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Relaxin Increases Elastase Activity and Protease Inhibitors in Smooth Muscle Cells from the Myometrium compared to Cells from Leiomyomas

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Abstract

Objective—Leiomyoma growth is dependent on ovarian steroids. Estrogen stimulates growth while progesterone can have both stimulatory and inhibitory effects. We sought to investigate the effect of relaxin on extracellular matrix metabolism (ECM) remodeling in myometrium compared to leiomyomas.

Design—Smooth muscle cells were cultured from large myomas and their corresponding myometrium and stimulated with different concentrations of relaxin. Total elastase activity, α -1 antitrypsin (ATT), TIMP-1, and TIMP-2 expressions were measured.

Setting—Medical school university hospital.

Patient(s)—Four premenopausal women undergoing hysterectomy.

Intervention(s)—Smooth muscle cells from leiomyomas and their corresponding myometrium were stimulated with different concentrations of relaxin.

Main Outcome Measure(s)—Elastase activity and protease inhibitor levels in smooth muscle cells.

Results—Relaxin-stimulated myometrial cells showed a positive response curve with respect to total elastase activity, ATT, TIMP-1, and TIMP-2 expressions, while leiomyoma cells showed no response to relaxin stimulation.

Conclusion—Relaxin stimulates ECM remodeling in the myometrium, while it does not seem to affect leiomyomas.

Keywords

Extracellular matrix; elastase; α -1 antitrypsin; TIMP-1; TIMP-2; myometrium; leiomyoma; smooth muscle cell

Leiomyomas are common uterine tumors that affect up to 77% of reproductive-aged women (1). Compared to normal myometrium, leiomyomas contain large amounts of collagen, elastin, hyaluronic acid, and glycosaminoglycans. Tumor growth is associated with extracellular matrix

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(ECM) remodeling which occurs via increased biosynthesis, decreased degradation or both. Collagen is degraded by a family of enzymes called the matrix metalloproteinases (MMPs), which are regulated by locally produced tissue inhibitors of metalloproteinases (TIMPs). MMP-2 expression, a protease capable of degrading collagen and elastin fibers, was found to be increased in leiomyoma compared to normal myometrium (2), suggesting active turnover.

Leiomyoma growth is also dependent on ovarian hormones. While estrogen tends to stimulate growth, progesterone appears to both stimulate and inhibit growth (3). Myoma growth occurs during the secretory phase of the menstrual cycle when circulating estrogen, progesterone, and relaxin are present (4). In this study, we sought to investigate the effect of relaxin on ECM remodeling using an *in vitro* cell model. Specifically, we examined elastase activity and protease inhibitor expressions [α -1 antitrypsin (ATT), TIMP-1, and TIMP-2] in relaxin-stimulated smooth muscle cells cultured from myometrium and leiomyoma.

The Institutional Review Board of the Stanford University School of Medicine approved this study. We selected women with leiomyoma (size 8–10 cm) in the secretory phase of the menstrual cycle. The phase of cycle was confirmed by endometrial histology. The participants had not received hormonal therapy for at least three cycles before surgery. Matched uterine myometrial and leiomyoma tissues were obtained from patients undergoing hysterectomy for leiomyoma. Uterine myometrial tissues from a fresh specimen were excised distal to the tumor and 1 cm in depth from the endometrium. A representative cross-section was fixed in 10% buffered formalin for 16 hours, processed with paraffin embedding, and used for immunohistochemistry. Myoma tissues were taken midway between the center and periphery of the tumor. After excision, the tissues were put into tubes containing Dulbecco's modified Eagle's medium (DMEM) for the isolation of smooth muscle cells for immunofluorescence staining and tissue culture as described previously (5).

Smooth muscle cells from the myoma or myometrium were cultured in a 4-well chamber slide. The cells were fixed with 4% PFA and treated with 5% Triton. After washing with 0.02% Triton in Tris-Buffered Saline (TBS-T) and blocking with 1% BSA in TBS-T, the slides were then incubated with rabbit anti-LGR7 or anti-LGR8 (1/100, Phoenix Pharmaceuticals, Belmont, CA, USA) and mouse anti-desmin (1/20, Sigma, St. Louis, MO, USA) primary antibody at 4° C overnight. Deletion of the primary antibody was used as a negative control. After washing, the slides were incubated with goat anti-mouse IgG-TRITC (1/50) and goat anti-rabbit-IgG-FITC (1/320, Sigma) at room temperature for 1 hour. DAPI staining was used to observe nuclei. They were visualized and photographed with AxioCam (Zeiss, Oberkochen, Germany).

