Abstract. The purpose of this study was to investigate the hypothesis that electrical stimulation regulates the levels of gene expression related to apoptosis in denervated muscle and prevents muscle atrophy after denervation. Nineteen rats were used in this study. To denervate soleus muscle, sciatic nerve was resected under aseptic condition. Electrical stimulation with 4 mA rectangular pulses of 0.5 ms duration at 2 Hz lasting for 1 hour was delivered to lower limb including the soleus muscle using two surface electrodes. After the stimulation periods of 4 weeks, the levels of gene expression related to apoptosis were evaluated. Electrical stimulation increased valosin-containing protein (VCP) expression and decreased cleaved caspase-12 expression in denervated muscles. These results indicated that electrical stimulation to denervated muscle suppresses ER-specific apoptosis by enhancing VCP expression. We proposed that electrical stimulation would be a potential treatment for preventing atrophy of denervated skeletal muscles.

Keywords: Denervation, muscle atrophy, apoptosis, electrical stimulation, valosin-containing protein

1. Introduction

Innervation is a critical factor in supporting the structural and functional integrity of skeletal muscles [14, 16]. Denervation results in a rapid and profound loss of mass [5,6,10] and force-generating ability in skeletal muscles [10]. Morphological studies have shown that the structure of denervated muscle undergoes significant changes, the most dramatic of which is the progressive atrophy of muscle fibers [2,28,30]. Recently, it was reported that muscle denervation induces apoptosis in myofibers [17,18]. Furthermore, denervated muscle increases the expression of muscle-specific genes [9, 15] as well as genes related to apoptosis in myofibers induced by activation of the ubiquitin/proteasome system [6,12]. This process is controlled by a series of genes such as Fas, the Bcl-2 family, the caspase family, c-myc, p-53, and c-fos [18]. In particular, the caspase protease family plays a central role in the implementation of apoptosis in skeletal muscle fibers [23,32].

It is well known that electrical stimulation can induce muscle contraction in denervated muscles. Previous investigations suggested that electrical stimulation to denervated muscle has a beneficial effect in preserving muscle appearance and function. A recent study indicated that electrical stimulation prevents muscle atrophy and maintains the contractile force in denervated rat hindlimb muscle [6,31]. In fact, electrical stimulation is already used to treat denervated muscles in the field of rehabilitation. Additionally, it has been reported that chronic electrical stimulation modifies gene expression rates, which regulate apoptosis of muscle fibers [9,21]. These reports raise the possibility that electrical stimulation may constitute a new form of gene therapy for preventing muscle atrophy after denervation. Howev-
er, the processes underlying the effects of electrical stimulation on denervated muscle are still uncertain.

The goal of this study was to clarify the effects of electrical stimulation in preventing muscle atrophy after denervation. We confirmed that denervation induces apoptosis of muscle fibers using immunohistochemical staining of single stranded DNA [13,33]. And we tested the hypothesis that electrical stimulation of denervated muscles regulates the levels of gene expression related to apoptosis and prevents muscle atrophy after denervation.

2. Materials and methods

2.1. Animal care and experimental groups.

Nineteen female Sprague-Dawley rats (150 ± 10 g) were used in this study. All experiments performed conform to the Guidelines for Animal Experiments of Asahikawa Medical College.

The animals were randomly divided in four groups; (1) normal untreated animals (N, n = 6); (2) denervated soleus (D, n = 5); (3) denervated soleus submitted to electrical stimulation (D + ES, n = 5), (4) normal soleus submitted to electrical stimulation (N + ES, n = 3). The rats were anesthetized with halothane, nitrous oxide and oxygen in an airtight chamber for the surgical denervation process and electrical stimulation of soleus.

2.2. Denervation process

In the denervated groups (D, D + ES), a small incision was made through the skin and fascia near the right trochanter between the gluteus maximus and biceps femoris muscles. The muscles were separated to isolate the sciatic nerve, and about 1 cm of the nerve was cut and removed.

