Down-Regulation of Collagen Synthesis and Matrix Metalloproteinase Expression in Myofibroblasts from Dupuytren Nodule Using Adenovirus-Mediated Relaxin Gene Therapy

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ABSTRACT: Dupuytren’s disease is a fibroproliferative connective tissue disorder characterized by contracture of the palmar fascia of the hand. The disease may cause flexion contracture of the fingers because of a metamorphosis of fibroblast phenotype and function and excessive deposition of extracellular matrix (ECM). The initial stage of the disease process is characterized by small nodular thickenings composed of hyperproliferative and fibroblastic cells in histology. In later stages, the nodules become aligned along lines of stress and cords are developed. Also, Dupuytren’s disease develops histological, surgical pathoanatomic changes, and biochemical alterations in the longitudinal fibers. Dupuytren’s disease eventually results in a palpable mass in the palm called Dupuytren nodule, which may be transient and mildly painful.

Molecular abnormalities associated with cell adhesion are one of the etiologic agents in Dupuytren’s disease. The disease involves the interaction of ECM components such as collagen, fibronectin isoforms, α5β1 integrin, periostin, β-catenin, glycosaminoglycans, proteoglycans, and tenasin-C via (1) the imbalance of the ECM and (2) abnormal expression of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-1 (TGF-β1), (3) a skewed ratio of collagen type I and III, and (4) increased expression of fibronectin and α-smooth muscle actin. It has also been shown that some proteinases associated with ECM degradation, such as matrix metalloproteinase-1 (MMP-1) and -2, and their inhibitors, including tissue inhibitor matrix metalloproteinase 1 (TIMP 1) and -2, were dysregulated in Dupuytren’s disease.

Relaxin (RLN) is a peptide hormone first described in 1926 by Frederick Hisawin as a member of the insulin superfamily and a substance influencing the reproductive tract. It consists of A and B chains and is connected by disulfide bridges similar to those of insulin. Also, RLN is a multifunctional factor that contributes to remodeling of the pelvic ligament, and inhibits fibrosis and inflammatory activities through the activation of G-protein-coupled receptor (GPCR) which acts to inhibit the inflammatory cell influx and ameliorate the effects of profibrotic factors, such as TGF-β1, in injured organs.

Matrix metalloproteinases can be classified into four subfamilies: interstitial collagenase, stromelysin, gelatinase, and membrane types of MMP, according to the traits of structure and function and involvement of the protein in disassembling ECM and basement membrane. MMPs are metal proteases containing zinc and are secreted as inactive pro-MMP types after being synthesized as prepro-enzymes. Active MMPs, which are induced by structural deformation for enzyme activity on the N-terminal of them is...
activated as well, are regulated by endogenous inhibitors such as α2-macroglobulin and tissue inhibitors of metalloproteinase (TIMPs). The expression of collagenases and their inhibitors in the normal ligament differs from that seen in the palmar fascia and nodule ligaments from Dupuytren's disease. The synthesis of MMPs and TIMP 1 is increased, despite the down-regulation of TIMP3 and 4 in the mRNA profile in the myofibroblasts from Dupuytren's patients, as compared to normal ligament.

Given that increased levels of collagen synthesis and reactive up-regulated MMPs and down-regulated TIMPs are major features of Dupuytren's disease, the biological approach of inhibiting collagen synthesis might serve as a novel therapeutic approach. Furthermore, the presence of a collagenolytic mechanism is of importance in deciding when to intervene, such as during the acute stage or late maturation stage, which specifically requires collagenolytic activity. The anti-fibrogenic effect of RLN might provide a therapeutic mechanism in the treatment of Dupuytren's disease via the interwoven pathways of MMPs, TIMPs, collagenesis, and collagenolysis. With proven efficacy at the molecular level, RLN therapy might prevent the progression of fibrosis or even resolve fibrosis via a minimally invasive injection approach, depending on the stage of Dupuytren's disease being treated.

