Regulation of connective tissue remodeling in the early phase of denervation in a rat skeletal muscle

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ABSTRACT
Denervation alters the metabolism of the extracellular matrix (ECM) in skeletal muscle; however, the underlying mechanisms of ECM remodeling are not fully understood. The aim of this study was to elucidate the dynamic features of the ECM regulatory process in the early phase of denervated skeletal muscle in male Wistar rats. We investigated the expression of collagens (total, type I, and type III), transforming growth factor beta 1 (TGF-β1), and matrix metalloproteases (MMPs) together with their endogenous inhibitors (TIMPs), at the mRNA and/or protein level in the soleus muscles of control animals and at days 3, 7, and 14 post-denervation. Expression of mRNA encoding collagens was decreased at days 3 and 7, and had recovered by day 14, in parallel with total collagen protein content. Content of TGF-β1 protein was elevated sequentially, up to a maximum of 158% at day 14 post-denervation (P < 0.05), as was TIMP-2 mRNA expression (272% at day 14), whereas MMP-1, MMP-2, and TIMP-1 mRNA expression was not affected at any stage. The initial reduction of collagen mRNA may be responsible for hypoactivity caused by the disappearance of contractile function. Recovery of collagen mRNA/protein at day 14 may be due mainly to the suppressive effects of TGF-β1 on collagen degradation via TIMP-2 upregulation.

Denervation causes degenerative changes in skeletal muscles, such as a rapid decrease in muscle volume followed by a reduction in the cross-sectional area of myofibers (2, 22, 24), alteration of myofiber types (22), and disarrangement of sarcomere structures (22). Denervation-induced changes are also observed in intramuscular connective tissue. Intramuscular connective-tissue collagen is mainly composed of types I and III (17). The connective tissue has an important role for muscle function. Length-force characteristics have been shown to be altered by the rupture of intramuscular connective tissue after fasciotomy (14). Therefore, changes in the components of intramuscular connective tissues may impair normal skeletal muscle function, often accompanied by a disturbance in joint mobility.

Several studies have reported quantitative changes in intramuscular connective tissue subsequent to non-physiological conditions, but results were inconsistent across studies. Denervation (2, 23), immobilization (13, 24), or unloading (19) lead to increases in collagen concentration in rat calf muscles. Deposition of type-I and -III collagen in the perimysium and epimysium of the gastrocnemius muscle was observed at day 7 post-denervation (23). Concentration of hydroxyproline, a major component of collagen protein, was also increased at 1 and

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The animals were anesthetized by administration of pentobarbital sodium (50 mg/kg). The left sciatic nerve was transected, and the animals were then sacrificed at day 3, day 7, and day 14 post-denervation. Untreated rats aged 8 weeks were used as controls. From 20 animals (n = 5 for each group), the soleus muscles were removed, weighed, and cut into two pieces, the proximal and distal halves. The midsections of the proximal halves were used for histological study, and the midsections of the distal halves were used for subsequent real-time PCR analysis. In addition, soleus muscles from another 20 animals (n = 5 for each group) were used for hydroxyproline analysis, and another 16 animals (n = 4 for each group) were used for ELISA analysis.

Hydroxyproline analysis. After the tendinous parts were carefully removed, whole muscles dissected from each animal were dried at 110°C for 1 h, weighed, and hydrolyzed in 6 N HCl at 130°C for 12 h. Muscle lysates were then neutralized by the addition of 2.5 N NaOH (pH 7), and were incubated with 0.05 M chloramine T for 20 min. The chloramine T was inactivated by the addition of perchloric acid before an equal volume of Ehrlich’s solution was added. After overnight incubation, absorbance of the lysates was read at 557 nm, and the concentration of hydroxyproline in the sample was calculated according to the standard curve. Collagen concentration was estimated using this measurement of hydroxyproline, on the assumption that hydroxyproline accounts for 13.8% of the total collagen (20). The amount of total collagen in each whole muscle sample was then calculated, and finally, total collagen concentration was determined by normalizing the collagen content to the dry mass of the tissue.

Immunohistochemistry. Transverse cryosections were stained with hematoxylin and eosin or type-I and -III collagen immunohistochemically. The cryosections were fixed with 3% formaldehyde in 0.1 M phosphate buffer (pH 7.4), the sections were incubated with a 1 : 4,000 dilution of mouse anti-human collagen type I (C2456, Sigma, USA) or a 1 : 16,000 dilution of mouse anti-human collagen type III (C7805, Sigma). After washing with PBS, the sections were incubated with biotinylated horse anti-mouse IgG secondary antibodies for 30 min, and then incubated with streptavidin-horseradish peroxidase for 30 min. The specimens were
then washed with PBS, and immunoreactivity was visualized with 0.05% 3,3'-diaminobenzidine and 0.01% H2O2 in 0.05 M Tris-HCl buffer (pH 7.2).

