MUSCULOSKELETAL PATHOLOGY

Glucocorticoid Steroid and Alendronate Treatment Alleviates Dystrophic Phenotype with Enhanced Functional Glycosylation of α -Dystroglycan in Mouse Model of Limb-Girdle Muscular Dystrophy with *FKRPP448L* Mutation

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Fukutin-related protein-muscular dystrophy is characterized by defects in glycosylation of α -dystroglycan with variable clinical phenotypes, most commonly as limb-girdle muscular dystrophy 2I. There is no effective therapy available. Glucocorticoid steroids have become the standard treatment for Duchenne and other muscular dystrophies with serious adverse effects, including excessive weight gain, immune suppression, and bone loss. Bisphosphonates have been used to treat Duchenne muscular dystrophy for prevention of osteoporosis. Herein, we evaluated prednisolone and alendronate for their therapeutic potential in the *FKRPP448L*-mutant mouse representing moderate limb-girdle muscular dystrophy 2I. Mice were treated with prednisolone, alendronate, and both in combination for up to 6 months. Prednisolone improved muscle pathology with significant reduction in muscle degeneration, but had no effect on serum creatine kinase levels and muscle strength. Alendronate treatment did not ameliorate muscle degeneration, but demonstrated a limited enhancement on muscle function test. Combined treatment of prednisolone and alendronate provided best improvement in muscle pathology with normalized fiber size distribution and significantly reduced serum creatine kinase levels, but had limited effect on muscle force generation. The use of alendronate significantly mitigated the bone loss. Prednisolone alone and in combination with alendronate enhance functionally glycosylated α -dystroglycan. These results, for the first time, demonstrate the efficacy and feasibility of this alliance treatment of the two drugs for fukutin-related protein-muscular dystrophy. (*Am J Pathol* 2016, 186: 1635–1648; <http://dx.doi.org/10.1016/j.ajpath.2016.02.015>)

Muscular dystrophies are a group of inherited diseases resulting from gene mutations, with progressive weakness and degeneration of skeletal and cardiac muscles. The diseases can also affect the gastrointestinal system, endocrine glands, bone, eyes, brain, and other organs, with considerable variation in the age of onset, severity, and pattern of affected muscles. Muscular dystrophies classically fall into nine major subgroups depending on the types of mutations, severity, patterns of manifestation, and shared phenotypes of the diseases. They include Duchenne (DMD), Becker, myotonic, congenital, Emery-Dreifuss, facioscapulohumeral, limb-girdle, distal, and

oculopharyngeal muscular dystrophy.¹ One specific group of muscular dystrophies known as dystroglycanopathy shares a common and secondary biochemical defect in glycosylation of α -dystroglycan (α -DG). The diseases are characterized by lack or reduced levels of glycosylated α -DG (hypoglycosylation) in body-wide tissues, especially in muscles. Mutations in a growing number of genes, either

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confirmed or putative glycosyltransferases, have now been identified as the causes of the biochemical defect. The genes involved include fukutin-related protein (*FKRP*), fukutin (*FKTN*), like-acetylglucosaminyl transferase (*LARGE*), protein-O-mannosyltransferase 1 (*POMT1*), protein-O-mannosyl transferase 2 (*POMT2*), protein-O-mannose-1,2-N-acetylglucosaminyltransferase 1 (*POMGNT1*), dolichyl-phosphate mannosyltransferase polypeptide 3 (*DPM3*), and isoprenoid synthase domain containing (*ISPD*).^{2–9} The most common form of dystroglycanopathies is caused by mutations in the *FKRP* gene, which are associated with a wide spectrum of disease severity from a milder form of limb-girdle muscular dystrophy to severe Walker-Warburg syndrome, muscle-eye-brain disease, and congenital muscular dystrophy type 1D.^{10–12} Structure analysis suggests that *FKRP* is a putative glycosyltransferase.¹³ However, no specific enzyme activity has been assigned to *FKRP* in the glycosylation pathway of the α -DG, and it remains to be investigated as to how mutations of the gene affect glycosylation of α -DG with such a great variability both in the levels of glycosylated α -DG and disease progression. Studies of *FKRP*-related muscular dystrophies during the past 15 years have been largely limited to the genetic diagnosis, genotype-phenotype correlation, and disease progression. Efforts have also been made to understand the protein expression and localization as well as mechanisms of *FKRP* in the pathway of glycosylation of α -DG.^{13–15} However, little progress has been made for the treatment of the diseases. There is no effective therapy available, and only physical therapy and palliative care are being routinely provided. Translational study aiming to develop experimental therapy to the most common limb-girdle muscular dystrophy and other dystroglycanopathies lags behind other types of muscular dystrophies. No clinical trial specifically for the diseases has been conducted.

