Tohoku J. Exp. Med., 2016, **238**, 49-56

Expression of TGF-β1 and CTGF Is Associated with Fibrosis of Denervated Sternocleidomastoid Muscles in Mice

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Injury to the recurrent laryngeal nerve often leads to permanent vocal cord paralysis, which has a significant negative impact on the quality of life. Long-term denervation can induce laryngeal muscle fibrosis, which obstructs the muscle recovery after laryngeal reinnervation. However, the mechanisms of fibrosis remain unclear. In this study, we aimed to analyze the changes in the expression of fibrosis-related factors, including transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), and α-smooth muscle actin (α-SMA) in denervated skeletal muscles using a mouse model of accessory nerve transection. Because of the small size, we used sternocleidomastoid muscles instead of laryngeal muscles for denervation experiments. Masson's trichrome staining showed that the grade of atrophy and fibrosis of muscles became more severe with time, but showed a plateau at 4 weeks after denervation, followed by a slow decrease. Quantitative assessment and immunohistochemistry showed that TGF-β1 expression peaked at 1 week after denervation ($p < 0.05$) and was maintained at its high level until 4 weeks. CTGF- and α-SMA-positive muscle cells were detected at 1 week after denervation, peaked at 2 weeks ($p < 0.05$), and remained at high levels with a subsequent slight decrease for 3-4 weeks. These results suggest that TGF-β1 and CTGF may be involved in the process of denervated skeletal muscle fibrosis. They may induce the differentiation of myoblasts into myofibroblasts, as characterized by the activation of α-SMA. These findings may provide insights on key pathological processes in denervated skeletal muscle fibrosis and develop novel therapeutic strategies.

Keywords: connective tissue growth factor; denervation; fibrosis; skeletal muscle; transforming growth factor-β1
Tohoku J. Exp. Med., 2016 January, **238** (1), 49-56.

Introduction

Surgical or traumatic disruption of the recurrent laryngeal nerve may cause a series of issues, such as unilateral vocal cord paralysis, leading to phonatory disorders, dyspnea, and aspiration, which greatly impair the quality of life (Koyanagi et al. 2015). With the development of microsurgical techniques, considerable success has been achieved in restoring the physiological function of denervated laryngeal muscles by laryngeal reinnervation, thereby enhancing phonation and optimizing vocal quality (Zheng et al. 1996; Donghui et al. 2010; Li et al. 2013, 2014). However, the efficacy of delayed reinnervation remains unsatisfactory. According to available data, the regenerative potential of denervated skeletal muscles may be dependent on denervation-related changes in the affected muscles, such as progressive myofiber atrophy, as well as persistent fibrotic changes, and eventually, irreversible pathological change (Jergovic et al. 2001; Sato et al. 2003). This fibrosis can obstruct the recovery of muscle fibers and prevent full strength recovery, which may be a primary factor in the tendency for injury to recur after denervation (Borisov et al. 2000, 2005). However, very little is known regarding the molecular mechanisms of fibrosis in denervated skeletal muscle.

Transforming growth factor-β1 (TGF-β1) and its downstream targets, especially the fibrotic effector connective tissue growth factor (CTGF), play important roles in

Received January 27, 2015; revised and accepted November 20, 2015. Published online December 19, 2015; doi: 10.1620/tjem.238.49.
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fibrotic lesions in multiple organs, such as the liver, kidneys, and lungs; namely, these factors may be potential targets for the prevention of fibrosis (Lijnen et al. 2000; Denton and Abraham 2001; Hersh et al. 2006; Lan 2011). TGF-β1 is also expressed and is associated with the onset of muscle fibrosis in dystrophic muscle (Gosselin et al. 2004) and chronic inflammatory muscle disease (Lundberg et al. 1997). The C2C12 mouse myoblast cell line and muscle laceration models were used to investigate the autocrine expression of TGF-β1 and its fibrotic effects. The results showed that overexpression of TGF-β1 stimulated myoblasts to differentiate into fibrotic cells in vivo, and that this differentiation process could be prevented with a TGF-β1 inhibitor (Li et al. 2004). Accordingly, we hypothesized that TGF-β1 and CTGF would also play a pivotal role in denervated skeletal muscle fibrosis.

