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Molecular Pathogenesis of Genetic and Inherited Diseases

Dysferlin Deficiency Shows Compensatory Induction of Rab27A/Slp2a That May Contribute to Inflammatory Onset

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Mutations in the dysferlin gene cause limb girdle muscular dystrophy 2B (LGMD2B) and Miyoshi myopathy. Dysferlin-deficient cells show abnormalities in vesicular traffic and membrane repair although onset of symptoms is not commonly seen until the late teenage years and is often associated with subacute onset and marked muscle inflammation. To identify molecular networks specific to dysferlin-deficient muscle that might explain disease pathogenesis, muscle mRNA profiles from 10 mutation-positive LGMD2B/MM patients were compared with a disease control [LGMD2I; (n = 9)], and normal muscle samples (n = 11). Query of inflammatory pathways suggested LGMD2B-specific increases in co-stimulatory signaling between dendritic cells and T cells (CD86, CD28, and CTLA4), associated with localized expression of both versican and tenascin. LGMD2B muscle also showed an increase in vesicular trafficking pathway proteins not normally observed in muscle (synaptotagmin-like protein Slp2a/SYTL2 and the small GTPase Rab27A). We propose that Rab27A/Slp2a expression in LGMD2B muscle provides a compensatory vesicular trafficking pathway that is able to repair membrane damage in the absence of dysferlin. However, this same pathway may release endocytotic vesicle contents, resulting in an inflammatory microenvironment. As dysferlin deficiency has been shown to enhance phagocytosis by macrophages, together with our findings of abnormal myofiber endocytosis pathways and dendritic-T cell activation markers, these results suggest a model of immune and inflammatory network over-stimulation that may explain the subacute inflammatory presentation. ***(Am J Pathol 2008, 173:1476–1487; DOI: 10.2353/ajpath.2008.080098)***

Limb-girdle muscular dystrophies are a group of heterogeneous disorders typically showing an autosomal recessive mode of inheritance, progressive muscle weakness, and high serum creatine kinase levels.[1] Two types of muscle disease, a distal myopathy (Miyoshi Myopathy, MM) and a form of limb girdle muscular dystrophy (LGMD2B) have both been shown to be caused by mutations of the dysferlin gene.[2,3] In both LGMD2B and MM, dysferlin gene mutations result in partial or complete loss of dysferlin protein in muscle as measured by immunoassays, although the reduction in dysferlin levels does not strictly correlate with clinical severity.[4] Both the proximal (LGMD2B) and distal (MM) phenotypes can be caused by identical mutations in the same family, suggesting the role of genetic modifiers and/or environmental influence on disease expression.[5] The dysferlin gene is localized to 2p13 with expression in various tissues ranging from kidney to monocytes, with highest levels in skeletal and cardiac muscle.[6] Dysferlin is localized predominantly to the muscle surface membrane, and is also associated with cytoplasmic vesicles.[7]

The dysferlin protein was originally named based on its similarity to the *Caenorhabditis elegans* protein FER-1. FER-1 is responsible for mediating fusion of intracellular vesicles with the spermatid plasma membrane.[8] Sequence homology between FER-1 and dysferlin includes

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tandem C2 domains and a C terminal transmembrane domain. The similarity in primary structure of the two proteins led to the suggestion that dysferlin may also play a role in membrane fusion events in skeletal muscle cells.[9] C2 domains are known to bind calcium, phospholipids or proteins to trigger signaling events and membrane trafficking, and this has led to speculation that dysferlin is important for membrane repair in skeletal muscle.[2,10] This hypothesis is supported by the finding that dysferlin deficient patient muscle shows numerous structural membrane defects when analyzed by electron microscopy, including tears in the plasma membrane and an accumulation of subsarcolemmal vesicles and vacuoles.[11] Also, laser-induced membrane damage in dysferlin-deficient myofibers has highlighted reduced membrane resealing capability compared to normal muscle myofibers.[12] These findings are consistent with a plasma membrane repair defect in dysferlin-deficient myofibers.

