Muscular Dystrophy: Disease Mechanisms and Therapies

Guest Editors: Sachchida Nand Pandey, Akanchha Kesari, Toshifumi Yokota, and Gouri Shankar Pandey
Muscular Dystrophy: Disease Mechanisms and Therapies
Muscular Dystrophy: Disease Mechanisms and Therapies

Guest Editors: Sachchida Nand Pandey, Akanchha Kesari, Toshifumi Yokota, and Gouri Shankar Pandey
Contents

Muscular Dystrophy: Disease Mechanisms and Therapies, Sachchida Nand Pandey, Akanchha Kesari, Toshifumi Yokota, and Gouri Shankar Pandey
Volume 2015, Article ID 456348, 2 pages

Primary Murine Myotubes as a Model for Investigating Muscular Dystrophy, Natalia Smolina, Anna Kostareva, Joseph Bruton, Alexey Karpushev, Gunnar Sjoberg, and Thomas Sejersen
Volume 2015, Article ID 594751, 12 pages

Volume 2015, Article ID 387090, 13 pages

Tyrosine 705 Phosphorylation of STAT3 Is Associated with Phenotype Severity in TGFβ1 Transgenic Mice, Eleonora Guadagnin, Jigna Narola, Carsten G. Bönnemann, and Yi-Wen Chen
Volume 2015, Article ID 843743, 7 pages

Genetic Engineering of Dystroglycan in Animal Models of Muscular Dystrophy, Francesca Sciandra, Maria Giulia Bigotti, Bruno Giardina, Manuela Bozzi, and Andrea Brancaccio
Volume 2015, Article ID 635792, 10 pages

Systemic Inflammation in Duchenne Muscular Dystrophy: Association with Muscle Function and Nutritional Status, Oriana del Rocio Cruz-Guzmán, Maricela Rodriguez-Cruz, and Rosa Elena Escobar Cedillo
Volume 2015, Article ID 891972, 7 pages
Editorial
Muscular Dystrophy: Disease Mechanisms and Therapies

Sachchida Nand Pandey, 1 Akanchha Kesari, 2 Toshifumi Yokota, 3 and Gouri Shankar Pandey 4

1 Research Center for Genetic Medicine, Children’s National Medical Center, Washington, DC 20010, USA
2 Miami Children’s Hospital, Miami, FL 33155, USA
3 Department of Medical Genetics, University of Alberta Faculty of Medicine and Dentistry, Edmonton, Canada T6G 2H7
4 Food and Drug Administration, Bethesda, MD 20993, USA

Correspondence should be addressed to Sachchida Nand Pandey; spandey@childrensnational.org

Progressive weakness and degeneration of skeletal muscles caused by genetic alterations fall into the category of muscular dystrophy. Muscular dystrophy occurs worldwide and affects all races. The overall incidence of muscular dystrophy varies among forms, as some forms are more common than others. Muscle loss and weakness are not necessarily caused by genetic alteration. Skeletal muscle inactivity, denervation, cancer-associated cachexia, and physiological responses to fasting or malnutrition cause skeletal muscle mass loss through imbalance in synthesis and breakdown of proteins. Several genes have been identified that are directly or indirectly involved in various muscle wasting. Studies performed in human and animal models have substantially contributed to our knowledge of molecular mechanism of muscle degeneration but still these findings are inadequate for developing effective therapy. Therefore, precise dissection of molecular mechanisms provides the way for the development of therapeutic interventions for muscular dystrophies as well as for skeletal muscle loss.

In this special issue, we intended to publish research and review articles on exploring molecular mechanisms and target identification for treatment of muscle diseases. This issue will give insight into cellular and molecular mechanisms, activation of signaling pathways, how activation of these pathways causes muscle dysfunction, and subsequent disease symptoms. The review article published in this special issue discusses the animal model for muscular dystrophy associated with dystroglycan (F. Sciandra et al.), followed by an article focusing on inflammation status and nutrition in Duchenne muscular dystrophy (DMD) patients (O. R. Cruz-Guzman et al.). The other three articles are related to cellular and molecular modeling of skeletal muscle loss. These articles describe recent advancement in the skeletal muscle research field as well as possibility for developing tools in therapeutic intervention. A study conducted by E. Guadagnin et al. provides new insight into the role of transforming growth factor beta 1 (TGFβ1) in skeletal muscles. TGFβ1 recently showed to be a key player in skeletal muscle atrophy and endomysial fibrosis. E. Guadagnin et al. demonstrated that TGFβ1 alone can induce Tyr705 phosphorylation of STAT3 in skeletal muscle cells, and higher pSTAT3 (Tyr705) leads to severe phenotype in transgenic TGFβ1 mice.

O. R. Cruz-Guzman et al. have shown that chronic inflammation in patients with DMD may be related to loss of muscle function or to obesity. It is not known whether circulating proinflammatory cytokines such as, IL-6, IL-1, and TNF-α levels are associated with muscle function. Therefore, the purpose of their study was to evaluate whether an association exists between systemic inflammation with muscle function and nutritional status in DMD patient. The study concluded that systemic inflammation is increased in patients with better muscle function and decreases in DMD patients with poorer muscle function; nevertheless, systemic inflammation is similar among different levels of nutritional status in DMD patients.

Dystroglycan (DG) is highly expressed in skeletal muscle and served as extracellular matrix receptor. Mutations in components of the DG complex are cause of several muscular...
dystrophies such as mutations in one of the DG complex genes, DAG1, which has been recently associated with two forms of muscular dystrophy. In this special issue, Sciandra et al. published a review focused on the animal model systems, conditional DG knockout, and knock-in mice development, in order to study the DG function in skeletal muscle as well as in other tissues.

Hampered calcium signaling has been often reported in muscular dystrophies, which led to proteolysis of muscle protein induced by calcium ions. The assessment of intracellular calcium event is important for understanding the molecular mechanisms underlying muscular dystrophies. To understand the precise mechanism of calcium signaling in muscle cells, there is a need of robust cellular model. N. Smolina et al. have examined myotubes as a model of adult skeletal muscle for studying evoked calcium release. In addition, the authors also assessed the possibility of this cellular model for studying functional mutation effects through lentiviral transduction. Therefore, primary murine myotubes may serve as a useful cellular model for investigating calcium signaling.

C. Baligand et al. identified the key molecular pathways activated during muscle remodeling after spinal cord injury (SCI) and locomotor training in a rat model since molecular events associated with changes in muscle mass after SCI are not known completely. In this study the authors have performed genome wide expression profiling of soleus muscles at multiple time points after SCI in the well-characterize rat model. Their expression data suggest the involvement of TGF-beta/smad3 signaling in association with decrease in muscle mass observed with SCI, while the BMP pathway was activated during treadmill training. This study may provide insight into effects of BMP signaling activation and TGFβ signaling on muscle regeneration with treadmill training in SCI through Smad3 downregulation, providing early indicators of efficient reloading in SCI model.

Articles published in this special issue will provide new insights into understanding the pathophysiology and novel therapeutic target identification of skeletal muscle diseases. As the understanding of skeletal muscle diseases improves with time, new findings will further enrich the current knowledge of these diseases, ultimately helping to develop effective therapy.

Acknowledgments

We would like to acknowledge all the authors for their contributions in this special issue. We would also express our gratitude to all the reviewers for their critical inputs to improve the articles content.

Sachchida Nand Pandey
Akanchha Kesari
Toshifumi Yokota
Gouri Shankar Pandey
Research Article

Primary Murine Myotubes as a Model for Investigating Muscular Dystrophy

Natalia Smolina,1,2,3 Anna Kostareva,1,3,4 Joseph Bruton,5 Alexey Karpushev,3 Gunnar Sjoberg,1,2 and Thomas Sejersen1,2

1Department of Women’s and Children’s Health, Karolinska University Hospital, Solna, 17176 Stockholm, Sweden
2Center for Molecular Medicine, Karolinska University Hospital, Solna, 17176 Stockholm, Sweden
3Federal Almazov Medical Research Centre, 2 Akkuratova Street, Saint Petersburg 197341, Russia
4Institute of translational Medicine, ITMO University, 14 Birzhevaya Line, Saint Petersburg 199034, Russia
5Department of Physiology and Pharmacology, Karolinska Institutet, 17177 Stockholm, Sweden

Correspondence should be addressed to Natalia Smolina; natalia.smolina@ki.se

Received 31 December 2014; Accepted 11 March 2015

Academic Editor: Sachchida Nand Pandey

Copyright © 2015 Natalia Smolina et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Muscular dystrophies caused by defects in various genes are often associated with impairment of calcium homeostasis. Studies of calcium currents are hampered because of the lack of a robust cellular model. Primary murine myotubes, formed upon satellite cell fusion, were examined for their utilization as a model of adult skeletal muscle. We enzymatically isolated satellite cells and induced them to differentiation to myotubes. Myotubes displayed morphological and physiological properties resembling adult muscle fibers. Desmin and myosin heavy chain immunoreactivity in the differentiated myotubes were similar to the mature muscle cross-striated pattern. The myotubes responded to electrical and chemical stimulations with sarcoplasmic reticulum calcium release. Presence of L-type calcium channels in the myotubes sarcolemma was confirmed via whole-cell patch-clamp technique. To assess the use of myotubes for studying functional mutation effects lentiviral transduction was applied. Satellite cells easily underwent transduction and were able to retain a positive expression of lentivirally encoded GFP up to and after the formation of myotubes, without changes in their physiological and morphological properties. Thus, we conclude that murine myotubes may serve as a fruitful cell model for investigating calcium homeostasis in muscular dystrophy and the effects of gene modifications can be assessed due to lentiviral transduction.

1. Introduction

Muscular dystrophies are a heterogenous group of genetic disorders characterized by muscle wasting and degeneration. Unraveling the pathogenesis of muscle dystrophies has great clinical and scientific importance and demands reliable cellular models for investigating underlying molecular mechanisms. Among various types of dystrophies Duchene muscular dystrophy (DMD) is well described due to availability of transgenic mice model, mdx mouse. These animals carry a point mutation in dystrophin gene, leading to appearance of premature stop codon which results in absence of full-length dystrophin [1]. It was shown that in murine model of DMD, mdx mouse intracellular calcium was twice greater than in wild type littermates. Calcium influx is increased since membrane is more permeable and cells undergo permanent calcium overload resulting in activation calcium dependent proteases [2]. Thus, calcium homeostasis is often hampered in muscular dystrophies, leading to enhanced proteolysis due to proteases activation by calcium ions [3]. Functional studies, especially assessment of calcium intracellular events, are of importance for clarifying molecular mechanisms underlying myodystrophies pathogenesis. However, data about calcium handling in muscular dystrophy were mostly obtained on single fibers isolated from mdx mice [4–6] or on primary myotubes formed from the mdx satellite cells [7]. Animal models are widely used as disease models; however, guided by 3R principles, the goal of scientists is to reduce animal usage in their studies and to rely on cell culture. The choice of relevant and informative cellular model is a key factor in...
successful analysis and dissection of signaling pathways in monogenic disorders. One of the major obstacles in skeletal muscle research is the lack of a good mature cell line model for studying neuromuscular disorders. A number of cell types have been traditionally used: primary mechanically [8–10] or enzymatically [11] isolated muscle fibers and satellite cells obtained from newborn animals and their subsequent differentiation and maturation into myotubes [12–15]. However, in the case of research attempting to identify the effects of mutations of calcium handling proteins, none of the hitherto used cell models is optimal.

Muscle fibers are terminally differentiated multinucleated cell that can be several centimetres long and are the basic repeating units of mature skeletal muscles. Primary isolated muscle fibres with tendons attached are the most reliable model for investigation of intracellular Ca\(^{2+}\) homeostasis and changes in muscle force production [8, 16]. However, due to the difficulty in isolating these cells in large numbers, the use of these cells in vitro experiments is limited and researchers have resorted to enzymatically dissociated fibres to be able to monitor changes in Ca\(^{2+}\) homeostasis [17–19]. In an attempt to overcome this limitation, use has been made of satellite cells. These cells located between the sarcolemma and basal lamina are a potent pool of muscle progenitor cells that can proliferate and fuse to repair or even form new muscles fibers in response to injury or increased physical activity and thus provide some regenerative capacity to muscle [20–26]. Satellite cells can be isolated easily from skeletal muscle biopsies using various enzyme digestion protocols and have been used for up to eight to ten passages in culture [27–29]. Myotubes formed upon satellite cells fusion have been frequently used to examine cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_\text{c}\)]) at rest and in response to stimulation [12–15]. These studies utilized satellite cells obtained from newborn mice and rats, which makes the universality and applicability of this model questionable.

Investigation of the role of individual proteins effect is often carried out by transgenic means whereby a protein of interest is expressed in a modified form, temporarily knocked down, or overexpressed. One important aspect in the choice of a suitable muscle cell model for analysis of calcium homeostasis is the ease with which cells can be genetically modified via viral transduction. Several different types of viral transduction have been tried including adenoviruses, adenoassociated viruses, herpes simplex viruses, and lentiviruses. At this point in time, efficient genetic modification via viral transduction of primary adult muscle fibers is difficult. Limited data exists regarding the effective adenoassociated viral transduction of muscle fibers in vivo when viral suspension was injected intramuscularly [30]. In another study high percentage of muscle fibres expressed reporter gene was achieved when 1.7 × 10\(^7\) transduction units of virus were applied for transduction; however, while protein expression from delivered virus was seen for several weeks, it was accompanied by a marked inflammatory and immune response [30]. When primary muscle fibers isolated from various muscle types were transduced via adenoviruses encoded β-Gal, the success rate 24 hours after transduction ranged from 64% (for animals at age two weeks) to 80% (for animals aged from one to three days). However, when the same approach was adopted in adult mice (six months), the efficiency of transduction dropped to 0% [31]. In contrast, the majority of satellite cells were amenable to adenoviral transduction regardless of the age of animal from which they were isolated. The level of expression of the introduced gene in the satellite cells was quite high (95% of cells expressed the β-Gal protein) [31].

Lentiviral (LV) transduction provides stable gene expression in postmitotic nondividing cells and is thus a promising tool for gene modification [32]. To date, positive transduction of muscle fibers was found only when virus was injected intramuscularly, that is, in vivo [33]. One other group has reported successful muscle fiber transduction via LV encoded β-Gal in vitro; however, these experiments utilized L6 myotubes and not adult muscle fibers [34]. There are reports confirming high efficiency of LV transduction for proliferating myoblasts, as well as myotubes, after 72 hours of differentiation [35].

In summary, an optimal muscle cell model for investigating molecular pathways underlying muscular dystrophy has to be morphologically and physiologically similar to mature muscle fibers and should undergo assessment of Ca\(^{2+}\) homeostasis. The goal of this study was to define a cell model of mature muscle cells that could be useful for studying Ca\(^{2+}\) homeostasis both with and without genetic modifications.

2. Materials and Methods

2.1. Muscle Fiber Isolation. Young (8–16 weeks old) C57BL/6 male mice were supplied by B&K Universal (Sollentuna, Sweden). All studies were approved by Stockholm North Local Animal Ethics Committee and Local Ethics Committee of Federal Almazov Medical Research Centre. Mice were sacrificed by cervical dislocation. Muscles were removed and placed in DMEM with 1% penicillin/streptomycin (Gibco, USA). Single muscle fibers were isolated from flexor digitorum brevis (FDB) muscle. Isolated muscles were cleaned of connective tissue and tendons and placed in 2 mL of filtered 0.3% collagenase I (C0130, Sigma, Germany) dissolved in DMEM (Gibco, USA) supplemented with penicillin-streptomycin (Gibco, USA) for 2 h at 37°C. After digestion, muscles were washed with DMEM supplemented with 20% FCS (Gibco, USA) to remove the residual enzyme. Muscles were gently triturated in 2 mL of DMEM supplemented with 20% FCS. After trituration, fiber suspension was incubated for 10 min in plastic dishes, which was found to be optional to reduce the amount of nonmuscle cells contamination. After the 10 min incubation, the fiber suspension was plated on Geltrex-coated (Gibco, USA) glass bottom Petri dishes (P35G–0–20-C, Mattek, USA), 500 μL of suspension per one dish. Geltrex was diluted in cold DMEM (1 : 100) and the glass bottom of the dishes were coated and incubated at 37°C for one hour after which the dish was washed with PBS several times to remove excess Geltrex. The fiber suspension was plated on the dish and left for 10 min to allow fibers to attach to the glass bottom before the addition of 2 mL of incubation media (DMEM supplemented with 20% FCS). The incubation media was renewed every two days by replacement of
half of medium. Cells were cultured in an incubator at 37°C under a 5% CO₂ atmosphere.

2.2. Primary Satellite Cell Isolation, Cultivation and Differentiation. Satellite cells were isolated via two strategies. In the first strategy, satellite cells were allowed to branch out of muscle fibers and attach to the dish bottom. In the second strategy, satellite cells were isolated as a "pure" culture by enzymatic dissociation of muscle fibers [28, 36, 37].

For the first strategy, muscle fibers were isolated from soleus and flexor digitorum brevis muscles by incubation in collagenase and subsequent trituration as described above and incubated until the satellite cells appeared in the dishes.

For the second strategy satellite cells were isolated enzymatically according to the protocol of Yablonka-Reuveni [38] with minor changes (Figure 1). In brief, isolated muscles were placed directly into enzyme solution, without any additional mechanical disruption with scissors. Digestion was done using collagenase type I instead of pronase. Muscle mincing was done using sterile blue pipette tips instead of glass Pasteur pipettes or serological pipettes; we did not filter the cell suspension through a strainer, since in our hands it decreased yield. The resultant satellite cells were plated on dishes coated with Geltrex instead of Matrigel. Thus soleus and FDB muscles were digested for 90 min at 37°C in 2 mL filtered 0.1% collagenase I (C0130, Sigma, Germany). To remove collagenase and cell debris after digestion, the cell suspension was centrifuged for 5 min at 400 x g and the supernatant containing enzyme solution was discarded. To release satellite cells from the fibers the pellet was resuspended in 2.5 mL of washing media (DMEM supplemented with 10% horse serum (HS) (Gibco, USA)). After the resuspension the fibers were let to settle for 5 min and then the supernatant containing satellite cells was removed to a fresh tube. To increase satellite cells yield purity this step was repeated twice. The double-collected supernatant was centrifuged for 10 min at 1000 x g, and the resultant supernatant was discarded and the pellet of cells was redissolved in 0.5 mL of proliferation media (DMEM supplemented with 20% FCS, 10% HS, and 1% chicken embryo extract (C3999, USBiological, USA)). Cells were plated on Geltrex-coated glass bottom petri dishes and cultured in proliferation medium until 80% confluence was reached. Fusion of some cells without external stimuli (differentiation media) was observed usually after 7 days of cultivation and served as a reliable indicator after which we induced differentiation. To induce satellite cell differentiation, the proliferation media was removed, cells were washed once with prewarmed PBS, and then differentiation media was added (DMEM supplemented with 2% HS). The differentiation media was renewed every other day by replacement of half of medium. Cells were cultured in an incubator at 37°C under a 5% CO₂ atmosphere.