In this study, we used a mouse model of accessory nerve transection to investigate the expression of and changes in fibrosis-related factors, namely TGF-β1, CTGF, and α-smooth muscle actin (α-SMA), in denervated sternocleidomastoid muscle. Due to experimental difficulties associated with the very small dimensions of the larynx and the recurrent laryngeal nerve in mice, we have opted for the sternocleidomastoid muscle as a head and neck skeletal muscle model for denervation experiments. A better understanding of the fibrosis-related factors TGF-β1, CTGF and α-SMA in muscle fibrosis may provide insights on key pathological processes in denervated skeletal muscle fibrogenesis, potentially leading to the development of novel therapeutic strategies.

Materials and Methods

Animals, surgical procedure, and tissue collections

All of the animal experiments were conducted in compliance with the Rules of Animal Experimental Ethics and the Guidelines for the Care and Use of Laboratory Animals in accordance with the requirements of the Ethics Committee of the Second Military Medical University. We also followed the National Institutes of Health Guidelines for the Care and Use of Animals. In total, 60 adult male C57BL/6 mice, weighing 20-30 g and between 8 and 10 weeks old, were purchased from the Experimental Animal Center of the Second Military Medical University (Shanghai, China). Mice were housed with free access to water and standard rodent chow. The 60 mice were divided randomly into 6 groups: control and 1-, 2-, 3-, 4-, and 8-week post-denervation groups. Mice were anesthetized with intraperitoneal injection of 4% chloral hydrate at a dose of 0.5 mL/100 g, and placed in a supine position. A surgical cut was made at the center of the anterior neck, the accessory nerves were exposed up to the skull base. The sternocleidomastoid muscles were denervated by cutting the accessory nerve trunk on the left side at a site 0.5 cm from the skull base. The nerve stump was ligated using a 5-0 silk thread. Accessory nerves were exposed but without cutting in the control mice. After recovery, the mice were housed under normal conditions. Mice were sacrificed and their sternocleidomastoid muscles were collected at each time point. Each muscle sample was divided into four quarters for histological staining, real-time quantitative PCR, and Western blot analysis.

Masson's trichrome staining

One quarter of each muscle was fixed in 4% paraformaldehyde (PFA) for 24 h, followed by routine dehydration, infiltration, and embedding in wax. Paraffin wax sections were cut at 8 μm. After dewaxing and rehydration, sections were washed in distilled water, after which Masson's trichrome staining was performed according to the kit's instructions (Jiancheng Bioengineering Institute, Nanjing, China). The staining showed the nuclei in black, muscles in red, and collagen in blue. Images were captured with a Nikon Eclipse 600 microscope (Tokyo, Japan) in three different fields within the injured area, and the absolute area of the scar was measured using IPP 5.0 software. We averaged the values of all tissue sections from the 10 mice in each group.

Immunofluorescence staining

One quarter of muscle was also fixed in 4% PFA overnight, gradually dehydrated through 15% and 30% sucrose in phosphate-buffered saline (PBS), and embedded in Tissue-Tek Optimum Cutting Temperature (OCT, Sakura Finetek Inc., Torrance, CA, USA). Cryosections (8 μm) were blocked in 20% normal goat serum in 0.5% Triton X-100/PBS for 15 min, and then incubated overnight at 4°C with primary antibody against TGF-β1 at 1:200 dilution (R&D Biotechnology, Minneapolis, MN), CTGF at 1:200 (R&D Biotechnology) and α-SMA at 1:100 dilution (Sigma Chemical, St. Louis, MO). After washing, sections were incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1h at 37°C, and counterstained with DAPI (Sigma Aldrich Inc., St. Louis, MO). Sections incubated with PBS instead of the primary antibodies were used as negative controls. Images were captured with Nikon Eclipse 600 fluorescence microscope (Tokyo, Japan) at a magnification of ×40, and the positive cells visible in the photomicrograph were counted in five random vision fields for three sections of each case.

Real-time quantitative PCR

Total RNA was extracted from the muscle samples using Trizol (Invitrogen, Grand Island, NY). Reverse transcription was performed according to the protocol with the PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd, Dalian, China). The locations and specific sequences of primers were chosen to exclude the detection of genomic DNA by placing one of the primers over a junction between two exons. The primer sequences for these target genes were as follows: CTGF: forward primer 5′-GGA CAC GAA CTC ATT AGA C-3′, reverse primer 5′-TCT CAC TTT GGT GGG ATA G-3′; TGF-β1: forward primer 5′-AAG GAC CTG GGT TGG AAG TG-3′, reverse primer 5′-TGG TTG TAG AGG GCA AGG AC-3′; α-SMA: forward primer 5′-AAC ACG GCA TCA TCA CCA AC-3′, reverse primer 5′-CAC AGC CTG AAT AGC CAC ATA C-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer 5′-ATC ACT GCC ACC CAG AAG-3′, reverse primer 5′-TCC ACG ACG GAC ACA TTG-3′.