Hence, in this study, we transferred a RLN gene construct into myofibroblasts isolated from Dupuytren nodules using an adenovirus system to investigate the effect of this gene on inhibition of fibrosis. We also sought to identify the mechanisms crucial to this process, such as a decrease in collagenesis or an increase in collagenolysis.

METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board.

Study Design

To test the anti-fibrotic effect of adenovirus-relaxin construct (Ad-RLN) on myofibroblasts in vitro, the cells from the nodules of patients with Dupuytren's disease were utilized. Myofibroblast cultures were exposed to Ad-RLN as a therapeutic gene and adenovirus-lacZ construct (Ad-LacZ) as a marker gene and cultured for 12 and 48 h. Myofibroblast cultures without adenoviral exposure served as saline control. The mRNA expression levels of type I collagen and type III collagen were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, MMP-1, and MMP-13 were measured using enzyme linked immunosorbent assay (ELISA), while MMP-2 and MMP-9 were analyzed by zymography. Also, fibroblastin levels were estimated using Western blotting and the total collagen synthesis was assayed.

Isolation and Culture of Cells

Nodules were isolated from four patients with Dupuytren's disease during partial fasciectomy. Myofibroblasts from the nodules of the involutional stage were utilized and maintained phenotypic characteristics. The dissected specimens were minced with a scalpel and the tissues were then digested for 60 min at 37˚C under gentle agitation in a medium composed of equal parts of Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL®, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) with 0.2% pronase (Sigma, St. Louis, MO) and 0.004% deoxyribonuclease type II (DNase, Sigma). The tissue was then washed three times with DMEM and digested 2 h under the same conditions, with the exception of pronase being replaced with collagenase type I (250 unit/ml, Sigma). Cells were filtered through a sterile nylon mesh filter (pore size: 75 μm, Falcon, Bedford, MA) and were then counted using a hemocytometer and plated in T25 plates (Nunc® Roskilde, Denmark) at a density of approximately 5 × 10^5 cells/ml. Primary cultures were sustained for 2–3 weeks in DMEM containing 10% FBS, 1%, v/v, penicillin, streptomycin, and nystatin (Gibco-BRL®, NY) in a 37˚C incubator with 5% CO₂ and humidity. Culture medium was changed twice a week.

Relaxin Constructs and Transfections

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 cytokemoglobin virus early promoter. Recombinant virus was grown in transformed human embryonic kidney 293 cells and purified by CsCl density gradient method. Titers were determined by optical density at 260 nm (OD260). At confluence, the myofibroblastic cells isolated from Dupuytren nodules were rinsed with Hank's Balanced Salt Solution (HBSS, Gibco-BRL®, NY) 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 1-h 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.

Beta-Galactosidase Activity Assay

For the efficiency of transfection, marker gene, that is, β-galactosidase activity was measured in transfected cells. In brief, the cells were transfected by Ad-RLN for 48 h at a density of 5 × 10^4 cells per well in culture medium, the medium was removed, and the cells were lysed in buffer containing 0.85% NaCl. And then the lysate was added Z-buffer consisting of Na₂HPO₄, NaH₂PO₄, H₂O, KCl, and MgSO₄·7H₂O and permeabilized with 0.1% SDS. The lysate was placed in 30˚C water bath, and added 4 mg/ml o-nitrophenyl-β-d-galactoside (ONPG). When a yellow color develops, the reaction was stopped with 1 M Na₂CO₃ and the supernatant was measured at 420, 550, and 650 nm. The activity was calculated.

Cell Viability Assay

After incubation for 12 and 48 h, the viability of myofibroblasts in each well was assessed using the EZ-Cytox Cell viability assay kit (Daelliga Service Co., Ltd, Seoul, Korea) as a manufacture instruments. Assays were done in triplicate. Two hundred microliters of culture medium and 30 μl WST assay reagent were added to each well and incubated for 1 h in 5% CO₂ at 37˚C with humidity. The supernatant was transferred to a 96-well plate which was measured at
Samples of medium were harvested from the cells cultured in 60-mm plates at a density of 2 x 10^6 cells per well for 12 and 48 h after transfection by Ad-RLN. MMP-1 and MMP-13 in the samples of cell-conditioned medium were monitored by a commercially available ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). The culture medium was concentrated using a collagen isolation and concentration protocol and measured at a 555-nm wavelength as a manufacturer instrument (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.

