Selective progesterone receptor modulator asoprisnil down-regulates collagen synthesis in cultured human uterine leiomyoma cells through up-regulating extracellular matrix metalloproteinase inducer

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BACKGROUND: A recent clinical trial demonstrated that selective progesterone receptor modulator asoprisnil is effective in reducing uterine leiomyoma volume. We investigated the effects of asoprisnil in vitro on the expression of the extracellular matrix (ECM)-remodeling enzymes and collagens in cultured leiomyoma and matching normal myometrial cells. METHODS: The expression of extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinases (MMPs), tissue inhibitors of MMP (TIMPs) and collagens were assessed by western blot analysis. RESULTS: Untreated cultured leiomyoma cells had significantly lower EMMPRIN (P<0.05), MMP-1 (P<0.05) and membrane type 1-MMP (MT1-MMP) (P<0.01) protein contents, but significantly higher TIMP-1 (P<0.05), TIMP-2 (P<0.01), type I (P<0.05) and type III (P<0.01) collagen protein contents compared with untreated cultured myometrial cells. Treatment with asoprisnil at concentrations ≥10⁻⁷ M for 48 h significantly (P<0.05) increased EMMPRIN, MMP-1 and MT1-MMP protein contents, and decreased TIMP-1 (P<0.05), TIMP-2 (P<0.01), type I (P<0.01) and type III (P<0.05 at 10⁻⁷ M; P<0.01 at 10⁻⁶ M) collagen protein contents in cultured leiomyoma cells compared with control cultures. However, asoprisnil treatment did not affect the protein contents of ECM-remodeling enzymes and collagens in cultured myometrial cells. CONCLUSIONS: These results suggest that asoprisnil may reduce collagen deposit in the ECM of cultured leiomyoma cells through decreasing collagen synthesis and enhancing the expression of EMMPRIN, MMPs and TIMPs without comparable effects on cultured myometrial cells.

Keywords: asoprisnil; extracellular matrix; extracellular matrix metalloproteinase inducer; leiomyoma; selective progesterone receptor modulator

Introduction

Uterine leiomyomas are sex-steroid-dependent benign neoplasms occurring in reproductive age women. Although it has been believed for a long time that estrogen is the main hormone contributing to the growth of leiomyomas, substantial new evidence indicates that progesterone also plays a vital role in the regulation of leiomyoma growth by affecting cell proliferation and apoptosis induction (Rein et al., 1995; Matsuo et al., 1997; Shimomura et al., 1998; Kurachi et al., 2001; Maruo et al., 2004).

Asoprisnil belongs to a new class of selective progesterone receptor modulators (SPRMs) that exert tissue-selective progesterone agonist, antagonist or mixed agonist/antagonist effects on progesterone-target tissues in vivo (Chwalisz et al., 2005). A recent clinical trial demonstrated that treatment with asoprisnil reduced leiomyoma volume and its symptoms in patients with symptomatic leiomyomata (Chwalisz et al., 2007). Although the precise mechanism by which asoprisnil reduces leiomyoma volume remains incompletely defined, we have recently demonstrated that asoprisnil inhibits the proliferation and induces apoptosis of cultured leiomyoma cells in the absence of comparable effects on normal myometrial cells (Chen et al., 2006; Wang et al., 2006; Sasaki et al., 2007), suggesting that asoprisnil inhibits the growth of cultured leiomyoma cells in a cell-type-specific manner in the uterus.

The extracellular matrix (ECM) is a fibrillar protein meshwork which provides a structural scaffold for tissue support and serves as a reservoir for growth factors (Chirco et al., 2006). Collagens are the major structural elements of the ECM and contribute to the stability and maintain the structural...
integrity of the tissues (Gelse et al., 2003). Fibril-forming collagens include collagen types I, II, III, V and XI. Type I collagen is the most abundant ECM protein, and type III collagen is distributed in type I collagen-containing tissues (Gelse et al., 2003). Collagen molecules are secreted into the extracellular space and self-assemble into fibrils in the ECM (Canty and Kadler, 2005).