2.3. Lentiviruses Production and Cell Transfection. The pLVTHM (20 μg), pMD2G (5 μg), and packaging pCMV-dR8,74psPAX2 (5 μg) plasmids were cotransfected into HEK-293T cells by a calcium phosphate method. The resultant production of lentivirus was concentrated by an ultracentrifugation method (20000 x g for 2 h at 4°C), resuspended in 1% BSA, frozen in aliquots at −80°C, and titered using HEK-293T cells as described previously [39] (http://tronolab.epfl.ch/).

Several different approaches were tested to successfully transduce primary muscle fibres. To facilitate transduction, polybrene (Sigma, Germany) at a final concentration 8 μg/mL was added to all transduced cells. We used (i) nonconcentrated virus and DMEM supplemented with 20% FCS as solution for muscle trituration and (ii) nonconcentrated and concentrated viral suspension as transduction agent and varied (iii) the incubation time with viruses and (iv) the type of plating surface (Figure 1). For transduction of the satellite cells, concentrated viral suspension at multiplicity of infection of 20 was added to the cells and incubated for 5 min before plating. Sixteen hours after transduction, the culture medium was completely replaced with fresh medium. To assess efficiency of viral transduction viruses coding for GFP were used in parallel.

2.4. Immunocytochemistry. The myogenic nature of the isolated cells was confirmed by immunocytochemical staining. Cells were fixed in 4% paraformaldehyde for 10 min at 4°C and then permeabilized with 0.05% Triton X-100 for 5 min. Nonspecific binding was blocked by incubation of permeabilized cells in 15% FCS for 30 min. Cells were incubated for one hour at room temperature with the following primary antibodies: anti-desmin (D33, DAKO, Denmark), anti-myosin heavy chain (MAB4470, R&D, USA), anti-ryanodine Receptor 1 (D4EI, Cell signaling, USA), anti-Mitofusin 2 (ab56889, Abcam, USA), anti-lamin A/C (NCL-LAM-A/C, Novocastra, UK). The secondary antibodies conjugated with Alexa Fluor 546 (Molecular Probes, USA) were applied for 45 min at room temperature. Nuclei were counterstained with DAPI (Molecular Probes, USA).

2.5. Whole-Cell Patch-Clamp. Ca²⁺ current was recorded in muscle fibres and myotubes using the whole-cell patch-clamp technique. Current recordings were performed with an Axopatch 200B amplifier and Digidata 1440A AD/DA converter (Molecular Device, USA). Data collection and analysis were done using pClamp 10.2 (Molecular Device, USA). Patch pipettes (1.5–4 MΩ) were pulled from borosilicate glass capillaries (World Precision Instruments, USA) by means of a micropipette puller P-1000 (Sutter Instruments, USA). The pipette solution had the following composition (mM): 120 CsCl, 5 MgATP, 10 EGTA, and 10 HEPES (adjusted to pH 7.4 using CsOH) and the bath solution contained the following (mM): 120 TEA-Cl, 10 CsCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPEs, 0.001 TTX, and 10 glucose, (adjusted to pH 7.4 using TEA-OH). Ca²⁺ current was evoked with a series of 200 ms depolarizing steps from –30 to 40 mV with 10 mV increments. In order to compare Ca²⁺ currents in different cells, Ca²⁺ current was normalized to the membrane capacitance.

2.6. Loading Cells with Calcium Indicators. Free intracellular Ca²⁺ was measured using the nonratiometric calcium indicator fluo-3 AM (Molecular Probes, USA). Rhod-2 AM (Molecular Probes, USA) was used to monitor free calcium in the mitochondrial matrix. Cells were incubated for 30 min with
Flow chart for primary muscle fibers isolation and transduction

Muscle isolation

Muscle digestion
90 min in 0.3% collagenase I

Muscle trituration
10 times up and down
1 mL plastic pipette tip

Nonconcentrated LV (MOI 1)
DMEM + 20% FCS

Concentrated LV (MOI 100)

Muscle fibers transduction
Incubation with LV

Nonconcentrated LV
5 min, 1.5 h, 3 h, 4.5 h

Concentrated LV
5 min, 1.5 h, 3 h

Muscle fibers plating

Noncoated dish overnight
Geltrex-coated dish

Geltrex-coated dish

(a)

Flow chart for satellite cells isolation, transduction, and cultivation

D.0  D.III  D.VII  D.IX  D.XIV  D.XXI

Muscle isolation in 0.1% collagenase I @400 xg

Muscle digestion in 2.5 mL of WM
20 times up and down
1 mL plastic pipette tip
Settle cells for 5 min @1000 xg

Muscle trituration in 2.5 mL of WM
×2

Cells resuspension in 0.5 mL of PM

Cells incubation with LV (MOI 20)

Positive GFP signal in satellite cells
Induce differentiation
First spontaneously contractile myotubes
ICC patch-clamp Ca^{2+} measurement
Degradation of myotubes

Stage duration
2 h 90 min 5 min 20 min 10 min 10 min 5 min

(b)

Figure 1: Flow chart for primary muscle fibers and satellite cells isolation and transduction. (a) Flow chart for primary muscle fibers transduction. Muscles after isolation underwent enzymatic digestion and then were triturated either in nonconcentrated LV or in DMEM supplemented with 20% FCS. After trituration in nonconcentrated LV (MOI 1), muscle fibers were transduced via nonconcentrated LV as well and were incubated with nonconcentrated LV for 5 min, 1.5 h, 3 h, or 4.5 h. Then muscle fibers were plated either directly to Geltrex-coated dish or cultivated on noncoated dish overnight and were then plated to Geltrex-coated dish. When cells were triturated in DMEM supplemented with 20% FCS, transduction was carried out either via nonconcentrated LV or via concentrated LV (MOI 100). Depending on LV type time of incubation varied, for nonconcentrated LV–5 min, 1.5 h, 3 h, and 4.5 h and for concentrated LV–5 min, 1.5 h, and 3 h. After transduction via concentrated LV cells were directly plated on Geltrex-coated dish. (b) Flow chart for satellite cells isolation, transduction, and cultivation. Satellite cells were isolated by means of enzymatic digestion and then centrifuged for 5 min at 400 xg and supernatant was discarded. Obtained cell pellet was twice resuspended in 2.5 mL of washing media (DMEM supplemented with 10% HS), suspension was settled for 5 min by gravity, and upper phase was transferred into fresh tube and spun down for 10 min at 1000 xg. Cells pellet was dissolved in 0.5 mL of proliferation media (DMEM supplemented with 20% FCS, 10% HS, 1% CEE) and transduced via concentrated LV (MOI 20); polybrene at final concentration 8 𝜇g/mL was added to cells. 72 hours after transduction positively transduced cells were observed. Seven days after isolation cells reached confluence and were induced to differentiation. 48 hours after differentiation first spontaneously contractile myotubes were detected. After seven days of differentiation myotubes were taken in analysis (immunocytochemistry, patch-clamp, and calcium measurement). Three weeks after isolation myotubes started to degrade. LV, lentivirus; MOI, multiplicity of infection; PM, proliferation media; WM, washing media.
2 μM fluo-3 AM or 5 μM rhod-2 AM and then washed for 20 min with Tyrode buffer at room temperature.

2.7. Stimulation of Sarcoplasmic Reticulum Ca$^{2+}$ Release and Laser Confocal Microscopy. Cells were stimulated chemically with 2 mM 2-chloro-m-cresol (CmC, Sigma, Germany) or electrically at 1 Hz, 10 Hz, or 100 Hz.

A BioRad MRC 1024 unit (BioRad Microscopy Division, Hertfordshire, England) with a dual Calypso laser (Cobolt, Solna, Sweden) mounted on a Nikon Diaphot 200 inverted microscope was used. In the majority of experiments, a Nikon Plan Apo 20x dry lens (N.A. 0.75) was used. The fluo-3 AM was excited with 491 nm light and emitted signal was collected at 515 nm, the rhod-2 AM was excited with 532 nm light and the emitted light collected through a 585 nm long-pass filter. Confocal images were captured every 7 s and a total of 42 images were obtained for every experimental condition.

3. Results

3.1. Muscle Fiber Isolation and Transduction. Dissociated flexor digitorum brevis muscle fibers demonstrated a cross-striated pattern and contracted in response to electrical stimulation in the same way as mechanically dissected muscle fibers; that is, a larger transient increase of fluo-3 was observed upon increasing the stimulation frequency from 1 to 10 to 100 Hz. However, we were not able to obtain effective positive transduction of these primary muscle fibers. Using non-concentrated virus, no GFP signal (to confirm that transfection has occurred successfully) was detected in the muscle fibers, although satellite cells branching off the muscle fibers expressed GFP (Figures 2(a) and 2(b)). Increasing the incubation time from 5 minutes to 90 minutes, 3 hours, and 4.5 hours with nonconcentrated viruses resulted in a reduced number of living muscle fibers. For example, in one experiment, the number of living muscle fibers plated immediately after isolation was twice as great as the number alive after 3 hours or 4.5 hours of incubation in the nonconcentrated viral media (40 and 20 living muscle fibers, resp.) and four times greater than after overnight incubation on noncoated dish (10 living muscle fibers) for both muscle fibers and satellite cells quantity (Figure 3). Similar results were seen when the experiment was repeated on two other occasions. When concentrated virus was used for transduction, GFP signal was observed in both muscle fibers and in satellite cells 72 hours after transduction (Figure 2(b)). However, positively transduced muscle fibers were unable to survive in culture for longer than 24 hours, lost their cross-striated pattern and did not contract in response to electrical stimulation. In contrast, fibers that were not exposed to LV retained their morphological appearance and physiological response to electrical stimulation during 72 hours of observation. Thus, concentrated virus provided a mild transduction effect but exhibited a very toxic effect on fibers and caused dedifferentiation, loss of cross striation, inability to respond to electrical stimulation, and death.

3.2. Satellite Cell Isolation and Transduction. Enzymatic digestion to obtain a “pure” satellite cell culture with satisfactory differentiation capacities resulted in more satellite cells in comparison to experiments where satellite cells were allowed to branch out of muscle fibers maintained in culture for four days. The numbers of enzymatically isolated satellite cells were far greater than in the case of branching out of muscle fibers satellite cells cultivated for similar times (Figure 4(a)). Moreover, enzymatic digestion was more efficient since it was possible to obtain satellite cells from any type of muscle, whereas satellite cells branching out from the muscle fiber were restrained by the numbers of intact fibers isolated. The best results were obtained for the FDB that consists overwhelmingly of short (about 600 μm in length) muscle fibers (Figures 4(b) and 4(c)). Muscle fiber isolation from the soleus muscle often resulted in severe fiber damage and, as a consequence, fewer satellite cells branched out of the surviving fibers.

In satellite cells culture isolated from soleus muscle by enzymatic digestion and transduced by exposure to concentrated LV, 95 ± 3% of the cells (n = 5 dishes) expressed GFP 72 h after transduction. The GFP signal remained stable up to and after fourteen days of differentiation when myotubes formation had occurred (Figure 4(d)), confirming the high efficiency and stability of transduction.

We assessed the myogenicity of isolated cells by anti-desmin immunostaining. The number of desmin-positive cells was divided by number of all analyzed cells. The percentage of myogenic cells was 74.3 ± 4.3% (n = 450 cells) (Figure 4(e)). Moreover, we estimated the myogenic potential of positively transduced cells. Cells were transduced via LV encoded human lamin and induced to differentiation. Obtained myotubes were stained with anti-lamin and anti-desmin. Positively stained myotubes displayed incorporating high percentage of nuclei expressing human lamin, thus confirming effective transduction of myogenic satellite cells (Figure 4(d), lower panel).

3.3. Characterization of Myotubes. For the study of cytosolic and intramitochondrial calcium homeostasis, satellite cells were enzymatically isolated from the slow-twitch soleus muscle. Upon differentiation satellite cells isolated from soleus muscle were able to fuse and form multinucleated myotubes that displayed spontaneous contractions already after 48 h of differentiation (Movie 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/594751). Immunocytochemistry confirmed that myotubes expressed proteins typical of late stages of muscle differentiation. Staining for principal sarcomere proteins, desmin and MyHC, gave a cross-striated pattern, similar to that seen in adult muscle fibers (Figures 5(a) and 5(b)). Staining with anti-Mitofusin 2 antibody to visualize mitochondria revealed a patchy staining cross-striated pattern in primary myotubes that was in contrast with the regular cross-striation pattern seen in adult muscle fibers (Figure 5(c)). Ryanodine receptors staining, indicating Ca$^{2+}$ channels in the membrane of the sarcoplasmic reticulum, was abundant in the cytoplasm of myotubes after seven days of differentiation and throughout the myoplasm of muscle fibers (Figure 5(d)). At seven days of differentiation myotubes responded to chemical (2-chloro-m-cresol, CmC) and electrical stimulation with release of Ca$^{2+}$...
from sarcoplasmic reticulum into the cytosol and subsequent uptake of Ca\(^{2+}\) by the mitochondria, confirming the presence of mechanisms of calcium pathways typical for mature muscle (Figure 6(a)). However, the changes in cytosolic Ca\(^{2+}\) were not greatly affected by changes in the stimulation frequency from 10 Hz to 100 Hz which is unlike the situation in adult muscle fibers where increasing the frequency causes a marked increase in cytosolic Ca\(^{2+}\). The presence of functional dihydropyridine receptors in the membrane of both muscle fibers and myotubes was confirmed by measurement of sarcolemmal Ca\(^{2+}\) currents using the patch-clamp. We observed inward current, corresponding with the given experimental conditions to Ca\(^{2+}\) current with characteristics typical of those of a L-type Ca\(^{2+}\) current in adult muscle fibers (Figures 6(b) and 6(c)). However, the peak current density was significantly smaller (\(P < 0.05\)) in myotubes than in muscle fibers.

**Figure 2:** Muscle fiber transduction via lentiviruses. (a) In 72 h after muscle fibers transduction via nonconcentrated LV encoded LMNA (1 hour incubation with LV) muscle fibers kept their cross-striated pattern (upper panel). When concentrated LV encoded GFP was applied muscle fibers acquired positive signal, however, lost their cross-striation (lower panel). Satellite cells in both applications were positively transduced. (b) In 72 h after muscle fibers transduction via nonconcentrated LV (1 hour incubation with LV) muscle fibers did not express GFP, while it expressed desmin (red), however, branching out satellite cells expressed GFP. (c) Nontreated muscle fibers retain their cross-striation pattern and did not differ from muscle fibers transduced via nonconcentrated LV. Scale bar corresponds to 50 \(\mu\)m.
with muscle fibers. Previously we showed that sarcoplasmic
presence of Ca\(^{2+}\) in the sarcoplasmic reticulum. Patch-clamp studies showed the
pathway linking L-type channel activation and the RyR in
tion indicating a functional excitation-contraction coupling

c a l c i u m i n r e s p o n s et o e l e c t r i c a n d c h e m i c a l s t i m u l a-
myotubes were able to contract and to release sarcoplasmic
indicating an extensive sarcoplasmic reticulum. Primary
in primary myotubes was found throughout the sarcoplasm
zation of mitochondria had not yet occurred. RyR staining
in myotubes with the typical cross-striated pattern found in adult muscle fibres. Mitofusin
2 was expressed throughout the cytoplasm of myotubes with no apparent cross-striation, indicating that the adult organization of mitochondria had not yet occurred. RyR staining
in primary myotubes was found throughout the sarcoplasm indicating an extensive sarcoplasmic reticulum. Primary
myotubes were able to contract and to release sarcoplasmic calcium in response to electrical and chemical stimulation indicating a functional excitation-contraction coupling
pathway linking L-type channel activation and the RyR in the sarcoplasmic reticulum. Patch-clamp studies showed the
presence of Ca\(^{2+}\) currents in plasma membrane of primary
myotubes although the L-type Ca\(^{2+}\) current density was less in myotubes than in adult muscle fibers. Probably due to less number of Ca\(^{2+}\) channels per cell in myotubes in comparison with muscle fibers. Previously we showed that sarcoplasmic Ca\(^{2+}\) release at 1 Hz stimulation was significantly lower than

4. Discussion

The goal of our study was to identify a robust and relevant cellular model for assessment of intracellular calcium homeostasis in mature muscle cells. We compared primary adult muscle fibers with myotubes formed by satellite cells fusion. We assessed their morphological and physiological properties and checked the ability of cells to undergo LV genetic modification. We demonstrated that primary myotubes formed after satellite cells fusion resembled primary adult muscle fibers in terms of morphology and physiology. Further, primary myotubes, in contrast to muscle fibers, can successfully undergo genetic modification via LV transduction and express the coded proteins in 72 hours after transduction for at least 14 days.

The immunocytochemical data show that primary myotubes expressed myosin and desmin filaments with the typical cross-striated pattern found in adult muscle fibres. Mitofusin 2 was expressed throughout the cytoplasm of myotubes with no apparent cross-striation, indicating that the adult organization of mitochondria had not yet occurred. RyR staining
in primary myotubes was found throughout the sarcoplasm indicating an extensive sarcoplasmic reticulum. Primary
myotubes were able to contract and to release sarcoplasmic calcium in response to electrical and chemical stimulation indicating a functional excitation-contraction coupling pathway linking L-type channel activation and the RyR in the sarcoplasmic reticulum. Patch-clamp studies showed the
presence of Ca\(^{2+}\) currents in plasma membrane of primary
myotubes although the L-type Ca\(^{2+}\) current density was less in myotubes than in adult muscle fibers. Probably due to less number of Ca\(^{2+}\) channels per cell in myotubes in comparison with muscle fibers. Previously we showed that sarcoplasmic Ca\(^{2+}\) release at 1 Hz stimulation was significantly lower than

that at 10 Hz stimulation, while increase of the stimulation frequency to 100 Hz did not result in any further increase in sarcoplasmic Ca\(^{2+}\) release [40], unlike the situation in adult muscle fibers where there is increasing [Ca\(^{2+}\)], with increasing stimulation frequencies [41].

Taken together, the results with primary myotubes are promising because of their morphological and physiological similarity to primary muscle fibers, though they do not completely replicate the situation in adult muscle fibers.

