Gonadotropin-releasing hormone (GnRH) agonist leuprolide acetate and GnRH antagonist cetrorelix acetate directly inhibit leiomyoma extracellular matrix production

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Objective: To determine the direct effect that GnRH analogues leuprolide acetate and cetrorelix acetate have on extracellular matrix in human leiomyoma and patient-matched myometrial cells.
Design: Laboratory study.
Setting: University hospital.
Patient(s): None.
Intervention(s): Cell culture, proliferation studies, and messenger RNA and protein analysis.
Main Outcome Measure(s): Expression of GnRHR1, *COL1A1*, fibronectin, and versican variant V0 in treated leiomyoma cells and patient-matched myometrial cells.
Result(s): Leiomyoma cells were treated with GnRH analogues for 6, 24, and 120 hours. Leuprolide treatment for 6 hours resulted in an increase in expression of GnRHR1 (4.02 ± 0.12-fold), *COL1A1* (6.41 ± 0.29-fold), fibronectin (9.69 ± 0.18-fold), and versican variant V0 (7.58 ± 0.43-fold). Leiomyoma cells treated with cetrorelix for 6 hours showed a decreased expression of GnRHR1 (0.5 ± 0.15-fold), *COL1A1* (3.79 ± 0.7-fold), fibronectin (0.92 ± 0.09-fold), and versican variant V0 (0.14 ± 0.07-fold). Leuprolide treatment of leiomyoma cells at high concentrations (10^{-5} M) did not result in an increase in protein production. Cetrorelix treatment of leiomyoma cells for 6 hours showed an increase in fibronectin protein production (3.14 ± 0.09-fold). Protein production of leiomyoma cells treated with cetrorelix for 120 hours demonstrated a decrease in GnRHR1 (0.51 ± 0.07-fold), *COL1A1* (0.35 ± 0.07-fold), fibronectin (1.94 ± 0.08-fold), and versican variant V0 (0.77 ± 0.19-fold).
Conclusion(s): Our findings demonstrate that GnRH analogue treatment directly regulated *COL1A1*, fibronectin, and matrix proteoglycan production. The reduction in versican variant V0 gene expression caused by cetrorelix treatment, and its association with the osmotic regulation of leiomyomas, presents a new and innovative approach to therapy for this disease. (Fertil Steril® 2012;98:1299–307. ©2012 by American Society for Reproductive Medicine.)
Key Words: Leiomyoma, leuprolide acetate, cetrorelix acetate, collagen 1A1, versican, fibronectin

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Uterine leiomyomas are highly prevalent, with 70%–80% of women burdened by the end of their reproductive years (1). For a significant proportion of these women, their leiomyomas will result in symptoms significant enough to warrant clinical intervention (2). Definitive surgical therapy, hysterectomy, is not an

option for women who desire future fertility. Women with leiomyomas are at increased risk of pregnancy loss (3), in addition to the morbid symptoms of menorrhagia, pelvic pain, and pelvic pressure. Radiologic interventions such as uterine artery embolization and MRI-guided high-frequency ultrasound ablation are either not indicated for women attempting pregnancy, or remain experimental (4, 5). As a result, therapeutic options are quite limited for the many women of reproductive age with uterine leiomyomas.

Currently, there is one medical intervention that is approved by the US Food and Drug Administration for presurgical treatment of uterine leiomyomas: leuprolide acetate (6). This GnRH agonist is believed to decrease the stimulatory effect of gonadal hormones on leiomyomas by decreasing gonadotropin stimulation of the ovary (7). There are convincing clinical data demonstrating effectiveness of GnRH analogues for decreasing leiomyoma size and symptoms (8, 9). However, the hypoestrogenic side effects of hot flashes and bone loss limit the therapeutic value of such treatment.

There is increasing evidence that GnRH analogues not only regulate gonadal hormone exposure but can also act directly on the leiomyoma. We and others have demonstrated the presence of GnRH receptors in surgical specimens of uterine leiomyomas and patient-matched myometrium (10, 11), and Chegini et al. (12) have demonstrated that leuprolide acetate and antide acetate directly stimulate the rate of [3H]thymidine incorporation and transforming growth factor (TGF)-β1 gene expression in leiomyoma cells. If GnRH analogues act directly, perhaps novel delivery systems could be designed to optimize therapeutic effect while minimizing systemic side effects.

