Up-regulation of matrix metalloproteinase expression in synovial fibroblasts from knee OA with flexion contracture using adenovirus-mediated relaxin gene therapy

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Directed by Professor Ick Hwan Yang

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ABSTRACT

Up-regulation of matrix metalloproteinase expression in synovial fibroblasts from knee osteoarthritis with flexion contracture using adenovirus-mediated relaxin gene therapy

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The aim of this study was to investigate the effect of the RLN gene on the inhibition of fibrosis in Synovial fibroblasts. Synovial fibroblast cells with adenovirus LacZ (Ad-LacZ) as a marker gene or adenovirus relaxin (Ad-RLN) as therapeutic gene showed transgene expressions in beta-galactosidase assay and Western blot analysis. Synovial fibroblasts with Ad-RLN demonstrated a 22% and 48% reduction in collagen I and III mRNA expressions respectively, a 50% decrease in MMP-1, 70% decrease in MMP-2, 80% decrease in MMP-9, and a 15% reduction in MMP-13 protein expression compared with cultures with viral control and saline control. In addition, the synovial fibroblasts with Ad-RLN showed a 40% decrease in TIMP 1 and a 15% increase in TIMP 2

protein expression at 48 h compared to cultures with viral control and saline control. Also, synovial fibroblasts with Ad-RLN demonstrated a 74% inhibition of fibronectin and a 52% decrease in total collagen synthesis at 48 h compared with cultures with viral control and saline control. In conclusion, the RLN gene render antifibrogenic effect on synovial fibroblasts from osteoarthritis with flexion contracture via direct inhibition of collagen synthesis not through collagenolytic pathway such as MMP-1, -13, TIMP 1, and 2. Therefore relaxin can be an alternative therapeutic strategy in initial stage of osteoarthritis with flexion contracture by its antifibrogenic effect.

Key words: Relaxin, Fibrosis, Matrix metalloproteinases, Total knee arthroplasty

Up-regulation of matrix metalloproteinase expression in synovial fibroblasts from knee osteoarthritis with flexion contracture using adenovirus-mediated relaxin gene therapy

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I. INTRODUCTION

A knee joint contracture is characterized by a restriction in the full range of motion (ROM) of a joint and occurs secondary to shortening of periarticular connective tissues and muscles.[1-3] Joint contractures restrict mobility, have a negative impact on quality of life, limit an individual's productivity and earning potential, and can prevent basic activities of daily living.[4-6] A restriction in full extension of the knee is called a knee flexion contracture. Patients were categorized as having mild, moderate, or severe flexion contracture. Flexion contractures between 5° and 15° were considered “mild”, contractures between 15° and 30° considered “moderate”, and contractures greater than 30° considered “severe”.[7] Osteoarthritis (OA) is the most common joint disorder. Knee OA affects 11%-15% of the US population 65 years of age or older, is a

leading cause of chronic disability, and often is treated with total knee arthroplasty (TKA) at end-stage.[8] Ritter et al reported that more than one third of patients with OA (5228 knees) who presented for TKA had a KFlC.[9,10] This proportion represents a significant number considering that 719,000 knee replacements were performed in the United States in 2010. Recently, knee OA with flexion contracture through biomechanical study, histological studies and imaging studies with MRI found that closely related to the pathological changes of synovial tissue. Several recent papers have begun to suggest what biochemical and structural processes may underlie the increase in connective tissue. Studies in this area are difficult because knee OA with flexion contracture is a dynamic process with pathological and biochemical findings which evolve as the disease progresses in severity.[11-13]

Stiffness with arthrofibrosis is a complication to inhibit post-operative joint mobility.[14] It is needed joint healing in patients with diabetes mellitus, poor compliance to exercise therapy, and joint immobilization as soon as possible because of high contracture possibilities.[15-17] Also, treatment to arthrofibrosis is costly and difficult and the patients are suffering. It is reported that joint stiffness resulted from intra-articular adhesion formation and capsular contracture by inflammation. The inflammation induces adhesion and contracture, which makes thick fibrotic scar tissue and immobilization and restricts mobility gradually.[18] The inflammatory cell infiltrate into synovium and capsular tissue during joint inflammation. The invaded cells release various

cytokines and other inflammatory signaling molecules. One of the representative cytokines is transforming growth factor factor beta 1 (TGF-β1).[18,19] The secretion of TGF-β1 leads to arthofibrosis with flexion contracture. the fibroblast in this fibrotic tissue expresses abnormal alpha smooth muscle actin (α-SMA)[20] and differentiate into contractile myofibroblast.[18,21] For examples, in dupuytren's contracture TGF-β1 with other inflammatory signal molecules plays an important role in this differentiation.[20] Large amount of extra cellular matrix (ECM) is accumulated by overexpression for collagen type I and fibronectin in the tissue containing myofibroblast, which make dense contractile connective tissue with maturation.