**Reverse-Transcription Polymerase Chain Reaction Analysis for LRG8 and Collagens**

Total RNA was isolated from myofibroblasts that were transfected by Ad-RLN for 12 and 48 h using the QIAGEN RNeasy mini kit (QIAGEN) following the manufacturer’s instructions. cDNA was prepared using the Maxime RT premix kit (Intron, Daejun, Korea). Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl using oligo (dT) primers. Collagen type I and III and LRG8 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).

**Enzyme Linked Immunosorbent Assay of Matrix Metalloproteinase-1 and -13**

Medium was harvested from each 60-mm plate of cells, which were cultured at a density of 2 x 10^6 cells per well for 12 and 48 h after transfection by Ad-RLN. MMP-1 and MMP-13 in the samples of cell-conditioned medium were monitored by gelatin substrate zymograms. In brief, 20 μl of the culture medium was mixed with 2 x sample buffer and electrophoresed on a 10% zymogram gel (Novex Zymogram gel, Invitrogen, Carlsbad, CA).

**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 10^6 cells per well for 12 and 48 h 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 2 x sample buffer and electrophoresed on a 10% zymogram gel (Novex Zymogram gel, Invitrogen, Carlsbad, CA).

**Protein Extraction and Western Analysis**

The cells were transfected by Ad-RLN for 12 and 48 h at a density of 2 x 10^6 cells per well in culture medium and were lysed in buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5 mM EDTA (pH7.4), 1 mM Tris–HCl (pH7.4), protease inhibitor (Complete Mini, Roche Diagnostics, Mannheim, Germany). 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, Pierce, Rockford, IL) membranes using a transfer system (Mini Trans-Blot Cell and systems, Bio-Rad, Hercules, CA). The blots were exposed to specific antibodies to fibronectin and TIMP 1, 3, and 4 (Abcam, Cambridge, England). After reacting with secondary antibody, immunoreactive bands were visualized using a Western blot detection system (West-Zol plus, iNtRON Biotechnology, Daejun, Korea). The blots were stripped of bound antibodies and were reprobed using antibodies to actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to verify loaded protein amounts.

**Total Collagen Content Assay**

Cells transfected with Ad-RLN were grown at a density of 4 x 10^4 cells per well in 12-well plates for 12 and 48 h in DMEM medium. Collagen was harvested from these cells after lysing in a buffer containing 0.5 mM EDTA (pH 7.4), 1 mM 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 manufacturer instrument (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 Analyses**

Data were compiled from three independent triplicate experiments on separate culture using the myofibroblasts 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 the results of the two groups. A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Beta-Galactosidase Activity on Dupuytren’s Disease Myofibroblasts**

Myofibroblasts from patients with Dupuytren’s nodule, transfected with Ad-LacZ demonstrated marked increase in beta-galactosidase activity compared to those cells with Ad-RLN and saline control, indicating highly efficient transduction rate of adenoviral marker gene construct (Fig. 1).

**Relaxin Protein Expression**

Myofibroblasts from patients with Dupuytren’s nodule, transfected with Ad-RLN showed increased expression of relaxin protein in Western blot analysis,
compared to cultures with Ad-lacZ and saline control (Fig. 2).

Cytotoxicity of Adenovirus on Dupuytren’s Disease Myofibroblasts
Myofibroblasts from patients with Dupuytren’s nodule when transfected with Ad-RLN and Ad-LacZ demonstrated no significant increase in cellular proliferation compared to those cells without Ad-RLN and Ad-LacZ, indicating there were no apoptotic, cytotoxic, or cellular proliferative effects of adenovirus on myofibroblasts from patients with Dupuytren’s nodule (Fig. 3).

