Effects of Alginate Gel-Encapsulated Adenoviruses Expressing Relaxin on Scar Remodeling in a Pig Model

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Directed by Professor Dong Kyun Rah

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I would also like to express my thanks to my beloved parents, wife, son, and daughter for unwavering support. I would like to dedicate my dissertation to all the people in

the above.

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ABSTRACT

Effects of Alginate Gel-Encapsulated Adenoviruses Expressing Relaxin on Scar Remodeling in a Pig Model

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To evaluate the effects of relaxin on scar remodeling, we investigated the scar remodeling process after injecting alginate gel-encapsulated adenoviruses expressing relaxin using a pig scar model.

In this study, we divided the scars into three groups and the injection was done according to the groups, respectively. Group I: alginate gel alone, group II: alginate gel + adenoviruses not containing the relaxin gene (dE1-RGD/LacZ), group III: alginate gel + adenoviruses expressing the relaxin gene (dE1-RGD/LacZ/RLX). The surface areas, color and pliability of scars were compared, and various factors influencing scar formation, collagen arrangement, and mast cell counts were quantitatively analyzed using RT-PCR and immunohistochemical staining.

The surface areas of scars decreased, color was improved, pliability increased, mast cell number decreased, and a more mature collagen

arrangement was observed in the group III. The expression levels of transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinase (TIMP)1, and α-smooth muscle actin (SMA) were reduced in the tissues of the group III, whereas the expression levels of TGF-β3 and matrix metalloproteinase (MMP)1 increased. Additionally, the expression of major extracellular matrix (ECM) components was decreased.

Alginate gel-encapsulated adenoviruses expressing relaxin improved the surface area, color, and pliability of scars. Myofibroblasts were likely inhibited by relaxin, which either reduces the expression of TGF-β1 and TIMP1, or increases the expression of TGF-β3 and MMP1; scar remodeling was also enhanced as mast cell number decreased.

Key words : scar remodeling, relaxin, adenovirus, alginate gel

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I. INTRODUCTION

Scars occurring as the result of collagen deposition during the wound healing process are able to elicit functional disability due to secondary scar contracture in addition to aesthetic and structural disorders. In cases of burn scars, scar contracture, in particular, can generate severe functional problems in the facial or joint regions.[1] Moreover, pathologic scars, such as keloids and hypertrophic scars enlarge drastically, resulting in an intractable disease, as it may relapse postoperatively. Fundamental treatments for scars have not yet been developed. In addition to wound healing, scar development remains an important research area in medicine. Although a large number of studies have investigated the mechanism of scar development to both minimize and alter scar formation, treatments for scars and collagen deposition need further investigations.[2]

Relaxin is a 6-kDa polypeptide hormone belonging to the insulin-like growth factor (IGF) family; it is primarily produced in the corpus luteum and

endometrium.[3] Recent studies revealed that relaxin exhibits biological activation in reproductive tissues and other organs.[4-6] Relaxin has anti-fibrotic effects that reduce inflammation and collagen synthesis. Relaxin is also known to reduce the activation of transforming growth factor (TGF)-β1, which plays an important role in scar formation. Through this process, the differentiation of fibroblasts into myofibroblasts is anticipated to slow. This anti-fibrotic effect of relaxin is thought to reduce scar formation. In addition, the effects of relaxin on mast cells, which play crucial roles in scar formation, are also anticipated to reduce scar formation.[7-9]

To overcome the very short biological half-life of relaxin (less than 4 min), adenoviruses expressing the relaxin gene were used as vectors. Gene therapy using adenoviruses has been primarily investigated for anti-tumor effects.[10] Instead of using the E1 gene, which is essential for adenovirus duplication, the relaxin gene was inserted into a non-duplicable adenovirus to prolong its effects by amplifying its expression both in and around adenovirus-infected cells. Since non-duplicable adenoviruses show anti-tumor effects in localized areas, they are considered to be suitable for the treatment of scars and other fibrotic diseases.[10,11]

The short half-life of relaxin was overcome, as adenovirus expressed relaxin for about 1 week. However, the maturation of the scar persisted for several months. For this reason, a longer-term drug delivery system must be developed. In this study, we used a delivery method that remarkably extended the

expression of relaxin using adenoviruses encapsulated in an alginate gel.[12,13] This study was designed to verify the effects of relaxin on scar remodeling by injecting alginate gel-encapsulated adenoviruses expressing relaxin in a pig scar model.

II. MATERIALS AND METHODS

1. Scar formation

The scar model was created using three Yorkshire pigs (4 months of age, 40 kg). Anesthesia was induced in each pig via an intramuscular injection of Zoletil (5 mg/kg, Virbac, Carros, France) and Rompun (2 mg/kg, Bayer, Seoul, Korea); all hairs were then removed from both the back and belly of each pig. Inhalation anesthesia was induced using isoflurane (IsoFlo, Abbott Laboratories, Abbott Park, IL). After anesthetizing the pigs, a total of 36 full-thickness skin defects (3×3 cm^2) were made on a back of each Yorkshire pig symmetrically, consisting of 18 wounds on each side of the midline. The depth of each wound reached the muscle fascia to mimic scar formation. The interval of each wound was maintained more than 3 cm to control the influence of the wounds on the process of wound healing. Both intravenous antibiotics and wound dressings with TegaDerm® (3M, St Paul, MN) were administered postoperatively for 5 days, and then open dressings were maintained. Fifty days later, scar tissues distinct from peripheral normal tissues formed (Figure 1).[14]

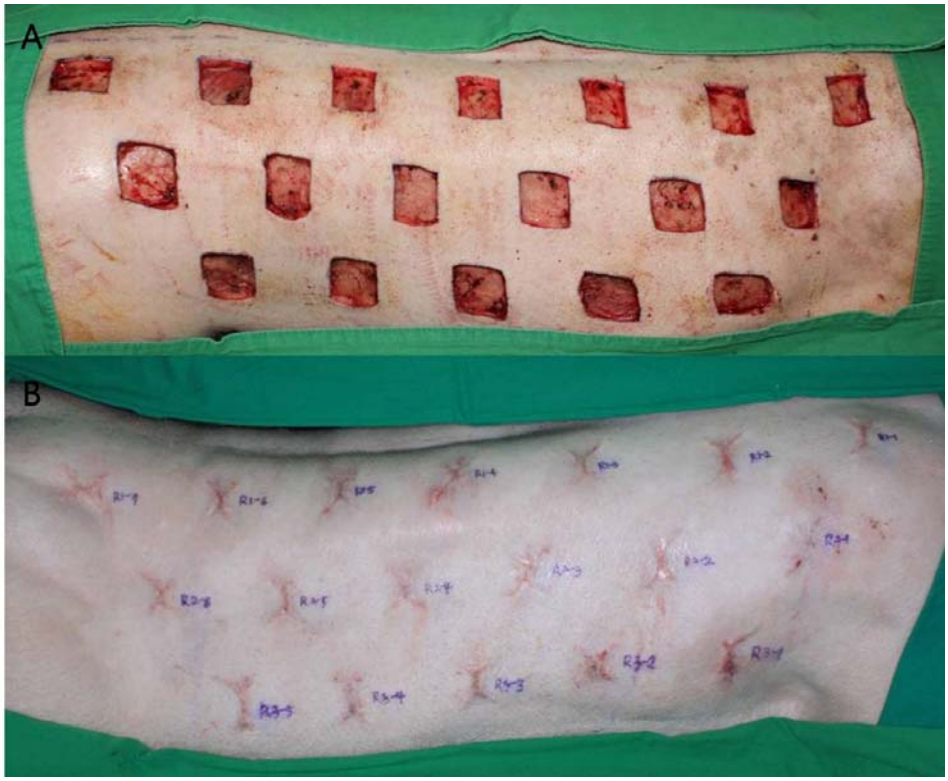

Figure 1. Pig Scar Model

(A) A total of 36 full-thickness skin defects (3×3 cm^2) were made on the backs of Yorkshire pigs symmetrically, consisting of 18 wounds on each side of the midline. (B) Fifty days later, scar tissues distinct from peripheral normal tissues formed.