Glucocorticoid steroids (steroids) have been available for treating muscular dystrophies for several decades and proved to be clinically beneficial for DMD, with increased life expectancy and improved muscle strength and function.¹⁶ In 1974, Drachman et al¹⁷ were the first reporting the use of steroids to treat DMD boys with positive outcome. In a randomized, double-blind, multicenter study in 1989, Mendell et al¹⁸ convincingly demonstrated improvement in muscle strength in a cohort of 103 DMD boys aged 5 to 15 years after 3 months of steroid treatment. Since then, steroids have become the standard treatment for DMD and been prescribed for other muscular dystrophies. Therapeutic consequence is believed to be achieved mainly through its anti-inflammatory effects with the inhibition of NF- κ B signaling, a mechanism called transrepression.^{19,20} However, benefits of steroids often last only a limited time period and are always associated with adverse effects, including dramatic weight gain and reduction in bone mineral density (osteoporosis) and growth retardation. This has led to the application of bisphosphonates, which have been widely used to treat postmenopausal osteoporosis, in conjunction with steroids to prevent bone loss for treating

muscular dystrophies. Bisphosphonates, analogs of endogenous pyrophosphates, have a high affinity for the hydroxyapatite of bone and prevent bone loss through inhibiting recruitment and promoting apoptosis of osteoclast to suppress bone reabsorption. One group of bisphosphonates, aminobisphosphonates, such as alendronate, are the first-line bisphosphonate drugs for osteoporosis and also affect calcium metabolism by lowering extracellular calcium levels.^{21,22} However, there have been no preclinical and clinical studies of steroids and bisphosphonates for *FKRP*-related MDs, except a case report with two patients treated with prednisolone.²³

We have recently generated several unique *FKRP* mutant mouse models containing L276I, P448L, and E310 mutations representing those observed in patients.²⁴ These *FKRP* mouse models recapitulate the extremely wide range of phenotypes seen in human *FKRP*-related diseases. One of the mouse models, *FKRPP448L*-homozygotes, exhibits a typical muscular dystrophy phenotype as seen in most of limb-girdle muscular dystrophy 2I, with dramatic reduction of functional glycosylation of α -DG in muscles and mild cardiac muscle pathology without obvious involvement in the central nervous system. Skeletal muscles of the mouse undergo progressive degeneration, especially in the diaphragm, although the life span of the mice can be beyond 16 months. In the current study, we used the *FKRPP448L*-mice to examine steroid and bisphosphonate separately and in combination for their efficacy in improving disease phenotype and muscle functions. The effect of the treatments on functional glycosylation of α -DG was also evaluated. Our results show that treatment with prednisone alone improves pathology, but is associated with severe immune suppression and unable to improve muscle function. A combined treatment with prednisolone and alendronate provides better improvement in muscle pathology, lowers serum creatine kinase (CK) levels, and enhances muscle function. Interestingly, prednisolone treatment leads to enhanced expression of functional α -DG (F- α -DG). The data indicate the higher potential of a combined use of the two classes of existing drugs for treatment of *FKRP*-related diseases.

Materials and Methods

Animals, Drugs, and *in Vivo* Delivery Methods

In each treatment group, 10 *FKRPP448L*- (*P448L*-) or *C57BL/6* mice, aged 4 to 5 weeks, were used. *P448L*- mice have a 1343C>T point mutation of the *FKRP* gene, resulting in an amino acid change from proline to leucine at position 448.²⁵ *P448L*- mice were orally administrated with 2 or 5 mg/kg prednisolone (Pharmaceutical Associates, Inc., Greenville, SC) or/and 0.1, 0.5, or 1 mg/kg alendronate (Northstar Rx LLC, Memphis, TN) daily or twice a week for 3 or 6 months, and control *P448L*- mice were gavaged with the same amount of saline only. Mice were sacrificed at scheduled time points, and muscles and organs were snap frozen in liquid nitrogen-cooled isopentane and stored at -80°C .

Experiments were approved by the Institutional Animal Care and Use Committee, Carolinas Medical Center (Charlotte, NC).

Histological Analysis and Fiber Size Determination

Sections (6 μ m thick) were cut from at least two thirds of the muscle length of tibialis anterior, quadriceps, biceps, and gastrocnemius muscles at 100- μ m spacing and from at least six levels from all other muscles (including cardiac, diaphragm, intercostal, and abdominal muscle) at 100- μ m spacing. The intervening muscle sections were collected for Western blot analysis. For the detection of F- α -DG, frozen cryosections were initially fixed in a cold (-20°C) ethanol/acetic acid (1:1) mixture for 1 minute and then immediately washed with phosphate-buffered saline four times, each for 15 minutes. Sections were blocked with 8% bovine serum albumin in phosphate-buffered saline for 30 minutes, and then incubated with monoclonal I1H6C4 (1:200) antibody against functionally glycosylated α -DG (Millipore, Temecula, CA). Rabbit polyclonal antibody P7 and monoclonal antibody to α -sarcoglycan were used. The primary antibody was detected by Alexa Fluor 594–labeled goat–anti-mouse IgM, goat–anti-mouse Igs, or goat–anti-rabbit Igs (Invitrogen, Eugene, OR). For detecting degenerating fibers, sections were directly incubated with goat–anti-mouse IgM Alexa Fluor 594 after blocking. Sections were also stained with hematoxylin and eosin and Masson's trichrome staining for histological assessment. Images were visualized using an Olympus BX51/BX52 fluorescence microscope (Opelco, Dulles, VA) and captured using the Olympus DP70 Digital Camera System (Opelco). The percentage of I1H6C4-positive fibers was determined from tibialis anterior muscles. Fiber size of tibialis anterior was determined using the MetaMorph Basic Offline software version 7.7 (Molecular Devices LLC, Sunnyvale, CA).