Relaxin is one of the uterine contraction hormones and involves in maintenance of pregnancy and contraction or relaxation of the pelvic ligaments. In addition to the reproductive hormone function, it acts on neoangiogenesis and vasodilation for antagonism, wound-healing, and the infarct of context under the fibrosis.[22] Also, relaxin is an insulin superfamily, blocks the influx of inflammatory cells through the activation of G-protein-coupled receptor (GPCR), and inhibits amelioration on profibrotic factors such as TGF-β1.[23,24]

Therefore, the aim of this study is to investigate the anti-fibrotic effect of relaxin in synovial fibroblast from patients with knee OA with flexion contracture activated with TGF-β1.

II. MATERIALS AND METHODS

Approval of Institutional Review Board was obtained this study and there was no research fund related to this study.

Study Design

To test the anti-fibrotic effect of adenovirus-relaxin construct (Ad-RLN) on synovial fibroblasts in vitro, the cells from the tissue of patients with knee osteoarthritis and 30°~150° flexion contracture were utilized. (Table 1) Synovial fibroblasts were activated by TGF-β1 (2ng/ml) and then exposed to Ad-RLN as a therapeutic gene, adenovirus-lacZ construct (Ad-LacZ) as a marker gene, and SB505124 as an inhibitor for TGF-β1 signal for 48 hours. Synovial fibroblast cultures without adenoviral exposure served as saline control. The mRNA expression levels of type I, III, IV, and V collagen were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, the mRNA expression for MMP -1, -3, -8, -9 and -13 were measured by RT-PCR. Also, fibronectin, phosphorylation of Smad2 and ERK1/2, alpha smooth muscle actin (α-SMA), TIMP-1,-2, MMP-1, -2, -9, and -13 levels were estimated using western blotting and the total collagen synthesis was assayed.

Isolation and culture of subsynovial fibroblast

Subsynovial tissue isolated from patients with knee OA with severe flexion

contracture was minced with a scalpel and the tissues were then digested for 2 hours at 37°C under gentle agitation in a medium composed of equal parts of Dulbecco's modified Eagle's medium containing collagenase type I (250 unit/ml). Cells were filtered through a sterile nylon mesh filter (pore size: 75□m) and were cultured for 2–3 weeks in DMEM containing 10% FBS, 1%, v/v, penicillin, streptomycin, and nystatin (Gibco-BRL®) in a 37°C incubator with 5% CO2 and humidity. Culture medium was changed twice a week.

Relaxin constructs and transfection

Two different adenoviral constructs were prepared for this study: Ad-LacZ expressing the lacZ gene as a viral control and Ad-RLN expressing the human relaxin gene. Each recombinant adenoviral vector originated from replication deficient type 5 adenovirus deleted the E1 and E3 regions of the genome. The RLN gene was cloned into the E1 region under the control of the human cytomegalovirus early promoter. Recombinant virus was grown in transformed human embryonic kidney 293 cells and purified by CsCl2 density gradient method. Titers were determined by optical density at 260nm (OD260). At confluence, the fibroblastic cells isolated from the rotator interval and anterior capsule were rinsed with Hanks' Balanced Salt Solution (HBSS) and exposed to HBSS containing one dose of Ad-LacZ and Ad-RLN with viral concentration of 80 multiplicity of infection (MOI). All cells were incubated in a 37°C incubator

with 5% CO2 and humidity to prevent drying during the 1h transfection. Then, culture medium (DMEM containing 10% FBS) was added to each well, and the cells were further incubated in a 5% CO2 incubator at 37°C, with humidity.

Reverse-Transcription Polymerase Chain Reaction Analysis for Collagens

Total RNA was isolated from synovial fibroblasts were transfected by Ad-RLN for 48h using the QIAGEN RNeasy® mini kit (QIAGEN) following the manufacturer's instructions. cDNA was prepared using the Maxime RT premix kit (Intron). Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl using oligo (dT) primers. Collagen type I and III and IV genes were amplified. Relative expression levels were normalized to the average beta-actin level. The data were analyzed using the Image J analyzer ver. 1.45 software (National Institutes of Health, MD, USA).

Protein Extraction and Western Analysis

The cells were transfected by Ad-RLN for 48h at a density of 2×105 cells per well and were lysed in buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5mM EDTA (pH7.4), 1mM Tris–HCl (pH7.4), protease inhibitor. Also, the culture medium was collected for demonstrating RLN protein expression by Ad-RLN. The lysates and culture medium were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes using a transfer system (Mini

Trans-Blot® Cell and systems). The blots were exposed to specific antibodies to α-SMA, fibronectin, relaxin and TIMP1, TIMP2, MMP1, MMP13, phosphor-Smad2, Smad2, phosphor-ERK1/2 and ERK1/2 (Abcam®). After reacting with secondary antibody, immunoreactive bands were visualized using a Western blot detection system (West-Zol® plus). The blots were stripped of bound antibodies and were reprobed using antibodies to actin (Abcam®) to verify loaded protein amounts.

Zymograms of Matrix Metalloproteinase-2 and -9

Samples of medium were harvested from the cells cultured in 60-mm plates at a density of 2 x 105 cells per well for 48 hours after transfection by Ad-RLN. MMP-2 and MMP-9 in the samples of cell-conditioned medium were monitored by gelatin substrate zymograms. In brief, 20 μl of the culture medium was mixed with 2x sample buffer and electrophoresed on a 10% zymogram gel (Novex® Zymogram gel, Invitrogen, CA, USA).