Real-time quantitative PCR was performed using the reagents and protocol supplied with a SYBR Premix Ex TagTM II kit (TaKaRa Biotechnology). All of the reactions were performed in triplicate for 40 cycles with an annealing temperature of 60°C and cDNA was amplified. Data were collected and analyzed on a Rotor Gene Q real-time PCR cycler (Qiagen Pty Ltd, Don caster, Victoria, Australia). Relative expression levels of target genes were analyzed using the 2-$\Delta\Delta$CT quantitative method. The mRNA level in the control group

was set at 1, and the other groups are shown as fold difference relative to the control group. GAPDH was used as the reference gene.

Western blot analysis

Muscle samples were homogenized in lysis buffer (Applied Biosystems, USA) containing 2 mM orthovanadate and protease inhibitors. Protein concentrations were determined with a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA). Proteins (30 μg) in each sample were denatured with the addition of Laemmli buffer, separated on a polyacrylamide (8%) gel, transferred to a PVDF (Millipore Co., Billerica, MA, USA) membrane, and probed with primary antibodies to TGF-β1, CTGF, and α-SMA at a 1:1,000 dilution. Signals were visualized and photographed using the Fluorchem FC2 system (Alpha Innotech Co., San Leandro, CA, USA) equipped with an imaging software that automatically analyzed the density of each band. GAPDH served as the internal control.

Statistical analysis

All of the data are presented as means ± standard deviations. The data was analyzed using the SPSS software (ver. 18.0) to examine if they satisfied the test of normality of the distribution, and the homogeneity test of variance, for which ANOVA and t-tests were used to determine significant differences between groups. Otherwise, the non-parametric Mann-Whitney U-test was used.

Results

Morphological studies on the denervated sternocleidomastoid muscle

Results of the Masson's trichrome staining showed that the sternocleidomastoid muscle samples in the C57BL/6 mice obviously underwent atrophy with denervation time, and were significantly smaller in the experimental groups when compared to the control group. The longer the time after denervation, the smaller the average diameter of muscle fibers and muscle cross sectional area. Simultaneously, the cross sectional area of collagen increased gradually (Fig. 1A). By statistical comparison, muscle cross sectional areas and average diameters of muscle fibers in the 3-, 4- and 8- week post-denervation groups were significantly smaller than those in the control group ($p < 0.05$) (Fig. 1B, C). The cross sectional areas of connective tissue in the 3-, 4- and 8-week post-denervation groups were significantly larger than those in the control group ($p < 0.05$) (Fig. 1B). Significant differences in the three parameters were observed between the 4-week post-denervation group and normal control group, the 1-week to 3-week post-denervation groups respectively (all $p < 0.05$). However, no signifi-