LRG8 and Collagens mRNA expression
Myofibroblasts with Ad-RLN demonstrated increased mRNA expression of LRG8, which is receptor for RLN at 48 h (Fig. 4). Expression of collagen type I and III mRNAs of myofibroblasts from patients with Dupuytren’s nodule and transfected with Ad-RLN showed a 22% decrease in collagen type I mRNA expression at 48 h and a 48% decrease in collagen type III mRNA expression at 12 h compared to saline control culture ($p < 0.05$) (Fig. 5).

Expression of MMP-1, -2, -9, and -13
Myofibroblasts from patients with Dupuytren’s nodule transfected with Ad-RLN showed a 50% decrease in MMP-1 and a 15% reduction in MMP-13 protein expression at 48 h compared to those cultures without Ad-RLN (Fig. 6). Also, the myofibroblasts transfected with Ad-RLN showed 70% and 80% decreases in levels of MMP-2 and -9 protein expression at 48 h compared to those cultures without Ad-RLN ($p < 0.05$) (Fig. 7).

Expression of TIMP 1, 3, and 4
Myofibroblasts from patients with Dupuytren’s nodule with Ad-RLN showed 40% decreases in the levels of TIMP 1 protein expression at 12 and 48 h compared to those cultures without Ad-RLN ($p < 0.05$). The myofibroblasts transfected with Ad-RLN showed a 37% decrease at 12 h and a 15% increase at 48 h in TIMP 3 protein expression compared to those cultures without Ad-RLN ($p < 0.05$). However, there was no significant difference in the expression of TIMP 4 at the protein level ($p < 0.05$) (Fig. 8).

Expression of Fibronectin
Myofibroblasts from patients with Dupuytren’s nodule with Ad-RLN showed 40% and 74% decreases in levels

Figure 2. Myofibroblasts from Dupuytren’s nodule transfected with adenovirus relaxin gene construct showed increased expression in Western blot analysis at 48 h.

Figure 3. Human myofibroblasts induced adenovirus relaxin gene construct and adenovirus LacZ gene construct were cultured for 12 and 48 h. DNA synthesis was analyzed by MTS assay. Cultures with adenovirus relaxin gene construct and adenovirus LacZ gene construct showed no significant change in DNA synthesis compared to cultures without adenovirus induction, indicating that virus particles had neither cellular proliferative nor cytotoxic effects on myofibroblasts from Dupuytren’s nodule in vitro. (A) MTS assay at 12 and 48 h culture, (B) crystal violet staining at 12 and 48 h culture (mean ± SD). Control = cultures with saline; Ad-RLN = adenovirus relaxin gene construct; Ad-LacZ = adenovirus LacZ gene construct.

Figure 4. Myofibroblasts from Dupuytren’s nodule transfected with adenovirus relaxin gene construct demonstrated increased mRNA expression of LRG8, which is receptor for relaxin, in RT-PCT at 48 h.
of fibronectin protein expression at 12 and 48 h respectively, compared to cells cultured without Ad-RLN ($p < 0.05$) (Fig. 9).

**Total Collagen Content**

Myofibroblasts from patients with Dupuytren’s nodule transfected with Ad-RLN showed a 52% decrease in total collagen protein expression at 48 h compared to those cultures without Ad-RLN ($p < 0.05$) (Fig. 10).

**DISCUSSION**

Dupuytren’s disease is characterized by finger contracture and dysfunction caused by the over-proliferation of myofibroblasts and the excessive formation of collagen fiber and ECM. Several nonsurgical treatments for this disease have been tried, such as radiation therapy and steroid injection, but those have ultimately been found to be unsuccessful. As an
alternative approach based on the pathomechanism of excessive fibrosis in Dupuytren's disease, Badalamente and Hurst tried collagenase injection to Dupuytren cord as a phase III clinical trials. This study showed that a significant reduction in contracture compared to placebo and treatment was well-tolerated with the majority of adverse events self-limited. However, improvement was quite less in patients with severe contractures and with contractures of the proximal interphalangeal joint. Even various medical treatments usually end up in less favorable clinical outcome. Hence, the best treatment for this disease so far is surgery, such as open fasciectomy, closed fasci-
The goal of surgical treatment is not to eliminate the disease, but to release joint contractures and improve hand function. Though there have been studies examining anti-fibrogenic effects in vivo, the etiopathogenesises and treatments of Dupuytren's disease were not clearly understood. Unfavorable medical and not uniformly successful surgical approaches have prompted the search for novel methods to treat Dupuytren's disease based on a pathomechanism.3,35,40