Total Collagen Content Assay

Subsynovial fibroblast transfected with Ad-RLN were grown at a density of 4×104 cells per well in 12-well plates for 48h in DMEM medium. Collagen was harvested from these cells after lysing in a buffer containing 0.5mM EDTA (pH 7.4), 1mM Tris–HCl (pH7.4), and protease inhibitor. The collagen samples were concentrated using a collagen isolation and concentration protocol and

measured at a 555-nm wavelength as a manufacture instruments (Sircol™, Biocolor Ltd, County Antrim, UK). The amounts of collagen were calculated based on a standard curve of soluble collagen provided by the collagen assay kit.

Statistical Analysis

Data were compiled from three independent triplicate experiments on separate culture using the fibroblasts isolated from four donors. Data are expressed as mean ± standard deviation (SD) from the results of three independent experiments. A two-tailed Student's t-test was used to compare between the results of the two groups. A value of $p<0.05$ was considered statistically significant.

III. RESULTS

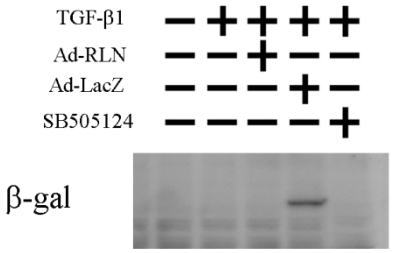


Figure 1. Beta galactosidase and relaxin expression on synovial fibroblasts

Synovial fibroblasts from patients with knee OA with severe flexion contracture, transfected with Ad-LacZ demonstrated beta galactosidase expression by western blot analysis, compared to those cells with Ad-RLN and saline control, indicating highly efficient transduction rate of adenoviral marker gene construct. Synovial fibroblasts from patients with knee OA with severe flexion contracture, transfected with Ad-RLN confirmed of relaxin protein expression in western blot analysis, compared to cultures with Ad-lacZ and saline control. (Figure.1)

