Adenovirus-Relaxin gene therapy on keloid : Attenuated proliferative response and collagen degradation

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제가 성형외과를 전공하여 지금의 자리에 있기까지 가르쳐 주시고 보살펴 주신 성형외과교실의 교수님 여러분들께도 깊은 감사를 드리며 실험과 연구과정에서 많은 도움을 주신 이원재 교수님께 특히 감사 드립니다.

성형외과 동문 선배이시자 제 인생의 모범이신 아버님, 오늘의 제가 있도록 사랑으로 보살펴 주신 어머님, 항상 힘이 되어주는 아내 김소영, 그리고 예원이 재원이와 이 기쁨을 함께 하고자 합니다.

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<ABSTRACT>

Adenovirus-relaxin gene therapy on keloids: Attenuated proliferative response and collagen degradation

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Keloid or hypertrophic scars are pathologic proliferations of the skin dermal layer that results from excessive collagen deposition. This pathologic scarring is mainly due to the abnormalities of keloid fibroblast. Relaxin (RLX), a pleiotropic hormone, is a member of the insulin and insulin-like growth factor (IGF) superfamily, and it inhibits collagen synthesis and expression of stimulated fibroblasts with a pro-fibrotic agent including TGF-β and IL-1, 6, when collagen is over expressed, but it does not affect the basal levels of collagen expression. However, the half-life of RLX is very short. In order to overcome this drawback, we made a replication-incompetent relaxin-expressing adenovirus (dl-lacZ-RLX-RGD). The purpose of this study is to investigate the effect of dl-lacZ-RLX-RGD on type I and III collagen mRNA expression, matrix metalloproteinase (MMP) -1, 3, and Smad-2, 3 mRNA expressions of human dermal fibroblasts cell line (HDFCs) and keloid fibroblasts (KFs). In an ex vivo study, we investigated the expression levels of various ECM such as collagen type III, fibronectin, and elastin using immunohistochemisty on the keloid spheroids

ex vivo cultures that were transduced with a relaxin-expressing adenovirus.

Keloid fibroblasts (5×10^5 cells), which were obtained from tissues excised during surgical procedures, and HDFCs were transfected with dl-lacZ-RGD or dl-lacZ-RLX-RGD at multiplicity of infection (MOI) 100 for 72 hours. Three days after transfection and incubation, a β-galactosidase stain was performed. The expression level of RLX protein was assessed by ELISA. The mRNA expressions of collagen type 1 and 3, and matrix metalloproteinases 1 and 3 were examined by RT-PCR. In an ex vivo study, we used keloid dermal tissue that had become spherical (i.e., spheroids) which was approximately 2 mm in diameter (n=3). For transduction of tumor spheroids, 50 μL of adenovirus dl-lacZ-RGF or dl-lacZ-RLX-RGD in a culture medium (i.e., 1×10^{9} viral particles) was added during the three days. We examined the expression levels of collagen type III, elastin, and fibronectin by immunohistochemistry and quantitatively analyzed the expression levels using the MetaMorph® image analyzer.

In the human dermal fibroblast cell line (HDFCs), under basal conditions, we observed stimulation of type I collagen and MMP-1, 3 mRNA expressions by relaxin-expressing adenovirus, whereas it decreased the type I and III collagen mRNA expression and MMP-1, 3 mRNA expressions by the addition of TGF-ß1. However, relaxin-expressing adenovirus decreased collagen types I and III and MMP-1, 3 on mRNA levels regardless of the addition of TGF-ß1 in the keloid fibroblasts and could significantly (more than 69%) reduce Smad-3 mRNA expression. However, Smad-2 mRNA expression was not changed. We think that dl-lacZ-RLX-RGD was shown to inhibit the phosphorylation of Smad-3, which was a key event in TGF-β signaling for collagen synthesis. In an ex vivo study, we investigated the expression levels of collagen type III, fibronectin, and elastin by immunohistochemisty on the keloid spheroids and showed that the expression of collagen type III, elastin, and fibronectin were decreased in keloid spheroids transduced with dl-lacZ-RLX-RGD.

Gene therapy using relaxin-expressing adenovirus could attenuate type I collagen mRNA expression and MMP-1, 3 mRNA expressions on keloid fibroblasts. We expect that relaxin gene transfer using adenovirus could be an effective treatment and may prevent keloid recurrence after surgical excision.

Key words: keloid, gene therapy, relaxin expressing adenovirus, collagen, MMP-1, 3

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I. INTRODUCTION

Keloid or hypertrophic scars are pathologic proliferations of the skin dermal layer that results from excessive collagen deposition. The etiological mechanism behind keloid pathogenesis is not understood. Ischemia theory[1], mechanical theory[2], hormones[3], autoimmune[4], and genetic theory[5] have been suggested for the pathogenesis of keloids, but none of these theories have been shown to be the definitive cause. The keloid fibroblast differs from fibroblasts within the normal dermis in several aspects. In keloids, the homeostasis of wound healing is not maintained, which results in excessive synthesis of the extracellular matrix (ECM) such as collagen, fibronectin, elastin, and proteoglycans[6-9]. Also, keloid fibroblasts react differently to the metabolic regulating factors involved in apoptosis[10], extracellular matrix metabolism, glucocorticoids, growth factors[9, 11], or phorbol esters[12] in comparison to normal dermal fibroblasts. There were other biochemical characteristics of keloids that suggested the expression of various types of MMPs (MMP-1, 2, 3, 8, 9, 13, etc.) is either increased or decreased in comparison with normal dermal fibroblasts and tissue inhibitors of metalloproteinase are increased[6, 7, 13, 14]. These abnormal keloid fibroblasts have been considered to be the cause of

abnormal scars, such as keloid or hypertrophic scars and pathologic fibrosis.

The pleiotopic hormone relaxin is a 6-kDa polypeptide that is classified as a member of the insulin and insulin-like growth factor (IGF)[15-19] superfamily and is naturally found in increased concentrations in the peripheral circulation during pregnancy. It is mainly made in the corpus luteum and endometrium, and is thought to have numerous biological functions, such as remodeling of the connective tissue of the reproductive tract and cervix. According to recent studies, relaxin has other biological effects on the connective tissues[20, 21], brain, heart[22, 23], kidney[23, 24], and lungs[25] and it is classified as a master hormone[18]. The effect of relaxin on the extracellular matrix is thought to inhibit collagen synthesis and expression of the stimulated fibroblasts with a pro-fibrotic agent, which causes collagen to be over expressed, but it does not affect the basal (normal) levels of collagen expression[20]. Also, it increases the expression of MMPs, such as MMP-1, 2, 3, 9, and 13[16, 17, 19, 23]. It is thought that relaxin induces collagen degradation by up-regulation of MMPs[16, 26], especially on cytokine-stimulated cells. Hence, we think that relaxin may cause collagen turnover by inhibiting collagen expression and synthesis and by stimulating matrix metalloproteinases on pathological fibrotic diseases such as keloids.

Gene therapy, using desired genes and expressing regulating factors transferred to cells, has been studied for anti-cancer effects[27] and congenital diseases related to gene deficiency[28]. Of various gene carriers, adenoviruses have been used widely for their ability to transfer relatively large sized genes to dividing or non-dividing cells[29, 30]. We generate the relaxin-expressing, replication-incompetent (dl-lacZ-RLX-RGD) adenovirus by inserting a therapeutic relaxin gene into the E3 adenoviral region, which is essential for the replication of genes, and transduce it into the human dermal fibroblast cell line and keloid fibroblasts. These replication-incompetent adenoviruses have limitations in anti-cancer gene therapy in that their anti-cancer effects are restricted to adjacent infected cells. However, they are well-suited and effective for gene delivery where there is high rate of, but transient, gene transfer and expression in benign conditions, such as in keloids and hypertrophic scars.

In our study, we used a replication-incompetent adenovirus to deliver relaxin genes to human dermal fibroblast cell lines and keloid fibroblasts. We then examined the effects of the relaxin-expressing adenovirus on type I and III collagen mRNA expression and MMP-1 and 3 mRNA expressions. The expression levels of collagen type III, fibronectin, and elastin were investigated by immunohistochemisty on the keloid spheroids, which were transduced with a relaxin-expressing adenovirus.

II. MATERIALS AND METHODS

1. Generation of relaxin-expressing adenoviruses

To generate relaxin-expressing adenoviruses at the E3 region, we first constructed an E3 shuttle vector[31]. The E3 left region of the adenovirus at 26591-28588 bp was amplified by polymerase chain reaction (PCR) with the following primer set: 5'-GCCCTCGAGGCCATAGTTGCTTGCTTGCA-3' as the sense primer and 5'-GCCCAGCTGACCTGAATTAGAATAGCCCG-3' as the antisense primer. The primers were designed to create *Xho* Ⅰ and *Pvu* Ⅱ sites (underlined), and the adenovirus total vector, vmdl324Bst, containing the Ad type 5 genome deleted in the E1 region (281-4640 in nucleotide of Ad 5) and the E3 region (28596-30470), was used as a template. The E3 right region of the adenovirus at 30504-31057 was amplified by PCR with the following primer set: '-GCCGAATTCCTGCTAGAAAGACGCAGGGC-3' as the sense primer and 5'-GCCGATATCGTCTTGCGCGCTTCATCTGC-3' as the antisense primer. The primers were designed to create *Eco*RI and *Eco*RV sites (underlined). The PCR product containing right or left regions of E3 was digested with *XhoI/ PvuII* and *Eco*RI/ *Eco*RV, respectively, and then subcloned into the corresponding sites of pSP72, which generated pSP72-E3. The relaxin gene was excised from pDNR-LIB-RLX (ATCC) using *Sal*I-*Hind*III and subcloned into the pCA14 vector (Microbix, Ontario, Canada), which generated pCA14-RLX. Then, a CMV-relaxin-polA expression cassette was excised from pCA14-RLX and subsequently cloned into the adenovirus E3 shuttle vector pSP72-E3 predigested with *BamH*I, which results in pSP72-E3/CMV-RLX. The newly constructed pSP72-E3/CMV-RLX shuttle vector was then linearized with *Pvu*I digestion. The replication-incompetent adenoviral vector pdl-lacZ, which expresses lacZ at the E1 region of the adenovirus, and pdl-GFP, which expresses GFP at the E1 region of the adenovirus, were linearized with *Spe*I digestion. The linearized pSP72-E3/CMV-RLX shuttle vector was then cotransformed into *Escherichia coli* BJ5183 together with the

*Spe*I-digested pdl-lacZ or pdl-GFP for homologous recombination[32], which generated pdl-lacZ-RLX and pAd-GFP-RLX adenoviral vectors.

To generate RGD-incorporated adenoviruses[33], the genome encoding the fiber in adenovirus serotype 5 (Ad5) (from 28592 to 30470) were cloned in to *Sac*II-*Kpn*I sites of pBluescript II SK(+) (Stratagene, La Jolla, CA), creating pSK5543 and genomes encoding the fiber in Ad5 (from 32123 to 32836) were cloned in to the *Eco*RI-*Hind*III site of pSP72 (Promega, Madison, WI), creating pSP72(713). For ease in incorporating RGD between the HI-loop (LNVTQETGDT**T**PSAYSMSFSWD) of the fiber knob, immediately downstream of the 11th Threonine, the new *BamH*I and *Mro*I sites were added following Threonine by PCR-mediated site directed mutagenesis. For PCR-mediated site directed mutagenesis, the following primer set was used: 5'- GAA ACA GGA GAC ACA *GGA TCC* GCG *TCCGGA* ACT CCA AGT GCA TAC -3' as the sense primer and 5'- GTA TGC ACT TGG AGT *TCCGGA* CGC *GGATCC* TGT GTC TCC TGT TTC -3' as the antisense primer. After PCR amplification, the resulting PCR product contained the *BamHI* and *MroI* sites (underlined) to make pSP72[713]. The nucleotide sequence of the mutated region was verified using an ABI PRISM 337 automatic DNA sequencer (Applied Biosystem, Foster City, CA). To construct a vector encoding RGD between HI-loop of the fiber knob, two complementary oligonucleotides were synthesized and annealed to form a DNA dulex. This DNA duplex was designed to contain a *BamH*I overhang 5' end and an *Mro*I overhang 3' end. The RGD consisted of 9-amino acid sequence CDCRGDCFC. The oligonucleotide sequences were 5'- ga tcc ***TGT GAC TGC CGC GGA GAC TGT TTC TGC*** t-3' and 5'- cc gga ***ACA ATG ACG GCG CCT CTG ACA AAG ACG*** g -3'. This oligonucleotide encode RGD (boldface and italicized). The resultant plasmid was then digested with *Nco*I and *Mfe*I, and cloned into pSK5543, generating an adenovirus fiber shuttle vector, pSK[5543-RGD].