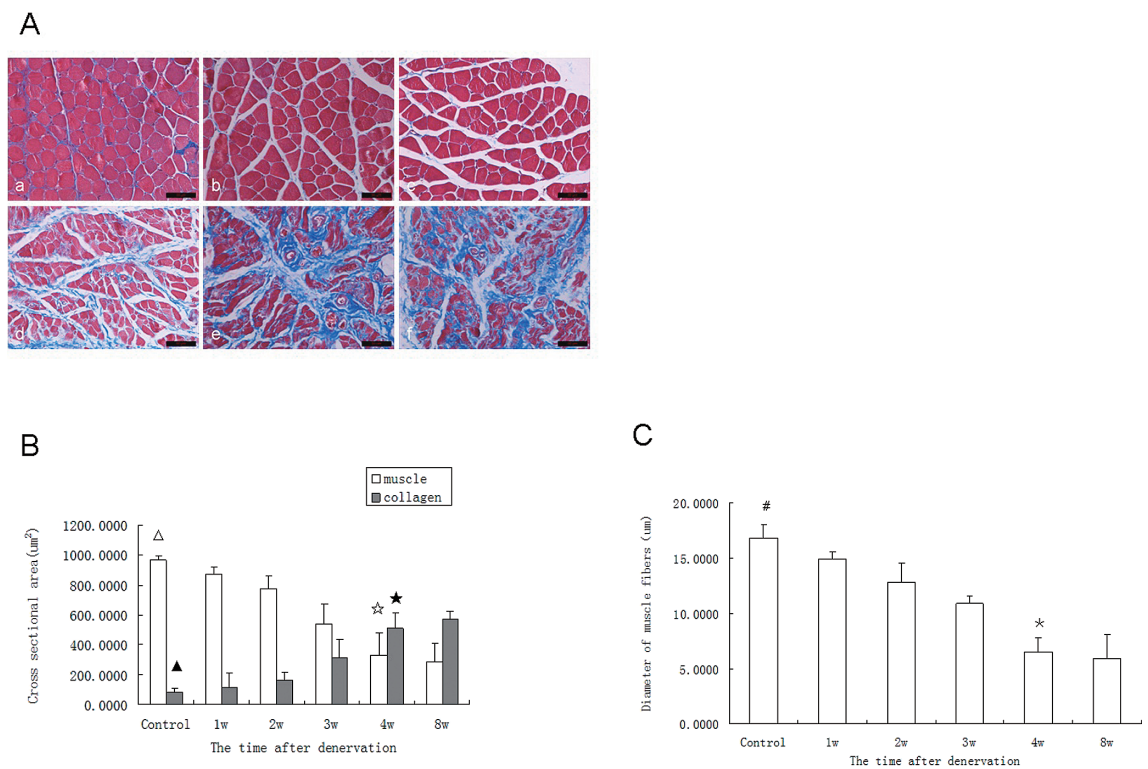

Fig. 1. Morphological changes in the sternocleidomastoid muscle after denervation.
A. Masson's trichrome staining shows the nuclei in black, muscle in red, and collagen in blue (40×). Parts a to f show normal muscle, and 1-, 2-, 3-, 4-, and 8-week post-denervation, respectively, with muscle fibrosis worsening with time after denervation. Cross sectional areas of muscles and average diameters of muscle fibers in the denervated groups correlated negatively with time post-denervation. whereas cross sectional areas of connective tissues correlated positively with time post-denervation. B, C. Quantitative analysis of cross sectional areas of muscles, cross sectional areas of connective tissues and average diameters of muscle fibers.
△, ▲, #: $p < 0.05$, vs. the 3-, 4-, and 8-week denervation groups for the three parameters respectively. ☆, ★, ⁎: $p < 0.05$, vs. the control, the 1-, 2-, and 3-week denervation groups, respectively.

cant differences in these three parameters were found between the 4- and 8-week post-denervation groups ($p > 0.05$) (Fig. 1B, C).

Expression of fibrotic factors in sternocleidomastoid muscle fibers in response to denervation

Immunofluorescence staining of cryostat sections revealed that TGF-β1, CTGF, and α-SMA were normally expressed at low levels in the control sternocleidomastoid muscle cells (Fig. 2A). For the experimental groups, however, there was strong expression of TGF-β1, reaching a peak (12 ± 3 per 100 myofibers) 1 week after denervation in the denervated muscle cells (Fig. 2B), compared with other denervation groups ($p < 0.05$). CTGF- and α-SMA-positive muscle cells were detected 1 week after denervation, and increased markedly in the number, reaching a peak (20 ± 2 and 34 ± 3 per 100 myofibers, respectively) at 2 weeks (Fig. 2C), compared with other denervation groups ($p < 0.05$). After that time point, the positive muscle cells decreased gradually in their number with time, but the staining intensity continued at a high level (Fig. 2D, E). The immunostaining showed that all of the three genes were located in the cytoplasm and membrane of muscle cells. These findings indicated that TGF-β1 was expressed within myofibers in denervated skeletal muscle at early time points after injury. As a downstream target, the fibrotic