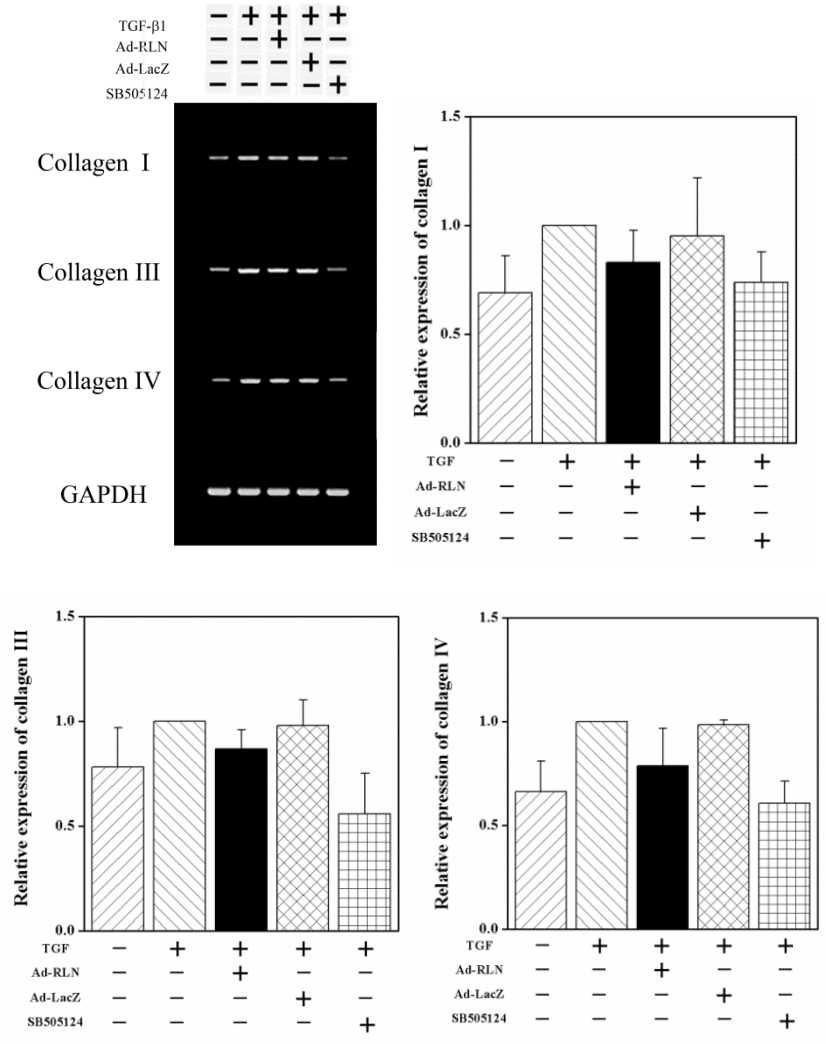
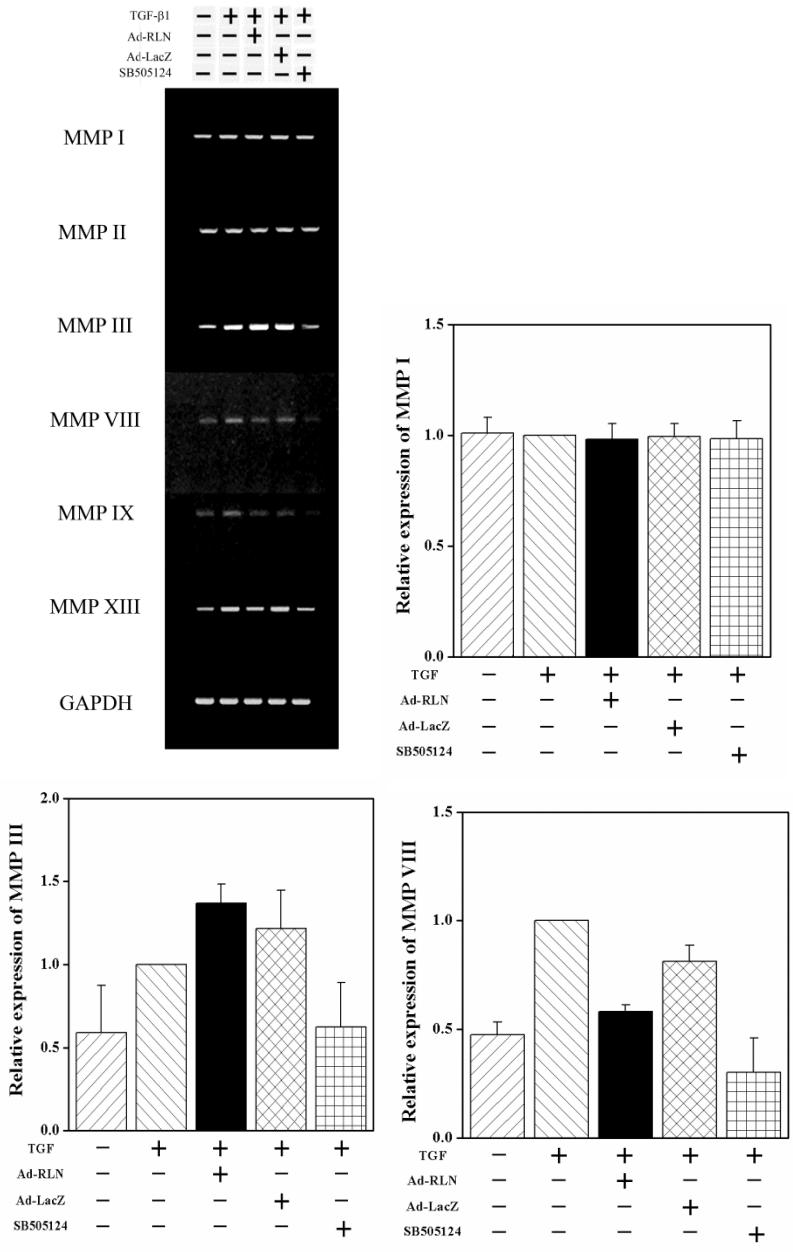


Figure 2. Collagen mRNA expression

Expression of collagen type I, III and collagen type IV mRNAs of Synovial fibroblasts from patients with knee OA with severe flexion contracture and transfected with Ad-RLN showed a 17%, 13% and 22% decrease at 48 hours compared to control culture (TGF-β1+, $p < 0.05$). However, there was no significant difference in the collagen type V expression at the mRNA level ($p <$

0.05).



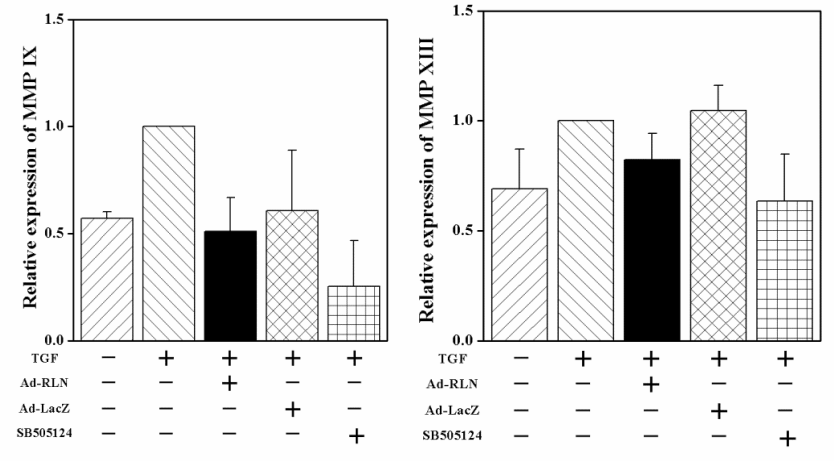


Figure 3. Matrix metalloproteinase mRNA expression

Synovial fibroblasts from patients with knee OA with severe flexion contracture transfected with Ad-RLN showed a 40% increase in MMP-3 mRNA expression compared to those cultures without Ad-RLN. However, the synovial fibroblasts from patients with knee OA with severe flexion contracture transfected with Ad-RLN showed a 40% decrease in MMP-8 mRNA expression at 48 hours compared to those cultures without Ad-RLN. Also, the synovial fibroblasts transfected with Ad-RLN showed a 50% reduction in MMP-9, and a 20% reduction in MMP-13mRNA expression at 48 hours compared to those cultures without Ad-RLN ($p < 0.05$).