Uterine leiomyomas are also known as fibroids owing to the excessive production of disorganized extracellular matrix (ECM) (13, 14). Because the majority of the tumor is made up of this disrupted matrix, resolution of the ECM is critical for resolution of bulk symptoms. In fact, in the absence of ECM dissolution, leiomyomas are highly stable, as demonstrated by a suspected leiomyoma that is more than 5,000 years old (15).

Clinically, GnRH analogues cause a rapid regression in leiomyoma size, but when therapy is discontinued, the leiomyomas rapidly return to their pretreatment size (16). Given the low mitotic rate of leiomyomas, this rapid growth is unlikely due to cell proliferation but is more likely due to regulation of leiomyoma ECM, which makes up the bulk of the tumor. However, little is known regarding the impact of GnRH analogues on leiomyoma ECM.

In this study, we hypothesized that the GnRH agonist leuprolide acetate and the GnRH antagonist cetrorelix acetate directly inhibit ECM gene and protein expression in human leiomyoma cells relative to untreated leiomyoma cells. To address this hypothesis, we evaluated ECM component expression, which is known to be altered between leiomyoma and myometrial cells. Leiomyoma cells treated with GnRH agonist or antagonist were compared with untreated cells, followed by confirmation and quantitation of ECM components.

MATERIALS AND METHODS

The study was carried out at the Uniformed Services University of the Health Sciences and the Walter Reed National Military Medical Center under a Human Use Committee–approved protocol.

Drugs and Chemicals

Leuprolide acetate (Sigma-Aldrich) and cetrorelix acetate (a generous gift of EMD Serono) were dissolved in water, aliquoted, and stored at −80°C.

Cell Proliferation Studies

Studies were conducted with patient-matched myometrial and leiomyoma cells that had been immortalized using the human papillomavirus (HPV 16) E6/E7 (17). Cells were plated in 48-well plates at an initial concentration of 5×10^3 cells per well. Growth media was defined as Dulbecco's Modified Eagle Medium (DMEM)/F12 media (Invitrogen) containing 10% fetal bovine serum (FBS), as well as antimicrobial and antifungal reagents. Cell growth was maintained at 37°C and 5% CO_2 until 30% confluence had been achieved. Cultures were then treated with cetrorelix acetate or leuprolide acetate at concentrations ranging from 10^{-9} M to 10^{-6} M. Myometrial and leiomyoma cells were treated for 48, 72, and 96 hours. The effect on proliferation of cetrorelix and leuprolide were assessed after each time point using the sulforhodamine-B assay. The assay measures total biomass by staining cellular proteins and is a marker for cell proliferation (Sigma-Aldrich).

RNA and Protein Protocol

Immortalized myometrial and leiomyoma cells (17, 18) were plated in six-well plates at a concentration of 2×10^4 cells per well and maintained in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO_2. When the cells reached 50% confluence, the media was replaced with phenol red–free DMEM/F12 containing 10% charcoal-stripped FBS. After 48 hours, the cells were exposed to serum-free media for 24–48 hours. Once monolayer cultures reached approximately 70%–80% confluence, they were treated with either leuprolide or cetrorelix at concentrations of 10^{-9} M to 10^{-6} M in phenol red–free DMEM/F12 containing 10% charcoal-treated FBS for 6 and 24 hours.

For 120 hours of exposure, fewer numbers of cells (1×10^4) were plated. The monolayer cultures were then treated with leuprolide after they had reached 30% confluence and after serum starvation for 24 hours. The cells were exposed to fresh media supplemented with specific concentrations of leuprolide every other day. At the end of 120 hours, the cells were at 85% confluence. After specified time points, the cells were either collected for RNA or protein for further analysis. The experiment was repeated three times, with two replicates for each experimental data point.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis

We used real-time reverse transcriptase–polymerase chain reaction (qRT-PCR) to evaluate the expression of GnRH receptor R1, and the ECM genes collagen 1A1, versican variant V0, and