Recent studies demonstrate that uterine leiomyomata are characterized by quantitative and qualitative abnormalities in several ECM components, including collagen and glycosaminoglycans (Stewart et al., 1994; Wolaniksa et al., 1998; Berto et al., 2003). Types I and III collagen mRNAs were shown to be elevated in leiomyomas relative to the adjacent myometrium in the proliferative phase of the menstrual cycle (Stewart et al., 1994). A microarray analysis demonstrated that pro-alpha1 (III) and pro-alpha1 (I) collagen mRNAs were increased in leiomyomas relative to the myometrium, suggesting the possible involvement of collagen deposition in leiomyoma enlargement (Wang et al., 2003). Electron microscopy revealed that leiomyomas contain abnormal collagen fibril structure and orientation (Leppert et al., 2004). Moreover, leiomyomas have decreased expression of dermatopontin, a collagen-binding ECM protein (Catherino et al., 2004; Arslan et al., 2005; Malik and Catherino, 2007), and increased expression of versican, a chondroitin sulfate proteoglycan involved in matrix assembly and structure (Catherino et al., 2004; Leppert et al., 2006; Malik and Catherino, 2007).

The highly regulated control of ECM turnover and homeostasis occurs in concert with the action of proteolytic enzymes known as matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) (Curry and Osteen, 2003; Visse and Nagase, 2003). MMPs participate in the degradation of the ECM, whereas TIMPs inhibit the activity of MMPs (Visse and Nagase, 2003). Extracellular matrix metalloproteinase inducer (EMMPRIN) is a transmembrane glycoprotein that regulates several MMPs (Guo et al., 1997). MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 were shown to be expressed in leiomyomas at mRNA and protein levels (Dou et al., 1997; Ma and Chegini, 1999). Dou et al. (1997) demonstrated that MMP and TIMP mRNA expression was increased in leiomyomas during the secretory phase of the menstrual phase compared with the proliferative phase, suggesting the hormonal regulation of MMP and TIMP.

However, the effects of asoprisnil on the ECM-remodeling enzymes and collagens in leiomyoma and normal myometrial cells have not been studied to date.

In the present study, we investigated the effects of asoprisnil on the protein levels of EMMPRIN, MMP-1, membrane type 1-MMP (MT1-MMP), TIMP-1, TIMP-2, type I and type III collagens in cultured human uterine leiomyoma cells in comparison with cultured normal myometrial cells.

**Materials and Methods**

**Tissue collection**

Twelve samples of leiomyoma tissues and myometrium were obtained from Japanese women with regular menstrual cycles who underwent hysterectomy at Kobe University Hospital. Informed consent was obtained from each patient before surgery for the use of uterine tissues. The Institutional Review Board approved the use of leiomyoma tissues and myometrium for culture experiments. The patients ranged in age from 31 to 48 years, with a mean age of 41.1 years, and had received no hormonal therapy for at least six months before surgery. The histological diagnosis of each uterine specimen was examined. Samples were excluded from the study if accurate menstrual cycle dates could not be assigned or if unexpected pathology was found (e.g. adenomyosis). The day of the menstrual cycle was determined by endometrial histological dating according to the method of Noyes et al. (1950). Four samples were collected from the proliferative phase and eight samples were from the secretory phase of the menstrual cycle.

**Cell culture**

Leiomyoma tissues and myometrium obtained were cut into small pieces and digested in 0.2% collagenase (wt/vol) at 37°C for 3–5 h (Matsuo et al., 1997; Chen et al., 2006). The cells were collected by centrifugation at 460g for 5 min and washed with phosphate-buffered saline. The cell viability was determined by trypan blue exclusion test.