The newly constructed pSK[5543-RGD]shuttle vector was then linearized with *SacI, XmnI* digestion. The replication-incompetent adenoviral vectors pdl-lacZ-RLX

and pdl-GFP-RLX were linearized with *Spe*I digestion. The linearized pSK[5543-RGD] shuttle vector was then cotransformed into *Escherichia coli* BJ5183 with the *Spe*I-digested pdl-lacZ-RLX or pdl-GFP-RLX for homologous recombination[32], which generated pdl-lacZ-RLX-RGD and pAd-GFP-RLX-RGD adenoviral vectors.

To verify the respective homologous recombinants, the plasmid DNA purified from overnight *E. coli* culture was digested with *Hind*III and the digestion pattern was analyzed. The proper homologous recombinant adenoviral plasmid DNA was digested with *Pac*I and transfected into 293 cells to generate pdl-lacZ-RLX-RGD and pAd-GFP-RLX-RGD adenoviruses[34]. All viruses were propagated in 293 cells, and the purification, titration, and quality analysis of all adenoviruses used were performed as previously described [34]. The titer (MOI) used in this study was determined by the absorbency of the dissociated virus at $A260_{nm}$ (one $A260_{nm}$ unit = 10^{12} viral particles/mL). The particle-to-MOI ratio was 100:1.

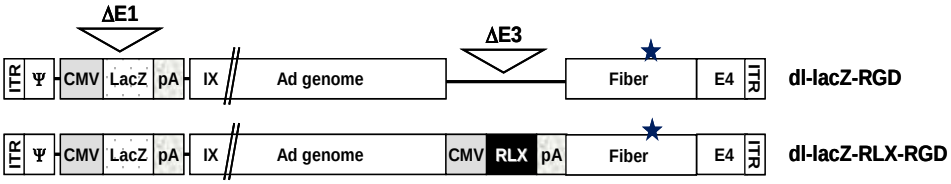


Figure 1. Schematic representations of the adenoviral vectors used in this study. All indicated adenoviral vectors were derived from full-length adenovirus genomes cloned and manipulated in *Escherichia coli* as bacterial plasmids. The replication-incompetent adenovirus that does not express relaxin, dl-lacZ, has the whole E1 region deleted and expresses the reporter gene lacZ (β -galactosidase protein) under the control of the constitutive cytomegalovirus (CMV) promoter inserted into the E1 region. The replication-incompetent relaxin-expressing adenovirus dl-lacZ-RLX carries a relaxin gene driven by the CMV promoter that was inserted into the E3 region. Δ E1 and Δ E3 denote the deletion of E1 and E3 genes, respectively. The RGD-incorporated adenovirus was generated between HI-loop of the fiber knob. ITR = inverted terminal repeat; Ψ = packaging signal; pA = polyA sequence; IX = protein IX; and RLX = relaxin.

2. Keloid-derived fibroblast culture and human dermal fibroblast cell line

Keloid-derived fibroblasts were obtained from tissues excised during surgical procedures. Cells were obtained from the central dermal layer of active stage keloid patients (Table 1). Keloids were identified by trained clinicians and pathologists. Using only the central dermal area, a dermal fibroblast cell culture was performed. The separated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and penicillin (30 U/mL), streptomycin (300μg/mL), and actinomysin. Culture medium was changed at 2 - 3 day intervals. When the primary culture became confluent, the culture medium in the primary culture flasks was removed and the cells were washed with PBS, detached completely by adding 2 mL of 0.025% trypsin, and collected. All cells used in this study were taken before passage 5. The human normal dermal fibroblast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (30 IU/mL), and streptomycin (300 μ g/mL).

Table 1. Profiles of keloid scar fibroblasts used in the study

	M/F	Race	Age	Origin
1	F	Korean	28	Earlobe
2	F	Korean	28	Shoulder
3	F	Korean	27	Earlobe
4	F	Korean	20	Earlobe
5	F	Korean	23	Earlobe
6	F	Korean	22	Earlobe
7	F	Korean	25	Earlobe

3. Transfection of keloid fibroblast with dl-lacZ-RGD and dl-lacZ-RLX-RGD adenovirus.

Keloid fibroblasts and HDF cell lines were infected in suspension with dl-lacZ-RGD and dl-lacZ-RLX-RGD adenoviruses at MOI 100 for 72 hours. The control group consisted of keloid fibroblasts with dl-lacZ-RGD adenovirus transfection and the experimental group was infected with dl-lacZ-RLX-RGD adenoviruses. Three days after transfection and incubation, β-galactosidase stain was performed. In all of the experimental groups, expressions of collagen types 1 and 3, matrix metalloproteinases 1 and 3, and Smad-3 mRNA were examined by RT-PCR. After the addition of TGF-ß1 (5 ng) on the keloid fibroblasts, which was transfected by dl-lacZ-RGD and dl-lacZ-RLX-RGD adenoviruses, the same procedures were performed. β-actin served as a loading control. In the HDFC lines the same experimental procedures were performed. The mRNA expression levels were quantified by densitometry and were corrected for loading differences performed on the β-actin results.

4. Enzyme-linked immunosorbent assay for relaxin and TGF-ß expressions

We infected 5×10^5 keloid-derived fibroblasts with dl-lacZ-RGD, dl-lacZ-RLX-RGD at 1, 10, 100, and 300 MOIs in 25-T culture flasks. Seventy-two hours later, the supernatant was collected by centrifugation at 15, 000 *g* for 10 minutes at 4 °C, and the level of relaxin protein was assessed by enzyme-linked immunosorbent assay (Endogen, Woburn, MA). Serial dilutions of a purified recombinant human relaxin preparation with a known concentration were used to generate a standard curve. The level of TGF-ß protein was assessed by ELISA kit (R & D Systems, USA) at 100 MOIs.

5. Reverse transcriptase-polymerase chain reaction

Total RNA was prepared with TRIZOL Reagent (Gibco BRL Life Technologies, Rockville, Maryland, USA), and complementary DNA was prepared from 1 μg of total RNA by random priming using a first-strand cDNA synthesis kit (Promega Corp., Madison, Wisconsin, USA), under the following conditions: 95℃ for 5 min, 37℃ for 2 hrs, and 75℃ for 15 min. The synthesized cDNA was added to a standard PCR reaction mixture using specific primers (Table 2). The cDNA was denatured for 5 min at 94℃, before 30 cycles of 94℃ for 30 sec, 52℃ for 40 sec, and 72℃ for 1 min. ß-actin cDNA was amplified in each sample to verify the successful completion of cDNA synthesis. After amplification, the RT-PCR products were electrophoresed on 2% agarose gel with 0.5 μg/mL of ethidium bromide.

Table 2. Primers for these studies

Primer	Sequence
Collagen I	**F 5' –GGTGGTGGTTATGACTTTGG- 3'**
	R 5'-GTTCTTGGCTGGGATGTTTT-3'
Collagen III	**F 5'-GCTCTGCTTCATCCCACTATTA-3'**
	R 5'-TGCGAGTCCTCCTACTGCTAC
MMP-1	**F 5'-GATGTGGAGTGCCTGATGTG-3'**
	R 5'-TGCTTGACCCTCAGAGACCT-3'
MMP-3	**F 5'-TCATTTGGCCATCTCTTCC-3'**
	R 5'-CTTATCCGAAATGGCTGCAT -3'
Smad-2	**F 5'-TTAAAGCACCTTGTGGAATC-3'**
	R 5'-AATAACGGAGAAGTGGGA-3'
Smad-3	**F 5'-CGAGGGGAGGTCTTTGCG-3'**
	R 5'-GCTCGGGGAACCCATCTG-3'
ß-actin	**F 5'-GTGCCACCAGACAGCACTGTGTTG-3'**
	R 5'-TGGAGAAGAGCTATGAGCTGCCTG-3'

6. Preparation of keloid spheroids and the spreading and penetration of relaxin-expressing replication-incompetent adenovirus in spheroids

For our experiments, we only used keloid dermal tissue that became spherical (i.e., spheroids) which was approximately 2 mm in diameter. For transduction of tumor spheroids, 100 μL of culture medium was removed from each spheroid culture dish by aspiration, and 50 μL of adenovirus dl-lacZ-RGF or dl-lacZ-RLX-RGD in culture medium (i.e., 1×10^{9} viral particles) was added. Three days later, β-galactosidase expression in each spheroid was assessed by use of 5-bromo-4-chloro-3-indolyl- β - d -galactopyranoside(Life Technologies, Rockville, MD), as described previously[35]. Each experiment was carried out three to four times and the results were similar in all experiments.

7. Evaluation of keloid spheroid by Histology and Immunohistochemistry

Keloid spheroid after adenovirus transfection and incubation was fixed in 10% formalin and embedded in paraffin (Wax-it, Vancouver, Canada), and 3- μm sections were cut. Representative sections were stained with Masson's trichrome and then examined by light microscopy. The same paraffin slides were also used in immunohistochemistry for evaluation of the expression levels of collagen type III, elastin, fibronectin, and TGF-ß. For the immunohistochemical staining method, in brief, keloid spheroids were washed several times with phosphate buffered saline (PBS), and the residual fixative was removed by treatment with 1% sodium borohydride for 1 hour. Spheroids were pretreated with a 3% hydrogen peroxide solution for 10 minutes, washed again with PBS several times, treated with the Histostain-plus kit (Zymade, CA, USA) at room temperature for 1 hour to prevent non-specific reactions, and then incubated overnight with anti-collagen type III (Sigma, St. Louis, MO), anti-elastin (Sigma, St. Louis, MO), anti-fibronectin or anti- TGF-ß (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies diluted to 1:1,000. The spheroids were then washed with PBS and incubated for 10 min at room temperature with biotinylated secondary antibody solution from the Histon-plus kit, which was followed by incubation at room temperature for 10 min with a horseradish peroxidase-conjugated tertiary antibody. Following this, the spheroids were stained with 0.05% diaminobenzidine (DAB, Sigma, St. Louis, USA) and 0.01% hydrogen peroxide, washed with distilled water, dehydrated, and clarified by a conventional method. The expression levels of collagen type III, fibronectin and elastin were quantitatively analyzed using the MetaMorph® image analysis software (Universal Image Corp.). Quantifications of positive-stained areas after the dl-lacZ-RLX-RGD and dl-lacZ-RGD transduced keloid spheroids were performed using MetaMorph imaging analyzer software. Threshold areas represent optical densities of the area on the section that is within the parameters considered to be a positive staining. Results are expressed as the mean optical density for six different digital images.

8. Statistics

Results expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made using the Kruskal-Wallis test and Wilcoxon Two Sample test to see the difference between keloid fibroblast infected by dl-lacZ-RGD and dl-lacZ-RLX-RGD. P values less than 0.05 were considered to be significant for all tests.

III. RESULTS

1. Quantitative ELISA results showing relaxin expression

We first examined the expression of relaxin in cultured keloid fibroblast cells with or without TGF-ß (5 ng) after their infection with dl-lacZ-RGD and dl-lacZ-RLX-RGD adenovirus at MOIs between 1 and 300. These were confirmed by β-galactosidase expression in keloid fibroblasts infected by dl-lacZ-RLX-RGD(Fig 2). Keloid fibroblasts were infected by dl-lacZ-RLX-RGD induced relaxin expression at 10, 100, and 300 MOIs. The expression of relaxin is increased by increasing MOIs. High levels of relaxin expression was induced at 100 MOI (114.98 pg/dl after 72 hours of incubation on the keloid fibroblast infected by dl-lacZ-RLX-RGD and 144.75 pg/dl after adding TGF-ß)(Fig. 3).

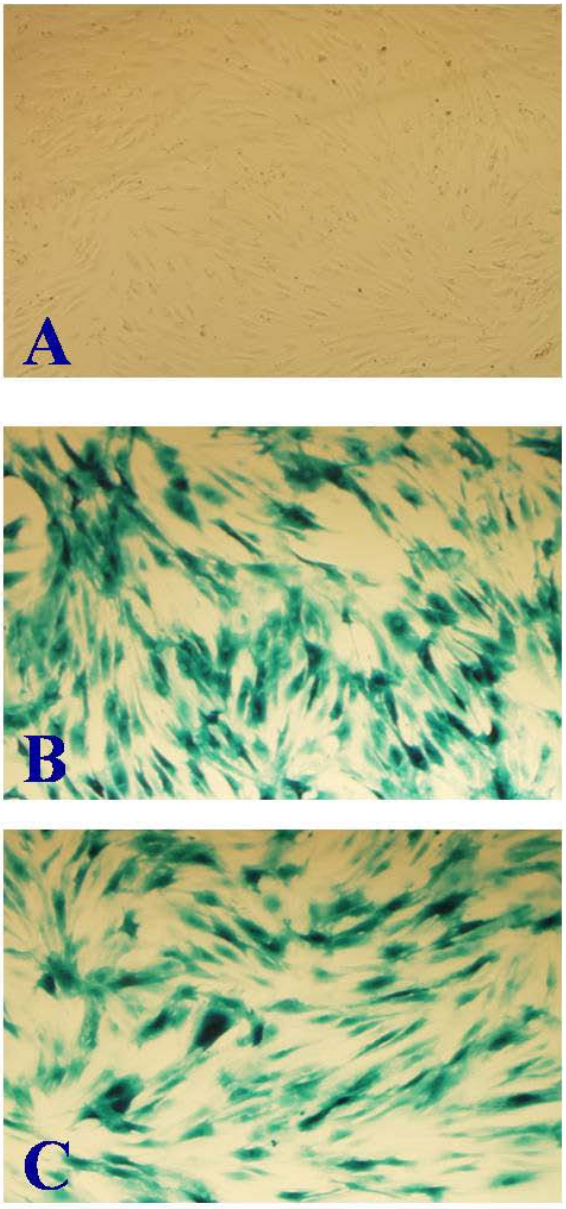


Fig. 2 β-galactosidase expression in keloid fibroblasts (A); Control group (B, C); keloid fibroblasts after their infection (100MOI) with dl-lacZ-RGD (B); and dl-lacZ-RLX-RGD (C).