Transduction experiments showed that satellite cells were easy to transduce with LV and were able to retain a GFP signal up to and after formation of myotubes. It is known that primary muscle fibers that are terminally differentiated muscle cells do not easily undergo LV transduction. Indeed in adult fibres 72 hours after transduction little GFP signal was detected but there was a loss of cross-striation pattern and inability to respond to electrical stimulation. It was previously shown that transduction of skeletal muscle fibers was more successful in young (<2 weeks) compared to older (>6 months) mice [31, 42]. High numbers of positively transduced muscle fibers were obtained for adenoviruses and herpes simplex viruses-1 but only in fibers in animals less than two weeks old [31, 42]. Several mechanisms coupled with aging appear to contribute to viral transduction resistance, including down-regulation of viral receptors, alteration of basal lamina properties, acquisition of immunological maturity, and decline of satellite cells number [30, 42–44]. Other work suggested that adenoviruses were effective for in vitro transduction of FDB muscles. However, the study on FDB muscle did not examine the retention of functional capacity of positively transduced muscle fibers [11]. In our experiments we used adult mice (at least ten weeks old) in order to test and develop a reliable model. We applied LV transduction due to its high transduction titer to genetically modify muscle fibers. However, when a positive signal in the muscle fibers was detected, the muscle fibers displayed a loss of cross-striation and had shortened, probably due to the high toxicity of viral application. On the other hand, LV was able to transduce satellite cells, both those free on the dish and also those attached to the muscle fiber. Moreover, we confirmed that LV efficiently transduced primary myoblasts both at the proliferation (myoblast) stage and at myotubes stage [34, 35]. To sum up while we were unable to achieve positive transduction of primary muscle fibers with retaining their functional activity, we demonstrated that satellite cells were easily transduced by LV and remained physiologically active, in line with previous works [35].

5. Conclusions

Muscle dystrophies are accompanied by impairment of intracellular calcium balance. Therefore, it is of particular importance to study calcium pathways within the muscle cells to elucidate precise molecular mechanisms underlying these disorders. Functional analysis of the myotubes formed upon primary satellite cells fusion confirmed their well-differentiated characteristics and their ability to react to electrical and chemical stimulations and the presence of functional L-type
Figure 4: Satellite cell isolation and transduction. (a) 96 hours after isolation enzymatic digestion to obtain a "pure" satellite cell culture resulted in more satellite cells in comparison to experiments where satellite cells were allowed to branch out of muscle fibers. (b) Satellite cell branching out primary muscle fiber, 96 hours after isolation. (c) Muscle fiber and branching out satellite cells 24 hours (left panel) and 96 hours (right panel) after isolation. (d) Enzymatically isolated satellite cells were transduced via concentrated LV (upper panel) encoded GFP. 72 hours after transduction via LV 95% of observed cells express GFP, thus confirming high transduction efficiency (lower panel) encoded human lamin A/C. Myotubes were stained anti-lamin and anti-desmin. Positive staining confirmed myogenicity of transduced cells. Nuclei are shown counterstained with DAPI. Scale bar corresponds to 50 μm (e) Satellite cells branching out primary muscle fiber stained anti-desmin. Positive staining confirms myogenicity of cells located on the muscle fiber surface.
Ca\textsuperscript{2+} channels in the plasma membrane. Moreover, unlike adult muscle fibres, satellite cells derived from adult mouse muscle were easily transduced via LV and were able to retain positive signal up to and after formation of myotubes. These results suggest that satellite cells constitute a promising cell model for further experiments aimed at exploring calcium pathways involved in muscle dystrophies caused by mutations in miscellaneous genes.
Figure 6: Physiological properties of primary myotubes. (a) Primary myotubes responded to chemical and electrical stimulation within an increase in cytosolic Ca\textsuperscript{2+} followed by mitochondrial Ca\textsuperscript{2+} uptake. Cytosolic Ca\textsuperscript{2+} increase evoked by CmC or electrical stimulations and myotubes responded to stimulations by contraction and [Ca\textsuperscript{2+}]\textsubscript{i} increase, confirming developed system of DHPR and RyR. Scale bar corresponds to 50 \(\mu\)m. (b) Representative L-type Ca\textsuperscript{2+} current traces recorded in myotubes after seven days of differentiation (upper panel) and in muscle fibers (middle panel) in response to a series of 200 ms depolarizing steps from –30 to 40 mV in 10 mV increments (lower panel). (c) Current-voltage relationship in adult FDB muscle fibers and myotubes at seventh day of differentiation. Data are presented as mean ± SD (n = 3 cells).

**Abbreviations**

Adeno: Adenovirus  
AAV: Adenoassociated virus  
CmC: 2-Chloro-\textit{m}-cresol  
DMD: Duchene muscular dystrophy  
FDB: Muscle flexor digitorum brevis  
GFP: Green fluorescent protein  
HSV: Herpes simplex virus
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments
This work was supported by the Swedish Heart-Lung foundation, Stifelsen Frimurare Barnhust, Stifelsen Samariten, ALF Grant no. 20120446, and Russian Federal program “Scientific and Educational Recourses of Russian Innovation,” “Russian Scientific Foundation,” Grant Agreement no. 14-15-00745.

References


Research Article

Transcriptional Pathways Associated with Skeletal Muscle Changes after Spinal Cord Injury and Treadmill Locomotor Training

Celine Baligand,1 Yi-Wen Chen,2,3 Fan Ye,4 Sachchida Nand Pandey,2 San-Huei Lai,2 Min Liu,4 and Krista Vandenborne4

1Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL 32610, USA
2Center for Genetic Medicine Research, Children’s National Health System, Northwest, Washington, DC 20010, USA
3Department of Integrative Systems Biology, George Washington University, Northwest, Washington, DC 20010, USA
4Department of Physical Therapy, University of Florida, Gainesville, FL 32610, USA

Correspondence should be addressed to Krista Vandenborne; kvandenb@phhp.ufl.edu

Received 11 February 2015; Accepted 19 May 2015

Academic Editor: Christian Guelly

Copyright © 2015 Celine Baligand et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The genetic and molecular events associated with changes in muscle mass and function after SCI and after the implementation of candidate therapeutic approaches are still not completely known. The overall objective of this study was to identify key molecular pathways activated with muscle remodeling after SCI and locomotor training. We implemented treadmill training in a well-characterized rat model of moderate SCI and performed genome wide expression profiling on soleus muscles at multiple time points: 3, 8, and 14 days after SCI. We found that the activity of the protein ubiquitination and mitochondrial function related pathways was altered with SCI and corrected with treadmill training. The BMP pathway was differentially activated with early treadmill training as shown by Ingenuity Pathway Analysis. The expression of several muscle mass regulators was modulated by treadmill training, including $Fst$, $Jun$, $Bmpr2$, $Actr2b$, and $Smad3$. In addition, key players in fatty acids metabolism ($Lpl$ and $Fabp3$) responded to both SCI induced inactivity and reloading with training. The decrease in $Smad3$ and $Fst$ early after the initiation of treadmill training was confirmed by RT-PCR. Our data suggest that TGF-$\beta$/Smad3 signaling may be mainly involved in the decrease in muscle mass observed with SCI, while the BMP pathway was activated with treadmill training.

1. Introduction

Spinal cord injury (SCI) is one of the most disabling health problems faced by adults today. One of the physiological changes secondary to SCI is progressive muscle atrophy. The loss of muscle mass after spinal cord injury has been well documented, with patients with complete SCI having about 20–55% muscle atrophy [1] and patients with incomplete SCI showing about 20–30% atrophy [2] within 6 months to 1 year after SCI. Muscle wasting does not only impact the care and lifestyle of patients after SCI but also has significant health implications, increasing the patients’ risk to develop secondary complications such as bone demineralization, diabetes, and cardiovascular disease [3, 4]. Maintenance of muscle mass is essential to metabolic health and is necessary to maximize functional gain from rehabilitation strategies after SCI.

A potential rehabilitation intervention in the treatment of individuals with SCI is the use of repetitive locomotor training to promote neural plasticity. This approach was derived from animal and human studies showing that stepping can be generated by virtue of the neuromuscular system’s responsiveness to phasic peripheral sensory information associated with locomotion, in the presence of central pattern generator (reviewed in [5]). Our group and others have shown that locomotor training can induce substantial
recovery in muscle size and muscle function in transected [6] and moderate contusion injury animal models of SCI [7–9]. Some studies suggest that this may reflect enhanced synthesis of growth factors within the central nervous system [10], but other potential activity-dependent molecular changes remain unknown, in particular the ones occurring locally in the muscle in parallel with atrophy and hypertrophy.

Muscle atrophy depends on the balance between protein breakdown, protein synthesis rates, and apoptosis. It has been attributed to the activation of various protein degradation pathways in several models of disuse, such as denervation, unloading, cachexia, or aging [11, 12]. Previous work in animal models showed that alterations in the protein ubiquitination and energy production related pathways are common features of the atrophy process [13–16]. The activation of growth factors such as insulin-like growth factors (IGFs), myogenic regulatory factors (MRFs) [17], transforming growth factors (TGFs) [18], and the bone morphogenic proteins (BMPs) [19] has also been shown to play an important role in muscle atrophy and hypertrophy. However, the activation or predominance of the different pathways involved can be specific to the condition inducing atrophy [20]. To our knowledge, only one study used gene profiling in human muscle samples to perform a general screening of the pathways activated in skeletal muscle after SCI [21]. However, the specific molecular signaling changes that occur after SCI and subsequent recovery and/or rehabilitation intervention remain largely unknown.

A better understanding of the molecular events regulating protein synthesis and degradation after SCI and locomotor training and their temporal relationship to changes in muscle mass is of considerable clinical importance and has far-reaching implications for posttraumatic health care. Therefore, the overall objective of this study was to identify key molecular pathways activated with muscle remodeling after SCI and during locomotor training. We implemented treadmill training in a well-characterized rat model of moderate SCI [9, 22–24] and performed genome wide expression profiling with microarray on soleus muscles at multiple time points during the course of SCI (prior and 3, 8, and 14 days after SCI) and during the course of a treadmill training intervention initiated 7 days after injury (8 and 14 days after SCI). We used two different approaches for data analyses. First, we took an unsupervised approach and identified molecular pathways affected most at each time point. We then targeted genes that are known to be involved in muscle remodeling and may play important roles in the process.

2. Material and Methods

2.1. Study Design. In this cross-sectional study, six groups of rats were studied, including a control group, three SCI groups at three time points (days 3, 8, and 14), and 2 SCI groups with treadmill training at two time points (days 8 and 14). In the latter groups, the treadmill was initiated 7 days after SCI. For the analysis of changes during the course of SCI, comparisons were done with reference to control samples for each time point after SCI (days 3, 8, and 14). For the analysis of the effects of treadmill training on SCI, samples from the treadmill trained SCI rats were compared to samples collected from SCI rats with no training. Differences between the trained muscles and untrained muscles at a specific time point (day 8 or day 14) were determined.

2.2. Animals. Thirty-six adult female Sprague Dawley rats (16 weeks of age, 260–280 g at the beginning of the study) were obtained from Charles River Laboratories and housed in a temperature (22 ± 1°C) and humidity (50 ± 10%) controlled room with 12:12 hours light:dark cycle. Animals were provided rodent chow and water ad libitum and were given 1 week to acclimatize to the environment. Animals were sacrificed at one of the following time points and conditions: 3 days after SCI (SCI13d, n = 6), 8 days after SCI (SCI18d, n = 6), 14 days after SCI (SCI14d, n = 6), 8 days after SCI and after 3 treadmill training sessions (SCI18d + TM, n = 6), and 14 days after SCI and after 5 days of repeated treadmill training sessions (SCI14d + TM). Age-matched control animals (CTR, n = 6) were used as the baseline. Experimental animals were given access to transgenic dough diet (Bio-Serv, NJ, product number S3472), placed on the bottom of the cage, to ensure adequate food intake. All procedures were performed in accordance with the US Government Principle for the Utilization and Care of Vertebrate Animals and were approved by the Institutional Animal Care and Use Committee at the University of Florida.

2.3. Spinal Cord Injury Procedure. All surgical procedures were performed under aseptic conditions. Moderate contusion SCI was produced using a NYU-MASCIS injury device as previously described [25, 26]. Briefly, the animals were deeply anesthetized with a combination of ketamine (90 mg/kg body weight) and xylazine (8 mg/kg body weight) and a dorsal laminectomy was performed at the thoracic vertebral level T7–T9 to expose the spinal cord [27]. Clamps attached to the spinous processes of T7 and T9 stabilized the vertebral column. Contusion was produced by dropping a 10 g cylinder from a height of 25 mm onto the T8 segment of the spinal cord. Analgesia was given in the form of buprinex (0.025 mg/kg) and ketoprofen (22 mg/kg) once daily over the first 36 hrs after SCI. The animals were housed individually and kept under vigilant postoperative care, including daily examination for signs of distress, weight loss, dehydration, and bladder dysfunction. Manual expression of bladders was performed 2-3 times daily, as required, and animals were monitored for the possibility of urinary tract infection.

2.4. Locomotor Training. Quadrupedal locomotor training was initiated on postoperative day 7. Training consisted of 20 min stepping sessions on a treadmill. Training was performed 3 times in the SCI18d + TM group (2 times on day 7 and one time on day 8) and was repeated twice a day for 5 days in the SCI14d – TM group. When necessary, body weight support was manually provided by the trainer. The level of body weight support was adjusted to make sure that the hindlimbs of the animals did not collapse and was gradually removed as locomotor capability improved. During
the first day of training, assistance was provided to place the rat hindpaws in plantar-stepping position during training. Typically, rats started stepping when they experienced some load on their hindlimbs.

2.5. Tissue Collection. Left soleus muscles were harvested in all groups. In the SCI8d + TM and SCI14d + TM groups, muscle samples were harvested 8 hours after the end of the last treadmill training session. Briefly, rats were anesthetized with isoflurane (3% for induction, 1-2% for maintenance), and a small dorsal, midline incision was made to expose the gastrocnemius-soleus complex. The soleus was carefully separated from the gastrocnemius, harvested, and weighed. The sample was rapidly frozen in isopentane, precooled in liquid nitrogen, and subsequently stored at −80°C.

$t$-test was used to determine the statistical significance ($p < 0.05$) of the changes in muscle mass.

2.6. Expression Profiling. GeneChip Rat Genome 230 2.0 Array microarrays containing approximately 30,000 transcripts were used for the expression profiling experiment. Standard procedures including total RNA isolation, cDNA synthesis, cRNA labeling, microarray hybridization, and image acquisition were done as described in the manufacturer's protocol and our previous publications [20, 28]. Briefly, total RNA was isolated with TRIzol reagent (Invitrogen) and then purified with RNeasy MinElute Cleanup Kit (Qiagen). Two hundred nanograms of total RNA from each sample was reverse-transcribed to double-stranded cDNA followed by in vitro cRNA synthesis using one-cycle target labeling and control reagents and protocol (Affymetrix). Biotin-labeled cRNA was then purified using GeneChip Sample Cleanup Module (Affymetrix) and fragmented randomly prior to hybridizing to the microarrays overnight. Each array was washed and stained using the Affymetrix Fluidics Station 450 and then scanned using the GeneChip Scanner 3000. The quality control criteria developed at Children’s National Medical Center Microarray Center for each array were followed [6].

Generation of hybridization signals of the microarrays was done using Microarray Suite 5.0 (MAS 5.0) (Affymetrix, CA) as previously described [21, 29–32]. After the absolute analysis, the gene expression values were imported into GeneSpring 11.0 (Silicon Genetics) for data filtering and statistical analysis. First, genes were filtered with numbers of present calls across the 36 arrays analyzed. Genes with at least 4 present calls (detected by more than 10% of the arrays) were selected for statistical analysis. We identified 31099 probe sets that met this filtering criterion. In GeneSpring, $t$-test was performed and probe sets showing significant ($p < 0.05$) expression changes were retained for pathway analysis. No additional fold change filters were used. The comparisons were done by comparing samples of each time point after SCI (days 3, 8, and 14) to the control time point (day 0), respectively. The treadmill trained samples were compared to the SCI samples collected at the same time point to obtain differences between the trained muscles and untrained muscles at a specific time point (day 8 and day 14).

To investigate molecular networks and pathways associated with gene lists in this study, Ingenuity Pathway Analysis (IPA) (Ingenuity Systems) was used with default settings to identify gene interactions and to prioritize molecular pathways differentially affected in different groups. Hierarchical clustering was performed using GeneSpring software to visualize transcripts showing coordinate regulation as a function of time.

The significance of the association between the genes in each dataset and the canonical pathway was determined by Fisher's exact test. The $p$ values were calculated to determine the probability of the association between the genes. All profiles have been made publicly accessible via NCBI GEO (number GSE45550) (http://www.ncbi.nlm.nih.gov/geo/).

2.7. Reverse Transcription and Quantitative RT-PCR Analysis. Reverse transcription and quantitative RT-PCR (qRT-PCR) were performed as previously described [17]. Briefly, total RNA (2 μg) was reverse-transcribed to cDNA using oligo(dT) primer (0.5 μg/μL) and reagents from Invitrogen. cDNA was amplified in triplicate in SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions included 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. Primer sequences were used for rat myogenic factor 6 (MYF6 6/MRF4), follistatin (FST), mothers against decapentaplegichomolog 3 or SMAD family member 3 (SMAD3), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as an internal control, are provided in Table 1. All primers were tested for nonspecific amplicons and primer dimers by visualizing PCR products on 2% agarose gels before performing qRT-PCR. The $\Delta\DeltaCT$ value method (where CT is cycle threshold) was used to determine fold differences as described previously [17].

### Table 1: Primer sequences used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>F-5′-TCGCCCCCTTCGGCTGATG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R-5′-CACGGGAAGGCTGAATCGTGA-3′</td>
</tr>
<tr>
<td>Smad3</td>
<td>Forward</td>
<td>F-5′-AAGATACCCAGGCTGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R-5′-CTGCTGCTCTGCTCTGATCTC-3′</td>
</tr>
<tr>
<td>Myf6</td>
<td>Forward</td>
<td>F-5′-CTAAGGAAGGAGGCAAG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R-5′-TGTGCCAATGCTGACTGAG-3′</td>
</tr>
<tr>
<td>Fst</td>
<td>Forward</td>
<td>F-5′-GTGGATCAGGAAGCTGTTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R-5′-GCTCATCGACAGAGCA-3′</td>
</tr>
</tbody>
</table>

3. Results

3.1. Changes in Soleus Muscle Wet Weight after SCI and Treadmill Training. Soleus muscle wet weights are presented in Figure 1. SCI resulted in a rapid loss in muscle weight 3 and 8 days after injury (−25%, $p = 0.0005$). By day 14, muscle weight was still lower than in controls without reaching significance (−16%, $p = 0.056$). However, 5 days of training significantly increased muscle wet weight (SCI14d + TM, $p = 0.005$).
Table 2: Five most significantly activated pathways for each comparison of the study.