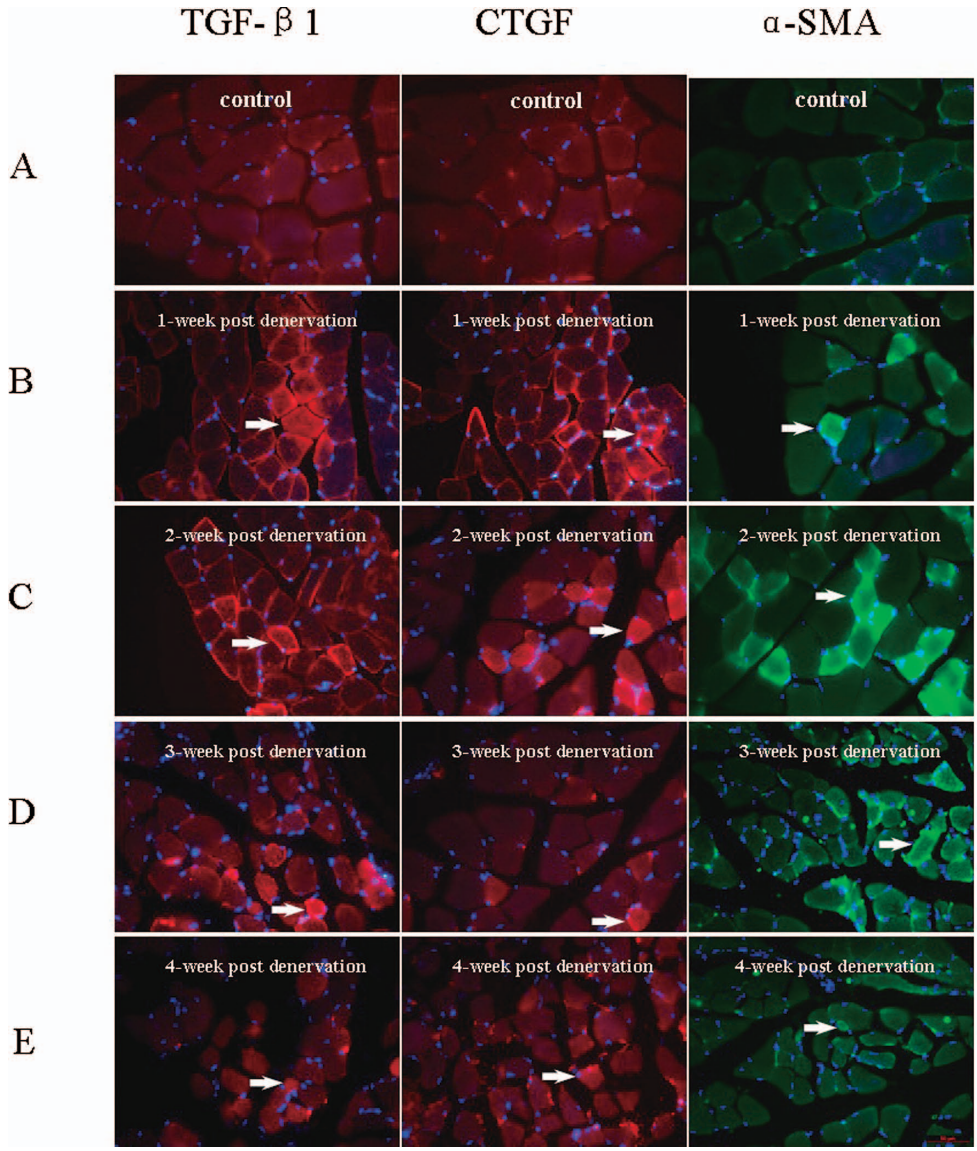

Fig. 2. Expression and localization of fibrosis-related factors.
The expression and location of TGF-β1 (red), CTGF (red), and α-SMA (green) as detected by immunofluorescent staining. DAPI is shown in blue. TGF-β1, CTGF, and α-SMA are shown at normal low levels in sternocleidomastoid muscle cells (A). TGF-β1 expressed strongly and reached a peak one week after denervation (arrowhead), and CTGF- and α-SMA-positive muscle cells (arrowhead) were also detected (B). Expressions of CTGF and α-SMA in muscle cells (arrowhead) reached a peak two weeks after denervation (C). Positive muscle Cells positive for the three fibrosis-related factors were still detected (arrowhead) 3 and 4 weeks after denervation (D) (E) (40×).

