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Effect of Neuromuscular Electrical Stimulation of Denervated Muscle on the mRNA Expression of IGFs in Rat Skeletal Muscle and Sciatic Nerve

KOUNOSUKE TOMORI, OTR[1)], RYUJI KOBAYASHI, OTR, PhD[2)], TOMOMI KOSEKI, OTR[3)], YUKIKO OHTA, PhD[1)]

[1)]*Kanagawa University of Human Services: 1–10–1 Yokosuka City, Kanagawa 238-8522, Japan. TEL: +81 46-828-2724, FAX: +81 46-828-2725, E-mail: tomori-k@kuhs.ac.jp*
[2)]*School of Health Science, KIBI International University*
[3)]*Yokosuka General Hospital, Uwamachi*

Abstract. [Purpose] In rehabilitation, it is important to prevent regressive change caused by denervated muscle atrophy. We determined the effect of neuromuscular electrical stimulation on expression of insulin-like growth factor-1 and 2, and their receptors' mRNA in skeletal denervated muscles and sciatic nerves of Wistar rats. [Methods] Denervation was performed by crushing the bilateral sciatic nerves. The day following the denervation, the bilateral gastrocnemius muscles were electrically stimulated percutaneously, for 30 min/day at 4 mA intensity and 10 Hz frequency. After 3 weeks, the muscles and crushed site of the sciatic nerve were dissected. [Results] Relative muscle weights were decreased by denervation; however, the decrease was less after electrical stimulation. Expression of insulin-like growth factor-1 and 2 mRNA in denervated muscle and sciatic nerve were increased by electrical stimulation. [Conclusion] In general, skeletal muscle growth and atrophy is regulated by insulin-like growth factor-1. The neurotrophic factors, including insulin-like growth factor-2, are produced in denervated muscle and initiate regeneration, and are transported retrogradely by axoplasmic transport. Therefore, our results demonstrate that electrical stimulation retards muscle atrophy and promotes nerve regeneration following denervation by increasing insulin-like growth factors' mRNA in the denervated muscle.
Key words: Neuromuscular electrical stimulation, IGF, Denervated muscle

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INTRODUCTION

Denervation-induced muscle atrophy is a major problem encountered in the rehabilitation of patients with spinal cord injury or peripheral nerve injury. As the muscle atrophy progresses, it interferes with activities of daily living due to reduction in neuromuscular functioning. Thus, it is important to prevent the regressive change caused by denervated muscle atrophy.

Neuromuscular electrical stimulation (NMES) is used to retard denervated muscle atrophy[1,2)]. In an earlier paper, we showed that the low-intensity (4 mA) NMES of denervated rat muscle retarded muscle atrophy, and increased axon diameter and nerve density in the sciatic nerve at the re-innervation phase[3)]. However, since we investigated only histological changes, the effect of NMES on the muscle at the molecular level was unclear.

In general, skeletal muscle growth and atrophy

are regulated by growth hormone and insulin-like growth factor (IGF)-1[4,5]. IGF-1 regulates muscle growth independently of growth hormones, and increases in concentration in response to mechanical stimulation[4], electrical stimulation and stretch[6]. Moreover, Day et al.[7] reported that injection of IGF-1 into denervated muscle decreased muscle atrophy, and maintained muscle force. On the other hand, IGF-2 mRNA encourages the re-establishment of synapses in denervated muscle until re-innervation is complete[8,9]. These reports suggest that IGFs play a critical role in functional recovery of denervated muscle. However, it is unknown whether expression of IGF mRNA in the denervated muscle is changed by NMES. Thus, we investigated the effect of NMES on the expression of mRNA of insulin-like growth factors in skeletal denervated muscle as well as the sciatic nerve.

METHODS

Eleven male Wistar specific pathogen free (SPF) rats (CLEA Japan, Tokyo, Japan; 7 weeks old, body weight, 172–197 g) were divided randomly into a control group (n = 3), a denervation group (D group, n = 4), and a denervation and electrical stimulus group (D+ES group, n = 4). All rats were housed in units of 2 or 3 rats per cage in our animal facility under a 12-h light-dark cycle at room temperature (24 ± 2 °C) and 55 ± 5% humidity. They were fed a diet of rodent chow (CE-2, CLEA Japan) and water *ad libitum*. This study was approved by the Animal Committee of Kanagawa University of Human Services.

The rats in the D and D+ES groups were anesthetized by an intraperitoneal injection of sodium pentobarbitone (40 mg/kg body weight). The skin covering the buttock was cut on both sides, and the sciatic nerve was crushed using pliers. This crushing procedure was chosen as the method of denervation since it uniformly and definitively damages nerve fibres with re-innervation[10]. Since the expression of mRNA of IGFs is changed by muscle stretching[6], the bilateral ankle joints of the rats were immobilized in the maximum plantar flexion position by a cast, to keep the muscle length constant.

