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Studying the role of dystrophin-associated proteins in influencing Becker muscular dystrophy disease severity

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Abstract

Becker muscular dystrophy is characterized by a variable disease course. Many factors have been implicated to contribute to this diversity, among which the expression of several components of the dystrophin associated glycoprotein complex. Together with dystrophin, most of these proteins anchor the muscle fiber cytoskeleton to the extracellular matrix, thus protecting the muscle from contraction induced injury, while nNOS is primarily involved in inducing vasodilation during muscle contraction, enabling adequate muscle oxygenation. In the current study, we investigated the role of three components of the dystrophin associated glycoprotein complex (beta-dystroglycan, gamma-sarcoglycan and nNOS) and the dystrophin homologue utrophin on disease severity in Becker patients. Strength measurements, data about disease course and fresh muscle biopsies of the anterior tibial muscle were obtained from 24 Becker patients aged 19 to 66. The designation of Becker muscular dystrophy in this study was based on the mutation and not on the clinical severity. Contrary to previous studies, we were unable to find a relationship between expression of nNOS, beta-dystroglycan and gamma-sarcoglycan at the sarcolemma and disease severity, as measured by muscle strength in five muscle groups and age at reaching several disease milestones. Unexpectedly, we found an inverse correlation between utrophin expression at the sarcolemma and age at reaching disease milestones.

Keywords: Becker muscular dystrophy; Dystrophin associated glycoprotein complex; Disease progression; nNOS; Utrophin

1. Introduction

Duchenne and Becker muscular dystrophies (D/BMD) are X-linked inherited disorders caused by mutations in the *DMD* gene, coding for dystrophin. This protein is part of the dystrophin-associated glycoprotein complex. In addition to dystrophin, this complex consists of several cytoplasmic (dystrobrevin, the syntrophins and neuronal nitric oxide synthase (nNOS)) and transmembraneous proteins (sarcoglycans, dystroglycans and sarcospan) [1,2]. Their interconnections and interaction with the muscle cytoskeleton and the extracellular matrix play an important role in maintaining muscle membrane stability. Since dystrophin has a key role in these interactions, absence of dystrophin, as in DMD, and (reduced quantity of) internally truncated dystrophin, as in BMD, lead to continuous muscle damage upon muscle contraction. Clinically, this results in progressive muscle weakness. In DMD, muscle weakness is severe, leading to loss of ambulation in the early teens, need for mechanical ventilation in the late teens/early twenties and death due to cardiac and/or respiratory failure in the mid-twenties and thirties [3–5]. The disease course in BMD is generally less severe, but also more diverse, ranging from patients with a DMD-like phenotype to patients remaining ambulant throughout life [6,7]. The reasons for this variability are not fully understood, although several factors have been implicated. One possible explanation for the clinical variability involves the expression of the other components of the dystrophin-associated complex at the sarcolemma. As the role of dystrophin is vital in localizing these proteins at the sarcolemma, most of them are absent in DMD patients [8]. However, in BMD their expression is variable. Because mutations in several of the proteins involved in the complex result in limb girdle muscular dystrophies, it is hypothesized that this expressional variability might influence disease severity in BMD [2]. As beta-dystroglycan is the only trans-membrane glycoprotein of this complex interacting directly with dystrophin, one could suggest expressional levels of this protein at the sarcolemma to be the most relevant in BMD.

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Furthermore, as the sarcoglycans stabilize the dystrophin-dystroglycan interaction, their expression levels and localization could influence severity as well. Finally, another protein considered possibly influential on disease severity in BMD is utrophin. This dystrophin homologue is normally expressed at the neuromuscular junction [9]. However, in the absence of dystrophin in both humans and animals, protein expression is upregulated and utrophin is located both at the neuromuscular junction and the sarcolemma [10–12]. Since in mice absence of both dystrophin and utrophin leads to a very severe phenotype, it is thought that utrophin is able to take over some of the stabilizing functions of dystrophin at the muscle membrane. Expression levels of utrophin at the membrane might therefore influence disease severity in BMD patients as well. In the current study, we examined if variable expression of several dystrophin associated glycoproteins (nNOS, beta-dystroglycan and gamma-sarcoglycan) or utrophin could be linked to the disease severity in a cohort of 24 BMD patients.

