Available online at www.sciencedirect.com

ScienceDirect

Neuromuscular Disorders ■■ (2015) ■■–■■

www.elsevier.com/locate/nmd

Dystroglycanopathy muscles lacking functional glycosylation of alpha-dystroglycan retain regeneration capacity

Hiroyuki Awano, Anthony Blaeser, Bo Wu, Pei Lu, Elizabeth Keramaris-Vrantsis, Qi Lu *

McColl-Lockwood Laboratory for Muscular Dystrophy Research, Cannon Research Center, Carolinas Medical Center, 1000 Blythe Blvd, Charlotte, NC 28203, USA

Received 27 August 2014; received in revised form 3 February 2015; accepted 11 March 2015

Abstract

In dystroglycanopathies, lack of glycosylated alpha-dystroglycan (α-DG) alters membrane fragility leading to fiber damage and repetitive cycles of muscle degeneration and regeneration. However the effect of the glycosylation of α-DG on muscle regeneration is not clearly understood. In this study, we examined the regenerative capacity of dystrophic muscles *in vivo* in *FKRP* mutant and *LARGE*^{myd} mice with little and complete lack of functionally glycosylated α-DG (F-α-DG) respectively. The number of regenerating fibers expressing embryonic myosin heavy chain (eMyHC) in the diseased muscles up to the age of 10 months is higher than or at similar levels to wild type muscle after notexin and polyethyleminine insults. The process of fiber maturation is not significantly affected by the lack of F-α-DG assessed by size distribution. The earlier appearance of a larger number of regenerating fibers after injury is consistent with the observation that the populations of myogenic satellite cells are increased and being readily activated in the dystroglycanopathy muscles. F-α-DG is expressed at trace amounts in undifferentiated myoblasts, but increases in differentiated myotubes *in vitro*. We therefore conclude that muscle regeneration is not impaired in the early stage of the dystroglycanopathies, and F-α-DG does not play a significant role in myogenic cell proliferation and fiber formation and maturation.

Keywords: Muscular dystrophy; Fukutin related protein; Muscle regeneration; Dystroglycanopathy

1. Introduction

Recently, autosomal recessive mutations in at least sixteen genes have so far been identified as the causes of a subset of muscular dystrophies, known as dystroglycanopathies, characterized by a common secondary defect in the glycosylation of dystroglycan. These genes include *fukutin*, fukutin-related protein (*FKRP*), *LARGE*, *POMGnT1*, *POMT1*, *POMT2*, Isoprenoid Synthase Domain Containing (*ISPD*), Transmembrane protein 5 (*TMEM5*), β1,3-N-acetylglucosaminyltransferase1 (*B3GNT1*), glycosyltransferase-like domain containing 2 (*GTDC2/POMGnT2*), β3-N-acetylgalactosaminyltransferase 2 (*B3GALNT1*), and *SGK196* [1–7]. A number of studies have demonstrated the function of some of these genes in the post-translational glycosylation modification of α-dystroglycan (α-DG) [8–14]. *POMT1* and *POMT2* catalyze the initial *O*-mannosylation of the protein [15]. *POMGnT1* acts as a protein *O*-mannose β-1,2-N-acetylglucosaminyltransferase [11] and *POMGnT2* acts as a protein *O*-mannose β-1,4-N-acetylglucosaminyltransferase [16]. *LARGE* has been described as a bifunctional glycosyltransferase (xylosyltransferase and glucuronyltransferase activities) producing repeating units of [-3-xylose-α1,3-glucuronic acid-β1-] [17]. However, functions of both *fukutin* and *FKRP* remain elusive [18].

Dystroglycan (DG) is one of the critical components of the dystrophin–glycoprotein complex (DGC) [19,20] and crucial for the maintenance of muscle membrane stability. The DG polypeptide is post-translationally cleaved into two subunits, the extracellular α-DG and the transmembrane β-DG that are non-covalently linked and provide a physical connection between the extracellular matrix (ECM) and the actin cytoskeleton [21–23]. Alpha-DG, through its extensively *N*- and *O*-linked glycans, acts as a cellular receptor for laminin and other ECM proteins, including agrin, perlecan, neurexin and pikachurin [24–30]. In dystroglycanopathies, lack of specific variants of glycosylated α-DG, defined largely by specific antibodies and binding affinity to laminin, destabilizes the DGC structure and alters membrane permeability, leading to fiber damage and degeneration. The clinic severity of dystroglycanopathies varies significantly from mild limb girdle muscular dystrophy 2I (LGMD2I) to severe

* Corresponding author. McColl-Lockwood Laboratory for Muscular Dystrophy Research, Cannon Research Center, Carolinas Medical Center, 1000 Blythe Blvd. Charlotte, NC 28203, USA. Tel.: +1 704 355 1701; fax: +1 704 355 1679.
E-mail address: qi.lu@carolinashealthcare.org (Q. Lu).

http://dx.doi.org/10.1016/j.nmd.2015.03.004
0960-8966/

congenital muscular dystrophy, Walker–Warburg syndrome and muscle–eye–brain disease. However, all diseases show progressive loss of muscle mass. Histologically, the affected muscles are eventually replaced by non-muscle fibrotic and fat tissues with loss of contractile function.

