

Development and progression of immobilization-induced skin fibrosis through overexpression of transforming growth factor- β 1 and hypoxic conditions in a rat knee joint contracture model.

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Key Words

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ABSTRACT

PURPOSE: The purpose of this study was to investigate the pathology and mechanism of immobilization-induced skin fibrosis in a rat joint contracture model.

METHODS: Rats were randomly divided into control and immobilization groups. In the immobilization groups, knee joints of the rats were immobilized for 1, 2, and 4 weeks. After each immobilization, skin was dissected. To assess fibrosis in the skin, the thickness and area of adipocytes and connective tissue fibers were measured. Myofibroblasts were analyzed by immunohistochemistry by using anti- α -SMA as a marker. Gene expression levels of type I and III collagen, TGF- β 1 and HIF-1 α were measured by using RT-PCR.

RESULTS:

One week after immobilization, there was a marked increase in the area of connective tissue fibers in the immobilization group. Type I and type III collagen were significantly increased with prolonged immobilization. Higher numbers of α -SMA-positive cells were noted in the immobilized group at 2 and 4 weeks after immobilization. The expression level of TGF- β 1 mRNA in the immobilization group increased after one week of immobilization. In contrast, the expression level of HIF1- α mRNA increased after 2 weeks of immobilization, and a greater increase was seen at 4 weeks after immobilization.

CONCLUSIONS: These results suggest that immobilization induces skin fibrosis with accumulation of types I and III collagen. These fibrotic changes may be evoked by upregulation of TGF- β 1 after one week of immobilization. Additionally, upregulation of HIF-1 α may related to skin fibrosis by accelerating the differentiation of fibroblasts to myofibroblasts starting at 2 weeks after immobilization.

Introduction

Joint contracture is generally recognized as a limitation of the range of joint motion induced by immobilization. This immobilization is often used as a medical treatment for reducing inflammation, pain, and tissue damage caused by trauma, burns, and surgery. Decrease of physical activity and poorer quality of life often result from joint contracture. Therefore, many studies have examined the alterations of soft tissues surrounding the joint, e.g. skeletal muscle [1], joint capsule [2, 3] and ligament [4, 5], and previous reports have revealed that fibrotic changes in the muscle [6] and capsule [7, 8] are one of the major pathologies of joint contracture.

Skin is the soft tissue that surrounds the joints and covers other soft tissues. Recent animal studies [9] have shown that, as skeletal muscle [6] and joint capsule [10] are involved in immobilization-induced joint contracture, the skin is one of the limiting factors for immobilization-induced joint contracture, which implies that some sort of dermatogenic alteration influences the development of joint contracture. However, there are no reports or studies involving the pathology and mechanism of immobilization-induced dermatogenic alteration.

Skin fibrosis is characterized by excessive accumulation of collagen, and is often observed in systemic sclerosis patients [11]. Histological examination of skin samples

from patients [12] and animals [13] showed that fibrosis is most prominent in the reticular dermis initially, but with progression, the subjacent adipose layer also becomes affected. Types I and III collagen are the main isoforms expressed in human skin and are maintained in a fixed proportion relative to one another in normal skin tissue [14]. Biochemical analysis of fibroblasts from systemic sclerosis patients demonstrated an increase of type I and type III collagen accumulation in the connective tissues [15]. These fibrotic changes are associated with thickening and stiffness of the skin, leading to the decline of skin extensibility and secondary joint contractures of the extremities [12]. Taking into account these reports and alterations of the joint capsule and muscle induced by immobilization, skin fibrosis appears to be one cause of immobilization-induced joint contracture.

The underlying molecular mechanisms of skin fibrosis in systemic sclerosis have been examined, and possible roles of multiple cytokines, growth factors, chemokines, and hypoxia have been identified. Transforming growth factor (TGF)- β 1 is the prominent key mediator in connective tissue remodeling during both normal wound healing and pathological fibrosis [16]. TGF- β 1 has a potent effect on fibroblast release of collagen, and differentiation of fibroblasts into myofibroblasts, which contribute to collagen secretion and accumulation in scleroderma, Dupuytren's disease, heart and kidney

fibrosis. In addition, tissue hypoxia, indicated by markedly increased hypoxia-inducible factor (HIF)-1 α , is an essential stimulus for excessive collagen synthesis via development of myofibroblasts [17]. Thus, we hypothesized that TGF- β 1 and hypoxia would be key factors regulating collagen synthesis and accumulation, particularly type I and type III collagen, in immobilization-induced skin fibrosis.

We aimed to clarify the pathology and mechanisms of skin fibrosis evoked by immobilization, to gain a better understanding as to the underlying molecular cause of immobilization-induced joint contracture.

Materials and Methods

Animals

Twelve-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 cm \times 40 cm \times 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 study, 66 rats were divided randomly into an immobilization groups ($n=36$) and a control group ($n=30$). Animals in the immobilization groups were anesthetized with pentobarbital sodium (40 mg/kg). The bilateral knee joints were

rigidly immobilized at full flexion with plaster casts, which were changed weekly because of loosening attributed to muscle atrophy. The knee joints were immobilized for 1, 2, and 4 weeks. Twelve rats were used for each immobilization period, and ten untreated control rats were tested for 1, 2, and 4 weeks. The experimental protocol was approved by the ethics review committee for animal experimentation of Nagasaki University (Authorization code: 1003241837).

Tissue sampling and preparation

The right and left popliteal skin of each rat was excised. Histological, immunohistochemical and biochemical analysis was performed in six rats of the immobilized group and in five rats of the control group. Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed on different rats than those who had undergone histological and immunohistochemical analysis, specifically six from the immobilized group and five from the control group. For histological analysis, the skin samples were fixed with prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). The skin was permeated with 10% sucrose in phosphate buffered saline (PBS) for one day and 30% sucrose in PBS for three days. These samples were then frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and stored

at -80°C until use. Serial frozen cross-sections of the skin, prepared using a cryostat (Leica, CM1950, Tokyo, Japan), were mounted on glass slides. For immunohistochemical analysis, these samples were also fixed with 4% paraformaldehyde in PBS, embedded in paraffin, prepared using a microtome (Yamato Kohki, LS-113, Saitama, Japan), prior to mounting on glass slides. Samples that were to be used in RT-PCR assays were treated with RNAlater (Ambion, Foster City, California) immediately after excision and stored in a deep freezer (-80°C).

Histological analysis

Cross-sections (20 μm) were stained with hematoxylin and eosin to distinguish the morphological characteristics of skin. Stained cross-sections of skin were observed with an optical microscope (Nikon, DS-Ri1, Tokyo, Japan). Thickness of dermal plus subcutaneous layers, area of connective tissue fibers and area of adipocytes in dermis plus subcutaneous tissue were determined using Scion image 4.0 software (W. Rasband, National Institutes of Health, Bethesda, Maryland). The individual performing the analysis was blind to the group in which the rats belonged.

Measurement of the thickness of dermal plus subcutaneous layers

Thickness of dermal plus subcutaneous layers, defined as the length from the top of the dermis to the inferior end of subcutaneous adipocytes, was examined in histological samples using Image J 1.45 software. Seven random measurements were taken per section (Fig. 1). The results were expressed in micrometers as mean values of the thickness of dermal plus subcutaneous layers for each group. Two investigators examined all of the sections independently in a blinded fashion.

Area occupied by adipocytes and connective tissue fibers in dermal plus subcutaneous layer

Using microscopy and standardized light conditions, the sections were magnified to 40 \times , and images were captured with a digital camera (Nikon, Tokyo, Japan). The area of dermal plus subcutaneous tissues was measured in each image. Then, using microscopy and standardized light conditions, the sections were magnified to 200 \times , and images were captured with a digital camera (Nikon, Tokyo, Japan). The area of adipocytes was calculated as the sum of the area of individual adipocytes. The ratio of adipocyte accumulation was calculated as the area of adipocytes in the area of dermal plus subcutaneous layer and expressed as a percentage. Then, the area of hair follicles, space between tissues, blood vessels, and adipocytes were measured in each image. The area

of connective tissue fibers was measured as the area of dermal plus subcutaneous layer minus the area of hair follicles, space between tissues, blood vessels, and adipocytes, and expressed as a percentage.

