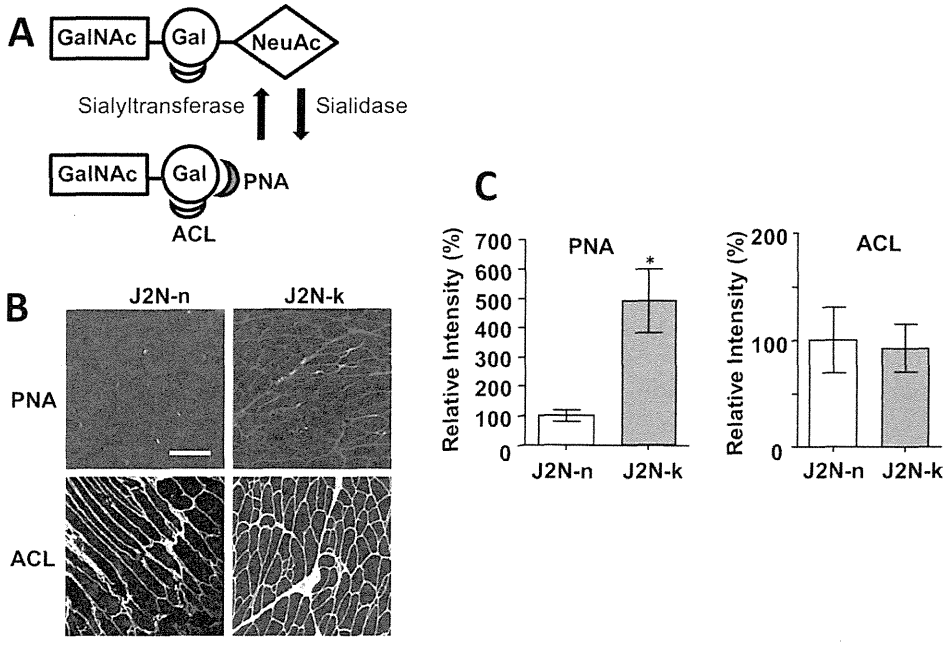

FIGURE 1. Increased PNA staining of J2N-k hamster muscles. (**A**) Schematic drawing representing the sialic acid–dependent or sialic acid–independent interaction of 2 types of lectins. Whereas ACL recognizes sialylated and nonsialylated sugars, PNA recognizes only nonsialylated sugars. NeuAc, neuraminic acid; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine. (**B**) Typical staining images with PNA or ACL. Frozen sections of quadriceps femoris muscle from dystrophic (J2N-k) and control hamsters (J2N-n) were stained with biotinylated PNA or ACL followed by FITC-avidin D. Scale bars: 50 μm. (**C**) Immunofluorescence images of myofiber cross-sections stained with lectins were analyzed by imaging software. Fluorescence intensity of each lectin staining was measured from 3 or 4 cross-sectional views of myofibers from 3 or 4 animals per group. Data were normalized with fluorescence in the J2N-n hamsters for each lectin. The data are presented as mean ± SD (n = 30–50 fibers/group). $^*P < 0.05$.

sarcolemmas from the J2N-k hamsters were more extensively stained with PNA compared with those from the J2N-n hamsters (Fig. 1B); the intensity of staining with PNA was approximately fivefold greater in the former than in the latter (Fig. 1C), indicating that the sarcolemmas from the J2N-k hamsters contained higher levels of nonsialylated sugars.

Stronger staining with PNA was also observed in muscle sections of *mdx* mice (Fig. 2A), with a PNA staining intensity that was approximately sixfold greater than that in the WT mice (Fig. 2B). Furthermore, when this staining method was applied to *dy/dy* mice, considerable staining with PNA was observed in the muscle sections (Fig. 2C); the staining intensity with PNA was approximately fourfold greater in *dy/dy* than in WT mice (Fig. 2D).

An important question is whether increased PNA staining occurs in necrotic fibers alone or throughout dystrophic muscles, which include normal and regenerating fibers. Intracellular IgG in the necrotic fibers was stained with anti-IgG antibody, as described previously,[8] whereas regenerating fibers with central nuclei were stained with DAPI. Intense PNA staining occurred everywhere in the *mdx* muscles (Fig. 3), including IgG-positive necrotic fibers, regenerating fibers with central nuclei, and normal fibers without central nuclei (see merged image in Fig. 3).

