Upregulation of IL-1β/TGF-β1 and hypoxia relate to molecular mechanisms underlying immobilization-induced muscle contracture

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Acknowledgments: This research was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (grant no. 21300200).

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Running title : Upregulation of IL-1β/TGF-β1 and hypoxia induced by immobilization in muscle contracture

Upregulation of IL-1β/TGF-β1 and hypoxia relate to molecular mechanisms underlying immobilization-induced muscle contracture

ABSTRACT: *Introduction:* We investigated the molecular mechanism underlying muscle contracture in rats. *Methods:* The rats were divided into immobilization and control groups, and soleus muscles of the right and left sides were selected for analyses. *Results:* The levels of CD-11b and α-SMA protein, IL-1β and TGF-β1 mRNA, and type I and III collagen protein and mRNA were significantly greater in the immobilization group than in the control group at all time points. HIF-1α mRNA levels were significantly higher in the immobilization group at 4 weeks. Moreover, HIF-1α, α-SMA, and type I collagen levels were significantly higher at 4 weeks than at 1 and 2 weeks in the immobilization group. *Conclusions:* In the early stages of immobilization, upregulation of IL-1β/TGF-β1 via macrophages might promote fibroblast differentiation that might affect muscle contracture. The soleus muscle became hypoxic in the later stages of immobilization, suggesting that hypoxia influences the progression of muscle contracture.

Key words: muscle contracture, fibrosis, IL-1β, TGF-β1, hypoxia

INTRODUCTION

Muscle contracture, caused by immobilization of joints with plaster casts or orthosis,[1,2] limits daily activities and interferes with rehabilitation. Reduced muscle extensibility decreases joint mobility, thus contributing to muscle contracture. Studies have shown that overexpression of collagen in the perimysium and endomysium is strongly associated with reduced muscle extensibility.[1,3-5] Although these lesions are known to progress via muscle fibrosis, the mechanism underlying immobilization-induced muscle fibrosis remains unclear.

Previous studies of the skin, lung, and liver have suggested that several factors are involved in fibrosis,[6-8] among which two are particularly important. The first is interleukin-1β (IL-1β)/transforming growth factor-β1 (TGF-β1) signaling via macrophages, which is closely associated with collagen overexpression during fibrosis. In particular, an increase in macrophages at the site of fibrosis contributes to IL-1β production,[9] which is implicated in fibrogenesis in various tissues.[10] IL-1β promotes fibroblast proliferation and collagen production via a TGF-β-dependent mechanism. Furthermore, IL-1β stimulation has been reported to increase TGF-β1 expression.[11] TGF-β1 signals fibroblasts to increase collagen synthesis; it is a potent inducer of fibroblast differentiation into myofibroblasts in several fibrotic diseases and is important in almost every step of tissue fibrosis.[12,13] Both *in vitro* and *in vivo* studies have demonstrated that TGF-β1 is overexpressed in fibrotic lesions, and that blocking TGF-β1 bioactivity suppresses collagen production and modulates the fibrotic process.[14,15]

The second important factor involved in fibrosis is hypoxia. Hypoxia has been related to fibrosis in various organs, where an increase in hypoxia-inducible transcription factor-1α (HIF-1α) has been observed in fibrotic tissue.[16] Moreover, hypoxia promotes fibrosis activation, and hypoxic exposure has been associated with the development of myofibroblasts.[17,18] In brief, the differentiation of fibroblasts to myofibroblasts induced by hypoxia might result in the overexpression of collagen, and these changes might contribute to immobilization-induced muscle fibrosis.

From previous reports, we hypothesized that the molecular mechanism of immobilization-induced muscle fibrosis involves changes in myofibroblast differentiation induced by IL-1β/TGF-β1

signaling via macrophages and/or by hypoxia. To test this hypothesis, we examined changes in the levels of CD-11b, a macrophage marker; IL-1β; TGF-β1; HIF-1α; α-smooth muscle actin (α-SMA), a myofibroblast marker; and type I and III collagen, the major collagen isoforms found in the perimysium and endomysium of the rat soleus muscle, after extended immobilization.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar rats were purchased from Kyudo Laboratories (Saga, Japan) and were bred in the Center for Frontier Life Sciences at Nagasaki University. The rats, which were maintained in 30 × 40 × 20-cm cages, were exposed to a 12-h light-dark cycle at an ambient temperature of 24°C. Food and water were available *ad libitum*. In this investigation, 50 rats (269.9 ± 9.2 g) were divided randomly into an immobilization group (n = 25) and a control group (n = 25). Animals in the immobilization group were anesthetized with pentobarbital sodium (40 mg/kg), and their right and left ankle joints were fixed in full plantar flexion with plaster casts to immobilize the soleus muscle in a shortened position. The plaster cast, which was fit from above the knee joint to the distal foot, was changed weekly because of loosening consequent to muscle atrophy. The ankle joint was immobilized for 1, 2, 4, 8, and 12 weeks. Five rats were used for each immobilization period, and five untreated control rats were tested weekly. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University.

Measurement of range of motion (ROM) of ankle joint dorsiflexion

At 1, 2, 4, 8, and 12 weeks after immobilization, the rats were anesthetized with pentobarbital sodium (40 mg/kg), and the ROM of ankle joint dorsiflexion was determined with a goniometer.[6,19] ROM was measured as the angle (0–180°) between the line connecting the fifth metatarsal to the malleolus lateralis of the fibula and the line connecting the malleolus lateralis of the fibula to the center of the knee joint, when the ankle was passively dorsiflexed with a tension of 0.3 N using a tension gauge (Shiro Industry Co., Osaka, Japan). In a pilot study, we confirmed that the minimum

tension required to achieve maximum dorsiflexion in the foot joint of control rats was 0.3 N, and the hip and knee joints of the rats were flexed maximally during ROM measurements.

Tissue sampling and preparation

Following ROM measurements for each immobilization period, the right and left soleus muscles in each rat were excised. The right soleus muscle was embedded in tragacanth, after which the muscle sample was frozen in isopentane cooled to its freezing point with liquid nitrogen and stored in a freezer at −80°C. Serial frozen cross sections of the muscles, prepared using a cryostat, were mounted on glass slides for histological, immunohistochemical, and immunofluorescence analyses. The left soleus muscle was treated with RNAlater® (Ambion, Foster City, CA, USA) immediately after excision and was stored in a deep freezer (−80°C) for use in reverse transcription-polymerase chain reaction (RT-PCR) assays.

