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Fast-Twitch Sarcomeric and Glycolytic Enzyme Protein Loss in Inclusion Body Myositis

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Abstract

Background—Inclusion body myositis (IBM) is an inflammatory disease of skeletal muscle of unknown cause. To further understand the nature of the tissue injury in this disease, we developed methods for large-scale detection and quantitation of proteins in muscle biopsy samples and analyzed proteomic data produced by these methods together with histochemical, immunohistochemical, and microarray data.

Methods—Twenty muscle biopsy samples from patients with inflammatory myopathies (N=17) or elderly subjects without neuromuscular disease (N=3) were profiled by proteomic studies using liquid chromatographic separation of peptides followed by mass spectrometry. Thirteen of the diseased samples additionally underwent microarray studies. Seventy muscle specimens from patients with a range of neuromuscular disorders were examined by ATPase histochemical methods. Smaller numbers of samples underwent immunohistochemical and immunoblot studies.

Results—Mass spectrometric studies identified and quantified approximately 300 total distinct proteins in each muscle sample. In inclusion body myositis and to a lesser extent in polymyositis, proteomic studies confirmed by histochemical, immunohistochemical, and immunoblot studies loss of many fast-twitch specific structural proteins and glycolytic enzymes despite relative preservation of transcript levels. Increased abundance of a nuclear membrane protein, immunoglobulins, and two calpain-3 substrates were present.

Conclusion—The atrophy present in inclusion body myositis muscle is accompanied by preferential loss of fast-twitch structural proteins and glycolytic enzymes, particularly glycogen debranching enzyme, with relative preservation of the abundance of their respective transcripts. Although muscle atrophy has long been recognized in IBM, these studies report the first specific proteins identified as reduced in quantity in IBM muscle.

Inclusion-body myositis (IBM) is a progressive inflammatory skeletal muscle disease of unknown cause and without effective treatment. The mechanisms of myofiber injury in IBM

are poorly understood. In biopsy samples examined by microscopy, some myofibers appear to be injured by invading cytotoxic T cells, while others have no apparent cause for their morphological abnormalities and have been called degenerative. At least 75 different proteins have been reported to be abnormally accumulated in IBM myofibers.

Almost all of these reports have been based on immunohistochemical evidence alone. Antibody reagents may react to a variety of targets, yet their immunoreactivity may be interpreted as indicative of the presence of only one specific protein. For example, the interpretation that β -amyloid ($A\beta$) protein accumulates in IBM myofibers is based entirely on reports of its presence by immunohistochemical methods using antibodies that may cross-react to β -amyloid precursor protein (β APP);¹⁶ no western blot study of IBM muscle that demonstrates a 4 kDa band (the approximate mass of $A\beta$) immunoreactive with any anti- β APP or anti- $A\beta$ antibody has ever been published. Similarly, the presence of antibody SMI-31 has been used to claim that phosphorylated microtubule associated protein tau is abnormally accumulated in IBM muscle,⁹ even though this antibody has published reactivity against a variety of other proteins, including neurofilaments H and M (manufacturer's datasheet, Covance, Inc.), microtubule associated protein 1b,¹² microtubule associated protein 2,³⁰ a lamin intermediate filament,⁴¹ and possibly sequestosome-1.⁴² The specific proteins to which SMI-31 binds in IBM muscle sections are unknown.

Because of the limitations of immunohistochemical studies, recent interest has developed in other methods for protein identification and quantitation in IBM muscle, using two-dimensional (2-D) electrophoretic gel protein separation and analysis of spot intensity,²¹⁻²⁶ peptide sequencing,⁷ and mass spectrometry.²⁶ Mass spectrometry has long been used to determine which proteins are the most abundant in preparations that contain small numbers of proteins.³² Recently, the technique of shotgun proteomics has been used to quantify large numbers of distinct proteins from biological materials.²²⁻⁴⁰ In this study, we developed and applied shotgun proteomic methods to the problem of protein identification and quantification in IBM and other inflammatory myopathies.

Methods

Patients and Samples

Mass spectrometry-based proteomic profiling was performed on muscle biopsy samples from 20 patients (IBM N=8; PM N=5; DM N=4; Normal N=3), and gene expression microarray studies were performed in parallel on 13 of the 17 diseased samples from this group (IBM N=5, PM N=4, DM N=4). Varying numbers of other histochemical, immunohistochemical, and microarray studies were performed on samples as listed (Supplementary Table). Because of limitations in quantity and availability of muscle tissue and other resources, not all studies were done on all biopsy specimens. Patients with IBM fulfilled criteria for definite or possible IBM;¹⁷ patients with PM or DM fulfilled criteria for definite or probable PM or DM.¹⁹ No patient with IBM received corticosteroids treatment at any time. None of the PM and 2 of the 4 DM patients whose samples underwent proteomic profiling were on immunosuppressive medications. Treatment status is detailed in the Supplementary Table. Normal subjects had no symptoms, signs, laboratory findings, or pathological abnormalities of a neuromuscular disease. Muscle biopsies were performed for diagnostic purposes, and patients provided informed consent for research studies, approved by our institutional review boards.