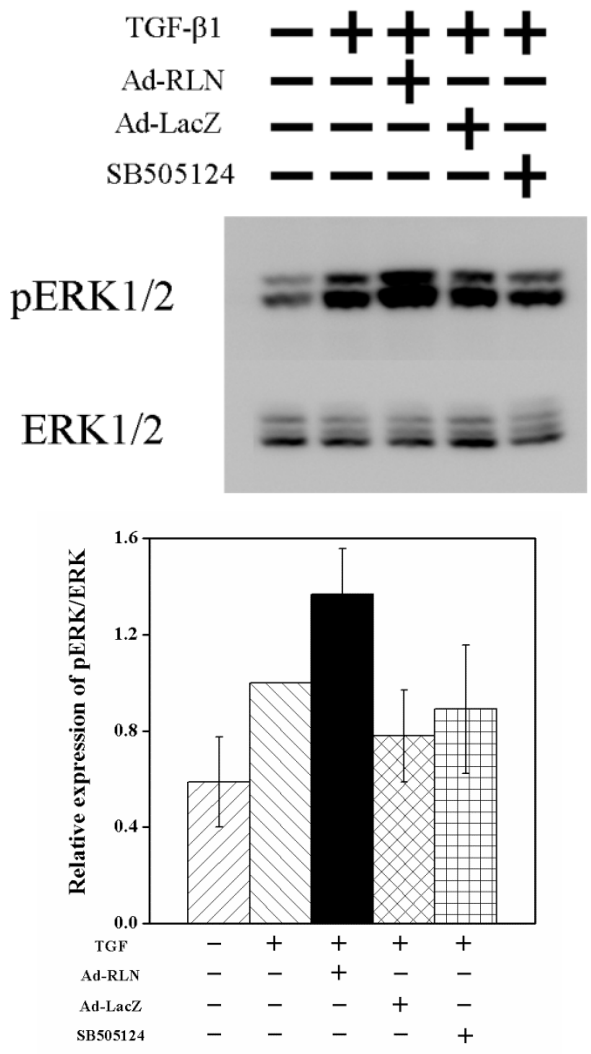
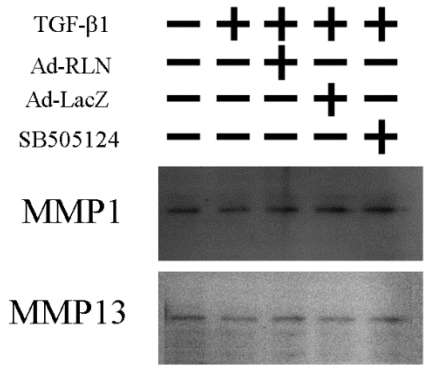
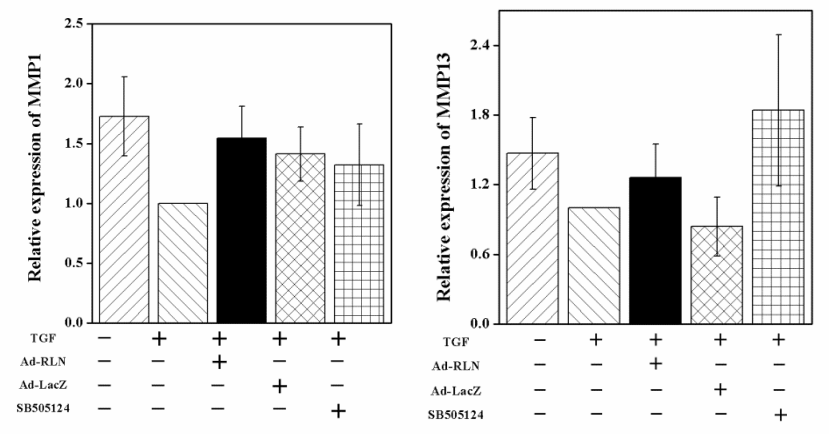
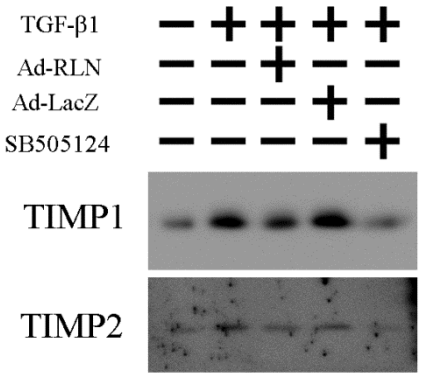


Figure 4. Phosphor-Smad2 and phosphor-ERK1/2 expression

Synovial fibroblasts from patients with knee OA with severe flexion contracture with Ad-RLN showed 70% increases in levels of phospho-ERK1/2 protein expression at 48 hours respectively, compared to cells cultured without Ad-RLN ($p < 0.05$).