Functional glycosylation of α-DG (F-α-DG) is required for normal development of the embryo. Defects of F-α-DG affects formation of the basement membrane in embryoid bodies as well as the development of many other tissues, including central nerve system (CNS) and eye [31]. Lack of dystroglycan in CNS disrupts the formation of Reichert's membrane during early development and causes embryonic lethality [32]. Furthermore, defects in neuronal migration and brain and eye development are the common phenotypes in severe end dystroglycanopathies with mutations in nearly all putative and confirmed glycosyltransferases. These defects appear to be related to the disruption of interactions between different cell types or between cells and ECM.

The effect of glycosylation of α-DG on muscle regeneration and specifically on the regenerative capacity of muscle-specific satellite cells is not clearly understood. An earlier study in conditional DG knockout mice suggested that expression of dystroglycan in the satellite cells may play an important role in maintaining muscle regeneration capacity [33]. This implies that loss of F-α-DG in dystroglycanopathies could limit the capacity of muscle regeneration, thus contributing to the dystrophic phenotype especially in the diseases with complete loss of F-α-DG. A more recent study however reported that the lack of functional glycosylation did not affect the number of satellite cells and their capacity to proliferate *in vitro* [34]. In this study, we investigated the regenerative capacity of diseased muscles in two distinct dystroglycanopathy model mice, the *FKRP Pro448Leu* (*P448L*) mutant and the *LARGE*^{myd} mice in comparison with wild type mice. The *FKRP P448L* mice present moderate disease phenotype and F-α-DG is lost in almost all muscle fibers except for a few revertant fibers [35]. The *LARGE*^{myd} mice show a more severe phenotype with clearly shortened life span and complete loss of F-α-DG in all muscle fibers. Our data suggest that myoblasts express little F-α-DG and muscles of both *FKRP* mutant and *LARGE*^{myd} mice retain a similar capacity of regeneration. Lack of F-α-DG does not significantly impede fiber maturation. The failure of dystroglycanopathy muscles to regenerate is likely related to the depletion of regenerating capacity and tissue remodeling as a result of progressive degeneration observed in muscular dystrophies in general.

2. Materials and methods

2.1. Animals and ethical statement

This study was carried out in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of Carolinas Medical Center (01-13-03A). All treatments were performed under isoflurane anesthesia, and every effort was made to minimize suffering.

The *P448L+*, *P448L−*, *LARGE*^{myd} (Jackson Laboratory, Bar Harbor, ME, USA) and *C57BL/6* were used. *P448L+* mice contain homozygous missense mutation (c.1343C>T, p.Pro448Leu) in the *FKRP* gene with the neomycin-resistant (Neo^r) cassette and their phenotype largely represents congenital muscular dystrophy with central nerve system involvement [36]. Another *FKRP* mutant mouse contains the same P448L mutation but with the Neo^r cassette removed from the insertion site (*P448L−* mice). The *FKRP P448L* mutant mice were backcrossed to more than 95% levels of congenicity to C57BL/6. *P448L−* homozygotes showed muscle weakness demonstrated by the limb muscle retraction, elevated serum CK and ALT levels, and dystrophic pathology associated with limb-girdle muscular dystrophy without clear defect in the central neural system [35].

2.2. Notexin and PEI experiments

In order to induce a skeletal muscle regeneration following degeneration, notexin (NTX; 40 µl at 5 µg/ml, Latoxan, Valence, France) [37] and polyethyleminine (PEI; branched, Mw; 25k, 40 µl at 0.5 µg/µl, Sigma, St. Louis, MO, USA) were injected into the tibialis anterior (TA) muscle. Injection of the regents was applied to only one leg of the mice. The contralateral leg, without treatment, was used as a control. After injection, TA muscle was examined at 2, 4, 7, 14 and 28 days for *P448L−* and *C57*, and at 4 and 14 days in *P448L+* and *LARGE*^{myd} due to limited mouse availability. Three mice were used for each treatment group.

2.3. Histology and immunohistochemistry

TA muscles were snap-frozen in isopentane chilled to freezing point in liquid nitrogen. Multiple cross sections of 6 µm thickness were cut from different level of frozen muscles and stained with hematoxylin and eosin (H&E). Immunohistochemical staining of embryonic myosin heavy chain (eMyHC) and Pax7 was performed on cross section of the muscle. Sections for Pax7 staining were fixed with formalin solution (10%, buffered neutral) for 5 minutes. After 1 hour blocking with 1% horse serum, primary antibodies against eMyHC (F1.652) and Pax7 (PAX7) (both from Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were incubated overnight at 4 °C, followed by washing for 10 minutes three times with PBS and finally incubated with Alexa Fluor 594-conjugated rabbit anti-mouse secondary antibodies (Life Technologies, Carlsbad, CA, USA) at 1:500 dilution. Sections were also stained with secondary antibody alone as negative controls. Muscle sections with maximal cross-sectional area were employed for cell counting and fiber size determination. For determination of percentage of central nucleated fibers, diameter of muscle fibers and number of Pax7 positive cells, three random 20× magnification images per section per animal were used (with more than 500 total number of fibers). The number of Pax7 positive cells per myofiber was obtained by dividing the total number of Pax7 positive cells by the total number of fibers counted. For eMyHC positive (eMyHC+) cell count and measurement of its diameter, muscle sections with the

Please cite this article in press as: Hiroyuki Awano, et al., Dystroglycanopathy muscles lacking functional glycosylation of alpha-dystroglycan retain regeneration capacity, Neuromuscular Disorders (2015), doi: 10.1016/j.nmd.2015.03.004