, providing further evidence that proper functioning of these two interdependent pathways is required for appropriate cellular development and differentiation.

Prior in vitro studies were completed in 2D conditions, which may not accurately depict in vivo conditions and provide no information of the formation of ECM. Our recently developed and validated 3D culture of uterine leiomyoma allows us to model in vivo conditions of excessive ECM secretion (8). In this study we evaluated a disease state that is characterized by excessive fibrosis. Using liarozole, a RA metabolizing blocking agent, we were able to demonstrate a reduction in TGF- β signaling and a consequential dissolution of ECM. Treatment of leiomyoma cells with liarozole demonstrated a decrease in TGF- β , its signaling pathway, and ultimately a reduction in collagen, versican, and fibronectin, ECM

proteins known contribute to the leiomyoma phenotype. Our study has demonstrated that the TGF- β cytokine and ECM proteins influenced by TGF- β are down-regulated by liarozole in 3D leiomyoma cultures, providing a potential novel therapy in the battle against this debilitating disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Levy G, Hill MJ, Beall S, Zarek SM, Segars JH, Catherino WH. Leiomyoma: genetics, assisted reproduction, pregnancy and therapeutic advances. *J Assist Reprod Genet.* 2012; 29:703–12. [PubMed: 22584729]
2. Baird DD, Dunson DB, Hill MC, Cousins D, Schectman JM. High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. *Am J Obstet Gynecol.* 2003; 188:100–7. [PubMed: 12548202]
3. Walker CL, Stewart EA. Uterine fibroids: the elephant in the room. *Science.* 2005; 308:1589–92. [PubMed: 15947177]
4. Payson M, Leppert P, Segars J. Epidemiology of myomas. *Obstet Gynecol Clin North Am.* 2006; 33:1–11. [PubMed: 16504803]
5. Myers ER, Barber MD, Gustilo-Ashby T, Couchman G, Matchar DB, McCrory DC. Management of uterine leiomyomata: what do we really know? *Obstet Gynecol.* 2002; 100:8–17. [PubMed: 12100798]
6. Lee J, Cuddihy MJ, Kotov NA. Three-dimensional cell culture matrices: state of the art. *Tissue Eng Part B Rev.* 2008; 14:61–86. [PubMed: 18454635]
7. Adissu HA, Asem EK, Lelievre SA. Three-dimensional cell culture to model epithelia in the female reproductive system. *Reprod Sci.* 2007; 14:11–9. [PubMed: 18089605]
8. Malik M, Catherino WH. Development and validation of a three-dimensional in vitro model for uterine leiomyoma and patient-matched myometrium. *Fertil Steril.* 2012; 97:1287–93. [PubMed: 22425196]
9. Malik M, Norian J, McCarthy-Keith D, Britten J, Catherino WH. Why leiomyomas are called fibroids: the central role of extracellular matrix in symptomatic women. *Semin Reprod Med.* 2010; 28:169–79. [PubMed: 20414841]
10. Leppert PC, Catherino WH, Segars JH. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Am J Obstet Gynecol.* 2006; 195:415–20. [PubMed: 16635466]
11. Liu Y. Renal fibrosis: new insights into the pathogenesis and therapeutics. *Kidney Int.* 2006; 69:213–7. [PubMed: 16408108]
12. Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol.* 2007; 293:L525–34. [PubMed: 17631612]
13. Yata Y, Gotwals P, Koteliensky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for anti-fibrotic therapy. *Hepatology.* 2002; 35:1022–30. [PubMed: 11981752]
14. Bonniaud P, Margetts PJ, Ask K, Flanders K, Gauldie J, Kolb M. TGF-beta and Smad3 signaling link inflammation to chronic fibrogenesis. *J Immunol.* 2005; 175:5390–5. [PubMed: 16210645]
15. Norian JM, Malik M, Parker CY, Joseph D, Leppert PC, Segars JH, et al. Transforming growth factor β regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. *Reprod Sci.* 2009; 16:1153–64. [PubMed: 19700613]
16. Joseph DS, Malik M, Nurudeen S, Catherino WH. Myometrial cells undergo fibrotic transformation under the influence of transforming growth factor beta-3. *Fertil Steril.* 2010; 93:1500–8. [PubMed: 19328471]