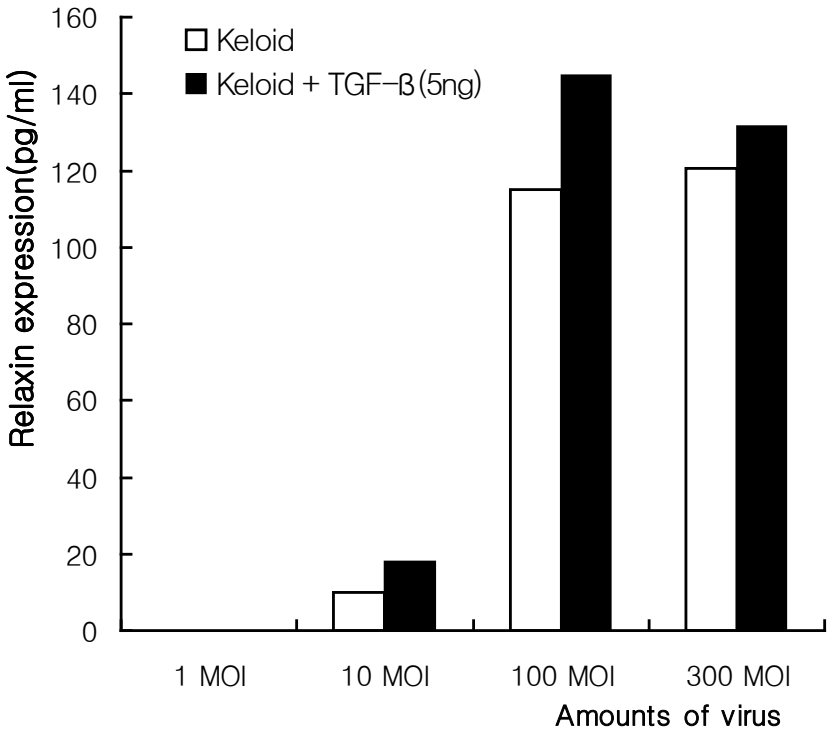


Fig. 3 Expression levels of relaxin protein. The levels of relaxin protein, which was examined by ELISA, were increased with increased MOIs. High levels of relaxin expression were induced at 100 MOI (114.98 pg/dL after 72 hours of incubation on the keloid fibroblast infected by dl-lacZ-RLX-RGD and 144.75 pg/dL after adding TGF-ß).

2. Relaxin-expressing adenovirus modulates a type I and III collagen mRNA expression.

Expression of type I and III collagen mRNA by keloid-derived fibroblasts (n=4) and HDFC after adenovirus transfection were examined by RT-PCR. In the human dermal fibroblast cell(HDFC) lines, basal expressions of type I and III collagen mRNA were moderately increased by the adenovirus dl-lacZ-RLX-RGD in comparison to the unstimulated fibroblast cell lines and adenovirus dl-lacZ-RGD. However, when TGF-ß1 (5 ng/mL) was added simultaneously with relaxin-expressing adenovirus, type I collagen mRNA expression was decreased by up to 68% (Fig. 4A). We think that TGF-ß induced collagen synthesis was inhibited by a relaxin-expressing adenovirus in HDFC lines.

In the keloid-derived fibroblasts, expressions of type I and III collagen mRNA were decreased by the dl-lacZ-RLX-RGD (Fig. 4B), which blocked type I collagen mRNA expression by 28% (keloid fibroblast infected with dl-lacZ-RGD : 1562.59±209.07, keloid fibroblast infected with dl-lacZ-RLX-RGD : 443.85±605.32) ($p<0.05$) and 77% for adding TGF-ß1 (keloid fibroblast infected with dl-lacZ-RGD: 1404.93±289.56, keloid fibroblast infected with dl-lacZ-RLX-RGD : 1090.11±732.48) (Fig. 5). Also, type III collagen mRNA expression was decreased by 59% (keloid fibroblast infected with dl-lacZ-RGD: 1187.29±369.1, keloid fibroblast infected with dl-lacZ-RLX-RGD : 699.78±279.57), and 84% for adding TGF-ß1 (keloid fibroblast infected with dl-lacZ-RGD : 1062.55±153.16, keloid fibroblast infected with dl-lacZ-RLX-RGD : 896.62±482.64)(Fig. 6). These results suggested that relaxin-expressing adenoviruses decreased type I and III collagens on mRNA levels regardless of adding TGF-ß1 to the keloid fibroblasts.

We think that gene therapy using relaxin-expressing adenovirus may lower collagen synthesis in keloids and increase basal collagen synthesis in the wound healing period of normal dermal tissue, which was a highly desirable transient effect for prevention of keloid recurrence after surgical excision.

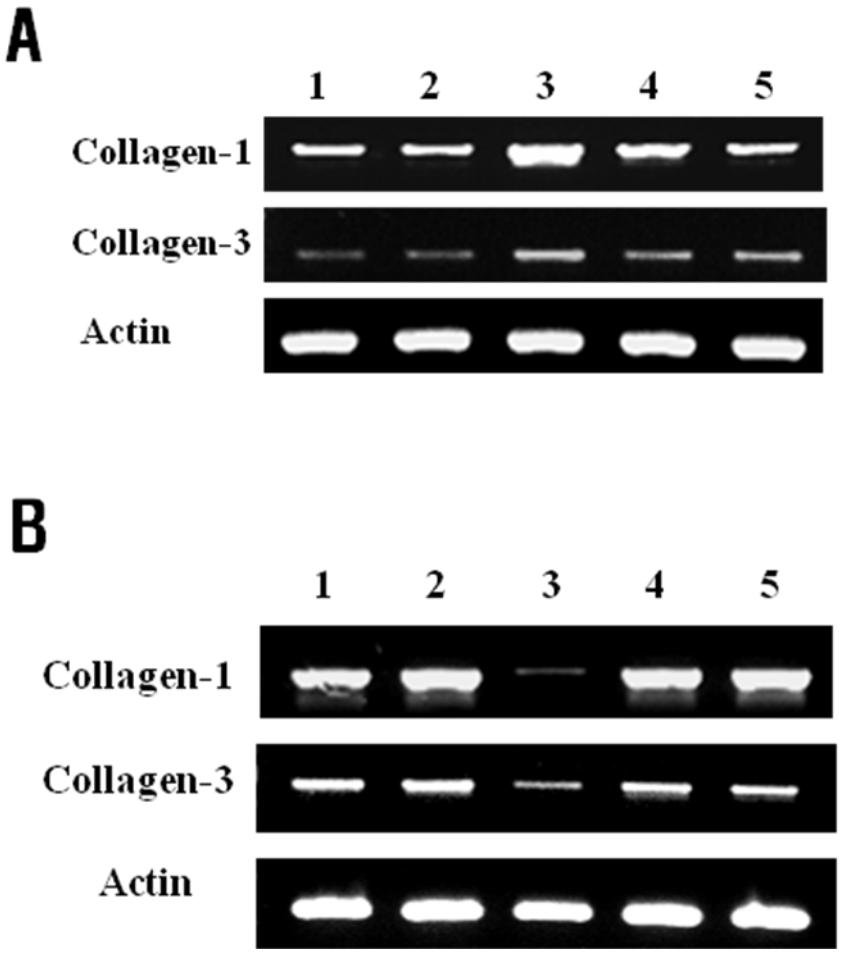


Fig. 4. Effect of relaxin-expressing adenovirus on the type I and III collagen mRNA in the human dermal fibroblast cell lines (A) and keloid-derived fibroblasts (B). Human dermal fibroblast cell lines and keloid fibroblasts were infected with dl-lacZ-RGD and dl-lacZ-RLX-RGD, with or without the addition of TGF-ß1 (5 ng/mL). Levels of actin mRNA were shown as a loading control. (1; unstimulated fibroblast, 2; dl-lacZ-RGD, 3; dl-lacZ-RLX-RGD, 4; dl-lacZ-RGD+ TGF-ß1, 5; dl-lacZ-RLX-RGD+ TGF-ß1).

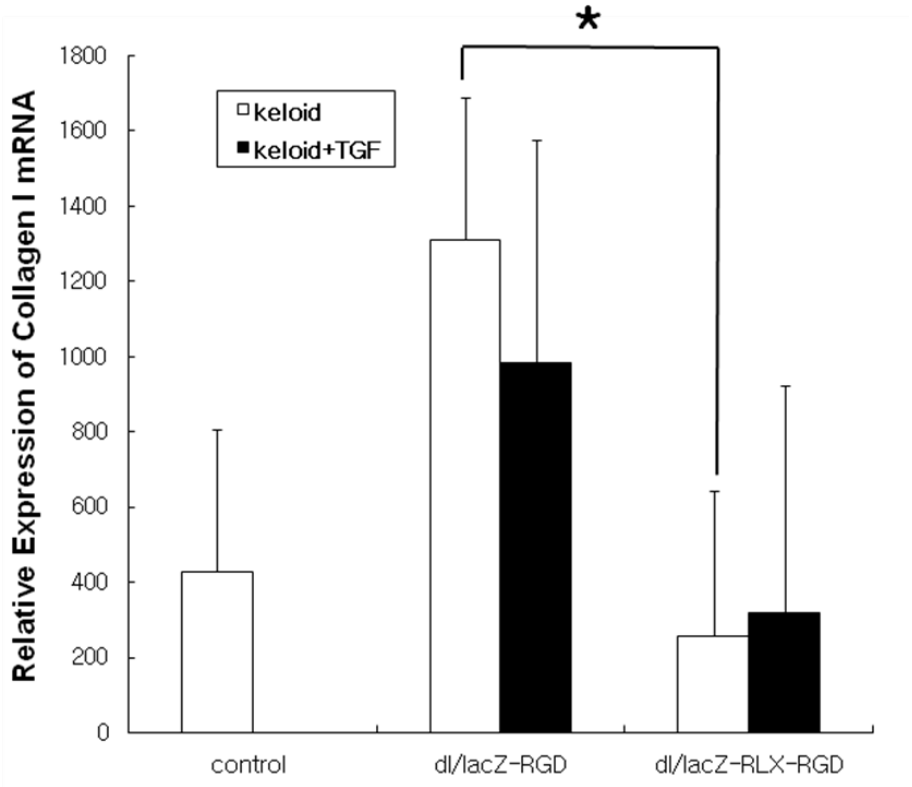


Fig 5. Relaxin-expressing adenovirus decrease type I collagen mRNA expression. Type I collagen mRNA levels quantified by scanning densitometry and were corrected for the levels of actin in same samples were shown to in keloid fibroblasts without TGF-ß1(open bars) or keloid fibroblasts with TGF-ß1(closed bars). (* : $p<0.05$)

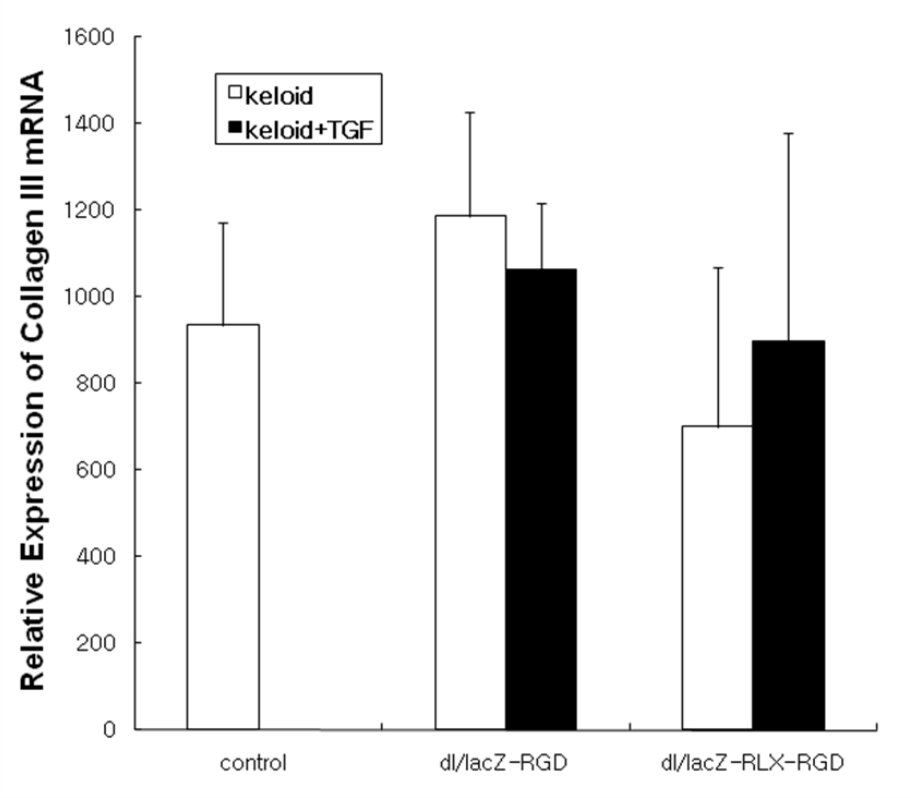


Fig. 6. Relaxin-expressing adenovirus decreased type III collagen mRNA expression. Type III collagen mRNA levels quantified by scanning densitometry and were corrected for the levels of actin in same samples were shown to in keloid fibroblasts without TGF-ß1 (open bars) or keloid fibroblasts with TGF-ß1 (closed bars). Relaxin-expressing adenoviruses decreased type III collagen mRNA expression, but without statistically significant differences.