RLN is an autocrine contraction hormone that affects the contraction and relaxation of ligaments around the pelvis for the maintenance of pregnancy and parturition. Also, RLN acts as an antagonist for fibrosis, wound-healing, neoangiogenesis, and vasodilation to the infarct of context in addition to its role as a reproductive hormone.22,23,41–43

In the present study, the effect of RLN in antifibrosis was investigated through genetic modification of myofibroblastic cells from patients with Dupuytren's nodule using Ad-RLN. We found that myofibroblastic cells from Dupuytren's nodule with Ad-RLN demonstrated 22% and 48% decreases in expression levels of collagen type I and III mRNA, respectively. Furthermore, cultures with Ad-RLN showed a 52% decrease in the synthesis of total collagen protein as compared with saline and viral control. These findings indicate that the RLN gene, when inserted into myofibroblastic cells, inhibits translation and transcription of collagen synthesis. Also, the synthesis of fibronectin, a non-collagenous protein, was suppressed about 74% compared to saline and viral control, at the protein level. Furthermore, protein expression levels of MMPs were significantly reduced depending on RLN transgene expression. In particular, the expression levels of MMP-1 and -13 at 48 h were clearly decreased. These observations suggest that RLN is not acting on the process of collagenolysis, but rather affects only the synthetic aspect of collagen, as in the translation and transcription of collagen. These results suggest a few areas of clinical significance in applying RLN gene therapy in Dupuytren's disease. Firstly, RLN intervention should be started at the initial or organizational phase rather than during the fully matured stage, as represented by cords and nodules, since inhibition of collagenesis is a crucial mechanism of this therapy, rather than collagenolysis via MMPs. Secondly, RLN plus collagenase therapy could be a useful future area of study. Thirdly, in terms of a pathomechanism in Dupuytren’s disease, increased collagenesis by myofibroblasts contributes significantly, rather than the decrease in collagenolysis via MMPs.

ECM remodeling is a negative feedback system in which cleaved and fragmented collagens in ECM negatively affect neocollagenesis. As a result, ECM degradation is increased. The MMPs are considered to be essential elements in this process since they are responsible for the first step of collagen degradation, in which the fibers are fragmented. Thereafter, gelatines and cystein proteases further degrade the collagen fragments.44,45 Hence in this study, inhibition of collagenesis via Ad-RLN and consequent down-regulation of MMPs in myofibroblasts from Dupuytren's disease (Figs. 6 and 7) supports aforementioned negative feedback mechanism, that is, decreased collagen synthesis by Ad-RLN adversely affects MMPs in down-regulatory way.

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.46 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.47 Furthermore, human dermal fibroblasts exposed to RLN modulate secretion of MMPs, collagenase inhibitors, and expression of TIMPs.48 The results from molecular phenotypic descriptors in tissues from Dupuytren's patients indicate that the expression levels of collagen type I and III, MMP-1, MMP-13, and TIMP 1 are increased and level of TIMP 3 is decreased, and while level of TIMP 4 is neutral, as compared to the control group.3,29,49

In conclusion, relaxin can be a novel therapeutic strategy in treating Dupuytren's disease by inhibiting collagenesis, but not collagenolysis, through an MMP-TIMP mechanism. Moreover, results of the current study should prompt more in-depth research into a cocktail therapy using both RLN and collagenase in the treatment of Dupuytren's disease, as well as into the relationship among collagens, MMPs, TIMPs, and their signaling.

ACKNOWLEDGMENTS
This work was supported, in part, by the Brain Korea 21 PLUS Project for Medical Science, Yonsei University and also by a faculty research from Yonsei University College of Medicine 6-2010-0612.

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