2. Production of Relaxin-Expressing Adenovirus and Encapsulation

Relaxin-expressing and E1- and E3-deleted adenovirus vectors were produced according to the conventional method.[8,10,15-17] An Arg-Gly-Asp (RGD) modification was conducted to facilitate the infiltration of adenovirus into the cells. Virus was diluted with PBS to 5×10^7 PFU. Diluted adenoviruses were mixed with a 5% alginate solution (0.7 cc) in a 1-cc syringe, and gelation and encapsulation were carried out using a 50 mM $CaCl_2$ solution (0.3 cc) before injection.[12,13]

3. Injection of Adenovirus

On the 50th postoperative day, wound epithelialization and scar formation occurred. Anesthesia was induced in each pig by an intramuscular injection of Zoletil (5 mg/kg) and Rompun (2 mg/kg); all hairs were then removed from both the back and belly of each pig. Virus injection was performed using a 27-gauge needle and a 1-ml syringe, and adenovirus/alginate mixtures were directly injected into the intradermal layer of the scar regions. We divided the scars into three groups; the alginate gel not containing adenoviruses was injected into group I. 5×10^7 PFU alginate gel-encapsulated adenovirus not containing the relaxin gene (dE1-RGD/LacZ) was injected into the group II. The same amount of alginate gel-encapsulated adenoviruses expressing relaxin (dE1-RGD/LacZ/RLX) was injected into the group III (Figure 2).

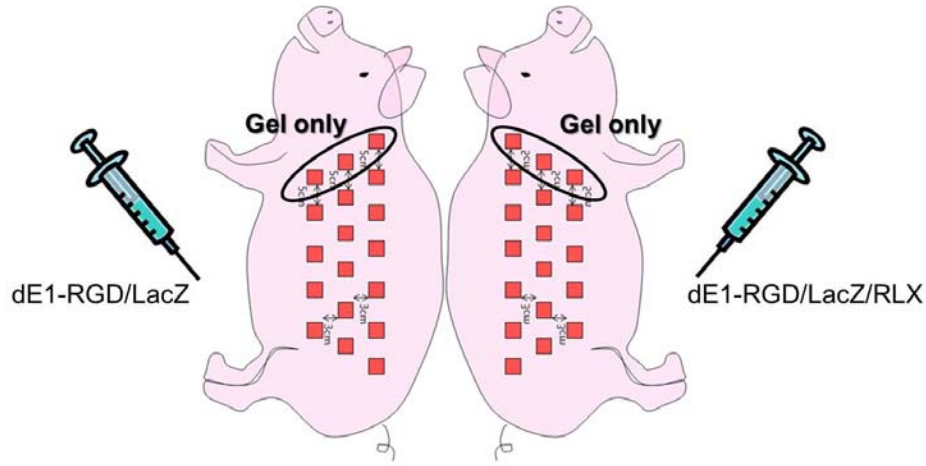

Figure 2. Injection of Adenovirus/Alginate Mixture

We divided the scars into three groups; the alginate gel not containing adenoviruses was injected into group I. 5×10^7 PFU alginate gel-encapsulated adenovirus not containing the relaxin gene (dE1-RGD/LacZ) was injected into the group II. The same amount of alginate gel-encapsulated adenoviruses expressing relaxin (dE1-RGD/LacZ/RLX) was injected into the group III.

4. Clinical Evaluation

The surface area, color, and pliability of scars were compared and evaluated in the three groups. A clinical evaluation was performed every 10 days until the 50th day after virus injection.

A. Scar Area

The surface areas of scars were measured by taking scar images using a digital camera. By using 1 cm^2 as the standard, measured with a ruler on the image, the surface areas of the scars were compared and assessed using Image J software (National Institutes of Health, USA).

B. Scar Color

The color of the scars was quantitatively analyzed with a spectrophotometer (CM-700D; Konica Minolta, Inc., Tokyo, Japan). The color of each scar was expressed using melanin (the degree of scar darkness) and erythema (the degree of scar redness) indices.[18]

C. Scar Pliability

Pliability was measured using a durometer (H1000 Mini-Dial, RexGauge Co., Buffalo Grove, IL). Each value was measured more than three times, and their average value was used for analysis.

5. Histological Analysis

For histological analysis, scars were biopsied in three different regions immediately and 10 days after virus injection in the three groups. Fifty days after injection, all of the remaining scars were biopsied. Collected specimens were fixed in 4% formaldehyde for 3 days and then embedded in a paraffin block. The block was cut into 3 μm-thick sections, which were then deparaffinized and hydrated.

A. Number of Mast Cells

For the specimens of immediately and 50 days after virus injection, toluidine blue (Sigma-Aldrich Co., St. Louis, MO) staining was performed, and the specimens were dehydrated and mounted. Using the stained tissues, the number of mast cells was compared with optical microscopy (BX51, Olympus, Tokyo, Japan). The number of mast cells was counted in five different areas, randomly chosen for each slide, and counting was done by magnifying the images under ×100 magnification.

B. Arrangement of Collagen

For the specimens of 50 days after virus injection, picrosirius red (Sigma-Aldrich Co., St. Louis, MO) staining was performed. The collagen arrangement was evaluated with optical microscopy (BX51, Olympus, Tokyo, Japan) under ×200 magnification.

C. Relaxin Immunofluorescence

Specimens collected 10 days after the administration of virus were embedded in paraffin blocks, which were then sectioned, deparaffinized, hydrated, and treated with blocking buffer (20% normal goat serum, S-100, Vector Lab Inc., Burlingame, CA) for 1 hour. After the removal of blocking buffer, the sections were immersed in primary antibody (anti-relaxin, Santa Cruz Biotechnology, Dallas, Texas) overnight at 4°C, and then incubated in secondary antibody (DyLight594, Vector Lab Inc., Burlingame, CA) at room temperature for 2 hours. The nucleus was stained with the mounting solution containing 4',6-diamidino-2-phenylindole (DAPI). The degree of relaxin expression in the tissues was evaluated using confocal microscopy (LSM700, Carl Zeiss, Oberkochen, Germany).

D. Real-time PCR (Q-PCR)

To evaluate the expression levels of TGF-β1, TGF-β3, tissue inhibitor of metalloproteinase (TIMP)1, matrix metalloproteinase (MMP)1, and α-smooth muscle actin (SMA) in the biopsied tissues, real-time PCR was performed immediately and 50 days after virus injection. First, cDNA was synthesized using AccuPower™ RT PreMix (Bioneer, Daejeon, Korea) after extracting total RNA using the TRIzol reagent (Invitrogen, Carlsbad, CA). PCR was performed in a 20-μl reaction volume containing the synthesized cDNA and 2× Taq

Premix (Solgent, Daejeon, Korea). Thermal cycling was performed initially for 5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C, and finally 5 min at 72°C. After electrophoresis using a 1% agarose gel, each band was evaluated using densitometry of Image J software (National Institutes of Health, USA). The standard value (β-actin) was converted to 1, and analysis was performed by obtaining the ratio with other measured values. PCR results of all factors were measured three times in each tissue, and their average value was used for analysis.