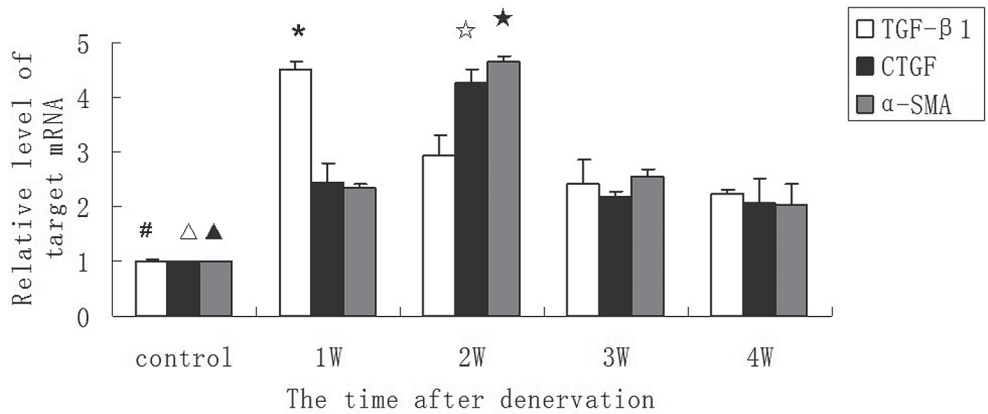

Fig. 3. Changes in TGF-β1, CTGF, and α-SMA mRNA levels in the sternocleidomastoid muscle after denervation. #, △, ▲: $p < 0.05$, vs. the 1-, 2-, 3-, and 4-week denervation groups for TGF-β1, CTGF, and α-SMA, respectively. *: $p < 0.05$, vs. the control, the 2-, 3-, and 4-week denervation groups, respectively. ☆, ★: $p < 0.05$, vs. the control, the 1-, 3-, and 4-week denervation groups, respectively.

effector CTGF was likely synthesized in myofibers in response to TGF-β1, and was thereby subsequently detected. During this process, myofibers became atrophic and when myofibers were gradually differentiated into myofibroblasts, as characterized by becoming positive for the fibrotic marker α-SMA.

mRNA expression of TGF-β1, CTGF, and α-SMA in sternocleidomastoid muscles after denervation

Real-time quantitative PCR showed that the relative transcript level of TGF-β1 significantly increased and reached a peak 1 week after denervation (Fig. 3), which was higher than the level in all of the other groups ($p < 0.05$). While CTGF and α -SMA were significantly upregulated 1 week after denervation, the highest relative transcript levels were observed in the group 2 weeks after denervation, as compared to the levels in all of the other groups ($p < 0.05$). Next, the relative transcript levels of the three factors declined, but were still higher at the following time points than in the control group ($p < 0.05$). No significant difference in relative expression levels of TGF-β1 were found between each pair of the denervation groups beyond 1 week ($p > 0.05$). There was no difference in relative expression levels of CTGF or α-SMA in the groups between the 3-week and 4-week post-denervation ($p > 0.05$).

Proteins levels of TGF-β1, CTGF, and α-SMA in sternocleidomastoid muscles after denervation

The levels of TGF-β1 protein expressions reached a peak 1 week after denervation, which were significantly higher than those in all other groups ($p < 0.05$). The levels then decreased, although they were still significantly higher than the control ($p < 0.05$). Optical density analyses of each band normalized to GAPDH showed that the highest mean proteins levels of CTGF and α-SMA were observed in the groups at 2 weeks post-denervation. These values were significantly different from those of all other groups ($p < 0.05$). After this time point, downregulation of both of these factors was observed (Fig. 4). The variation patterns in protein levels were consistent with the PCR results.

Discussion

The negative effects of denervation in skeletal muscles, such as irreversible atrophy, extracellular matrix (ECM) formation, and connective tissue accumulation, may be among the major reasons for the poor prognosis in reinnervation (Borisov et al. 2000, 2005). Although protecting denervated skeletal muscles from fibrosis is key to successful reinnervation, including laryngeal reinnervation, the mechanisms of fibrosis remain unclear, and to date, there is no effective method.

Many investigators have focused on the mechanisms underlying hepatic and renal fibrosis and methods to prevent fibrosis. In recent years, studies on TGF-β1 showed that it is an effective factor in inducing tissue fibrosis, and plays an important role in the process of fibrosis in the kidney, liver, lung, and heart (Lijnen et al. 2000; Hersh et al. 2006; Lan 2011). Its functions in muscle fibrosis are also significant (Li and Huard 2002; Li et al. 2004; Andreetta et al. 2006). In addition, activated TGF-β1 can eventually lead to fibrosis (Gressner and Weiskirchen 2006). TGF-β1 also has multiple functions as a cytokine, and is indispensable in various processes with anti-inflammatory and antitumor effects (Massague et al. 2000). TGF-β1 knockout mice showed loss-of-function in inhibiting inflammation and died of severe global inflammation (Letterio and Bottinger 1998). Prenatal lethality of TGF-β1 knockout mice, which occurred about 10 days post-conception, was due to abnormal vasculogenesis during the embryonic period (Dickson et al. 1995). Because of the multiple target cell types and complex biological effects, the outcomes of blocking or inhibiting TGF-β1 to prevent fibrosis are unpredictable, which has restricted the development of therapies targeting this protein.