**Real-time PCR.** Stabilized muscle tissues were homogenized with TRIzol reagent (Invitrogen, USA). After homogenization, chloroform was added to each tube and mixed. The samples were then centrifuged and the aqueous phase was transferred to fresh tubes. Samples of total mRNA were extracted using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer’s instructions. The integrity of the RNA was determined by electrophoresis. Using total mRNA and the SuperScript III First-strand synthesis system (Invitrogen), cDNA was prepared by reverse transcription. Quantitative analysis of mRNA was performed with the ABI-7300 Fast Real-time PCR system (Applied Biosystems, USA) using Taqman Gene Expression Assays (Applied Biosystems) for type-I collagen (COL1A1; Rn01463849_m1), type-III collagen (COL3A1; Rn01437681_m1), TGF-β1 (Rn00572010_m1), MMP-1 (Rn01772484_g1), MMP-2 (Rn01538170_m1), TIMP-1 (Rn00587558_m1), TIMP-2 (Rn00573232_m1) mRNA, and ribosomal protein S18 rRNA (Rn01428913_g). All mRNA levels were determined as relative ratios to the level of ribosomal protein S18 RNA in the same cDNA sample.

**Enzyme-linked immunosorbent assay (ELISA).** Midsections of each muscle were homogenized with a 20-fold volume of buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA) and 0.01% Triton X-100. TGF-β1 protein concentration was measured using the Quantikine Mouse/Rat/Porcine/Canine TGF-β1 immunoassay (R & D Systems, USA) according to the manufacturer’s instruction.

**Statistics.** To compare the differences between denervated and intact muscles, the Mann-Whitney U test was applied to muscle wet weight data. The Kruskal-Wallis test was applied to mRNA data to compare the differences between muscles from untreated control rats, and muscles at different stages of post-denervation. Post hoc comparisons with the control and the various stages of denervation were performed using Dunnett’s T3 test. Muscle wet weight and TGF-β1 protein expression were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey HSD test. For all tests, statistical significance was accepted as P < 0.05.

**RESULTS**

The wet weights of the denervated muscles decreased progressively from day 3 to 14 (P < 0.05), while that of the intact contralateral muscles increased, resulting in a progressive decrease in the wet-weight ratio of the right to left soleus muscles (R/L) (Table 1).

Histologically, the soleus muscle fibers showed an orderly and tightly packed arrangement in the controls (Fig. 1A). At day 3 post-denervation, muscle fibers were slightly smaller than those in the controls, a trend that progressed until day 14 (Figs. 1B–D). Both type-I collagen (Fig. 1E) and type-III collagen (Fig. 1I) were observed in the intermuscular connective tissue surrounding the nerves and blood vessels, and the perimysium in the control muscles. In the endomysium, low levels of type-III collagen were seen. At day 3 post-denervation, expression of type-I (Fig. 1F) and -III collagen (Fig. 1J) was very similar to controls. At day 7, expression of type-I and -III collagen was increased in the intramuscular spaces, accompanied by thickening of the perimysium and endomysium (Fig. 1G, K). Collagen deposition was more pronounced at day 14 (Fig. 1H, L).

Collagen protein concentration, expressed as collagen weight per dry mass of muscle, was significantly increased at day 14 (99%, 104%, and 142% at days 3, 7, and 14 post-denervation, respectively) (Fig. 2A). On the other hand, total collagen protein content in whole muscles was transiently decreased as compared with the level of the controls at day 3.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Changes in the body weight and the muscle mass</th>
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<tr>
<td>Group</td>
<td>body weight, g</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>260 ± 6</td>
</tr>
<tr>
<td>3 days</td>
<td>262 ± 2</td>
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<tr>
<td>7 days</td>
<td>290 ± 5</td>
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<tr>
<td>14 days</td>
<td>324 ± 4</td>
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Values are means ± SE (14 animals in all groups except control with 11 animals).

*: Significantly different from intact sides (P < 0.05).
and 14 (P < 0.05), respectively (Fig. 4B).

MMP-1 mRNA expression was nearly absent in muscles from all groups (data not shown). MMP-2 mRNA expression was 42%, 167%, and 60% at days 3, 7, and 14, respectively (Fig. 5A). TIMP-1 mRNA expression was 48%, 97%, and 98% at days 3, 7, and 14, respectively (Fig. 5B). MMP-2 mRNA and TIMP-1 mRNA expression was not significantly affected by denervation at any time point. On the other hand, TIMP-2 mRNA expression was suppressed at day 3 (38%), and subsequently increased at day 7 (136%) and day 14 (272%, P < 0.05) (Fig. 5C) compared to controls.

DISCUSSION

The present study revealed that type-I and -III colla-
Collagen remodeling in muscle expression was increased in the perimysium, endomysium, and the intramuscular spaces surrounding the nerves and blood vessels at day 7 and day 14 post-denervation. These temporal histological changes are in agreement with a previous study in denervated rat gastrocnemius muscles (23). However, in our study, total collagen protein content, as calculated by the measurement of hydroxyproline, decreased at day 3 and day 7 ($P < 0.05$) post-denervation. This paradox can be explained by the rapid decrease in the relative volume of the soleus muscle post-denervation. The fibrotic changes characterized by immunohistochemistry do not reflect an increase in total collagen protein content, but rather a decrease in the concentration of collagen protein in atrophied skeletal muscle.