E. Immunohistochemical Staining

To analyze the expression levels of elastin and fibronectin, identical paraffin blocks used for histological analysis were used for immunohistochemical staining. The paraffin blocks were treated overnight at 4°C with anti-elastin (Sigma-Aldrich Co., St. Louis, MO) and fibronectin (Santa Cruz Biotechnology, Dallas, Texas) as primary antibodies, and then secondary antibodies (Dako, Glostrup, Denmark) were added. Five different areas, randomly chosen on each slide, were observed with optical microscopy (BX51, Olympus, Japan) to identify the degree of expression under ×200 magnification. Semi-quantitative analysis was performed on the images using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA).

6. Statistical Analysis

All test results were presented as the means ± SEM, and the results of the quantitative analysis were statistically analyzed in three groups using one-way ANOVA and the multiple-comparison Bonferroni correction method. A p value less than 0.05 ($p<0.05$) was considered statistically significant. Statistical analysis was performed using SAS software (version 9.1.3, SAS Institute Inc., Cary, NC).

III. RESULTS

1. Clinical Evaluation

A. Scar Area

After virus injection, decreases were shown in the surface areas of scars in all groups over time. However, a more significant decrease in scar area was found in the group III compared with other groups (Figure 3). The sizes of the initial scar were 3.15±0.15 cm^2, 3.12±0.11 cm^2, and 3.18±0.08 cm^2 in the group I, II and III, respectively. However, the sizes were 1.61±0.15 cm^2 in the group I and 1.70±0.10 cm^2 in the group II 50 days after virus injection. The size was reduced to 1.37±0.05 cm^2 in the group III, which was a significant decrease ($p<0.05$).

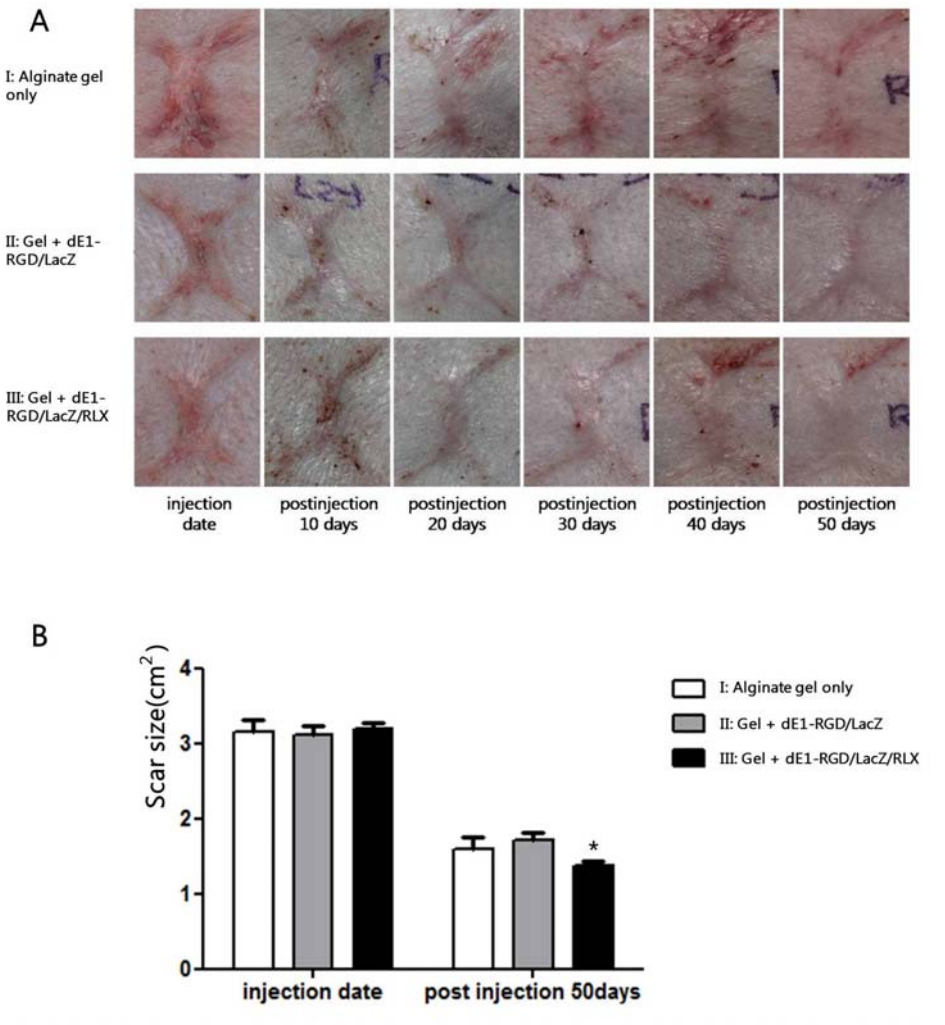

Figure 3. Scar Area

(A) The surface area of the scars decreased in all three groups and persisted for 50 days after virus injection. A greater decrease was observed in the group III.

(B) The sizes of the initial scars were 3.15±0.15 cm^2, 3.12±0.11 cm^2, and

3.18±0.08 cm^2 in the group I, II and III, respectively. However, the sizes were 1.61±0.15 cm^2 in the group I and 1.70±0.10 cm^2 in the group II, 50 days after virus injection. The size was reduced to 1.37±0.05 cm^2 in the group III (*p<0.05).

B. Scar Color

Immediately after scar formation, erythema indices representing the redness of the scar were 1.97±0.20, 2.01±0.10, and 2.01±0.09 in the group I, II and III, respectively, with no significant differences among the three groups. Fifty days after virus injection, erythema indices were 2.07±0.35, 2.05±0.08, and 1.52±0.15, respectively, in the group I, II and III.

Moreover, melanin indices indicating the degree of scar darkness of scar were 0.23±0.06, 0.23±0.02, and 0.23±0.03 in the group I, II and III, respectively, with no significant differences among the three groups. Fifty days after virus injection, melanin indices were 0.21±0.05, 0.21±0.04, and 0.08±0.02, respectively, in the group I, II and III.

No significant differences were shown between the erythema and melanin indices measured immediately after scar formation and 50 days after injection using spectrophotometry in the group I, II and III. In contrast, significant decreases were seen in the erythema and melanin indices in the group III ($p<0.05$, Figure 4).