Determination of collagen content

In general, hydroxyproline is thought to be exclusive to collagen; thus, the concentration of collagen was determined by measuring the hydroxyproline levels. Popliteal skin were analyzed for hydroxyproline content. Collagen content was estimated via the modified technique of Reddy et al. [18]. Samples were weighed after lyophilization for 24 hours, were hydrolyzed in 6 N HCl for 15 h at 110 °C. Next, the samples were hydrolyzed in an alkali for 120 min at 90 °C. The hydrolyzed specimens were then mixed with buffered chloramine-T reagent and left to stand to allow oxidation at room temperature (22 °C). The chromophore was developed by adding Ehrlich's aldehyde reagent, after which the absorbance of each sample was measured at 540 nm with a spectrophotometer (Molecular Devices, SpectraMax 190 Microplate Reader, Sunnyvale, USA). Absorbance values were plotted against the concentration of standard hydroxyproline; the presence of hydroxyproline in sample extracts was determined from the standard curve. The hydroxyproline concentration of samples was calculated as the

content per dry weight ($\mu\text{g}/\text{mg}$ dry weight).

Immunohistochemical analysis

Myofibroblasts have features that are intermediate between those of fibroblasts and smooth muscle cells. Alpha-smooth muscle actin (α -SMA) is one of the most useful markers for myofibroblasts. Skin cross-sections (5 μm) were air-dried. To inhibit endogenous peroxidase, the sections were then incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature (RT). After washes in 0.01 M phosphate-buffered saline (PBS; pH 7.4), the sections were blocked with 5% bovine albumin in PBS for 60 min and incubated overnight at 4 °C with a mouse monoclonal anti- α -smooth muscle actin (α -SMA) primary antibody (1:1000; Exalpha Biologicals, Inc., Shirley, Massachusetts). The sections were rinsed in PBS for 15 min, after which biotinylated goat anti-mouse IgG (1:500; Vector Laboratories, Burlingame, California) 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 30 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 (pH 7.4) at RT. After a final washing step, α -SMA sections were stained

with 1% methyl green and covered. The sections were observed under an optical microscope.

Using microscopy and standardized light conditions, the sections were magnified to 400 \times , and images were captured with a digital camera (Nikon, Tokyo, Japan). The number of myofibroblasts was determined from the images by counting the number of α -SMA–positive cells at 10 randomly selected sites. We calculated the ratio of the α -SMA positive cells to the total number of cells except vascular endothelial cell and hair follicle cells. Vascular and hair follicle areas were omitted from the analysis. The individual performing the analysis was blinded to the groups to which the rats belonged.

Reverse transcription polymerase chain reaction (RT-PCR)

The left popliteal skin was used for this analysis. Total RNA was extracted from skin samples (RNeasy Fibrous Tissue Mini Kit; Qiagen, Valencia, California) according to the manufacturer's protocol. Total RNA was used as template for the reverse transcription reaction (QuantiTec; Qiagen) to synthesize cDNA. PCR was then performed following the manufacturer's instructions (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 58 °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. Gene-specific primers for TGF- β 1, HIF-1 α , types I and III collagen, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used in the 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 expressions of TGF- β 1, HIF-1 α , and types I and III collagen mRNA were normalized to the expression of the internal control (GAPDH).

Statistical analysis

All data are presented as mean \pm 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 the Scheffe's method. Differences were considered significant at $p < 0.05$.

Results

Histological Findings

Cell infiltration indicating tissue inflammation was not seen in either the control or the immobilization group (Fig. 2). Histological findings in the immobilization group showed marked thickening of the dermis and excessive accumulation of connective tissue fibers from 1 week after immobilization (Fig. 2D), which extended toward subcutaneous tissue. On the other hand, adipose tissue in the subcutaneous tissue of the same group was decreased from 1 week after immobilization (Fig. 2D). These histological alterations became pronounced with prolonged immobilization. At 4 weeks after immobilization, adipose tissue in the immobilization group was not present (Fig. 2F).

Thickness of dermal plus subcutaneous layers

The thickness of the dermis plus subcutis was $1207.6 \pm 454.3 \mu\text{m}$ in 1 week control, $928.4 \pm 279.5 \mu\text{m}$ in 2 weeks control, and $1227.7 \pm 212.7 \mu\text{m}$ in 4 weeks control. In the immobilization groups, thickness was $1072.0 \pm 300.9 \mu\text{m}$ at 1 week, $1048.3 \pm 350.6 \mu\text{m}$ at 2 weeks, and $1242.4 \pm 290.0 \mu\text{m}$ at 4 weeks (Fig. 3A). There were no significant differences between the thicknesses of dermal plus subcutaneous layers in immobilized

group versus the control group.

Area occupied by connective tissue fibers in dermal plus subcutaneous layer

The ratio of area occupied by connective tissue fibers in dermal plus subcutaneous layer was $64.2 \pm 11.7\%$ for 1 week control, $59.4 \pm 7.6\%$ for 2 weeks control, and $69.0 \pm 12.7\%$ for 4 weeks control in the control group. In the immobilization groups, the ratio was $74.1 \pm 6.7\%$ at 1 week, $82.8 \pm 8.0\%$ at 2 weeks, $83.0 \pm 5.4\%$ at 4 weeks (Fig. 3B).

The ratio of area occupied by connective tissue fibers in the dermal plus subcutaneous layer in the immobilization group was significantly greater than that in the control group for each immobilization period ($p < 0.05$). In addition, in the immobilization groups, the ratio of area occupied by connective tissue fibers in the area of dermal plus subcutaneous layer at 4 weeks was significantly higher than that at 1 week ($p < 0.05$).

Area occupied by adipocytes in dermal plus subcutaneous layer

The ratio of area occupied by adipocytes in dermal plus subcutaneous layer was $28.8 \pm 11.0\%$ for 1 week control, $30.0 \pm 11.0\%$ for 2 weeks control, and $23.3 \pm 12.5\%$ for 4 weeks control in the control group. In the immobilization groups, the area ratio was $16.3 \pm 5.7\%$ at 1 week, $7.6 \pm 7.4\%$ at 2 weeks, and $4.7 \pm 3.0\%$ at 4 weeks (Fig. 3C). The area

ratio of immobilization group were not only significantly less than that of the control for each immobilization period ($p < 0.05$), but also decreased significantly with an increase in the duration of immobilization ($p < 0.05$).

Hydroxyproline content

Hydroxyproline content were 3.0 ± 0.6 $\mu\text{g}/\text{mg}$ dry weight for 1 week control, 3.0 ± 0.4 $\mu\text{g}/\text{mg}$ dry weight for 2 weeks control, 3.0 ± 0.5 $\mu\text{g}/\text{mg}$ dry weight for 4 weeks control in the control group. In the immobilization groups, content were 4.3 ± 0.3 $\mu\text{g}/\text{mg}$ dry weight at 1 week, 6.0 ± 1.6 $\mu\text{g}/\text{mg}$ dry weight at 2 weeks, 7.0 ± 1.7 $\mu\text{g}/\text{mg}$ dry weight at 4 weeks (Fig.4). Hydroxyproline content in the immobilization group was significantly greater than that in the control group for each immobilization period ($p < 0.05$). In addition, in the immobilization groups, hydroxyproline content at 4 weeks was significantly higher than that at 1 week ($p < 0.05$).

Ratio of myofibroblasts

Alpha-SMA positive cells were observed faintly in dermal plus subcutaneous layer in both the control and immobilization group at 1 week after immobilization. However, increased α -SMA positive cells in the immobilized skin were observed at 2 and 4 weeks

after immobilization. The ratio of α -SMA positive cells per randomly selected ten sites were 7.1 ± 5.8 % in 1 week control, 7.7 ± 8.1 % in 2 weeks control, and 6.3 ± 3.6 % in 4 weeks control in the control group. In the immobilization groups, the ratio of cells was 8.2 ± 8.4 % at 1 week, 11.3 ± 7.1 % at 2 weeks, 16.7 ± 12.2 % at 4 weeks (Fig. 5E). The ratio of α -SMA positive cells in the immobilization group were significantly higher than those in the control group at 2 and 4 weeks ($p < 0.05$). Moreover, in the immobilization groups, the ratio of myofibroblasts at 4 weeks were significantly higher than those at 1 and 2 weeks ($p < 0.05$).

