SHORT COMMUNICATION

Xin Is a Marker of Skeletal Muscle Damage Severity in Myopathies

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Xin is a striated muscle-specific protein that is localized to the myotendinous junction in skeletal muscle. However, in injured mouse muscle, Xin expression is up-regulated and observed throughout skeletal muscle fibers and within satellite cells. In this study, Xin was analyzed by immunofluorescent staining in skeletal muscle samples from 47 subjects with various forms of myopathy, including muscular dystrophies, inflammatory myopathies, mitochondrial/metabolic myopathy, and endocrine myopathy. Results indicate that Xin immunoreactivity is positively and significantly correlated ($r_s = 0.6175$, $P = <0.0001$) with the severity of muscle damage, regardless of myopathy type. Other muscle damage measures also showed a correlation with severity [Xin actin-binding repeat-containing 2 ($r_s = -0.7108$, $P = 0.0006$) and collagen ($r_s = 0.4683$, $P = 0.0783$)]. However, because only Xin lacked immunoreactivity within the healthy muscle belly, any detectable immunoreactivity for Xin was indicative of muscle damage. We also investigated the expression of Xin within the skeletal muscle of healthy individuals subjected to damaging eccentric exercise. Consistent with our previously mentioned results, Xin immunoreactivity was increased 24 hours after exercise in damaged muscle fibers and within the activated muscle satellite cells. Taken together, these data demonstrate Xin as a useful biomarker of muscle damage in healthy individuals and in patients with myopathy. The strong correlation between the degree of muscle damage and Xin immunoreactivity suggests that Xin may be a suitable outcome measure to evaluate disease progression and treatment effects in clinical trials. (*Am J Pathol* 2013, 183: 1703–1709; <http://dx.doi.org/10.1016/j.ajpath.2013.08.010>)

Xin is a striated muscle-specific protein localized at the intercalated disks of the heart and the myotendinous junctions (MTJs) of skeletal muscle.^{1,2} It is normally not detectable in the belly of healthy, uninjured skeletal muscle.^{3,4} However, this subcellular localization pattern changes dramatically in response to muscle injury, suggesting that Xin contributes significantly to the remodeling/repair process of skeletal muscle.³

Xin is encoded by the Xin actin-binding repeat-containing 1 (*XIRP1*) gene [previously referred to as cardiomyopathy-associated 1 (*CMYA1*)] and is a member of the Xin-repeats actin-binding protein family, along with Xin actin-binding repeat-containing protein 2 (*XIRP2*), which shares similar amino acid repeats with Xin.^{1,5} Xin's characteristic structural motifs allow for a variety of potential protein-binding partners. Xin displays the ability to bind and cross-link F-actin, making them extraresistant to depolymerization.⁵ Xin also possesses

several highly conserved proline-rich regions, allowing for binding to proteins containing consensus Ena/VASP homology 1 (EVH1) domains.¹ Mammalian enabled/vasodilator stimulated phosphoprotein (MENA/VASP; both are EVH1 domain containing proteins) colocalize with, and directly bind, the amino-terminus of Xin, whereas filamin C, a muscle-specific myotilin- and sarcoglycan-interacting protein, interacts with the C-terminus of Xin.¹

In adult skeletal muscle, the predominant theory regarding Xin's function suggests that it is a multiadapter protein, involved in F-actin organization and stabilization, specifically at sites that involve considerable mechanical

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strain.^{1,4} The role of Xin within regenerating skeletal muscle, however, has yet to be fully characterized. Given the expression pattern of Xin within regenerating mouse skeletal muscle⁶ and its role as a multiadapter protein within the cytoskeleton, we hypothesized that Xin would serve as a useful biomarker of muscle damage within myopathic or damaged skeletal muscle.