The expression of $COL1A1$ and $COL3A1$ mRNA was decreased at day 3 post-denervation, although gradual recovery of mRNA levels was observed during the following period, up to day 14. In the literature, type-I collagen mRNA expression in unloaded soleus muscle decreased transiently at day 3 (12), or remained unchanged at day 7 and day 14 (11), and day 28 (19). Type-III collagen mRNA expression tended to be reduced (11) or was unchanged at day 28 (19). In immobilized rat models, type-I collagen mRNA expression was decreased transiently at day 3, and type-III collagen mRNA expression was decreased at day 3 and day 7 in the soleus muscle (10). In addition, type-I and -III collagen mRNA expression increased 10-fold in the soleus muscle after hindlimb reloading following 14 days of unloading (11). These data indicate that mRNA expression of collagens may depend on muscle activity and/or mechanical tension. Thus, it is suggested that rapid reduction of collagen mRNA expression is responsible,
In the present study, TGF-β1 expression was increased progressively in denervated muscles with concomitant suppression of collagen mRNA expression. However, the suppression of collagen mRNA was transient and exhibited a tendency towards recovery between day 3 and day 14 post-denervation, which was well synchronized with an increase in TGF-β1 expression. TGF-β1 might be active in stimulating collagen synthesis as well as inhibiting collagen degradation.

Recovery of collagen protein content at day 14 post-denervation may also be explained by the low degradation rate of collagen proteins. The rate of collagen degradation is determined by the activities at least in part, for the hypoactivity caused by contractile dysfunction in the initial phases of denervated muscles.

Generally, collagen metabolism is due to either increased synthesis or decreased degradation, or a combination of both mechanisms. In this study, COL1A1 and COL3A1 mRNA expression appeared to decrease transiently at day 3 or 7, but had recovered at day 14 post-denervation. TGF-β1 acts as a potent inhibitor of collagen degradation, whilst also acting as a stimulator of collagen synthesis. The stimulating action of TGF-β in collagen synthesis was confirmed by the suppressive action of TGF-β neutralizing antibody on the expression of type-I and -III collagen mRNA in hypertrophied myocardial tissue (18), or the suppressive action of a TGF-β antagonist on fibroblast proliferation and fibrotic protein expression in lacerated skeletal muscles (6).

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Recovery of collagen protein content at day 14 post-denervation may also be explained by the low degradation rate of collagen proteins. The rate of collagen degradation is determined by the activities
of MMPs relative to their inhibitors, such as TIMPs (27). Whereas MMP collagenases (MMP-1, -8, -13, and -18) have an ability to cleave types I, II, and III collagen, MMP gelatinases (MMP-2 and -9) degrade denatured collagen (29). In skeletal muscles, MMPs and TIMPs have been mainly studied during stages of degeneration and inflammation, and scar tissue formation (4). In denervated muscle, especially in the early phases when it has not yet been reinnervated, there is little information about the role of MMPs and TIMPs. Whereas several kinds of MMP were studied using gene expression (7), enzymatic activity (7), and localization by immunohistochemistry at muscle fibers, neuromuscular junctions, vessels, nerves, and fibroblast-like cells (7, 15, 21), the expression of TIMPs has never been examined in previous studies. In accordance with the findings that MMP-2 mRNA expression and immunohistochemical localization were unchanged up to 30 days after denervation or reinnervation (7, 15), our results showed no significant modification of MMP-2 mRNA expression by denervation. On the other hand, expression of TIMP-2 mRNA was significantly increased at day 14 post-denervation. These results suggest that increased TIMP-2 may inhibit collagen degradation via inhibition of the activity of MMPs, presumably including MMP-2. In addition, it is important to note that TGF-β1 regulates TIMP-2. A previous study reported that the expression of TIMP-2 was increased in fibrotic myocardium induced by overexpression of TGF-β1 in transgenic mice (25). In the present study, we demonstrated a progressive increase in TGF-β1 and TIMP-2 expression until day 14 post-denervation, with suppressed expression of mRNA encoding collagen proteins. Taken together, increased collagen deposition in denervated muscles may be established mainly by TGF-β1’s suppressive effects on collagen degradation via TIMP-2 upregulation.

Previous studies have reported that mechanical stretching modulates TGF-β1 and type-I collagen in various type of cells. The expression of TGF-β1 and type-I collagen mRNA and protein was upregulated by mechanical stretching in cultured intestinal smooth muscle cells (9) and fibroblasts (3). In contrast, brief static stretching could decrease TGF-β1 and type-I collagen in subcutaneous connective tissue (5). Therefore, an appropriate amount of stretch needs to be given to denervated muscle in order to inhibit skeletal muscle fibrosis through the action of TGF-β1. Further studies are required to identify the effective amount and timing of such therapeutic intervention for fibrosis in skeletal muscle.

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