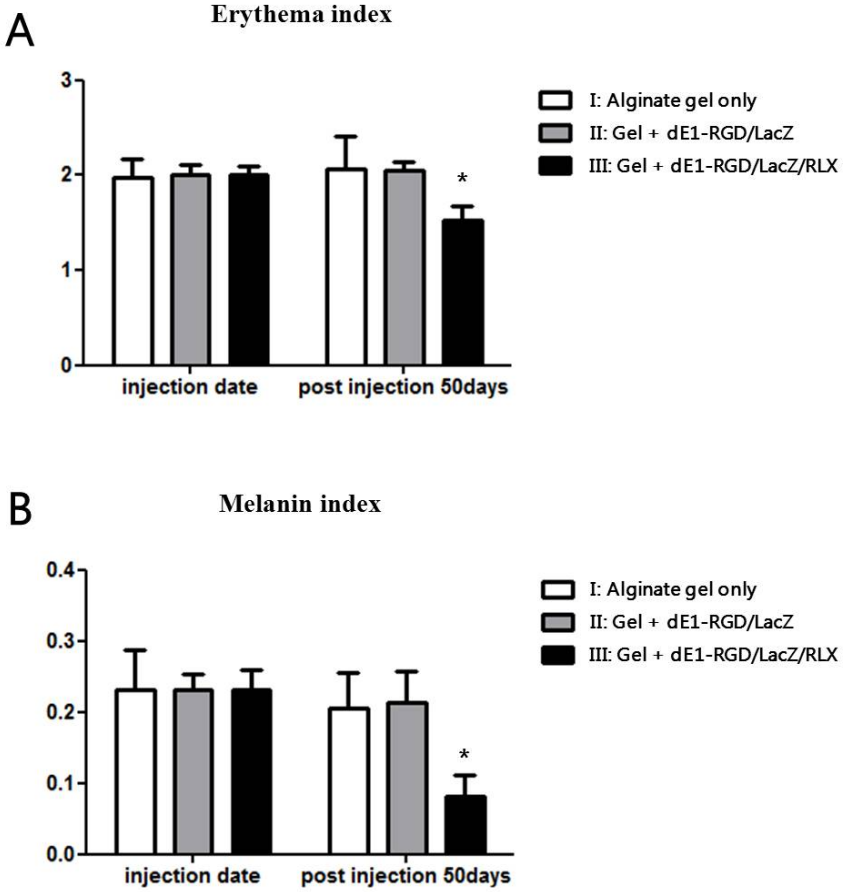

Figure 4. Scar Color

(A) Erythema indices immediately after scar formation were 1.97±0.20, 2.01±0.10, and 2.01±0.09 in the group I, II and III, respectively, with no significant differences. Fifty days after virus injection, erythema indices were 2.07±0.35, 2.05±0.08, and 1.52±0.15 in the group I, II and III, respectively. Significant decreases in erythema indices were seen in the group III (*p<0.05).

(B) Melanin indices immediately after scar formation were 0.23±0.06,

0.23±0.02, and 0.23±0.03 in the group I, II and III, respectively, with no significant differences. Fifty days after virus injection, melanin indices were 0.21±0.05, 0.21±0.04, and 0.08±0.02 in the group I, II and III, respectively. Significant decreases in melanin indices were seen in the group III (*p<0.05).

C. Scar Pliability

The pliability of the scars increased as measured durometry values decreased. Immediately after the formation of the scar, durometry values were 20.50±1.42, 19.73±0.88, and 19.08±1.06 in the group I, II and III, respectively, with no significant differences. Fifty days after injection of virus, the values decreased to 18.00±0.58, 17.46±1.62, and 11.15±1.72, respectively, in the group I, II and III. Pliability increased in all groups over time. Pliability significantly increased in the group III compared with the group I and II ($p<0.05$, Figure 5).

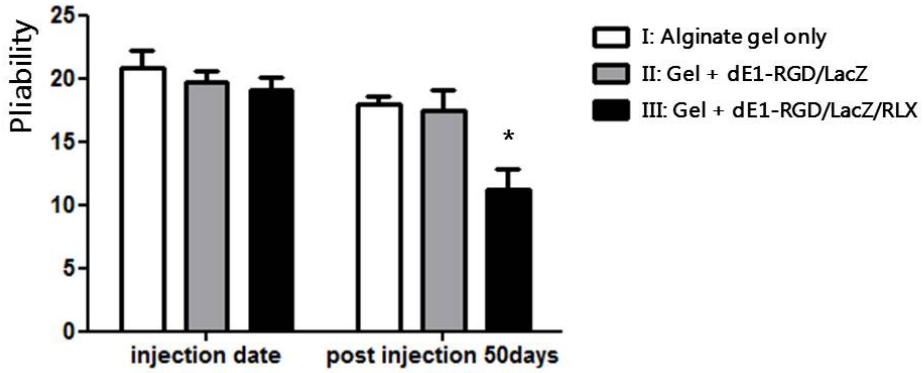

Figure 5. Scar Pliability

Immediately after scar formation, durometry values were 20.50±1.42, 19.73±0.88, and 19.08±1.06 in the group I, II and III, respectively, with no significant difference. Fifty days after the injection of virus, the values were 18.00±0.58, 17.46±1.62, and 11.15±1.72 in the group I, II and III, respectively. A statistically significant increase was observed in the pliability of the group III compare with the group I and II (*p<0.05).

2. Histological Evaluation

A. Number of Mast Cells

The number of mast cells was counted in five different areas, randomly chosen on each slide, and counting was carried out using an optical microscope (×100). Immediately after scar formation, mean numbers of mast cells were 6.20±1.30, 7.00±0.80, and 6.30±0.70 in the group I, II and III, respectively, with no significant differences. Fifty days after injection of the virus, mean numbers of observed mast cells were 6.80±0.42 in the group I and 7.09±0.68 in the group II. A significant decrease was seen in the mean number of mast cells in the group III (4.89±0.40; $p<0.005$, Figure 6).