Leiomyomas and myometrial cells were plated at densities of ~1 x 10⁶ cells/dish in 10-cm² culture dishes and 1 x 10⁶ cells/well in 6-well tissue culture plates. The isolated cells were subcultured at 37°C for 120 h in a humidified atmosphere of 5% CO₂-95% air in phenol-red free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (vol/vol; Invitrogen Life Technologies, Inc., Grand Island, NY, USA). The monolayer cultures reaching ~70% confluence were treated with graded concentrations (10⁻⁸ M, 10⁻⁷ M and 10⁻⁶ M) of asoprisnil (TAP Pharmaceutical Products Inc., IL, USA) in serum-free, phenol red-free DMEM. Asoprisnil was dissolved in absolute ethanol. The final concentration of ethanol in culture media was <0.01%, and the same concentration of ethanol was used as a vehicle in control cultures.

**Western blot analysis**

Proteins were extracted from cultured leiomyoma and myometrial cells as described previously (Shimomura et al., 1998; Chen et al., 2006). The cells were lysed at 4°C for 20 min using a lysis buffer (150 mM NaCl, 2 mM phenylmethylsulphonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/ml aprotinin, 0.1% sodium dodecyl sulfate and 50 mM Tris–HCl, pH 7.5). Whole cell lysates were centrifuged at 13 000g for 30 min at 4°C, and the supernatants were collected. Each 100-μg aliquot of the protein extract was electrophoresed on a 3–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. The proteins were transferred from gels to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were exposed overnight to primary antibodies, followed by incubation for 1 h with horse-radish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). The antigen–antibody complexes were detected with the enhanced chemiluminescence detection system (Amersham Biosciences). The membranes were visualized by exposure to X-OMAT film (Eastman Kodak Co., Rochester, NY, USA). The radioautograms were scanned and quantified with Chemilmage 4400 (Astec Co., Ltd., Osaka, Japan). The experiments were repeated with at least three different specimens in triplicate with similar results, and the reported results are representative. The relative values of each protein were normalized with β-actin values from the same samples. The following primary antibodies were used in this study: EMMPRIN (sc-9753), MMP-1 (sc-6837), MT1-MMP (sc-12 367), TIMP-1 (sc-6832), TIMP-2 (sc-9905), type I collagen (sc-12 367), TIMP-1 (sc-6832), TIMP-2 (sc-9905), type I collagen...
Statistical analysis
The data were expressed as the mean ± SD from at least three independent experiments. Statistical significance was determined using Student’s *t*-test and one-way analysis of variance (ANOVA). A difference with a *P* < 0.05 was considered statistically significant.

Results
EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen protein levels in untreated cultured leiomyoma cells and normal myometrial cells
We examined the basal protein contents of EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen in cultured leiomyoma cells and normal myometrial cells by western blot analysis after subculture in phenol red-free DMEM supplemented with 10% FBS for 120 h (Fig. 1). The stage of the menstrual cycle when the samples were obtained did not affect the protein levels of the ECM-remodeling enzymes in either the untreated leiomyoma or normal myometrial cells (data not shown).

Table I shows the fold change of the value of EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen in cultured leiomyoma cells relative to cultured myometrial cells. EMMPRIN, MMP-1 and MT1-MMP protein levels in untreated leiomyoma cells were significantly lower than those in untreated normal myometrial cells (EMMPRIN and MMP-1, *P* < 0.05; MT1-MMP, *P* < 0.01). TIMP-1 and TIMP-2 protein levels in untreated leiomyoma cells were significantly higher than those in untreated normal myometrial cells (TIMP-1, *P* < 0.05; TIMP-2, *P* < 0.01). The levels of types I and III collagen proteins in untreated leiomyoma cells were also significantly higher than those in untreated normal myometrial cells (type I collagen, *P* < 0.05; type III collagen, *P* < 0.01).

