Life Sciences 146 (2016) 15–23

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Myostatin propeptide gene delivery by gene gun ameliorates muscle atrophy in a rat model of botulinum toxin-induced nerve denervation☆

Sen-Wei Tsai [a,b,c], Yu-Tang Tung [a], Hsiao-Ling Chen [d], Shang-Hsun Yang [e,f], Chia-Yi Liu [a,g], Michelle Lu [a], Hui-Jing Pai [a], Chi-Chen Lin [h], Chuan-Mu Chen [a,i,j,*]

[a] Department of Life Sciences, Agricultural Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan
[b] Department of Physical Medicine and Rehabilitation, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung 404, Taiwan
[c] Department of Physical Medicine and Rehabilitation, School of Medicine, Tzu Chi University, Hualien 970, Taiwan
[d] Department of Bioresources, Da-Yeh University, Changhua 515, Taiwan
[e] Department of Physiology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[f] Institute of Basic Medical Sciences, National Cheng Kung University, Tainan 701, Taiwan
[g] Department of Nursing, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan
[h] Institute of Biomedical Sciences, National Chung Hsing University, Taichung 402, Taiwan
[i] Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung 402, Taiwan
[j] Integrative Evolutionary Galliform Genomics (iEGG) Center, National Chung Hsing University, Taichung 402, Taiwan

ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form 14 December 2015
Accepted 31 December 2015
Available online 5 January 2016

Keywords:
Myostatin propeptide
Gene therapy
Muscle atrophy
Muscle regulatory factors
Acetylcholine receptor

ABSTRACT

Aim: Muscle atrophy is a common symptom after nerve denervation. Myostatin propeptide, a precursor of myostatin, has been documented to improve muscle growth. However, the mechanism underlying the muscle atrophy attenuation effects of myostatin propeptide in muscles and the changes in gene expression are not well established. We investigated the possible underlying mechanisms associated with myostatin propeptide gene delivery by gene gun in a rat denervation muscle atrophy model, and evaluated gene expression patterns.
Main methods: In a rat botulinum toxin-induced nerve denervation muscle atrophy model, we evaluated the effects of wild-type (MSPP) and mutant-type (MSPPD75A) of myostatin propeptide gene delivery, and observed changes in gene activation associated with the neuromuscular junction, muscle and nerve.
Key findings: Muscle mass and muscle fiber size was moderately increased in myostatin propeptide treated muscles ($p < 0.05$). And enhancement of the gene expression of the muscle regulatory factors, neurite outgrowth factors (IGF-1, GAP43) and acetylcholine receptors was observed. Our results demonstrate that myostatin propeptide gene delivery, especially the mutant-type of MSPPD75A, attenuates muscle atrophy through myogenic regulatory factors and acetylcholine receptor regulation.
Significance: Our data concluded that myostatin propeptide gene therapy may be a promising treatment for nerve denervation induced muscle atrophy.

1. Introduction

Muscle atrophy is a common symptom associated with reduced skeletal muscle activity following nerve injury. To date, there are no pharmacological strategies for preventing or treating muscle atrophy [36]. Recent studies suggest that myostatin has an important role in the regulation of skeletal muscle and ligament growth [11,22,23]. Myostatin, a member of the TGF-β superfamily, was identified as a negative regulator of skeletal muscle growth [39,47]. As a precursor protein, two proteolytic processing events are necessary to transform the inactive precursor myostatin into the biologically active form [19,26]. In the first step, the signal propeptide targeting the protein to the secretary pathway is removed, thereby generating a latent form of the myostatin complex composed of myostatin propeptide (MPro) and a disulfide-linked C terminal dimer (myostatin). In the second step, the MPro is removed and results in the generation of a biologically active disulfide-

Abbreviations: AChR, acetylcholine receptor; BoNT-A, botulinum toxin A; cdk, cyclin-dependent kinase; CMV, cytomegalovirus; DEPC, diethylpyrocarbonate; EGFP, enhanced green fluorescent protein; GAP-43, growth associated protein of 43 kDa; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; IGF-1, insulin-like growth factor I; MPro, myostatin propeptide; MRF4, myogenic regulatory factor 4; MSPP, wild-type myostatin propeptide; MSPPD75A, mutant-type myostatin propeptide; Myf5, myogenic factor 5; MyoD, myogenic differentiation factor D; NMJ, neuromuscular junction; P21, cyclin-dependent kinase inhibitor P21; RT-PCR, reverse-transcription polymerase chain reaction; SV40, simian virus 40.