Preparation of samples for and performance of liquid chromatography and mass spectrometry (LC/MS)

For proteomic studies, ten 20 μm cryostat-cut sections of frozen muscle tissue were collected in microcentrifuge tubes at -20°C , giving at least 300 μg of protein wet weight. A solution of 400 μL of buffer containing 1% SDS, 10mM DTT, and 50mM triethanolamine bicarbonate (TEAB) was added to the muscle tissue and boiled at 100°C for 20 minutes. The micro BCA assay (Pierce Biotechnology, Rockford, IL) was used to determine protein concentration. Muscle lysate was alkylated with 50 mM iodoacetamide, and the excess iodoacetamide was inactivated with 50 mM DTT. Following acetone precipitation, the pellet was resuspended in 20mM TEAB. Sequencing Grade Modified Trypsin (#V5113, Promega) was added, and the sample was incubated overnight at 37°C .

Sample clean-up was performed with Vydac Solid Phase Extraction (SPE) C18, 13 μm columns. Samples were acidified after digestion with 0.5% TFA. Cartridges were conditioned with 2mL of 100% acetonitrile, followed by 3mL of 0.25% TFA (v/v). Samples were loaded at 2mL/min followed by rinsing with 5mL of 0.25% TFA (v/v). Samples were eluted in 200 μL of 75% acetonitrile, 0.1% formic acid (FA).

Reverse-phase HPLC was carried out as follows. Samples (2.5 μg) in 5% acetonitrile, 0.1% FA were injected with a Famos Autosampler onto a 75 $\mu\text{m} \times 25\text{cm}$ fused silica capillary column packed with C18 media (Magic C18 beads, Michrom Bioresources Inc, Auburn, CA). Samples were eluted at 250 $\mu\text{L}/\text{min}$, with a split ratio of about 1:200. The gradient was from 5% acetonitrile 0.35% FA to 30% acetonitrile 0.35% FA over 190 minutes for a total run length of 240 minutes. A Thermo Finnigan LTQ mass spectrometer was run in a "Top Nine" configuration at 200K resolution for a full scan m/z 395-1500 Da, with +1 charge state rejection, and monoisotopic precursor selection.

Mass Spectrometry Data Analysis

Approximately 10,000 spectra were collected per LC/MS run. Raw files were converted to Mascot generic files using custom software (courtesy of Tom Patterson, Children's Hospital). Mascot searches were performed against the human International Protein Index (IPI) database (release v3.25 from <ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/human>), allowing for oxidized methionine and N-terminal pyroglutamic acid. All matches for important proteins were manually curated, as described.³¹ Proteins were considered credible if two or more distinct peptides were identified. Each identified peptide was mapped to a manually curated human protein database that had been mapped to the human gene index number so that it could be identified using the internationally accepted gene symbol. Thus, alternatively spliced isoforms of the same gene were counted as one protein. Except where noted, peptides were assigned solely to the most abundant protein to which they could be mapped. Identified peptides were assigned consistently to the smallest possible list of identified proteins because of the large number of peptides that are shared by related muscle proteins.

23

Microarray Experiments and Data Analysis

Gene expression profiling of muscle was performed on 31 samples (9 IBM, 8 PM, 4 DM, and 10 normal), including 13 diseased samples that also underwent proteomic profiling (5 with IBM, 4 with PM, 4 with DM) using Affymetrix HG-U133A microarrays representing approximately 18,000 genes. Data were analyzed using MAS5.0 software as previously described.¹⁴ Normalization of microarray datasets was performed by linear regression to a reference sample. When more than 1 probeset measured a single gene's transcript, the mean of the MAS5 computed expression values for these probesets was used. Symbols for measured transcript levels are represented in italics (i.e., *MYH7*).

Histochemistry and Immunohistochemistry

Histochemistry using ATPase methods after preincubation at pH 9.4 was performed on 70 samples, including 19 of the 20 samples studied by proteomic profiling plus an additional 51 samples across a range of neuromuscular diseases (49 with inflammatory myopathies, 15 with muscular dystrophies, 2 with neurogenic disease, and 4 normal). Cryostat sections, air dried at room temperature, were placed into a Coplin jar containing a room temperature solution of 0.084 M glycine buffer, pH 9.4, 0.12 M calcium chloride, 0.084 M sodium chloride, and 0.91 mM adenosine 5'-triphosphate (ATP). The Coplin jar was heated to 37°C in a warm air oven for 45 minutes. The sections were rinsed in distilled water, soaked in 2% (w/v) cobalt(II) chloride hexahydrate for 5 minutes, and washed with 3 changes of distilled water. Sections were stained for 30 seconds in 2% (w/v) ammonium sulfide and rinsed in running tap water. Sections were counterstained for 1 minute with Harris hematoxylin, dipped in 1.0% (w/v) sodium tetraborate hexahydrate and rinsed in distilled water. After dehydration with alcohols and clearing with xylenes, sections were coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Tiled images of entire muscle sections were obtained with a Zeiss Axioimager microscope and analyzed with Axioimager image analysis software.