Protein Extraction and Western Blot Analysis

Total proteins were extracted from tibialis anterior muscles and cultured cells using TX-100 buffer (1% Triton X-100, 50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were homogenized in TX-100 buffer, and the supernatants were collected by centrifugation at $16,000 \times g$ for 10 minutes. Protein concentration was determined by modified Lowry assay (Bio-Rad DC protein assay; Bio-Rad, Hercules, CA). The lysates were then loaded onto 4% to 20% Tris-glycine gel (Invitrogen, Carlsbad, CA). The proteins were transferred to polyvinylidene difluoride membranes with constant ampere at 200 mA for 2 hours in a cold room (4°C). Polyvinylidene difluoride specimens were incubated with protein-free T20 blocking buffer (Pierce, Rockford, IL). The antibodies against α -DG (I1H6C4) and α -actin (Sigma, St. Louis, MO) were incubated in 20 mmol/L Tris, pH 7.4,

150 mmol/L NaCl, and 0.1% Tween 20 at 1:2000 dilutions. α -DG and α -actin antibodies were detected by horseradish peroxidase–goat anti-mouse IgM (Invitrogen) and goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad, Hercules, CA), respectively. Blots were developed with electrochemiluminescence (PerkinElmer, Waltham, MA), and the images were exposed and processed by a LAS-4000 imaging system (Fujifilm, Valhalla, NY). The intensity of the bands obtained from the treated muscle was measured and compared with that from normal muscle of *C57BL/6* mice (ImageJ software version 1.42; NIH, Bethesda, MD).

Bone Density

Femurs and tibias of mice were digital X-rayed on the Portable Pixarray 100 X-ray machine (BioOptics Software, Tucson, AZ). Bone density was measured using ImageJ software version 1.42. The same bones from age-matched normal *C57BL/6* mice were used as controls.

Grip Strength Test

Grip strength was assessed using a grip strength meter consisting of horizontal forelimb mesh and an angled hind limb mesh (Columbus Instruments, Columbus, OH). Five successful forelimb and hind limb strength measurements within 2 minutes were recorded, and data were normalized to body weight, then divided by those from similar ages of control saline-treated mice.

Echocardiogram

Animals anesthetized with 1% to 4% isoflurane were placed on a mouse monitor pad with nose cone supplying 1% to 2% isoflurane and oxygen. Electrode gel was applied to the paws that were taped down over the electrocardiogram pads on the monitor platform. Hair was removed from the chest of the mouse, and ultrasound gel was added. Data were gathered using the Bioscan SonixTablet Ultrasound System (Analogic Ultrasound, Peabody, MA). The heart was imaged under M and B mode, with the B mode placement imaging the heart at the level of the aortic sphincter. Ejection fraction was calculated using the equation: Ejection Fraction (%) = $[(\text{EDV} - \text{ESV})/\text{EDV}] \times 100$, where ESV indicates end-systolic volume and EDV indicates end-diastolic volume. Stroke volume was calculated as follows: Stroke Volume = EDV – ESV. Cardiac output was calculated as follows: Cardiac Output = Stroke Volume $\times$ Heart Rate.

Measurement of Serum CK and Other Components

Mouse blood was taken immediately after cervical dislocation and centrifuged at $1500 \times g$ for 15 minutes. Serum was separated and stored at -80°C . The level of serum

components was determined by Charles Riverside Laboratories International (Wilmington, MA).

Statistical Analysis

All of the results were expressed as means $\pm$ SEM, and the data were analyzed using a two-tailed *t*-test. $P < 0.05$ was considered significant.

Results

Daily and Twice a Week Administration of 5 mg/kg Prednisolone Improves Muscle Histology and Enhances Glycosylation of α -Dystroglycan

To evaluate the potential therapeutic effect, we first tested prednisolone in the *P448L*- mutant mice with a regimen of daily and twice a week oral administration of 5 mg/kg dosage with 10 mice for each cohort for 3 months. Saline-treated mice were used as controls. Body weight and illness were recorded daily. During the treatments, no death occurred

and weight changes of the treated mice were similar to the saline-treated group. Grip force measurement showed that both 5 mg/kg treatments increased the muscle strength compared with the saline-treated group during the first 2 months (but without statistical significance). However, no difference was observed by the third months (Figure 1A). Serum test at the end of the treatment showed no significant difference in levels of CK among all groups (Figure 1B). No differences were observed in gross appearance and histology of the liver and kidney between the treated mice and the control group. However, one most noticeable change was the reduction in spleen size, only 2.5 mg/g body weight in the treated mice compared with approximately 3.5 mg/g body weight in both wild-type and saline-treated mutant mice. Histologically, there was a significant decrease in size of lymphoid follicles with nearly a complete loss of germinal centers compared with the saline group (Figure 1, C and D). This is consistent with an early report in *mdx* mice treated with prednisolone.²⁶

Treatment for 3 months with both daily and twice a week 5 mg/kg prednisolone showed a clear improvement in

Figure 1 Short-term effects of prednisolone treatment on muscle strength, serum markers, and spleen histology in *FKRP448L*- mice (*P448L*-). **A:** Grip strength measurement. **B:** Serum testing. Creatine kinase, creatinine, total bilirubin (Total Bili), calcium, alanine transaminase (ALT), alkaline phosphatase (ALP), urea nitrogen, and γ -glutamyltransferase (GGT). No significant reduction in creatine kinase levels and differences of other serum components are observed in all treatment groups. **C:** Histology (hematoxylin and eosin staining) of spleen. **D:** Weight of spleen. **E:** Size of lymphoid follicles in spleen. The 5 mg/kg daily and twice a week prednisolone significantly reduces spleen size and lymphoid follicles with nearly a complete loss of germinal centers compared with saline group. Included were normal C57 mice (C57), *P448L*- mice treated with saline only (saline), and 2 mg/kg daily, 5 mg/kg twice a week, and 5 mg/kg daily prednisolone-treated *P448L*- mice. $n = 10$ mice in each group. * $P < 0.05$ versus saline-treated mice (two-tailed *t*-test).