We applied the aforementioned staining procedure to the muscle biopsy samples from patients with muscular dystrophy (Fig. 4A and B). Whereas faint staining with PNA was observed in normal control muscles, muscle samples from patients with muscular dystrophy (Becker and/or limb-girdle muscular dystrophy) were strongly stained with PNA (Fig. 4A); the staining intensity with PNA was more than fourfold greater in muscles from patients with muscular dystrophy than in normal muscles (Fig. 4B). These data suggest that, similar to dystrophic animal models, muscle samples from patients with muscular dystrophy possess markedly reduced levels of sialic acids.

Muscular dystrophy results in sarcolemmal damage, which accelerates the release of cytosolic enzymes into the extracellular space. Therefore, it is probable that a sufficient amount of sialidase is released from injured muscles to remove sialic acids from the sarcolemma. To measure the catalytic action of sialidase, frozen sections of skeletal muscles from healthy mice were treated with externally added sialidase and subsequently stained with PNA. Sialidase treatment resulted in extensive staining of the muscle surface with PNA (Fig. 5A)

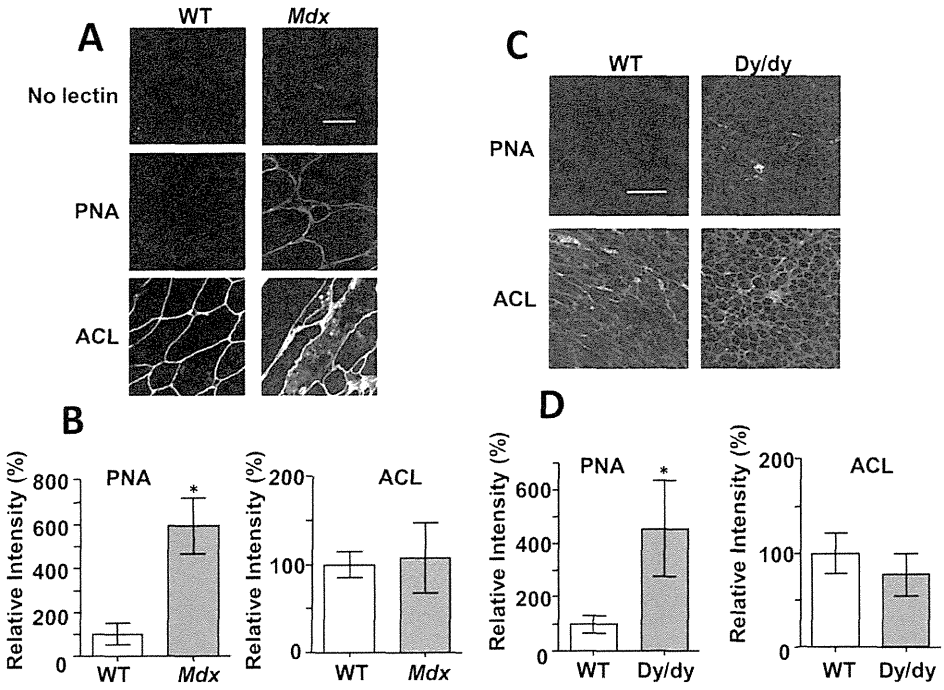

FIGURE 2. Increased PNA staining in the muscles of *mdx* and *dy/dy* mice. (**A**) Frozen sections of quadriceps femoris muscle from *mdx* (*Mdx*) and control mice (WT) were stained with biotinylated PNA or ACL followed by FITC-avidin D. Scale bars: 50 μm. (**B**) Immunofluorescence images of myofiber cross-sections stained with lectins were analyzed by imaging software, and fluorescence intensity was normalized to that of healthy mice. The data are presented as mean ± SD (n = 20–50 fibers/group); $^*P < 0.05$. (**C**) Frozen sections of quadriceps femoris muscle from *dy/dy* (*Dy/dy*) and control mice (WT) stained with biotinylated PNA or ACL, followed by FITC-avidin D. Scale bars: 50 μm. (**D**) Immunofluorescence images of myofiber cross-sections stained with lectins were analyzed by imaging software. Fluorescence intensity of each lectin staining was measured from 3 or 4 cross-sectional views of myofibers from 3 or 4 animals per group and normalized to that of healthy mice. The data are presented as mean ± SD (n = 40–50 fibers/group). $^*P < 0.05$.