Table I. EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen protein levels in untreated cultured leiomyoma cells and myometrial cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMMPRIN</td>
<td>1.00</td>
<td>0.61 ± 0.11*</td>
</tr>
<tr>
<td>MMP-1</td>
<td>1.00</td>
<td>0.67 ± 0.28*</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>1.00</td>
<td>0.82 ± 0.13**</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.00</td>
<td>1.28 ± 0.18*</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>1.00</td>
<td>1.50 ± 0.54**</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>1.00</td>
<td>1.31 ± 0.12*</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>1.00</td>
<td>1.26 ± 0.22**</td>
</tr>
</tbody>
</table>

The relative intensities of EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen proteins were normalized to the respective *β*-actin. Results represent the mean ± SD of the fold change of the value in leiomyoma cells relative to myometrial cells of at least three independent experiments performed in triplicate. *P* < 0.05 versus untreated myometrial cells; **P* < 0.01 versus untreated myometrial cells.

Effects of graded concentrations of asoprisnil on EMMPRIN, MMP-1 and MT1-MMP protein levels in cultured leiomyoma cells and myometrial cells cultured for 48 h, as assessed by western blot analysis
Figure 2 shows the effects of graded concentrations of asoprisnil on EMMPRIN, MMP-1 and MT1-MMP protein levels in leiomyoma cells and myometrial cells cultured for 48 h, as assessed by western blot analysis.

Effects of graded concentrations of asoprisnil on EMMPRIN, MMP-1 and MT1-MMP protein levels in cultured leiomyoma cells and normal myometrial cells
To examine whether asoprisnil treatment modulates the expression of the ECM-remodeling enzymes in cultured leiomyoma cells and normal myometrial cells, EMMPRIN, MMP-1, MT1-MMP protein levels were assessed by western blot analysis in the cells cultured for 48 h in the absence or presence of asoprisnil (Fig. 2).
Asoprisnil modulates extracellular matrix in leiomyomas

Table II. Effects of graded concentrations of asoprisnil on EMMPRIN, MMP-1 and MT1-MMP protein levels in leiomyoma cells and myometrial cells cultured for 48 h.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Asoprisnil (10^-8 M)</td>
<td>1.06 ± 0.07</td>
<td>1.02 ± 0.12</td>
<td>1.08 ± 0.12</td>
<td>1.21 ± 0.21</td>
<td>1.04 ± 0.05</td>
<td>1.11 ± 0.12</td>
</tr>
<tr>
<td>Asoprisnil (10^-7 M)</td>
<td>1.07 ± 0.02</td>
<td>1.34 ± 0.35*</td>
<td>1.02 ± 0.08</td>
<td>1.39 ± 0.35*</td>
<td>1.03 ± 0.13</td>
<td>1.32 ± 0.35*</td>
</tr>
<tr>
<td>Asoprisnil (10^-6 M)</td>
<td>1.02 ± 0.10</td>
<td>1.39 ± 0.32*</td>
<td>0.87 ± 0.15</td>
<td>1.47 ± 0.50*</td>
<td>1.00 ± 0.16</td>
<td>1.38 ± 0.44*</td>
</tr>
</tbody>
</table>

The relative intensities of EMMPRIN, MMP-1 and MT1-MMP proteins were normalized to the respective β-actin. Results represent mean ± SD of the fold change of the value of at least three independent experiments performed in triplicate. *P < 0.05 versus untreated control cultures.

Table III. Effects of graded concentrations of asoprisnil on TIMP-1 and TIMP-2 protein levels in leiomyoma cells and normal myometrial cells cultured for 48 h.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
<th>Myometrial cells</th>
<th>Leiomyoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Asoprisnil (10^-8 M)</td>
<td>1.00 ± 0.00</td>
<td>0.79 ± 0.17</td>
<td>1.02 ± 0.01</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>Asoprisnil (10^-7 M)</td>
<td>0.97 ± 0.03</td>
<td>0.63 ± 0.41*</td>
<td>1.02 ± 0.06</td>
<td>0.92 ± 0.04**</td>
</tr>
<tr>
<td>Asoprisnil (10^-6 M)</td>
<td>0.97 ± 0.03</td>
<td>0.46 ± 0.24*</td>
<td>0.99 ± 0.07</td>
<td>0.85 ± 0.05**</td>
</tr>
</tbody>
</table>

The relative intensities of TIMP-1 and TIMP-2 proteins were normalized to the respective β-actin. Results represent mean ± SD of the fold change of the value of at least three independent experiments performed in triplicate. *P < 0.05 versus untreated control cultures; **P < 0.01 versus untreated control cultures.