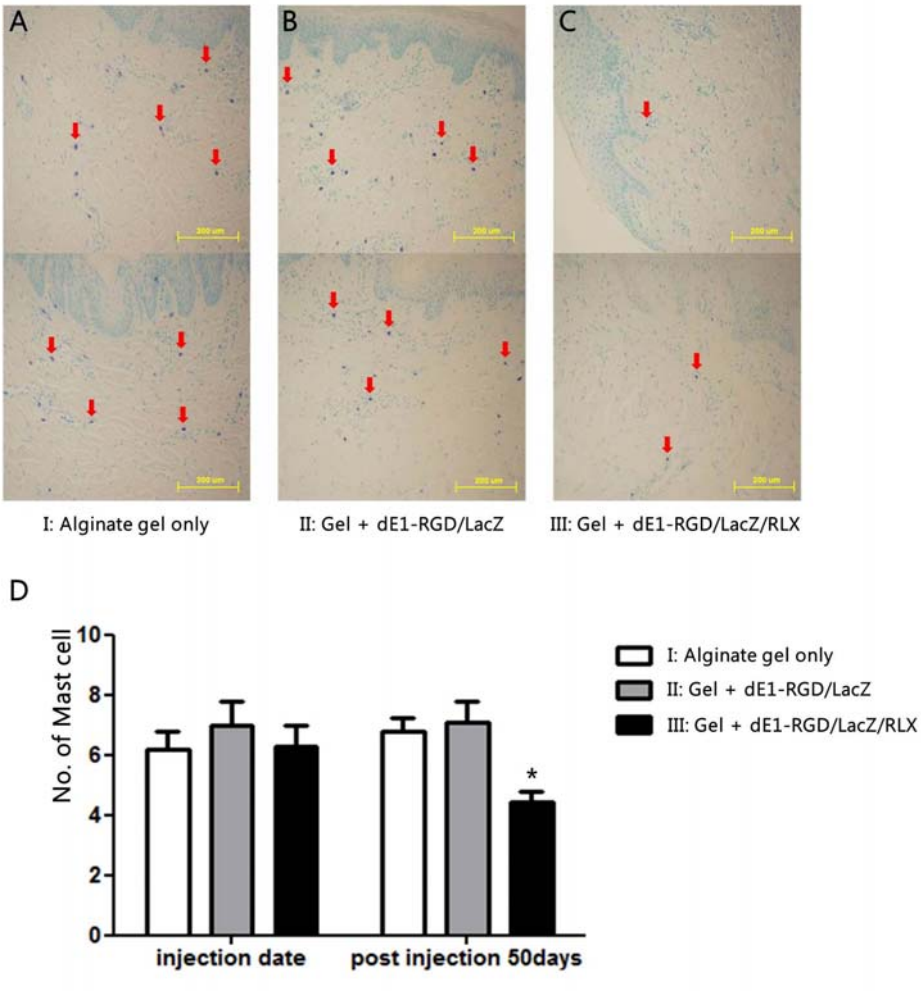

Figure 6. Mast Cell Count

Mast cells in the group I (A), group II (B), and group III (C) after toluidine blue staining (×100 magnification); values were quantified at the time of injection and 50 days after injection of virus. (D) Immediately after scar formation, the average numbers of mast cells were 6.20±1.30, 7.00±0.80, and 6.30±0.70 in the group I, II and III, respectively, with no significant differences.

Fifty days after the injection of virus, the average numbers of mast cells were 6.80±0.42 in the group I and 7.09±0.68 in the group II. A statistically significant decrease was found in the average number of mast cells in the group III (4.89±0.40; *p<0.005).

B. Arrangement of Collagen

Tissues collected 50 days after virus administration were stained with picrosirius red for the analysis of collagen arrangement. A more matured bundle-shaped collagen arrangement was identified in the group III compared with the group I and II (Figure 7).

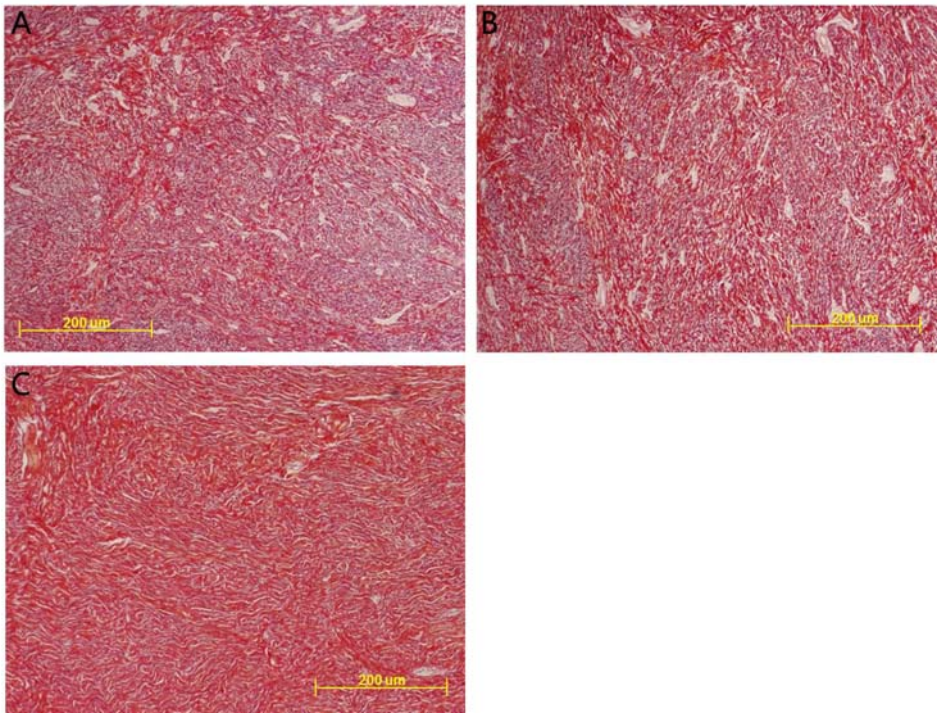

Figure 7. Collagen Arrangement

Collagen arrangement in the group I (A), group II (B), and group III (C) after picrosirius red staining (×200 magnification); more matured bundle shape of collagen arrangement was identified in the group III compare to the group I and II.

C. Relaxin Immunofluorescence

To examine the expression levels of relaxin in the tissues after virus injection, we biopsied and immunofluorescently stained the tissues 10 days after virus administration. Almost no staining of relaxin was observed in the group I and II. In contrast, relaxin was expressed in the group III (Figure 8).

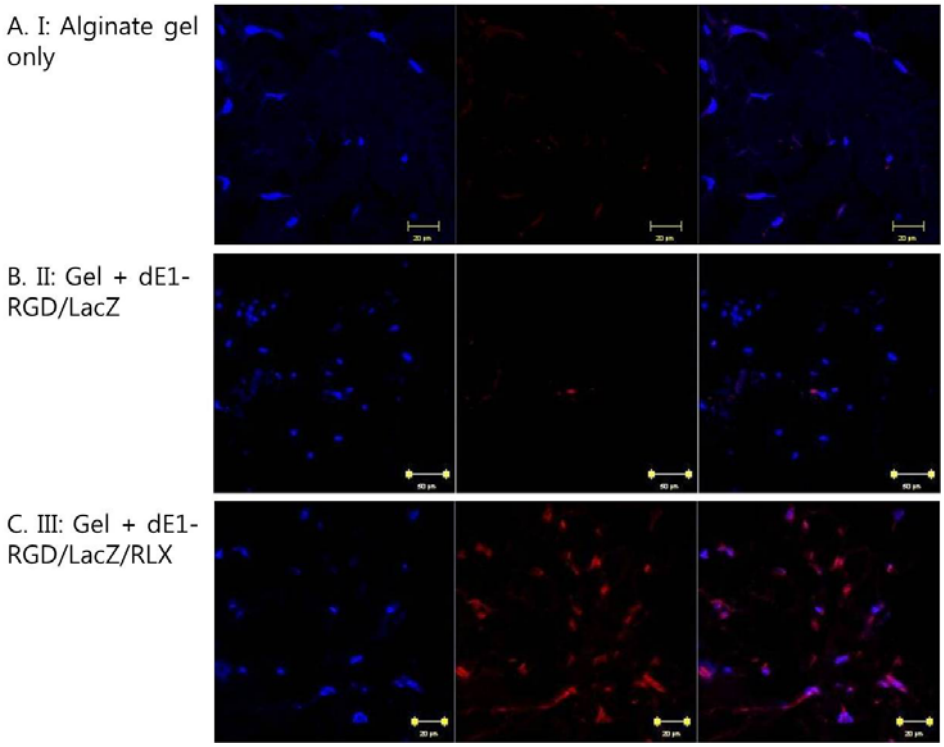

Figure 8. Relaxin Immunofluorescence

Relaxin immunofluorescence staining in the group I (A), group II (B), and group III (C), 10 days after virus injection; blue represents the nuclei stained with DAPI, red represents relaxin expression. Almost no staining of relaxin was observed in the gel and control groups. In contrast, relaxin was highly expressed in the relaxin group.

D. Real-time PCR (Q-PCR)

To evaluate the expression levels of TGF-β1, TGF-β3, TIMP1, MMP1, and α-SMA, we performed real-time PCR on the day of virus injection and 50 days after virus injection.

