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Muscle degeneration in neuraminidase 1-deficient mice results from infiltration of the muscle fibers by expanded connective tissue

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ABSTRACT

Neuraminidase 1 (NEU1) regulates the catabolism of sialoglycoconjugates in lysosomes. Congenital NEU1 deficiency in children is the basis of sialidosis, a severe neurosomatic disorder in which patients experience a broad spectrum of clinical manifestations varying in the age of onset and severity. Osteoskeletal deformities and muscle hypotonia have been described in patients with sialidosis. Here we present the first comprehensive analysis of the skeletal muscle pathology associated with loss of Neu1 function in mice. In this animal model, skeletal muscles showed an expansion of the epimysial and perimysial spaces, associated with proliferation of fibroblast-like cells and abnormal deposition of collagens. Muscle fibers located adjacent to the expanded connective tissue underwent extensive invagination of their sarcolemma, which resulted in the infiltration of the fibers by fibroblast-like cells and extracellular matrix, and in their progressive cytosolic fragmentation. Both the expanded connective tissue and the juxtaposed infiltrated muscle fibers were strongly positive for lysosomal markers and displayed increased proteolytic activity of lysosomal cathepsins and metalloproteinases. These combined features could lead to abnormal remodeling of the extracellular matrix that could be responsible for sarcolemmal invagination and progressive muscle fiber degeneration, ultimately resulting in an overt atrophic phenotype. This unique pattern of muscle damage, which has never been described in any myopathy, might explain the neuromuscular manifestations reported in patients with the type II severe form of sialidosis. More broadly, these findings point to a potential role of NEU1 in cell proliferation and extracellular matrix remodeling.

1. Introduction

Compartmentalized degradation of sialoglycoconjugates is initiated in lysosomes by the glycosidase *N*-acetyl-α-neuraminidase-1 (NEU1), a pivotal enzyme required for the removal of terminal sialic acid residues [1]. NEU1 is transported to lysosomes and activated therein by associating with the serine carboxypeptidase protective protein/cathepsin A (PPCA) [2]. NEU1 is a component of a multiprotein complex containing PPCA, β-galactosidase, and *N*-acetylgalactosamine-6-sulfate sulfatase. Two genetically distinct lysosomal storage diseases are associated with NEU1 deficiency: sialidosis, which results from structural mutations at the *NEU1* locus on chromosome 6p21 [3], and galactosialidosis, which is caused by a primary defect of PPCA, leading to secondary deficiencies of NEU1 and β-galactosidase [4]. Patients with sialidosis experience a broad spectrum of clinical manifestations, varying in the timing of onset and severity of the symptoms and mostly correlating with the levels of residual enzyme activity [5]. Type I sialidosis, the attenuated form of the disease, occurs during the second decade of life and results in progressive loss of vision associated with nystagmus, ataxia, and grand-mal seizures but not dysmorphic features [3]. Type II sialidosis, the severe form of the disease, is characterized by hydrops foetalis and neonatal ascites, the presence of abnormal somatic features, and severe neurologic involvement. A subset of patients with sialidosis presents with symptoms of profound muscle dysfunction, including muscle hypotonia, atrophy, and osteoskeletal deformities [5]. However, a detailed characterization of the muscle involvement in these patients has never been reported.

Abbreviations: Neu1, neuraminidase 1; PPCA, protective protein/cathepsin A; Lamp-1, lysosomal-associated membrane protein-1; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; PM, plasma membrane; SytVII, synaptotagmin VII

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Neu1$^{-/-}$ mice develop a systemic disease that closely mimics the pathologic manifestations seen in patients with the early-onset forms of sialidosis, including growth retardation, splenomegaly, severe neurologic deterioration, and premature death [6]. Spleen enlargement in these mice coincides with the time-dependent occurrence of extramedullary hematopoiesis. Our previous studies of the molecular bases of this phenotype identified Neu1 as a negative regulator of lysosomal exocytosis [7]. The latter is a Ca^{2+}-dependent process that entails the recruitment/docking of lysosomes to the plasma membrane (PM), the fusion of the lysosomal membrane with the PM, and the release of lysosomal contents into the extracellular matrix (ECM) [8]. We found that the lysosomal-associated membrane protein-1 (Lamp-1), which has been implicated in the recruitment/docking of lysosomes to the PM, is a substrate of Neu1. In the absence of Neu1, oversialylated Lamp-1 has a longer half-life, and this increases the number of lysosomes poised to dock at the PM and to engage in lysosomal exocytosis [7]. The downstream effect is the enhanced release of catalytically active proteases and glycosidases extracellularly, a phenomenon that affects the processing of cell surface proteins and ECM components [7]. In the fibroblasts of patients with sialidosis who have different clinical phenotypes, we demonstrated an inverse correlation between the levels of the residual NEU1 activity and the increase in lysosomal exocytosis. Given the ubiquitous tissue distribution of this lysosomal enzyme, it is conceivable that the process of lysosomal exocytosis in absence of Neu1 activity is deregulated in other tissues and underlie many of the pathologic manifestations characteristic of this disease, albeit the downstream effects of this process may be cell type-specific.