Figure 3: Effects of graded concentrations of asoprisnil on TIMP-1 and TIMP-2 protein levels in leiomyoma cells and myometrial cells cultured for 48 h, as assessed by western blot analysis.

Effects of graded concentrations of asoprisnil on TIMP-1 and TIMP-2 protein levels in cultured leiomyoma cells

To explore whether asoprisnil treatment modulates the expression of the ECM-remodeling enzymes in cultured leiomyoma cells and normal myometrial cells, asoprisnil (10^-8 M to 10^-6 M) was added to the cell cultures for 48 h, as assessed by western blot analysis in the cells cultured for 48 h in the absence or presence of asoprisnil (Fig. 3).

Table III shows the fold change of the value of TIMP-1 and TIMP-2 in cultured leiomyoma cells relative to normal myometrial cells. One-way ANOVA of the indices for TIMP-1 and TIMP-2 showed significant effects of asoprisnil concentration (P < 0.05). TIMP-1 and TIMP-2 protein levels in cultured leiomyoma cells were significantly (P < 0.05) increased by the treatment with asoprisnil at concentrations ≥ 10^-7 M compared with untreated control cultures (Fig. 2, Table II). In cultured normal myometrial cells, treatment with graded concentrations of asoprisnil did not affect EMMPRIN, MMP-1 and MT1-MMP protein levels (Fig. 2, Table II).

Effects of graded concentrations of asoprisnil on TIMP-1 and TIMP-2 protein levels in cultured leiomyoma cells and normal myometrial cells

Effects of graded concentrations of asoprisnil on type I collagen and type III collagen levels in cultured leiomyoma cells and normal myometrial cells

To examine whether asoprisnil treatment modulates the expression of the ECM component in cultured leiomyoma cells and normal myometrial cells, type I collagen and type III collagen protein levels were assessed by western blot analysis in the cells cultured for 48 h in the absence or presence of asoprisnil (Fig. 3).

Table IV shows the fold change of the value of type I collagen and type III collagen in cultured leiomyoma cells and normal myometrial cells. One-way ANOVA of the indices for type I collagen and type III collagen showed significant effects of asoprisnil concentration (TIMP-1, P < 0.05; TIMP-2, P < 0.01) (Fig. 3, Table III). In cultured normal myometrial cells, treatment with graded concentrations of asoprisnil did not affect TIMP-1 and TIMP-2 protein levels (Fig. 3, Table III).

Effects of graded concentrations of asoprisnil on type I collagen and type III collagen levels in cultured leiomyoma cells and normal myometrial cells


analysis revealed that EMMPRIN, MMP-1, MT1-MMP, TIMP-1, TIMP-2, type I collagen and type III collagen proteins were present in cultured leiomyoma and myometrial cells. We found that compared with untreated normal myometrial cells, EMMPRIN, MMP-1 and MT1-MMP protein levels were significantly lower in untreated leiomyoma cells, whereas TIMP-1, TIMP-2, type I collagen and type III collagen protein levels were significantly higher in those cells. The results presented here suggest that the dysregulation of EMMPRIN, MMPs and TIMPs with the excess of collagen synthesis in leiomyoma cells may contribute to the accumulation of collagens in the ECM, leading to the expansion of uterine leiomyomas in vivo.