Immunohistochemistry using antibodies against myosin heavy chains (16 patients; fast MHCf; slow MHCs; developmental MHCd; and neonatal MHCn) and against glycogen debranching enzyme (22 patients; AGL) was performed. Cryostat sections of muscle cut at 10 µm were fixed in cold acetone (-10° ± 5°C) for 5 minutes, although for myosin heavy chains, unfixed tissues was also used. Sections were soaked consecutively in room temperature 0.05 M tris(hydroxymethyl)aminomethane buffer (TRIS), pH 7.5, for 5 minutes, and in 0.05 M TBS-T (TRIS buffered saline with Tween 20; Signet Laboratories, Inc./Covance Research Products, Inc., Dedham, MA) supplemented with 4% porcine serum for 30 minutes.

Mouse monoclonal antibodies (Novocastra/Vision Biosystems, Inc., Norwell, MA) used were MHC-fast (clone WB-MHCf, isotype IgG1, 1:60 dilution, 1 hour incubation), MHC-slow (clone WB-MHCs, isotype IgG1, 1:80 dilution, 1 hour incubation), MHC-developmental (clone RNMy2/9D2, isotype IgG1, 1:40 dilution, 4 hours incubation), and MHC-neonatal (clone WB-MHCn, isotype IgG1, 1:20 dilution, 4 hours incubation); the rabbit polyclonal antibody AGL (1:50 dilution, 5µg/ml, 4 hours incubation, cat. no. AP2402a, Abgent, San Diego, CA) was also used. Following primary antibody treatment, slides were rinsed with 0.025M TBS-T, soaked in same, and soaked in 0.05M TBS-T-porcine serum prior to incubation for 30 minutes with horseradish peroxidase labelled polymer conjugated to either goat anti-mouse immunoglobulins (mouse Envision+, Dako, Carpinteria, CA) or goat anti-rabbit immunoglobulins (rabbit PowerVision, ImmunoVision Technologies, Co./Vision BioSystems, Inc., Norwell, MA). Slides were washed and soaked as before. Antibody localization was effected using a peroxidase reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB+, Dako) as the chromogen. Slides were washed with water, counterstained with methyl green (usually omitted with AGL antibody stained slides), dehydrated through alcohols and xylenes, and coverslipped with mounting medium (Richard-Allan Scientific, Kalamazoo, MI).

Immunofluorescent studies with dual staining using antibodies against α -actinin-3 (ACTN3) and MHCf were performed on 11 samples, 10 of which underwent proteomic profiling. ACTN3 expression was detected using crude 5B2 rabbit antisera at 1:200 dilution, overnight at 4°C. MHC-fast was used at 1:40 dilution overnight at 4°C. Secondary antibodies used were donkey anti-rabbit alexa 594 and goat anti-mouse alexa 488 (Invitrogen Corp., Carlsbad, CA), both at 1:200 dilution for 30 minutes at room temperature.

Comparisons of Transcriptome and Proteome Data

Thirteen disease samples (5 with IBM, 4 with PM, 4 with DM) had both proteomic and microarray data. For calculation of transcript fold-ratios and their comparison with protein fold-ratios, these data were analyzed in comparison to normal data consisting of proteomic data from 3 samples and microarray data from a separate 10 samples. Comparison of quantitative protein and transcript data was made through mapping of gene symbols from MASCOT determined protein to Affymetrix probeset identifications.

Debranching enzyme immunoblots

Whole muscle lysates (WML) were prepared using 5 mg of cryostat sectioned muscle homogenized in 200 μ l of lysis buffer (containing 20 mM Tris pH 7.6, 2% SDS, 5 mM DTT), centrifuged at 10,000 g for 10 min at 4°C and the supernatant removed. The micro BCA assay (Pierce, Rockford, IL) was used to determine protein concentration, and the fractions were stored at -80°C. For SDS-PAGE, 30 μ g of WML from each sample was diluted with NuPAGE LDS Sample Buffer (4X) (Invitrogen, Carlsbad, CA), reduced with 10 mM DTT, heated at 95°C for 10 minutes, centrifuged at 2000 g for 10 minutes, loaded onto 4-12% Bis-Tris Gels (Invitrogen), and resolved using MOPS running buffer (Invitrogen) at a voltage of 100-150 mV. The gels were transferred to a nitrocellulose membrane using NuPAGE Transfer Buffer (Invitrogen) at 30 mV for 1.5 hours, washed in PBS including 0.1% Tween-20 (PBST0.1%), blocked for 1 hr in 5% fat free milk in PBST0.1% (5% milk/PBST0.1%) and stored at 4°C.

Blots were incubated with rabbit polyclonal anti-AGL C-terminal antibody (# AP2402B, Abgent, San Diego, CA) at the dilution of 1:600 in 5% milk/PBST0.1% at 4°C overnight with shaking (except for the first two lanes that served as negative control for the primary antibody). After 5 washes of 5 minutes in PBST0.1%, the blots were incubated with secondary HRP-tagged goat anti-rabbit antibody IgG (# ab6721, Abcam, Cambridge, MA) at the dilution of 1:5000 in 5% milk/PBST0.1% for 1 hour at room temperature (RT), with shaking, and washed as above. SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford IL) and Kodak films were used for visualization of the bands. The blots were stripped using Restore Western Blot Stripping Buffer (#21062, Pierce) for 45 minutes at 37°C, washed twice for 10 minutes with PBST0.1% and blocked for 1 hr in 5% milk/PBST0.1%. To determine reactivity against actin, immunoblotting was repeated as above using rabbit polyclonal anti-actin primary antibody (# sc1616, Santa Cruz Biotechnology, sc1616, Santa Cruz, CA) at the dilution of 1:4000 for 1 hour at RT and secondary HRP-tagged goat anti-rabbit antibody IgG (# ab6721, Abcam) at the dilution of 1:10000 for 1 hour at RT.