Histological analysis

Cross sections (7 μm) were stained with hematoxylin and eosin to identify myofiber morphological characteristics and signs of previous muscle injury, such as centralized nuclei. Dyed cross sections of muscle were evaluated with an optical microscope. The cross-sectional area of the myofibers was determined using Scion image software (W. Rasband, National Institutes of Health, Bethesda, MD, USA). More than 100 myofiber measurements were recorded per animal. The analysis was conducted using the double-blind method.

Immunohistochemical analysis

Cross sections (7 μm) were air-dried and were fixed in ice-cold acetone for 10 min. To inhibit endogenous peroxidase, the sections were then incubated with 0.3% H_2O_2 in methanol for 40 min at room temperature (RT). After washes in 0.01 M phosphate-buffered saline (PBS; pH 7.4), the sections were incubated for 10 min at RT with 0.1% Triton X-100 in PBS. The sections were blocked with 5% bovine albumin in PBS for 20 min and incubated overnight at 4°C with a mouse

monoclonal anti-CD-11b primary antibody (1:2000; BMA Biomedicals, Augst, Switzerland) or a mouse monoclonal anti-α-SMA primary antibody (1:2000; Exalpha Biologicals Inc., Shirley, MA, USA). The sections were rinsed in PBS for 15 min, after which biotinylated goat anti-mouse IgG (1:1000; Vector Laboratories, Burlingame, CA, USA) was applied for 60 min at RT. The sections were then rinsed in PBS and reacted with avidin-biotin peroxidase complexes (VECTASTAIN Elite ABC kit; Vector Laboratories) for 60 min at RT. Horseradish peroxidase binding sites were visualized with 0.05% 3,3′-diaminobenzidine (the reaction product was dark brown) and 0.01% H_2O_2 in 0.05 M Tris buffer at RT. After a final washing step, the CD-11b sections were covered according to the conventional method, while α-SMA sections were covered after staining with 1% methyl green. The sections were observed under an optical microscope.

Using microscopy and standardized light conditions, the sections were magnified to 400×, and images were captured with a digital camera (Nikon, Tokyo, Japan). The number of macrophages and myofibroblasts was determined from the images by counting the number of CD-11b- and α-SMA-positive cells per 100 myofibers. Vascular areas were omitted from the analysis, which was conducted using the double-blind method.

Immunofluorescence analysis

Cross sections (10 μm) were air-dried, fixed in ice-cold acetone for 10 min, and blocked with 5% bovine albumin in 0.01 M PBS for 2 h. The sections were then incubated overnight at 4°C with primary antibodies, either mouse monoclonal anti-type I collagen (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-type III collagen (1:1000; LSL, Shiga, Japan). The sections were rinsed in PBS for 15 min and incubated for 60 min at RT with secondary antibodies, either fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:50; Millipore, Billerica, MA, USA) or Texas red-conjugated anti-rabbit IgG (1:1500; Vector Laboratories).

The stained sections were used for semi-quantitative analysis of type I and III collagen in the perimysium and endomysium. Magnified images (200×) of each section were imported into the computer, and the fluorescence intensity of FITC and Texas red in the perimysium and endomysium

was measured with imaging software (NIS-Elements; Nikon, Tokyo, Japan). The images were trimmed to 150 × 150 pixels, with 50 sites per muscle sample. The brightness of the pixels in the images was measured within the range of 0 (minimum) to 255 (maximum), total brightness in the images was calculated, and the data were divided by the fluorescent area. The analysis was conducted using the double-blind method.

RT-PCR

The left soleus muscle was used for this analysis. Total RNA was extracted from muscle samples using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Total RNA was used as template with a QuantiTect® Reverse Transcription Kit (Qiagen) to prepare cDNA, and PCR was performed using TaKaRa Taq™ Hot Start Version (TaKaRa, Shiga, Japan). The thermal cycling conditions were 94°C for 5 min; 28–37 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 2 min to ensure complete product extension. The synthetic gene-specific primers for IL-1β, TGF-β1, HIF-1α, types I and III collagen, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used in RT-PCR are listed in Table 1. The optimal cycle numbers were determined by examining multiple criteria, and each target gene was amplified accurately without saturation. Samples of each reaction product were separated by 2.0% agarose gel electrophoresis, visualized with ethidium bromide staining, and photographed under ultraviolet illumination. The density of each band was measured using densitometry. The relative expression of IL-1β, TGF-β1, HIF-1α, and type I and III collagen mRNA was normalized to the expression of the internal control (GAPDH).

Statistical analysis

All data are presented as mean ± standard deviation (SD). The difference between groups for each immobilization period was assessed using the non-paired *t*-test, whereas differences within the same group were assessed using one-way analysis of variance (ANOVA) followed by Scheffé's

method. Differences were considered significant at $p < 0.05$.

RESULTS

ROM for ankle joint dorsiflexion

ROM for dorsiflexion in the control groups was 160° at all time points. In the immobilization group, ROM was 124.0° ± 3.2° at 1 week, 108.0° ± 6.3° at 2 weeks, 73.0° ± 4.8° at 4 weeks, 59.0° ± 3.9° at 8 weeks, and 47.5° ± 2.7° at 12 weeks after immobilization. ROM on dorsiflexion in the immobilization group was not only significantly lesser than that in the control for each immobilization period, but also decreased significantly with an increase in the duration of immobilization.

Histological changes

The cross-sectional area of myofibers ranged from 2521.0 ± 631.2 μm^2 to 4256.7 ± 1046.2 μm^2 in the control group. The cross-sectional area of myofibers in the immobilization group was 1979.3 ± 527.3 μm^2 at 1 week, 1758.6 ± 601.3 μm^2 at 2 weeks, 1547.1 ± 615.4 μm^2 at 4 weeks, 2135.1 ± 550.5 μm^2 at 8 weeks, and 2049.0 ± 512.6 μm^2 at 12 weeks (Fig. 1). The cross-sectional area of myofibers in the immobilization group was significantly lesser than that in the control group at each time point, and the atrophic changes progressed through 4 weeks of immobilization. Although the atrophic changes diminished at 8 and 12 weeks, the cross-sectional area of the myofibers did not return to control levels. However, with the exception of atrophic changes, abnormal findings were not apparent in the immobilization group.