Expression levels of type I and III collagen, TGF- β 1, and HIF-1 α mRNA

Type I collagen

In 1 week control, 2 weeks control and 4 weeks control rat, relative expression levels of type I collagen mRNA was 0.81 ± 0.10 , 0.80 ± 0.14 , and 1.10 ± 0.23 , respectively. At 1, 2 and 4 weeks, relative expression levels of type I collagen mRNA in the immobilization groups was 0.97 ± 0.10 , 1.31 ± 0.32 , and 2.10 ± 0.31 , respectively (Fig. 6A). Type I collagen mRNA expression levels in the immobilization group was not only significantly higher than that of the control at each immobilization period ($p < 0.05$), but also increased significantly with an increase in the duration of immobilization ($p <$

0.05).

Type III collagen

In 1 week control, 2 weeks control and 4 weeks control, relative expression levels of type III collagen mRNA was 1.03 ± 0.17 , 0.84 ± 0.10 , and 1.13 ± 0.24 , respectively. At 1, 2 and 4 weeks, relative expression levels of type III collagen mRNA in the immobilization groups was 1.64 ± 0.15 , 1.71 ± 0.20 , and 2.01 ± 0.34 , respectively (Fig. 6B). Type III collagen mRNA expression levels in the immobilization group was significantly higher than in the control group for each time-point ($p < 0.05$). Furthermore, in the immobilization groups, type III collagen mRNA at 4 weeks was significantly higher than that at 1 week ($p < 0.05$).

TGF- β 1

In 1 week control, 2 weeks control rats and 4 weeks control, relative expression levels of TGF- β 1 mRNA in the control group was 0.79 ± 0.30 , 0.80 ± 0.12 , and 1.06 ± 0.33 , respectively. At 1, 2 and 4 weeks, relative expression levels of TGF- β 1 mRNA in the immobilization groups was 1.74 ± 0.17 , 1.84 ± 0.52 , and 2.18 ± 0.52 , respectively (Fig. 7A). TGF- β 1 mRNA expression levels in the immobilization group was significantly

higher than in the control group for each time-point ($p < 0.05$). However, in the immobilization groups, there was no significant difference in TGF- β 1 mRNA expression levels at the different immobilization time-points.

HIF-1 α

In 1 week control, 2 weeks control and 4 weeks control, relative expression levels of HIF-1 α mRNA in the control group was 0.63 ± 0.13 , 0.87 ± 0.23 , and 1.03 ± 0.35 , respectively (Fig. 7B). At 1, 2 and 4 weeks, relative expression levels of HIF-1 α mRNA in the immobilization groups was 0.75 ± 0.13 , 1.29 ± 0.27 , and 1.82 ± 0.36 , respectively. HIF-1 α mRNA expression levels was significantly higher in the immobilization group than that in the control group at 2 and 4 weeks ($p < 0.05$). In addition, within the immobilization groups, HIF-1 α mRNA expression levels at 4 weeks was significantly higher than that at 1 week ($p < 0.05$).

Discussion

Previous studies have described that scleroderma-associated skin fibrosis occurred in the dermis and that reduction of adipocytes was confirmed in the subcutaneous tissue with thickening of the skin [19, 20]. Furthermore, contractures of fingers, elbows, and

knees joints developed in advanced skin fibrosis [21]. Therefore, we examined the histological changes in the dermal plus subcutaneous layers using microscopic observation and semi-quantitative image analysis. Our histological findings in the immobilization group showed no tissue damage or increase of inflammatory cells, which indicated that immobilization with plaster cast did not cause skin inflammation in this knee joint contracture model. In addition, increase in fibrous connective tissue along with an atrophy and reduction of adipocytes were confirmed in the dermal plus subcutaneous layers from 1 week after immobilization, which progressed at 4 weeks after immobilization. Meanwhile, no significant change was observed in thickness of the dermis plus subcutis between the control and immobilization group in any immobilized period. Generally, skin thickening is a defining feature in systemic sclerosis, which is characterized by excessive accumulation of collagen in the dermal plus subcutaneous layer and involving decrease of adipocytes [22]. In this study, deposition of fibro connective tissue and decrease of adipocyte were observed; however thickening of the dermis plus subcutis was not observed in immobilized skin. The difference between systemic sclerosis skin and immobilized skin is the presence or absence of skin inflammation, which may be associated with thickening of the dermis plus subcutis. In immobilized skin, we hypothesize that fibrous connective tissue

increased in the space created by atrophy and/or reduction of adipocytes in the subcutaneous skin layer, which may be one of the reason why skin thickening does not develop and characteristic features of immobilization-induced skin fibrosis, differing from scleroderma-associated skin fibrosis.

We examined alterations of skin collagen content by immobilization by measuring hydroxyproline content in the skin. Increase of hydroxyproline content by immobilization was similar to that of connective tissue fibers in image analysis. These results suggested that increase of fibrous connective tissue observed in immobilized skin was derived from the overexpression of collagen in dermis plus subcutis, and that the overexpression of collagen in the skin may be one of the major pathologies of immobilization-induced joint contracture, similar to skeletal muscle and joint capsule.

Types I and III collagen are the main components of dermal and subcutaneous layer. It is well known that excessive deposition of the type I collagen is the hallmark of skin fibrosis in systemic sclerosis patients [23, 24]. The results of RT-PCR in this study is in agreement with the results of our collagen content and histological analysis mentioned above. These results suggest that overproduction of both type I and III collagen induced by immobilization results in an increase of fibrous connective tissue in the immobilized skin. On the other hand, type I collagen mRNA was significantly increased with each

immobilization period while no time-dependent change was observed regarding type III collagen mRNA expression levels. Our previous study revealed that although type I collagen mRNA expression levels in the immobilized rat soleus muscle significantly increased with immobilization period, no time-dependent increase of type III collagen mRNA expression levels was observed [6]. These differences of the synthesis of types I and III collagen are difficult to explain without speculation.

It is generally believed that the content of collagen correlates with skin tensile strength [25, 26]. Indeed, previous studies have reported that the tensile strength of the skin from systemic sclerosis patients is substantially increased compared to that of normal skin [27]. These reports indicated that excessive accumulation of collagen in skin led to decline of skin extensibility and secondarily, to joint contractures. Therefore, we hypothesize that immobilization-induced skin fibrosis derived from overproduction of types I and III collagen in dermal plus subcutaneous layer is one pathological cause of immobilization-induced joint contracture.

It is well known that TGF- β 1 can lead to pathologic tissue fibrosis through fibroblast activation and the induction of fibroblast differentiation to myofibroblasts, characterized by α -SMA [28-30]. Several previous reports have demonstrated that TGF- β 1 is a key player in the process of skin fibrosis formation by inducing the overexpression of type I

and type III collagen through increasing myofibroblasts numbers [24, 29]. In this study, TGF- β 1 mRNA expression levels increased in the immobilization group at 1 week after immobilization despite the absence of histological findings of inflammation. After that, there was no significant change up to 4 weeks after immobilization when comparing each immobilization period. Although, in the previous study, there was no inflammation histologically in the immobilized skeletal muscle, TGF- β 1 mRNA upregulation that may be associated with increases in macrophages and IL-1 β mRNA expression levels was observed after 1 week of cast immobilization [6]. It is likely that these alterations were related to the execution of skeletal muscle cell apoptosis followed by immobilization-induced muscle atrophy [6, 31]. Adipocyte atrophy and/or reduction observed in our current study might be a consequence of adipocyte apoptosis, which may be related to increase in TGF- β 1 mRNA expression levels. However, we did not confirm the changes of macrophage, IL-1 β mRNA expression levels, and adipocyte apoptosis, which we plan to do in the future.