Materials and Methods

Patients and Ethics Statement

A total of 47 muscle samples from patients with various myopathies were included in the study (Supplemental Figure S1 and Supplemental Table S1). Skeletal muscle biopsy specimens were taken from the vastus lateralis using a 5-mm Bergström needle, as previously described.⁷ Control muscle biopsy specimens were taken from two healthy male individuals from 18 to 25 years of age. Biopsy specimens were also taken from three healthy individuals 24 hours after 300 maximal eccentric contractions.⁸ Control and eccentrically exercised subjects were informed of the procedures and the potential risks associated with the study and gave written informed consent. This portion of the study was approved by the Hamilton Health Sciences Research Ethics Board (08-413) and conformed to the Declaration of Helsinki regarding the use of human subjects as research participants. The myopathy portion of the study was performed on archival tissue by the approval of the Hamilton Health Sciences Research Ethics Board (10-327).

Histochemical and Immunofluorescence Analyses

Frozen muscle biopsy specimens were cut into cross sections (6 μ m thick) and adhered to charged glass slides for histochemical and immunofluorescence staining. Sections from all biopsy specimens were analyzed for Xin expression to determine whether a correlation existed between Xin and muscle damage. A subset of samples that displayed a strong Xin-to-damage correlation were further analyzed for collagen, filamin C, and Xirp2 expression.

Picrosirius Red

To stain for collagen content, sections were incubated for 1 hour in picrosirius red solution containing 0.1% Direct Red 80 (no. 365548; Sigma, St. Louis, MO) in a saturated aqueous solution of picric acid (p6744; Sigma). Sections were then rinsed in acidified water (0.5% acetic acid in distilled H₂O) twice. Afterwards, sections were dehydrated in three changes of 100% ethanol and one change of xylene and coverslipped using Permount mounting media (Fisher Scientific, Toronto, ON, Canada).

Immunofluorescence Staining

Xin, filamin C, and Xirp2 expression levels were evaluated by immunofluorescence staining. First, sections were dried, fixed

in cold methanol and acetone, and then incubated for 30 minutes in blocking solution (10% normal goat serum, 1.5% bovine serum albumin, and 0.3% triton). Primary antibodies were applied as follows: Xin for 1 hour at room temperature (mouse monoclonal; dilution, 1:5¹), filamin C at 4°C overnight (rabbit polyclonal; dilution, 1:1000; custom made by BioGenes, Berlin, Germany), and Xirp2 at 4°C overnight (mouse monoclonal; dilution, 1:10⁹). A biotin-streptavidin detection system was used as recommended (Vector Labs, Burlingame, CA). After incubation in the appropriate biotinylated secondary antibody, a tertiary streptavidin antibody conjugated to dyelight Alexa 594 (1:250; Abcam, Burlingame, CA) was applied for 30 minutes at room temperature. Blocking solution was reapplied before costaining the sections with laminin (ab14055; Abcam), diluted 1:250, and incubated at 4°C overnight. Laminin was detected using an anti-chick Alexa 488 antibody (1:250; Abcam), after which DAPI (1:10,000; Abcam) was applied to the sections for 5 minutes to identify nuclei.

In eccentrically damaged muscle, Xin was costained with dystrophin (15277; Abcam), diluted 1:250, or a custom-made affinity-purified Rad (Ras associated with diabetes) chicken polyclonal antibody (1 mg/mL; Genway Biotech, San Diego CA), diluted 1:150, and incubated at 4°C overnight. The use of Rad antibody was for the detection of activated satellite cells, as previously described.¹⁰ Sections were then incubated in anti-rabbit (1:250) or anti-chick (1:300) Alexa 488 antibody (Abcam), followed by DAPI.