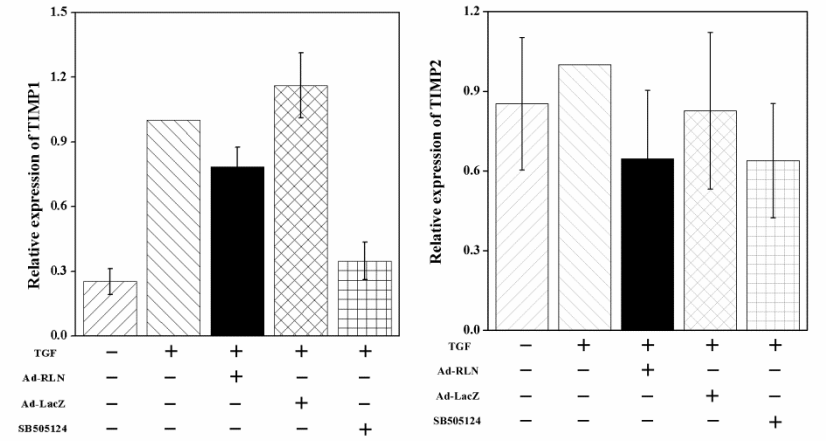


Figure 5. TIMPs and MMPs expression

Synovial fibroblasts from patients with knee OA with severe flexion contracture with Ad-RLN showed 25% decreases in the levels of TIMP 1 and 65% decrease at 48 hours in TIMP 2 protein expression compared to those cultures without Ad-RLN. ($p < 0.05$). However, Synovial fibroblasts from patients with knee OA with severe flexion contracture with Ad-RLN showed 50% increases in the levels of MMP 9 and 50% increase at 48 hours in MMP 13 protein expression compared to those cultures without Ad-RLN. ($p < 0.05$) There was no significant difference in the MMP 1 expression at the protein level ($p < 0.05$)

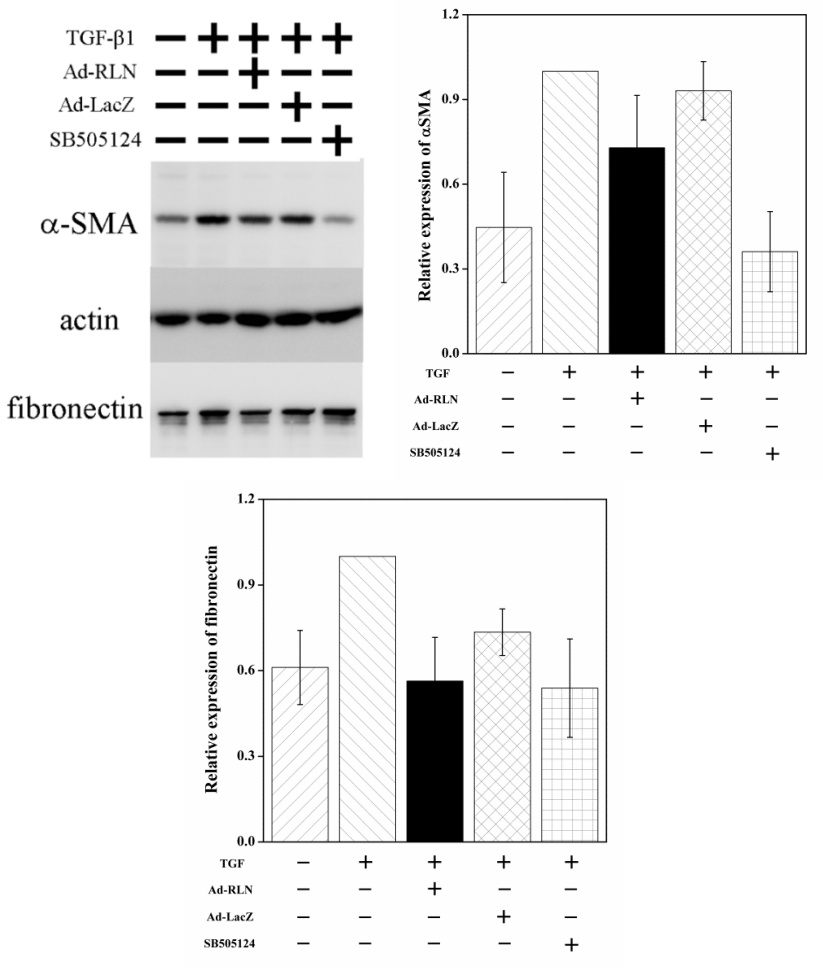


Figure 6. Alpha smooth muscle actin and fibronectin expression

Synovial fibroblasts from patients with knee OA with severe flexion contracture with Ad-RLN showed 70% decreases in levels of alpha smooth muscle actin expression at 48 hours respectively, compared to cells cultured without Ad-RLN ($p < 0.05$). Also there were 45% decreases in the fibronectin expression at the protein level ($p < 0.05$).