Results

A Protocol for Human Muscle Proteomic Profiling

We developed a protocol for quantitative proteomic profiling of human muscle biopsy specimens, consisting of trypsin digestion of the entire sample, followed by 75 μ m reverse phase HPLC separation coupled to the collection of high mass accuracy MS-MS spectra, without the use of gel electrophoresis. Approximately 300 distinct proteins were identified from each biopsy specimen. To examine biological and technical reproducibility, a single muscle sample was sectioned into 4 adjacent 200 μ m pieces, and LC/MS was performed individually on each piece. Protein quantitation was highly reproducible (mean of pairwise Pearson's correlation coefficients=0.998). However, when samples from different individuals were compared, many of the protein expression values differed significantly between individuals. In some cases, these changes correlated with disease, but in many cases there was evidence of individual-specific patterns of protein expression.

Loss of Fast-Twitch Structural Proteins by Proteomic Quantitation in IBM Muscle

The set of most differentially reduced proteins in IBM compared to normal (Table 1) was notable for containing many fast-twitch muscle proteins. Structural proteins found to be decreased in IBM muscle included α -actinin-3 (present at 1/10th the normal amount), myosin binding protein C 2 (MYBPC2) (1/5th normal), troponin T type 3 (TNNT3), myozenin 1 (MYOZ1), myomesin 2 (MYOM2), and myosin heavy chain 1 (MYH1). MYH1 is the most abundant myosin heavy chain isoform present within the fastest-twitch type 2b fibers. The reduction in fast-twitch structural proteins in IBM was not due to a generalized or non-specific reduction in muscle structural proteins. Comparison of corresponding fast-twitch with slow-twitch structural protein isoforms showed the protein loss was greater for fast-twitch isoforms. Specific reduction in fast-twitch isoforms was present in all the inflammatory myopathy categories. It was reduced to a greater extent in IBM than PM, and it was only mildly reduced in DM (Figure 1).

Histochemical and Immunohistochemical Evidence Concurs with Proteomic Evidence for Depletion of Fast Myosin Heavy Chains

To separately validate proteomic data that suggested depletion of fast-twitch myofiber proteins in IBM muscle, we examined tissue sections stained for ATPase after preincubation at pH 9.4. In normal muscle, this approach has been widely used to identify fiber types. Fast-twitch myofibers containing myosin heavy chain 1 (MYH1) and myosin heavy chain 2 (MYH2) stain dark, while slow-twitch fibers containing myosin heavy chain 7 (MYH7) stain light.

For 19 of the 20 samples profiled by proteomic methods, we examined whole muscle sections stained with the ATPase-pH-9.4 method using tiling imaging and image analysis software (Zeiss Axiovision). We computed the relative proportions of the total surface area of dark compared with light staining as a reflection of the total quantity of dark-staining myosin heavy chain isoforms present in muscle. The proportion of ATPase-pH-9.4 dark-staining myofiber area was significantly reduced in IBM (36%) compared to normal (58%; $p=0.02$), but it was not reduced in PM (53%; $p=0.59$) or DM (50%; $p=0.19$ Student's t-test) in comparison with normal muscle.

To further understand the specificity of fast-twitch myosin heavy chain depletion in IBM compared with other muscle diseases, we extended these studies to 70 muscle biopsy samples across a wide range of muscle pathology. Specimens were chosen based on availability of samples and independent of knowledge of fiber type analyses. Thirteen of 18 IBM samples clustered among the samples with the lowest proportion of dark-staining myofiber surface area on ATPase-pH-9.4 studies (Figure 2). The reduction in dark-staining myofiber area was statistically significant for IBM (32%) in comparison to normal (60%; $p=0.0002$). PM (49%; $p=0.08$) dark staining myofiber area showed a non-significant trend toward reduction, while DM (53%; $p=0.20$) and muscular dystrophies (50%; $p=0.12$) showed no difference from normal. The loss of fast-twitch myosin heavy chains in IBM was not simply a consequence of disease chronicity, as patients with muscular dystrophies had similar duration of symptoms (mean IBM 48 months compared to muscular dystrophy 45 months; $p=0.86$) and similar degree of weakness of the biopsied muscle ($p=0.19$). Patients with PM also had similar degree of weakness of the biopsied muscle ($p=0.77$) and did not differ statistically significantly in age (mean IBM 63 years, mean PM 56 years, $p=0.23$) or in the choice of muscle sampled (IBM 61% biceps, 22% quadriceps, 17% other; PM 61% biceps, 15% quadriceps, 24% other).

To further examine these findings, immunohistochemistry of adjacent 10 μ m sections using antibodies against fast (MHCf) and slow (MHCs) myosin heavy chains was performed on 9

of the 20 cases studied through the proteomic approach (5 IBM, 3 PM, 1 DM). Correlation of MHCf-positive staining fiber area ($r^2 = 0.79$) and MHCs-negative staining fiber area ($r^2 = 0.84$) with ATPase-pH-9.4 staining was high. IBM muscle frequently contained many small MHCf-positive staining fibers (Figure 3).