Muscle Damage Scoring System

Patient biopsy specimens were assessed using three histopathological categories of muscle damage to determine an overall numerical damage score to which protein expression could be correlated (Figure 1). Muscle damage scores were assigned for each category (of 4), allowing for a total score of 12 based on multiple $\times 20$ magnification images taken of each muscle section, stained with laminin and DAPI. The first category was the degree of nuclear accumulation within the muscle (assessed using DAPI), where, with increasing damage severity, nuclei begin to aggregate at the periphery of myofibers (scored as 1 to 2), within the extracellular matrix (scored as 2 or 3), and within myofibers (scored as 3 or 4) (Figure 1). Laminin can be used to identify signs of myopathy because it is a component of myofiber basement membranes¹¹ and is up-regulated in the extracellular matrix (ECM) during muscle repair.^{12,13} Therefore, damage was assessed by the increase in dysregulation of laminin expression directly surrounding each myofiber (scored as 1 or 2) and increased density of laminin in the ECM (scored as 3 or 4). Last, as muscle damage becomes more severe, myofiber size and shape become more variable, as seen in Figure 1 and in the study by Kley et al.¹⁴ Increased myofiber size variability was assessed with a score from 1 to 4. By using this system, biopsy specimens from myopathy patients were compared with control (score, 0), and the most damaged muscle subsequently received scores closer to 12.

Figure 1 Muscle damage scoring system. The degree of skeletal muscle damage was assessed based on three histopathological indices: the degree of nuclear accumulation, laminar dysregulation, and the variability of myofiber shape/size. Myopathic muscle sections were stained for laminin (green) and nuclei marker, DAPI (blue), and given a score of 4 for each category, where 4 is the most severe dysregulation. (Image contrast is enhanced for clear visualization of staining.)

Image Analysis

Images of the immunostained muscle sections were captured in high resolution using a Nikon (Melville, NY) 90i Eclipse microscope. Threshold detection on Nikon Elements software (NIS Elements AR, Melville, NY) was used to determine the percentage of positively stained area in the total muscle section, determined as signal above background threshold. Background threshold was determined by defining the exposure time in which an absence of signal was noted in the negative control sections (ie, samples not stained with primary antibody).

Statistical Analysis

Spearman's rank correlation (r_s) was used to determine whether a significant relationship (at $P < 0.05$) existed between the severity of damage and the expression of Xin, collagen, filamin C, and Xirp2 within the muscle, using GraphPad Prism 5 software (La Jolla, CA). $P < 0.05$ was considered significant.

Results

Immunoreactivity for Xin Positively Correlates with Severity of Muscle Damage

Immunoreactivity for Xin as a percentage of total muscle cross-sectional area was determined in muscle sections from patients with various forms of myopathy (Figure 2A and Supplemental Figure S2). The degree of muscle damage in each subject, as assessed by nuclei infiltration, laminar dysregulation, and fiber morphological characteristics (Figure 1),

when correlated with Xin levels, revealed a significant and positive relationship ($r_s = 0.6175$, $P < 0.0001$) (Figure 2A). Control muscle from healthy subjects showed baseline levels of Xin at levels not different from 0 (Figure 2A and Figure 3A). In muscle with a low degree of damage, Xin was detected at the intracellular periphery of the myofibers (Figure 3B). In some instances, Xin colocalized with nuclei underneath the basal lamina, which is consistent with the documented expression pattern of Xin within activated satellite cells.^{3,6} As the degree of muscle damage increased, as noted by increases in nuclei around the fiber periphery and fiber size/shape variability, Xin expression also increased around the myofiber periphery (Figure 3C). In severely damaged muscle, where laminin expression in the ECM is increased and macrophages infiltrate damaged myofibers, Xin is highly expressed at the fiber periphery and within muscle fibers (Figure 3D). In healthy muscle that was damaged through eccentric contractions, Xin was also observed to be up-regulated 24 hours after exercise in the sublamellar region, assessed with dystrophin and DAPI. This is indicative of the localization of Xin to the activated satellite cell (Figure 4A). Furthermore, Xin was colocalized with Rad, a marker of activated satellite cells in eccentrically damaged muscle (Figure 4C). The expression of Xin within the muscles of healthy subjects after eccentric exercise (Figure 4, A and B) and within activated satellite cells (Figure 4C) further supports the use of Xin as a biomarker for muscle damage.