ATT staining was performed on paraffin-embedded myoma and myometrium using the avidin-biotin-peroxidase (ABC) method as described previously (6). The slides were stained with rabbit anti- α -1-antitrypsin (1/20 Sigma) primary antibody overnight at 4° C. Deletion of the primary antibody was used as a negative control. After rinsing with TBS-T, slides were incubated with a secondary antibody, goat anti-rabbit biotin conjugate (1/50, Vector Laboratories). The slides were incubated with Vectastatin ABC Kit (Vector Laboratories) reagent for 30 minutes at room temperature. Slides were counterstained with 25% of hematoxylin. They were visualized and photographed with AxioCam. Weigert's solution and Van Gieson's mixture of picric acid and acid fuchsin were used to stain elastin and collagen respectively on paraffin-embedded myometrium and myoma tissues as described previously (6).

Smooth muscle cells (SMC) were isolated from leiomyoma and myometrial tissues as described previously (5). Briefly, the tissues were minced into 1–2 mm explants and digested with 200u/ml collagenase (Invitrogen, Carlsbad, CA, USA). Dispersed cells were then

centrifuged at 1000 rpm for 10 minutes and washed twice in serum-free media. The cells were plated on a 6-well plate in DMEM with 10 % FBS, 100 μ /ml of penicillin and 100 μ /ml of streptomycin in an atmosphere of 5% CO₂/95% air. The purity of the cells was assessed by immunofluorescence using mouse anti-desmin monoclonal antibody (Sigma, St. Louis, MO, USA (7). When cells were confluent, they were primed with 1 μ M 17 β -estradiol in 0.2% lactalbumin hydrolysate (Sigma) (8) in DMEM for 72 hours to induce relaxin receptor expression. The medium was then removed and replaced with 0.2% lactalbumin hydrolysate in DMEM without or with various amounts of relaxin (0–100 ng/ml, a gift of Dr. Aaron Hsueh, Stanford University) for 48 hours. The conditioned media were concentrated with Centricon (Millipore Corp., Bedford, MA, USA) and stored at – 80° C until experiments were performed.

Elastase activity in the conditional media was determined by the generation of free amino groups from succinylated elastin (6). Briefly, 50 μ l of conditional media was added to 100 μ g succinylated elastin in 50 μ M sodium borate buffer (pH 8.0, Sigma) and incubated at 37° C for 1 hour. Fifty μ l of a 0.03% solution of TNBSA (Sigma) were added to each reaction and left for 20 minutes at room temperature. The optical density of each reaction was determined using Molecular Devices microplate reader at 450 nm. The elastase activity was standardized by protein concentration.

Ten micrograms of total protein from conditional media were separated by 10% SDS-PAGE under reducing condition (TIMP-1 and TIMP-2) or non-reducing condition (ATT) and blotted onto nitrocellulose membranes (Pierce, Rockford, IL) in an electrophoretic transfer cell (Bio-Rad, Hercules, CA) (9,10). Blots were blocked with 5% non-fat milk at 4 C overnight. After blocking, the membrane was washed three times in PBS-T (PBS, pH 7.4 and 0.1% Triton), then incubated with monoclonal anti-TIMP-1, TIMP-2 (1 μ g/ml Oncogene Research Products, Cambridge, MA, USA) α -1 or 1:5000 rabbit anti-human α -1 antitrypsin (Sigma) for 1 hour at room temperature, followed by 3 washes in PBS-T. The membrane was incubated in 1:5000 dilutions of sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK) conjugated to HRP for 1 hour at room temperature, followed by three washes in PBS-T. Blots were developed by chemiluminescence. The optical density was determined by GEL-DOC 2000(Bio-Rad).

Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC, USA). For each dose response curve, analysis of variance and *post hoc* analysis (Tukey-Kramer HSD) were applied to determine whether there was a statistically significant difference between the means of cell groups treated and untreated with relaxin. A probability value of < 0.05 denoted a statistical difference between groups.

Four premenopausal women with large myomas (greater than 8cm) were recruited. Given that leiomyomas grow during the secretory phase, we only obtained tissues from women in the secretory phase of the menstrual cycle (confirmed by endometrial histology). We confirmed expression of relaxin receptors, LGR7 and LGR8, and ATT in our cultured smooth muscle cells and tissues. We also verified the presence of substrate (collagen and elastin) for the ECM proteases in both myometrium and myoma.