2.3. Electrical stimulation procedures

In the electrical stimulation groups (N + ES, D + ES), electrical stimulation with 4 mA rectangular pulses of 0.5 ms duration at 2 Hz lasting for 1 hour was obtained by interfacing an electrical stimulator (SEN-7203, NIHON KOHDEN, Tokyo) with the surface electrode (6 mm in diameter). One electrode was positioned on the back of animal as an indifferent electrode. Another active electrode was placed on the skin over the soleus muscle. Electrical stimulation delivery was started the day after nerve resection and performed once every 2 days for 4 weeks. During the period of stimulation, animals were anesthetized with halothane, nitrous oxide and oxygen. After the stimulation periods of 4 weeks, bilateral soleus muscles were removed under deep anesthesia with ketamine. The removed soleus muscle was immediately frozen in isopentane at −80°C.

2.4. Immunohistochemistry staining of single stranded DNA

Frozen sections of each muscle were made with a cryotome to a thickness of 8 µm. These sections were incubated with 3% hydrogen peroxidase in methanol for 30 min to block endogeneous peroxidase activity. Following a 5-min incubation at room temperature with Protein Block (DAKO, Denmark), tissue sections were sequentially incubated with anti-single stranded DNA (ssDNA) antibody (diluted 1:200; DAKO, Denmark) overnight at 4°C and with peroxidase-labeled dextran polymer (EnVision; DAKO, Carpinteria, CA) for 30 min at room temperature. The sections were visualized by immersing the slides in freshly prepared 0.02% diaminobenzidine solution for 10 min. The sections were finally counterstained with hematoxylin and mounted. Stained tissue sections were examined microscopically in a coded manner by three different persons. The ssDNA positive nuclei were noted by being strongly stained relative to normal nuclei. More than 500 nuclei were counted and used to calculate the percentage of ssDNA positive nuclei.

2.5. cDNA array analysis.

Total RNA was extracted from the resected muscles of two animals (one each from group 1 (N), group 2 (D) and group 3 (D + ES)) using Sepasol-RNA I (Nacalai Tesque, Japan). Total RNA was further treated with DNase I (Message Clean kit; GenHunter, Nashville, TN) to remove contaminating DNA. 32P-labeled cDNA probes were synthesized using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). These probes were hybridized to the BD Atlas Gene Lists rat (Clontech) according to the manufacturer’s protocols. The arrays were then exposed to a phosphorimaging screen at room temperature for 30 min and scanned using a BAS2000 phosphorimager (Fuji Photo Film, Tokyo, Japan). A grid was applied to images of the hybridization spots, and spot intensities were quantified using BASStation, version 1.31 (Fuji Photo Film).
Background signals were defined as the average of the hybridization signals produced by negative controls on the array. All hybridization signals were normalized by the mean of the internal control signals.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from each resected muscle using Sepasol-RNA I (Nacalai Tesque, Japan) and treated with DNase I (Message Clean kit). The RNA was reverse-transcribed for 60 min at 37°C using Moloney murine leukemia virus reverse transcriptase (GeneHunter, Nashville, TN) with 2 µM oligo(dT) primers (Applied Biosystems, Foster City, CA) according to the manufacturers’ protocols. The following primers were used for valosin-containing protein (sense, 5'-TGGCTGTGGGAAAACCTTAC-3'; antisense, 5'-TCAGCTCCAGAAAAGCCATT-3'), and GAPDH (sense, 5'-GTCATCAATGGGAAGCCTGT-3'; antisense, 5'-CCCATCATCAAAAGTGGGAAG-3') (Sigma Genosis Japan, Hokkaido, Japan). Hot-start PCR was performed in a 10 µl reaction mixture containing 4.95 µl H2O, 1 µl 10x PCR buffer (containing 15 mM MgCl2), 1 µl of 2 mM dNTP mixture, 1 µl each of 5 µM sense primer and antisense primers, 0.05 µl of 5 U/ml AmpliTag Gold DNA polymerase (Applied Biosystems), and 1 µl of 100 ng/µl cDNA. The reaction was carried out as follows: initial denaturation at 94°C for 4 min; followed by 38 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and a final elongation step of 12 min at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

2.7. Western blot analysis

Muscle from each animal was homogenized in ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, and 0.1% SDS, pH 8.0). Protein concentrations were determined using a BCA assay. 100 µg of protein of each sample was run on 4% to 12% Bis-Tris SDS-PAGE gels (Invitrogen, San Diego, CA). Proteins were then transferred to Immobilon-P (Millipore, Bedford, MA) by semi-dry blotting. The membrane was blocked in blocking agent (5% skim milk in 2% BSA-PBS). Then the membrane was incubated in primary antibody overnight at 4°C followed by incubation with peroxidase-conjugated secondary antibody for 1 hour at room temperature. Immnoreactivity was visualized by a chemiluminescent method using ECL plus (Amer sham Biosciences, UK).