17. Arici A, Sozen I. Transforming growth factor- β 3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. *Fertil Steril.* 2000; 73:1006–11. [PubMed: 10785229]
18. Chegini N. Proinflammatory and profibrotic mediators: principal effectors of leiomyoma development as a fibrotic disorder. *Semin Reprod Med.* 2010; 28:180–203. [PubMed: 20414842]
19. Levens E, Luo X, Ding L, Williams RS, Chegini N. Fibromodulin is expressed in leiomyoma and myometrium and regulated by gonadotropin-releasing hormone analogue therapy and TGF-beta through Smad and MAPK-mediated signalling. *Mol Hum Reprod.* 2005; 11:489–94. [PubMed: 16123076]
20. Luo X, Xu J, Chegini N. Gonadotropin releasing hormone analogue (GnRHa) alters the expression and activation of Smad in human endometrial epithelial and stromal cells. *Reprod Biol Endocrinol.* 2003; 1:125. [PubMed: 14678567]
21. Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M, et al. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. *J Immunol.* 2004; 173:2099–108. [PubMed: 15265946]
22. Malik M, Webb J, Catherino WH. Retinoic acid treatment of human leiomyoma cells transformed the cell phenotype to one strongly resembling myometrial cells. *Clin Endocrinol (Oxf).* 2008; 69:462–70. [PubMed: 18248652]
23. Catherino WH, Malik M. Uterine leiomyomas express a molecular pattern that lowers retinoic acid exposure. *Fertil Steril.* 2007; 87:1388–98. [PubMed: 17276435]
24. Axel DI, Frigge A, Dittmann J, Runge H, Spyridopoulos I, Riessen R, et al. All-trans retinoic acid regulates proliferation, migration, differentiation, and extracellular matrix turnover of human arterial smooth muscle cells. *Cardiovasc Res.* 2001; 49:851–62. [PubMed: 11230985]
25. Zirn B, Samans B, Spangenberg C, Graf N, Eilers M, Gessler M. All-trans retinoic acid treatment of Wilms tumor cells reverses expression of genes associated with high risk and relapse in vivo. *Oncogene.* 2005; 24:5246–51. [PubMed: 15897880]
26. Jakowlew SB, Zakowicz H, Moody TW. Retinoic acid down-regulates VPAC(1) receptors and TGF-beta 3 but up-regulates TGF-beta 2 in lung cancer cells. *Peptides.* 2000; 21:1831–7. [PubMed: 11150643]
27. Van Wauwe J, Coene MC, Cools W, Goossens J, Lauwers W, Le Jeune L, et al. Liarozole fumarate inhibits the metabolism of 4-keto-all-trans-retinoic acid. *Biochem Pharmacol.* 1994; 47:737–41. [PubMed: 8129749]
28. De Coster R, Wouters W, Van Ginckel R, End D, Krekels M, Coene MC, et al. Experimental studies with liarozole (R 75,251): an antitumoral agent which inhibits retinoic acid breakdown. *J Steroid Biochem Mol Biol.* 1992; 43:197–201. [PubMed: 1525060]
29. Smets G, Van Ginckel R, Daneels G, Moeremans M, Van Wauwe J, Coene MC, et al. Liarozole, an antitumor drug, modulates cytokeratin expression in the Dunning AT-6sq prostatic carcinoma through in situ accumulation of all-trans-retinoic acid. *Prostate.* 1995; 27:129–40. [PubMed: 7567691]
30. Van heusden J, Borgers M, Ramaekers F, Xhonneux B, Wouters W, De Coster R, et al. Liarozole potentiates the all-trans-retinoic acid-induced structural remodelling in human breast carcinoma MCF-7 cells in vitro. *Eur J Cell Biol.* 1996; 71:89–98. [PubMed: 8884182]
31. Liarozole. Liarozole fumarate, Liazal, R 75251, R 85246. *Drugs R D.* 1999; 2:427–30. [PubMed: 10763459]
32. Van heusden J, Wouters W, Ramaekers FC, Krekels MD, Dillen L, Borgers M, et al. The antiproliferative activity of all-trans-retinoic acid catabolites and isomers is differentially modulated by liarozole-fumarate in MCF-7 human breast cancer cells. *Br J Cancer.* 1998; 77:1229–35. [PubMed: 9579827]
33. Pavez Lorie E, Ganemo A, Borgers M, Wouters L, Blockhuys S, van de Plassche L, et al. Expression of retinoid-regulated genes in lamellar ichthyosis vs. healthy control epidermis: changes after oral treatment with liarozole. *Acta Derm Venereol.* 2009; 89:12–20. [PubMed: 19197536]