α -Actinin-3 Reduction Parallels Fast-Twitch Myofiber Loss

Proteomic data also indicated depletion of α -actinin-3 (ACTN3), a fast-twitch fiber specific actinin isoform, in IBM samples. Since up to 20% of some populations normally express no ACTN3 due to a common nonsense codon polymorphism,²⁹ we performed immunofluorescent studies for this protein in 10 samples (IBM N= 5, PM N=3, DM N=4) for which proteomic data was available. All five IBM samples had visible ACTN3 expression present in existing type 2 fibers, excluding homozygous null mutation of ACTN3 as a possible reason for the loss of this protein. The three samples with the lowest proteomic quantified ACTN3 levels, all with IBM, had decreased ACTN3 staining intensity, while the other seven samples had qualitatively normal ACTN3 intensities (Supplementary Figure 1).

The Magnitude of IBM Fast-Twitch Protein Depletion is Greater than the Corresponding Transcript Depletion

We compared proteomic data with gene transcriptional data for fast- and slow-twitch proteins from 5 IBM samples for which both proteomic and microarray transcriptional profiling was performed. Although fast-twitch proteins were reduced in comparison to slow-twitch proteins, transcript levels were relatively similar (Figure 4A).

We also compared fiber type proportion histochemical data with gene transcriptional data in IBM and PM. For 17 patients (9 with IBM, 8 with PM), we compared the ratio of dark to light staining on ATPase-pH-9.4 sections, a measure of the proportion of fast myosin heavy chain proteins MYH1 and MYH2 to the slow myosin heavy chain protein MYH7, with the corresponding measure for transcripts, $(MYH1+MYH2)/MYH7$. Linear regression ($R^2=0.66$; $p<.0001$) or cubic polynomial ($R^2=0.73$; $p=.0006$) fitting between histochemical data measuring protein and microarray data measuring transcript showed high correlation; for a middle range of fiber-type proportions, the fast-to-slow *MYH* transcript ratio was constant. However, at the extreme of type 2 fiber loss (at 14-17%), this fast-to-slow *MYH* transcript ratio was reduced (Figure 4B). These findings suggest that a narrow ratio of fast-to-slow *MYH* transcript can provide for a wide range of fast-to-slow MYH protein, but extreme reduction of *MYH* fast transcript results in reduction of MYH fast protein.

“Regenerating” Forms of Myosin Heavy Chain in Inflammatory Myopathies

Normal adult human skeletal muscle contains three known mature myosin heavy chains: MYH1, MYH2, and MYH7. Muscle regeneration is accompanied by production of the alternative myosin heavy chain 3 (“embryonic myosin”; MYH3) and myosin heavy chain 8 (“neonatal myosin”; MYH8). We compared relative abundances of these five myosin heavy chain isoforms in inflammatory myopathy compared to normal muscle. Proteomic analysis showed substantial upregulation of MYH3 and MYH8 protein in IBM (18-fold and 18-fold) and PM (58-fold and 30-fold) (Supplementary Figure 2). Comparison of transcriptional data with proteomic data showed that increased transcription of *MYH3* and *MYH8* was present in all 3 diseases, IBM (33-fold and 28-fold), PM (71-fold and 30-fold), and DM (17-fold and 6-fold). Immunohistochemistry confirmed the presence of MYH3 and MYH8 protein in IBM, PM, and DM (Supplementary Figure 2).

Consideration of MYH3 and MYH8 isoform abundance is important in interpreting ATPase-pH-9.4 histochemical data. MYH3 and MYH8 expressing myofibers (generally called “regenerating”) were dark in our ATPase-pH-9.4 studies, so that deficiency of MYH1 and

MYH2, combined with a surplus of MYH3 and MYH8, could account for no apparent loss of type 2 fibers in ATPase-pH-9.4 studies. This may be the case in PM, where a reduction in proteomic quantified MYH1 (Table 1) of the same magnitude as is present in IBM is however, accompanied by a much greater abundance of MYH3 and MYH8. This could explain the relatively similar total dark-staining areas of PM and normal muscle in our ATPase-pH-9.4 studies.

Deficiency of Debranching Enzyme and Other Glycolytic Enzymes May be Present in IBM Muscle

Proteomic studies showed significant reduction ($p < .05$) in four glycolytic enzyme proteins in IBM compared to normal (Figure 5 and Table 1). The greatest reduction was seen for the glycogen debranching enzyme amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL), mutations in which produce a systemic disorder that includes a distal vacuolar myopathy with some clinical and pathological features similar to IBM. AGL reduction was relatively specific to IBM compared to PM and DM. AGL was the single most reduced protein identified in IBM compared to PM. In addition, phosphofructokinase mutase (PFKM), fructose-1,6-bisphosphatase 2 (FBP2), and phosphoglycerate mutase 2 (PGAM2) were significantly reduced in IBM compared to normal. As with structural proteins, we compared transcript levels with protein levels for glycolytic enzymes. Although AGL protein was reduced 5-fold that of normal, AGL transcript was less reduced at 1.4-fold decreased from normal.