<table>
<thead>
<tr>
<th>Group/comparison</th>
<th>3 days</th>
<th>8 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein ubiquitination pathway</td>
<td>Mitochondrial dysfunction</td>
<td>Mitochondrial dysfunction</td>
</tr>
<tr>
<td>No treadmill training versus control</td>
<td>Mitochondrial dysfunction</td>
<td>Valine, leucine, and isoleucine degradation</td>
<td>Synthesis and degradation of ketone bodies</td>
</tr>
<tr>
<td></td>
<td>Oxidative phosphorylation</td>
<td>Propanoate metabolism</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td></td>
<td>Regulation of eIF4 and p70S6K signaling</td>
<td>Butanoate metabolism</td>
<td>Citrate cycle</td>
</tr>
<tr>
<td></td>
<td>Ubiquinone biosynthesis</td>
<td>Pyruvate metabolism</td>
<td>Ephrin receptor signaling</td>
</tr>
<tr>
<td>Treadmill training versus no training</td>
<td>Protein ubiquitination pathway</td>
<td>Estrogen receptor signaling</td>
<td>EIF2 signaling</td>
</tr>
<tr>
<td></td>
<td>Amyloid processing</td>
<td>BMP signaling pathway</td>
<td>Regulation of eIF4 and p70S6K signaling</td>
</tr>
<tr>
<td></td>
<td>Role of BRCA1 in DNA damage response</td>
<td>Hereditary breast cancer signaling</td>
<td>Glutamate metabolism</td>
</tr>
<tr>
<td></td>
<td>Hereditary breast cancer signaling</td>
<td>Granzyme B signaling</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Muscle wet weight was significantly decreased on days 3 and 8 as compared to day 0 and significantly increased in the treadmill training group as compared to untrained animals on day 14. *p < 0.05.

Figure 2: Number of probe sets differentially expressed in control and SCI rat soleus muscles decreased 8 and 14 days after SCI.

3.2. The Highest Number of Differentially Expressed Transcripts Was Observed 3 Days after SCI. The largest number of genes differentially expressed compared to controls was found 3 days after SCI (Figure 2). In the treadmill trained groups, the largest changes were found on day 14, compared to untrained animals (Figure 2). In the untrained group, about 60% of the genes were downregulated at each time point, which was reversed in the treadmill training group on day 8 with 63% of the genes upregulated. To identify the major molecular pathways affected in each condition, Ingenuity Pathway Analysis (IPA) was performed. Table 2 shows the top 5 canonical pathways for each comparison.

3.3. Protein Ubiquitination and ATP Production Related Genes Were Altered following SCI. At the early time point (day 3), we found that the protein ubiquitination pathway was largely activated. Indeed, 90% of the genes identified by IPA showed increase in gene expression. In particular, the expression of many proteasome subunits (PSMs) and ubiquitin specific peptidases and enzymes was increased (Suppl. Table 2 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/387090). However, changes in this pathway were no longer significant on days 8 and 14 as compared to control levels. On the other hand, the mitochondrial dysfunction and oxidative phosphorylation pathways were consistently ranked among the top 5 most significant pathways at all time points of the study (days 3, 8, and 14) (Table 2). More than 95% of the genes identified in these two pathways were downregulated 3 days after SCI (Suppl. Tables 2, 3, and 4). In particular, the expression of the NADH dehydrogenase subunits (NDUF) genes, the succinate
3.4. Treadmill Training Rapidly Reversed the Changes in Protein Ubiquitination, Translation Factors, and Mitochondrial Function Pathway. The protein ubiquitination pathway was rapidly affected by treadmill training. While the majority of the PSMs were upregulated on day 3, an overall significant decrease was observed after 3 sessions of treadmill training. In the Eif2 and the eif4/p70sk6 pathways, the expression of the eukaryotic translation initiation factors (EIFs) subunits and kinases underwent overall negative fold changes on day 3 (Suppl. Table 2), which was corrected with training (Suppl. Table 4). In addition, the expression of NDUFs ATP synthases, Cox8A and Cox7A2L, was upregulated in trained animals on day 14, also showing a corrective effect of TM on muscle oxidative metabolism.

3.5. TGF-β/Smad and BMP Signaling Pathways Are Involved in the Muscle Remodeling Process during the Course of SCI and Early Treadmill Training. Because the bone morphogenic proteins (BMPs) signaling pathway was significantly altered on day 8 in the trained group (ranked number 3, p < 0.005), we examined the changes in gene expression of some key players in this pathway and in the TGFβ pathway. To explore possible downstream changes, we also looked into the gene expression of the different smads associated with these pathways (Smad1, Smad3, Smad4, Smad5, Smad6, and Smad8), in particular on day 3, and with acute training on day 8.

On day 3 in the BMP pathway, Smad4 (+1.8, p < 0.05), Smad3 (+1.3, p < 0.05) gene expression was upregulated. Smad3 did not show any significant changes on day 3, while the activin receptor 2B (Acvr2B) was downregulated (~1.6, p < 0.05). In parallel, Smad6, inhibitor of Bmpr2 activation, was decreased (~2.3, p < 0.05).

In SCI8d, Smad1 gene expression was increased (+1.6 fold, p < 0.05). In addition, both follistatin (Fst) and Smad3 expression were increased (+2.5 fold, p < 0.05, +2.0 fold, p < 0.05, resp.) as compared to controls (Figure 3).

In SCI + TM on day 8, Fst was significantly decreased (~1.5 fold, p < 0.05). Smad3 was also found to be significantly downregulated (~1.3 fold, p < 0.05). The expression of the BMP complex 3 (Bmp3) was decreased (~2.35, p < 0.05). Importantly, Bmpr2 was concomitantly overexpressed with treadmill training as compared to SCI only (+1.6 fold, p < 0.05). As part of the genes reported in the BMP pathway activated with training on day 8, Jun followed the same large and transient increase (+1.5, p < 0.05) as seen in Bmpr2 (Figure 3).

3.6. Genes Involved in Myogenesis and Muscle Regeneration Were Affected by Treadmill Training. In addition to genes identified by IPA, we specifically studied genes involved in myogenesis, lipid metabolism, and fiber type switches, with a particular focus on those that were affected by 5-day treadmill training.

Igfbp5, modulator of IGF1 function in skeletal muscle, showed a significant decrease 3 days after SCI, followed by a large increase on day 14. This upregulation was not observed in the treadmill training group.

Three days after injury, dramatic changes were observed in myogenic regulatory factors (MRFs), including Myf6 and myogenin (Myog) (Figure 4). The Myog expression pattern was not affected by treadmill training. On the other hand, treadmill training induced an additional increase (p < 0.05) in Myf6 after the first 3 sessions as compared to untrained animals.

3.7. Fatty Acid Metabolism and Fiber Type Switch Related Genes Showed High Sensitivity to SCI and Treadmill Training. Lipoprotein lipase (Lpl) and Fabp3 gene expression were dramatically decreased 3 days after SCI as compared to controls and maintained low expression levels on days 8 and 14 after SCI. While the first sessions of locomotor training did not significantly affect Lpl expression, it was completely restored on day 14 (+2.32 fold, p < 0.005) as shown in Figure 5.

As early as 3 days after SCI and throughout the experiment, several fast-twitch fiber markers genes (Mhyl, Mybph, and Myh4) showed large and significant changes (Figure 6). In parallel, the expression of several slow-twitch and vasculature smooth muscles markers (Myh3 and Myl2) was decreased (p < 0.04) (Figure 6). Treadmill training had a significant reverse effect on the expression of some fiber type related genes, such as the fast-twitch markers Myhl and Myh4.

3.8. RT-qPCR Validation. To validate microarray findings, we selected 3 genes (Fst, Smad3, and Myf6) that were found to be significantly affected by SCI and showed reversed changes after the initial treadmill training. RT-qPCR confirmed that Fst mRNA levels were significantly higher on day 8 after SCI compared to controls (+2.5 fold, p < 0.05) and decreased in the trained group compared to untrained (Figure 7). Smad3 expression was also significantly decreased with acute treadmill training (~1.5 fold, p < 0.05). On the other hand, the increase in Myf6 in SCI on day 8 was not confirmed (Figure 7(c)).

4. Discussion

The genetic and molecular events associated with changes in muscle mass and function after SCI and after the implementation of candidate therapeutic approaches are still not completely known. We used a well-characterized rat model of moderate SCI combined with treadmill training as a rehabilitation strategy to explore the pathways and genes involved in these conditions. The unique design of our study and the use of genome wide analysis allowed the identification of several major canonical pathways involved in protein synthesis and muscle metabolism regulation after SCI and treadmill training. In particular, the activity of the protein ubiquitination and mitochondrial function related pathways was altered with SCI and corrected with treadmill training. Of particular interest, the BMP pathway was differentially activated with early treadmill training as shown by IPA. The expression of several muscle mass regulators was modulated
Figure 3: BMP/TGF-β pathway related genes. Gene expression level changes in Fst (a), Smad3 (b), Acvr2b (c), Smad1 (d), Bmpr2 (e), Smad6 (f), Jun (g), and Smad4 (h) in soleus of trained and untrained SCI animals. All expression levels are referenced to a control sample's GAPDH levels. *Significantly different from controls (p < 0.05). #Significantly different from untrained animals (p < 0.05).
by treadmill training including \( Fst, \) \( Jun, \) \( Bmpr2, \) \( Actr2b, \) and \( Smad3. \) In addition, key players in fatty acids metabolism (\( Lpl \) and \( Fabp3) \) proved to be major sensors of SCI induced inactivity and reloading with training. The decrease in \( Smad3 \) and \( Fst \) early after the initiation of treadmill training was confirmed by RT-PCR. Our data suggest that TGF-\( \beta/Smad3 \) signaling may be mainly involved in the decrease in muscle mass observed with SCI, while the BMP pathway was activated with treadmill training. We also identified changes in fiber type markers, consistent with a switch towards type II fibers with SCI, and a reverse effect of treadmill training at the gene expression level as early as 1 day after initiation.

4.1. Acute Response from the Protein Ubiquitination Pathway to SCI and Training. The protein ubiquitination pathway is essential to the control of protein breakdown and turnover in the cell. It has been established that the activation of this pathway contributes largely to muscle wasting in multiple

**Figure 4:** Myogenic regulatory factors. Gene expression level changes in \( Myf6 \) (a), \( Myog \) (b), in soleus of trained and untrained SCI animals. All expression levels are referenced to a control sample’s GAPDH levels. *Significantly different from controls (\( p < 0.05 \)). #Significantly different from untrained animals (\( p < 0.05 \)).

**Figure 5:** Fatty acids metabolism. Gene expression level changes in \( Lpl \) (a), \( Fabp3 \) (b), in soleus of trained and untrained SCI animals. All expression levels are referenced to a control sample’s GAPDH levels. *Significantly different from controls (\( p < 0.05 \)). #Significantly different from untrained animals (\( p < 0.05 \)).
conditions [33], including denervation [34, 35], age and sarcopenia [36], and hindlimb suspension in rats [37] and in spinal cord injury in humans [21, 38]. In our model of moderate SCI, the protein ubiquitination pathway was activated as early as 3 days after injury, with the expression of 56 genes significantly upregulated. This upregulation was most likely responsible for the unbalance in protein synthesis/degradation ratio resulting in the large muscle wasting observed in the soleus on day 3. Conversely, the expression of genes in the protein ubiquitination pathway was very rapidly decreased on day 8 after only 3 sessions of treadmill training in SCI animals and the expression of several 20S proteasome subunits was significantly reduced. This was in accordance with previous observations in a hindlimb suspension rat model [39] 10 days after reloading. However, in another study in humans, Reich et al. [38] could not detect any reverse effect with 24 h of reloading after lower limb suspension. Here, we were able to observe a significant and early response within 36 h after the first training session, with a significant decrease in the expression of the genes related to protein ubiquitination (Suppl. Table 5).

4.2. Normalization of Mitochondrial Function Related Pathways with Training. Genes involved in the mitochondrial dysfunction and oxidative phosphorylation related pathways were also significantly changed. Together, the large number of NUDF, SDH, and ATP synthases with changing gene expression observed in SCI demonstrates a strong metabolic activity disturbance associated with SCI that does not completely recover within 14 days after surgery. Mitochondrial dysfunction after SCI was observed in vivo in several studies performed in humans with SCI [40, 41]. Our own work using 31P magnetic resonance spectroscopy to assess mitochondrial function in vivo in the same rat model of moderate SCI [24]
Figure 7: FST (a), SMAD3 (b), and MYF6 (c) mRNA expression in the soleus muscle of controls, untrained SCI animal on day 8 after surgery (SCI8d), and trained SCI animal on day 8 after surgery (SCI8d-TM). *p < 0.05.

Figure 8: Schematic of the changes in soleus muscle gene expression within the TGFβ and BMP pathways, with SCI (a) and acute response to treadmill training (b). Panel (a) shows the combined changes observed on day 3 and 8 after SCI, where several Smads (Smads 3, 4, and 1/5) and Fst gene expression was increased compared to control in parallel with muscle atrophy. Panel (b) summarizes the changes observed 8 days after SCI after 3 sessions of treadmill training compared to untrained animals. The changes in Smad3 and Fst were reversed, and Bmpr2 expression was increased, suggesting a role for the BMP pathway in the initiation of muscle mass recovery process observed with treadmill training.
showed that ATP production by mitochondria was affected 1 week after SCI and recovered in 3 weeks without training. It should be noted that, in the present study, no acute effect of treadmill training on mitochondrial dysfunction and oxidative phosphorylation pathways related genes was detected on day 8. However, our results suggest that treadmill training and the concomitant muscle hypertrophy are accompanied by partial correction of the mitochondrial function pathway genes (NUDFs and ATP synthase) at a later time (day 14), making mitochondria a target for early therapy in moderate SCI.

4.3. Role of TGF-β/Smad and BMP Pathways in Atrophy following SCI. Follistatin (FST) is well known as an inhibitor of the myostatin signaling within the TGFβ pathway. It has been shown that Fst overexpression leads to a large increase in muscle mass in different animal models [42, 43]. Our results showed a large significant increase of FST mRNA levels with SCI after 8 days. It is possible that this increase reflects a protective mechanism intended to stimulate muscle growth. However our data showed that muscle wet weight did not further decrease after the initial 28% loss on day 3. One possibility is that the increase in FST mRNA levels may not have been sufficient to produce a recovery of muscle mass. Interestingly, we also observed a concomitant increase in Smad3 and Smad4 gene expression, downstream the Act2b receptor, which might be responsible for atrophy. Indeed, phosphorylated SMAD3 can mediate the activation of ubiquitin ligases that induces proteasomal degradation of contractile proteins [44]. More importantly, Winbanks et al. [45] recently showed that SMAD3 protein expression prevents skeletal muscle growth induced by follistatin and may suppress Akt/mTOR/S6K signaling. In addition, Smad1 expression downstream the BMP pathway was increased, potentially competing with SMAD3 for the recruitment of SMAD4. If translated at the protein level, the balance between FST inhibition of the TGFβ signaling and increased SMAD3 and SMAD1 expression may have led to a plateau in soleus muscle mass after the initial drop (Figure 8).

4.4. Role of TGFβ/Smad and BMP Pathways in Hypertrophy following Treadmill Training. On the other hand, treadmill training was able to restore the initial muscle mass by day 14 after SCI. Our main associated findings were a large and rapid increase in the expression of Bmpr2 with treadmill training 36 hours after initiation, in parallel with the significant decrease in FST and SMAD3 mRNA levels. The increased Bmpr2 (BMPs receptor) levels could favor its activation by BMPs and the subsequent phosphorylation of Smad1, Smad5, and Smad8, which in turn bind with Smad4 leading to hypertrophy [19]. Whereas a decrease in FST expression has the ability to enable MSTN signaling and muscle growth, a parallel decrease in SMAD3 protein expression could contribute to an increase in SMAD4’s availability to other binding proteins that can lead to hypertrophy, such as SMADs 1, 5, and 8. A concomitant and rapid increase in Jun was observed. It was shown that this transcription factor acts downstream the TGFβ [46] and that overexpression of JUN results in dephosphorylation of SMAD3 [47]. This body of observations is suggestive of a deactivation of the TGFβ/Smad pathway and a larger role for the BMP axis in the hypertrophy process following SCI and treadmill training.

4.5. Other Growth Factors (IGFs, MRFs). The IGF1-Akt-mTOR pathway is known as a positive regulator of muscle mass [18, 48–50]. This pathway was not highly ranked by the present microarray analysis. However, the group previously demonstrated the impact of moderate SCI and treadmill training on several IGF proteins and binding proteins, but not on MYF5 in skeletal muscle [23]. Consistently with these previous results, here we observed that Igfbp5 and Myog were overexpressed after SCI and that Igfbp5 expression levels were corrected with treadmill training. In addition, we explored the possible impact of treadmill training on MRF4/MYF6 as indicated by the microarray results and found no significant increase in mRNA levels. This discrepancy may be due to experimental variations or slight biological differences between the batches of muscle samples used for microarray and RT-qPCR validation.

4.6. Fatty Acid Metabolism and Fiber Type Related Genes as a Sensor of Muscle Activity in SCI and Treadmill Training. Lipoprotein lipase (LPL) is a major enzyme involved in fatty acid (FA) metabolism and transport. After transport into the cytoplasm, FA binds to the fatty acid binding protein 3 (FABP3). Both LPL and FABP3 have been shown to be highly sensitive to contractile activity in muscle [51, 52]. Here, both Lpl and Fabp3 genes showed high sensitivity to SCI induced disuse and to locomotor training. The downregulation of these fatty acid transporters in SCI rats was consistent with the recent observation by Long et al. [53] in muscle biopsies obtained from human subjects with SCI. On the other hand, we measured a large positive change in Lpl and Fabp3 gene expression in response to locomotor training, with expression levels nearly corrected (92% of control levels) by day 14. Upregulation of LPL and FABP3 has been previously demonstrated in conjunction with increased muscle activity, such as muscle endurance training in humans [54], reloading after hindlimb suspension [28], and electrostimulation in denervated muscles [55] in rats. We established that treadmill training, as performed in our study, was sufficient to restore control levels of gene expression within 5 days. Our results further demonstrated the high sensitivity of Lpl and Fabp3 gene expression to muscle activity and reloading achieved by treadmill training leading to muscle hypertrophy in moderate SCI rats.

Fiber type related genes also showed a great sensitivity to SCI induced disuse. It has been well established in the literature that muscle fibers distribution undergoes a shift towards fast fibers with disuse after SCI [56]. Consistent with this, we observed a fast change in the expression of myosin heavy and light chains genes, with an increase in some of the fast type (Myh1, Myhbp) and a decrease in the slow type (Myl2 and Myh3). Interestingly, the expression level of Myh1 was corrected in response to treadmill training on day 14, showing the effect of training on SCI muscles.
In summary, using genome wide analysis, we were able to identify some of the main pathways responsible for muscle wasting following moderate SCI and more importantly the corrective effect of treadmill training on these pathways. Here, we chose to focus on the BMP and TGF-β signaling and established a key role for some of the genes within these pathways. Our observations suggest that Smad3, Bmpr2, and Fst are genes of interest in the study of moderate SCI. Protein expression and phosphorylation need to be investigated to allow further interpretation, although beyond the scope of this study. It would make it possible to test the hypotheses that Fst overexpression in SCI competes with Smad3 regulation and that this effect is reversed by treadmill training leading to muscle mass recovery. More importantly, this would elucidate whether or not treadmill training activates the BMP/Smad pathway contributing to hypertrophy. This would establish the effect of BMP signaling activation and TGFβ signaling on muscle regeneration with treadmill training in SCI via Smad3 downregulation proving early indicators of efficient reloading in SCI rat and as a promising therapeutic approach. The body of data presented in this study constitutes a comprehensive guide to future studies targeting muscle mass preservation or recovery in moderate SCI.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was funded by the Paralyzed Veterans Administration-2347, NIH/NICHD ROIHD048051, and IR24HD050846. Yi-Wen Chen and Sachchida Nand Pandey were partially supported by NIH/NIAMS under Award number 1R01AR052027 and FSHD Society under Award number FSHS-82013-01.