On the other hand, the ratio of α -SMA positive cells was not increased at 1 weeks after immobilization; however, types I and III collagen mRNA expression levels and hydroxyproline content were significantly increased at 1 week after immobilization. These results raise the possibility that overexpression of TGF- β 1 activates the

fibroblasts, rather than promoting differentiation to myofibroblasts. A direct stimulation of fibroblasts by TGF- β 1 to produce more collagen could be achieved earlier than the differentiation of fibroblasts to myofibroblasts. In this study, we did not confirm the early stage of alteration of TGF- β 1 mRNA transcript levels, the ratio of myofibroblast, and collagen expression. More precise analysis in the early stage of immobilization is needed to clarify this question.

HIF-1 α is well known as an indicator of tissue hypoxia that promote the differentiation of fibroblasts to myofibroblasts [32, 33], similar to the role of TGF- β 1, in scleroderma-associated skin fibrosis. In the current study, we performed RT-PCR, which showed HIF-1 α mRNA expression levels increasing at 2 weeks after immobilization, and then, rising higher at 4 weeks than at 1 week, which were similar to the changes in α -SMA positive cells, fibrous connective tissue, hydroxyproline content and type I and III collagen mRNA expression levels. Namely, more severe hypoxic conditions at 4 weeks after immobilization indicate the acceleration of excessive accumulation of types I and III collagen through the noted increase myofibroblasts in addition to overexpression of TGF- β 1. Altintas et al. described that 72 h splint immobilization of the forearm resulted in a decrease of blood cell flow and density of functional dermal capillaries, and they concluded that immobilization induces the decline of skin

microcirculation [34]. Our results regarding HIF-1 α mRNA expression levels were supported indirectly by that previous findings. To identify hypoxic conditions, further examination including measurement of skin blood flow by near-infrared spectroscopy or number of capillaries identified by CD31 expression is needed.

Recent studies have described a novel link between intradermal adipose tissue loss and dermal fibrosis, which was that adiponectin-positive intradermal progenitors give rise to dermal myofibroblasts [35]. This indicates that there is a possibility that the increase of myofibroblasts observed in immobilized skin occurred not only due to fibroblast differentiation, but potentially also from the differentiation of adipocytes. Therefore, the increase in number of myofibroblasts can in part be potentially explained by differentiation from not only fibroblasts but also adipocytes, which may be explained by decreased adipose tissue in our image analysis.

This study has several limitations. First, we had not confirmed the protein level changes of TGF- β 1, HIF-1 α in immobilized skin. To clarify the pathology and mechanism of immobilization-induced skin fibrosis, quantitative analysis of these proteins is necessary. The data relating the cause and effect of cellular and molecular events are insufficient in our study. Additionally, we must confirm the upstream regulatory mechanism of upregulation of TGF- β 1 and HIF-1 α . The mechanism underlying the decrease in adipose

tissue in immobilized skin is unclear. Previous studies reported that Wnt10b/ β -catenin signaling and macrophages were related to the decline of adipocytes [24, 36]. Further studies on Wnt10b/ β -catenin signaling changes are required. There is the potential that the decrease of adipose tissue is associated with an increase in the ratio of myofibroblasts. Finally, since hypoxia may be one of main key factors in immobilization-induced skin fibrosis, multidimensional analysis is needed.

In conclusion, deposition of fibrous connective tissue attributed to overexpression of type I and III collagen in dermal plus subcutaneous layer is one of main pathology of immobilization-induced skin fibrosis. At 1 week after immobilization, overproduction of collagen associated with TGF- β 1 overexpression resulted in the development of immobilization-induced skin fibrosis. At 2 weeks post-immobilization, the skin became hypoxic, which may promote an increase in both the ratio of myofibroblasts and type I collagen mRNA transcript levels. At 4 weeks after immobilization, marked increases in HIF-1 α were observed, which may accelerate the overexpression of type I and III collagen mRNA transcript levels and increase of the ratio of myofibroblasts.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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

Gene target	F/R	Arrangement	Gene Bank No.
Type I collagen	F	5'-GAAGACCTATGTGGGTATAAGTC-3'	Z78279.1
	R	5'-CCCTGCTTTATGCCAGCAATCA-3'	
Type III collagen	F	5'-TGCTGCCATTGCTGGAGTTGGAG-3'	BC087039.1
	R	5'-GTTGGTCACTTTCACTGGTTGAC-3'	
TGF- β 1	F	5'-ATACAGGGCTTTTCGCTTCAG-3'	BC076380.1
	R	5'-GTGTGTCCAGGCTCCAAATG-3'	
HIF-1 α	F	5'-CCATCCATGTGACCATGAGG-3'	AF057308.1
	R	5'-GCAAGCATCCTGTACTGTCC-3'	
GAPDH	F	5'-CCTTCCGTGTTCCCTACC-3'	M17701.1
	R	5'-AGGATGCCCTTTAGTGG-3'	

F, forward; R, reverse.

Fig 1. Thickness of dermal plus subcutaneous layers.

We measured the thickness of dermal plus subcutaneous layer that was defined as the length from the top of the dermis to the inferior end of subcutaneous adipocytes (shown with a double-headed arrows).

Fig 2. Histological findings of popliteal skin using hematoxylin and eosin (H&E) staining. (A) 1 week control (B) 2 week control (C) 4 week control (D) 1 week after immobilization (E) 2 weeks after immobilization (F) 4 weeks after immobilization. In the control group (A, B, C), the dermis and subcutaneous tissue were observed clearly. In the immobilization group, connective tissue fibers were increased in the area of subcutaneous tissue at 1 week after immobilization (arrow heads in D). Adipocytes in the subcutaneous tissue appeared to be replaced with connective tissue fibers until 4 weeks after immobilization (arrows in F). Scale bar = 200 μ m.

Fig 3. Semi-quantitative histological analysis using H&E stained images in popliteal skin. The open bar is the control group and the filled bar is the immobilization group. (A) Thickness of dermis plus subcutis. There were no significant differences between the immobilized group and the control group. (B) Ratio of area occupied by connective

tissue fibers in dermal plus subcutaneous layers. In the immobilization group, a significantly higher ratio was seen than in the control group at each immobilization period. In the immobilization groups, ratio of connective tissue fibers at 4 weeks after immobilization was significantly higher than at 1 week after immobilization. (C) Ratio of area occupied by adipocyte in dermal plus subcutaneous layers. Ratio of adipocytes in the immobilization group was significantly decreased compared to that of the control at each immobilization period and decreased significantly with duration of immobilization. Data are expressed as mean \pm SD. * $p < 0.05$ vs control; # $p < 0.05$ vs 1 week immobilization group; † $p < 0.05$ vs 2 weeks immobilization group.