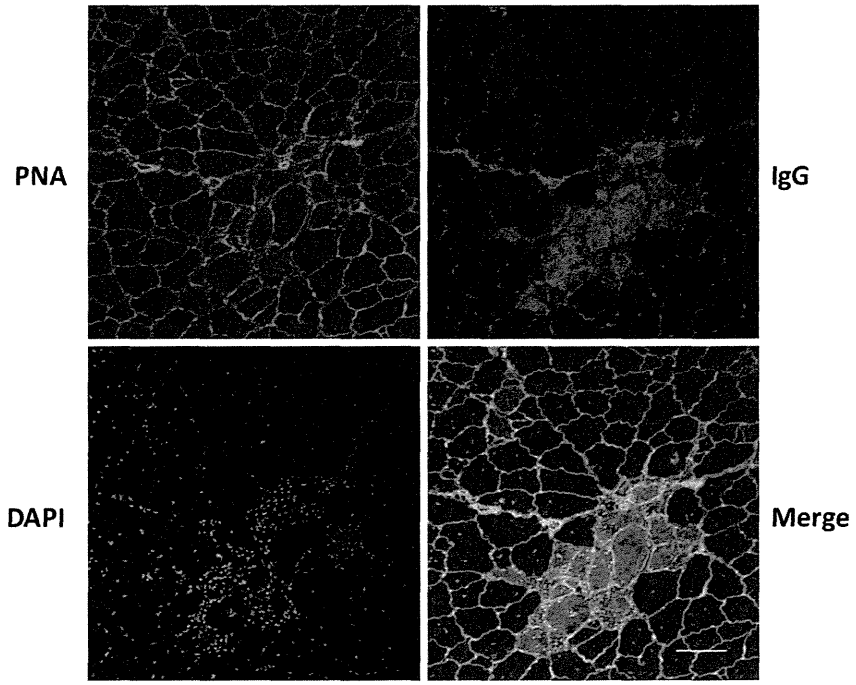

FIGURE 3. PNA staining is observed in all states of muscles, including necrotic fibers. Frozen sections of quadriceps femoris muscle from *mdx* mice were stained with FITC-PNA for sialic acids, DAPI for nuclei, and anti-mouse IgG for necrotic fibers. Scale bars: 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