In addition to their role in the intralysosomal catabolism of sialylated macromolecules, NEU1 and PPCA are found at the cell surface together with the elastin-binding protein as part of the elastin receptor protein complex [9,10]. It has been postulated that within this complex NEU1 participates in the regulation and assembly of the elastic fibers [9,11]. Recently, Starcher et al. [12] demonstrated that elastic fiber deposition is altered in the aorta and lung of *Neu1*-null mice and that the impaired extracellular assembly of the elastic fibers causes this effect. In skeletal muscle, the interplay between the ECM and individual muscle fibers is crucial for the normal function and integrity of this tissue. In addition to providing mechanical support, the ECM and, in particular, the basement membrane/basal lamina function as a scaffold during muscle regeneration. Components of these structures and the muscle fibers, such as collagens, laminins, integrins, and dystroglycans, represent bona fide signaling molecules that play key roles in muscle development, maintenance, and regeneration. Disturbing any of these components may adversely affect muscle strength and integrity and result in muscle disease [13–15]. The concept that NEU1 may directly or indirectly influence the behavior of extracellular components by altering the sialic acid content of intracellular and extracellular substrates could be a common determinant of many of the systemic abnormalities characteristic of sialidosis.

Here we show that in *Neu1*$^{-/-}$ mice, muscle fibers undergo progressive disruption associated with profound alterations of ECM components and infiltration by connective tissue. Many of these abnormalities can be attributed to excessive expansion of perimysial and endomysial connective tissue, coupled to increased proliferation of fibroblast-like cells, abnormal deposition of collagen fibers, and enhanced proteolytic activity in the ECM. These findings explain, in part, the neuromuscular signs and symptoms described in patients with sialidosis and indicate a primary role of NEU1 in the pathogenesis of muscle diseases.

2. Materials and methods

2.1. Animals

Neu1$^{-/-}$ and wild-type (WT) mice (FVB/NJ background), aged 15 days to 7 months, were included in this study. The *Neu1*$^{-/-}$ mice were previously generated in our laboratory by targeted disruption in the *Neu1* locus [6]. Animals were housed in a fully AAALAC-accredited animal facility with controlled temperature (22 °C), humidity, and lighting (alternating 12-hour light–dark cycles). Food and water were provided *ad libitum*. All procedures in mice were performed according to animal protocols approved by our Institutional Animal Care and Use Committee and NIH guidelines.

2.2. Histology

The gastrocnemius, tibialis anterior, quadriceps, and diaphragm were excised for analysis. The gastrocnemius muscles were weighed for comparison between *Neu1*$^{-/-}$ [1–2 months ($n=6$), 3–4 months ($n=5$), and 5–6 months ($n=5$)] and WT [1–2 months ($n=8$), 3–4 months ($n=4$), and 5–6 months ($n=5$)] mice. The muscle tissues were dissected, frozen immediately in isopentane cooled in liquid nitrogen, and stored at −80 °C until processed for histologic analysis. Fragments of muscle tissues were also fixed in 4% paraformaldehyde (PFA) for immunohistochemical analysis and 4% glutaraldehyde for electron microscopy. Longitudinal or transverse sections (8 μm) of the frozen muscles were cut on a cryostat (Leica CM3050). The sections were stained with hematoxylin and eosin (H&E) for overall morphologic assessment. Cross-sectional areas of gastrocnemius muscles from three *Neu1*$^{-/-}$ mice and three matching controls (5–6 months) stained with H&E were measured by tracing each individual myofiber by using the ImageJ software (NIH Software). At least 200 fibers were measured per animal in three different fields of gastrocnemius muscles.

2.3. Immunohistochemistry

The indirect immunofluorescence method was done for the analyses of the following proteins: transcription factor 4 (TCF4; rabbit monoclonal, Cell Signalling #2565, 1:100), dystrophin (mouse monoclonal, Sigma D8168, 1:400), β-dystroglycan (mouse monoclonal, Abcam ab49515, 1:200), and laminin (rabbit polyclonal, Sigma L9393, 1:200). Frozen sections were blocked with blocking solution containing 2% BSA and 10% normal serum in PBS for 30 min. Sections used for monoclonal antibodies were incubated with AffiniPure Fab Fragment Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch) in PBS for 1 hour. Sections were incubated with primary antibodies diluted in blocking solution for at least 1 hour at room temperature or overnight at 4 °C, washed three times for 10 min with PBS, and blocked with blocking solution for 10 min. The sections were incubated with Alexa-488 or Cy3 (anti-mouse or anti-rabbit), with or without 488-phalloidin for 40 min, washed in PBS (five times for 5 min), and mounted with Vectashield mounting medium/DAPI (Vector, H1000). As an internal control, samples were incubated with only the secondary antibody. The slides were examined under a fluorescent microscope (Olympus BX50, NY, USA) or confocal microscope (Zeiss LSM510 Meta, NY, USA, or Nikon C1Si, NY, USA).

The immunoperoxidase procedure was done to analyze the levels of collagen IV (rabbit polyclonal, Abcam ab13966, 1:400), collagen VI (rabbit polyclonal, Santa Cruz sc-20649, 1:100), reticulin (rat monoclonal, Acris BM4018, 1:100), tenascin (rabbit polyclonal, Chemicon International AB19013, 1:500), cathepsin B (rabbit polyclonal, Upstate #06-480, 1:100), Lamp-1 (rat polyclonal, BD Pharmingen #553793, 1:100), Lamp-2 (rabbit polyclonal, Zymed #51-2200, 1:200), pax7 (mouse monoclonal, Developmental Studies Hybridoma Bank), and proliferating cell nuclear antigen (PCNA; rabbit polyclonal, Abcam ab15497, 1:200). Slides were washed in PBS and incubated for 30 min with blocking solution (1% BSA, 0.5% Tween-20, and 10% normal goat serum in PBS). Subsequently, the primary antibody was diluted in blocking solution, and slides were incubated overnight at 4 °C. The sections were then rinsed in PBS, incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies for 60 min, rinsed in PBS,