3. Relaxin-expressing adenovirus decrease the expression of Smad-3 mRNA but not TGF-β1 expression.

To examine the effect of relaxin-expressing adenovirus on Smad-3 mRNA expression, keloid fibroblasts were transfected with dl-lacZ-RGD and dl-lacZ-RLX-RGD. As shown in Figure 7, the relaxin-expressing adenovirus could significantly reduce Smad-3 mRNA expression over 69% ($p<0.05$). However, Smad-2 mRNA expression was not changed (data not shown). We think that dl-lacZ-RLX-RGD inhibited the phosphorylation of Smad-3, which was a key event in TGF-β signaling for collagen synthesis.

The effects of dl-lacZ-RGD and dl-lacZ-RLX-RGD on the expression of TGF-β1 were examined. There was no significant difference between baseline and dl-lacZ-RGD or dl-lacZ-RLX-RGD induced expression of TGF-β1 (Fig. 8).

From these results, dl-lacZ-RLX-RGD does not affect TGF-β1 expression but does have effects on Smad-3 mRNA expression, which is another key factor of collagen synthesis. Therefore, the effects of dl-lacZ-RLX-RGD, which reduced collagen synthesis in keloid fibroblasts, is regulation in the intracellular pathway other than regulation of TGF-β1 expression, which is a typical fibroactive growth factor. No differences in Smad-3 mRNA expression were noted on HDFC lines (data not shown).

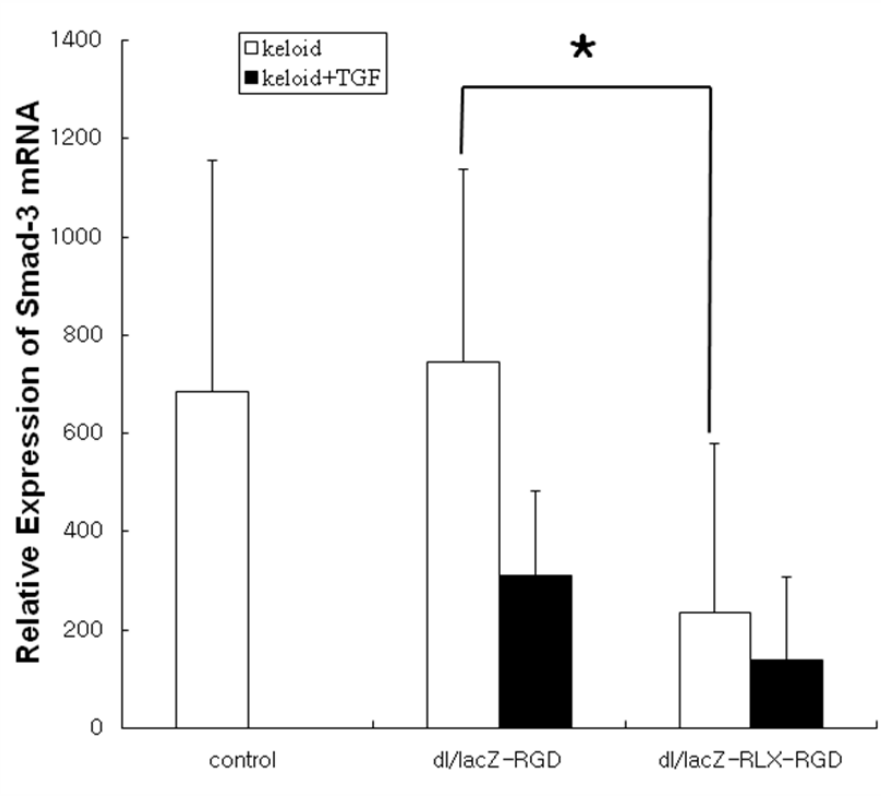


Fig. 7. Effects of relaxin-expressing adenovirus on Smad-3 mRNA expression of keloid fibroblasts. The relaxin-expressing adenovirus could reduce Smad-3 mRNA expression more than 69% (* : $p<0.05$) on keloid fibroblasts. After adding TGF-β1 (5 ng), Smad-3 mRNA expression was decreased, but without statistical significance.

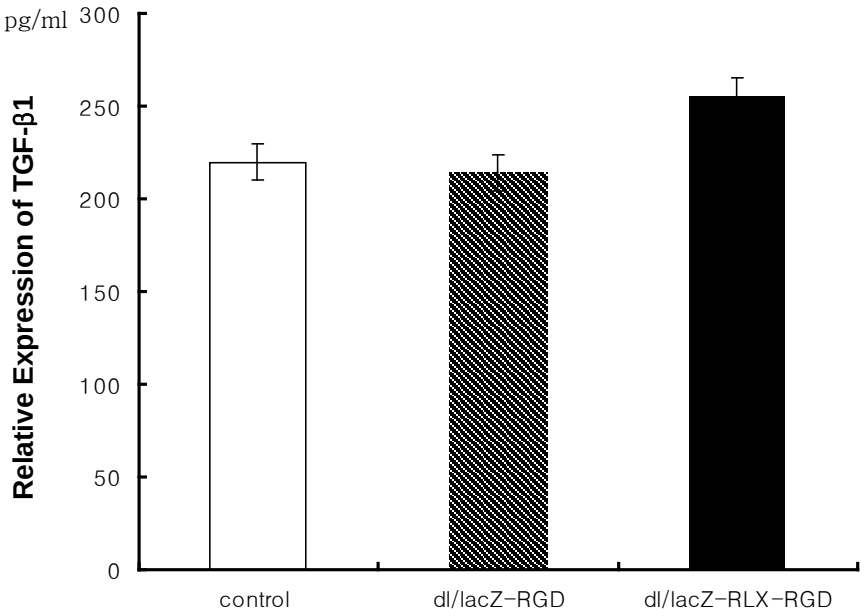


Fig. 8. TGF- β1 protein expression by ELISA. The differences between the relaxin-expressing adenovirus infected group (experimental group) and control group revealed no statistically significance.

4. Relaxin-expressing adenovirus decrease the MMP-1 and 3 mRNA expression.

The effects of relaxin-expressing adenovirus on MMP-1 and 3 mRNA expression were examined by RT-PCR. In the unstimulated human dermal fibroblast cell lines, dl-lacZ-RLX-RGD increases MMP-1, 3 mRNA expression, but after treatment of TGF-ß1 (5 ng/mL) the expressions of MMP-1, 3 mRNA decreased.(Fig. 9A) Under basal conditions, we observed stimulation of type I collagen and MMP-1, 3 mRNA expressions by relaxin-expressing adenovirus, whereas it decreased the type I and III collagen mRNA expression and MMP-1, 3 mRNA expressions by the addition of TGF-ß1 (5 ng/mL)(Fig. 9A).

In the keloid fibroblast dl-lacZ-RLX-RGD significantly decrease the expression of MMP-1 (keloid fibroblast infected with dl-lacZ-RGD : 1412.78±385.71, keloid fibroblast infected with dl-lacZ-RLX-RGD : 390.52±560.48)($p<0.05$), MMP-3 (keloid fibroblast infected with dl-lacZ-RGD : 983.91±317.57, Keloid fibroblast infected with dl-lacZ-RLX-RGD : 317.57±396.87)($p<0.05$) mRNA regardless of treatment of TGF-ß1 (5 ng/mL)(Fig. 9B and 10). In other words, the expression of MMP-1, 3 mRNA was down-regulated by relaxin-expressing adenovirus on keloid fibroblast and stimulated HDFC cell lines by cytokine such as TGF-ß1.

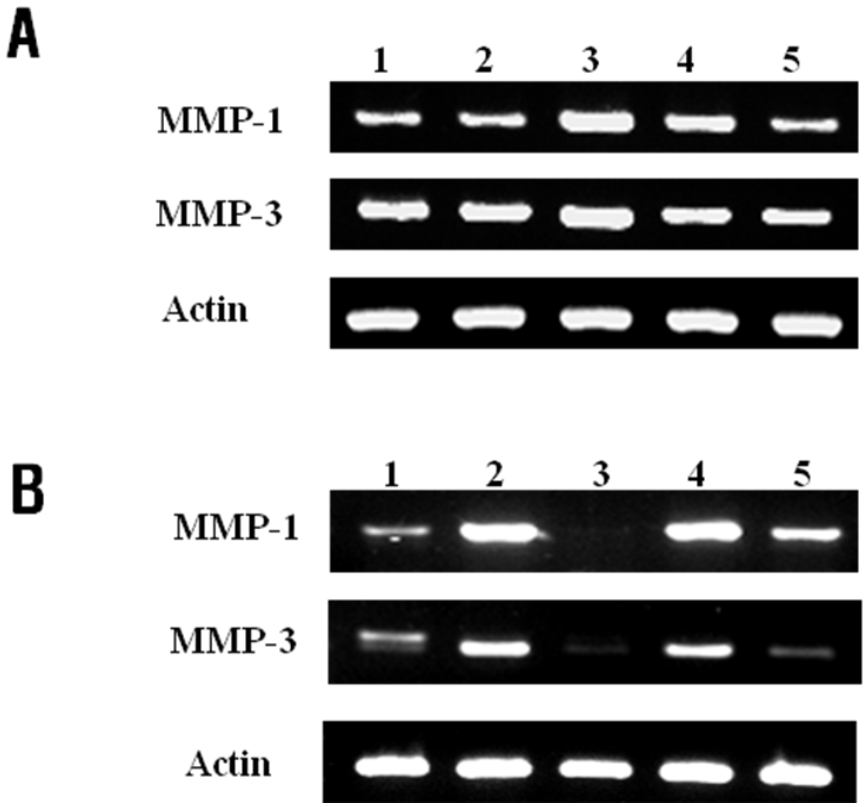
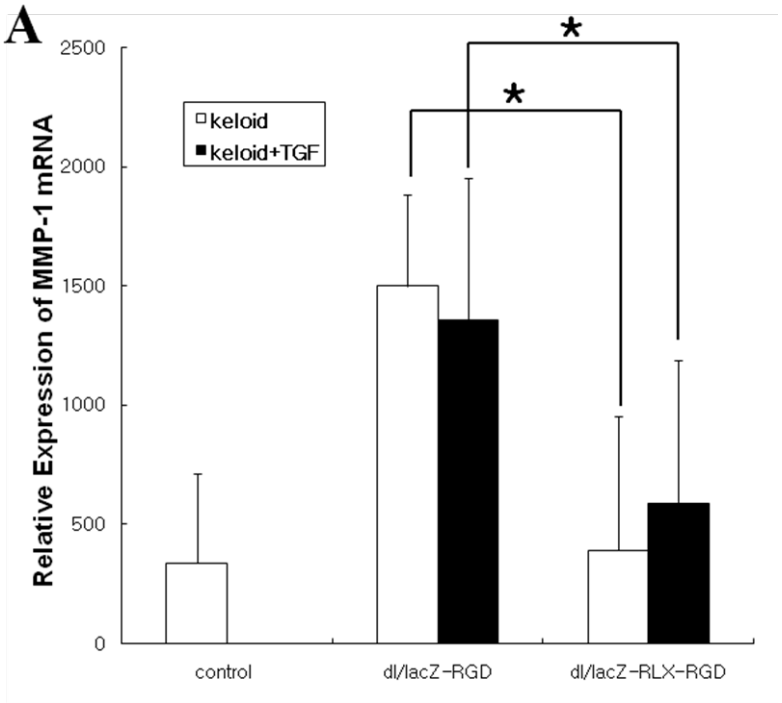