34. Gilden M, Malik M, Britten J, Delgado T, Levy G, Catherino WH. Leiomyoma fibrosis inhibited by liarozole, a retinoic acid metabolic blocking agent. *Fertil Steril*. 2012; 98:1557–62. [PubMed: 22925684]
35. Malik M, Catherino WH. Novel method to characterize primary cultures of leiomyoma and myometrium with the use of confirmatory biomarker gene arrays. *Fertil Steril*. 2007; 87:1166–72. [PubMed: 17222838]
36. Catherino W, Salama A, Potlog-Nahari C, Leppert P, Tsibris J, Segars J. Gene expression studies in leiomyomata: new directions for research. *Semin Reprod Med*. 2004; 22:83–90. [PubMed: 15164303]
37. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29:e45. [PubMed: 11328886]
38. Lee BS, Nowak RA. Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-beta 3 (TGF beta 3) and altered responses to the antiproliferative effects of TGF beta. *J Clin Endocrinol Metab*. 2001; 86:913–20. [PubMed: 11158066]
39. Leppert PC, Baginski T, Prupas C, Catherino WH, Pletcher S, Segars JH. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil Steril*. 2004; 82(Suppl 3):1182–7. [PubMed: 15474093]
40. Vahlquist A, Blockhuys S, Steijlen P, van Rossem K, Didona B, Blanco D, et al. Oral liarozole in the treatment of patients with moderate/severe lamellar ichthyosis: results of a randomized, double-blind, multinational, placebo-controlled phase II/III trial. *Br J Dermatol*. 2014; 170:173–81. [PubMed: 24102348]
41. Verfaillie CJ, Vanhoutte FP, Blanchet-Bardon C, van Steensel MA, Steijlen PM. Oral liarozole vs. acitretin in the treatment of ichthyosis: a phase II/III multicentre, double-blind, randomized, active-controlled study. *Br J Dermatol*. 2007; 156:965–73. [PubMed: 17263800]
42. Berth-Jones J, Todd G, Hutchinson PE, Thestrup-Pedersen K, Vanhoutte FP. Treatment of psoriasis with oral liarozole: a dose-ranging study. *Br J Dermatol*. 2000; 143:1170–6. [PubMed: 11122017]
43. Miller VA, Rigas JR, Muindi JR, Tong WP, Venkatraman E, Kris MG, et al. Modulation of all-trans retinoic acid pharmacokinetics by liarozole. *Cancer Chemother Pharmacol*. 1994; 34:522–6. [PubMed: 7923564]
44. Xu Q, Kopp JB. Retinoid and TGF-beta families: crosstalk in development, neoplasia, immunity, and tissue repair. *Semin Nephrol*. 2012; 32:287–94. [PubMed: 22835460]
45. Wen X, Li Y, Hu K, Dai C, Liu Y. Hepatocyte growth factor receptor signaling mediates the anti-fibrotic action of 9-cis-retinoic acid in glomerular mesangial cells. *Am J Pathol*. 2005; 167:947–57. [PubMed: 16192631]
46. Hoover LL, Burton EG, O'Neill ML, Brooks BA, Sreedharan S, Dawson NA, et al. Retinoids regulate TGF β signaling at the level of Smad2 phosphorylation and nuclear accumulation. *Biochim Biophys Acta*. 2008; 1783:2279–86. [PubMed: 18773928]

FIGURE 1.