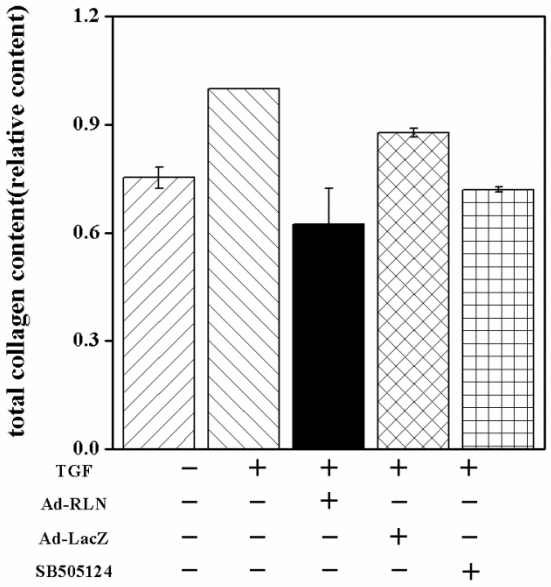


Figure 7. Total collagen content

Synovial fibroblasts from patients with knee OA with severe flexion contracture transfected with Ad-RLN showed a 38% decrease in total collagen protein expression at 48 hours compared to those cultures without Ad-RLN ($p < 0.05$) .

Table 1. Sample information

No.	Age/Sex	Contracture level
#1	63/Female	Severe/30-120
#2	77/Female	Severe/35-130
#3	69/Female	Severe/30-115
#4	77/Female	Severe/40-125
#5	76/Female	Severe/35-130

Table 2. The mRNA and protein expression on the cells with Ad-RLN and TGF-β1 vs. TGF-β1. ↑: increase; ↓: decrease; -: no change; *: $p<0.05$

mRNA expression		Protein expression	
Collagen I	↓↓	pERK/ERK	↑↑↑(*)
Collagen III	↓↓	MMP1	↑↑↑ (*)
Collagen IV	↓↓↓(*)	MMP13	↑↑↑
MMP1	-	TIMP1	↓↓↓(*)
MMP3	-	TIMP2	↓↓↓(*)
MMP8	↓↓↓	a-SMA	↓↓↓(*)
MMP9	↓↓↓	fibronectin	↓↓↓(*)
MMP13	↓↓	Total collagen content	↓↓↓(*)

IV. DISCUSSION

Etiology of stiffness after TKA is inflammatory arthropathy and prior knee surgery with poor preoperative range of motion (ROM) and osteoarthritis.[25-27] Intraoperative factors that may cause the stiff TKA is improper balancing from overstuffing in patellofemoral joint, inadequate sizing in component, bone resection in osteophytes, and failure in normal tibial slope and contracted capsule.[28-30] Also, after operation, patients may lead to secondary stiffness due to factors such as infection, arthrofibrosis, heterotopic ossification, inadequate rehabilitation, and complex regional pain syndrome. Most of patients who have unclear cause are diagnosed as arthrofibrosis that lead into fibroplasia from progressive scarring on joint.[31-34] Primary arthrofibrosis results in a painful impairment of joint flexibility during fibrotic tissue remodeling after joint trauma or surgery. It is separated from secondary arthrofibrosis by inaccurate implant positioning[35] and diagnosis in the arthrofibrosis depends on histopathological finding and clinical symptoms such as loss of motion since inaccurate diagnostic markers.[36]

The reparative inflammatory mechanism in arthrofibrosis is not clear although it is similar to other fibrotic disorders.[35] In general, fibrosis is informed that profibrotic molecules i.e. TGF-β1 is secreted, fibroblast is differenced into myofibroblast, and stiffening is preceded with matrix accumulation. The activated myofibroblast by fibrosis is not occurred in apoptosis after wound healing and makes pathologic scar with continuous ECM production.

Unbalancing in synthesis and degradation of ECM gets molecules such as collagens and proteoglycan in the intercellular space.[37,38]

TGF-β1 is representative profibrotic molecule with connective tissue growth factor (CTGF) and major factor on fibrosis occurred in liver of kidney. It is reported that the capsule in the immobilized knee model has a potency to control TGF-β1 or CTGF and may prevent contracture in the joint by somehow blocking the fibrotic process.[39] Therefore we investigated the proteins which involve in the accumulation and abatement of the ECM in the fibroblast transformed with TGF-β1 from knee synovium. Furthermore we identified that the relaxin gene using adenovirus may regulate molecules to control ECM components such as collagens, MMPs and TIMPS. The expression of collagen IV was decreased significantly on the cell with Ad-RLN and TGF-β1 compared to cells without Ad-RLN in mRNA level. Also, the expression of collagen I and III was decreased insignificantly in mRNA expression. The expression of MMP1 and 13 was increased on the cell with Ad-RLN and TGF-β1 compared to cells without Ad-RLN in protein level. However, the expression of TIMP1 and 2 was significantly reduced on the cell with Ad-RLN and TGF-β1 compared to cells without Ad-RLN in protein level. The expression α-SMA and fibronectin was remarkably cut back on the cell with Ad-RLN and TGF-β1 compared to cells without Ad-RLN in protein level. It is confirmed that reduction of the molecules are through phosphorylation of the ERK1/2. These

investigations suggest that RLN is acting on the process of collagenolysis by increasing the MMP expression. Furthermore the expression decrease of extracellular matrix is positive feedback system in which produced collagenases and reduced inhibitors of collagenases. Therefore, fibrosis was cut back. (Table 2)