Fig 9. Effect of relaxin-expressing adenovirus on the MMP-1, 3 mRNA in the human dermal fibroblast cell lines (A) and keloid fibroblast (B). Human dermal fibroblast cell lines and keloid fibroblasts were infected with dl-lacZ-RGD and dl-lacZ-RLX-RGD and with or without the addition of TGF-ß1 (5 ng/mL). In the HDFC lines (A), relaxin-expressing adenovirus increased the mRNA expression of MMP-1, 3. However, it decreased MMP-1, 3 mRNA expression by the addition of TGF-ß1 (5 ng/mL). In keloid fibroblast (B), relaxin-expressing adenovirus decrease the expression of MMP-1, 3 mRNA regardless of treatment of TGF-ß1 (5 ng/mL). (1; unstimulated fibroblast, 2; dl-lacZ-RGD, 3; dl-lacZ-RLX-RGD, 4; dl-lacZ-RGD+ TGF-ß1 (5 ng/mL), 5; dl-lacZ-RLX-RGD+ TGF-ß1 (5 ng/mL))


A
Relative Expression of MMP-1 mRNA
2500
2000
1500
1000
500
0
keloid
keloid+TGF
*
*
control
dl/lacZ-RGD
dl/lacZ-RLX-RGD

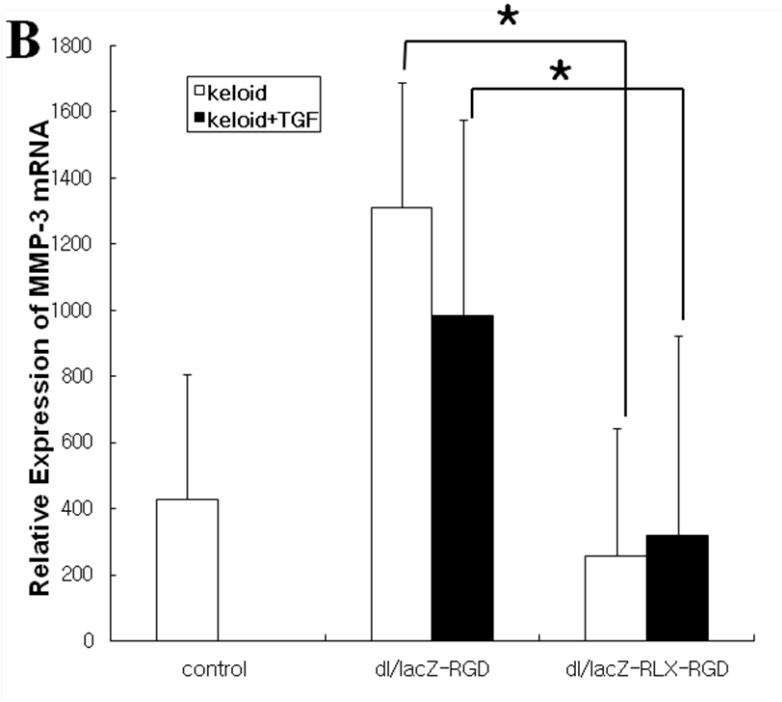

B
Relative Expression of MMP-3 mRNA
1800
1600
1400
1200
1000
800
600
400
200
0
keloid
keloid+TGF
*
*
control
dl/lacZ-RGD
dl/lacZ-RLX-RGD

Fig 10. Relaxin-expressing adenovirus decrease a MMP-1, 3 mRNA expression regardless of the addition of TGF-ß1 in keloid fibroblasts. Keloid fibroblasts were infected with dl-lacZ-RGD and dl-lacZ-RLX-RGD and with or without the addition of TGF-ß1 (5 ng/mL). The expressions of MMP-1 and MMP-3 mRNA were decreased in the keloid fibroblast which infected with dl-lacZ-RLX-RGD compared to keloid fibroblast infected with dl-lacZ-RGD. With the addition of TGF-ß1, the same results were obtained. (* : $p<0.05$, A: MMP-1, B: MMP-3)

5. Transduction efficiency and viral spread of relaxin-expressing adenovirus in keloid spheroids.

Keloid spheroids were used to evaluate the transduction efficiency and tissue penetration of relaxin-expressing adenovirus. Spheroids transduced with dl-lacZ-RLX-RGD expressed β -galactosidase more strongly on their peripheral surface than spheroids transduced with dl-lacZ-RGD.(fig. 11) We thinked that the relaxin-expressing adenovirus, dl-lacZ-RLX-RGD, was transduced and spread to the core of the spheroid more efficiently than the control vector dl-lacZ-RGD.

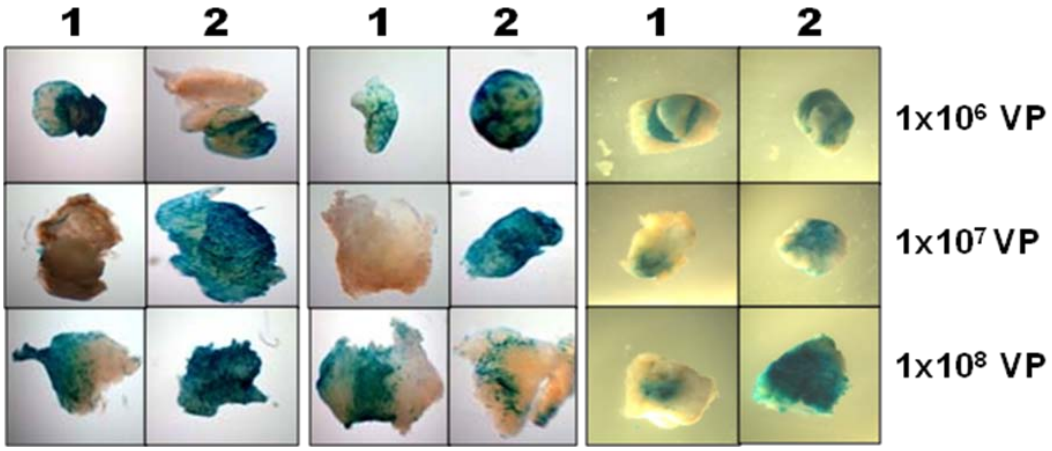


Fig 11. Viral spread of relaxin-expressing adenovirus in keloid spheroids. Spheroids were transduced with dl-lacZ-RGD, which did not express relaxin (**columns 1**), or dl-lacZ-RLX-RGD, which expressed relaxin (**columns 2)**, at 1×10^6 , 1×10^7 , and 1×10^8 viral particles per 50 μ L. Three days after infection, spheroids were processed to visualize β -galactosidase expression (**blue color**).

6. Evaluation of keloid spheroid by histology and immunohistochemistry

Upon using Masson's trichrome staining, we found that keloid spheroid had a dense and excessive deposition of extracellular matrix(ECM) such as collagen in spite of dl-lacZ-RLX-RGD transfection.(Fig. 12) However, in some areas of keloid spheroid infected by dl-lacZ-RLX-RGD, loosening and smooth appearance of ECM were showed. However, three days of spheroid culture were insufficient for effects of relaxin in pathological collagen accumulated keloid tissues.

Immunohistochemical staining showed that the expression of collagen type III(Fig. 13), elastin(Fig. 14), fibronectin(Fig. 15) were decreased in the keloid spheroid transduced with dl-lacZ-RLX-RGD compared to keloid spheroid transduced with dl-lacZ-RGD. Quantification of positive-stained areas were performed using MetaMorph Imaging Analysis Software(Universal Image Cor.). Within keloid spheroid tranduced by dl-lacZ-RLX-RGD, over-expression of fibronectin and elastin were observed (statistically significant differences are indicated by $*p<0.01$). The expression of collagen type III was increased in keloid spheroid tranduced by dl-lacZ-RLX-RGD. but, the difference in collagen type III levels between the keloid spheroid infected by dl-lacZ-RLX-RGD and dl-lacZ-RGD was not statistically significant.(Fig. 16)

The major components of ECM, elastin, fibronectin, and collagen type III, were showed the decreased expression by relaxin-expressing adenovirus transduction and incubation.

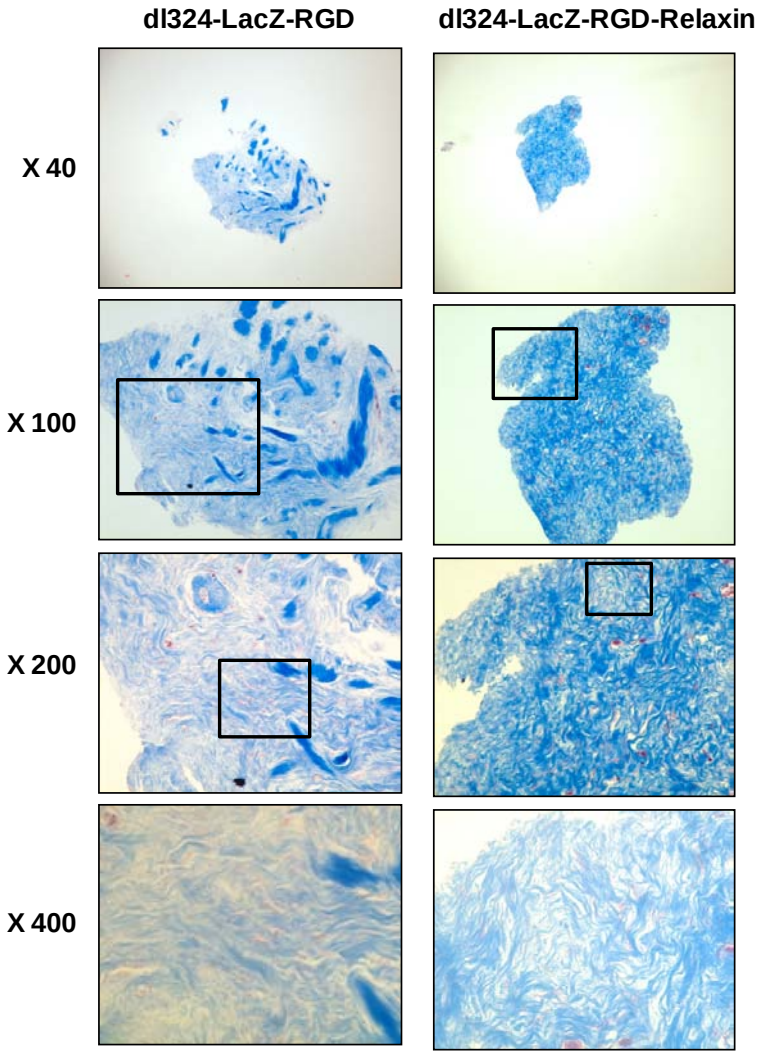


Fig 12. Masson's and trichrome staining of keloid-spheroid. The keloid spheroid had a dense and excessive deposition of extracellular matrix(ECM) such as collagen in spite of dl-lacZ-RLX-RGD transfection. But, in some area of keloid spheroid (x400) infected by dl-lacZ-RLX-RGD, loosening and smooth appearance of ECM was showed. But, other area of keloid spheroid had a dense and compact appearance.

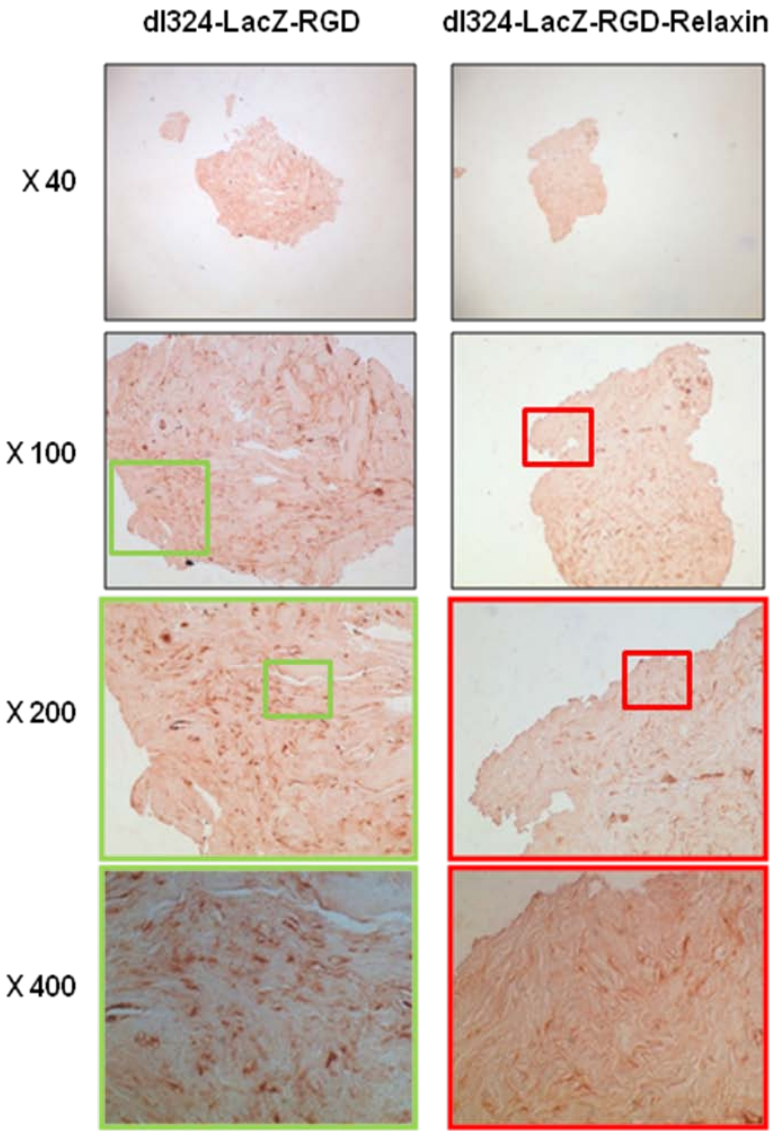


Fig 13. Immunohistochemical staining for type III collagen of keloid spheroid treated with dl-lacZ-RGD and dl-lacZ-RLX-RGD. The expression of collagen type III was lower on the keloid spheroid infected by dl-lacZ-RLX-RGD than dl-lacZ-RGD.