References


Tyrosine 705 Phosphorylation of STAT3 Is Associated with Phenotype Severity in TGFβ1 Transgenic Mice

Eleonora Guadagnin, 1 Jigna Narola, 1 Carsten G. Bönnemann, 2 and Yi-Wen Chen 1,3

1Research Center for Genetic Medicine, Children’s National Medical Center, 111 Michigan Avenue, NW, Washington, DC 20010, USA
2Neuromuscular and Neurogenetic Disorders of Childhood Section, Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 35 Convent Drive, Building 35, Room 2A116, Bethesda, MD 20892, USA
3Department of Integrative Systems Biology and Department of Pediatrics, George Washington University, 2121 I Street Northwest, Washington, DC 20052, USA

Correspondence should be addressed to Yi-Wen Chen; ychen@childrensnational.org

Received 20 January 2015; Accepted 30 March 2015

Academic Editor: Toshifumi Yokota

Copyright © 2015 Eleonora Guadagnin et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Transforming growth factor beta 1 (TGFβ1) is a key player in skeletal muscle degenerative and regenerative processes. We previously showed that conditionally overexpressing TGFβ1 in skeletal muscles caused myofiber atrophy and endomysial fibrosis in mice. However, the disease severity varied significantly among individual mice. While 40% of mice developed severe muscle pathology and lost body weight within 2 weeks of TGFβ1 transgene induction in muscles, the rest showed milder or no phenotype. This study aims at determining whether signal transducer and activator of transcription 3 (STAT3) plays a role in the phenotypic difference and whether it can be activated by TGFβ1 directly in muscle cells. Our results show that while total STAT3 was not differentially expressed between the two groups of mice, there was significantly higher pSTAT3 (Tyr705) in the muscles of the mice with severe phenotype. Immunohistochemistry showed that pSTAT3 (Tyr705) was localized in approximately 50% of the nuclei of the muscles. We further showed that TGFβ1 induced Tyr705 phosphorylation of STAT3 in C2C12 cells within 30 minutes of treatment while total STAT3 was not affected. Our findings suggest that TGFβ1 alone can induce Tyr705 phosphorylation of STAT3 in skeletal muscle cells and contribute to disease severity in transgenic TGFβ1 mice.

1. Introduction

TGFβ1 belongs to the TGFβ superfamily and has been shown to regulate a wide variety of biological processes, including promotion of apoptosis, inhibition of cell growth, and induction of cell differentiation, migration, and extracellular matrix (ECM) deposition [1, 2]. Several studies showed that persistent expression and activation of TGFβ1 act as negative regulator of muscle repair by inducing apoptosis in myoblasts, suppressing muscle differentiation, and causing fibrosis in the muscles [3–5]. TGFβ1 is believed to be responsible for the ECM deposition in skeletal muscle [6–9], which leads to endomysial and perimysial fibrosis in muscular dystrophies, including Duchenne muscular dystrophy and congenital muscular dystrophies [10–12]. Using animal models, we and others demonstrated that TGFβ1 alone can cause muscle atrophy and fibrosis in vivo [5, 13, 14]. However, TGFβ1 is also recognized to play critical roles in muscle regeneration process by recruiting macrophages to clean up the damaged tissues after muscle injury and in muscle diseases [15].

The signal transducer and activator of transcription (STAT) family are composed of latent cytoplasmic proteins with a dual molecular role: signal transducer and transcription activator [16, 17]. One member of the STAT family, STAT3, is expressed in most of tissue types and responds mainly to IL-6, IL-10, and EGF signals [18, 19]. Phosphorylation of specific receptor tyrosine residue (Tyr705 or Ser727) in response to ligand stimulation determines the activities of STAT3. Tyr705 phosphorylation of latent cytoplasmic STAT3 promotes STAT3 homodimerization or heterodimerization with other STATs, which leads to nucleus translocation and DNA binding. Ser727 phosphorylation takes place at the
C-terminal transactivation domain of STAT3 and allows maximal activation of transcription of its target genes [20]. Within hours, STAT3 is exported back to the cytoplasm and the signaling cascade is terminated [21–23]. Previous studies showed that TGFβ1 directly activates STAT3 in other cell types, including proximal tubular cells, T-cells, and pancreas [24–26]. One study showed that STAT3 activation by TGFβ1 plays a major role in the pathological connective tissue induction and fibrosis by TGFβ1 [24–26]. One study showed that STAT3 activation by TGFβ1 types, including proximal tubular cells, T-cells, and pancreas showed that TGFβ1 directly activates STAT3 in skeletal muscle cells.

To study the effects of TGFβ1 on muscle fibrosis and atrophy, we generated a tet-repressible muscle-specific TGFβ1 transgenic mouse model [5]. In this model, withdrawal of oral doxycycline induces the expression of TGFβ1 transgene. The study showed that TGFβ1 overexpression in skeletal muscles causes muscle atrophy and endomyosial fibrosis. Interestingly, we observed that a subgroup of the TGFβ1 transgenic mice showed more severe muscle weight loss while the rest exhibited milder pathology. The size of the myofibers was significantly smaller and the endomyosial fibrosis was significantly higher in the subgroup with severe phenotypes, suggesting that activation of additional signaling pathways leads to more severe phenotypes. In this study, we investigated whether the STAT3 and phosphorylation of the protein in the mice were associated with more severe phenotype. In addition, we conducted an in vitro study using C2C12 myoblasts to determine whether TGFβ1 can activate STAT3 in muscle cells.

2. Materials and Methods

2.1. Mouse Model and Muscle Collection. All muscle samples used in this were collected as described previously [5]. Briefly, the tet-repressible muscle-specific TGFβ1 transgenic mice (TRE-TGFβ1/mCK-tTA) were generated by crossing two transgenic mouse lines (TRE-TGFβ1 and mCK-tTA). The TRE-TGFβ1 line carries a porcine TGFβ1 cDNA containing a double mutation where cysteines at positions 223 and 225 are converted to serines, which is regulated by the tetrO recognition element (TRE). The mCK-tTA line carries a construct containing the tetracycline-controlled transactivator (tTA) protein driven by a muscle-specific creatine kinase promoter (mCK). The presence of doxycycline in cells inhibits binding of tTA to the TRE and blocks TGFβ1 transgene expression. After crossbreeding the TRE-TGFβ1 and mCK-tTA lines, the pregnant female mice received drinking water with doxycycline (200 μg/mL in 5.0% sucrose) in order to suppress the TGFβ1 transgene expression in the pups in utero. After weaning, all pups were maintained on water treated with doxycycline until the transgene was induced. In this study, doxycycline was removed from water to induce transgene expression in the TRE-TGFβ1/mCK-tTA mice when the mice were 6 weeks old. The muscles were collected 2 weeks after the TGFβ1 transgene was induced. Littermates with only one of the transgenes, which do not express TGFβ1 were used as controls.

2.2. Immunoblotting. Vastus lateralis muscles were sectioned with a Leica CM 1900 cryostat (Walldorf, Baden-Württemberg, Germany). Thirty 10 μm cryosections were lysed in 50 μL of RIPA buffer (0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 150 mM sodium chloride, and 50 mM Tris HCl pH 7.5) for 30 minutes on ice, with protease inhibitor cocktail (Complete, Roche, Mannheim, Germany) as well as phosphatase inhibitor cocktail (PhosStop, Roche, Mannheim, Germany). At the end of the incubation, the cell extracts were centrifuged for 10 minutes (12,000 g) at 4°C. The amount of protein was calculated using the Quick Start Bradford Protein Assay Kit 1 (Bio Rad Laboratories, Hercules, CA). Then 30 μg of protein in NuPAGE LDS Sample Buffer (Life Technologies, Grand Island, NY) and NuPAGE Sample Reducing Agent (Life Technologies, Grand Island, NY) was loaded to SDS-PAGE gel for immunoblotting analysis. The primary antibodies used were pSTAT3 (Y705, 1:1000; Cell Signaling Technology, Danvers, MA), pSTAT3 (S727, 1:1000; Cell Signaling Technology, Danvers, MA), and Total STAT3 (1:1000; Cell Signaling Technology, Danvers, MA). Bound antibodies were detected using ECL reagents. The results were normalized to GAPDH (1:5000; Millipore, Billerica, MA). Band intensity was evaluated by densitometry analysis, normalized to its total content, and reported as fold increase relative to respective control set as 1.

2.3. Immunofluorescence Staining. To detect pSTAT3 (Tyr705), muscle sections of 5 μm were fixed in 4% paraformaldehyde, washed 3 times in 1x PBS, permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature, and blocked with 5% goat serum. The slides were incubated overnight at 4°C with the primary antibody against pSTAT3 Tyr705 (1:100 diluted in 5% goat serum). Secondary antibody only was used as the negative control. After 3 washes in PBS for 15 minutes each, the slides were incubated with the secondary antibody Alexa Fluor 680 Donkey Anti-Rabbit IgG (Life Technologies, Grand Island, NY) for 1 hour at room temperature and then washed again 3 times in 1x PBS. Finally, the slides were mounted with the appropriate mounting medium (ProLong Gold Antifade Reagent with DAPI, Molecular Forbes, Life Technologies, Grand Island, NY). Images of the tissue sections (20x, 40x) were taken using Nikon Eclipse E800 microscope (Nikon, Chiyoda-ku, Tokyo, Japan), RT slider camera (Diagnostic Instrument, Sterling Height, MI), and SPOT advanced software.

2.4. Cell Culture and Treatment. Murine C2C12 myoblasts were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin at 37°C in 5% CO2. Cells were seeded in 6-well plates, and when they were 70% confluent, they were induced to differentiate with DMEM supplemented with 5% horse serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin at 37°C in 5% CO2. TGFβ1 (R&D SYSTEMS, Minneapolis, MN) was reconstituted at 20 μg/mL in sterile 4 mM HCl containing...
Figure 1: STAT3 phosphorylation level following TGFβ1 overexpression. Representative western blot of vastus lateralis muscle protein samples of controls (n = 6), LO (n = 6), and EO (n = 6), with anti-phospho-STAT3 (Tyr705), anti-phospho-STAT3 (Ser727), anti-total-STAT3, and anti-GAPDH. Graphs show normalized expression values of pSTAT3 (Tyr705), pSTAT3 (Ser727), and total STAT3 normalized to GAPDH ± SEM. All the values are normalized for the average of the control. * indicates p < 0.05.

1 mg/mL BSA, according to the manufacturer’s instructions. C2C12 were then treated with TGFβ1 10 ng/mL after 7 days of differentiation for 30 minutes, 2 hours, and 24 hours. The cells were harvested and lysed in 30 μL of RIPA buffer for immunoblotting.

2.5. Statistical Analysis. Data are shown as mean ± SEM. The Kruskal-Wallis test was used for determining statistical significance among different groups of mice. Values of p < 0.05 were considered significant. Student's t-test was used for determining statistical significance in treated cells. Values of p < 0.05 were considered significant.

3. Results

3.1. Tyr705 Phosphorylation of STAT3 Is Associated with the Severe Phenotypes Induced by TGFβ1. After the TGFβ1 transgene was induced for two weeks, approximately 40% of mice developed severe phenotypes, including early body weight loss and severe myofiber atrophy and fibrosis [5]. In the study, the mice in this group were defined as mice with early onset (EO). The rest of mice were grouped into the late onset (LO) group. To determine whether STAT3 activation, which is known to be involved in muscle atrophy induced by IL-6, is involved in the variation of phenotypic presentations, we first examined the protein expression of total STAT3 as well as two phosphorylated STAT3, pSTAT3 (Tyr705), and pSTAT3 (Ser727), in muscles collected from the two groups of mice. Littermates of these mice, which did not express TGFβ1, were used as baseline control.

Immunoblotting analysis showed that while the total STAT3 was not significantly different among the EO, LO, and control groups, pSTAT3 (Tyr705) was significantly induced in the muscles of EO mice. No pSTAT3 (Tyr705) was detected in the control or LO mice (Figure 1). The expression of pSTAT3 (Ser727) was observed in muscles of all 3 groups but no significant difference among them. Variations of expression levels of total STAT3 and pSTAT3 (Ser727) were observed among different samples in all three groups. However, no correlation between the total STAT3 and pSTAT3 (Ser727) was observed.

3.2. pSTAT3 (Tyr705) Is Localized in the Nucleus of Myofibers in the TGFβ1 Mice with Severe Phenotype. After examining the phosphorylation status of STAT3, we investigated the cellular localization of pSTAT3 (Tyr705). Immunofluorescence staining using a pSTAT3 (Tyr705)-specific antibody showed that expression of pSTAT3 (Tyr705) was visible and was localized in nuclei of the muscles of the EO mice, but was not detectable in the LO mice and controls (Figure 2). Approximately 50% of nuclei in the vastus lateralis muscles of the EO mice were positive of pSTAT3 (Tyr705). When costained with PAX7, a satellite cell marker, no pSTAT3 positive cells were costained. Our previous studies showed no overt inflammatory infiltration in the muscles of these mice [5]; therefore, most of the positive nuclei are likely
myonuclei. To confirm that, we costained muscle sections with CD14 and CD11b (monocyte/macrophage markers) and CD3 (lymphocytes marker), respectively. In the few positive cells, no nuclei were costained with pSTAT3 (Tyr705).

3.3. TGFβ1 Induces Tyr705 Phosphorylation of STAT3 in C2C12 Myoblasts. In order to determine whether TGFβ1 can directly activate STAT3 in muscle cells, we treated the murine myogenic cell line, C2C12, with recombinant TGFβ1 protein. The pSTAT3 (Tyr705) level was determined by immunoblotting. The results showed that TGFβ1 significantly increased pSTAT3 (Tyr705) 30 minutes after the treatment (7.9-fold, p < 0.001). The pSTAT3 (Tyr705) level descended to baseline level after 2 hours of treatment. There was no change of the total STAT3 protein during the time course examined between the treated and control groups (Figure 3).

4. Discussion
In this study, we explored the relationship between TGFβ1 and STAT3 activation using a tet-repressible muscle-specific
TGFβ1 transgenic mouse and C2C12 cells. The phosphorylation of the Tyr705 residue is required for STAT3 dimerization, nuclear translocation, and DNA binding [28, 29]. Phosphorylation of the Ser727 residue is believed to promote STAT3 transcriptional activity through the enhanced recruitment of transcriptional cofactors, which is not required for functional activation of STAT3. Our data showed that overexpression of TGFβ1 increased the amount of pSTAT3 (Tyr705) significantly in the EO mice but not in the LO mice. In addition, the effect was mediated through the phosphorylation of Tyr705 but not Ser727. While TGFβ1 mediated activation of STAT3 in skeletal muscles was not reported previously, TGFβ1 activated STAT3 by phosphorylating Tyr705 has been reported in hepatic cells and a mouse model of hepato-cellular carcinoma [27, 30]. Both of these studies reported a direct activation of STAT3 by TGFβ1. Interestingly, a recent study showed that hepatitis C virus (HCV) activates TGFβ1 expression via STAT3 in hepatic stellate cells [31]. These findings suggested a potential positive feedback loop between TGFβ1 and STAT3 in the hepatic cells. In our TGFβ1 transgenic model, we previously reported that expression of endogenous TGFβ1 was induced in mice with more severe phenotypes, suggesting potential involvement of a positive feedback loop [5]. Whether the STAT3 activation directly modulates genes involved in muscle atrophy and fibrosis as reported in previous studies or it induces endogenous TGFβ1 expression which is responsible for more severe phenotypes needs to be further examined.

STAT3 was originally reported for its capacity to mediate signaling predominantly from cytokines such as IL-6, IL-11, leukemia inhibitory factor (LIF), and oncostatin M. It is expressed in a large number of tissues and its activation drives the transcription of genes encoding proteins involved in angiogenesis, inflammation, apoptosis, extracellular matrix deposition, and cellular signaling [32]. IL-6 is well known for its crucial role in maintenance of skeletal muscle metabolism [33–35]. IL-6-induced STAT3 has been shown to promote satellite cells proliferation and myoblasts differentiation. Acute and transient activation of STAT3 via Tyr705 phosphorylation by IL-6 was reported to be associated with muscle hypertrophy after 10 weeks of resistance training in rats [36]. The hypertrophic effect was associated with the early upregulation of the IL-6/STAT3 signaling pathway and the downregulation of myogenic regulatory factors, including Pax7, MyoD, Myf5, and myogenin, in the satellite cells. While well controlled IL-6 expression plays a critical role in maintaining the homeostasis of skeletal muscles, studies also showed that persistent Tyr705 phosphorylation is associated with impairment of metabolism by negatively affecting skeletal muscle insulin signaling and glucose uptake [37] and is believed to be responsible for the IL-6-induced cancer cachexia [33, 38]. Our study showed that overexpression of TGFβ1 for 2 weeks induced pSTAT3 (Tyr705) in skeletal muscles of the mice with severe phenotypes. Approximately half of the nuclei were positive for pSTAT3 (Tyr705). Since TGFβ1 was the only gene overexpressed in the mouse model and is the driving force of the disease phenotype [5], the data suggested that TGFβ1 is able to activate the STAT3 signaling directly. However, whether IL-6 signaling is involved in the process is not clear and needs further investigation. To examine the possibility and demonstrate that TGFβ1 directly activates STAT3 in skeletal muscle cells, we treated the C2C12 cells with recombinant TGFβ1 and examined the activation of STAT3 at 3 time points (30 min, 2 hrs, and 24 hrs) within 24 hours. Our findings showed that the pSTAT3 Tyr705 was transiently activated within 30 minutes. This result is in agreement with a recent study using immortalized rat hepatic stellate cells (HST) [27]. In this study, it was also shown that JAK1 is necessary for the Tyr705 phosphorylation and activation of STAT3. Knockdown of JAK1 but not JAK2 or Tyk2 is sufficient to attenuate TGFβ1 mediated STAT3 activation. STAT3 is canonically activated by JAKs (JAK1, JAK2, JAK3, and Tyk2), which in turn are activated by a large number of cytokine and growth factors, including IL-10, IL-6, and EGF, and it is well known to play a crucial role in myogenic proliferation and differentiation [39, 40]. Whether the activation of STAT3 is mediated through JAK1 in our models needs to be investigated further.