Measurement of macrophages and myofibroblasts

1) Macrophages

The number of CD-11b-positive cells per 100 myofibers was 9.0 ± 5.8 to 13.4 ± 10.0 in the control group. In the immobilization group, the number was 33.6 ± 20.0 at 1 week, 33.3 ± 17.9 at 2 weeks, 28.5 ± 16.2 at 4 weeks, 37.4 ± 17.1 at 8 weeks, and 33.7 ± 17.5 at 12 weeks after

immobilization (Fig. 2). The number of CD-11b-positive cells was significantly higher in the immobilization group than that in the control group for each time point. Within the immobilization group, there was no significant difference in the number CD-11b-positive cells at the different time points.

2) Myofibroblasts

The number of α-SMA-positive cells per 100 myofibers was 4.8 ± 5.5 to 7.1 ± 8.4 in the control group. In the immobilization group, the number was 10.4 ± 12.2 at 1 week, 11.6 ± 12.3 at 2 weeks, 20.4 ± 14.1 at 4 weeks, 19.5 ± 11.7 at 8 weeks, and 19.8 ± 14.1 at 12 weeks after immobilization (Fig. 2). The number of α-SMA-positive cells in the immobilization group was significantly greater than that in the control group for each immobilization period. Furthermore, in the immobilization group, the number of α-SMA-positive cells at 4, 8, and 12 weeks was significantly higher than that at 1 and 2 weeks.

Expression of IL-1β, TGF-β1, and HIF-1α mRNA

1) IL-1β

At 1, 2, 4, 8, and 12 weeks, IL-1β mRNA expression in the immobilization group was 1.4 ± 0.3-fold, 1.5 ± 0.3-fold, 1.5 ± 0.2-fold, 1.5 ± 0.2-fold, and 1.4 ± 0.2-fold higher, respectively, than the expressions in the control group (Fig. 3A). At all the time points, IL-1β mRNA expression was significantly higher in the immobilization group than that in the control group. However, within the immobilization group, there was no significantly difference in IL-1β mRNA expression at the different immobilization time points.

2) TGF-β1

At 1, 2, 4, 8, and 12 weeks, the expression of TGF-β1 mRNA in the immobilization group was 1.6 ± 0.4-fold, 1.5 ± 0.3-fold, 1.5 ± 0.3-fold, 1.8 ± 0.3-fold, and 1.7 ± 0.4-fold higher, respectively, than the expressions in the control group (Fig. 3B). TGF-β1 mRNA expression in the immobilization

group was significantly higher than that in the control for each time point. However, within the immobilization group, there was no significant difference in TGF-β1 mRNA expression at the different immobilization time points.

3) HIF-1α

HIF-1α mRNA expression in the immobilization group was 1.1 ± 0.1-fold, 1.1 ± 0.3-fold, 1.6 ± 0.3-fold, 1.7 ± 0.2-fold, and 1.7 ± 0.5-fold higher than the expressions in the control group at 1, 2, 4, 8, and 12 weeks, respectively (Fig. 3C). HIF-1α mRNA expression was significantly higher in the immobilization group than that in the control group at 4, 8, and 12 weeks after immobilization. Additionally, within the immobilization group, there was significant difference in HIF-1α mRNA expression at 4, 8, and 12 weeks relative to that at 1 and 2 weeks.

Quantification of type I and III collagen

1) Type I collagen

Figure 4 shows immunofluorescence images of type I collagen. Staining in the perimysium and endomysium was brighter in the immobilization group than that in the control group. In the immobilization group, type I collagen luminescence in the endomysium increased gradually until 4 weeks after immobilization. Figure 5 shows quantitative and semi-quantitative measurements of type I collagen expression for all immobilization periods. Type I collagen mRNA levels increased 1.3 ± 0.1-fold at 1 week, 1.5 ± 0.1-fold at 2 weeks, 1.9 ± 0.2-fold at 4 weeks, 1.8 ± 0.3-fold at 8 weeks, and 1.8 ± 0.2-fold at 12 weeks after immobilization, relative to the levels in the control group (Figure 5A). Image analysis showed that type I collagen protein levels in the immobilization group increased 1.1 ± 0.2-fold to 1.2 ± 0.3-fold in the perimysium (Fig. 5B) and 1.2 ± 0.7-fold at 1 week, 2.6 ± 1.8-fold at 2 weeks, 4.1 ± 2.0-fold at 4 weeks, 4.4 ± 2.0-fold at 8 weeks, and 4.7 ± 2.0-fold at 12 weeks after immobilization in the endomysium, relative to the levels in the control group (Fig. 5C). Type I collagen mRNA and protein levels in the immobilization group were significantly higher than the levels in the control for each immobilization period. Furthermore, in the immobilization

group, type I collagen protein expression in the endomysium and mRNA expression were higher at 4, 8, and 12 weeks than those at 1 and 2 weeks after immobilization (Fig. 5A, C).

2) Type III collagen

Figure 6 shows immunofluorescence images of type III collagen. Staining in the perimysium and endomysium was brighter in the immobilization group than in the control group. Figure 7 shows type III collagen expression for each immobilization period. Quantitative analysis showed that type III collagen mRNA levels increased 1.4 ± 0.3-fold to 1.6 ± 0.3-fold, relative to the levels in the control group, for all immobilization periods (Figure 7A). Semi-quantitative analysis of the immunofluorescence images showed that type III collagen protein levels increased 1.1 ± 0.2-fold to 1.2 ± 0.3-fold in the perimysium (Figure 7B) and 1.1 ± 0.2-fold to 1.2 ± 0.2-fold in the endomysium (Figure 7C), relative to levels in the control group, for all immobilization periods. mRNA and protein levels of type III collagen were significantly higher in the immobilization group than the levels in the control group, for each immobilization period. However, type III collagen expression in the immobilization groups was no significantly different between the immobilization periods.