CTGF is a specific downstream effector of TGF-β1; its fibrogenic effects have attracted much attention. Recently,

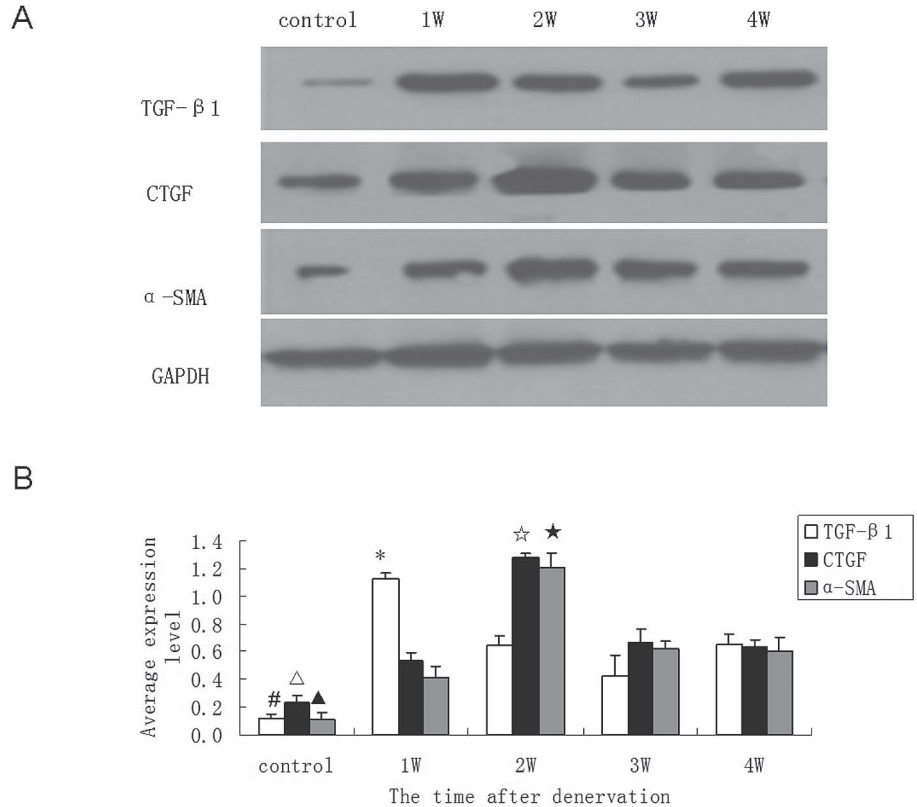

Fig. 4. Western blot analysis for expression of TGF-β1, CTGF and α-SMA in denervated sternocleidomastoid muscles. (A) A representative image of a Western blot, repeated at least three times, showed that TGF-β1, CTGF, and α-SMA proteins were detected after denervation. (B) Further optical density analyses showed that the highest transcription level of TGF-β1 was 1-week post-denervation, whereas the highest levels of CTGF and α-SMA proteins were observed in 2-week post-denervation group.
#, △, ▲: $p < 0.05$, vs. the 1-, 2-, 3-, and 4-week denervation groups for TGF-β1, CTGF, and α-SMA, respectively. *: $p < 0.05$, vs. the control, the 2-, 3-, and 4-week denervation groups, respectively. ☆, ★: $p < 0.05$, vs. the control, the 1-, 3-, and 4-week denervation groups, respectively.

there have been increasing reports about the upregulation of CTGF in many fibrotic diseases, and the association between CTGF and fibrosis in many tissues and organs (Lasky et al. 1998; Gupta et al. 2000; Leask et al. 2002). However, there have been few studies on the function of CTGF in skeletal muscle fibrosis. CTGF is overexpressed in human muscles in diseases such as Duchenne muscular dystrophy, with TGF-β1 upregulation (Sun et al. 2008). Maeda et al. (2005) showed that CTGF was expressed in L6 rat skeletal myotubes, and could be induced by TGF-β1 in vitro. Researchers demonstrated in vitro experiments that, under TGF-β1 treatment, myoblasts and myotubes expressed CTGF through autocrine and paracrine routes, which inhibited myoblast differentiation by downregulating the early muscle regulatory factors myogenin and myosin, and induced myoblast dedifferentiation by decreasing MyoD and desmin, two markers of committed myoblasts (Mochizuki et al. 2005). However, the precise role of CTGF in skeletal muscle remains unclear.