Figure 2 Effect of 3-month prednisolone treatments on muscle pathology of *P448L* mice. **A:** Hematoxylin and eosin staining of tibialis anterior (TA), quadriceps, gastrocnemius, and diaphragm from normal *C57* mice (*C57*), saline-treated *P448L* mice (saline), 2 mg/kg daily, 5 mg/kg twice a week, or 5 mg/kg daily prednisolone-treated *P448L* mice. **B:** Distribution of fiber sizes of TA muscles in diameter (x axis). The two 5 mg/kg treatment regimen significantly increases the number of small fibers (<40 μm in diameter), but with a significant reduction in the number of hypertrophic fibers (>80 μm in diameter). **C:** The number of infiltration sites (with >10 monocytes in one focal area) in TA muscles. **D:** Percentages of centranucleated muscle fibers in TA muscles. A significant decrease in percentage of centranucleated fibers is observed in the 5 mg/kg prednisolone-treated groups in comparison with saline controls. **E:** Detection of degenerating fibers (red stained) in TA muscles by immunostaining with Alexa 488-conjugated rabbit anti-mouse Igs. **F:** The number of degenerating fibers stained by Alexa 488-conjugated rabbit anti-mouse Igs in TA muscles. Prednisolone treatment significantly reduces the number of degenerating fibers. *n* = 10 mice in each group (**B**, **C**, **D**, and **F**). **P* < 0.05 versus saline-treated mice (two-tailed *t*-test).

muscle pathology with a significant reduction in degenerating fibers demonstrated by staining for cytoplasmic immunoglobulins (Figure 2). Clusters (more than five fibers) of degenerating fibers were rarely identified in the muscles of the treated mice, although isolated individual degenerating fiber remained in most muscles. This is in contrast with saline-treated muscles in which the number of degenerating fibers was significantly higher in nearly every skeletal muscle (Figure 2, A, E, and F). Treatment of 5 mg/kg prednisolone

also greatly reduced inflammation with almost elimination of focal accumulation of infiltrates (Figure 2C). Improved pathology was further supported by significant reduction in the number of hypertrophic fibers (>80 μm in diameter); thus, there was less variation in fiber size in the two 5 mg/kg treatment groups. However, the number of small fibers (<40 μm in diameter) increased significantly, almost doubled in the saline-treated groups (Figure 2B). Interestingly, the percentage of centranucleated fibers in the 5 mg/kg treated

groups decreased with significance in comparison with saline controls (Figure 2D). The only histological change observed in the cardiac muscles of the mutant mice was an increase in limited focal fibrosis, and there was no noticeable difference between the treated and control groups.

Daily 2 mg/kg prednisolone treatment for 3 months showed limited effect on muscle function and histology of *P448L*- mice when compared with the saline-treated controls (Figures 1A and 2A). No reduction in CK levels and no change in grip force generation were observed (Figure 1, A and B). Histologically, 2 mg/kg treatment did not significantly reduce the number and the cluster of degenerating fibers (Figure 2, A, E, and F). The number of hypertrophic fibers decreased considerably but remained closer to the saline controls and was higher than that in the 5 mg/kg treated groups (Figure 2, A and B). Although the overall size of spleen and its germinal center was smaller than those from the control group, the difference was not statistically significant (Figure 1, C–E). The considerable differences between 2 and 5 mg/kg treatment groups suggest that improvement in pathology and degree of immune suppression with prednisone treatment are dose dependent.

Interestingly, we detected an increase in number of fibers expressing F- α -DG in both 5 mg/kg prednisolone treated muscles (Figure 3). As reported previously,²⁴ most skeletal muscle fibers of the *P448L*- mutant mice lacked detectable F- α -DG. However, a small proportion of muscle fibers express detectable levels of F- α -DG in close association with regeneration indicated by their small size and centranucleation.²⁵ Prednisolone treatment clearly increased

the number of fibers expressing F- α -DG, from <6% in the saline control group to >20% in the 5 mg/kg treated groups. Treatment with 2 mg/kg only marginally increased F- α -DG–positive fibers (Figure 3). In contrast to the control group, most of the F- α -DG–positive fibers with moderate to strong signal in the treated muscles were near normal sizes (>40 μ m in diameter) and often in large clusters and lacking expression of embryonic myosin; thus, they were not newly regenerated fibers (Figure 3, A and B).²⁵ Weaker, yet higher than background levels of staining were also detected in large areas of the remaining fibers of all skeletal muscles, including diaphragm. Again, muscles after 2 mg/kg treatment did not show a similar degree of enhancement in expression of F- α -DG (Figure 3).