The day following the denervation, the bilateral gastrocnemius (GAS) muscle in the D+ES group was electrically stimulated, percutaneously. The NMES was performed using an electrostimulator (Torio300, Sakai, Japan) and silver surface electrodes (5 mmϕ and 3 mmϕ). The rats in the D+ES group were anesthetized, and surface electrodes were bilaterally attached to the shaved posterior surface of each leg. The bilateral GAS muscle was stimulated using a frequency of 10 Hz, and the current intensity was set to 4mA. The wave form used was a symmetric rectangle with duration of 250 μsec. In an earlier paper, we showed that low frequency electrical stimulation affects hypertrophy of muscle fiber in superficial and deep strata more than at high frequency[11]. The stimulation intensity was chosen, because it is the stimulation intensity required to obtain mild plantar flexion in the rat ankle. The rats in the ES group were stimulated 30 min/day, 6 day/week, for 3 weeks.

The rats were sacrificed by decapitation under light anesthesia with diethylether after 3 weeks. The bilateral GAS muscles and the crushed site of the sciatic nerves were quickly excised, and weighed. A segment of GAS muscle was transversely removed from the mid-belly of each muscle. The segment of GAS muscle and sciatic nerves were frozen in liquid nitrogen and stored at –80 °C until analysis in a quantitative polymerase chain reaction (PCR).

Total RNA was isolated from the GAS muscle and sciatic nerve using TRIzol reagent (Invitrogen, Japan) according to the manufacturer's instructions. The concentration of RNA was estimated by spectrophotometer at 260 nm, and the ratio of absorbance at 260 nm to that at 280 nm was checked. The RNA sample was stored at –80 °C in a freezer.

The RNA samples (1 μg) were converted into cDNA by reverse transcription using Super ScriptTM III RT (Invitrogen, Japan). To quantitatively estimate the mRNA expressions of several target genes, IGF-1, IGF-2, IGF-1 receptor (IGF-1R), and IGF-2 receptor (IGF-2R), real-time PCR amplification was performed on a Light-Cycler instrument system (Roche, Japan), and FastStart DNA MasterPLUS SYBR Green I (Roche, Japan). The amplification program included initial denaturation at 95 °C for 10 min, 40 to 50 cycles of denaturation at 95° C for 15 sec, annealing at 60° C for 15 sec, and extension at 72° C for 10 sec. We checked that these amplifications did not detect specific amplification by melting curve analysis. The

Table 1. Primers used for real-time PCR

Gene	Sequence*
IGF-1	5'-AGCTGAGATAGTGTTTCCCAAAGG-3'
	5'-TTCCAAACGCGAAATGAATG-3'
IGF-2	5'-AAATCGATGTTGGTGC-3'
	5'-CCTGCTGAAGTAGAAGCC-3'
IGF-1R	5'-GCCAACAAGTTCGTCC-3'
	5'-GTAGTCCGTCTCGTAGAT-3'
IGF-2R	5'-GAAGATGGCGAGCCCT-3'
	5'-CGCAGACATCCGGTGGTG-3'
GAPDH	5'-CCTTCCGTGTTCCTACC-3'
	5'-AGGATGCCCTTTAGTGG-3'

*Upper: forward primer; lower: reverse primer.

Table 2. Muscle wet weight relative to body weight

Group	Muscle Wet Weight
CON	2.08 ± 0.14
D	0.86 ± 0.03**
D+ES	0.97 ± 0.03**, †

Values are relative muscle weight (muscle wet weight / body weight 10^4); means and standard deviation (Mean ± SD) are shown. CON: Control; D: Denervation; D+ES: Denervation and Electrical Stimulation. Significant differences from control are denoted ** , for $p < 0.01$ and † denotes a significant difference from denervation alone ($p < 0.05$).

Table 3. The expression of IGF mRNA in denervated muscle and sciatic nerve

	Group	IGF-1	IGF-2	IGF-1R	IGF-2R
Denervated Muscle	CON	0.045 ± 0.026	4.13 ± 2.28	0.092 ± 0.022	0.223 ± 0.053
	D	0.098 ± 0.099	7.50 ± 2.82	0.606 ± 0.391	1.996 ± 1.253
	D+ES	0.546 ± 0.382 **, ††	13.79 ± 6.52 **, †	1.056 ± 0.704 **	4.440 ± 3.333 **
Sciatic Nerve	CON	0.202 ± 0.162	0.221 ± 0.094	0.061 ± 0.069	0.011 ± 0.006
	D	0.299 ± 0.134	0.232 ± 0.125	0.068 ± 0.031	0.041 ± 0.023 *
	D+ES	0.494 ± 0.130 **, †	0.746 ± 0.094 **, †	0.097 ± 0.030	0.052 ± 0.012 **

Values are mean and standard deviation (Mean ± SD) are shown. CON: Control; D: Denervation; D+ES: Denervation and Electrical Stimulation. Significant differences from control are denoted **, for $p < 0.01$. Significant differences from denervation alone are denoted † , ††, for $p < 0.05$ and $p < 0.01$, respectively.

quantitations of target genes were performed by the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method[12]. The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was estimated as an internal control. Each primer pair (Table 1) was synthesized by Invitrogen (Japan).