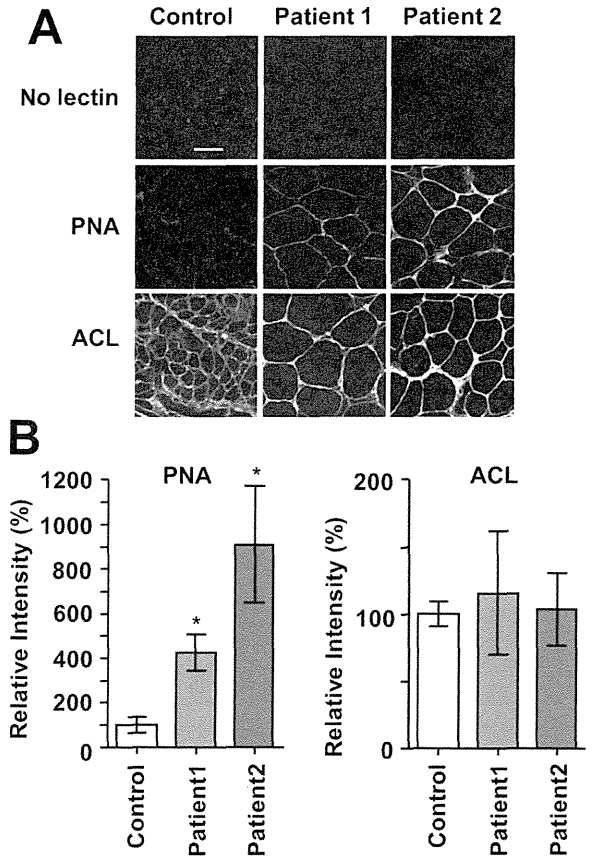

FIGURE 4. Increased PNA staining in the muscles of patients with muscular dystrophy. (**A**) Frozen sections of skeletal muscle from dystrophic patients and non-dystrophic controls stained with biotinylated PNA or ACL lectin or without any lectin, followed by FITC-avidin D. Scale bars: 50 μm. Patient 1: Becker muscular dystrophy; patient 2: limb-girdle muscular dystrophy. (**B**) Immunofluorescence images of myofiber cross-sections stained with lectins were analyzed by imaging software and normalized to that of control muscles. The data are presented as mean ± SD (n = 10–20 fibers/group). *$P < 0.05$.

that was detected even upon treatment with a very low concentration of sialidase (50 mU; Fig. 5B).

DISCUSSION

In this study, we demonstrated that the cell surface content of sialic acids was markedly reduced (by 60–80%) in skeletal muscles from dystrophic model animals—J2N-k hamsters, *mdx* mice, and *dy/dy* mice—and from patients with muscular dystrophy. By contrast, the amount of Galβ1,3GalNAc stained with ACL was not altered in dystrophic muscles. Glycosylation status has a critical pathologic significance in the genesis of dystrophy. For example, several types of dystrophic muscles show aberrant glycosylation of α-DG, which is a key molecule linking the sarcolemma to the extracellular matrix.[23] Therefore, the reduced sialic acid levels observed in this study may contribute to the severity of muscle damage in various subtypes of muscular dystrophy. A recent study reported that transgenic overexpression of a specific type of sialyltransferase (ST3Gal-II), which could lead to extensive sialylation, resulted in development of dilated cardiomyopathy.[22] However, surface sialic acid content in skeletal muscles was unexpectedly reduced because of muscle damage.[22] The pathological importance of sialic acids was suggested in distal myopathy with rimmed vacuoles,[18,20] and treatment with sialic acid metabolites prevented development of the myopathic phenotype in a mouse model.[24] Recently, sialic acids attached to ion channels through *O*-linkages were reported to modulate the gating of potassium channels[25] or the Na^+ channel NaV1.4,[26] thereby causing significant shifts in their voltage dependences. Thus, reduced sialylation in ion channel proteins may contribute to reduced muscle performance via altered action potentials in muscular dystrophy.

In a model of limb-girdle muscular dystrophy, premature senescence of satellite cells was