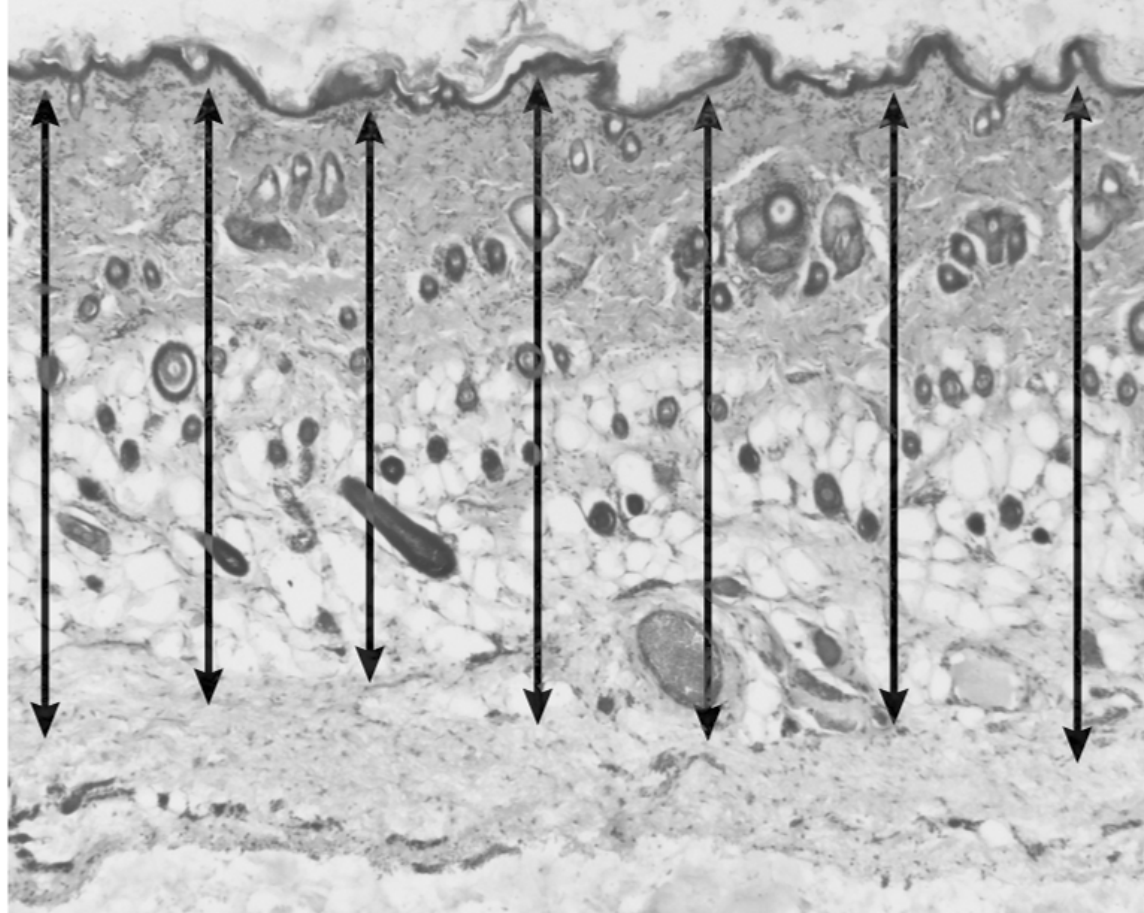
Fig 4. The concentration of collagen was determined via measurement of hydroxyproline content. The open bar is the control group and the filled bar is the immobilization group. Popliteal skin were analyzed for hydroxyproline content. Collagen content in the immobilization group significantly higher than that of the control group at each immobilization periods. Furthermore, collagen content after 4 weeks immobilization was significantly greater, compared with either 1 or 2 weeks of immobilization. Data is expressed as mean \pm SD. * $p < 0.05$ vs control; # $p < 0.05$ vs 1 week immobilization group

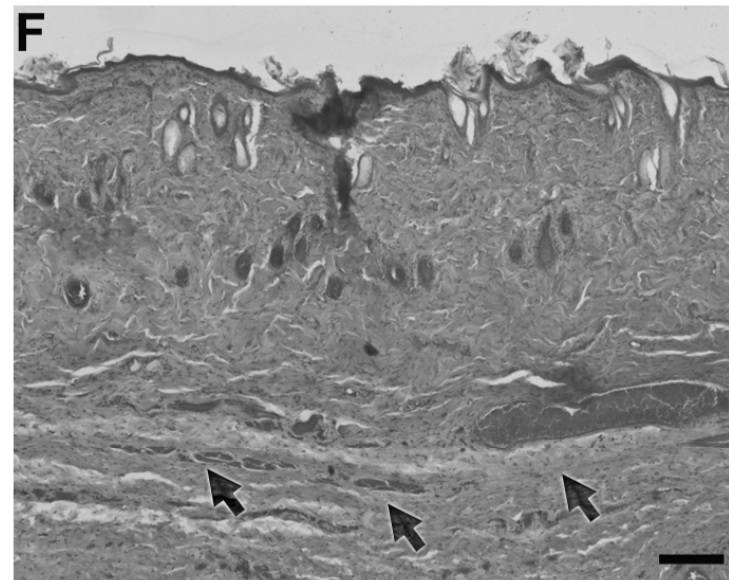
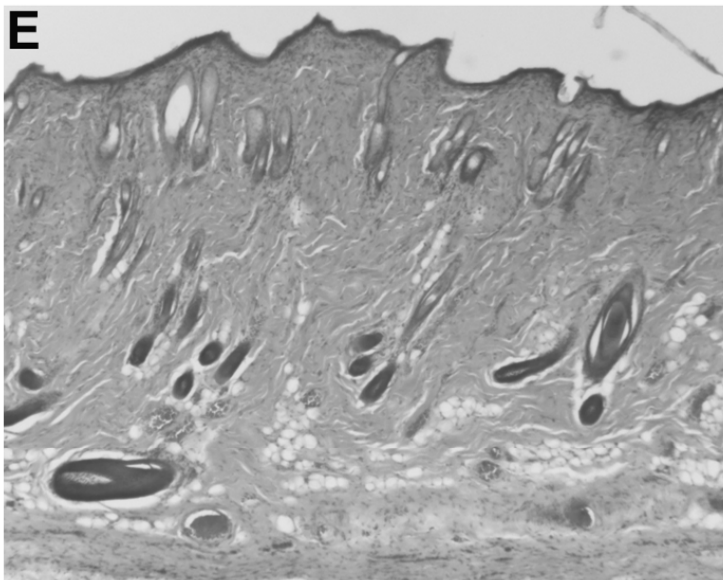
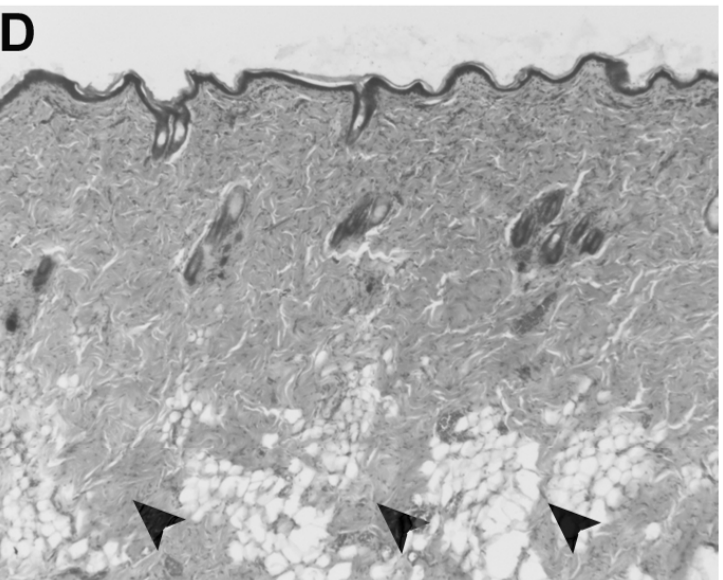
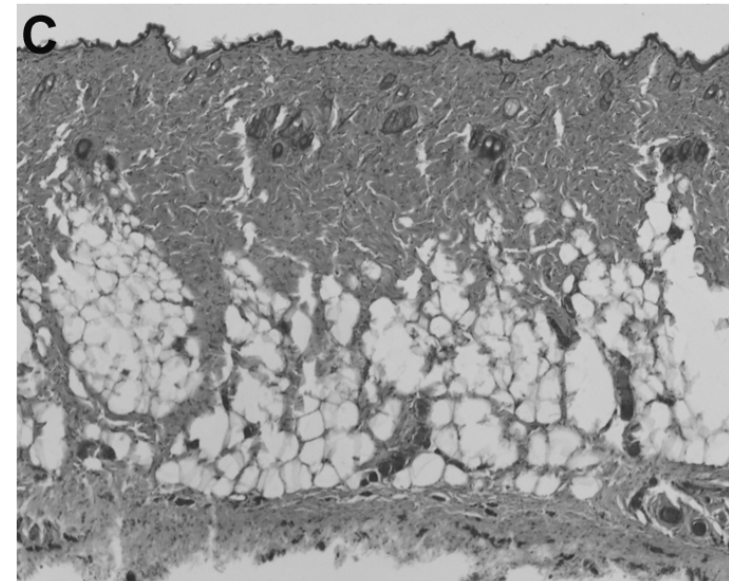
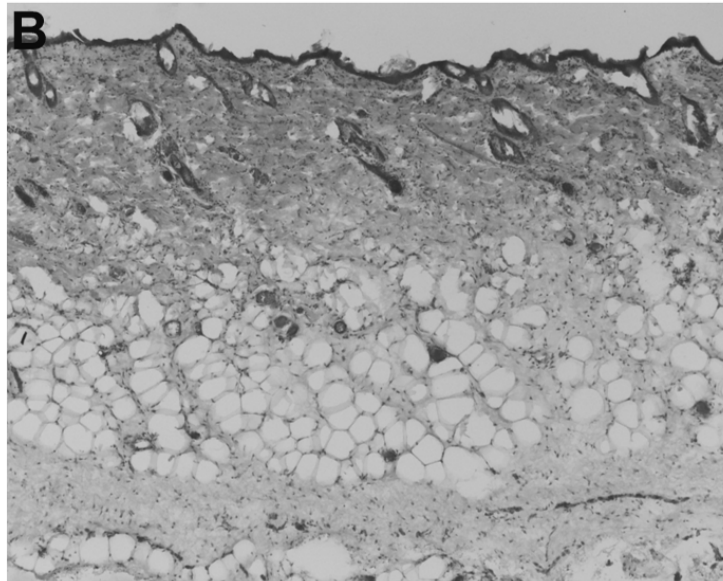
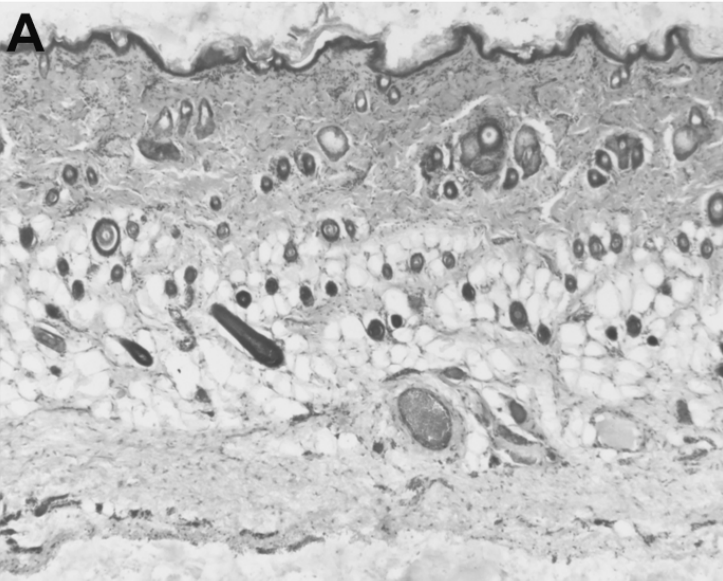
Fig 5. Immunohistochemical analysis of myofibroblasts in the popliteal skin. (A) 1 week control, (B) 1 week after immobilization, (C) 2 weeks after immobilization, (D) 4 weeks after immobilization. (E) The open bar is the control group and the filled bar is the immobilization group. Immobilization significantly increased the ratio of α -SMA positive cells in the dermal plus subcutaneous layers after 2 weeks. Additionally, an increase in the ratio of α -SMA positive cells was observed with immobilization. The arrow indicates α -SMA positive cells. Data is expressed as mean \pm SD. * $p < 0.05$ vs. control; # $p < 0.05$ vs. 1 week immobilization group; † $p < 0.05$ vs 2 weeks immobilization group. Scale bar 20 μ m.