The reduction in proteomic quantified AGL in IBM was further studied through immunohistochemistry and immunoblots. We examined serial frozen sections using antibodies against AGL and fast and slow myosin heavy chains (MHCf and MHCs) in 10 IBM, 10 other inflammatory myopathy (5 PM and 5 DM), and 2 normal samples. Most IBM samples showed similar staining intensity, despite proteomic quantified reduction in AGL. However, 2 of 10 IBM samples, but none of 10 other inflammatory myopathy samples, showed faint or absent staining (Figure 6). As fast-twitch fibers are generally believed to have greater abundances of glycolytic enzymes than slow-twitch fibers, it is tempting to argue that the reduction in AGL was a by-product of fast-twitch myofiber loss. However, we found that in normal, DM, and PM muscle AGL was qualitatively more abundant in slow-twitch than fast-twitch myofibers (Figure 6F), suggesting that AGL was lost from slow-twitch fibers in IBM. Serial sections of these 2 IBM muscle samples indeed showed numerous slow-twitch fibers (expressing MHCs but not MHCf) devoid of AGL staining (Figure 6E). Immunoblots for AGL were performed on whole muscle lysates from the 2 IBM muscle samples with reduced AGL by immunohistochemistry and confirmed reduction of AGL (Figure 6G).

Proteins Increased in IBM muscle

In comparison to normal muscle, the most differentially increased proteins in IBM muscle (Table 2) included the nuclear membrane protein lamin A/C, immunoglobulin heavy chains (IGHM and IGHA), and filamin alpha (FLNA). Lamin A/C is present in the lining of rimmed vacuoles. This may reflect nuclear degeneration in IBM muscle,¹⁵ though its abundance in these studies could reflect infiltrating immune system or proliferating fibroblast cells. Although it has long been thought that there was no significant B-cell infiltration into muscle, recent studies have demonstrated the presence of antigen-matured plasma cells in IBM muscle,^{5,13} and the abundance of immunoglobulin heavy chains might reflect this process. Filamin A (FLNA), an actin-binding protein, and AHNAK nucleoprotein are both substrates of the proteolytic enzyme calpain-3. Other overexpressed proteins in IBM muscle include several components of the extracellular matrix, in agreement with increased fibrosis and connective tissue seen histologically in these specimens.

Discussion

Using proteomic, histochemical, and immunohistochemical methods, we found substantial type 2 myofiber (fast-twitch) protein loss in most IBM muscle samples. These were among the greatest amounts we have seen across a range of muscle diseases. We attempted to understand if this loss is specific to IBM or a reflection of patient age, muscle inflammation, or disease chronicity through comparisons with samples from elderly normal individuals and patients with PM and muscular dystrophy. Loss of type 2 myofiber proteins in muscle specimens from healthy elderly individuals has been reported in proteomic studies;¹¹ we found proteomic quantified type 2 myofiber protein loss in IBM (8 patients, mean age 63 years) even in comparison to elderly normal individuals (3 subjects, mean age 70 years). With regard to effects of inflammation, PM samples had significant reduction in some type 2 proteins (e.g., MYH1) suggesting that inflammation of the type seen in both IBM and PM of sufficient duration might produce type 2 protein loss in both diseases. As PM muscle biopsy samples almost always are obtained from patients with shorter duration of disease, there are no adequate control samples available to discern any additional effects of inflammation separately from disease chronicity. Nevertheless, the effects of disease chronicity alone in other muscle diseases can be assessed through comparisons with matched samples from patients with muscular dystrophy who have equivalent degrees of weakness and symptom duration as IBM. Here, we found reduction in type 2 fiber staining area by ATPase studies that was significantly greater in IBM muscle than in muscular dystrophy samples.

Little attention has previously been directed to type 2 fiber protein loss in IBM. One single study of 11 IBM patients reported type 2 fiber atrophy using ATPase methods, but it did not study other muscle diseases; comparison was made to normal and neurogenic diseases.¹ In contrast, no pattern of fiber type atrophy was found in published studies that specifically examined this issue in 10 IBM patients¹⁰ and 28 IBM patients,³⁹ and major textbooks of muscle disease do not report a specific pattern of fiber type atrophy or predominance in IBM.^{3,24,27,33,35}

Although gross atrophy of quadriceps, forearm finger flexors, and wrist flexors is a well recognized feature of IBM, to date not a single specific protein has been identified as being reduced in quantity in IBM compared with normal muscle. The approach taken here allows for the study of IBM muscle atrophy at a molecular level. Atrophy in most patients with IBM occurs through a process that preferentially results in loss of fast-twitch protein isoforms. There is no *a priori* reason to assume that all fast-twitch proteins are equally depleted in IBM muscle with type 2 atrophy based on ATPase histochemistry, given that this method reflects only proportions of myosin heavy chains. Indeed, the proteomic approach here suggests that certain specific fast-twitch proteins (e.g., ACTN3 and MYBPC2) are more severely depleted than others. The mechanism of this loss is unknown, but we have some evidence that a failure of gene expression is not the cause, as we found that transcript abundance is less affected than protein abundance. Potential other mechanisms involve failure of protein translation or excessive protein degradation; our studies provide no indication of which of these mechanisms might be involved.