2.8. Statistical analysis

The Steel-Dwass’ test (multiple comparison test) was used to determine the significance of the differences between each group.

3. Results

3.1. Immunohistochemistry staining of ssDNA

Representative immunohistochemical staining of ssDNA positive nuclei in soleus muscle is shown in Fig. 1. The normal nuclei and ssDNA positive nuclei are stained with blue and brown, respectively. In normal soleus muscle (N), ssDNA positive nuclei were
very rare. In the denervated muscles (D), ssDNA positive nuclei remarkably increased and were uniformly distributed throughout the entire muscle. These findings revealed that apoptosis in the soleus muscle was induced by denervation of the sciatic nerve. The expression of ssDNA positive nuclei in each group is summarized in Fig. 2. The percentages of ssDNA positive nuclei in the denervated group (D: mean 52%) was significantly higher than those in the control group (N: mean 27%) and the denervated group with electrical stimulation (D + ES: mean 39%).

3.3. RT-PCR analysis

To confirm the result of the microarray analysis, the level of mRNA for VCP was evaluated by RT-PCR. In RT-PCR, VCP expression in group (D) was lowest out of all groups and the variation among this group was very small compared with the other groups. VCP expression in group (D) was significantly lower than those of group (N) and group (D + ES) (p < 0.05). Expression in group (N + ES) was not changed compared with that of group (N) (Fig. 3).

3.4. Western blot analysis

Western blot analysis showed that VCP expression in group (D + ES) was highest among the groups. VCP expression in group (D + ES) was significantly higher than those in group (N) and group (D) (p < 0.05). Expression in group (N + ES) was not changed relative to group (N) (Fig. 4).

Cleaved caspase-12 expression in group (D) was highest in all groups. Expression of cleaved caspase-12 in group (D + ES) was significantly lower than that of group (D) (p < 0.05), but there was no significantly difference between group (N) and (N + ES). Also no difference in cleaved caspase-12 expression was observed between group (N) and group (N + ES) (Fig. 5).

3.2. cDNA array analysis

Microarray analysis indicated four genes with great differences (higher than 3.0-fold) in expression. All four genes were upregulated by 4 weeks electrical stimulation after denervation, and the magnitude of the difference varied from 3.9-fold to 13.6-fold. Two of these genes (cholinergic receptor and superoxide dismutase) were upregulated by denervation alone. Conversely, valosin-containing protein (VCP) and heat shock protein were downregulated by denervation. Electrical stimulation to denervated muscles produced the greatest changes in the level of expression of the genes for VCP (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank Accession No.</th>
<th>ratio: (D)/(N)</th>
<th>ratio: (D + ES)/(D)</th>
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</thead>
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<tr>
<td>valosin-containing protein</td>
<td>U11760</td>
<td>0.2</td>
<td>13.6</td>
</tr>
<tr>
<td>cholinergic receptor, nicotinic</td>
<td>X74835</td>
<td>25.5</td>
<td>8.9</td>
</tr>
<tr>
<td>delta polypeptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heat shock 27 kd protein</td>
<td>M86389</td>
<td>0.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Superoxide dismutase 1, soluble</td>
<td>Y00404</td>
<td>1.2</td>
<td>7.4</td>
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</table>

Fig. 2. Percentages of ssDNA positive nuclei in the denervated group (D: mean 52%) was significantly higher than those in the control group (N: mean 27%) and the denervated group with electrical stimulation (D + ES: mean 39%).
Fig. 3. In RT-PCR, VCP expression in group (D) was significantly lower than those of group (N) and group (D + ES) ($p < 0.05$).

Fig. 4. Western blot analysis for VCP. VCP expression in group (D + ES) was significantly higher than those in group (N) and group (D) ($p < 0.05$).