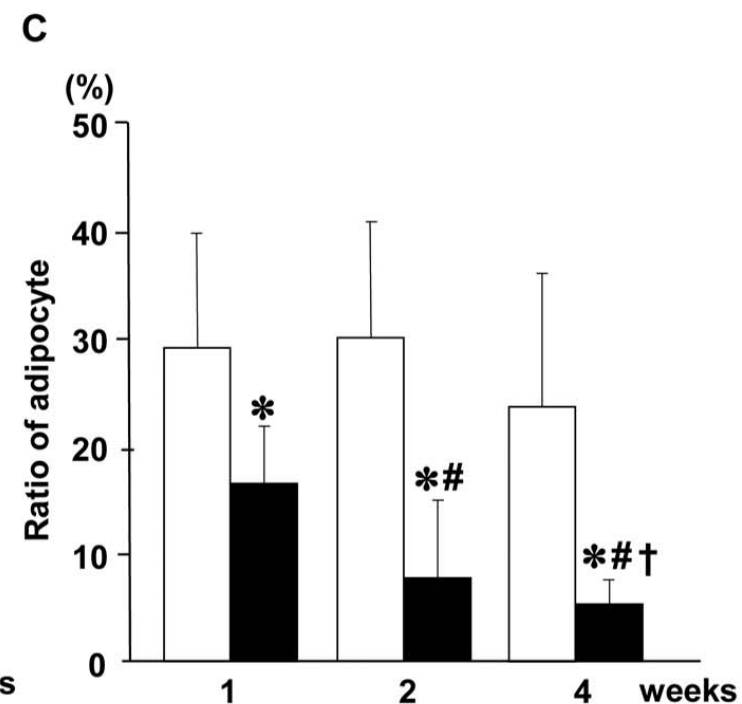
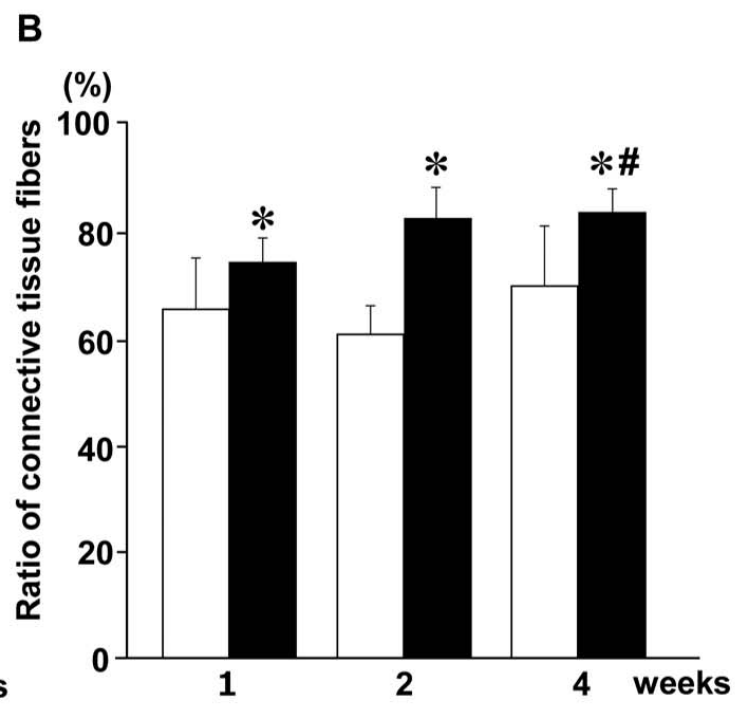
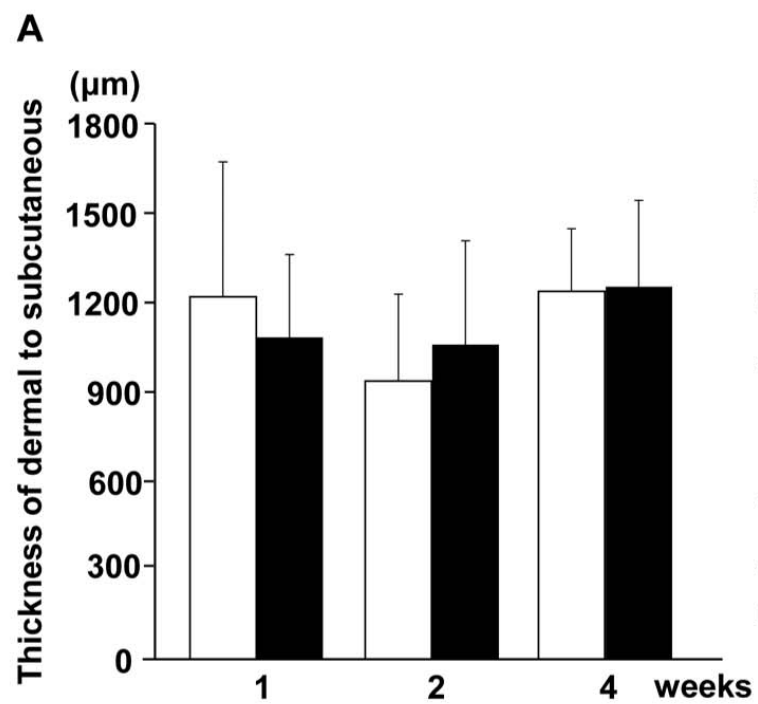
Fig 6. Expression levels of types I and III collagen mRNA in the popliteal skin. The open bar is the control group and the filled bar is the immobilization group. (A) Type I collagen mRNA expression levels in the immobilization group was significantly increased compared to that in the control group was significantly at 1 week after immobilization. Furthermore, time-dependent increase of mRNA expression levels was seen in the immobilization group until 4 weeks after immobilization. (B) Type III collagen mRNA expression levels in the immobilization group was significantly higher