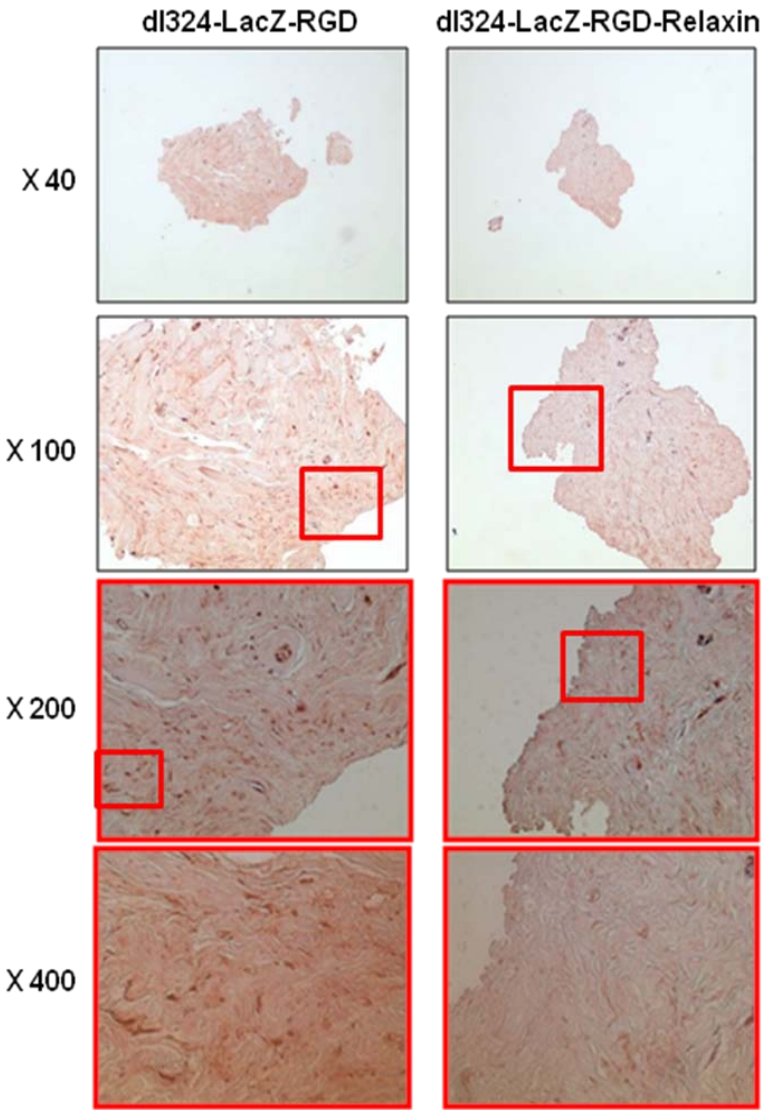


Fig 14. Immunohistochemical staining for elastin of keloid spheroid treated with dl-lacZ-RGD and dl-lacZ-RLX-RGD. The expression of elastin was lower on the keloid spheroid infected by dl-lacZ-RLX-RGD than dl-lacZ-RGD.

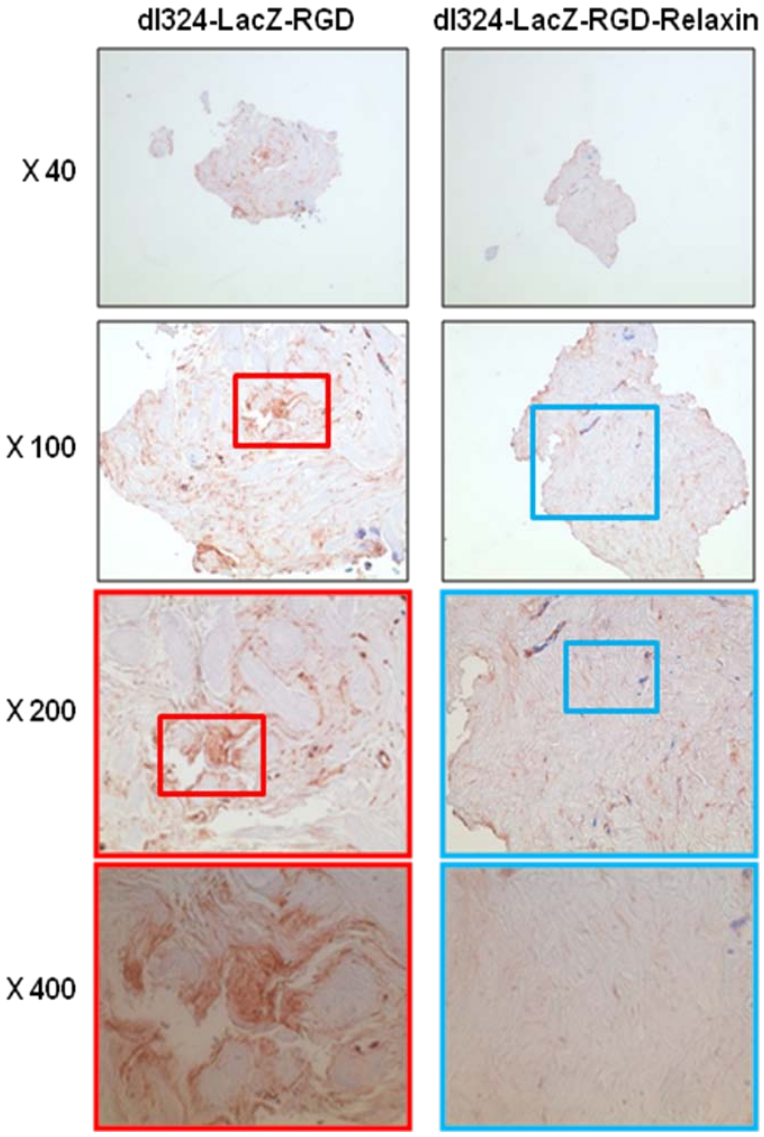


Fig 15. Immunohistochemical staining for fibronectin of keloid spheroid treated with dl-lacZ-RGD and dl-lacZ-RLX-RGD. The expression of fibronectin was lower on the keloid spheroid infected by dl-lacZ-RLX-RGD than dl-lacZ-RGD.

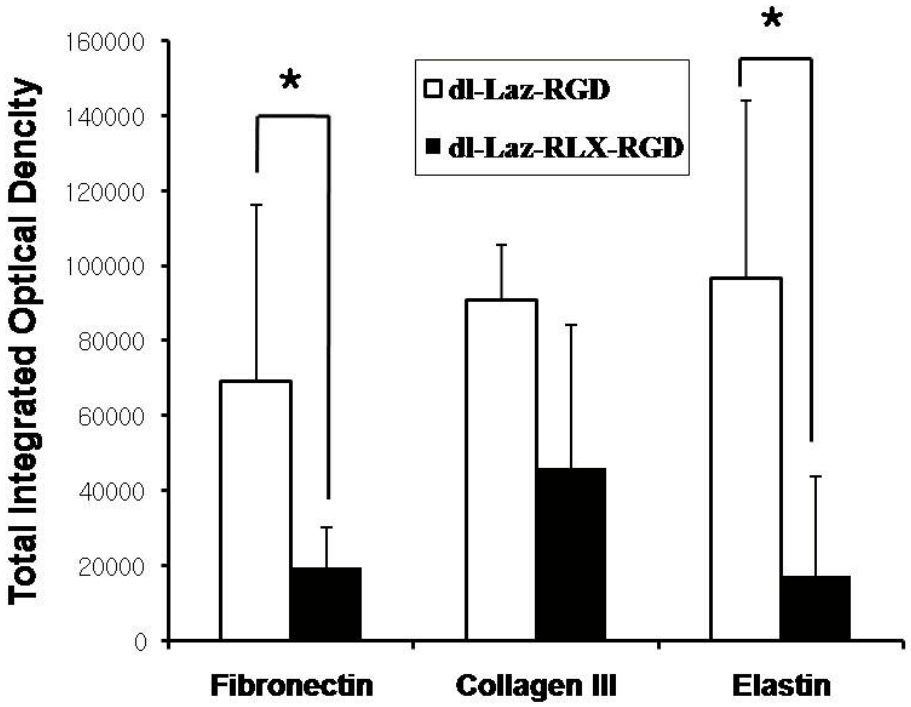


Fig 16. Quantitative analysis of fibronectin, collagen III and elastin expression using immunohistochemical staining. Within keloid spheroid tranduced by dl-lacZ-RLX-RGD, over-expression of fibronectin and elastin were observed (statistically significant differences are indicated by $*p<0.01$). The expression of collagen type III was increased in keloid spheroid tranduced by dl-lacZ-RLX-RGD. but, the difference in collagen type III levels between the keloid spheroid infected by dl-lacZ-RLX-RGD and dl-lacZ-RGD was not statistically significant.

IV. DISCUSSION

In this study, we showed that there was decreased collagen I and III mRNA expression which are major extracellular matrix components, and decreased the expressions of matrix metalloproteinase(MMP)-1, 3 mRNA on the human dermal fibroblast cell lines and keloid fibroblasts which transfected by dl-lacZ-RGD and dl-lacZ-RLX-RGD adenoviruses(relaxin expressing adenovirus).

Gene therapy involves the correction of genetic aberration of host DNA or introduction of new function to the host cells by the delivery of a therapeutic or deficient genes[28, 29] which using recombinant DNA technology. The introduction of natural macromolecules into the patient cells in this sense, it is similar to the protein replacement therapy in Diabetes and hemophilia. The research of gene therapy to correct genetic aberrations of tumors[27], infectious disease and autoimmune disease or to delivery of a therapeutic gene and its associated regulatory elements into the cell nucleus to express the gene at therapeutic or physiological levels is now under way[29].

In order to deliver a particular therapeutic genes into the cell, an appropriate vector is necessary[29, 30]. An ideal gene delivery vectors have to meet several requirements: it has to be innocuous to the human body, its mass production should be feasible, have a bearing on the efficiency of gene transfer to the cell. Vectors can involve either nonviral transfection or viral transduction. Some examples of vectors include both virus such as adenovirus, retrovirus , and adeno-associated virus and nonviral vectors such as the naked DNA/plasmids, liposomes, and basic proteins. There are distinguishing advantages and disadvantages over each other in terms of structural stability and efficiency.

Among these viral vectors, adenovirus type 5 being its prototype, adenovirus(Ad) vector is highly efficient in yielding transduction both in dividing and nondividing cell[29, 30]. Therefore, it is a widely used for gene delivery in up to 25% of clinical gene therapy trials[29]. The relaxin expressing adenovirus used in our study is highly efficient and safe for gene transfer. The adenovirus transgene is expressed in epichromosomal fashion, which may exhibit random chromosomal integration and insertion

mutagenesis. However, the transgene has disadvantage due to its epichromosomal location since it makes immune response inevitable. Therefore, the duration of expression is rather limited. Given these characteristics, the replication-deficient recombinant adenovirus becomes most useful under conditions where high but transient gene transfer and expression is necessary. The treatment of Keloid is the most suitable candidate for applying this therapy among the fields of plastic surgery.

One of the disadvantages of Ad mediated gene transfer is the fact that Ad infectivity is dependent upon the extent of Coxsackie adenovirus receptor(CAR) expression. Many cells including tumor cells (advanced tumor cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, and dermal fibroblasts etc.) lack CAR expression and therefore gene transfer is insufficient. To overcome this problem capsid-modified Ad vector had been developed[28]. Adding RGD peptides to the HI loop of fiber knob has shown the highest transduction efficiency among different modifications of this Ad vector and this type of modified adenovirus[28, 36] was used in our study.