2. Patients and methods

2.1. Patients

This study was part of a larger study investigating possible causes for the diversity in the disease course of BMD patients, performed in Leiden in 2011. Adult BMD patients were recruited from the Dutch Dystrophinopathy Database as previously described by van den Bergen et al. [13]. A questionnaire was used to grade patients' clinical status by noting ages at which the following disease milestones were reached; first motor symptoms, difficulty walking stairs, use of a walking aid, wheelchair dependence and use of mechanical ventilation. Additionally a fresh percutaneous conchotome muscle biopsy of the anterior tibialis (TA) muscle was obtained if patients consented to this.

2.2. Muscle strength

Bilateral quantitative muscle testing (QMT) of five muscle groups (elbow flexion and –extension, hip flexion and –extension and handgrip) was performed on a quantitative muscle assessment system using the protocol described by Hogrel et al. [14]. To correct for confounding effects of age and body composition of participants, an individual z-score was calculated for all tested muscles [14]. From these z-scores an average for all five muscle groups was subsequently calculated.

2.3. Immunohistochemistry

Sections of 10 μm were cut from the TA biopsies using a Shandon Cryotome (Thermo Fisher Scientific Co., Pittsburgh, PA, USA). Sections were fixed for 1 min with ice-cold acetone. Each section was stained with dystrophin antibodies and either nNOS, beta-dystroglycan, gamma-sarcoglycan or utrophin antibodies. Goat polyclonal dystrophin (SC-7461, Santa Cruz Biotechnology, USA) diluted 1:50, rabbit polyclonal NOS1 (SC-8309, Santa Cruz Biotechnology, USA) diluted 1:50, and mouse monoclonals B-DG (SC28535, Santa Cruz Biotechnology, USA), G-SARC (Ab104478, Abcam, the Netherlands) and Mach07 (SC-81557, Santa Cruz Biotechnology, USA) diluted 1:50 were used to detect dystrophin, nNOS, beta-dystroglycan, gamma-sarcoglycan and utrophin, respectively. Alexa-fluor 488 and Alexa-fluor 594 conjugates (both from Invitrogen, the Netherlands) diluted 1:1000 were used as secondary antibodies for dystrophin (488) and nNOS, beta-dystroglycan, gamma-sarcoglycan or utrophin (all 594). Slides were analyzed using a fluorescence microscope (DM RA2; Leica Microsystems, Wetzlar, Germany), and digital images were taken using a CCD camera (CTR MIC; Leica Microsystems). A section of healthy muscle was used as a positive control, and a section of healthy muscle where the incubation with the first antibody was omitted was used as a negative control. nNOS was assessed qualitatively according to presence in muscle membrane and/or cytoplasm. Blinded scoring was conducted as follows: 1) primarily localized in the cytoplasm; 2) localized both at muscle membrane and cytoplasm; 3) primarily membrane bound, one being the worst and three the best score (Supplementary Fig. S1).

For dystrophin, beta-dystroglycan and gamma-sarcoglycan, the following scoring system was used: 1) absent (no positive fibers observed), 2) moderate staining (some weak positive fibers and/or cytoplasmatic staining observed), 3) reasonable staining (the majority of fibers are positive but at lower levels than control), 4) good staining (fibers are positive comparable to control levels). For utrophin, the scoring system consisted of: 1) neuromuscular junction staining only, 2) light membrane staining and 3) clear membrane staining. Dystrophin scoring was performed for the dystrophin co-stainings done for nNOS, beta-dystroglycan and gamma-sarcoglycan, so this was scored 3 times for each sample, where the most frequently observed score was taken.

2.4. Statistics

Correlations between the different QMT-scores were calculated using Pearson's R. Differences in mean z-scores of QMT-scores between the different expression patterns of nNOS, beta-dystroglycan, gamma-sarcoglycan dystrophin and utrophin respectively were calculated using a Kruskal–Wallis Test. Differences in age at reaching several disease milestones and expression patterns of nNOS, beta-dystroglycan, gamma-sarcoglycan dystrophin and utrophin were calculated using a Kaplan–Meier Survival Curve. Significance levels were set at $p = 0.05$.

3. Results

3.1. Patients

Thirty-two BMD patients participated in our study. Of these, muscle biopsy material for further analysis was available for 24 patients. Mean age of these patients was 39 years (SD 13; range 19–66). Seven participants were wheelchair dependent at the time of study, at an average age of 38 years (SD 16; range 10–60). See Table 1 for all disease milestones. The QMT scores of all individually tested muscle groups showed good correlation ($R > 0.6$ and $P < 0.05$ for all). The average z-scores for muscle strength ranged from –6.1 to 3.9 (SD 2.2). See Supplementary Table S1 for all patient characteristics.