(A) TGF- β 3 protein expression in myometrial cells, leiomyoma cells, and leiomyoma 3D cultures treated with increasing concentrations of liarozole. *Statistically significant difference ($P < .05$) between leiomyoma and myometrial cultures. +Statistically significant difference ($P < .05$) between treated and untreated leiomyoma 3D cultures. Representative sample of (B) myometrial surgical specimen, (C) leiomyoma surgical specimen, (D) myometrium 3D culture, (E) leiomyoma 3D culture, and (F) liarozole-treated leiomyoma 3D culture at $\times 40$ magnification, demonstrating scant TGF- β 3 (stained brown) in myometrial cultures. This is increased in leiomyoma culture and decreased with liarozole treatment. Levy. Liarozole inhibits fibroid ECM formation. Fertil Steril 2014.

FIGURE 2.

Assessment of (A) TGF- β , (B) fibronectin, (C) COL1A1, and (D) versican expression after treatment of myometrial and leiomyoma 3D cultures with TGF- β and graded concentrations of liarozole. $P < .05$; *Statistically significant difference ($P < .05$) between leiomyoma and myometrial cultures. +Statistically significant difference ($P < .05$) between treated and untreated leiomyoma 3D cultures. #Difference between fibroid treated with TGF- β and TGF- β + liarozole.

Levy. Liarozole inhibits fibroid ECM formation. Fertil Steril 2014.

FIGURE 3.

(A) Fibronectin protein expression in myometrial cells, leiomyoma cells, and leiomyoma 3D cultures treated with increasing concentrations of liarozole. *Statistically significant difference ($P < .05$) between leiomyoma and myometrial cultures. (B) COL1A1 protein expression in myometrial cells, leiomyoma cells, and leiomyoma 3D cultures treated with increasing concentrations of liarozole. (C) Versican protein expression in myometrial cells, leiomyoma cells, and leiomyoma 3D cultures treated with increasing concentrations of liarozole. *Statistically significant difference ($P < .05$) between leiomyoma and myometrial cultures. +Statistically significant difference ($P < .05$) between treated and untreated leiomyoma 3D cultures.

Levy. Liarozole inhibits fibroid ECM formation. Fertil Steril 2014.

TABLE 1Gene expression of TGF- β 3 and ECM genes in 3D cultures treated with liarozole.

Gene	Untreated myometrium	Untreated leiomyoma	Liarozole-treated leiomyoma	
			10 ⁻⁹ M	10 ⁻⁸ M
TGF- β 3	1.00 $\pm$ 0.15	1.86 $\pm$ 0.49 ^a	0.62 $\pm$ 0.07 ^{a,b}	0.21 $\pm$ 0.05 ^{a,b}
Fibronectin	1.00 $\pm$ 0.08	8.53 $\pm$ 0.60 ^a	2.06 $\pm$ 0.10 ^b	0.47 $\pm$ 0.03 ^{a,b}
COL1A1	1.00 $\pm$ 0.22	2.00 $\pm$ 0.44 ^a	2.45 $\pm$ 0.78 ^a	0.98 $\pm$ 0.35 ^b
Versican	1.00 $\pm$ 0.81	3.87 $\pm$ 0.79 ^a	3.70 $\pm$ 0.76 ^a	2.24 $\pm$ 0.29 ^{a,b}

Note: Data are presented as mean and standard error of the mean. Data are normalized to untreated myometrial cells. COL1A1 = collagen 1A1; ECM = extracellular matrix; TGF- β 3 = transforming-growth factor- β 3.

^a $P < .05$ compared with myometrial control.

^b $P < .05$ compared with untreated fibroid control.

Levy. Liarozole inhibits fibroid ECM formation. Fertil Steril 2014.