Alendronate Does Not Improve Muscle Pathology

P448L- mice treated with 0.1, 0.5, or 1 mg/kg of alendronate daily for 3 months did not show any change in behaviors and body weight, and no treatment-related death occurred. No significant difference in grip strength was demonstrated between alendronate-treated and control groups, although force production of skeletal muscle showed a trend of dose-dependent increase with alendronate compared with saline group (Supplemental Figure S1A). In contrast to prednisolone treatment, all three dose treatments did not significantly improve pathology of any skeletal muscle, and degeneration and infiltration remained similar to the control group (Supplemental Figure S1, D and E, and Supplemental Figure S2). This was reinforced by the pattern

Figure 3 Improvement of functionally glycosylated α -dystroglycan (F- α -DG) after 3-month prednisolone treatment in *P448L*- mice. **A:** Immunohistochemical staining of F- α -DG with monoclonal IIIH6C4 antibody. Blue nuclear staining with DAPI. **B:** The number of positive F- α -DG muscle fibers in tibialis anterior muscles. Both 5 mg/kg prednisolone increase the number of fibers expressing F- α -DG. Included were normal C57 mice (C57), saline-treated *P448L*- mice (saline), and 2 mg/kg daily, 5 mg/kg twice a week, and 5 mg/kg daily prednisolone-treated *P448L*- mice. $n = 10$ mice in each group. * $P < 0.05$ versus saline-treated mice (two-tailed t -test).

of fiber size distribution, which showed no statistical difference in small regenerating and large hypertrophic fibers between two lower-dose alendronate-treated groups and the saline control group. However, there is a significant increase in the number of small fibers with the 1 mg/kg treated group (Supplemental Figure S1C). Similarly, the number of degenerating and percentage of centranucleated fibers were the same in all groups (Supplemental Figure S1, D and F). Immunohistochemistry with IIH6 showed more fibers expressing F- α -DG in most skeletal muscles, but not as conspicuous as in those prednisolone-treated muscles (Supplemental Figure S3 and Figure 3). Liver and kidney were normal in histology, and the levels of serum enzymes indicating the organ's functions remained similar to the control groups (Supplemental Figure S1B). As expected, change in spleen size was not detected.

Alendronate Plus Prednisolone Improve Muscle Pathology and Function, and Enhance Glycosylated α -Dystroglycan

We next examined the effect of combined treatment of 5 mg/kg prednisolone with the three doses of alendronate through daily oral administrations for 3 months in comparison with prednisolone or alendronate alone. No death and change in behavior were observed with all of the treatments. However, the body weight of the mice with combined treatments was clearly lighter than those treated with prednisolone or alendronate alone. This was dose related because the mice with higher-dose alendronate showed slightly smaller size, which was closer to that of wild-type *C57* mice (but without statistical significance) (Supplemental Figure S4A). Muscle strength was only slightly increased in the two higher-dose groups, as indicated by the change in grip force measurement, although without statistical significance (Supplemental Figure S4B). However, the mean serum CK levels were clearly reduced in all three combine-treated groups in a dose-dependent manner, with significance for the highest-dose group (Figure 4). More important, the combined treatments significantly reduced monocyte infiltration and fiber degeneration with almost no necrotic fiber and focal infiltration in the leg muscles of high-dose alendronate-treated mice (Figure 4A and Supplemental Figure S4, C and D). This was associated with a clear reduction in fibrotic tissues and the percentage of centrally nucleated fibers (Figure 4A and Supplemental Figure S4E). The dose-dependent improvement in muscle pathology was supported by the distribution in fiber size toward normal *C57* control in the combined treatment groups, especially with higher doses of alendronate (Supplemental Figure S4F). This is in sharp contrast with either of the drugs alone, both increasing small regenerating fibers (Figure 2, A and B, Supplemental Figure S1C, and Supplemental Figure S2).

As expected, alendronate did not mitigate prednisolone-induced immunosuppression. The size of the spleen in all

three combined treatment groups was significantly smaller than the saline control and alendronate only treated groups. This was consistent with diminished germinal centers histologically. No change in gross appearance and histology of the liver and kidney was observed, and levels of serum markers representing the two organs remained similar in all groups (Figure 4B).

The combination treatments also increased F- α -DG-positive fibers in all skeletal muscles, reaching up to 35% in the mice treated with the highest dosage (1 mg/kg alendronate and 5 mg/kg prednisolone). Most of the IIH6-positive fibers were of normal size. Also, the IIH6 signals on these fibers were generally weaker than that observed in the newly regenerated fibers with small caliber (Figure 5).