EMMPRIN is a cell surface glycoprotein with N-linked oligosaccharides that belongs to a family of the immunoglobulin superfamily (Biswas et al., 1995). It is well documented to stimulate the production of MMP-1, MMP-2, MMP-3 and MT1-MMP in fibroblasts and tumor cells (Guo et al., 1997; Sameshima et al., 2000; Sun and Hemler, 2001; Caudroy et al., 2002). It is expressed as a core protein (27 kDa), a less glycosylated form (~32 kDa), and a highly glycosylated (HG) form (45–65 kDa) (Tang et al., 2004; Nabeshima et al., 2006). HG form of EMMPRIN stimulates MMP production (Sun and Hemler, 2001; Tang et al., 2004). The present study is the first to demonstrate the presence of EMMPRIN protein in cultured leiomyoma cells and normal myometrial cells, suggesting a role of EMMPRIN in the regulation of tissue remodeling through the induction of MMPs. Moreover, we demonstrated that asoprisnil treatment induced the HG form of EMMPRIN with 45–70 kDa in cultured leiomyoma cells. Thus, it seems likely that the asoprisnil-induced increase in HG form of EMMPRIN may augment the production of MMP-1 and MT1-MMP in cultured leiomyoma cells. Furthermore, we provided the first evidence that asoprisnil treatment significantly up-regulated EMMPRIN, MMP-1 and MT1-MMP protein levels in cultured leiomyoma cells, but significantly attenuated TIMP-1, TIMP-2, type I collagen and type III collagen protein contents in those cells without affecting the levels of the ECM-regulating enzymes and collagens in cultured normal myometrial cells.

MMPs represent a family of zinc-dependent endopeptidases which are grouped into collagenases, gelatinases, stromelysins, matrilysins, MT-MMPs and others (Nagase et al., 2006).

Table IV. Effects of graded concentrations of asoprisnil on type I collagen and type III collagen protein levels in leiomyoma cells and myometrial cells cultured for 48 h.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Type I collagen</th>
<th>Type III collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myometrial cells</td>
<td>Leiomyoma cells</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Asoprisnil (10⁻⁸ M)</td>
<td>1.02 ± 0.21</td>
<td>0.95 ± 0.11</td>
</tr>
<tr>
<td>Asoprisnil (10⁻⁷ M)</td>
<td>0.93 ± 0.30</td>
<td>0.78 ± 0.12*</td>
</tr>
<tr>
<td>Asoprisnil (10⁻⁶ M)</td>
<td>0.93 ± 0.10</td>
<td>0.75 ± 0.13**</td>
</tr>
</tbody>
</table>

The relative intensities of type I collagen and type III collagen proteins were normalized to the respective β-actin. Results represent the mean ± SD of the fold change of the value of at least three independent experiments performed in triplicate. *P < 0.05 versus untreated control cultures; **P < 0.01 versus untreated control cultures.

Discussion

We have previously demonstrated that asoprisnil inhibits cell proliferation and induces apoptosis in cultured leiomyoma cells in the absence of comparable effects on cultured normal myometrial cells (Chen et al., 2006; Wang et al., 2006; Sasaki et al., 2007). These in vitro data suggest that the shrinkage of leiomyomata, as observed in clinical trial, may be related to anti-proliferative and pro-apoptotic actions of asoprisnil. Another possible mechanism underlying this effect could be via tissue-specific modulation of the ECM production. The effects of asoprisnil on the expression of ECM-remodeling enzymes and collagens in cultured leiomyoma cells and normal myometrial cells, however, have not been investigated until now.

In the present study, we first examined the expression of the ECM-remodeling enzymes and collagens in untreated cultured leiomyoma cells and normal myometrial cells. Western blot
Collagenases include MMP-1, MMP-8, MMP-13 and MMP-18 that cleave interstitial collagen types I, II and III (Tang et al., 2004). MT1-MMP also cleaves collagen types I, II and III (Nagase et al., 2006). MMPs are synthesized as pro-MMPs, which are activated intracellularly, and secreted from the cells, or cell surface-bound as active enzymes (Nagase et al., 2006). The data presented here revealed that asoprisnil treatment augmented MMP-1 and MT1-MMP protein contents in cultured leiomyoma cells. The increases in MMP-1 and MT1-MMP protein contents in cultured leiomyoma cells caused by the asoprisnil treatment may be attributable to the up-regulation of EMMPRIN levels.