Thanks to the technological advancements of molecular biology, it has been elucidated that cytokines play a central role as modulator in tissue regeneration and healing. Therefore we can speculate that gene therapy targeting variable cytokines which ensure persistent and stable expression of these cytokines or gene products will become applicable in treating various clinical problems, involving soft-tissue repair or wound healing, bone formation, nerve regeneration, cranial suture development[29]. Especially, the transient gene therapy which is similar to proper protein replacement deserve much attention and has great potential in its clinical applicability in the plastic and reconstructive surgery[37] because it does not permanently alter tissue or organ. Tissue repair through genetic approach is based on delivering therapeutic molecules into the cells using either vector or modified cell. Proteins or polypeptides is insufficiently delivered into target sites(due to the short half-life), and delivering of intracellular signal transduction molecules such as Smads in soluble form is not feasible, gene transfer can be the key to tissue repair.

The pleiotropic peptide hormone, relaxin is thought to play a role in structural remodeling of reproductive tract, and inhibits the collagen synthesis and expression of the stimulated fibroblasts with a pro-fibrotic agent, which the collagen is over expressed, but it does not affect the basal(normal) levels of collagen expression in contrast to the interferon gamma[15-17, 23, 26, 38]. However, according to our experimental results, on the human dermal fibroblast cell(HDFC) line transfected by adenovirus expressing relaxin mRNA expression of type I and III collagen were not decreased, but slightly up-regulated. The relaxin expressing adenovirus apparently induces both collagen synthesis as well as activation of MMP-1 and 3 on the unstimulated HDSC. After TGF-β1 stimulation, we observed that relaxin expressing adenovirus reduced the over expression of collagen and MMP-1 and 3. Increasing of collagen synthesis on unstimulated HDSC can be attributed to the following reasons: First, there may have been differences in the effectiveness between delivering relaxin in protein forms and delivering in a stable and persistent form through genetic delivery. Second, activation of intracellular adenylate cyclase and cAMP by relaxin[16, 26, 39] may have served as a fibrotic factor thereby increasing collagen synthesis in HDFC. Although some literature have been documented demonstrating correlation between intracellular concentration of cAMP and increased collagen synthesis[16, 40], there is no explaining its impact upon dermal fibroblast yet, which needs further studies. There are some evidence postulating that the increased collagen synthesis through relaxin is a de novo synthesis rather than direct effect of relaxin[41]. Congruently, our results also corroborate this theory since the expressions of MMP-1, 3 mRNA have increased with respect to increased collagen synthesis on unstimulated dermal fibroblast. Therefore, we concluded that this phenomenon was due to either direct or indirect regulatory effect of relaxin upon the extracellular matrix. However, there is still the possibility of relaxin directly exerting its effect on the synthesis or the damage of collagen and further study is needed to elucidate this fact. Keloid is the result of persistent collagen synthesis by dermal fibroblasts thereby accumulating excess amount of extracellular matrix. Therefore our results of decreasing the expression of

collagen-I, III mRNA by relaxin expressing adenovirus can be applied to treat keloids through gene therapy. Also we thinked that relaxin expressing adenovirus have a highly effective anti-fibrotic agent because these gene therapy does not affect or slightly increased basal collagen expression

Relaxin acts directly on the TGF-ß stimulated fibroblasts to inhibit myofibroblast accumulation and collagen synthesis/secretion[17, 19, 20, 23]. The collagen synthesis by TGF-ß can be summarized as phosphorylation of Smad-2 and Smad-3, formation of complex with Smad-4, and finally the translocation of complex from cytoplasm to nucleus of the cell. Recent studies suggested that relaxin inhibited the phosphorylation and translocation of Smad-2 to the nucleus[24, 42, 43]. However, according to our results, relaxin expressing adenovirus decreased the expression of Smad-3 mRNA regardless of TGF-ß expression, which in turn decreased functions of TGF-ß to collagen synthesis. Because Smad-3 signaling plays an important role in keloid pathogenesis[44-46], such as abnormal collagen over-accumulation, antifibrotic effect of relaxin expressing adenovirus which are mediated by inhibition of Smad-3 pathway is beneficial in keloid fibroblast.

It is important to acknowledge the fact that relaxin may exert tissue specific or organ specific effects despite its similar effects throughout the entire body. Recent studies suggest that maximal stimulation of collagen expression is achieved through a complex set of interplay between different multiple signal transduction pathways. Integrating all these results, we now know that the mechanism behind relaxin lays numerous complex pathways influencing each other and many factors govern the effect. Therefore, further investigation on individual pathways and factors is crucial to a better understanding of this material.

The matrix metalloproteinase(MMP) is a family of over 20 Zn-dependent metalloproteinases which is responsible for cell adhesion, migration, proliferation and differentiation[47-49]. They can be categorized into collagenase (MMP-1), gelatinase A (MMP-2), matrilysin (MMP-7), gelatinase B (MMP-9). They are inhibited by tissue-derived inhibitors(TIMPs) and also involved with the degradation of

extracellular matrix such as collagen, elastin, fibronectin, and proteoglycans. Many factors including growth factors, cytokines, tumor oncogenes, retinoids, dexamethazone, colchicine can affect the expression of MMPs[48, 49]. Different types of MMPs(MMP-1, 2, 3, 8, 9, 13 etc.) are either increased or decreased in their expression in fibroblast disorders such as keloid or hypertrophic scars when compared to normal dermal fibroblasts[6, 7, 13, 14]. The increased expression or activity of MMPs could be associated with keloid spreading to adjacent normal tissues and the high metabolic activity of keloid tissues.

The expression of MMP-1, which plays an important role in fibroblast migration, is higher(6 fold) in keloid fibroblast compared to the normal dermal fibroblast[14, 20, 25, 50]. The increased collagen synthesis and MMPs(especially MMP-1) expression enhance the migratory and invasive activity into neighboring normal tissue. MMP-1, interstitial collagenase, is part of a subfamily of MMPs that plays a central role in the pathogenesis of abnormal collagen turnover[20]. By degrading the collagen triple helix, it is susceptible to proteolysis by gelatinases(MMP-2). The degraded collagen is a specific substrate of the MMP-2, and it is thought that increased production of MMP-1 and 2 play pivotal role in eliminating abnormal or unfolded collagen. Our results examined that the expression of MMP-1, 3 mRNA was down-regulated by relaxin expressing adenovirus on keloid fibroblast and stimulated HDFC by cytokine such as TGF-ß1, but under basal conditions, we observed stimulation of type I collagen and MMP-1, 3 mRNA expressions by relaxin expressing adenovirus. These results were implies that gene therapy using relaxin-expressing virus may inhibit migratory effect of keloid preventing invasion into neighboring normal tissue and could decrease the accumulated abnormal collagen. In addition, relapse of keloid occur via increased collagen synthesis, fibroblast migration and destruction of peripheral normal skin after surgical excision or inflammatory stimulation. And during this process MMP-1 expression is increased. Therefore, we can expect that gene therapy using relaxin expressing adenovirus decrease postoperative recurrence rates of keloid by inhibiting both collagen synthesis and expression of MMPs.

Recent studies in various cell types and in animal models have demonstrated that relaxin induce degradation of collagen, fibronectin[51], and elastin through increasing the expression of MMP-1, 2, 3, 9, 12, and 13. In contrast, MMP-1,3 mRNA expression had been decreased by relaxin expressing adenovirus on keloid fibroblast and stimulated HDFC. We think that this discrepancies may be explained by increased levels of intracellular cAMP by persistent release of relaxin by gene therapy. Cyclic nucleotide cAMP is an important signaling transduction molecule that influences cell proliferation, differentiation, and migration as well as metalloproteinase expression[40, 52, 53]. Increased intracellular cAMP levels can decrease fibroblast function and inhibit conversion of fibroblasts into myofibroblasts, which is a potential target to blunt fibrosis[40]. There are contradictory results for MMPs expression. It is reported that cAMP elevation inhibits expression of MMP-2,9 and keratinocyte migration[52], while in others stating inhibitory effects on the expression and activity of MMP-2. The intracellular cAMP pathway seems to regulate the fibrosis on dermal fibroblast and keloid fibroblast, but further study explaining the mechanisms of myofibroblasts on keloid is needed.

Relaxin has been demonstrated to reverse pathological fibrosis in vivo model of lung, heart, or kidney. Despite its role, it cannot be delivered through oral administration and limitations such as short half-life, low bioavailability, enzymatic inactivation, and only a transient effect by protein replacement therapy. However, the skin and wounds topically transduced with adenoviral vectors could produce transgene expression for up to 10 days. Therefore we think that the virus mediated relaxin delivery has been suggested as an alternative way for prevention of abnormal fibrosis and recurrence of keloid after the surgical excision.

V. CONCLUSION

We have investigated the effects of relaxin expressing adenovirus(dl-lacZ-RLX-RGD) on the expression of many substances that comprise the ECM such as collagen, fibronectin, elastin and MMP-1, 3 on human dermal fibroblast cell lines and keloid fibroblast.

In the human dermal fibroblast cell(HDF) lines, under basal conditions, we observed stimulation of type I collagen and MMP-1, 3 mRNA expressions by relaxin expressing adenovirus, whereas it decreased the type I and III collagen mRNA expressions and MMP-1, 3 mRNA expressions by the addition of TGF-ß1. However, relaxin-expressing adenovirus decreased type I, III collagen and MMP-1, 3 on mRNA levels regardless of adding TGF-ß1 in the keloid fibroblasts and could significantly reduce Smad-3 mRNA expression over 69%. But, Smad-2 mRNA expression was not changed. We thinked that dl-lacZ-RLX-RGD was shown to inhibit the phosphorylation of Smad-3 which was key events in TGF-β signaling for collagen synthesis.

In ex vivo study we investigated the expression levels of collagen type III, fibronectin and elastin by immunohistochemisty on the keloid spheroids and showed that the expression of collagen type III, elastin, fibronectin were decreased in the keloid spheroid transduced with dl-lacZ-RLX-RGD.

Therefore, we could expect that gene therapy using relaxin expressing adenovirus decrease postoperative recurrence rates of keloid by inhibiting both type I collagen synthesis and expression of MMP-1 and 3.

REFERENCES

1. Kischer C, Thies A, Chvapil M. Perivascular myoblast and microvascular occlusion hypertrophic scars and keloids. Human pathology 1982;13(8):8-19.
2. Ehrlich HP, Needle AL. Wound healing in tight-skin mice: delayed closure of excised wounds. Plastic and reconstructive surgery 1983;72(2):190-8.
3. Janssen de Limpens AM, Cormane RH. Studies on the immunologic aspects of keloids and hypertrophic scars. Archives of dermatological research 1982;274(3-4):259-66.
4. Ford LC, King DF, Lagasse LD, Newcomer V. Increased androgen binding in keloids: a preliminary communication. The Journal of dermatologic surgery and oncology 1983;9(7):545-7.
5. Omo-Dare P. Genetic studies on keloid. Journal of the National Medical Association 1975;67(6):428-32.
6. Niessen FB, Spauwen PH, Schalkwijk J, Kon M. On the nature of hypertrophic scars and keloids: a review. Plastic and reconstructive surgery 1999;104(5):1435-58.
7. Tuan TL, Nichter LS. The molecular basis of keloid and hypertrophic scar formation. Molecular Medicine Today 1998;4(1):19-24.
8. Burd A, Huang L. Hypertrophic response and keloid diathesis: two very different forms of scar. Plastic and reconstructive surgery 2005;116(7):150-7e.
9. Tredget EE, Shankowsky HA, Pannu R, Nedelec B, Iwashina T, Ghahary A, et al. Transforming growth factor-beta in thermally injured patients with hypertrophic scars: effects of interferon alpha-2b. Plastic and reconstructive surgery 1998;102(5):1317-28; discussion 29.
10. Luo S, Benathan M, Raffoul W, Panizzon RG, Egloff DV. Abnormal balance between proliferation and apoptotic cell death in fibroblasts derived from keloid lesions. Plastic and reconstructive surgery 2001;107(1):87-96.
11. Kikuchi K, Kadono T, Takehara K. Effects of various growth factors and histamine on cultured keloid fibroblasts. Dermatology 1995;190(1):4-8.
12. Myles ME, Russell JD, Trupin JS, Smith JC, Russell SB. Keloid fibroblasts are refractory to inhibition of DNA synthesis by phorbol esters. Altered response is accompanied by reduced sensitivity to prostaglandin E2 and altered down-regulation of phorbol ester binding sites. The Journal of biological chemistry 1992;267(13):9014-20.
13. Uchida G, Yoshimura K, Kitano Y, Okazaki M, Harri K. Tretinoin reverses upregulation of matrix metalloproteinase-13 in human keloid-derived fibroblasts. Experimental dermatology 2003;12:35-42.
14. Fujiwara M, Muragaki Y, Ooshima A. Keloid-derived fibroblasts show increased secretion of factors involved in collagen turnover and depend on