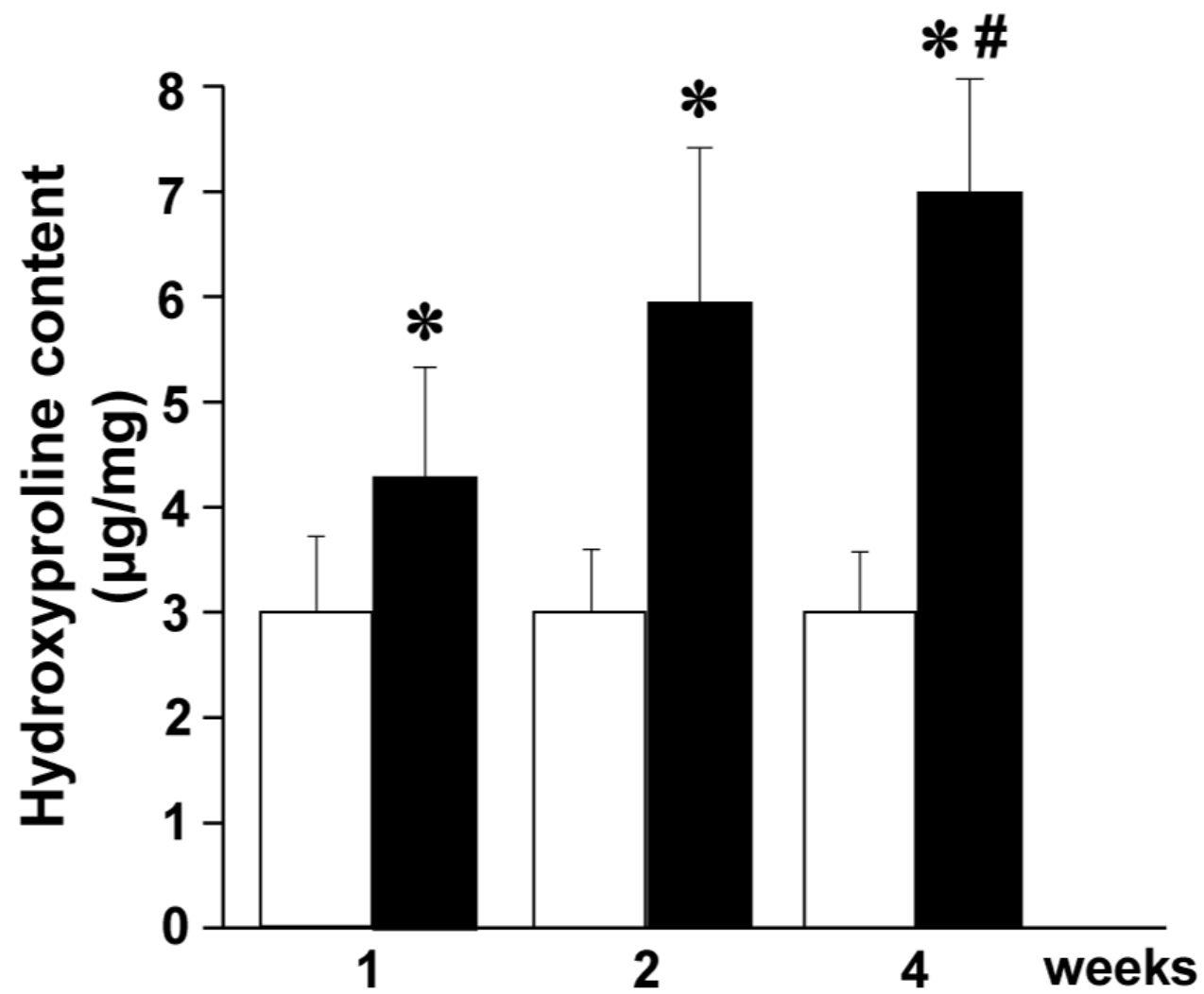
than that of the control group at each immobilization periods. Furthermore, the expression levels after 4 weeks immobilization was significantly greater, compared with either 1 or 2 weeks of immobilization. Data is expressed as mean \pm SD. * $p < 0.05$ vs control; # $p < 0.05$ vs 1 week immobilization group; † $p < 0.05$ vs 2 weeks immobilization group.

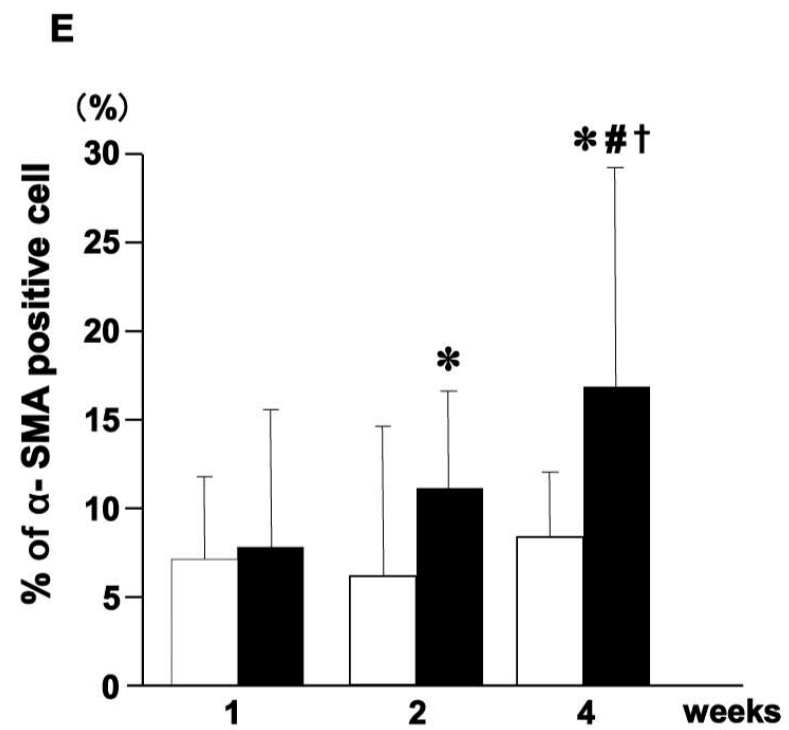
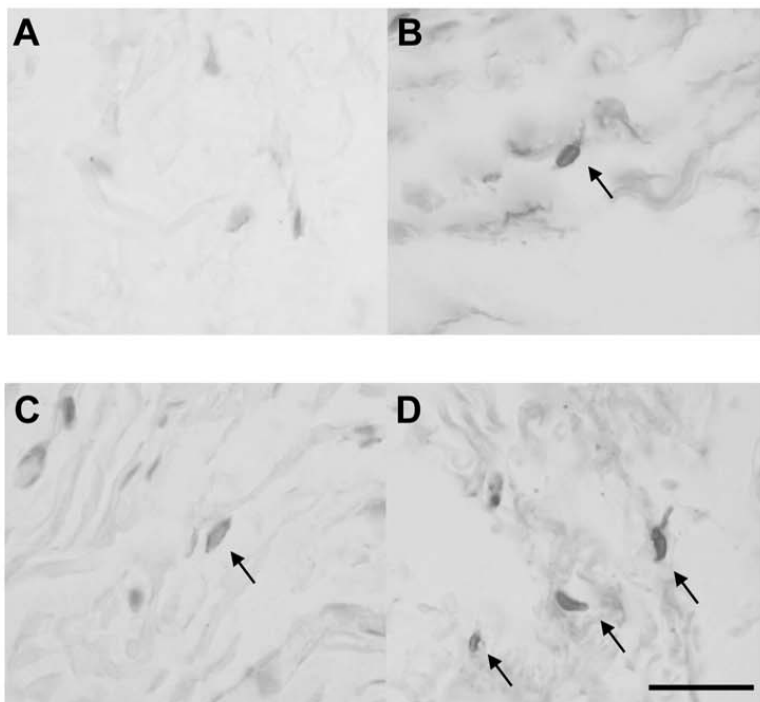
Fig 7. Expression levels of TGF- β 1 and HIF-1 α mRNA in the popliteal skin. The open bar is the control group and the filled bar is the immobilization group. (A) TGF- β 1 mRNA expression levels in the immobilization group was significantly increased compared to that in the control group at 1 week after immobilization; however, immobilization period-depended changes were not observed in the immobilization group. (B) HIF-1 α mRNA expression levels in the immobilization group was significantly higher than that of the control group at each immobilization period. Furthermore, the expression levels after 4 weeks immobilization was significantly greater, compared with either 1 or 2 weeks of immobilization. Data is expressed as mean \pm SD. * $p < 0.05$ vs control; # $p < 0.05$ vs 1 week immobilization group; † $p < 0.05$ vs 2 weeks immobilization group.