DISCUSSION

Our findings showed that ROM for dorsiflexion in the immobilization group decreased to 77.5% of that in the control group at 1 week after immobilization and continued to decrease gradually as the duration of immobilization increased. These results are similar to those described in a previous report.[1] We assume that muscle contracture occurred and progressed in the soleus muscle of the immobilization group. The results of histological analysis showed that muscle fiber atrophy accompanied immobilization. The atrophic changes progressed through 4 weeks of immobilization and diminished after 8 weeks of immobilization. We surmise that the recovery was induced by adaptation to the stress of immobilization. Although our findings are consistent with those of previous studies,[19] the details of immobilization-induced muscle fiber atrophy were unclear in this study. Our results indicate that the changes in ROM and cross-sectional area of myofibers did not

occur in parallel. Additionally, the present study showed that the protein and mRNA levels of type I and III collagen increased over time. Furthermore, type I collagen mRNA expression was higher at 4, 8, and 12 weeks than that at 1 and 2 weeks after immobilization. These results indicate that immobilization-induced muscle fibrosis occurred during the early stages of immobilization (1 to 2 weeks after immobilization), and that fibrosis progressed in the later stages of immobilization (4 weeks after immobilization).

In the early stages of immobilization, CD-11b-positive cells, IL-1β mRNA, TGF-β1 mRNA, and α-SMA-positive cells increased in the immobilization group. CD-11b is a marker of macrophages. The number of CD-11b-positive cells in the immobilization group was higher than the number in the control group from the first week after immobilization. These findings indicate that the number of macrophages increased in the immobilized soleus muscle. The mechanism underlying the increase in macrophages has been described in previous studies. MCP-1 plays a key role in the migration of monocytes/macrophages.[20] Previous studies have shown that MCP-1 mRNA expression in the gastrocnemius muscle is higher on the immobilized side than on the control side after 2 weeks of immobilization.[20] Caspase-3, an effector caspase involved in the execution of apoptosis, is essential for MCP-1 mRNA upregulation and increased macrophage infiltration in skeletal muscles.[21] Moreover, immobilization has been associated with caspase-3 activation in the rat gastrocnemius muscle after 8 days of immobilization.[20] Previous research has shown that macrophages are the main source of IL-1β in fibrotic lesions.[22] In other words, macrophages can induce the production of IL-1β.[23] Our study showed that IL-1β mRNA expression increased in the soleus muscle after 1-week immobilization. Therefore, we concluded that the increase in macrophages was related to the upregulation of IL-1β mRNA in immobilized skeletal muscle. IL-1β is a potent inducer of TGF-β1 synthesis[9] that induces fibroblast activation and collagen production in a TGF-β-dependent mechanism.[24] Several reports have shown that TGF-β1 a key player in fibrotic diseases[25,26] and an important component of muscle fibrosis formation.[27,28] Moreover, TGF-β1 is a key factor promoting the conversion of fibroblasts into myofibroblasts, a differentiation process commonly associated with pathological conditions such as fibrosis.[29,30] Myofibroblasts produce large amounts of

collagen[31] and play a major role in pathological contracture, such as in Dupuytren contracture, plantar fibromatosis, and frozen shoulder.[30] In this study, TGF-β1 mRNA expression and the number of α-SMA-positive cells increased in the rat soleus muscle from the first week of immobilization. These results indicate that TGF-β1 mRNA upregulation affects the differentiation of fibroblasts into myofibroblasts early in the immobilization period, and that these alterations are associated with the incidence of immobilization-induced muscle fibrosis.

In the immobilization group, HIF-1α mRNA levels and the number of α-SMA-positive cells were higher at 4, 8, and 12 weeks than those at 1 and 2 weeks. HIF-1α regulates gene expression, and HIF-1α expression increases with hypoxia.[32, 33] Hypoxic changes were induced by a decrease in the volume of blood flow to inadequate levels, known as ischemia,[34] which in turn was triggered by a decrease in the number of capillaries. In our pilot studies, the number of capillaries in immobilized rat soleus muscles was significantly lower than the number in control muscles after a 4-week immobilization.[35] Therefore, we hypothesized that ischemia was caused by a reduction in the number of capillaries in the immobilized muscles.

Previous research has shown that α-SMA protein expression increases in hypoxic fibroblasts, indicating that hypoxia promotes fibroblast differentiation into myofibroblasts.[20,21] Our study demonstrated that HIF-1α mRNA expression was higher in the immobilization group than in the control group at 4 weeks after immobilization. In short, immobilized soleus muscles became hypoxic at 4 weeks. Additionally, in the immobilization group, the number of α-SMA-positive cells increased further at 4, 8, and 12 weeks. Thus, the acceleration of myofibroblast differentiation induced by hypoxia was related to the overexpression of type I collagen at 4 weeks after immobilization. We surmised that the lesions promoted immobilization-induced muscle fibrosis in muscle contracture.

This study has several limitations. First, the mechanism underlying the increase in the number of macrophages in the immobilized soleus muscle is unclear. Further studies on changes in MCP-1 and caspase-3 are required. Additionally, we were unable to confirm whether hypoxia in immobilized soleus muscles began after 4 weeks. Further studies to examine blood flow volume and capillaries in the immobilized soleus muscle are needed to address this issue. Furthermore, we focused on changes

in the skeletal muscle as the major factor of muscle contracture in our study. However, in addition to skeletal muscle, the skin, capsules, and ligaments have also been associated with contracture lesions.[36] Thus, a comprehensive investigation of these structures is be required to understand the mechanism of contracture in its entirety. Finally, data relating cause and effect of cellular and molecular event are insufficient in our study. Future studies will use an antagonist or inhibitor to address this limitation.

In summary, our study indicates that, in the early stages of immobilization of the soleus muscle, an increase in the number of macrophages induces IL-1β mRNA upregulation, which in turn upregulates TGF-β1 mRNA expression, causing the differentiation of fibroblasts into myofibroblasts. These changes result in the overexpression of type I and III collagen. Consequently, we surmise that IL-1β/TGF-β1 signaling via macrophages affects the incidence of immobilization-induced muscle fibrosis in muscle contracture. Furthermore, in the late stages of immobilization, the soleus muscle became hypoxic. The promotion of myofibroblast differentiation by hypoxia accelerates the overexpression of type I collagen. Thus, we conclude that hypoxia promotes immobilization-induced muscle fibrosis in muscle contracture.