☆ Author contributions: H.L.C. and C.M.C. conception and design of research, S.W.T., Y.T.T., M.L., H.J.P. and C.Y.L. performed experiments; Y.T.T., S.H.Y., C.C.L. and C.M.C. analyzed data and prepared figures; S.W.T., S.H.Y. and C.M.C. interpreted results of experiments; S.W.T. drafted manuscript; H.L.C., Y.T.T. and C.M.C. edited and revised manuscript; S.W.T., H.L.C., Y.T.T., S.H.Y., C.Y.L., M.L., H.J.P., C.C.L. and C.M.C. approved the final version of manuscript.

* Corresponding author at: Department of Life Sciences, Office of Research and Development, National Chung Hsing University, No. 250, Kuo Kuang Road, Taichung 402, Taiwan.
E-mail address: chchen1@dragon.nchu.edu.tw (C.-M. Chen).

http://dx.doi.org/10.1016/j.lfs.2015.12.056
0024-3205/

linked myostatin protein [13,19]. One important role of MPro is proposed to be associated with the regulation of the biologically active myostatin [20].

A recently developed pharmacological treatment for muscle atrophy is the administration of myostatin inhibitor. Using myostatin-binding protein is therefore a potential treatment strategy for muscle disease [36,46]. The use of recombinant synthesized MPro as an endogenous inhibitor of myostatin was previously shown to improve the pathophysiological performance of the *mdx* mouse model [5,6]. MPro gene delivery was also suggested as another method to block the effect of myostatin and to enhance myofiber growth [28,32].

Several animal models, such as hindlimb casting and Achilles tendon laceration, have been shown to induce muscle atrophy [4]. The injection of botulinum toxin A (BoNT-A) is another approach for investigating muscle atrophy [42]. BoNT-A is a bacterial endopeptidase, and the light chain of BoNT-A acts specifically on the SNAP-25 of SNARE protein in the neuromuscular junctions. Focal intramuscular injection of BoNT-A can denervate muscles by inhibiting the release of acetylcholine at the neuromuscular junction (NMJ), which decreases the ability of a muscle to generate a forceful contraction and results in local paresis and atrophy [10,43]. The temporal blockade of neuromuscular function allows us to investigate the modification of muscle physiological activity from paralysis to recovery.

The mechanism of myostatin-mediated atrophy involves inhibition of myogenic regulatory factor gene expression, inhibition of IGF-1 related myotube hypertrophy, reduction of muscle protein synthesis, and enhancement of the Smad3-induced ubiquitin-proteosome pathway [18,22,29,40]. Previous studies showed that myostatin inhibition by MPro gene delivery can enhance the physiological performance of muscle by increasing myofiber growth and muscle force [5,14,32], but the underlying mechanism is not well studied. Although the neuromuscular junction is important in maintaining muscle function, whether MPro gene delivery has an effect on the regeneration of acetylcholine receptors remains to be elucidated. In this study, we investigated the effect of wild-type (MSPP) and mutant-type (MSPPD75A) MPro gene delivery on the BoNT-A-induced atrophied gastrocnemius muscle. We hypothesized that MPro gene delivery would prevent (or attenuate) the atrophy process in muscle mass. We investigated the expression of genes associated with neuromuscular recovery such as acetylcholine receptors (AChR α, β, δ, and ε), neurite outgrowth (IGF-1 and GAP-43), and myogenic regulatory factors (Myf-5, MRF4, Myogenin, MyoD, and myostatin). We further determined the effects of MSPP and MSPPD75A on muscle atrophy treatment and assessed the cellular changes in atrophied muscle after treatment with the MPro gene.