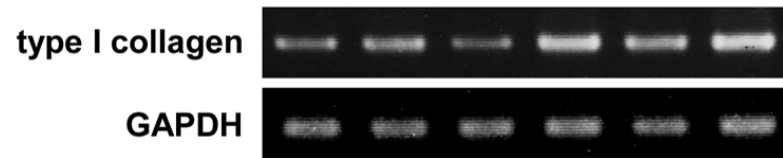
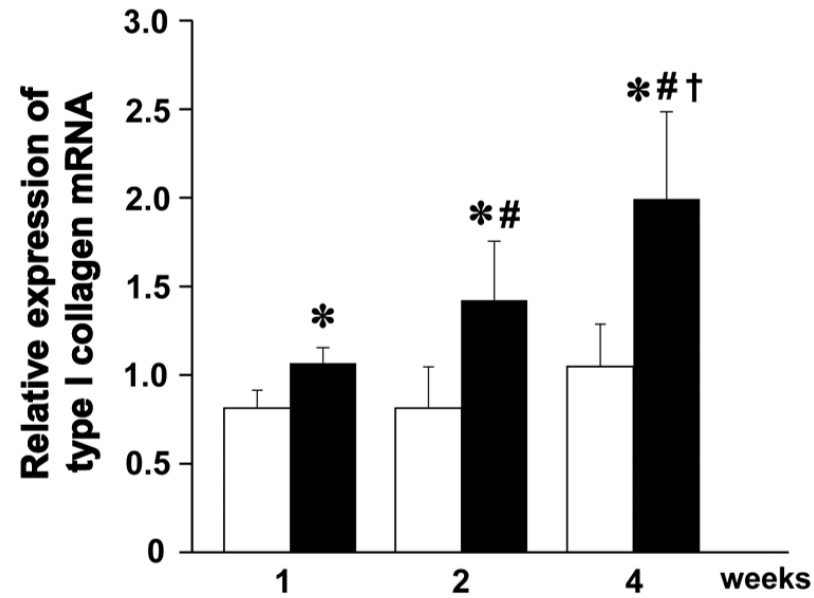
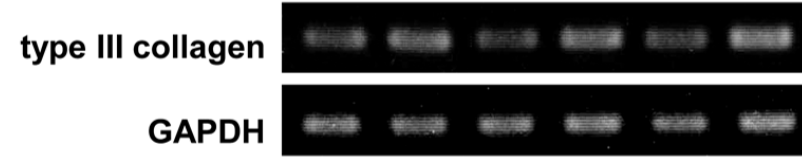
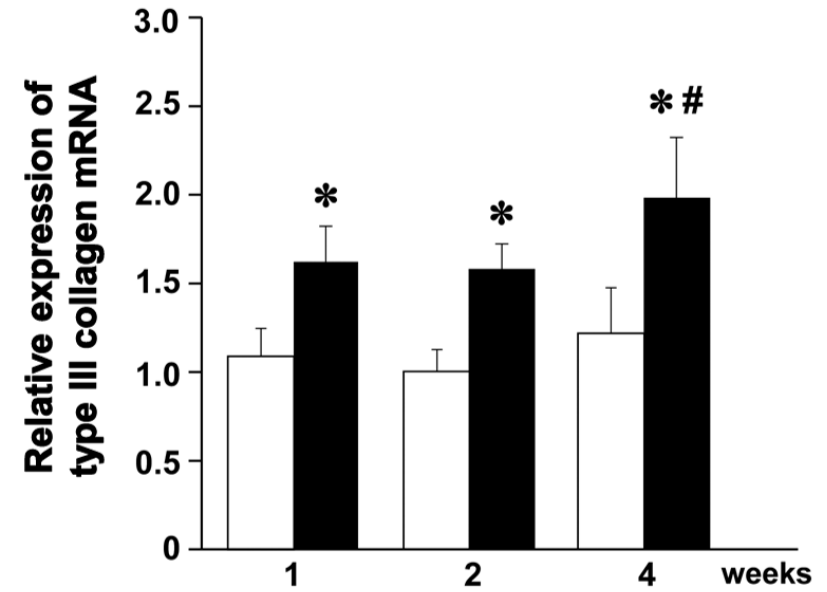


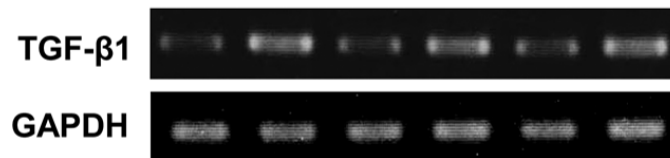
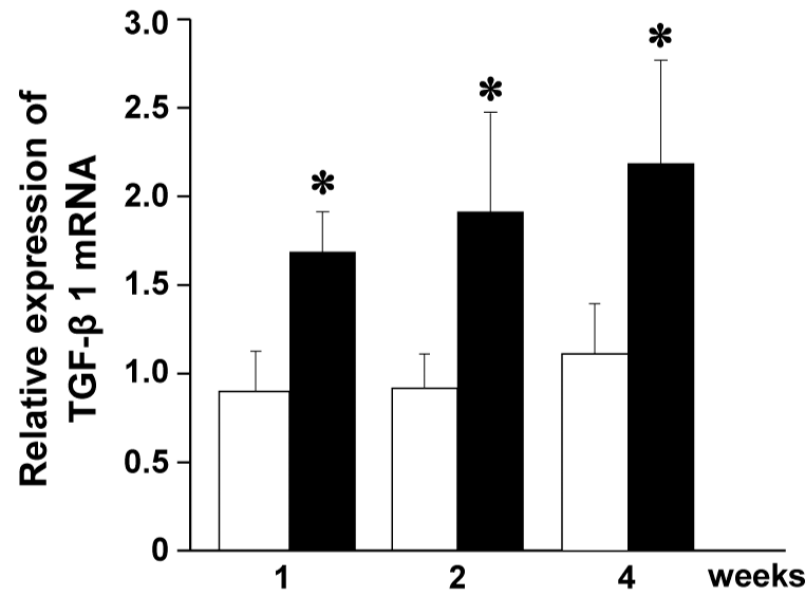








A**B**

A**B**