Li et al. (2004) demonstrated that in a mouse model of lacerated skeletal muscle, myoblasts differentiated into myofibroblasts, expressed α-SMA, and thus induced fibrosis. Recent studies showed that myoblasts and myotubes expressed and secreted CTGF under TGF-β1 or lysophosphatidic acid treatment in vitro, and myoblasts differentiated into myofibroblasts under the effects of recombinant CTGF, which was associated with an overproduction of ECM (Mochizuki et al. 2005). In addition, recombinant CTGF inhibited myoblast differentiation, but in contrast, promoted dedifferentiation. These various findings indicate that CTGF plays a key role in injured skeletal muscle. However, it is rarely reported whether the expression and function in denervated skeletal muscle may be similar to those in lacerated muscle or congenital myopathy models.

In the present study, we investigated morphological changes and the expression of the fibrosis-related factors, TGF-β1, CTGF, and α-SMA in denervated sternocleidomastoid muscles. We first demonstrated that the sternocleidomastoid muscles gradually developed fibrosis with time after denervation, but this process slowed following 4 weeks. We also observed that denervation caused continued high expression of fibrosis-related factors. TGF-β1 expression reached a peak first, and the activation of downstream factors, such as CTGF and α-SMA, was obviously

slower than that of TGF-β1, suggesting that denervation first activated the expression of TGF-β1, then perhaps through cascade reactions induced CTGF and α-SMA expression, ultimately resulting in atrophic muscle fibrosis. As a marker protein of myofibroblasts, the increase in α-SMA indicated that fibrosis occurred in the muscle tissues. These results suggested that TGF-β1 and CTGF play important roles during the process of denervated skeletal muscle fibrosis, and are positively correlated with fibrosis. However, the mechanism by which TGF-β1 and CTGF induce fibrosis in denervated skeletal muscle is not yet clear.

Although active regeneration of skeletal muscle was found to occur at 7 and 10 days after laceration injury in a murine model, the lesion area was later occupied by newly formed scar tissues, which may be primarily produced by cells from the ECM cells (Menetrey et al. 1999; Huard et al. 2002; Li and Huard 2002). Fibroblast-like circulating cells that originate in the bone marrow migrate (Li and Huard 2002) into the injured muscle and participate in scar tissue formation, as described before (Bucala et al. 1994; Abe et al. 2001). It has been suggested that the release of TGF-β1 from lacerated muscle can induce the pathological differentiation of marrow-derived stem cells and other types of muscle cells (Li et al. 2004). The continued upregulation of TGF-β1, via an autocrine mechanism, not only contributed to the infiltration of lymphocytes, but also triggered muscle cells to differentiate into fibroblasts and underwent atrophy during muscle healing, and had effects in various other types of cells, including cardiomyocytes and hepatocytes (Taimor et al. 1999; Schrum et al. 2001). TGF-β1 can induce collagen deposition by increasing synthesis of most matrix proteins and decreasing the production of matrix degrading proteases via the p38 MAPK pathway (Rodriguez-Barbero et al. 2002). Such positive feedback cycle of TGF-β1 expression would promote atrophy and fibrosis, and hinder proper regeneration in skeletal muscle healing.

We showed that skeletal muscle denervation caused sustained activation of the fibrosis-related factors TGF-β1 and CTGF. Moreover, muscle fibers expressed α-SMA in response to TGF-β1 and CTGF. This might have induced the differentiation of myoblasts into myofibroblasts, leading to accelerated muscle fibrosis, which is a long process. According to the morphological results, we found that muscular atrophy occurred first, followed by progressive connective tissue and ECM deposition. We speculate that these results may be not only be attributable to the differentiation of myoblasts into myofibroblasts, but may also be related to the migration, homing, differentiation, and functioning of bone marrow-derived cells and muscle tissue-derived interstitial cells (Mochizuki et al. 2005). Further studies are needed to provide insights on these issues and to demonstrate whether the molecular mechanisms in denervation might be similar to those in lacerations models.

Acknowledgments

This work was supported by Grant No. 81070774, No. 81170899 for science research from National Natural Science Foundation of China and the research grant 2012Y052 from the Shanghai Municipal Health Bureau foundation for the Youth.

Conflict of Interest

The authors declare no conflict of interest.

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