Long-Term Combined Treatment of Alendronate and Prednisolone Improves Muscle Histology and Enhances Glycosylated α -Dystroglycan

To confirm the therapeutic benefit of the combined treatment, we further investigated the longer-term (6 months) effect with 1 mg/kg alendronate and 5 mg/kg prednisolone in the *P448L*-mutant mice in comparison to the treatments of prednisolone alone. Similar to the shorter-term treatments, the 6-month regimen did not affect general behavior of the mice in all groups. Pattern in body weight of the mice with the combined treatment grew slower than those treated with alendronate, prednisolone, and controls up to 4 months and then, gradually increased to the levels similar to the other three groups by the end of 6-month treatment (without statistical difference between groups) (Supplemental Figure S5A). Similar to 3-month treatment, both prednisolone and combined treatments only marginally increased skeletal muscle function during the first 3 to 4 months, as indicated by grip force measurement, and the difference was no longer observed by the end of 6-month treatments (Supplemental Figure S5B). Ultrasound scanning examination showed no significant difference in cardiac functions and structure parameters between the treated groups, although heart rate of the combined treatment group was lower than that of the saline group (Supplemental Table S1). Long-term combined treatment reduced serum CK levels significantly. In contrast, CK levels were persistently high in the prednisolone-treated group, despite significant reduction in monocyte infiltration and fiber degeneration (Figure 6, A and B). A higher number of small fibers and decreased proportion of hypertrophic fibers persisted compared with saline controls (Supplemental Figure S5C and Figure 6A). The combined treatment of alendronate and prednisolone significantly reduced monocyte infiltration and degenerating fibers (Supplemental Figure S5, D and E, and Figure 6A). Fiber size was also considerably normalized with only a few small newly regenerating and large hypertrophic fibers (Supplemental Figure S5C and Figure 6A), and the number of centranucleated fibers was the lowest within the groups (Supplemental Figure S5F). Consistently, Masson's trichrome staining demonstrated

Figure 4 Improvement in muscle pathology after 3-month alendronate (Alen) plus prednisolone (Pred) treatment in *P448L*- mice. **A:** Hematoxylin and eosin staining of skeletal and cardiac muscles. The combined treatments significantly reduce monocyte infiltration and fiber degeneration with almost no necrotic fiber and focal infiltration in the leg muscles of high-dose alendronate-treated mice. **B:** The levels of serum enzymes: creatine kinase, creatinine, total bilirubin (Total Bili), calcium, alanine transaminase (ALT), alkaline phosphatase (ALP), urea nitrogen, and γ -glutamyltransferase (GGT). Combined treatments dose-dependently reduce creatine kinase levels with significance for the highest dose group. Included were normal *C57* mice (*C57*), saline-treated *P448L*- mice (saline), and 0.1, 0.5, and 1 mg/kg alendronate plus 5 mg/kg prednisolone daily-treated *P448L*- mice. $n = 10$ mice in each group. $*P < 0.05$ versus saline-treated mice (two-tailed *t*-test).

reduced area of collagen accumulation in muscles from the prednisolone and combined treatment groups (Supplemental Figure S6).

Functionally glycosylated α -DG remained elevated after 6-month treatment in both prednisolone treatment groups, with a lower level in alendronate-treated group (Figure 7, A and B). The relative levels of F- α -DG were confirmed by Western blots (Figure 7C). The sizes of the IIH6-positive fibers remained consistent to those described in the muscles after 3-month treatment, with most of the fibers being normal size in prednisolone-treated groups. Expression of dystrophin and α -sarcoglycan was homogeneous and did not show difference between wild-type *C57* and

P448L- mice with or without treatments (Supplemental Figure S7). Immunosuppression indicated by small size of spleen and germinal centers remained evident in both prednisolone-treated groups (Supplemental Figure S5G). Histology of the liver and kidney and levels of serum enzyme remained indistinguishable among all groups (Supplemental Figure S5, G and H).

Effect of Alendronate and Prednisolone Treatment on Bone Density

Both alendronate and prednisolone have a profound effect on bone health. We, therefore, X-rayed all leg bones

Figure 5 Improvement of functionally glycosylated α -dystroglycan (F- α -DG) after 3-month combined treatments of alendronate and prednisolone in *P448L*- mice. **A:** Immunohistochemical staining of F- α -DG with monoclonal IIH6C4 antibody. Blue nuclear staining with DAPI. **B:** The number of positive F- α -DG muscle fibers in tibialis anterior muscle. All of three combined treatments significantly increase the number of fibers expressing F- α -DG. Included were normal *C57* mice (*C57*), saline-treated *P448L*- mice (saline), and 0.1, 0.5, and 1 mg/kg alendronate plus 5 mg/kg prednisolone daily-treated *P448L*- mice. $n = 10$ mice in each group. * $P < 0.05$ versus saline-treated mice (two-tailed t -test).

of 6-month treated mice. As expected, bone density of both femur and tibia of the dystrophic *P448L*- mutant mice was significantly lower than the age-matched normal *C57* mice. Prednisolone treatment further aggravated the bone loss with the bone density lower than those of saline-treated control *P448L*- mutants (Figure 8, A and B). Alendronate alone showed higher bone density of the *P448L*- mutants when compared with the saline-treated group, although the difference did not reach statistical significance. However, the use of alendronate alleviated severity of prednisolone-associated bone loss, with bone density of the combined treatment group higher than that of the prednisolone group, and no longer

showed significant difference to the saline control group (Figure 8, A and B).