Abbreviations: α-SMA, α-smooth muscle actin; ANOVA, analysis of variance; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF-1α, hypoxia-inducible transcription factor-1α; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; PBS, phosphate-buffered saline; ROM, range of motion; RT, room temperature; RT-PCR, reverse-transcription polymerase chain reaction; SD, standard deviation; TGF-β1, transforming growth factor-β1

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TABLE 1. Sequences of primers used in RT-PCR

FIGURE LEGENDS

FIGURE 1. Changes in the cross-sectional area of the soleus muscle. Open bars show control groups. Gray bars show immobilization groups. Data are mean ± S.D.; * $p < 0.05$ compared with the control groups; #$p < 0.05$ between immobilization periods for the immobilization group.

FIGURE 2. Analysis of macrophages (upper) and myofibroblasts (lower) in the soleus muscle. Immunohistochemical staining for CD-11b and α-SMA in the soleus muscle. (A) Nine-week-old control rats and rats (B) 1 week, (C) 2 weeks, (D) 4 weeks, (E) 8 weeks, and (F) 12 weeks after immobilization. Arrowheads indicate α-SMA-positive cells. Scale bar: 20 μm. Figures on the right show the number of CD-11b- and α-SMA-positive cells in 100 myofibers. Open bars show control groups. Gray bars show immobilization groups. Data are mean ± S.D.; *$p < 0.05$ compared to control; #$p < 0.05$ between immobilization periods for the immobilization group.

FIGURE 3. Expression of IL-1β (A), TGF-β1 (B), and HIF-1α (C) mRNA. Open bars show control groups. Gray bars show immobilization groups. Data are mean ± SD; *$p < 0.05$ compared to control; #$p < 0.05$ between immobilization periods for the immobilization group.

FIGURE 4. Immunofluorescence staining for type I collagen in the soleus muscle. (A) Nine-week-old control rats and rats at (B) 1 week, (C) 2 weeks, (D) 4 weeks, (E) 8 weeks, and (F) 12 weeks after immobilization. Arrows indicate the perimysium, and arrowheads indicate the endomysium. Scale bar: 50 μm.

FIGURE 5. Analysis of type I collagen in the soleus muscle. (A) Type I collagen mRNA expression. Open bars show control groups. Gray bars show immobilization groups. (B, C) Data are expressed relative to control values for each immobilization period. Semi-quantitative measurements of type I

collagen in the perimysium (B) and endomysium (C). Data are mean ± SD; *$p < 0.05$ compared to control; #$p < 0.05$ between immobilization periods for the immobilization group.

FIGURE 6. Immunofluorescence staining for type III collagen in the soleus muscle. (A) Nine-week-old control rats and rats at (B) 1 week, (C) 2 weeks, (D) 4 weeks, (E) 8 weeks, and (F) 12 weeks after immobilization. Arrows indicate the perimysium, and arrowheads indicate the endomysium. Scale bar: 50 μm.

FIGURE 7. Analysis of type III collagen in the soleus muscle. (A) Type III collagen mRNA expression. Open bars show control groups. Gray bars show immobilization groups. (B, C) Data are expressed relative to control values for each immobilization period. Semi-quantitative measurements of type III collagen in the perimysium (B) and endomysium (C). Data are mean ± SD; *$p < 0.05$ compared to control.

Figure 1

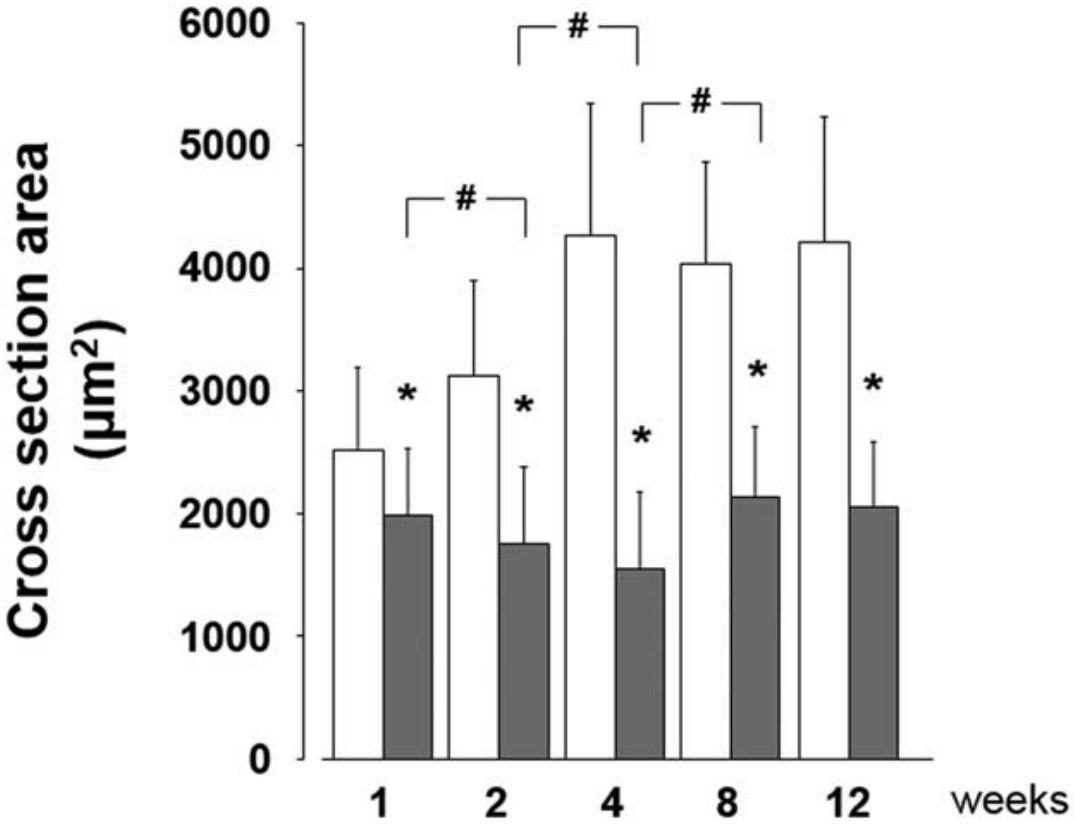
Cross section area
(μm²)
6000
5000
4000
3000
2000
1000
0
#
#
#
*
*
*
*
*
*
1
2
4
8
12
weeks