Muscle Damage Scores Correlate with Collagen Content

Collagen expression is elevated in damaged muscle, resultant, in part, from increased need for ECM remodeling and

the associated inflammatory response¹¹; therefore, it was reasonable to validate the muscle damage scoring system against collagen content. By using a picrosirius red stain on a subset of muscle cross sections from the 47 patients, our findings are consistent with previous findings^{12,13,15,16} demonstrating a positive correlation between muscle damage scores and collagen content ($N = 15$, $r_s = 0.4683$, $P = 0.0783$) (Figure 2B). In undamaged (healthy control) and low-score damaged muscle, collagen is located within the lamina of the myofiber, stained in red (Figure 3, E and F). As muscle damage becomes more extensive, collagen is deposited in the extracellular space between damaged muscle fibers shown in Figure 3, G and H, thereby increasing the percentage of collagen content in the total cross-sectional area.

Immunoreactivity for Filamin C Expression Does Not Significantly Correlate with Severity of Muscle Damage

Filamin C is located in the myofibrillar Z-disk and at the MTJ of skeletal muscle, where it binds to Xin.¹ Immunofluorescence staining on muscle cross sections was performed to determine whether immunoreactivity for filamin C was correlated with the degree of damage in muscle. In low to moderately damaged muscle, immunoreactivity for filamin C was similar to that found in controls when analyzed as a percentage of total cross-sectional area (Figure 3, I–K). Conversely, in highly damaged muscle, filamin C was found to aggregate in the subsarcolemmal area and within muscle fibers (Figure 3L); the percentage of total cross-sectional area did not significantly correlate with muscle damage severity ($N = 17$, $r_s = -0.2244$, $P = 0.3866$) (Figure 2C).

Xirp2 Is Redistributed in Myopathic Muscle and Is Negatively Correlated with Severity of Muscle Damage

Xirp2 consists of Xin repeats that are capable of binding actin, and is localized to the Z-disk region in skeletal muscle.¹⁷ Our results indicate that Xirp2 immunofluorescence is highest as a percentage of area in control muscle, where it is consistently expressed in differing degrees of intensity in each myofiber (Figure 3M). As muscle damage increased, Xirp2 expression became less homogenous throughout the cross-sectional area (Figure 3, N and O). In severely damaged muscle (highest scored), Xirp2 was redistributed to form high-intensity stained accumulations that were of a reduced area fraction (Figure 3P). Therefore, Xirp2 expression as a percentage of total cross-sectional area decreased as muscle damage increased, resulting in a significant negative correlation ($N = 19$, $r_s = -0.7108$, $P = 0.0006$) (Figure 2D).

Discussion

In the present study, skeletal muscle biopsy specimens from 47 patients with various forms of myopathy were acquired

Figure 2 Correlation between proteins of interest and degree of muscle damage in myopathic patients. **A:** Xin expression as a percentage of total area in myopathic muscle is significantly and positively correlated with muscle damage severity. Xin expression levels of subjects a (control), b, c, and d (red) are shown in Figure 3 (A–D, respectively). Xin expression in controls (a) is negligible. **B:** Collagen expression was also analyzed as a percentage of total muscle section area, and is positively correlated with degree of muscle damage. Representative images of subject e (control), f, g, and h (red) are shown in Figure 3. Collagen expression is evident in control samples. **C:** There is no significant relationship between filamin C expression and muscle damage severity. Images of subject i (control) to l (red) are shown in Figure 3. Because filamin C is located in myofibrillar Z-disks, it is expressed in differing intensities in all myofibers in cross section, with only the highest-intensity myofibers considered positive. Therefore, control samples display the most uniform and, thus, highest expression of filamin C. **D:** Xirp2 expression in total muscle section area is significantly, but negatively, correlated with degree of muscle damage. As noted herein, the redistribution of Xirp2 from diffuse staining throughout the myofiber (with no damage) to focal accumulations (with severe damage) is largely responsible for the negative correlation observed between Xirp2 and muscle damage. Expression in control subject (m, red) is consistent throughout the muscle section, which results in a high percentage expression in the total area. As the degree of muscle damage increases (subjects n to p, red) (Figure 3), Xirp2 aggregates to specific areas; therefore, expression is less uniform throughout the entire muscle section.