Statistical analysis was performed using SPSS software (SPSS Inc., Japan). The quantitative data of relative muscle weight (muscle wet weight / body weight) and mRNA expression were evaluated statistically using analysis of variance. When differences were observed, a post hoc Scheffe test was performed. For all tests, the significance level was considered to be 5% ($p < 0.05$).

RESULTS

The relative weight of the GAS muscles (Table 2) in the D and D+ES groups were decreased compared to those in the control group ($p < 0.01$). However, the relative weight of the GAS muscles in the D+ES group was greater than that in the D group ($p < 0.05$).

In the GAS muscles (Table 3), mRNA expression of all IGFs in the D+ES group was increased compared to the control group ($p < 0.01$). IGF-1 and IGF-2 mRNA in the D+ES group were increased compared to the D group ($p < 0.01$, 0.05, respectively).

In the sciatic nerves (Table 3), IGF-1, IGF-2 and IGF-2R mRNA in the D+ES group were increased compared to the control group ($p < 0.01$). IGF-1 and IGF-2 mRNA in the D+ES group were increased compared to the D group ($p < 0.05$). IGF-2R mRNA in the D group was increased compared to the control group ($p < 0.01$).

DISCUSSION

The decrease in the relative muscle weight in the experimental groups, compared to the control group, suggests that 3 week denervation of rat GAS muscle induces muscle atrophy. The lower decrease in muscle mass observed in the D+ES group compared to the D group suggests that NMES of the denervated muscle retards the progression of

muscle atrophy. Although many previous studies[13,14] have suggested that NMES of denervated muscle retards muscle atrophy, the results were based only on morphological evaluation and the therapeutic mechanism whereby NMES reduces skeletal muscle atrophy remained unclear.

The expression of IGF-1 mRNA in the GAS muscle of the D+ES group was increased, compared with the D group. IGF-1 mRNA is expressed in normal skeletal muscle cells, and its expression is increased by *in vivo* activity models such as loading[4], stretch[6], electrical stimulation[6], and eccentric contraction[15]. It is postulated that the increase in IGF-I stimulates myogenic processes such as satellite cell proliferation, differentiation, and fusion, via an autocrine or paracrine mechanism[4]. In our study, non-NMES muscle contraction and stretch was limited as much as possible by immobilizing the ankle of the animals using a cast. Our results suggest that NMES-evoked muscle contraction increases expression of IGF-1 mRNA, and retards the progress of denervated muscle atrophy.

The expression of IGF-2 mRNA was increased in denervated muscle, and further enhanced by NMES. It is conceivable that IGF-2 may support nerve regeneration in denervated muscle, because expression of IGF-2 mRNA increased significantly compared with IGF-2 in the denervated muscle cells, and was found to a much greater extent in the midregion of the denervated muscle, which is rich in end-plates[9]. Thus, NMES for denervated muscle may promote intramuscular nerve regeneration by increasing expression of IGF-2 mRNA.

In the crushed site of the sciatic nerve, the expression of IGF-1 and IGF-2 mRNA was increased in the D+ES group compared to the control and D groups. In previous studies, endogeneous IGF-1 and IGF-2 appeared to promote regeneration after crush lesions, because expressions of both were increased in the distal nerve, and infused IGF-1 and IGF-2 promoted motor axon regeneration[16]. Moreover, in our previous study, NMES of denervated muscle increased axon diameter and nerve density in the crushed site of the sciatic nerve[3]. These results suggest that the promotion of nerve regeneration by NMES in denervated muscle may be secondary to an increase in the IGFs. The mechanism whereby NMES promotes expression of IGFs in the crushed site of sciatic nerve is not yet clear. However, in general, the neurotrophic factors, including IGFs, are produced in peripheral targets, and transported retrogradely by axoplasmic transport. At 3 weeks after denervation, re-innervation of the muscle in our model began. Thus, the increase in IGFs produced by NMES may have been transported to the crushed site via axoplasmic transport.

In conclusion, NMES of denervated muscle increases mRNA expression of IGFs in both skeletal muscle and the crushed site of peripheral nerves, and may retard muscle atrophy and promote nerve regeneration.

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