Figure 2

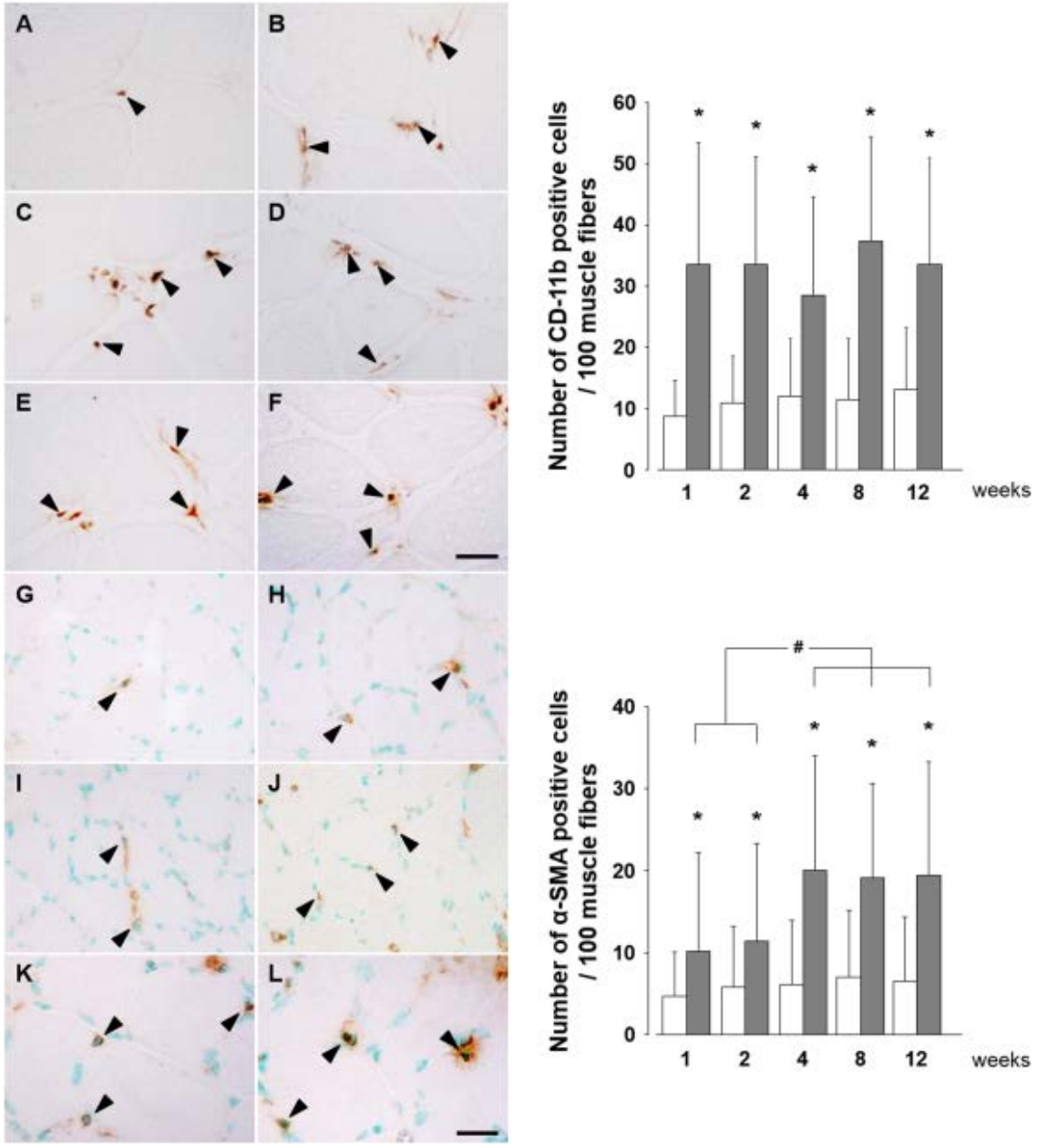
A
B
C
D
E
F
G
H
I
J
K
L
Number of CD-11b positive cells / 100 muscle fibers
60
50
40
30
20
10
0
1
2
4
8
12
weeks
Number of α-SMA positive cells / 100 muscle fibers
#
40
30
20
10
0
1
2
4
8
12
weeks

Figure 3

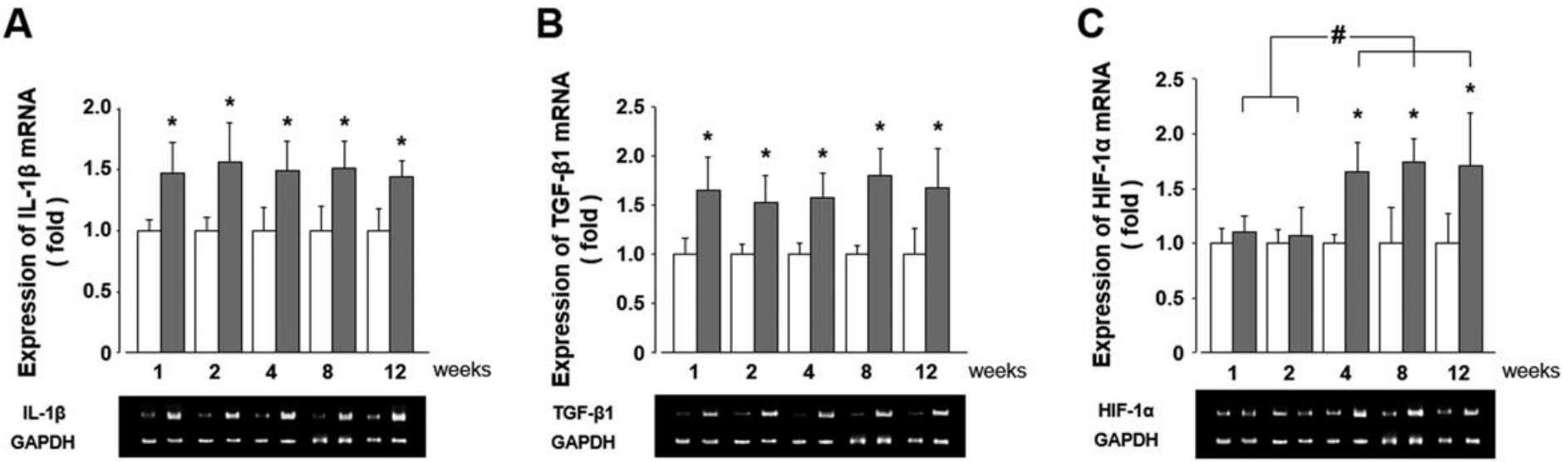
A
Expression of IL-1β mRNA (fold)
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
IL-1β
GAPDH
B
Expression of TGF-β1 mRNA (fold)
2.5
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
TGF-β1
GAPDH
C
#
Expression of HIF-1α mRNA (fold)
2.5
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
HIF-1α
GAPDH