No significant differences were shown in TGF-β1 levels in the early stage of scar formation. Fifty days after virus injection, a significant decrease was detected in the group III (0.54±0.05) compared with the group I and II (0.83±0.10 and 0.95±0.19, respectively) ($p<0.05$, Figure 9A).

No significant differences were observed in the expression levels of TGF-β3 in the early stage of scar formation. Fifty days after virus injection, TGF-β3 levels were 0.66±0.12, 0.68±0.64, and 0.86±0.04 in the group I, II and III, respectively, with a significant increase in the group III ($p<0.05$, Figure 9B).

There were no significant differences in MMP1 levels among the three groups in the early stage of scar formation. The expression levels of MMP1 were 0.74±0.11, 0.72±0.04, and 0.78±0.02 in the group I, II and III, respectively. Fifty days after virus injection, MMP1 levels were 0.74±0.11, 0.72±0.04, and 0.78±0.02 in the group I, II and III, respectively. Although an increase was detected in the group III, no statistical differences were found ($p>0.05$, Figure 9C).

No significant differences were shown in TIMP1 levels among the three groups in the early stage of scar formation; however, 50 days after injection, TIMP1 levels were 0.91±0.14, 0.86±0.06, and 0.69±0.03 in the group I, II and

III, respectively. A statistically significant decrease was found in the group III (p<0.05, Figure 9D).

Regarding α-SMA levels, which indirectly represent the amount within myofibroblasts, there were no significant differences among the three groups in the early stage of scar formation. Moreover, α-SMA levels were 1.65±0.53, 1.97±0.60, and 0.62±0.12 in the group I, II and III, 50 days after virus injection. A significant decrease was observed in the group III (p<0.05, Figure 9E).

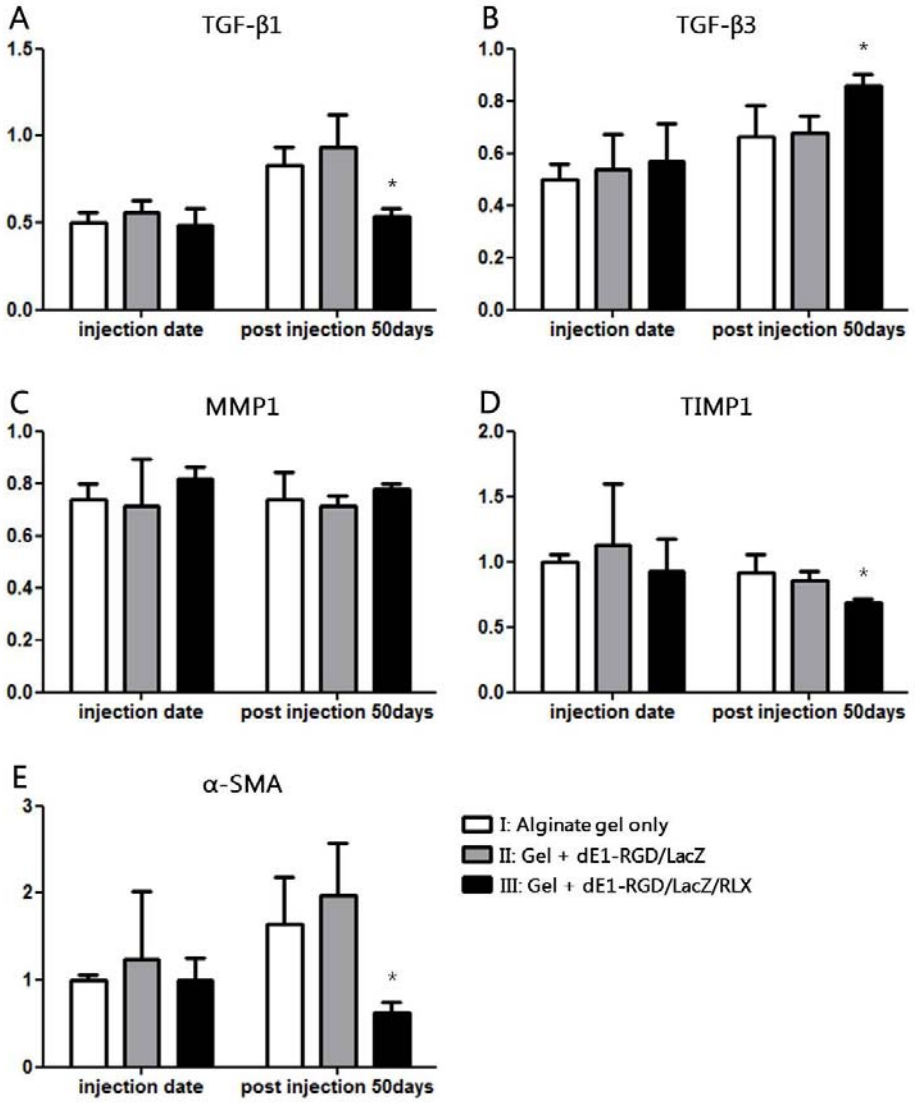

Figure 9. Comparison of mRNA Expression

(A) TGF-β1, (B) TGF-β3, (C) MMP1, (D) TIMP1, (E) α-SMA.

In the group III, the expression of TGF-β1, TIMP1, and α-SMA was significantly decreased 50 days after virus injection as determined by real-time

Q-PCR analysis. The expression of TGF-β3 was significantly increased in the group III (*p<0.05).

E. Immunohistochemical Staining

We performed immunohistochemical staining to evaluate the amount of elastin and fibronectin in the scar tissue of each group.

After immunohistochemical staining of the tissues, elastin values were 22,331±1673, 24,810±1329, and 14,257±1034 in the group I, II and III, respectively, according to the semi-quantitative analysis performed using MetaMorph image analysis software. A significant decrease was observed in the group III compared with the other groups ($p<0.0001$, Figure 10).

Fibronectin values were 959.2±97.5, 1732.3±141.9, and 941.2±125.0 in the group I, II and III, respectively. Although a significant decrease was shown in the group III compared to the group II ($p<0.0001$), no statistically significant decrease was seen between the group I and group III ($p>0.05$, Figure 11).