and analyzed for immunoreactivity to Xin, collagen, filamin C, and Xirp2. Results indicate Xin expression is positively and significantly correlated with the degree of muscle damage, regardless of the type of myopathy. Although collagen, filamin C, and Xirp2 also display a distinct expression pattern in highly damaged muscle, our results indicate that Xin is the most sensitive and reliable marker for muscle damage throughout the spectrum of muscle damage severity. Furthermore, Xin expression was also observed to be up-regulated in the muscle belly and within muscle satellite cells of eccentrically damaged muscle of healthy individuals. Thus, Xin can be considered a useful marker of damaged muscle regardless of etiology, whose expression is strongly and positively correlated with damage severity. Unlike other candidate proteins measured herein, Xin immunoreactivity was not detectable in healthy muscle

Figure 3 Xin, collagen, filamin C, and Xirp2 display differential expression patterns as muscle damage increases. Xin (A–D, red) (Figure 2A) expression is not detectable in control muscle; however, expression increases and is displayed around the myofiber periphery in low and moderately damaged muscle. In highly diseased muscle, Xin expression is seen as aggregates within muscle fibers. Collagen (E–H, red) (Figure 2B) is found in the basal lamina of each muscle fiber in control subjects, and as damage increases and ECM remodeling ensues, collagen content increases. Filamin C (I–L, red) (Figure 2C) is found in myofibrillar Z-disks and, therefore, expressed in differing degrees of intensity in myofibers in muscle cross sections, where the fibers with the most intense expression were considered positive (Figure 2C). In severely damaged muscle, intense filamin C aggregation is noticed around and within some myofibers. Xirp2 (M–P, red) (Figure 2D) is also found in the muscle Z-disk and, therefore, is expressed in all myofibers with differing intensity in cross sections in control subjects. As muscle damage increases, Xirp2 begins to aggregate and, therefore, expression of total cross-sectional area decreases, although expression is more intense within specific myofibers. Scale bars: 100 μm (A–H); 200 μm (I–P).

fibers outside of the MTJ and, therefore, could be considered a useful diagnostic tool to determine even modest amounts of muscle damage in research studies or in clinical trials as a primary outcome measure.^{18–21}

Skeletal Muscle Regeneration

Determining Severity of Muscle Damage

Skeletal muscle has the capacity to undergo a relatively rapid regenerative process in response to muscle damage that is characterized by two general phases: degeneration of disrupted myofibers and subsequent repair of damaged

fibers and/or *de novo* synthesis of new myofibers. In myopathic muscle, this process is generally an ongoing process and, therefore, severity of muscle damage can be determined by assessing the presence of factors associated with regeneration.

During the initial phase of muscle degeneration, myofiber integrity is compromised, which is evident by increased serum levels of creatine kinase, a typical marker of muscle damage.²² The myofiber external lamina is made up of collagen IV, laminin, and heparin sulfate proteoglycans.¹⁶ Therefore, in this study, disruption of myofiber integrity was determined by the degree of laminin dysregulation

Figure 4 Xin expression in eccentrically damaged muscle from healthy subjects. Xin expression is increased 24 hours after exercise, and localized to muscle satellite cells. This is shown because Xin (red) is localized with activated satellite cell marker, Rad (green) in **C**, and nuclei marker, DAPI (blue), in the sublamellar region (**A**), assessed with dystrophin. Localization (**yellow arrowheads**) of Xin (red) and dystrophin (green) in **B** suggests Xin is found in the membrane of eccentrically damaged muscle. **Arrows** in **C** highlight Xin expression colocalizing with the activated satellite cell marker, Rad, and the nuclear marker, DAPI. Scale bar = 20 μ m (**A**; consistent for all panels).