matrix metalloproteinase for migration. British journal of dermatology 2005;153(2):295-300.
15. Samuel CS. Relaxin: antifibrotic properties and effects in models of disease. Clinical medicine & research 2005;3(4):241-9.
16. Samuel CS, Hewitson TD, Unemori EN, Tang ML. Drugs of the future: the hormone relaxin. Cellular and molecular life sciences 2007;64(12):1539-57.
17. Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocrine reviews 2004;25(2):205-34.
18. Samuel CS, Hewitson TD, Unemori EN, Tang ML. Drugs of the future: the hormone relaxin. Cell Mol Life Sci 2007 Jun;64(12):1539-57.
19. Dschietzig T, Bartsch C, Baumann G, Stangl K. Relaxin-a pleiotropic hormone and its emerging role for experimental and clinical therapeutics. Pharmacology & therapeutics 2006;112(1):38-56.
20. Unemori EN, Amento EP. Relaxin modulates synthesis and secretion of procollagenase and collagen by human dermal fibroblasts. The Journal of biological chemistry 1990;265(18):10681-5.
21. Unemori EN, Beck LS, Lee WP, Xu Y, Siegel M, Keller G, et al. Human relaxin decreases collagen accumulation in vivo in two rodent models of fibrosis. J Invest Dermatol 1993 Sep;101(3):280-5.
22. Samuel CS, Unemori EN, Mookerjee I, Bathgate RA, Layfield SL, Mak J, et al. Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis in vivo. Endocrinology 2004 Sep;145(9):4125-33.
23. Samuel CS, Hewitson TD. Relaxin in cardiovascular and renal disease. Kidney international 2006;69(9):1498-502.
24. Heeg MH. The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway. Kidney international 2005;68(1):96.
25. Unemori EN, Pickford LB, Salles AL, Piercy CE, Grove BH, Erikson ME, et al. Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts in vitro and inhibits lung fibrosis in a murine model in vivo. J Clin Invest 1996 Dec 15;98(12):2739-45.
26. van der Westhuizen ET, Halls ML, Samuel CS, Bathgate RA, Unemori EN, Sutton SW, et al. Relaxin family peptide receptors--from orphans to therapeutic targets. Drug discovery today 2008;13(15-16):640-51.
27. Kim JH, Lee YS, Kim H, Huang JH, Yoon AR, Yun CO. Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy. Journal of the National Cancer Institute 2006;98(20):1482-93.
28. Flotte TR. Gene therapy: the first two decades and the current state-of-the-art. Journal of cellular physiology 2007;213(2):301-5.

29. Ghali S, Dempsey MP, Jones DM, Grogan RH, Butler PE, Gurtner GC. Plastic surgical delivery systems for targeted gene therapy. Annals of plastic surgery 2008;60(3):323-32.
30. St George JA. Gene therapy progress and prospects: adenoviral vectors. Gene therapy 2003;10(14):1135-41.
31. Yun CO, Kim E, Koo T, Kim H, Lee YS, Kim JH. ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis. Cancer Gene Ther 2005 Jan;12(1):61-71.
32. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J Virol 1996 Jul;70(7):4805-10.
33. Ganesh S, Gonzalez Edick M, Idamakanti N, Abramova M, Vanroey M, Robinson M, et al. Relaxin-expressing, fiber chimeric oncolytic adenovirus prolongs survival of tumor-bearing mice. Cancer Res 2007 May 1;67(9):4399-407.
34. Lee H, Kim J, Lee B, Chang JW, Ahn J, Park JO, et al. Oncolytic potential of E1B 55 kDa-deleted YKL-1 recombinant adenovirus: correlation with p53 functional status. Int J Cancer 2000 Nov 1;88(3):454-63.
35. Yun CO, Yoon AR, Yoo JY, Kim H, Kim M, Ha T, et al. Coxsackie and adenovirus receptor binding ablation reduces adenovirus liver tropism and toxicity. Hum Gene Ther 2005 Feb;16(2):248-61.
36. Ganesh S, Gonzalez Edick M, Idamakanti N, Abramova M, Vanroey M, Robinson M, et al. Relaxin-expressing, fiber chimeric oncolytic adenovirus prolongs survival of tumor-bearing mice. Cancer research 2007;67(9):4399-407.
37. Xu B, Liu ZZ, Zhu GY, Yang JF, Zhao JP, Wang JC, et al. Efficacy of recombinant adenovirus-mediated double suicide gene therapy in human keloid fibroblasts. Clinical and experimental dermatology 2008;33(3):322-8.
38. Jaffe HA, Gao Z, Mori Y, Li L, Varga J. Selective inhibition of collagen gene expression in fibroblasts by an interferon-gamma transgene. Experimental lung research 1999;25(3):199-215.
39. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci U S A 2005 Jan 11;102(2):437-42.
40. Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, Insel PA. The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. Proceedings of the National Academy of Sciences of the United States of America 2008;105(17):6386-91.
41. McGuane JT, Parry LJ. Relaxin and the extracellular matrix: molecular mechanisms of action and implications for cardiovascular disease. Expert

reviews in molecular medicine 2005;7(21):1-18.
42. Bathgate RA, Lekgabe ED, McGuane JT, Su Y, Pham T, Ferraro T, et al. Adenovirus-mediated delivery of relaxin reverses cardiac fibrosis. Molecular and Cellular Endocrinology 2008;280(1-2):30-8.
43. Mookerjee I, Hewitson TD, Halls ML, Summers RJ, Mathai ML, Bathgate RA, et al. Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. The FASEB journal 2009;23(4):1219-29.
44. Wang Z, Gao Z, Shi Y, Sun Y, Lin Z, Jiang H, et al. Inhibition of Smad3 expression decreases collagen synthesis in keloid disease fibroblasts. Journal of plastic, reconstructive & aesthetic surgery 2007;60(11):1193-9.
45. Phan TT, Lim IJ, Aalami O, Lorget F, Khoo A, Tan EK, et al. Smad3 signalling plays an important role in keloid pathogenesis via epithelial-mesenchymal interactions. The Journal of pathology 2005;207(2):232-42.
46. Phan TT, Lim IJ, Sun L, Chan SY, Bay BH, Tan EK, et al. Quercetin inhibits fibronectin production by keloid-derived fibroblasts. Implication for the treatment of excessive scars. Journal of dermatological science 2003;33(3):192-4.
47. Nagase H, Woessner JF. Matrix metalloproteinases. The Journal of biological chemistry 1999;274(31):21491-4.
48. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biologic activity and clinical implications. Journal of clinical oncology 2000;18(5):1135-49.
49. Soo C, Shaw WW, Zhang X, Longaker MT, Howard EW, Ting K. Differential expression of matrix metalloproteinases and their tissue-derived inhibitors in cutaneous wound repair. Plastic and reconstructive surgery 2000;105(2):638-47.
50. Palejwala S, Stein DE, Weiss G, Monia BP, Tortoriello D, Goldsmith LT. Relaxin positively regulates matrix metalloproteinase expression in human lower uterine segment fibroblasts using a tyrosine kinase signaling pathway. Endocrinology 2001 Aug;142(8):3405-13.
51. Babu M, Diegelmann R, Oliver N. Fibronectin is overproduced by keloid fibroblasts during abnormal wound healing. Molecular and cellular biology 1989;9(4):1642-50.
52. Lee C, Lee J, Choi YA, Kang SS, Baek SH. cAMP elevating agents suppress secretory phospholipase A(2)-induced matrix metalloproteinase-2 activation. Biochemical and biophysical research communications 2006;340(4):1278-83.
53. Schiller M, Verrecchia F, Mauviel A. Cyclic adenosine 3',5'-monophosphate-elevating agents inhibit transforming growth factor-beta-induced SMAD3/4-dependent transcription via a protein kinase

A-dependent mechanism. Oncogene 2003;22(55):8881-90.

ABSTRACT(IN KOREAN)

Adenovirus-Relaxin을 이용한 켈로이드의 유전자 치료; 켈로이드 섬유모세포의 교원질 합성과 metalloproteinases(MMPs) 의 합성에 미치는 영향

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설 철 환

켈로이드(keloid)나 비후성 반흔(hypertrophic scar)은 외상이나 수술적 자극에 의해서 진피와 피하지방층에 과도한 세포외 기질 등이 침착 되는 피부의 병적 상태이다. 이의 병인기전에 대해서는 명확하게 밝혀지지 않고 있으며 확실한 치료법도 없는 상태이다.

Relaxin 은 Insulin 과 insulin-like growth factor(IGF) 부류에 속하는 6 kDa 의 호르몬으로 세포외 기질에 대하여 MMP-1,2,3,9 등과 같은 여러 종류의 MMPs 의 발현을 촉진하여 교원질의 분해를 촉진시키고, 새로운 교원질 합성을 억제하는 것으로 알려져 있다. 하지만 relaxin 은 과다 발현된 교원질의 분해만을 촉진 시키며, 인터페론 감마(IFN-γ)와 같은 싸이토카인과는 달리 최소량의 교원질 합성을 유지시킨다. 단백질 형태의 relaxin 은 혈중에서 반감기가 짧기 때문에 직접적 투여로는 효과가 떨어지므로 본 연구는 켈로이드 섬유모세포와 정상 인체 진피 섬유모세포주에 relaxin을 발현하는 복제 불능 아데노바이러스를 감염시켜 제한적으로 섬유모세포 내에서 relaxin 을 발현 시켰을 때 교원질

합성(Collagen type I, III)과 MMP-1, 3 mRNA 발현을 RT-PCR 을 이용하여 어떻게 변화하는가를 알아보고자 하였으며 켈로이드에서 유전자 치료의 가능성을 알아보고자 하였다. 또한 켈로이드 조직구(spheroid)를 relaxin 을 분비하는 아데노바이러스로 감염시킨 후 조직 배양한 후에 엘라스틴, 피브로넥틴, 제 3 형 콜라젠 등의 세포외기질의 발현 정도 및 변화를 면역화학조직염색을 통해서 알아보고자 하였다.

켈로이드 섬유모세포(5 x 10^5 개)와 정상 인체 진피 섬유모세포에 dl-lacZ-RGD(대조군 바이러스), dl-lacZ-RLX-RGD(relaxin 을 분비하는 실험군 바이러스)를 100 multiplicities of infection (MOIs)로 감염시키고 72 시간 동안 배양한 후 β-galactosidase 염색을 통해서 바이러스의 감염여부를 확인하였으며 relaxin 발현 여부는 ELISA 법으로 확인하였다. 각각의 섬유모세포에서 제 1 형 및 3 형 collagen mRNA 의 발현 정도와 Smad-2,3 mRNA 의 발현 정도를 RT-PCR 방법으로 측정하였으며 MMP-1, 3 mRNA 의 발현 정도도 측정하였다. Ex vivo 연구로서 켈로이드 조직구를 만든 후 dl-lacZ-RGD, dl-lacZ-RLX-RGD 바이러스($1x10^9$ viral particles)을 감염시킨 후 3 일간 배양하였다. 조직구내에서 엘라스틴, 피브로넥틴, 제 3 형 콜라겐 등의 세포외기질의 발현 정도를 면역화학조직염색을 통해서 확인한 후 MetaMorph® 이미지 분석을 통해서 정량적으로 알아보았다.

정상 인체 진피 섬유모세포주(HDFCs)의 경우 TGF-ß 으로 stimulation 시키지 않았을 경우 제 1 형 콜라젠 및 MMP-1, 3 mRNA 발현의 증가를 관찰할 수 있었지만 TGF-ß 를 첨가하였을 때는 제 1 형 및 3 형 콜라겐 mRNA 의 발현이 감소되는 것과 함께 MMP-1, 3 mRNA 의 발현도 역시 감소되는 결과를 얻을 수 있었다. 하지만 켈로이드 섬유모세포에서는 TGF-ß 의 첨가 여부에 관계없이 제 1 형 및 제 3 형

콜라젠 및 MMP-1, 3 mRNA 발현의 감소를 관찰할 수 있었으며 이때 Smad-2 mRNA 발현은 변화가 없었지만 Smad-3 mRNA 발현은 의미 있게 감소되는 것을 관찰할 수 있었다. 즉, relaxin 을 발현하는 아데노바이러스는 Smad-3 mRNA 의 발현을 감소시키며 결국은 TGF-ß 의 작용을 방해하여 교원질 합성을 저하시키는 결과를 나타낸 것으로 생각되었다. Ex vivo 연구에서는 켈로이드 조직구내의 엘라스틴, 피브로넥틴, 제 3 형 콜라겐 등의 세포외기질의 발현 정도가 면역화학조직염색상에서 relaxin 을 분비하는 아데노바이러스를 감염시켰을 때 저하되는 것을 알 수 있었으며 MetaMorph® 이미지 분석에서도 의미 있게 발현이 저하된 것을 알 수 있었다.

켈로이드에서 relaxin을 분비하는 아데노바이러스를 이용한 유전자 치료는 켈로이드에서 과다하게 발현되는 제 1형 콜라겐의 발현을 감소시키고 수술적 절제 후 켈로이드의 재발 시 중요 역할을 하는 MMP-1, 3 mRNA의 발현을 억제시킴으로써 켈로이드의 치료와 예방에 좋은 효과를 보일 것으로 생각된다

핵심 되는 말 : 켈로이드, 유전자 치료, Relaxin, 콜라겐, 세포외 기질, MMP-1, 3