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Relaxin Signaling in Uterine Fibroids

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Abstract

Uterine fibroid is the most common tumor of female reproductive organs. The role of relaxin signaling in leiomyoma development was analyzed. We used 23 matched pairs of leiomyoma and normal myometrium samples to compare the expression of relaxin family peptide receptors RXFP1, RXFP2, caveolin 1, desmin, steroid receptors and their co-factors NCOR1 and NCOR2. The expression of RXFP1 evaluated by quantitative RT-PCR was down-regulated in fibroid tissues. Relaxin or INSL3 treatment suppressed TGF- β induced phosphorylation of SMAD2 in rat leiomyoma ELT-3 cells in vitro suggesting a possible involvement of relaxins in etiology of leiomyoma.

Keywords

leiomyoma; transforming growth factor; relaxin; INSL3; phospho-SMAD2

INTRODUCTION

Uterine fibroid tumors (leiomyoma) are the most common gynecological neoplasms clinically affecting up to 30% of all women during reproductive age. The primary characteristics of uterine fibroids are the increased proliferation of myometrial cell, production of the abundant extracellular matrix (ECM), and the downregulation of ECM-degrading enzymes. Recent progress in cDNA microarray analysis revealed a distinct similarity of leiomyoma with other fibrotic processes.¹ The crucial role of several cytokines and growth factors including TGF- β is well-recognized in this disease.² Myometrium is a well-established target tissue of relaxin. Relaxin, a member of insulin-relaxin peptide family, signals through its G protein-coupled receptor RXFP1. It regulates ECM remodeling through alterations of collagen deposition, cell migration and proliferation, and overall tissue homeostasis.³ Relaxin suppression of TGF- β induced signaling and gene expression as well as myofibroblast differentiation was previously demonstrated in other organs.^{4, 5, 6, 7} The aims of this study were to analyze the gene expression of RXFP1, RXFP2, caveolin-1, ER1, PR and other genes in human leiomyoma and normal myometrium specimen and to determine whether relaxin and INSL3 were involved in the TGF- β signaling pathway.

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MATERIAL AND METHODS

Tissue collection

Human leiomyoma and myometrium tissue were obtained from patients undergoing hysterectomy for symptomatic leiomyoma at the Baylor Clinic of Baylor College of Medicine, Houston, Texas, under the Institutional Review Board approved human subjects protocol. The average age of the patients was 43.2 years old (37–49), 21% had single and 79% had multiple fibroids. The collected tissue were immediately placed in RNAlater (Ambion, Austin, TX) and stored at -80°C for further use.

RNA extraction

Total RNA was extracted from the leiomyoma and myometrium tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) and oligo-dT primers according to the manufacturer's recommended conditions.

Gene expression analysis

We evaluated different genes expressed in leiomyoma and myometrium using quantitative real-time PCR using SYBR Green detection protocol and iQ5 thermal cycler (Bio-Rad Labs, Hercules, CA). The primer sequences and PCR conditions are listed in Table 1. Expression of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used to normalize the expression of tested genes. Gene expression was calculated as a ratio between paired leiomyoma and myometrium samples from the same patient. The t-test was used to evaluate the statistical significance of differences.

Protein extraction

Eker rat uterine fibroid cells (ELT-3) were cultured in DMEM containing 10% FBS and 1% P-S. After being starved overnight, the cells were treated for 1 hour with human relaxin, porcine relaxin, INSL3, TGF- β , TGF- β + human relaxin, TGF- β + INSL3. Proteins were prepared by standard techniques.

Western blot analysis

The tissue lysates were subjected to SDS-PAGE (Invitrogen, 4–12%) and transferred to polyvinylidene difluoride membrane. Western blot hybridization was performed with anti-phospho-SMAD2 antibody (1:1000, Cell Signaling Technology, Danvers, MA) at 4°C overnight, followed by HRP goat anti-rabbit secondary antibody for 1 hour at room temperature. Finally, the SuperSignal West Pico kit (Thermo Fisher Scientific Inc., Rockford, IL) was used for detection.

Immunohistochemistry

The expression of RXFP1 in fibroid and myometrial tissue sections was analyzed using monoclonal anti-human RXFP1 antibody (1:200, Abcam, Cambridge, MA).

RESULTS

We analyzed the expression of 8 genes in 23 pairs of human leiomyoma and matched normal myometrium using quantitative RT-PCR. Among these genes, the *RXFP1* expression in leiomyoma was significantly down-regulated compared with that in normal myometrium ($p < 0.05$) (Fig. 1).