Figure 4

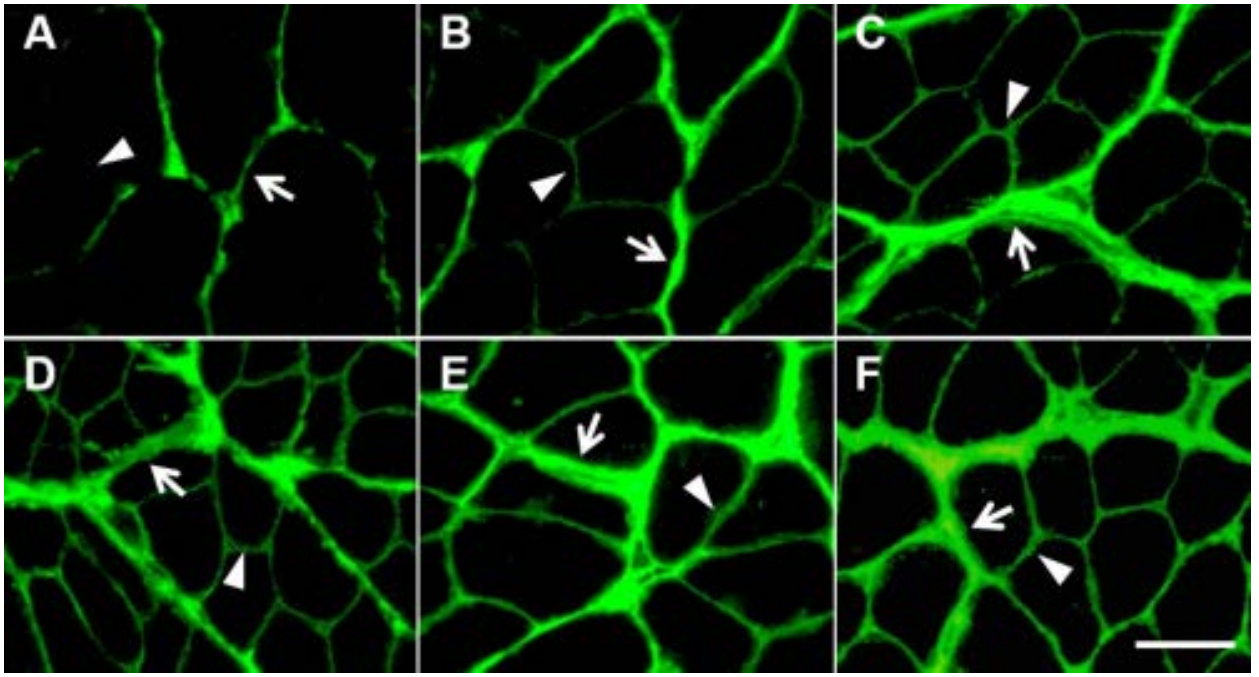

Figure 5

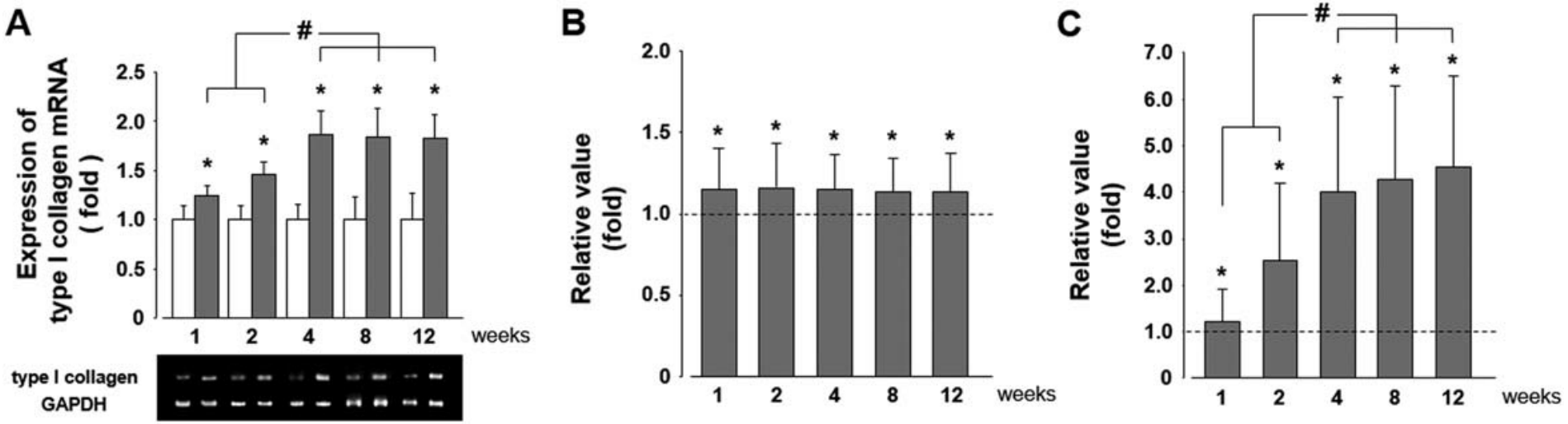
A
Expression of type I collagen mRNA (fold)
2.5
2.0
1.5
1.0
0.5
0
#
*
1
2
4
8
12
weeks
type I collagen
GAPDH
B
Relative value (fold)
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
C
Relative value (fold)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
#
1
2
4
8
12
weeks