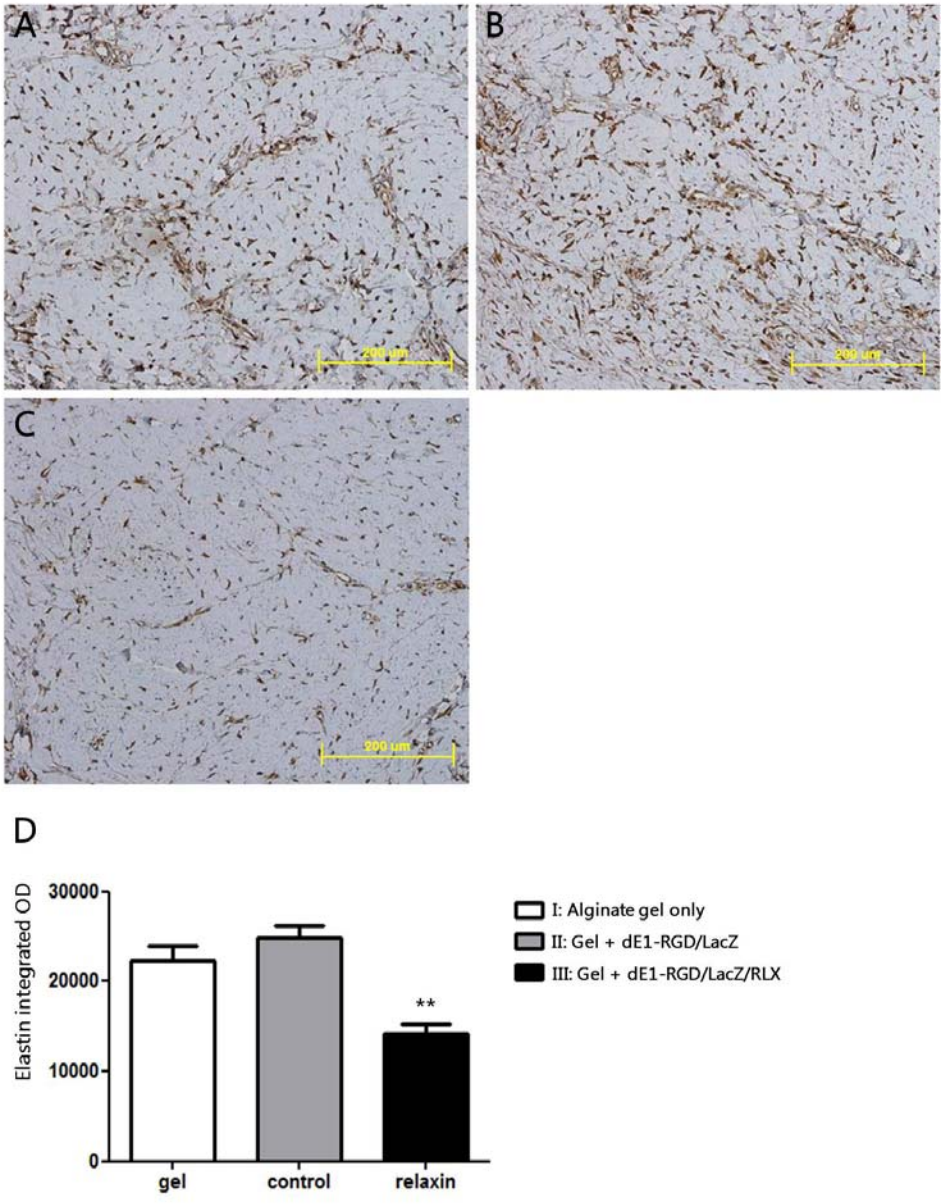

Figure 10. Elastin Immunohistochemical Staining

Elastin expression in the group I (A), group II (B), and group III (C); elastin expression was decreased in the group III compared with the other groups. (D)

Elastin values were 22,331±1673, 24,810±1329, and 14,257±1034 in the group I, II and III, respectively, according to the semi-quantitative analysis performed with MetaMorph image analysis software. A significant decrease was observed in the group III compared with the other groups (**p<0.0001).

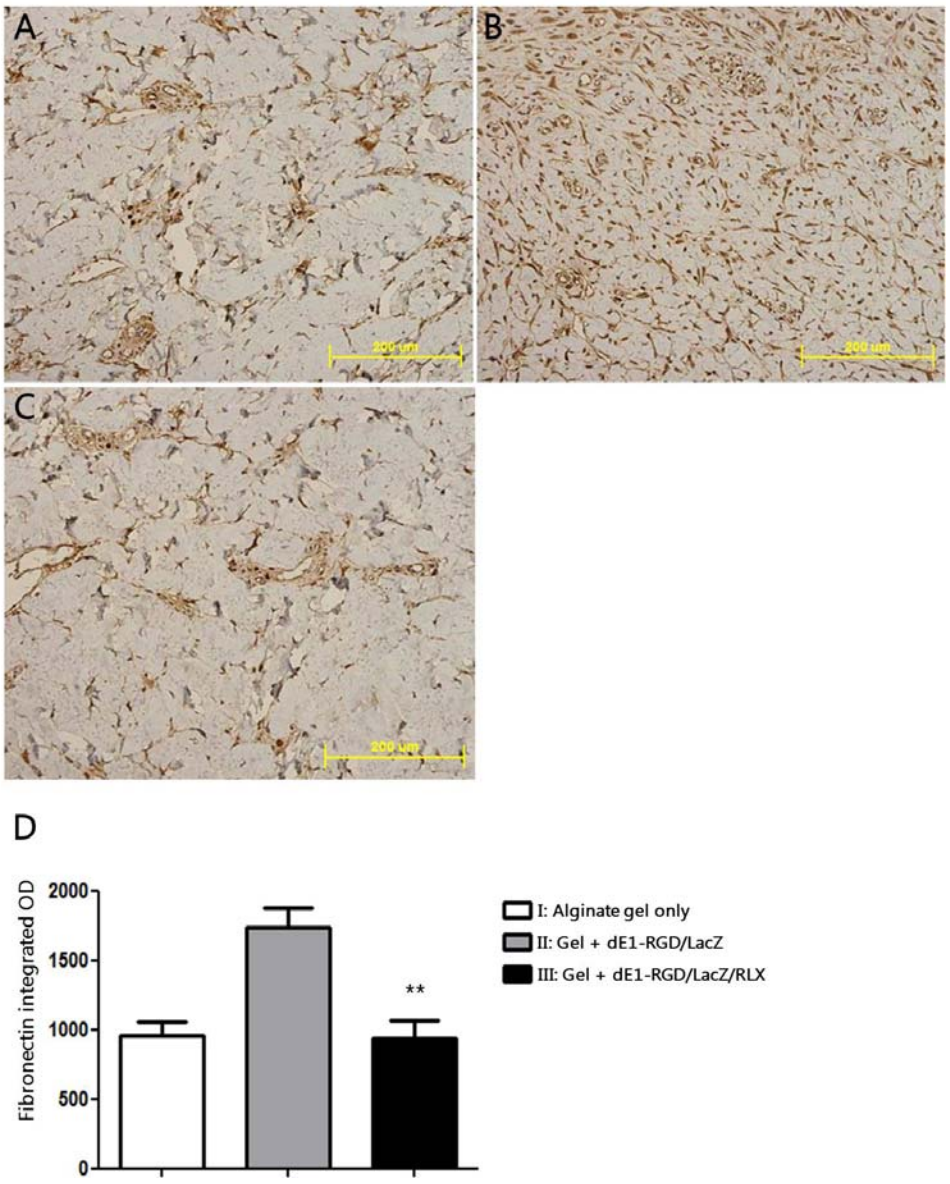

Figure 11. Fibronectin Immunohistochemical Staining

Fibronectin expression in the group I (A), group II (B), and group III (C); fibronectin expression was decreased in the group III compared with the group II. (D) Fibronectin values were 959.2±97.5, 1732.3±141.9, and 941.2±125.0 in

the group I, II and III, respectively. Although a significant decrease was shown in the group III compared to the group II (**p<0.0001), no statistically significant differences were found between the group I and group III (p>0.05).