Two other published studies have used proteomic approaches to study IBM muscle. One study used 2-dimensional gel electrophoresis to separate proteins and identify regions of interest in the gels based on quantitative measures of gel-spot intensity, and used mass spectrometry to identify but not quantitate proteins.²⁶ From among approximately 900 spots per gel, 22 were identified as having different intensities in 4 IBM compared to other inflammatory myopathy samples; western blot and immunohistochemical studies provided further confidence for increased amounts of 3 proteins (Apo-A1, HSP27, and DJ-1). Though

an immunohistochemical study had provided evidence for increased amounts of alpha-B crystallin (α BC) protein in IBM muscle compared with other inflammatory myopathy muscle,⁴ this gel-spot intensity study reported decreased amounts of α BC in IBM muscle. No western blot studies that assess α BC quantity in IBM muscle have been reported. Poor reproducibility of the 2-D gel spot intensity approach to protein quantitation in IBM muscle was noted in a highly detailed study analyzing between 2272 and 4522 gel spots in each of 51 silver-stained gels from 6 patients with IBM and 5 without neuromuscular disease; no uniquely increased or decreased spots were present.²¹ Our approach is distinct from these studies in that we have used mass spectrometry to both identify and directly quantitate protein species without the use of gel electrophoresis. For this reason, we are able to detect many poorly soluble myofibrillar proteins whose detection is troublesome using 2-D gels.

Our studies also provide evidence for reduction in the quantity of several glycolytic pathway proteins in IBM muscle. As these enzymes are generally believed to be more abundant in type 2 than type 1 myofibers, their loss is in agreement with the data that demonstrate loss of type 2 structural proteins. Reduction in debranching enzyme AGL protein from most but not all IBM muscle samples is of unknown significance and consequence. We are unaware of any previous studies that demonstrate which, if either, fiber type predominantly expresses AGL; our studies of normal and diseased muscle consistently demonstrated more intensely staining immunoreactive AGL in type 1 than in type 2 fibers. The proteomic quantified reduction of AGL in IBM was present even in some specimens with normal immunohistochemical staining of muscle sections. Two IBM patients had severely reduced quantity of AGL by immunohistochemistry and by immunoblots, and the loss in these IBM patients occurs from both fiber types. A vacuolar myopathy with distal weakness, features also seen in IBM, may occur in patients with AGL mutations. The functional consequences for this deficiency in IBM, such as whether this may contribute to the loss of strength in this disease, are unknown.

Of the previously reported proteins accumulated in IBM muscle, we identified only one (lamin A/C) as increased in abundance. We did not detect even one peptide from β APP under conditions that allowed us to identify approximately 2000 gene products (data not shown) even though β APP is readily detectable in platelet preparations using similar methods (K. Parker, data not shown). The muscle lysate resuspension and digestion approach used here should favor the detection of poorly soluble and heterogeneous proteins (like collagen and titin), which were found in abundance in diseased and normal samples. Although mass spectrometric profiling of protein abundances is less sensitive than the immunohistochemical methods that have been used to conclude that there are many overexpressed proteins in IBM myofibers, the complete lack of detection of any of these by our methods is of concern. With regard to β APP, we can only say that we did not detect it through our less sensitive method. We note, however, that several published immunohistochemical studies have also failed to visualize β APP and A β in IBM muscle (3 independent laboratories studying a combined 35 patients);^{25,28,37} β APP protein and transcript have been reported in “regenerating” muscle fibers in all control diseases studied, including dermatomyositis, polymyositis, Duchenne muscular dystrophy, and amyotrophic lateral sclerosis, by the same laboratory that reported a specific pathogenic role for these molecules in IBM;^{2,34} and β APP transcript was found to be at least as highly expressed in polymyositis muscle as in IBM and higher in dermatomyositis muscle than IBM.³⁶ The continued emphasis on β APP and A β as pathogenic elements in IBM in the face of this already existing evidence, most of which was published more than 13 years ago, seems puzzling.

Two proteins we found overabundant in IBM muscle - filamin A (FLNA), an actin-binding protein, and AHNAK nucleoprotein - have not previously been implicated as being relevant

to IBM. These proteins are both calpain-3 substrates and are believed to accumulate in response to calpain-3 functional deficiency.²⁰⁻³⁸ Their expression was significantly increased in IBM in comparison with PM and DM. These findings could indicate functional calpain-3 deficiency in some IBM muscle samples.

The methods we have applied to inflammatory myopathy muscle have many variations and some limitations. Liquid chromatography followed by mass spectrometry requires peptides in solution, so that varying methods of solubilization of muscle result in differing peptide fractions and differing protein identifications. In a recent study, human vastus lateralis muscle was separated by SDS gels, resulting in the identification of 954 different proteins.¹⁸ Using similar methods on the soluble compartment alone, we have extended this number to about 2000 distinct protein identifications per sample (unpublished data), but the expense of such approaches rise rapidly with the number of proteins identified. One of the limitations of our approach is variation in protein abundances even between samples within the same disease category, just as pathological examination of specimens under the microscope may differ substantially among specimens from patients within the same disease category. This likely reflects the many confounding variables, such as age, severity, disease chronicity, the small volume of muscle tissue sampled compared to the total volume of a person's affected skeletal muscle, and other factors unique to each particular patient.