(Figure 1). Laminin expression was further used to examine myofiber size and shape variability, which is characteristic of myopathic muscle.¹⁴ The degenerative phase is also marked by an activation of mononucleated inflammatory and myogenic cells. These cells include neutrophils and macrophages that remove cellular debris.²² This allows for remodeling of the ECM and an increase of ECM protein deposition, such as collagen I, III, and V, and fibronectin, necessary for structural stability of the muscle.^{15,16} Therefore, collagen content was used as an identifier of muscle damage, and used to validate the muscle damage scoring system. A significant positive correlation exists between muscle damage scores and collagen content, indicating it is appropriate to determine myopathic muscle damage severity based on laminar dysregulation, fiber variability, and nuclei infiltration.

After the degenerative process, muscle regeneration occurs where activated satellite cells proliferate, and differentiate to form new myofibers, or fuse to existing damaged fibers in an attempt to repair them.¹² Characteristic of these fibers are centrally located myonuclei, stained with DAPI in Figure 1, indicative of severely damaged muscle.^{12,22}

Xin Is the Most Suitable Candidate for a Marker of Muscle Damage Severity

In uninjured skeletal muscle, Xin expression is negligible, and localized to the MTJ.⁴ In this study, we analyzed Xin expression in the skeletal muscle of subjects with a wide array of myopathies: muscular dystrophies [limb-girdle muscular dystrophy (LGMD) 2A, LGMD2B, LGMD2I, LGMD2G, fasciocalapulohumeral muscular dystrophy, myotonic dystrophy, and congenital muscular dystrophy], inflammatory myopathies, mitochondrial myopathy, metabolic myopathy, neuromyopathy, and endocrine myopathy. The importance of these findings is that Xin is a marker of damage severity in a variety of myopathies, and there appeared to be no significant relationship between Xin expression and any one disease. Therefore, Xin may be used to determine degree

of muscle damage in any of the previously mentioned myopathies.

Results also indicate that Xin expression is up-regulated in damaged healthy muscle 24 hours after eccentric exercise. In eccentrically damaged muscle, and slightly damaged myopathic muscle, Xin expression is found at the inner periphery of the myofiber and colocalizing with the activated satellite cell marker, Rad.¹⁰ This expression/distribution profile is also observed in moderately damaged muscle (Figure 3C) with the intensity of immunoreactivity increasing. In severely damaged myopathic muscle, Xin is expressed within regenerating fibers, consistent with the expression pattern observed in injured mouse skeletal muscle.⁶ Therefore, Xin expression as a percentage of total muscle area is positively correlated with the degree of muscle damage in myopathic patients, regardless of the type of myopathy exhibited.

Other candidate proteins examined include collagen, filamin C, and Xirp2. Collagen content is known to increase as ECM remodeling ensues during regeneration. However, collagen is consistently expressed throughout healthy skeletal muscle and, therefore, its expression pattern may be less capable of deciphering between healthy, low, and moderate degrees of muscle damage compared with Xin. For the same reason, filamin C and Xirp2 may not be as clinically sensitive for the degree of muscle damage in myopathic patients, because they are expressed in uninjured myofibers. However, Xirp2 expression did display a significant redistribution with increasing damage to the muscle. These Xirp2 accumulations within and around the periphery of regenerating fibers [much like that observed for Xin with severe damage (Figure 3)] are of particular interest and certainly require further study.

The expression of Xin within damaged skeletal muscle suggests a novel alternative to other used methods for assessing muscle damage (eg, serum creatine kinase, myoglobin, and troponin). Although these serum measures are less invasive, they are highly variable in both quantity and temporal expression.²³ The clinical advantage of Xin as a muscle damage marker is the ability to determine the

overall degree of damage in various myopathies, because plasma protein levels of the previously mentioned proteins may not necessarily increase linearly with disease severity. Xin is a unique marker of damage as the location of expression, either at the myofiber periphery or in accumulations within damaged fibers, is pathologically significant in determining disease severity. Taken together, Xin expression can be used to determine whether muscle is damaged and ascertain the degree of damage in both healthy muscle after injury and in various myopathic conditions. Furthermore, the absence of Xin immunoreactivity in the muscle belly makes the identification of damage much easier to assess than other proteins examined whose expression was present within uninjured skeletal muscle.

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Supplemental Data

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