Figure 6

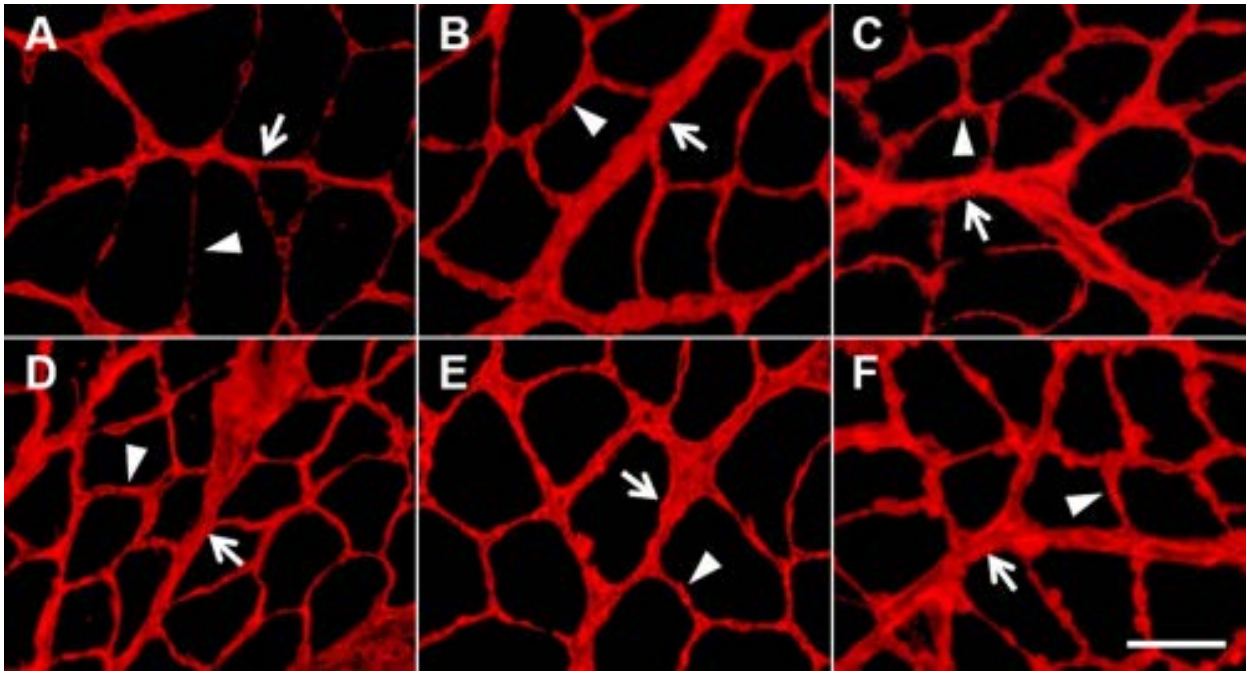

Figure 7

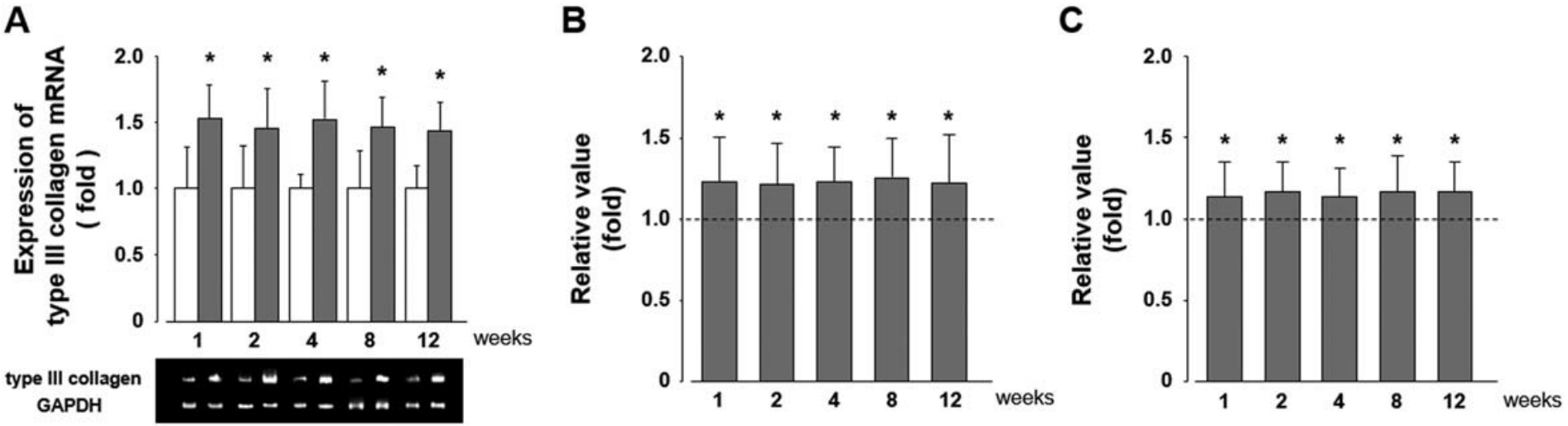
A
Expression of type III collagen mRNA (fold)
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
type III collagen
GAPDH
B
Relative value (fold)
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks
C
Relative value (fold)
2.0
1.5
1.0
0.5
0
1
2
4
8
12
weeks

Table 1. Sequences of primers used in RT-PCR.

Object gene	F/R	Arrangement	Gene Bank no.
IL-1β	F	5′-AATGACCTGTTCTTTGAGGCTGAC-3′	BC091141.1
	R	5′-CGAGATGCTGCTGTGAGATTTGAA-3′	
TGF-β1	F	5′-ATACAGGGCTTTCGCTTCAG-3′	BC076380.1
	R	5′-GTGTGTCCAGGCTCCAAATG-3′	
HIF-1α	F	5′-CCATCCATGTGACCATGAGG-3′	AF057308.1
	R	5′-GCAAGCATCCTGTACTGTCC-3′	
Type I collagen	F	5′-GAAGACCTATGTGGGTATAAGTC-3′	Z78279.1
	R	5′-TGATTGCTGGCATAAAGCAGGG-3′	
Type III collagen	F	5′-TGCTGCCATTGCTGGAGTTGGAG-3′	BC087039.1
	R	5′-GTTGGTCACTTTCACTGGTTGAC-3′	
GAPDH	F	5′-CCTTCCGTGTTCCTACC-3′	M17701.1
	R	5′-CCACTAAAGGGCATCCT-3′	

F, forward; R, reverse.