The current understanding of molecular and cellular defects in LGMD2B/MM does not explain a number of the enigmatic features of the presentation and progression of patients with dysferlin deficiency in muscle. First, dysferlin-deficient patients are typically quite healthy until their late teens, and in our patient cohort some patients showed impressive athletic skill at young age, before disease onset. Second, disease onset is typically in late teens or early twenties, and is often associated with a subacute onset of weakness, with marked muscle inflammation on muscle biopsy.[13,14] The onset and inflammation often leads to the misdiagnosis of polymyositis.[14,15] Finally, the relatively wide inter- and intrafamilial variation in clinical phenotype, subacute onset, and marked inflammation suggest that environmental factors, other genetic modifiers, or both may play a stronger role in LGMD2B than in the other dystrophies.

The aggressive inflammation often observed in dysferlin-deficient muscle distinguishes it from other Limb-girdle dystrophies. Systematic studies of the inflammatory cell content showed a predominance of macrophages, and $CD4^+$ cells, however CD8+ cytotoxic T cells are also abundant.[11,13,14] Normal monocytes contain dysferlin, and LGMD2B/MM patients lack dysferlin in their monocytes.[16,17] We have recently shown that dysferlin-deficient monocytes display abnormal signaling and phagocytotic activity that could contribute to excessive inflammation in patient muscle.[18] Further, human LGMD2B and mouse (SJL) dysferlin-deficient muscle showed macrophage and dendritic cell activation markers, including HLA-DR, HLA-ABC, and CD86 (human), and MOMA-2, CD11c, and ICAM-1 (mice) suggesting that mild myofiber damage in dysferlin-deficient muscle may result in an exaggerated monocyte/dendritic cell response secondary to dysferlin protein loss.

In the present study, we sought to identify the novel networks induced as a direct consequence of dysferlin deficiency. Key to our approach is the filtering out of molecular changes resulting from non-specific muscle pathology (degeneration, regeneration, fibrosis, and inflammation). To accomplish this, we compared a series of dysferlin-deficient, mutation-positive LGMD2B/Miyoshi patient muscle biopsy profiles to a muscular dystrophy of similar age of onset and clinical severity, LGMD2I, involving partial loss-of-function of FKRP (fukutin-related protein). We also included muscle biopsies from normal volunteers, Becker muscular dystrophy, and amyotrophic lateral sclerosis as additional controls. This approach led to the identification of both vesicle trafficking and inflammatory changes that were specific to or strongly exaggerated in dysferlin-deficient muscle.

Materials and Methods

Patients

The patient population in the study was taken from a molecular diagnostic referral population. Frozen muscle biopsies from patients with a tentative diagnosis of muscular dystrophy were sent to the Hoffman laboratory at Children's National Medical Center, Washington DC. All samples were subjected to a standardized set of biochemical and histological assays. Tests included hematoxylin and eosin histological stains, dystrophin immunostaining, α-sarcoglycan immunostaining, merosin (laminin alpha2) immunostaining, dystrophin immunoblotting and dysferlin immunoblotting, as previously described.[19] Patients were recruited and samples were analyzed under protocol 2405, which has been reviewed and approved by the Office for the Protection of Human Subjects at Children's National Medical Center.

Muscle biopsies from four groups of subjects were studied for mutation and expression profiling studies: dysferlin deficient, FKRP (LGMD2I), Becker muscular dystrophy, normal volunteers (exercise studies), and amyotrophic lateral sclerosis (ALS). For dysferlin-deficient biopsies used in this study, twenty five patient biopsies showed complete or greatly reduced dysferlin signal by duplicate immunoblots (Table 1), and these patients were then used for subsequent dysferlin DNA mutation studies, mRNA profiling, and protein characterizations. For LGMD2I subjects, approximately 1000 frozen muscle biopsies from the diagnostic muscle tissue bank were selected, genomic DNA prepared from muscle cryosections, and mutation screening for the FKRP gene was done (see Materials and Methods). For Becker muscular dystrophy, patient biopsies showed dystrophin of abnormal size and quantity by duplicate immunoblots, and showed dystrophin gene deletion mutations. For ALS, patients were diagnosed by El Escorial criteria. Normal controls were from normal volunteers enrolled in exercise studies, and different biopsies used for mRNA profiling and protein validation studies.

Genomic DNA Analysis

For dysferlin-deficient patients, twenty five patients showing complete or greatly reduced dysferlin by duplicate immunoblots were selected, and genomic DNA was isolated from 10 mg of flash-frozen muscle biopsy using Genomic DNA Purification Kit (Gentra Systems Minneapolis, MA). 10 ng of the genomic DNA was used as a template for amplification of each of the 55 exons of the dysferlin gene using