We performed immunohistochemical staining for RXFP1 on human leiomyoma and normal myometrium tissue sections. The expression of RXFP1 was detected in both tissues, however in fibroid samples the RXFP1 staining was consistently weaker (data not shown). Rat leiomyoma ELT-3 cells were treated with different combinations of TGF- β , relaxins and INSL3 for 1 hour. The phosphorylation of SMAD2 in these cells was analyzed using Western blot hybridization. Compared with the negative control group, the phosphorylation of SMAD2 was dramatically up-regulated when treated with TGF- β . The SMAD2 phosphorylation was significantly down-regulated when leiomyoma cells were treated with relaxin or INSL3 even without TGF- β stimulation. Furthermore, the up-regulation of phosphorylated SMAD2 induced by TGF- β was attenuated by the treatment of either relaxin or INSL3 (Fig. 2).

DISCUSSION

Uterine leiomyoma, or uterine fibroid, is a benign myometrial tumor of uterine smooth muscles. It has been demonstrated that some cytokines and growth factors play an important role in the progression of uterine fibroids.² Among those, transforming growth factor- β (TGF- β) appear to be the most important fibrosis-promoting cytokine. Some studies strongly suggest that TGF- β plays a central role in the pathogenesis of this disease by contributing to tumor growth through stimulation of both myometrial cell proliferation and production of abundant extracellular matrix.^{8,9} TGF- β signaling is initiated by binding of the ligand to type I and II receptor complex, which activates the phosphorylation of signal transducers SMAD2 and 3, their nuclear transition, and activation of the target genes.¹⁰

The antifibrotic properties of relaxin have already been demonstrated for pulmonary, cardiac, dermal, and renal fibroblasts.^{4, 5, 6, 7} In renal fibroblasts relaxin prevents TGF- β induced collagen synthesis by suppression of SMAD2 phosphorylation and complex formation of SMAD3.¹¹ Our results show that the RXFP1 expression in human uterine fibroid tumors is reduced compared with the expression in matched normal myometrium from the same patient. The RT-PCR data were further corroborated by immunohistochemistry with RXFP1 specific monoclonal antibody. Interestingly we did not find differences in the expression of estrogen receptor alpha, progesterone receptor, their corepressors NCOR1 and NCOR2, INSL3 receptor RXFP2, caveolin 1, or desmin, a marker of smooth muscle cells.

We have demonstrated also that both relaxin (human recombinant and native porcine) might affect the TGF- β signaling *in vitro*. The Western blot analysis showed that both these peptides suppressed TGF- β signaling pathway by suppression of SMAD2 phosphorylation. The decrease expression of relaxin receptor in fibroids may therefore lead to the increase of TGF- β signaling and thus progression of the tumor.

In summary, our data suggest that the suppression of relaxin signaling in uterine fibroid might play a role in tumor etiology and the stimulation by relaxin may have an antifibrotic effect on leiomyoma.

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FIGURE 1. Relative gene expression in uterine fibroids and normal myometrium analyzed by quantitative RT-PCR. **A.** The ratio of gene expression in fibroid versus normal myometrium from the same patient. The gene expression in each sample was normalized to the expression of GAPDH. **B.** The *RXFP1* gene expression in individual fibroid and myometrium samples. The difference is significant ($p<0.05$).

FIGURE 2.

Relaxin and INSL3 decrease phosphorylation of SMAD2 in rat leiomyoma ELT-3 cells. Representative Western blot shows reduced phosphorylation of SMAD2 after treatment with relaxins (hrRLN, human recombinant relaxin 2; pRLN, porcine relaxin) and INSL3 with and without TGF- β stimulation.

Table 1

Primer sequences and PCR conditions for tested genes

Gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
RXFP1/Relaxin receptor	F: GAGTATTTGTCTGGGTTGTATCTGC	193	60
	R: TTGTATTCTCCACGAACCTTAGGTC		
RXFP2/INSL3 receptor	F: CAACTCACGCTATGTCCATC	120	57
	R: GCAAGGCATACTTCTGATACTG		
CAV1/Caveolin 1	F: GTAGACTCGGAGGGACATC	105	57
	R: CACTTGCTTCTCGCTCAG		
ESR1/Estrogen receptor alpha	F: TGGCTACATCATCTCGGTTTC	152	60
	R: CAGGACTCGGTGGATATGG		
PR/Progesterone receptor	F: TCACAGCGTTTCTATCAACTTAC	145	60
	R: CAGCAATAACTTCAGACATCATTTTC		
NCOR1/Nuclear receptor corepressor 1	F: GCAGCTAACTTCATAGACGTGATCATC	102	60
	R: AGATAAGCTACTAGAAGAGTCTGAACTTTGAGA		
NCOR2	F: GAGGACTGGACTGTTTTTTCACA	101	60
	R: AAGGTATCAAAAATATACCCTGTAAACCA		
DES/Desmin	F: TGAAGGAAGAAGCAGAGAAC	82	54
	R: CGCTCCAGGTCATCAA		