Fig. 5. Western blot analysis for cleaved caspase-12. Expression of cleaved caspase-12 in group (D + ES) was significantly lower than that of group (D) ($p < 0.05$), but there was no significantly difference between group (N) and (N + ES).
4. Discussion

Denervation results in a rapid and profound loss of mass [5,6,10] and force-generating ability in skeletal muscles [10]. Morphological studies have shown that changes of the muscle structure are drastic within the first 4 weeks after denervation and gradually reach a plateau [6,12]. Similarly, another study indicated that the changes in expression of muscle-specific genes persist for up to 4 weeks after denervation [10]. These results indicate that the first 4 weeks is a critical period for analyzing the mechanisms of muscle atrophy after denervation. Thus, we speculated that electrical stimulation from early phase after denervation would be more important to prevent muscle atrophy. For this reason, we started electrical stimulation the day after nerve section and delivered it to denervated soleus muscle for 4 weeks. This stimulation procedure is quite similar to that of previous description [29]. Our results revealed that ssDNA positive nuclei in denervated muscles significantly decreased with electrical stimulation. This indicates that electrical stimulation influences the regulation of apoptosis in myofibers of denervated muscles.

Recent studies show that apoptosis mainly contributes to muscle atrophy after denervation [1]. It has been reported that cell death in denervated skeletal muscle is distinct from classical apoptosis [8]. Denervation induces apoptosis in myofibers via activation of the ubiquitin/proteasome system [6,12], and is mainly triggered by endoplasmic reticulum (ER) stress, such as the storage of misfolded proteins in the ER. This ER-specific apoptosis is mediated by specific activation of caspase-12, which is specifically activated by ER stress [24]. It has been reported that caspase-12-deficient mice are resistant to ER stress-induced apoptosis [24]. These investigations indicate that caspase-12 may play an important role in ER-specific apoptosis after denervation of skeletal muscle. On the other hand, misfolded proteins in the ER are removed to the cytosol and degraded by 26S proteasomes in the normal cell cycle [7]. Valosin-containing protein (VCP), a member of AAA ATPase family, is one of the important proteins that has a role in the reduction of misfolded protein storage in ER [20]. A previous report indicated that VCP is released by ER stress regulated by caspase-12 [27]. VCP transports proteins from the ER to the cytosol, and contributes to ubiquitin/proteasome-dependent proteolysis [20,34]. For these reasons, we analyzed the changes in caspase-12 and VCP to confirm apoptosis after denervation and to evaluate the effect of electrical stimulation on denervated muscles. The present results show that caspase-12 expression increased and VCP expression decreased in the muscles of the denervated groups. These results indicate that ER stress is an important factor for apoptosis of denervated muscle fibers and is consistent with previous investigations.

Some studies indicate that electrical stimulation is effective in preventing muscle atrophy after denervation [4,6,26,31]. However, the influence of such stimulation in regulating apoptotic gene expression in denervated muscles has barely been studied. We hypothesized that electrical stimulation might prevent muscle atrophy by inhibiting ER-specific apoptosis after denervation. If this hypothesis is true, then protein expression related to ER-specific apoptosis must be changed by electrical stimulation. The results of our study indicated that electrical stimulation increases VCP expression and decreases caspase-12 activation in denervated muscles. These results suggest that electrical stimulation to denervated muscle suppresses ER-specific apoptosis by enhancing VCP expression. We propose that electrical stimulation plays an important role in the reduction of ER stress and prevention of apoptosis after denervation.

It is well known that electrical stimulation induces contraction of denervated muscles. Electrical stimulation has been used to improve dysfunctions such as acroparalysis [3,11,22,25] or neurogenic bladder [19] caused by spinal cord injury. Furthermore, electrical pacing to human paralyzed laryngeal muscles restores normal respiration and vocalization [35,36]. Preservation of muscle excitability is necessary for recovering from motor dysfunctions. Additionally, preservation of motor function is accompanied by the morphological preservation of muscle appearance. Our study indicates that electrical stimulation might have a beneficial effect in reducing apoptosis after denervation of muscles. Although further studies are necessary to clarify how stimulation influences the muscle regeneration process, we propose that electrical stimulation has potentially beneficial effects in the preservation of morphological and functional properties of denervated muscles.

Abbreviations

BCA, bicinchoninic acid; BSA, bovine serum albumin; ER, endoplasmic reticulum; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; VCP, Valosin-containing protein.
References