IV. DISCUSSION

Scars which are caused by collagen deposition incur a large number of functional problems. With improvements in economic status, many patients want to minimize scar formation and enhance wound healing. For this reason, a large number of treatments, including scar revision, laser therapy and medications, have been applied to prevent scar formation. However, the successful treatment of scars has not been fully resolved.[19-21]

Several factors are involved in the formation of a scar. Continued inflammation in a wound increases the expression of TGF-β1, which leads to the accelerated differentiation of fibroblasts into myofibroblasts. The major cause of fibrotic scar contraction is considered the elevated α-SMA levels in myofibroblasts. Furthermore, TGF-β1 is believed to be involved in abnormal fibrosis by synthesizing extracellular matrix and infiltrating the interstitial tissues.[21-26]

Mast cells, also involved in the process of wound healing, are reported to be involved in the proliferation and contraction of fibroblasts, and the synthesis of extracellular matrix. They are also thought to play a crucial role in scar formation. The amount of mast cells was four times higher in hypertrophic scars than in normal skin tissues.[27-29]

Animal models are essential for research of scar prevention. However, scars are rarely formed in animals, unlike in humans. We have created an animal scar model by making full-thickness skin defects on the backs of pigs that are similar

to human scars.[14,30-32] In this study, we used human relaxin-expressing adenoviruses in the pig scar model. Both human and porcine relaxin showed similar biological activity in a previous study.[33] Additionally, human relaxin was also effective in a porcine scar model.[34]

We carried out a relaxin gene therapy study on keloids and local ischemic flaps using adenoviruses expressing relaxin.[15-17] Relaxin gene therapy on keloids was the previous stage of this study. Relaxin is anticipated to have positive effects on the treatment and prevention of keloids by reducing the expression of collagen I and III, both excessively expressed in keloids, and by decreasing the levels of elastin and fibronectin, which are both increased in keloids.

This study was performed using an *in vivo* experimental model by adopting a pig scar model used previously in *in vitro* studies. The aim of this study was to verify the anti-fibrotic effect of relaxin on scars. To achieve this goal, a modification of the conventional relaxin delivery method with adenovirus vectors was necessary to extend the effects of relaxin. The survival of the viral gene carrier was confirmed up to 24 days after injecting alginate gel-encapsulated adenoviruses expressing relaxin.[12,13]

When alginate gel-encapsulated adenoviruses expressing relaxin were injected into pig scars, reduction in the surface area of the scar was accelerated clinically and skin color was normalized. Moreover, the pliability of the scars was improved.

Furthermore, we found that the number of mast cells decreased histologically in the group III. Fifty days after injection of the virus, the average number of mast cells was 6.80±0.42 and 7.09±0.68 per high power field (×100) in the group I and II, respectively. A significant decrease in the number of mast cells was found in the group III (4.89±0.40 at ×100 magnification). In normal porcine dermis, the average number of mast cells was 5.3 per high power field (×100).[14] Thus, we estimated that the number of mast cells in the group III was in the normal range.

A more mature collagen arrangement was observed in the group III through strengthened collagen remodeling compared to ordinary scars. Relaxin was found to prevent the fibrosis of scar tissues by inhibiting the expression of major ECM components, such as elastin and fibronectin. Relaxin reduced the expression levels of TGF-β1, TIMP1, and α-SMA, which are profoundly involved in scar formation, and elevated the expression levels of TGF-β3 and MMP1, which block scar formation. In this study, the effects of relaxin on scars aligned with the *in vitro* results of previous studies performed on keloid tissues.[15]

These clinical and histological improvements of scars were believed to be caused by inhibiting TGF-β1 expression and decreasing the number of mast cells, which play crucial roles in scar formation. The inhibition of TGF-β1 by relaxin in scar tissues was reported previously.[8,15,16,22] Relaxin was shown to decrease mast cell numbers in a swine model of acute myocardial infarction.[35,36]

The inhibition of TGF-β1 and the decrease in mast cell number by relaxin may prevent the conversion of fibroblasts into myofibroblasts during the scar remodeling process.

In addition to scars on the skin, the anti-fibrotic effects of relaxin could be applied to chronic pulmonary fibrosis and liver cirrhosis, which abnormally incur fibrosis and scars in major organs.[37-39] In this study, we used adenovirus vectors encapsulated in an alginate gel to prolong the effects of relaxin over time. This delivery method is anticipated to be useful for various research fields and applications of gene therapy.

Despite this anticipation, the potential therapeutic use of relaxin-expressing adenovirus possesses limitations for safe and effective gene therapy because of acute inflammatory responses and innate immune response. Exhaustive research efforts has prompted the development of novel strategies to overcome these limitations.[40] Therefore, we think that further refinement studies will be needed for more efficient and safe gene transfer in patients.

V. CONCLUSION

When alginate gel-encapsulated adenoviruses expressing relaxin were injected into pig scars, the expression levels of TGF-β1, TIMP1, and α-SMA, which play critical roles in scar formation, were reduced. Additionally, the expression levels of TGF-β3 and MMP1, which inhibit scar formation, were increased. Moreover, the number of mast cells decreased in scar tissues, and a more mature collagen arrangement was observed compared to ordinary scars with facilitated scar remodeling. Furthermore, the expression of major ECM components, such as elastin and fibronectin, was decreased. These outcomes suggest that alginate gel-encapsulated adenoviruses expressing relaxin clinically enhance the reduction of scar surface area and improve the color and flexibility of scars.

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ABSTRACT(IN KOREAN)

Alginate gel에 포획된 Relaxin을 분비하는 아데노바이러스가 돼지의 반흔 성숙 과정에 미치는 영향

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Relaxin이 가지고 있는 항섬유화 효과가 피부에 생기는 반흔을 감소시킬 수 있는지 확인하고자, alginate gel에 포획된 relaxin을 분비하는 아데노바이러스를 돼지 반흔 모델에 주입하여 반흔 성숙과정에 미치는 영향을 연구하였다.

세마리의 돼지 등에 전층피부결손을 만들어 반흔 모델을 제작하였으며, 이를 세 군 (I, II, III)으로 나누었다. 수술 후 50일 창상이 상피화 되고 반흔이 형성된 후, 제 I군에는 alginate gel만을 주입하였고, 제 II군에는 relaxin을 분비하지 않는 아데노바이러스를 alginate gel에 포획하여 주입하였으며, 제 III군에는 relaxin을 분비하는 아데노바이러스를 alginate gel에 포획하여 주입하였다. 이후 반흔의 면적, 색상, 유연성 등의

변화를 50일간 비교하였으며, 조직검사를 통해 교원질의 배열상태, 비만세포(mast cell)의 개수를 측정하고, 반흔 형성에 영향을 미치는 여러 인자들을 정량분석하였다.

바이러스 주입 50일 후, 제 III군에서 반흔의 면적이 감소하고, 색이 호전 되었으며, 유연성이 증가하였다. 또한 교원질의 배열이 더 성숙한 형태로 관찰되었으며, 비만세포의 개수가 감소하였다. 그리고 조직 내 TGF-β1, TIMP1, α-SMA의 발현이 감소되었고, TGF-β3, MMP1의 발현이 증가되었다. 또한 elastin이나 fibronectin 등 주요한 세포외 기질 구성성분의 발현이 억제되었다.

Alginate gel에 포획된 relaxin을 분비하는 아데노바이러스는 반흔의 면적, 색, 유연성 등을 향상시키는 효과가 있었다. 이 같은 결과는 relaxin이 조직 내 TGF-β1, TIMP1를 감소시키고 TGF-β3, MMP1 등의 발현을 증가시켜 근섬유모세포(myofibroblast)의 발현이 억제되고, 비만세포가 감소되어 이를 통해 반흔의 성숙을 촉진하는 것으로 생각된다.

핵심되는 말 : scar remodeling, relaxin, adenovirus, alginate gel