2. Materials and methods

2.1. Animals

Thirty-six Sprague–Dawley rats (8 weeks of age) were used in this study. Twelve rats were assigned to 4 groups ($n = 3$, Control, EGFP, MSPP and MSPPD75A) for constructs expression analysis. And twenty-four rats were randomly assigned to another 4 groups ($n = 6$) for muscle atrophy study: Control/EGFP (EGFP construct injection), BoNT-A/EGFP (BoNT-A gastrocnemius injection and EGFP construct injection), BoNT-A/MSPP (BoNT-A gastrocnemius injection and MSPP-EGFP plasmid injection) and MSPPD75A (BoNT-A gastrocnemius injection and MSPPD75A-EGFP plasmid injection). The animals were housed in an animal resources facility in a room with a controlled temperature (20–22 °C) and a 12-h light/dark cycle. Rat chow and water were provided *ad libitum*. All of the animal trials were approved by the Animal Care and Use Committee at Chung Hsing University.

2.2. Gastrocnemius localization and BoNT-A injection

Sonography (Visual Sonics Vevo 770 plus RMV 704) was used for injection site localization. After the anesthesia (1.0% isoflurane) was administered, the hind legs were shaved and cleaned with a hair removal cream (Nair, Church & Dwight Corp., Mississauga, ON, Canada). The animals were placed in the supine position with their hind legs in the flexed position. Ultrasound gel was placed on the skin of hind legs to serve as a coupling fluid before using the transducer. The location of the gastrocnemius was identified by sonography (Fig. 1A), and the injection site for the other rats was at a depth of approximately 5–6 mm at about 50% of the length of the hind limb. Vials of lyophilized botulinum toxin A (BOTOX®, Allergan, Irvine, CA) were reconstituted with an instillation of 4 ml of normal saline solution in a 100 unit vial (25 units/ml). The toxin was injected into the left gastrocnemius muscle at a dosage of 3 units/kg bodyweight in an injection volume of approximately 20 μl using a Hamilton syringe (Fisher Scientific, Pittsburgh, PA) with a 27-gauge needle (Fig. 1B). Equal volumes of toxin were injected into both the medial and lateral heads of the left gastrocnemius muscle. An equivalent volume of saline was injected into the right gastrocnemius muscle to serve as a contralateral control.

2.3. Construction of myostatin propeptide expression plasmid

The porcine myostatin propeptide (pMPro) cDNA (bases 1–795, GenBank accession no. NM_214435) was generated by reverse-transcription PCR. The forward and reverse primers utilized for MPro were listed in Table 1. The forward primer contained the sequence GCCACC at the translation initiation site. The propeptide gene was then cloned into pGEM-T vector (Promega) with the CMV promoter to form the pGEMT-MPro (MSPP). To obtain a mutant MPro construct (MSPPD75A) with resistance to proteinase, the mutated propeptide gene D75A was constructed by performing PCR-based site-directed mutagenesis at nucleotide position 223 (counting from the ATG start codon). The proteolytic cleavage site of MPro aspartate at position 75 was replaced with an alanine residue (D75A). The genes of propeptide MSPP and mutated propeptide MSPPD75A were inserted into the *EcoR* I sites of pEGFP-N2 (Catalog #6081-1; Clontech Laboratories, Inc., Mountain View, CA) to generate the MSPP-EGFP and MSPPD75A-EGFP fusion constructs. The pEGFP-N2 was served as the control (EGFP). Plasmids were amplified in *Escherichia coli* DH 5α and purified with an EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA).

2.4. Myostatin propeptide plasmid gene gun injection

The rats were anesthetized (1.0% isoflurane) and their abdomens shaved prior to being weighed and receiving a plasmid injection into their abdominal skin *via* a helium-powered gene gun (BioWare; Fig. 1C) at a pressure of 50 psi [21]. The injection dosage of the plasmid was 0.35 mg/kg body weight (approximately 100 μg), and the plasmid was injected into 5 points separately over the abdomen skin (Fig. 1B). To test the expression of our constructs, 12 rats ($n = 3$, Control, EGFP, MSPP and MSPPD75A) for constructs expression analysis were sacrificed 3 days after gene gun injection, and the frozen section of rat abdomen muscles were examined by fluorescent microscopy for observing EGFP signals. And the other 24 rats received gene gun plasmid injection on day 14 post-BoNT-A injection.

2.5. Histological analysis

The rats were weighed every 10 days after treatment and sacrificed 7 weeks later. Gastrocnemius muscles from both sides of the rats under study were dissected, weighed, and prepared for hematoxylin and eosin (H&E) staining [44]. For the morphometric analysis, transverse muscle sections of gastronemius were processed, and muscle fiber diameters were measured as the distance between the opposite sides of the