Discussion

Benefits of corticosteroid to muscular dystrophies were initially demonstrated 40 years ago in DMD boys and later by randomized, double-blind, multicenter study.^{17,18} However, significant long-term adverse effects, specifically immune suppression and disturbance to metabolism, severely limit its appeal to many DMD patients and physicians. For the same reasons, use of steroid has not been

Figure 6 Improvement in muscle pathology in *P448L*- mice after 6-month alendronate plus prednisolone treatments. **A:** Hematoxylin and eosin staining of skeletal and cardiac muscles. Combined treatment significantly reduces monocyte infiltration and degenerating fibers. Fiber size is considerably normalized with only a few small newly regenerating and large hypertrophic fibers. **B:** The levels of serum enzymes: creatine kinase, creatinine, total bilirubin (Total Bili), calcium, alanine transaminase (ALT), alkaline phosphatase (ALP), urea nitrogen, and γ -glutamyltransferase (GGT). Serum creatine kinase levels are significantly reduced in combine-treated groups. Creatine kinase levels are persistently high in the prednisolone-treated group. Included were normal *C57* mice (*C57*), saline-treated *P448L*- mice (saline), 5 mg/kg prednisolone daily-treated *P448L*- mice (Pred), 1 mg/kg alendronate daily-treated *P448L*- mice (Alen), 1 mg/kg alendronate + 5 mg/kg prednisolone daily-treated *P448L*- mice (Alen + Pred). $n = 10$ mice in each group. * $P < 0.05$ versus saline-treated mice (two-tailed *t*-test).

generally considered as an effective treatment for many other types of muscular dystrophies. Currently, another reason for the reluctance of both physicians and non-DMD patients to use steroid is the uncertainty of its potential efficacy to specific types of muscular dystrophies that have unique pathogenesis. *FKRP*-related muscular dystrophies have a distinctive biochemical nature, with a secondary hypoglycosylation of α -DG as the direct cause responsible for the degeneration of muscle fibers. Because no effective treatment is available, use of steroid to alleviate muscle pathology and delay disease progression remains a valuable option. However, there have been no designed pre-clinical and clinical studies of steroids for *FKRP*-related

diseases, except a case report with two patients treated with variable doses of prednisolone over a 5-year period.²³ This report described benefits, especially within the 10 months of the treatment, to skeletal muscles judged with isometric muscle strength in knee extensors, motor function by SCOTT test, and 46 minutes walking time. The current study of prednisolone treatment in the *FKRPP448L*-mutant mouse demonstrates that steroid treatment has clear positive effects by reducing inflammation and damage in the diseased muscles. However, prednisolone treatment only minimally and temporary improves muscle strength, and severely suppresses immune system in the mouse model. These results are, therefore, consistent with clinical

Figure 7 Improvement of functionally glycosylated α -dystroglycan (F- α -DG) after 6-month treatments with alendronate plus prednisolone in *P448L*- mice. **A:** Immunohistochemical staining of F- α -DG with monoclonal IIH6C4 antibody. Blue nuclear staining with DAPI. **B:** The number of positive F- α -DG muscle fibers in tibialis anterior (TA) muscle. **C:** Western blot for F- α -DG protein expression and α -actin as loading control. Prednisolone and combined treatments significantly increase F- α -DG. Included were normal *C57* mice (*C57*), saline-treated *P448L*- mice (saline), 5 mg/kg prednisolone daily-treated *P448L*- mice (Pred), 1 mg/kg alendronate daily-treated *P448L*- mice (Alen), and 1 mg/kg alendronate + 5 mg/kg prednisolone daily-treated *P448L*- mice (Alen + Pred). $n = 10$ mice in each group. * $P < 0.05$ versus control saline mice (two-tailed *t*-test).

observation and the results from DMD animal model study.²⁶ One interesting observation of the study is that the desirable effect can be equally achieved with both daily and twice a week treatment with the same dosage, suggesting that an optimized regimen could achieve equivalent benefits with minimized adverse effects for treating *FKRP*-related muscular dystrophy.²⁶

One severe consequence of reduced and loss of mobility as a result of disease progression in muscular dystrophy is the osteoporosis. Corticosteroid treatment can further exacerbate the already fragile bone structure; thus, bone loss and subsequent fracture have been one major concern for the muscular dystrophy population. For this reason, bisphosphonates have been investigated in many clinical trials, mostly in DMD patients to combat osteoporosis.²²

In 2011, Gordon et al²⁷ reviewed steroid and bisphosphonate treatment in DMD cases from 1963 to 2006 and reported that patients with a combined treatment resulted in improved survival rate compared with patients with steroids alone. Srinivasan et al²⁸ reported that use of bisphosphonate was able to stabilize bone mineral density in patients with steroid. Sbrocchi et al²⁹ conducted a retrospective observational study of intravenous bisphosphonate therapy and reported improved back pain and stabilization or increases in the height ratios of fractured vertebrae. Sarkozy et al³⁰ studied a total of 52 DMD patients in Newcastle, UK, and reported that prophylactic oral bisphosphonate therapy could prevent steroid-induced osteoporosis. Although the benefits of bisphosphonate to bone density have been widely supported, the current