5. Conclusion

Our study demonstrated that pSTAT3 (Tyr705) activation is associated with severity of phenotypes of our mouse model overexpressing TGFβ1 in skeletal muscles. The activated STAT3 was localized in the nuclei of myofibers, suggesting transcription activation. Both in vivo and in vitro data suggested that pSTAT3 (Tyr705) can be induced by TGFβ1. These data thus point to a novel signaling pathway that may modulate and contribute to the molecular and cellular mechanism of skeletal muscle fibrosis and atrophy in various diseases.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Acknowledgments

Research reported in this paper was supported by the Department of Defense under Award no. W81XWH-10-1-0659 and the NIH/NICHD under Award no. 1R24HD050846. Yi-Wen. Chen was partially supported by NIH/NIAMS under Award no. 1R01AR052027. Carsten G. Bønnemann is supported by intramural funds of NIH/NINDS.

References


[33] A. Bonetto, T. Aydogdu, X. Jin et al., “JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6...


Genetic Engineering of Dystroglycan in Animal Models of Muscular Dystrophy

Francesca Sciandra, 1 Maria Giulia Bigotti, 2 Bruno Giardina, 3 Manuela Bozzi, 3 and Andrea Brancaccio 1,2

1Istituto di Chimica del Riconoscimento Molecolare, CNR c/o Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, 00168 Roma, Italy
2School of Biochemistry, Bristol University, Bristol BS8 1TD, UK
3Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Roma, Italy

Correspondence should be addressed to Francesca Sciandra; francesca.sciandra@icrm.cnr.it
and Andrea Brancaccio; andrea.brancaccio@icrm.cnr.it

Received 3 October 2014; Accepted 11 March 2015

Academic Editor: Gouri Shankar Pandey

Copyright © 2015 Francesca Sciandra et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In skeletal muscle, dystroglycan (DG) is the central component of the dystrophin-glycoprotein complex (DGC), a multimeric protein complex that ensures a strong mechanical link between the extracellular matrix and the cytoskeleton. Several muscular dystrophies arise from mutations hitting most of the components of the DGC. Mutations within the DG gene (DAG1) have been recently associated with two forms of muscular dystrophy, one displaying a milder and one a more severe phenotype. This review focuses specifically on the animal (murine and others) models system that have been developed with the aim of directly engineering DAG1 in order to study the DG function in skeletal muscle as well as in other tissues. In the last years, conditional animal models overcoming the embryonic lethality of the DG knock-out in mouse have been generated and helped clarifying the crucial role of DG in skeletal muscle, while an increasing number of studies on knock-in mice are aimed at understanding the contribution of single amino acids to the stability of DG and to the possible development of muscular dystrophy.

1. Introduction

The extracellular matrix receptor dystroglycan (DG) is highly expressed in skeletal muscle and in several developing and adult tissues, typically in cell types that adjoin basement membranes, such as epithelial and neural tissues [1–3].

DG is composed of two subunits, α- and β-DG, deriving from a posttranslational cleavage of a single mRNA species encoded by a single gene (DAG1) [4]. α-DG is an extracellular protein characterized by an extensive and heterogeneous glycosylation mainly concentrated within an elongated central mucin-like region which separates two globular domains, the N- and C-terminal domains [5]. α-DG binds with high affinities to the LG domains-containing extracellular proteins, such as laminin-α2, perlecan, and agrin, and in turn interacts noncovalently with the β-subunit, a transmembrane protein [6]. The cytosolic domain of β-DG is anchored to actin through the interaction with dystrophin [7–9], and β-DG also constitutes a scaffold for proteins involved in signal transduction such as Gbr2 and ERK [10, 11].

In skeletal muscle, DG is the central component of the dystrophin-glycoprotein complex (DGC), a multisubunit protein complex which links the actin cytoskeleton to the extracellular matrix [12] (Figure 1). Other members of the DGC include transmembrane proteins such as sarcoglycans and sarcospan and multiple cytoplasmic proteins, including dystrobrevin and syntrophins.

The role of the DGC in muscle is to provide mechanical reinforcement to the sarcolemma and to maintain membrane integrity during cycles of contraction and relaxation. In fact, mutations in any components of the DGC cause distinct forms of muscular dystrophy [13]. In humans, mutations in dystrophin lead to Duchenne and Becker muscular dystrophy [14], mutations in sarcoglycans cause limb-girdle muscular...
dystrophy [15–19], and mutations in laminin-α2 cause congenital muscular dystrophy [20]. Recently, mutations in DAG1 have been reported in three patients, affecting DG function by impairing glycosylation of α-DG or by presumably disrupting the α/β-DG binding interface [21–23].

Moreover, several mutations in 12 proteins involved in the O-mannosyl-glycosylation pathway of α-DG have been identified so far which lead to a variety of clinical symptoms, including severe muscular dystrophy and abnormal central nervous system development and function. These diseases are defined as “secondary dystroglycanopathies” (for recent reviews see [24, 25]). The defective O-mannosyl glycosylation of α-DG impairs its multiple interactions with its extracellular partners, eventually destabilizing the link between the cytoskeleton and the extracellular matrix. Secondary loss of α- and β-DG at the muscle membrane also occurs in Duchenne and Becker muscular dystrophies [26] and in some forms of limb-girdle muscular dystrophy [18].

The recovery of DG glycosylation state via transgenic overexpression of LARGE, a putative enzyme involved in the first steps of the posttranslational processing of α-DG, has been proposed as a therapeutic strategy for muscular dystrophy [27], although with conflicting outcomes [28–30].

The recently emerging data on patients affected by primary and secondary dystroglycanopathies reinforce the notion that a correct expression and modification of DG are crucial for muscle fibres stability and function. A relevant amount of genetic engineering work has been carried out so far on the DAG1 gene in several laboratories. This review will be focused on the animal models generated to understand the function of DG in skeletal muscle, as well as in other tissues, and to better understand its involvement in neuromuscular disorders (Tables 1 and 2).
Table 1: DG mouse models characterized by a muscle and/or central nervous system phenotype.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Muscular dystrophy</th>
<th>CNS involvement</th>
<th>NMJs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimaeric mice [34]</td>
<td>Progressive</td>
<td>—</td>
<td>Disorganized and disrupted</td>
</tr>
<tr>
<td>MCK-Cre/DG-null [38]</td>
<td>Mild</td>
<td>—</td>
<td>Normal</td>
</tr>
<tr>
<td>GFAP-Cre/DG-null [45]</td>
<td>—</td>
<td>Neuronal migration errors, brain malformation</td>
<td>—</td>
</tr>
<tr>
<td>MORE-DG-null [38, 39]</td>
<td>Severe</td>
<td>Neuronal migration errors, brain malformations, and ocular defects (WWS phenotype)</td>
<td>—</td>
</tr>
<tr>
<td>Nestin-Cre/DG-null [47] and Crx-Cre/DG-null [50]</td>
<td>—</td>
<td>Abnormal retinal physiology</td>
<td>—</td>
</tr>
<tr>
<td>DG&lt;sup&gt;TMSM&lt;/sup&gt;/DG&lt;sup&gt;TMSM&lt;/sup&gt; [21]</td>
<td>Mild</td>
<td>Some neurological impairments</td>
<td>Compromised</td>
</tr>
<tr>
<td>DG&lt;sup&gt;Y90OF/Y90OF&lt;/sup&gt; [56]</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>DG&lt;sup&gt;Y90OF/Y90OF&lt;/sup&gt;/mdx [56]</td>
<td>Ameliorated</td>
<td>—</td>
<td>Normal</td>
</tr>
<tr>
<td>DG&lt;sup&gt;WT&lt;/sup&gt; overexpression [40]</td>
<td>Normal</td>
<td>Normal</td>
<td>25% smaller than normal but only 1% are aberrant</td>
</tr>
<tr>
<td>DG&lt;sup&gt;WT&lt;/sup&gt; overexpression/mdx [40]</td>
<td>Not ameliorated</td>
<td>—</td>
<td>Not ameliorated</td>
</tr>
<tr>
<td>DG&lt;sup&gt;S654A&lt;/sup&gt; overexpression [63]</td>
<td>Mild</td>
<td>—</td>
<td>Compromised</td>
</tr>
<tr>
<td>DG&lt;sup&gt;Δβcyt/Δβcyt&lt;/sup&gt; [47]</td>
<td>—</td>
<td>Mild effects in the retina</td>
<td>—</td>
</tr>
</tbody>
</table>

—: not analysed.

Table 2: Mouse models in which DG was targeted in tissues other than skeletal muscle and brain and additional DG animal models with muscle and central nervous system defects.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney specific DG knock-out mouse (podocin-Cre/DG-null, Pax2-Cre/DG-null, Pax3-Cre/DG-null, HoxB7-Cre/DG-null) [64]</td>
<td>Normal</td>
</tr>
<tr>
<td>Schwann cells specific DG knock-out mouse (P0-Cre/DG-null) [66]</td>
<td>Severe neurological dysfunctions</td>
</tr>
<tr>
<td>DG knock-out in Caenorhabditis elegans [68]</td>
<td>Defects in gonad and vulval epithelium and in motoneurons</td>
</tr>
<tr>
<td>RNAi knock-out of DG in Drosophila melanogaster [73, 74]</td>
<td>Muscle degeneration and neuronal defects</td>
</tr>
<tr>
<td>Inhibition of DG translation via morpholino antisense in zebrafish [78]</td>
<td>Muscle defects</td>
</tr>
<tr>
<td>Zebrafish patchytail [79]</td>
<td>Dystrophic muscles,ocular and central nervous system defects</td>
</tr>
<tr>
<td>Zebrafish dag&lt;sup&gt;1&lt;/sup&gt; iso702  [81]</td>
<td>Muscular dystrophy</td>
</tr>
<tr>
<td>Inhibition of DG translation via morpholino antisense in Xenopus laevis [82–86]</td>
<td>Defects in the somitogenesis, epidermal differentiation, the renal and renal developing</td>
</tr>
<tr>
<td>Overexpression of DG in Xenopus laevis embryos [86]</td>
<td>Aberrant neuromuscular junctions</td>
</tr>
</tbody>
</table>

2. From Knock-Out Mice to the Different Strategies to Circumvent Embryonic Lethality

In 1997 the DG knock-out mouse was generated and analyzed in Kevin Campbell's Laboratory [31]. The targeting vector was designed to replace a portion of the DAG1 second coding exon with the neo-cassette following homologous recombination. DAG1-null allele resulted in a deletion in the exon including the 3' splice acceptor site and a large portion of the coding sequence of α-DG. Animals that were heterozygous for the targeted allele appeared healthy and bred normally. Interestingly, DG transcripts in skeletal muscle of heterozygous mice were only 10–20% lower than those in wild-type mice, suggesting a compensatory increase in the expression level of the untargeted allele. Accordingly, DG protein levels in skeletal muscle were also comparable between wild-type and heterozygous animals. However, the DG knock-out was lethal for homozygous mice embryos that died at the embryonic day 6.5 because of the disorganization of Reichert’s membrane, one of the first specialized extraembryonic basement membranes. The absence of laminin receptor precluded the assembly of laminin in a network and the distribution of laminin and collagen-IV appeared patchy, suggesting a crucial role of DG in the organization of the basement membranes [31]. This conclusion was further confirmed by the molecular analyses...
of the embryoid bodies derived from homozygous Dagi-null ES cells in which an ordered basement membrane failed to form [32].

As DG is involved in the development of basement membranes, it certainly is fundamental for normal human development, and the failure to identify null mutations in Dagi linked to muscular dystrophies in humans is probably due to early embryonic lethality of such mutations. Interestingly, Frost et al. described a patient affected by a mild myopathy with central nervous system involvement who was heterozygous for a DNA deletion which included also the DG gene [33]. In this patient, only 50–60% of native DG is produced and correctly glycosylated thus showing a much lower degree of compensation compared to the heterozygous DG-null mouse. Although other genes present in the same deleted region could account for the phenotype, this case report suggests the possibility that the heterozygosis for DG-null mutations (haploinsufficiency) could produce pathological consequences in humans. A substantial genetic screening effort, carried out on an enlarged number of patients, would be necessary for the identification of additional cases that may be related to the haploinsufficiency of DG.

To circumvent the embryonic lethality of the DG knock-out mouse, highly chimaeric mice, generated with ES cells targeted for both Dagi alleles, were generated [34]. In chimaeric mice deficient in DG rescued from the embryonic lethality, skeletal muscle differentiated normally but they developed a progressive muscular dystrophy reminiscent in many respects of that of mice with double mutations in dystrophin and utrophin [35]. Significant differences in fibre size, central nuclei, and connective tissue infiltration characterized the skeletal muscle histology of DG-null chimaeric mice that died at 13 months [34]. DG plays a crucial role also in stabilizing acetylcholine receptors [36] and consequently in chimaeric mice the neuromuscular junctions (NMJs) were grossly disorganized and disrupted [34]. In the most severely affected mice, the heart appeared dilated and with an extensive connective tissue hyperplasia. At the sarcolemma of DG-null chimaeric mice the entire DGC complex was disassembled, with dystrophin and sarcoglycans absent in many fibres. However, laminin-α2, perlecain, and agrin were expressed at wild-type levels and the basement membrane appeared organized in an ordered network. At the sarcolemma of DG-null chimaeric mice the entire DGC complex was disassembled, with dystrophin and sarcoglycans absent in many fibres. However, laminin-α2, perlecain, and agrin were expressed at wild-type levels and the basement membrane appeared organized in an ordered network. It is likely that, in differentiated skeletal muscle, the expression of integrins or other extracellular matrix receptors exert an important compensatory effect in supporting the skeletal muscle differentiation and basement membrane assembly [37]. However, the DG-null chimaeric mice pointed out the central role of DG in the maintenance of the DGC and muscle integrity.

An additional step forward in understanding the functional role of DG in skeletal muscle came from the conditional inactivation of skeletal muscle DG using the Cre-loxP system under the muscle creatine kinase (MCK) promoter [38]. The MCK-Cre/DG-null mice were viable and born with the expected frequency; they developed muscular dystrophy around 4–6 weeks of age but the phenotype became milder with advanced age. As a matter of fact, satellite cells, which had not been targeted by the Cre recombinase, supported the muscle regeneration and formation of novel fibres expressing DG and the other components of DGC. Moreover, in old mice, muscle fibres appeared hypertrophic and larger, as compared with controls and with the other mouse models of muscular dystrophy. Like in chimaeric DG-null mice, also in MCK-Cre/DG-null mice, laminin-α2 was expressed and the basement membrane was correctly assembled. However, while in chimaeric DG-null mice the NMJs were disrupted, in MCK-DG-null mice they were preserved.

In MORE-DG-null mice [38], the inactivation of Dagi was driven by Cre recombinase under the control of the Mox 2 promoter enabling the targeting of DG in all tissues of the embryo, while DG was still expressed in extraembryonic membranes to circumvent embryonic lethality. MORE-DG-null mice were significantly smaller than control littermates, a majority of the mice died within 48 h after birth, and the remaining mice typically failed to survive the fourth postnatal week. In addition, MORE-DG-null mice exhibited profound muscle weakness and muscular dystrophy was present at birth, reminiscent of a secondary dystroglycanopathy phenotype (see next paragraph) [39]. Consistent with the results obtained with MCK-Cre/DG-null mice, MORE-DG-null mice displayed severe impaired regeneration capacity since satellite cells were also targeted by Cre recombinase under the control of the Mox 2 promoter [38].

The phenotype observed in chimaeric and conditional knock-out mice demonstrated the importance of DG for the stability of the DGC and for the structural integrity of the sarcolemma. However, the overexpression of DG in transgenic mice onto an mdx background did not inhibit muscular dystrophy; on the contrary, it exacerbated the phenotype by decreasing the utrophin and sarcoglycans expression at the sarcolemma [40].

3. Conditional DG Knock-Out in the Brain Recapitulates the Outcome of Secondary Dystroglycanopathies

Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS) are congenital muscular dystrophies (CMDs) with associated developmental brain defects [41–43]. The genes that are mutated in these disorders are those of the enzymes involved in the O-mannosyl glycosylation of α-DG, in particular protein-O-mannosyl transferase 1 (POMT1), protein-O-mannosyl transferase 2 (POMT2), protein-O-linked mannose beta 1,2-N-acetylgalactosaminyltransferase (POMGnT1), and fukutin, an enzyme indirectly implicated in a pathway to further modify the phosphorylated O-linked mannose located in the mucin-like domain of α-DG [44].

The conditional DG knock-out mouse in the brain was produced, using the Cre-LoxP methodology, in order to analyse the function of DG in the central nervous system and to demonstrate the role of DG in the brain malformations seen in CMDs [45]. Brain-selective expression of Cre recombinase was accomplished using a human glial fibrillary acid protein (GFAP) promoter expressed as early as embryonic day 13.5. GFAP-Cre/DG-null mice followed the expected Mendelian distribution and were fertile.
In the GFAP-Cre/DG-null mice cerebral cortex, DG was not expressed in the astrocytes abutting the brain surface (glia limitans) and cerebral microvessels, in radial glia and in a subset of neurons that are the progeny of radial glia [45, 46]. Also the localization of dystrophin isoforms was impaired in these cells. The basal lamina of the glia limitans that plays a critical role for normal cortical development was severely disrupted. The results were a number of brain structural developmental defects similar to those seen in MEB, WWS, and FCMD patients. The abnormalities of the glia limitans permitted the overextended migration of neurons in the developing brain, which is the most important diagnostic feature of cobblestone lissencephaly observed in the most severe cases of secondary dystroglycanopathy. Such mice also lacked the usual fissure between the brain's hemispheres, a characteristic of WWS, and suffered from an overabundance of gli.

Despite the large similarities with CMDs, GFAP-Cre/DG-null mice did not recapitulate the most severe characteristics observed in the brain of patients affected by WWS. On the contrary, the earlier suppression of DG expression in MORE-DG-null mice (in which Cre recombinase under the control of the Mox2 promoter operates at E7.5) was sufficient to cause malformations that broadly resembled the clinical spectrum of WWS, including hydrocephalus and ocular malformations with structural defects of both the anterior and posterior chambers of the eye [39].

The DAGI gene was targeted to generate other mouse models for the detailed analysis of the role of DG in the central nervous system and in the visual function. For example, the following two novel DG mouse models were created and analyzed by Satz and colleagues [47]: (1) the conditional knock-out nestin-CRE/DG-null mouse, in which Cre recombinase was under the control of the rat nestin enhancer expressed in neuroepithelial precursor cells and in the retina as early as embryonic day 9.5 (further analysed in [48, 49]) and (2) a knock-in mouse expressing a truncated DG lacking the entire β-DG cytodomain (in which a premature stop codon was inserted after Lys778 resulting in β-DG). Surprisingly, according to the authors, a reasonable number of knock-in mice were obtained [47]. It remains unclear whether these mice would display some pathologic effect in their skeletal muscle.