Several limitations should be noticed in this study. First, the interpretation of the results is limited by the small patients. Also, it is not investigated that the relaxin in directly involved in the expression of MMPs and TIMPs and the specific ways to reduce fibrosis. Further evaluation with a larger sample size would be recommended. Second, the gender distribution of our study should be considered when comparing our findings to other study group. In our study, all 5 patients were women. Female gender dominance has been noted in many published and, for some reason, this female dominance is even more notable in Korean patients undergoing total knee arthroplasties.[40-44]

RLN can promote matrix remodeling by increasing cell proliferation, reducing the expression of α-SMA, and decreasing the synthesis of collagen in the renal fibroblasts.[45] Recombinant human RLN can alter the connective tissue phenotype of human lung fibroblasts, decrease overexpression of procollagen type I and III induced by TGF-β1, and reduce synthesis and secretion of MMP-1. Also, RLN controls excessive collagen deposition by blocking bleomycin-induced pulmonary fibrosis in human lung fibroblasts.[46] Furthermore, human dermal fibroblasts exposed to RLN modulate secretion of MMPs,

collagenase inhibitors, and expression of TIMPs.[47] Also, we reported that the relaxin can regulate the expression of collagens and MMPs in the myofibroblast isolated from dupuytren's contracture. [48] In this study, it can be demonstrated that the relaxin also may control the synthesis and degradation of ECM in the myofibroblast transformed by TGF-β1.

V. CONCLUSION

The RLN gene renders antifibrogenic effect on synovial fibroblasts from osteoarthritis with flexion contracture via direct inhibition of collagen synthesis not through collagenolytic pathway such as MMP-1, -13, TIMP 1, and 2. Therefore relaxin can be an alternative therapeutic strategy in initial stage of osteoarthritis with flexion contracture by its antifibrogenic effect. In summary, this is the first report to evaluate the anti-fibrotic effect in the fibroblast isolated from patients with arthrofibrosis. It needs to identify that the molecules involved in relaxin through the fibrosis process and to validate for preventive therapeutic agent.

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ABSTRACT(IN KOREAN)

굴곡구축을 동반한 슬관절 관절염환자에서 아데노바이러스를 이용한 릴렉신 유전자치료의 효과

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고 재 한

슬관절의 구축은 슬관절 주위 수술 전,후에 흔히 관찰되는 합병증 중의 하나로 슬관절 주위의 근육과 결합조직 등의 연부조직의 변형 등의 이차적인 변화 후에 발생하게 된다. 우리는 본 연구에서 슬관절 구축을 동반한 퇴행성 관절염 환자에서 최근 널리 진행되고 있는 아데노바이러스 릴렉신 유전자 치료의 효과를 알아보고자 했다. 30도 이상의 중증의 굴곡구축을 동반한 환자 5명의 슬관절 윤활조직을 채취하였고, TGF-β1을 이용하여 슬관절 윤활조직에서 분리한 섬유아세포를 근육섬유아세포로 변환하여 세포외기질의 합성과 분해에 관여 하는 분자의 발현 변화를 관찰하였다. 우리는 아데노바이러스 릴렉신 유전자 처리를 시행한 그룹과 아데노바이러스 릴렉신 유전자 치료 처리를 하지 않은 그룹과의 비교대조 연구를 시행하였다. 아데노바이러스 릴렉신 유전자 치료 처리를 한 그룹에서는 collagen IV의 유전자 발현은 의미있게 감소하였으나 반면에 collagen I, III, V의 발현은 유의하지 않음을 확인하였다. TGF-β1에 의해 감소된 MMP3 유전자는 발현이 증가하였으나, 반면에 MMP 8 9 13은 감소하였다. 또한 세포외기질의 주요 성분인 α-SMA과 fibronectin 의 단백질 발현은 아데노바이러스 릴렉신에 의해서 감소되었음을 확인하였다. TIMP1과 2의 발현은 감소하였고, MMP1, 9, 13의 단백질 발현은 증가하였다. 더불어 이러한 분자의 발현이 Smad2와 ERK1/2의 인산화 과정을 통해 이루어지는 것을

확인할 수 있었다. 본 연구는 슬관절 굴곡구측이 있는 환자로부터 분리된 윤활조직의 섬유아세포의 항섬유화 효과를 증명한 첫번째 실험으로. 추후 이러한 긍정적인 아데노바이러스 릴렉신의 효과와 더불어 릴렉신 물질이 섬유화 과정 중 어디에 관여하는지는 더 확인해야 하며, 더 많은 연구를 통해서 릴렉신 물질의 치료제 및 예방제로써의 가능성을 확인하여야 한다.

핵심되는 말 : 릴렉신, 퇴행성 관절염, 슬관절 인공관절치환술, 섬유화