Figure 8 Bone density after 6-month treatments with alendronate (Alen) and prednisolone (Pred) in *P448L*- mice. **A:** Digital X-rayed images of femurs and tibias of mice on Portable Pixarray 100 (BioOptics Software) X-ray machine. **B:** Bone density was measured using ImageJ software version 1.42 (NIH, Bethesda, MD). Same bones from age-matched normal *C57BL/6* mice were used as controls. Bone density of both femur and tibia of *P448L*- mutant mice is significantly lower than the age-matched normal *C57* mice. Prednisolone treatment further reduces bone density significantly compared with the saline-treated control *P448L*- mutants. Alendronate improves bone density of the *P448L*- mutants and eliminates prednisolone-related significant bone loss in the combine-treated group. Included were normal *C57* mice (*C57*), saline-treated *P448L*- mice (saline), 5 mg/kg prednisolone daily-treated *P448L*- mice (Pred), 1 mg/kg alendronate daily-treated *P448L*- mice (Alen), and 1 mg/kg alendronate + 5 mg/kg prednisolone daily-treated *P448L*- mice (Alen + Pred). $n = 10$ mice in each group. * $P < 0.05$ versus *C57* mice (two-tailed *t*-test).

study also investigated the effect of bisphosphonate on dystrophic muscles. Our results show that alendronate alone dose-dependently improved, although limited, skeletal muscle force production. This was associated with an increase in the levels of functional glycosylated α -DG.

A similar finding has not been available in patients, likely because clinical evaluation of biochemical markers of dystrophic muscles could not be conducted. Mechanisms behind these changes and relationship between the two events are, therefore, not understood. Although major pharmaceutical applications of bisphosphonates are for inhibition of bone resorption in treatment of osteoporosis and various conditions of hypercalcemia, the resulting decrease in serum-free calcium can, to some extent, benefit dystrophic muscles from further damage caused by relatively higher levels of free serum calcium as a result of enhanced bone resorption in patients. This is supported by reports that drug-induced reduction in free calcium levels can improve muscle function in models of muscular dystrophies.^{31,32} The effect of alendronate on cellular metabolism, such as the HMG-CoA reductase pathway, could also play a role by affecting diseased muscles. This might also explain the unexpected enhancement in functional glycosylation of α -DG, although direct evidence is yet to be demonstrated. Nevertheless, the potential dual positive effect of bisphosphonate on protection of bone loss and muscle damage merits further investigation. However, it is also clear from the result that significant therapeutic effect on muscles could not be achieved with bisphosphonate alone because no clear reduction in CK levels and improvement in muscle pathology were observed with any dosage used.

One interesting finding of the current study is the detection of enhanced expression of functional glycosylation of α -DG in the prednisolone-treated skeletal muscles, including the diaphragm, but not in cardiac muscle. The enhancement remains clearly detected after 6 months of treatment. This is associated with considerable improvement in muscle pathology with reduced fiber degeneration, variation in fiber sizes, and number of hypertrophic fibers. Interestingly, there is no reduction in the number of small regenerating fibers. The results, therefore, present a complex picture for interpretation of the mechanisms involved for the enhanced production of F- α -DG. As we reported earlier, the *FKRPP448L*- mutant mice lack F- α -DG in all muscles, except for some revertant fibers, which express detectable to normal levels of F- α -DG. We recently identified that these revertant fibers are related to muscle regeneration, with newly regenerating fibers expressing strong F- α -DG within the first 2 weeks. The levels of expression then decrease and become largely undetectable by 4 weeks. Therefore, one possible explanation is that prednisolone enhances muscle regeneration, thus the expression of F- α -DG in the treated muscles. This will be consistent to the fact that there is hardly any revertant fiber in the cardiac muscle, which exhibits little regeneration capacity. However, muscle regeneration is considered a consequence of muscle degeneration, which is clearly diminished at both 3 and 6 month time points of the treatment. Furthermore, the overall number of fibers with small caliber remains a minority and does not increase significantly when compared with the control groups. Therefore, increase in de novo muscle regeneration is unlikely to be the major cause for the observed

phenomenon. Because a small number of fibers with normal size can also be identified with expression of F- α -DG in the untreated mutant muscles, these results indicate factors other than regeneration could also be involved in the process.²⁵ We, therefore, hypothesize that the use of steroid affects metabolism of the diseased muscles, leading to a delay in the process of fiber maturation, and thus the prolonged expression of revertant F- α -DG. This hypothesis would be consistent to the fact that use of steroid affects wound healing.³³ Inhibition of inflammation in diseased muscles could further impair the fiber regeneration and maturation.³⁴ Nevertheless, expression of F- α -DG is expected to have a protective effect against damage to the dystroglycanopathy muscles, thus delay disease progression.

The results from individual patient and different animal models of muscular dystrophies with same or similar steroid treatment regimen may vary because of yet unknown reasons. This can be appreciated from a comparison of the results from an early study in mdx mouse, a model of human DMD, to that of the current study. Both studies applied a nearly identical dose regimen of prednisolone treatment, but with different methods of administration. Both studies result in early improvements in muscle functions, but the initial benefits disappeared with prolonged treatment of 6 months. However, although the early study found a significant increase in heart fibrosis and weight gain associated with a significant deterioration in cardiac systolic function after 100 days of treatment, the current study shows that cardiac functions of the *FKRP* mutant mice remained similar to the control group. Furthermore, our study showed a severe immune suppression at both 3 and 6 month treatment time points. This is in contrast to the early study, which described a significant decrease in spleen weight only at 100 days ($P < 0.05$), but unexpectedly not at 50 or 180 days of treatment.

In summary, the current study demonstrates that a combined use of bisphosphonate and steroids provides better therapeutic effect than steroid alone to *FKRP*-related muscular dystrophy.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.02.015>.

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