Moreover, Omori and colleagues developed a retinal photoreceptor-specific DG conditional knock-out (Crx-Cre/DG-null) mice, in order to analyze the role of DG localized at the presynaptic elements of photoreceptor cells [50]. The results showed the crucial role of presynaptic DG for both the formation of proper photoreceptor ribbon synaptic structures and normal retinal electrophysiology.

4. DG Knocking-In: A Powerful Tool to Dissect the Role Played by Specific Amino Acids

4.1. DG$^{T190M}$/$DG^{T190M}$, the First Case of Primary Dystroglycanopathy. The first case of a homozygous missense mutation in the DAGI gene was described in a 16-year-old patient [21] originally described as affected by a mild form of limb-girdle muscular dystrophy associated with mental retardation but normal brain imaging (recently classified as limb-girdle muscular dystrophy 2P [51]). Immunofluorescence and immunoblot of muscle biopsies showed that α-DG was hypoglycosylated and had a reduced affinity toward laminin. The mutation (T192M), located within the RNA binding protein-like domain of the α-DG N-terminus [52], is supposed to reduce, via a mechanism that needs to be fully elucidated yet, the binding between α-DG and the glycosyltransferase LARGE, an interaction that is essential for the posttranslational modification of α-DG and for the DG’s laminin-binding activity [53]. Recently, a mild form of muscular dystrophy characterized by hypoglycosylated α-DG was associated with the compound heterozygous missense mutations (V74I and D111N) with both mutated sites located within the N-terminal domain of α-DG and a pathological molecular mechanism similar to the one described for the T192M mutation was hypothesized [22].

A knock-in mouse was generated introducing the mutation T192M (that in mouse corresponds to T190M) by homologous recombination [21]. Heterozygous mice were normal, while homozygous knock-in mice presented abnormalities consistent with those observed in the patient. Analysis of muscle biopsies revealed hallmarks of muscular dystrophy, such as centrally nucleated fibres, and a hypoglycosylated α-DG with a decreased laminin-binding activity. The T190M mutation interfered also with the organization of the NMJ. Although no structural abnormality was evident in the brains of the knock-in mice, these mice had abnormal hind limb clamping, a phenotype common to mouse models featuring neurologic impairment. Interestingly, in the heart, the mutated α-DG still bound to laminin and no obvious signs of any pathological abnormality were observed.

Recently, a novel homozygous mutation in DAGI has been identified in a Libyan family with two siblings affected by a dystroglycanopathy resembling a MEB-like condition [23]. The mutation (C669F) hits an amino acid already identified as a signal for proteasome degradation of DG [57–59]. The cytodomain of β-DG represented a tool for dissecting the role of the phosphorylation of DG in the skeletal muscle and for testing novel therapeutic ideas [56].

In fact, previous studies suggested that tyrosine phosphorylation of DG at the site Y892 may be an important mechanism for modulating the association of DG with its cellular binding partners dystrophin and utrophin and may also work as a signal for proteasome degradation of DG [57–59].

The knock-in mouse DG$^{T890F/Y890F}$/mdx. Although not directly linked to a specific disease, a knock-in mouse hitting a tyrosine within the cytodomain of β-DG represented a tool for dissecting the role of the phosphorylation of DG in the skeletal muscle and for testing novel therapeutic ideas [56]. The knock-in mouse DG$^{T890F/Y890F}$/mdx appeared normal and healthy. Skeletal muscle analysis of knock-in mice did not reveal any differences or abnormalities compared to the wild-type mice.
In order to assess whether the inhibition of tyrosine phosphorylation in DG had any beneficial effect on dystrophic skeletal muscle, DG\(^{Y890F/Y890F}\)mdx mice were further generated [56]. Interestingly, the expression of Y890F mutant DG in an mdx background significantly improved the muscle phenotype, reducing the number of centrally nucleated fibres and the levels of creatine kinase. Moreover, also an improvement in resistance to eccentric contraction-induced injury was observed in DG\(^{Y890F/Y890F}\)/mdx mice. Changing a single phosphorylation site in DG reinforced the DGC sarcolemma localization preventing the proteasome degradation of DG. In fact, the inhibition of tyrosine phosphorylation of DG in mdx mice was sufficient to restore the sarcolemma localization of DG, α-sarcoglycan, and sarcospan also in the absence of dystrophin, while utrophin was confined at the NMJ [56]. Moreover, an increase in the plectin expression/localization at the sarcolemma was also observed. Plectin is a cytolinker protein that binds β-DG at different sites, thus providing a stabilizing link between DG and the cytoskeleton [60]. It was already known that treatment with proteasomal inhibitors improves the muscle pathophysiology in some mouse models, such as mdx mice [61] or d\(^{yNK}\) mice [62], and in this context the DG\(^{Y890F/Y890F}\)mdx mouse highlights novel targets for therapeutic intervention.

5. Mice Overexpressing DG

The overexpression of multiple and randomly integrated copies of the coding sequence of DG was obtained by microinjection of a pBS-HSAvpA cDNA construct into fertilized CB6 oocytes. Mice overexpressing wild-type DG were normal compared to control [63]. Interestingly, transgenic lines overexpressing DG mutated in the cleavage site S654 were also created in order to understand the role of the posttranslational cleavage resulting in the production of the two interacting subunits [63]. In the transgenic DG\(^{S654A}\) mice only the uncleaved DG precursor was correctly expressed, while the expression of endogenous and processed DG was inhibited. In DG\(^{S654A}\) mice, most muscles were dystrophic with increased levels of central nuclei. The lack of the DG cleavage and the presence of muscular dystrophy correlate with altered glycosylation of α-DG. In addition, the expression of dystrophin and α-sarcoglycan decreased, while utrophin and laminin-α5 were upregulated, probably as a secondary effect of muscle regeneration. Abrerrant NMJs were observed in DG\(^{S654A}\) mice, although also DG\(^{WT}\) mice occasionally showed fragmented NMJ [63].

6. Conditional DG Knock-Out Mice in Other Tissues Compared to Skeletal Muscle

DG is also highly expressed in epithelia and in the peripheral nervous system (Table 2). To study the role of DG in the kidney, different conditional knock-out mice were created to selectively delete DG from podocytes, ureteric bud, metanephric mesenchyme derivatives, and all renal epithelial cells using the Cre-lox system under the control of podocin, HoxB7, Pax-3, and Pax-2 promoters, respectively [64]. Surprisingly, DG deletion from kidney resulted in no aberrant phenotypes. Kidney formation and function proceeded normally in the absence of DG and the only detectable abnormality was a mild increase of the glomerular basement membrane thickness. This observation was further confirmed in chimaeric mice generated with fukutin-null embryonic stem cells expressing a hypoglycosylated α-DG, in which minor glomerular structure abnormalities were found without functional renal defects [65]. These results suggest that DG and its correct glycosylation may be important in the maintenance of podocyte architecture and extracellular matrix assembly; however, the presence of integrin α3β1 as an additional laminin receptor and basement membrane organizer in podocytes may preserve the structure and functionality of the kidney [65]. Accordingly, there are no reported renal dysfunctions in human patients with impaired DG glycosylation.

To analyse the role of DG in peripheral nerves, DG was disrupted selectively in Schwann cells using the P0 protein promoter and Cre-loxP technology [66]. The loss of DG caused severe neurological dysfunctions, including a slow nerve conduction. In P0-Cre/DG-null mice the myelin sheaths around the nerves were structurally abnormal and extended throughout the internodal segments. The DG-interacting proteins, sarcospan, sarcoglycan, and α-dystrobrevin, were lost from the membrane and laminin was not deposited around the nerves. These findings point to the crucial role of DG in myelin integrity and in node of Ranvier structure and function [67].

7. Other Animal Models to Study the DG Functions in Muscle and Central Nervous System

DG and most of the members of DGC are highly conserved in vertebrates, including fish, and invertebrates. Therefore, DG offers a wide range of possible animal models aside from mouse to understand the role of DG in the pathogenesis of muscular dystrophies.

7.1. Caenorhabditis elegans. Interestingly, in Caenorhabditis elegans, DG is expressed in epithelial and neuronal tissues but not in muscle. Indeed, a deletion, cg121, that removes most of the coding and some of the 3′ untranslated region of DGNI gene, resulted in viable but sterile animals, with neuronal defects and normal muscles [68]. DGNI contains the N-terminal immunoglobulin-like domain of vertebrate α-DG and a shorter mucin-like region, while the α/β proteolytic cleavage region and the residues involved in binding WW and SH3 domain-containing proteins such as dystrophin are all missing [68].

7.2. Drosophila melanogaster. All known components of the DGC are present in the fruit fly [69]. Several Drosophila DG isoforms are generated via alternative splicing [70, 71]. Only one of these contains the full mucin-like domain, characterized by significant levels of glycosylation, while DG isoforms that lack the mucin-like domain are required to
maintain polarity in the follicular epithelium, suggesting that the isoforms can have different functional roles in Drosophila [71]. Moreover, Drosophila DG is not cleaved in α- and β-subunits but it is expressed as a single polypeptide [72].

Interestingly, muscle-specific RNAi-mediated knockdown of DG, as well as of dystrophin isoforms, led to age-dependent, progressive climbing deficits, severe muscle degeneration in adult flies, and defects in neurons migration and eye development [73]. Haines and colleagues, analysing larvae carrying mutant alleles of DG, also established that DG is required in Drosophila larval muscles to maintain integrity [74].

The similar defects observed in both flies and humans make Drosophila an attractive model for further studies on clarifying the role of the DGC. In particular, using the RNAi knockdown mutants of DG, many genes had been identified as possible regulatory genes of DG and dystrophin, such as genes involved in muscle function and components of Notch, TGF-β, and EGFR signalling pathways [75]. Recently, it was also shown that in Drosophila the expression level of DG may be buffered in a homeostatic fashion via a mechanism mediated by the miR-310s complex which acts directly on the alternative DG 3′-UTR [76]. Deficiencies in the miR-310s complex resulted in cobblestone brain, a phenotype reminiscent of human lissencephaly type II [76]. This evidence represents a seminal result, paving the way for identifying similar regulation mechanisms also in higher vertebrates and mammals.

7.3. Danio rerio. In recent years, Danio rerio (zebrafish) has emerged as a powerful genetic tool to study muscle diseases [77]. Disruption of DG translation using an antisense morpholino oligonucleotide (MO) approach, led to the destabilization of the dystrophin muscle, with loss of sarcomere organisation and necrosis of the developing muscle [78]. The NMJ and central nervous system appeared normal. The lack of the DG protein impaired also the localization of dystrophin. Interestingly, in an ENU (N-ethyl-N-nitrosourea) mutagenesis screen aimed at identifying genes responsible for skeletal muscle disorders, a DG homozygous mutant, patchy-tail, was found to show impaired locomotion behaviour, dystrophic muscles and ocular and central nervous system defects [79]. The point mutation resulted in a missense amino acid change of valine to aspartic acid (V567D) within the Ig-like domain in the C-terminal region of a-DG, leading to a destabilization and degradation of the protein [79, 80]. The absence of DG led to a reduced expression of dystrophin and laminin-a2. The sarcolemma appeared grossly disorganized and detached from the extracellular matrix. NMJs were normal in patchytail fish, but severe abnormalities of brain and eyes were observed and the embryos did not survive more than 10 days post fertilization. This case represents so far the first case in which a single point mutation is able to induce the complete depletion of DG from tissues, highlighting the importance that even single aminoacids may have for the stability and/or folding pathway of the DG precursor [56, 80]. An additional loss of function mutation was identified in a zebrafish affected by muscular dystrophy (dag1hau072) due to a nonsense mutation (R398>Stop) within the mucin-like region of a-DG, causing the complete loss of DG [81]. The absence of DG led to the dislocation of dystrophin and to muscle fibre detachment, followed by disruption of sarcolemma integrity.

7.4. Xenopus laevis. The possible DG functions in different tissues of Xenopus laevis were analysed using the morpholino knock-out approach. It was shown that, during Xenopus development, DG is important for the somitogenesis and that the interaction between DG and the extracellular matrix is indispensable for the alignment of the myoblasts in the somites [82]. The loss of DG influenced the epidermal differentiation in the retinal and renal development [83–85]. Moreover, the injection of rabbit DG RNA into Xenopus embryos produced an overexpression of DG that altered the acetylcholine receptors aggregation and the NMJ structure [86].

8. Perspectives

Starting from the 1997 knock-out mouse [31], during the last 17 years, an impressive amount of work has been carried out already on DG at the genetic level. It is likely to expect in the next few years a further increase both in the number of patients/families identified who carry mutations specifically within the Dagi gene and in the number of animal genetic models that will be generated and analyzed. In particular, it could be particularly interesting to focus on the 3′ region of Dagi, corresponding to the α/β-DG interface, the genetic clinical screenings carried out on still unassigned cases of myopathy presenting with symptoms that may suggest the presence of a dystroglycanopathy. In this specific and very small region (that could be therefore analyzed inexpensively) in fact, mutations have been found to grossly affect the stability and the maturation of the complex, in zebrafish as well as in human patients [23, 79, 81].

Via such comparative studies, a full circle will be completed in the elucidation of the function(s) of DG in muscle and nonmuscle tissues. Moreover, the genetic data on human patients and the generation of novel animal models will certainly boost the research on potential therapeutic approaches for human muscular dystrophies.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

Maria Giulia Bigotti is the recipient of a Research Career Re-entry Fellowship from the Wellcome Trust, which is hereby gratefully acknowledged.

References


[64] G. Jarad, J. W. Pippin, S. J. Shankland, J. A. Kreidberg, and J. H. Mîner, “Dystroglycan does not contribute significantly to...


Research Article

Systemic Inflammation in Duchenne Muscular Dystrophy: Association with Muscle Function and Nutritional Status

Oriana del Rocío Cruz-Guzmán,1 Maricela Rodríguez-Cruz,1 and Rosa Elena Escobar Cedillo2

1Laboratorio de Biología Molecular, Unidad de Investigación Médica en Nutrición, Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS, 4th Floor, Avenida Cuauhtémoc No. 330, Colonia Doctores, Delegación Cuauhtémoc, 06725 México, DF, Mexico
2Servicio de Electrodiagnóstico y Distrofia Muscular, Instituto Nacional de la Rehabilitación, México, DF, Mexico

Correspondence should be addressed to Maricela Rodríguez-Cruz; maricela.rodriguez.cruz@gmail.com

Received 2 February 2015; Revised 18 March 2015; Accepted 27 March 2015

Academic Editor: Akanchha Kesari

Copyright © 2015 Oriana del Rocío Cruz-Guzmán et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Inflammation described in patients with Duchenne muscular dystrophy (DMD) may be related to loss of muscle function or to obesity. It is unknown if circulating proinflammatory cytokines (IL-6, IL-1, and TNF-α) levels are associated with muscle function. The purpose was to evaluate whether an association exists between systemic inflammation with muscle function and nutritional status in DMD patients. In 66 DMD patients without corticosteroid treatment, the following were evaluated in serum: cytokines (IL-1, IL-6, and TNF-α), C-reactive protein (CRP), leptin, adiponectin, and creatine kinase (CK). Muscle function was evaluated using Vignos Scale. Patients with better muscle function had the highest concentration of CK, IL-1, and TNF-α compared with less muscle function. No differences in IL-6 and adiponectin concentration were identified among groups with different levels of muscle function. Also, no differences were observed in the concentration of cytokines among groups with different nutritional status levels (underweight, normal weight, and overweight/obese). However, CRP and leptin were increased in the obese group compared with normal and underweight subjects. Systemic inflammation is increased in patients with better muscle function and decreases in DMD patients with poorer muscle function; nevertheless, systemic inflammation is similar among different levels of nutritional status in DMD patients.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked muscle disease [1] caused by mutations in the DMD gene that code for dystrophin. This protein has an important structural role during muscle contraction. Fragile muscle fiber and instable sarcolemma are some of the outcomes of the protein mutation. These alterations lead to chronic inflammation, which is an important feature of the disease pathophysiology. First, if the fragile muscle suffers a stretch process, the sarcolemma experiences a mechanical stress. Consequently, Ca²⁺ levels increase and activate the necrosis [2]. Next, necrotizing myofibers are attacked and removed principally by M1 macrophages. At the same time, M1 cells secrete proinflammatory cytokines and recruit additional inflammatory cells, increasing the inflammation process. It has been reported that biopsy of the dystrophic muscle shows overexpression of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) in comparison with healthy muscles, leading to chronic inflammation [3–6]. Because muscle biopsy is an invasive method, there is no information about the inflammation during the progression of muscle damage in DMD patients. Despite this, it has been demonstrated that DMD patients have increased levels of cytokines (TNF-α, IL-6) in serum compared with healthy subjects [3, 4, 7]. There are no studies about circulating biomarkers of inflammation throughout evolution of dystrophy.

DMD patients usually present generalized motor delays and gait difficulties in early childhood. Muscle weakness is progressive, causing loss of ambulation by early adolescence (between 9 and 12 years of age) [1]. Some assessment scales,
such as the Vignos Scale, have been used to evaluate the motor function of neuromuscular diseases. The Vignos Scale provides information about the degree of functional dependence of the patient and measures lower extremity function [8]. To support this assessment, clinical circulating biomarkers such as creatine kinase (CK) are used where high levels of CK reflect muscle damage. However, it is important to consider that CK levels are not reliable because they vary considerably under several independent stress conditions, not necessarily associated with muscle damage [9].

Additionally, the inflammation process might exhaust the degeneration and regeneration cycles of the muscle fiber, provoking the replacement of muscle fibers by connective and adipose tissue [10]. Adipose tissue accumulated in muscle tissue may lead to metabolic alterations such as obesity. It is well known that DMD patients develop obesity from the age of 7 years [11] not only due to glucocorticoid treatment as we previously reported. A sample of DMD patients (n = 66) who were not taking medication, including glucocorticoids, demonstrated 18.3% underweight, 22.7% overweight, and 68% obesity [12]. It is well known that obesity is associated with low-grade inflammation of white adipose tissue due to the secretion of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 [13–15]. In the same way, C-reactive protein (CRP) is also increased in the serum of obese subjects. Although CRP is not a cytokine, it is a useful inflammatory marker for obesity [15, 16].

Other molecules associated with obesity are leptin and adiponectin. Leptin has been demonstrated to be increased in obese children, whereas adiponectin is decreased. Also, leptin has a positive relationship with CRP and is related to the amount of body fat [16].

Chronic inflammation caused by muscle damage or due to obesity in DMD patients has an important impact on disease progression [10]. Identification of inflammatory status during the progression of the dystrophy is clinically important because it will be useful for the detection of the degree of muscle damage, increasing the possibility to propose early interventions regardless of the origin of the inflammation, whether muscle damage or obesity. It has an important impact on the progress of the clinical characteristics in DMD patients.