The proteolytic activity of MMPs is inhibited by TIMPs (Lambert et al., 2004). TIMP-1 and TIMP-2 amino acid sequences share 40% homology in the N-terminal domain (Lambert et al., 2004). TIMP-1 expression is inducible by external stimuli, while TIMP-2 is constitutive (Lambert et al., 2004). Although untreated leiomyoma cells had elevated TIMP-1 and TIMP-2 protein levels compared with untreated normal myometrial cells, asoprisnil treatment induced a significant decrease in TIMP-1 and TIMP-2 protein levels in cultured leiomyoma cells. This suggests that the reduced activity of TIMPs leads to the inhibition of the proteolytic activity of MMP-1 and MT1-MMP in the extracellular space. Thus, it seems likely that asoprisnil-induced induction of MMPs and reduction of TIMPs may promote the proteolysis of interstitial collagens and result in the remodeling of the ECM in leiomyomas.

Actually, in the present study, the synthesis of types I and III collagens was significantly down-regulated in cultured leiomyoma cells in response to asoprisnil treatment. The mechanism by which asoprisnil decreases types I and III collagen expression remains poorly understood. The possible explanation includes an intracellular degradation of collagens by activated MMPs and the suppressed transcription or translation of collagens in cultured leiomyoma cells in response to asoprisnil treatment. Several factors are documented to regulate type I collagen gene expression in scleroderma skin fibroblasts, including protein factors like CCAAT-binding factor, Sp1, nuclear factor-1, Smad signaling molecules, coactivators CBP/p300, transforming growth factor β3 (TGFβ3) and its receptors, and connective tissue growth factor (Ghosh, 2002). TGFβ3 is shown to play an important role in type I and III collagen production in leiomyoma cells (Lee and Nowak, 2001). In this context, we previously demonstrated that asoprisnil treatment caused a decrease in TGFβ and TGFβ type II receptor levels in cultured leiomyoma cells at 72 h, but not at 48 h (Wang et al., 2006). Therefore, it seems unlikely that TGFβ3 is involved in asoprisnil-induced inhibition of types I and III collagen synthesis in cultured leiomyoma cells at 48 h of treatment. Nevertheless, we cannot exclude the possibility that asoprisnil treatment represses collagen gene transcription in cultured leiomyoma cells through modulating, in an unknown way, the factors linked to collagen gene expression.

On the basis of our results, we can speculate that asoprisnil-induced decrease in collagen synthesis in cultured leiomyoma cells could reduce the collagen deposition in the ECM, which in turn causes the shrinkage of the ECM volume and the subsequent impairment of the stability and structural integrity of leiomyoma tissues and the suppression of leiomyoma expansion in vivo. In addition, the reduction of collagen accumulation in the ECM may disturb the function of collagens as a reservoir for growth factor such as platelet-derived growth factor (PDGF). PDGF has been shown to bind to collagen I–VI in the mesenchymal cells (Somasundaram and Schppan, 1996) and increase mRNA levels of proliferating cell nuclear antigen, collagen α1 (I) and collagen α1 (III) in uterine leiomyomata (Liang et al., 2006). Therefore, a possible decreased storage of PDGF in the ECM of leiomyomas in response to asoprisnil treatment may inhibit the potential proliferative activity of leiomyoma cells and an accumulation of collagens in the ECM. Thus, it is likely that asoprisnil may clinically contribute to the suppression of the growth and expansion of leiomyomas through modulating the metabolism of the collagens and a stored growth factor in the ECM. Further study is necessary to investigate the effects of asoprisnil on the expression of collagens and a growth factor in the ECM of leiomyoma tissues by using hysterectomy specimens from patients with leiomyomata after treatment with asoprisnil. Taken together, our data strongly suggest that asoprisnil treatment modulates the balance between MMPs and TIMPs in favor of the collagenolytic activity in concert with a decreased synthesis of interstitial collagens in cultured leiomyoma cells, thereby most likely causing an increased degradation of the ECM and a decreased deposition of collagens in the ECM.