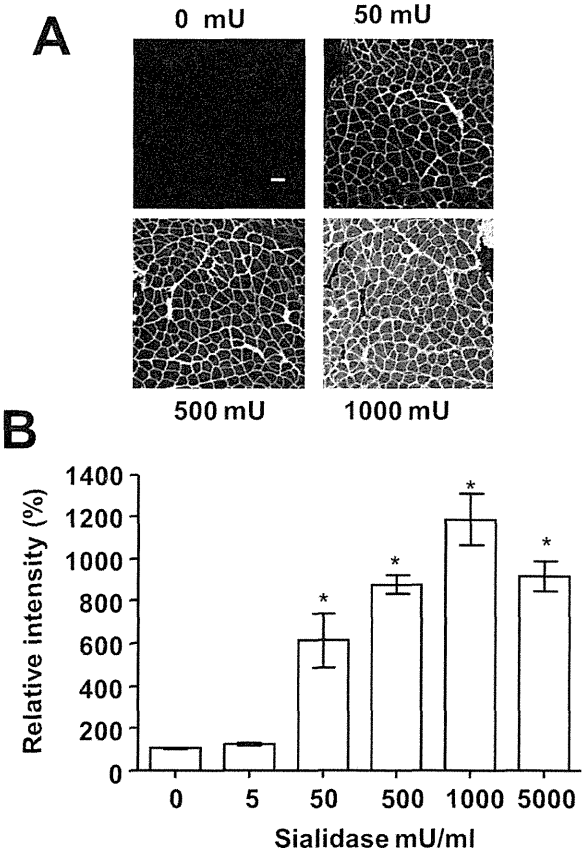

FIGURE 5. Sialidase effectively removes sialic acids and thus increases PNA staining in normal muscles. (**A**) Frozen sections of skeletal muscle from control mice were treated at 37°C for 2 h with various concentrations of sialidase and then stained with biotinylated PNA lectin, followed by FITC-avidin D. Scale bars: 50 μm. (**B**) Immunofluorescence images of myofiber cross-sections stained with PNA were analyzed by imaging software and normalized to cross-sections without sialidase treatment. The data are presented as mean ± SD (n = 50 fibers). *$P < 0.05$.

suggested to be involved in the genesis of muscular dystrophy.[27] Therefore, the aging phenomenon could be related to degeneration/regeneration cycles occurring in several myopathies. Indeed, sialic acid content has also been reported to be reduced during aging in the brain[28] and erythrocytes.[29] However, we did not observe significant differences in sialic acid content between normal skeletal muscles from 4-week-old and 6-month-old mice, and loss of sialic acid from membranes was observed whenever muscle fibers were damaged, so the reduction of sialic acid in dystrophic muscle is not likely to be due to muscle aging.

We observed that a low concentration (0.05 U/ml) of exogenously added sialidase is sufficient to remove sialic acid moieties from muscle sections of healthy mice (Fig. 5). Furthermore, PNA staining was observed in all types of muscle fibers, including necrotic, regenerating, and normally growing fibers (Fig. 3). In addition, we easily detected enhanced PNA staining in mechanically and chemically injured muscles, but not in contralateral muscles separated from injured muscles (Y. Iwata, unpublished data). Therefore, it is likely that a trace amount of sialidase is released from damaged muscle fibers, thereby cleaving surface sialic acids on and near their fibers. In contrast to low-dose sialidase (0.05 U/ml), which is sufficient for detecting changes in surface sialic acid content, we typically detect nearly 100-fold enzyme units of released CK (approximately 5 U/ml) in the serum of *mdx* mice or J2N-k hamsters.[30] Reductions in sialic acid content were also observed in the *dy/dy* mice, where CK release was not detected, despite the evident symptoms of muscle damage. Therefore, whereas surface sialic acids reduction is easily detected *in vitro*, even when only a small amount of sialidase is released from damaged muscle fibers, CK is reliably detected only when a relatively large quantity of CK is released from muscle fibers.

In addition to the accelerated release of sialidase, reduced biosynthesis of sialic acids and inhibition of sialyltransferase activity may also contribute to reduced levels of surface sialic acids. It has been reported that sialic acid concentrations *in vivo* were normal in erythrocyte membranes of patients with limb-girdle muscular dystrophy,[31] suggesting that the biosynthesis pathway of sialic acids and sialyltransferase activity were not dramatically affected by this type of muscular dystrophy. However, another study reported decreased sialyltransferase activity in the hearts of cardiomyopathic hamsters.[32]

In this study, we did not analyze in detail whether the sarcolemmal sialic acid contents are changed in heart muscles with cardiomyopathy or heart failure. However, our preliminary experiments have suggested that, similar to skeletal muscle fibers, cardiac muscles from symptomatic 11-week-old J2N-k hamsters also showed increased PNA staining compared with their healthy counterparts (Y. Iwata et al., unpublished data). Thus, sialic acid reduction may also be a marker of cell damage in cardiac and skeletal muscles, although further studies are required for generalizing the hypothesis of reduced sialic acid content to other animal models or patients with heart failure. There is a long-lasting debate concerning the involvement of stem cells in heart regeneration.[33] Given that such regenerating cells are expected to preserve surface sialic acids, positive staining of sialic acids may be useful as a method for identification of such cells.

In conclusion, we have observed that levels of sarcolemmal sialic acids were markedly reduced in dystrophic muscles, presumably via the release of cytosolic sialidase from damaged muscle fibers. Surface sialic acids are useful markers and novel therapeutic targets for damaged muscle. This highly sensitive and potentially versatile procedure may be useful for diagnostic purposes in humans, as well as for the detection of muscle damage in animal models.

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