Thus, the purpose of this investigation was to evaluate whether an association exists among systemic inflammation with muscle function and nutritional status in DMD patients.

2. Materials and Methods

2.1. Subjects and Methods. The study was conducted at the Laboratory of Molecular Biology, Medical Research Unit in Nutrition, Pediatric Hospital, Centro Medico Nacional Siglo XXI (CMN SXXI), Instituto Mexicano del Seguro Social (IMSS). All DMD patients seen at the outpatient Electrodagnostic Muscular Dystrophy Service at the National Institute of Rehabilitation were recruited for the cross-sectional study between January 2011 and December 2013. Subjects with a clinical diagnosis of DMD were candidates to participate in the study and confirmatory molecular diagnosis of dystrophy was carried out as described in a recent report in the same patients [12]. Therefore, 66 patients (aged 4–18 years) with confirmed diagnosis of dystrophy were included in the present study. Children were not included if they received therapy with corticosteroids. None of the patients was taking medications during the study.

The study was approved by the institutional ethics committee of the IMSS. Parents and patients received an explanation of study fundamentals, procedures, benefits, right to confidentiality, and right to withdraw from the study if they wished. All parents provided written informed consent in adherence with the human subjects’ guidelines of the institutional ethics committee.

After a 12 h overnight fast, peripheral blood was collected from each patient in a Vacutainer without anticoagulant to quantify the cytokines TNF-α, IL-6, IL-1, CRP, and CK. Serum samples were kept at –70°C until analysis. A medical history was then obtained by trained personnel using the Vignos Scale. Finally, anthropometric measurements such as weight, height, and body composition by dual-energy X-ray absorptiometry (DEXA) were measured.

2.2. Vignos Scale Measurement. There are different procedures to evaluate muscle function in DMD patients. One of the most utilized is the Vignos Scale, which measures lower extremity function. It is painless and easy to assess and completion time is short. The range of the scale has been classified from 1 to 10 as follows [19]:

(1) walks and climbs stairs without assistance,
(2) walks and climbs stairs with aid of railings,
(3) walks and climbs stairs slowly with aid of railings (>25 sec for seven standard steps),
(4) walks unassisted and rises from chair but cannot climb stairs,
(5) walks unassisted but cannot rise from chair or climb stairs,
(6) walks only with assistance or walks independently with long leg braces,
(7) walks in long leg braces but requires assistance for balance,
(8) stands in long leg braces but unable to walk even with assistance,
(9) confined to wheelchair,
(10) confined to bed.

Based on the chronological functional stages, patients were classified into three groups according to the Vignos Scale: Group A that includes patients with independent ambulation (range 1–5); Group B that includes patients with assisted ambulation (range 6–8); and Group C that includes patients with wheelchair-limited mobility (range 9–10) [20].

2.3. Anthropometric Measurements. Body weight (kg) and height (m) measurements were all performed by trained personnel. For subjects who were able to stand erect, height was measured with a wall-mounted stadiometer (Model 208,
2.4. Body Composition. Body composition measurements were carried out using DEXA (Lunar Prodigy, GE Medical Systems, Madison, WI). enCore software, v. 2004 (Lunar Corporation), was used to analyze whole-body DEXA scans.

2.5. Biochemical Assays. CRP and proinflammatory cytokines IL-1, IL-6, and TNF-α (pg/mL and mg/dL) were quantitated by a solid-phase chemiluminescent immunometric assay using commercial kits (IMMULITE 1000 Analyser, DPC, Siemens, Malvern, PA). Leptin (ng/mL) and adiponectin (pg/mL) were measured using a commercial kit (Linco Research, St. Louis, MO) based on radioimmunoanalysis. CK (U/L) was determined by chemiluminescent immunometric assay by a commercial kit (Spinreact, Sant Esteve, Spain).

2.6. Statistical Analysis. Statistical analysis was performed using SPSS Statistics software (v. 22.0; SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD) or median (minimum, maximum) as appropriate. Statistical significance was defined at $P < 0.05$. Distribution of the studied variables was examined using the Shapiro-Wilk test. Analysis of differences among groups was conducted with one-way analysis of variance using Bonferroni’s or Dunnet post hoc. For data not normally distributed, nonparametric statistics such as Kruskal-Wallis and Mann-Whitney U test to identify differences between groups were used.

3. Results

3.1. Study Subjects. In this study, 66 patients with clinical and molecular diagnosis of DMD were included. Table 1 summarizes the clinical, anthropometric and biochemical parameters of DMD patients. In this sample study, patient ages were $9.4 ± 3.1$ years (mean ± SD); 18 patients were wheelchair-bound. The mean of Vignos Scale value indicates that patients are capable of walking and climbing stairs but with difficulties. According to the CDC, normal-weight patients have a BMI of $46.3 ± 35.6$. On the other hand, as expected, the average concentration of CK ($11475.0 ± 6830.5$ U/L) is elevated with respect to normal values.

### Table 1: Clinical, anthropometric, and biochemical parameters in DMD patients.

<table>
<thead>
<tr>
<th>DMD patients $n = 66$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Wheelchair bound (number of boys)</td>
</tr>
<tr>
<td>Vignos Scale</td>
</tr>
<tr>
<td>Body weight (kg)</td>
</tr>
<tr>
<td>Height (m)</td>
</tr>
<tr>
<td>Body fat mass (%)</td>
</tr>
<tr>
<td>Body fat mass (kg)</td>
</tr>
<tr>
<td>Lean body mass (kg)</td>
</tr>
<tr>
<td>Percentile of BMI</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
</tr>
<tr>
<td>IL-1 (pg/mL)</td>
</tr>
<tr>
<td>TNF–α (pg/mL)</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
</tr>
<tr>
<td>CK (U/L)</td>
</tr>
</tbody>
</table>

DMD: Duchenne muscular dystrophy; IL-6: cytokine 6; IL-1: cytokine 1; BMI: body mass index; TNF–α: tumor necrosis factor; CRP: C-reactive protein; CK: creatine kinase. Data are expressed as mean ± SD. Values reported in healthy subjects of *IL–6, 1.93 ± 1.38 pg/mL; **IL-1, 3.6 ± 1.0; ***TNF–α (pg/mL) 3.6 ± 0.9 pg/mL [7,17,18].

3.2. Systemic Inflammation and Muscle Function in DMD. Because chronic inflammation is associated with muscle damage impacting on the progress of Duchenne dystrophy, we decided to classify the study population according to muscle functional status using the Vignos Scale.

As expected, our results show that patients with independent ambulation (Vignos Scale A) (Table 2) had a higher concentration of proinflammatory cytokines IL-1 and TNF-α in comparison with wheelchair-limited mobility patients (Vignos Scale A). In the same sense, CK, the biomarker of muscle damage, is also increased in the same group compared with the Vignos Scale A and B groups. In contrast, patients with wheelchair-limited mobility (Vignos Scale C) have a lower concentration of proinflammatory cytokines (IL-1 and TNF-α) and CK in comparison with Vignos Scale A group. Nonetheless, no differences in IL-6 were observed among groups.

IL-1 concentration decreased four times in Vignos Scale C group in comparison with Vignos Scale A group and decreased six times in Vignos Scale B group. Regarding TNF-α, its concentration decreased by one in Vignos Scale C group compared with Vignos Scale A group. CK concentration was reduced four times in Vignos Scale C group with respect to Vignos Scale A group (Table 2).

Leptin and CRP increased three and five times, respectively, in Vignos Scale C group compared with Vignos Scale A group. In contrast, no differences in adiponectin concentration were detected among groups (Table 2).

Accordingly, patients from Vignos Scale C group have the highest percentage of body fat, body weight, and lean body...
mass compared with patients with better muscle function (Table 2). Although both lean and fat mass showed a higher increase in Vignos Scale C group than Vignos Scale A group, it does not occur in the same proportion. In Vignos Scale A group, we can observe that the amount of lean body mass (kg) is almost three times higher than body fat mass (kg), but in Vignos Scale C group the proportion between lean and fat body mass (kg) is similar (Table 2).

3.3. Systemic Inflammation and Nutritional Status in DMD. We did not observe significant differences in the concentration of proinflammatory cytokines such as IL-1, IL-6, and TNF-α among groups with different nutritional status. These results suggest that the inflammatory status produced by these cytokines is similar, independent of the nutritional status of patients. In the same sense, no differences were found in age, concentration of CK, and Vignos Scale among levels of nutritional status. In contrast, as expected, the concentration of CRP in overweight/obese patients was higher than that in the other two groups (Table 3). Accordingly, body weight, body fat mass, lean mass, and leptin increased significantly (P < 0.05) in overweight/obese boys in comparison with patients with healthy weight (Table 3). Increase of these parameters in obesity is well known, although no differences in adiponectin concentration among nutritional status groups were observed (Table 3).

4. Discussion

Inflammation plays a main role in the development of the pathology in DMD. Although it is well known that chronic and exacerbated inflammation can provoke the loss of muscle regeneration, currently there are insufficient human studies that identify whether an association exists between systemic inflammation with muscle function and nutritional status in DMD patients. The results of this study suggest that systemic inflammation is associated with muscle function in patients with DMD.

Because muscle biopsy is an invasive procedure, there is no information about the inflammation during muscle damage progress of DMD patients. Consequently, in an attempt to identify the inflammation process of dystrophy through evolution in a noninvasive manner, we analyzed inflammatory molecules in serum. Although it is well known that the inflammation in DMD is mainly localized in muscle fibers from damaged muscle, the findings of this investigation lead us to consider some cytokines as biomarkers of systemic inflammation that may indicate muscle injury.

4.1. Systemic Inflammation and Muscle Function in DMD. Inflammation has an important impact on the physiopathology of DMD, where the immunology cells secrete cytokines that amplified the inflammation process. Exacerbation of inflammation can provoke the loss of muscle regeneration and decrease muscle mass; consequently, there is a loss of muscle function.

Our results show that proinflammatory cytokines (IL-1 and TNF-α) are decreased in the serum of patients with less muscle function. It is well known that cytokines are increased in dystrophic patients compared to healthy subjects. For instance, TNF-α concentration in serum of DMD patients is increased eight times in comparison with healthy boys (30.2 ± 9.5 versus 3.6 ± 0.9 pg/mL) of the same age (8.1 ± 1.9 years old) [18]. IL-6 is also increased in serum of DMD individuals (3.77 ± 2.71 pg/mL) compared with healthy subjects (1.93 ± 1.38 pg/mL) [7]. Therefore, cytokines may have a significant

### Table 2: Inflammatory parameters and body composition in DMD patients with different levels of muscle function.

<table>
<thead>
<tr>
<th>Vignos Scale A (1–5)</th>
<th>Vignos Scale B (6–8)</th>
<th>Vignos Scale C (9–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.04 ± 2.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.04 ± 3.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.43 ± 1.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>IL-6 (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.99 (2.00, 9.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.27 (2.00, 4.23)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.46 (2.00, 4.37)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>IL-1 (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.18 (0.23, 28.30)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.46 (7.76, 10.30)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.44 (1.22, 2.82)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>TNF-α (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.24 ± 2.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.53 ± 1.86&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>8.71 ± 2.17&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Adiponectin (ng/mL)</strong></td>
<td>51.83 ± 33.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.62 ± 15.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Leptin (ng/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.59 ± 786&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.00 ± 6.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.10 ± 18.63&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CRP (mg/dL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.72 (0.30, 7.44)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.31 (0.30, 5.46)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.94 (2.52, 6.96)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CK (U/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14332.14 ± 6382.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6241.66 ± 3090.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>398750 ± 1818.74&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Body weight (kg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.24 ± 7.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.50 ± 13.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.00 ± 18.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Height (m)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.20 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.34 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.52 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Body fat mass (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23.25 ± 11.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.88 ± 8.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.93 ± 6.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Lean body mass (kg)</strong></td>
<td>18.78 ± 4.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.44 ± 6.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Body fat mass (kg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.00 ± 4.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.80 ± 8.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.10 ± 11.91&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DMD: Duchenne muscular dystrophy; IL-6: cytokine 6; IL-1: cytokine 1; TNF-α: tumor necrosis factor; CRP: C-reactive protein; CK: creatine kinase.

The parameter of Vignos Scale was grouped according to Vignosis scale A (0–5 points), Vignos Scale B (6–8 points), and Vignos Scale C (9-10 points).

Values are presented as mean ± SD.

Different superscript letters (a, b, and c) indicate statistical significance. P < 0.05 (ANOVA, comparison among Vignos Scale groups using Bonferroni post hoc test). Some columns show two letters indicative of statistical significance (no difference or difference with other groups).
impact on the inflammatory process. There are some studies that suggest a correlation between the inflammatory cytokine expression and muscle function in DMD. de Pasquale et al. demonstrated a higher expression of proinflammatory cytokine (IL-17) in muscle biopsy from DMD patients with the lower motor outcome evaluated by North Star Ambulatory Assessment score (NSAA), suggesting that IL-17 mRNA expression and muscle function in DMD. de Pasquale et al. demonstrated a higher expression of proinflammatory cytokine (IL-17) in muscle biopsy from DMD patients with the lower motor outcome evaluated by North Star Ambulatory Assessment score (NSAA), suggesting that IL-17 mRNA levels and functional outcome are negatively associated [6].

According to those findings, CK was also reduced in the same patients. It has been reported that CK decreases in older patients [21, 22]. Saito et al. reported in young patients (<20 years old) that TNF-α concentration (60.7 ng/L) was approximately five times higher compared with patients >20 years of age (12 ng/L) [4].

Based on these studies and according to our results, we propose that the concentration of cytokines (serum inflammatory markers) is particularly increased in the early stage of the disease such as in younger DMD patients with independent ambulation. This proposal is also based on other studies [23–25] where the authors described that the number of inflammatory cells decreases in muscle tissue of dystrophic patients after the age of 8 years and the quantity of these cells is greater in the first years of the patient’s life. However, it is important to consider that muscle fibers contribute to inflammation due to their ability to produce cytokines as demonstrated in cells of dystrophic and nondystrophic mice. Cell lines of myocytes yield and secrete different cytokines into the medium such as IL-1, IL-6, and TNF-α and send signals to the muscle to synthesize additional cytokines [26–28]. Therefore, younger patients with better muscle function have more muscle mass with a higher production of cytokines. The concentration of these cytokines in serum is increased in comparison with patients with less muscle function.

Based on these studies and because we observed that patients with the most affected muscle function (wheelchair-limited mobility) have the lowest concentration of IL-1, TNF-α, and CK compared with patients with less muscle damage (independent ambulation), we hypothesized that the decrease in concentration of these cytokines and CK may result from muscle loss in DMD patients. This hypothesis is also supported by Emery and Muntoni who suggest that the declining CK levels in serum of DMD patients is associated with a decrease in ambulatory skills and disease progression [22].

### 4.2. Systemic Inflammation and Nutrition Status in DMD

Cytokines are secreted by adipose tissue and this secretion is increased in obese subjects. The muscle of dystrophic boys is replaced by fat mass. Therefore, inflammation in DMD patients may also result from obesity.

We observed that concentration of inflammatory molecules, Vignos Scale, and age was similar among groups with different levels of nutritional status, suggesting a similar inflammatory status independent of the obesity grade. For instance, underweight and obese children have similar cytokine values. Interestingly, our results suggest that, in underweight patients, systemic inflammation may be caused in part by the pathology of DMD because these patients have less body fat mass. However, in patients with overweight or obesity, this systemic inflammation may be originated by adipose tissue. It has been reported that proinflammatory cytokines are increased in obese children. The authors reported a higher concentration of serum IL-6 (0.14 versus 0.05 pg/mL) and TNF-α (9.2 pg/mL versus 8.1) in obese Mexican children (13 years old) than in nonobese children [29].

Others molecules associated with obesity are CRP, adiponectin, and leptin. Liver and adipose tissue produce

---

**Table 3: Anthropometric, clinical, and inflammatory parameters in DMD patients with different levels of nutritional status.**

<table>
<thead>
<tr>
<th>Underweight</th>
<th>Healthy weight</th>
<th>Overweight/obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>9.31 ± 3.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.32 ± 3.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>19.51 ± 4.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.32 ± 10.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.22 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.27 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body fat mass (%)</td>
<td>14.89 ± 5.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.87 ± 13.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>15.63 ± 3.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.91 ± 4.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body fat mass (kg)</td>
<td>2.62 (0.88, 5.42)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.30 (1.58, 30.47)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>3.00 ± 1.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.13 ± 6.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adiponectin (ng/mL)</td>
<td>48.17 ± 26.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.63 ± 20.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>2.71 (2.00, 4.21)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.09 (2.00, 9.00)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1 (pg/mL)</td>
<td>7.20 (0.34, 14.00)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.60 (0.23, 28.50)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>11.12 ± 2.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.76 ± 3.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.47 (0.30, 1.98)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01 (0.30, 6.96)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>11650.00 ± 6217.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12389.45 ± 7404.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vignos Scale</td>
<td>3.0 (2.0, 5.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 (1.0, 9.0)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DMD: Duchenne muscular dystrophy; IL-6: cytokine 6; IL-1: cytokine 1; TNF-α: tumor necrosis factor; CRP: C-reactive protein; CK: creatine kinase. Values are presented as mean ± SD. Different superscript letters (a, b, and c) indicate statistically significant difference. P < 0.05 ANOVA, comparison among different levels of nutritional status using Bonferroni’s post hoc test. Some columns show two letters indicative of statistical significance (no difference or different from other groups).
CRP and both may contribute to elevated plasma CRP levels in obesity. A high amount of body fat may produce an important part of the circulating CRP [17, 30]. As expected, CRP and leptin were increased in overweight and obese boys because body fat was increased. These two molecules (CRP and leptin) were also increased in the Vignos Scale C group, possibly explained because subjects in the Vignos Scale C group had the highest values of fat mass [11].

However, it is important to consider that this study has some limitations. We evaluated these cytokines in blood, but not in muscle. However, we should consider that these are circulating cytokines and this may offer an explanation in regard to muscle inflammatory status. Another limitation is the lack of control subjects of the same age to evaluate cytokine concentrations. The main strength of this study is that we demonstrated, for the first time, differences in inflammatory status among groups with different levels of muscle function and nutritional status. Because inflammation plays a key role in the pathological progress, it is necessary to evaluate other contributors to the inflammatory process during the course of dystrophy such as other cytokines and immunological cells to propose different management procedures in these patients.

5. Conclusions

To our knowledge, these novel results present evidence regarding inflammatory status in DMD patients with different levels of muscle function. In the first state of the pathology when patients have better muscular function, the systemic inflammation status is increased. This inflammation is decreased in patients with poorer muscle function. Concentration of systemic inflammatory molecules is independent of nutritional status.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors express their appreciation to the patients and families for participation in the study. This work was supported by Consejo Nacional de Ciencia y Tecnología, México (grant no. Salud-2012-180058). The authors acknowledge Sharon Morey, Scientific Communications, for providing editorial assistance.

References