In the present study, we demonstrated a cell type-specific action of asoprisnil as an anti-fibrotic agent in cultured leiomyoma cells. Unlike cultured leiomyoma cells, asoprisnil did not affect EMMPRIN, MMPs, TIMPs, type I collagen and type III collagen protein levels in cultured normal myometrial cells. The mechanism underlying the differential actions of asoprisnil between cultured leiomyoma cells and normal myometrial cells remains undetermined. The possible explanations may be the difference in the progesterone receptor (PR) isoform expression (Chen et al., 2006; Xu et al., 2006) and the differential recruitment of nuclear receptor coactivators and corepressors between the two types of cells. We previously showed that PR-B protein expression was elevated in untreated cultured leiomyoma cells compared with cultured normal myometrial cells, but no difference was noted in PR-A expression between the two types of cells (Chen et al., 2006). Furthermore, we found that a progesterone antagonist CDB-2914 that exhibited the similar anti-proliferative and pro-apoptotic effects as asoprisnil decreased PR-B and increased PR-A protein levels in cultured leiomyoma cells without affecting these PR isoform levels in cultured myometrial cells (Xu et al., 2006). A recent study demonstrates that asoprisnil weakly recruited coactivators such as steroid receptor coactivator and amplified in breast cancer 1, but strongly recruited the corepressors, nuclear corepressors, in a manner similar to RU486 (Madauss et al., 2007). However, it remains unknown whether asoprisnil treatment differently recruits nuclear receptor coactivators and corepressors in cultured leiomyoma cells and normal myometrial cells. Further study will be necessary to elucidate the precise mechanism responsible for the differential effects of asoprisnil in the expression of the
ECM-remodeling enzymes and collagens between the two types of cells.

In summary, the results of this study demonstrated that asoprisnil selectively increased EMMPRIN, MMP-1 and MT1-MMP protein contents and decreased TIMP-1, TIMP-2, type I collagen and type III collagen protein contents in cultured leiomyoma cells in the absence of comparable effects on cultured normal myometrial cells.

In conclusion, we demonstrated in an in vitro system that the SPRM, asoprisnil, targets various important ECM pathways in leiomyoma cells that may lead to the reduction in collagen synthesis and increase in its degradation. We previously showed that asoprisnil exerts cell-specific anti-proliferative and pro-apoptotic activities on leiomyoma cells in vitro. The results of the present study suggest that SPRM effects on the leiomyoma cells are more complex and include changes in the ECM-remodeling enzymes and collagens that play an important role in the integrity of leiomyoma tissue. These observations may have clinical implication. They suggest that the changes in ECM metabolism may contribute to leiomyoma volume reduction, which was observed in clinical trials with asoprisnil (Chwalisz et al., 2007). However, this implication should be interpreted with caution, since various additional mechanisms may play a role in an in vivo situation, including tissue concentration of asoprisnil in leiomyomata after in vivo exposure, the effects of active metabolites such as J912, storage of growth factor in the ECM, changes in ovarian steroid levels and decrease in uterine artery perfusion. These mechanisms would need to be evaluated in clinical studies.

Authors’ contribution
All authors have met contributions (i) substantial contributions to conception and design, or acquisition of data or analysis and interpretation of data, (ii) drafting the article or revising it critically and (iii) final approval of the version to be submitted.

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References
Lee BS, Nowak RA. Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-ß3 (TGFß3) and altered responses to the antiproliferative effects of TGFß. J Clin Endocrinol Metab 2001;86:913–920.
Ma C, Chegini N. Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-ß. Mol Hum Reprod 1999;5:950–954.