Total elastase activity was significantly higher in smooth muscle cells cultured from the myometrium compared to leiomyoma ($P < 0.05$ at relaxin concentration of 10 ng/ml, Figure 1A). Interestingly, total elastase activity remained consistently low in smooth muscle cells cultured from leiomyomas, in spite of increasing relaxin concentration.

With respect to ATT, an elastase inhibitor, smooth muscle cells cultured from myometrium showed a significant positive dose response curve with increasing concentrations of relaxin. The highest expression occurred at the 10ng/ml relaxin concentration dose ($P < 0.05$, Figure 1B), which corresponds to the dose of highest total elastase activity. This pattern suggests

increased ECM remodeling, with active degradation and inhibition occurring with relaxin stimulation. Again, smooth muscle cells cultured from leiomyomas did not show any dose response to relaxin. Both TIMP-1 and TIMP-2, inhibitors of MMP-2, showed increased expression with increasing relaxin concentrations in myometrial cells compared to myoma cells. Myoma smooth muscle cells remained consistently unchanged.

ECM remodeling is dependent on the constant interplay between proteases and their inhibitors. This complex system is further modulated by reproductive hormones, growth factors, and cytokines. Estrogen and/or progesterone have both been associated with leiomyoma growth (3,11). However, myomas have been observed to both increase and decrease in size with the use of a levonorgestrel-releasing intrauterine device, which creates a progestational environment in the uterus (12). Likewise, some leiomyomas grow during pregnancy while many shrink or remain stable in size (13–15). Maruo et al. suggested that in addition to its stimulatory effects, progesterone may also exert inhibitory effects on leiomyoma cell growth and survival. This dual role appears to be modulated by the local growth factor environment. There is currently scant data on the effects of relaxin – a peptide hormone present during the secretory phase of the menstrual cycle and in pregnancy – on leiomyoma growth.

Relaxin increases MMPs (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in the pig uterus and cervix (16,17). Serine protease activity in these tissues is also increased during pregnancy (18). These changes in the ECM are thought to contribute to uterine growth. To further understand the role of reproductive hormones on the pathophysiology of leiomyomas, we investigated the effect of relaxin on ECM remodeling in myomas compared to normal myometrium. Given that MMP-2 activity has been reported to be higher in myoma compared to myometrium (2,19), we investigated the expression of inhibitors of MMP-2 (TIMP-1 and TIMP-2). Similarly, due to the observed increase in serine protease activity with relaxin, we examined total elastase activity and the associated inhibitor, ATT.

There are two potential areas of weakness in this preliminary study. Bourlev et al. have documented that leiomyoma growth tends to occur in the peripheral area of the tumor (4). The peripheral area of the leiomyoma is also where angiogenesis is most active (20) and may exhibit higher levels of degradation due to this. Because of this concern, we biopsied in the middle one-third of the myoma, away from the area of increased angiogenesis, rather than the outer one-third or central portions. This may contribute to the lack of response seen in the cultured myoma cells. Furthermore, estrogen and progesterone receptor levels have been shown to be reduced in cell cultures compared to whole tissues (21). Cells were taken during the secretory phase and used at first pass. Estrogen stimulation was used to induce relaxin receptor expression. It is possible that our culture condition may have artificially altered relaxin receptor expression in the myoma cells and, thus, cell response. Future tissue studies are necessary for verification.

In this *in vitro* study, we observed an increase in total elastolytic activity and a concurrent increase in protease inhibitor (ATT, TIMP-1, TIMP-2) expression in relaxin-stimulated myometrial smooth muscle cells compared to leiomyoma smooth muscle cells. This is consistent with increased ECM remodeling activity with active degradation and inhibition in the myometrium. Interestingly, the smooth muscle cells cultured from the leiomyomas did not show any response to relaxin, suggesting a stable and quiescent ECM. The interactions among estrogen, progesterone and relaxin require further examination.

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Figure 1.

Cultured SMCs isolated from myomas (>8cm) and corresponding myometrium were treated with increasing concentrations of relaxin (0–100ng/ml). (A), Total elastase activity in supernatant was determined by elastase assay. Each bar is the mean $\pm$ SE of total activity (u/mg/ml) from 5 experiments of two patients. (B), Western blotting of α 1-antitrypsin (ATT) in the supernatant of cultured SMCs. The expression of ATT in the supernatant from myometrium increased with relaxin (10ng/ml), no change was observed in the supernatant from myoma SMCs. Each bar is the mean $\pm$ SE of ATT from 5 experiments of two patients. *, significantly different from non-treated groups ($P < 0.05$).