Lastly, the findings of greater loss of type 2 fiber proteins in IBM muscle suggest that corticosteroids could have potentially harmful effects in this disease. It is widely recognized that these drugs lack efficacy in IBM, but their known effects on producing type 2 fiber atrophy might be predicted to worsen this aspect of pathology. It is possible that potential modest benefits of one therapy may be masked by use of corticosteroids as a second concurrent therapy, as occurred in a controlled trial of intravenous immunoglobulin with prednisone.⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Specific reduction of fast-twitch but not slow-twitch proteins in inclusion body myositis muscle

Corresponding protein isoforms present in fast-twitch (type 2) and slow-twitch (type 1) myofibers compared in inclusion body myositis (IBM), polymyositis (PM), and dermatomyositis (DM). In inclusion body myositis, whereas multiple fast-twitch isoforms are reduced, most of the corresponding slow-twitch isoforms are either normal or present at levels greater than normal. Error bars indicate 1 standard error of the mean.

Figure 2. Whole section tiled images stained with ATPase-pH-9.4 method show reduced fast-twitch myosin heavy chain staining
(A,B) IBM shows reduced area of dark-staining fast-twitch myofibers, compared with age and disease duration matched PM (C,D) and age matched normal (E,F) muscle. All samples A-F are from biceps brachii muscle. (G) Proportion of fast-twitch to slow-twitch (dark to light staining) myofiber area of total section images computed with image analysis software for 70 muscle samples from a wide range of neuromuscular diseases. The loss of dark-staining fast-twitch myofiber area in IBM is substantial and to a degree greater than seen in other classes of disorders. Thirteen of 18 IBM samples (marked with *) cluster among the cases with the lowest percentage of fast-twitch myofiber surface area.

Figure 3. Immunohistochemistry for myosin heavy chains fast (MHCf) and slow (MHCs) in inflammatory myopathy muscle

Comparison of paired serial sections. (A) IBM. There are many small MHCf staining myofibers. The MHCs-positive myofibers are larger. Most of the larger MHCf-positive myofibers are hybrid fibers, expressing slow myosin heavy chains as well. Comparison with DM and PM in (B and C). Representative images shown. Grayscale version of original color image.

Figure 4. Comparisons of microarray measured transcripts with proteins measured by proteomics and by histochemistry

(A) Proteomic quantitated fast-twitch proteins are less abundant in IBM muscle than slow-twitch proteins, but transcript abundances are relatively similar. (B) Histochemically quantitated myosin heavy chains (MYH) compared to transcripts in IBM and PM. With ATPase-pH-9.4 histochemistry, the proportion of dark to light staining represents the proportion of fast-twitch myofibers (containing either MYH1 or MYH2 proteins) to slow-twitch myofibers containing MYH7 protein. This proportion was therefore compared with a fast-to-slow transcript ratio computed as the $(MYH1+MYH2)/MYH7$ transcript abundances. Cubic polynomial modeling of this relationship suggests that transcript levels vary little

across a middle range of fiber-type proportions (approximately 20-50% fast-twitch), but at the extremes of fiber-type depletion, transcript ratios drop or rise accordingly. Error bars indicate 1 standard error of the mean.

Figure 5. Reduction in glycolytic enzyme protein levels in inflammatory myopathy muscle
 (A) Proteomic quantification of enzymes involved in glycolysis (listed sequentially from left to right on the x-axis) in IBM, PM, and DM muscle compared to normal muscle. An * indicates proteins for which there is significant reduction ($p < .05$) in IBM compared to normal: the glycogen debranching enzyme amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL), phosphofructokinase mutase (PFKM), fructose-1,6-bisphosphatase 2 (FBP2), and phosphoglycerate mutase 2 (PGAM2). FBP2 peptides were identified at low levels in normal (therefore not included in Table 1) but were still significantly lower in IBM ($p=.007$) and so included here. No protein was detected in normal muscle for hexokinase 1 (HK1). (B) Comparison of transcripts and proteins for IBM

for glycolytic enzymes shows greater reductions in protein than transcript for AGL and FBP2.

Figure 6. Decreased debranching enzyme AGL in some IBM muscle samples

(A-D) Muscle from 2 patients with IBM (P366 and P356) have regions of large fibers devoid of AGL immunoreactivity in comparison to normal and other inflammatory myopathy (dermatomyositis – DM). (E) Adjacent 10 µm sections show that in IBM the AGL depleted fibers are all type 1 (containing MHC-slow). (F) Dermatomyositis muscle and normal muscle (not shown) have a checkerboard pattern of AGL staining with greater intensity in fibers containing slow myosin heavy chain isoform, either type 1 fibers (marked “s”) or hybrid fibers (marked “h”), compared with fast myosin heavy chain (marked “f”). (G) Immunoblot shows reduction in the amount of AGL in IBM patient samples P356 and 366 compared to normal.

Columns indicate fold-ratios and p-values of IBM, polymyositis (PM), and dermatomyositis (DM) compared to normal. Proteins were included for which 5 or greater peptides in normal muscle were identified, and for which the fold-ratio for IBM compared to normal was 0.75 or less. p-values were calculated with Student's t-test without correction for multiple hypothesis testing. Shaded boxes indicate a p-value of less than 0.05.

Table 1
Most reduced proteins in inclusion body myositis (IBM) compared to normal

Symbol	Name	IBM/ Norm Fold	PM/ Norm Fold	DM/ Norm Fold	IBM/ Norm p-value	PM/ Norm p-value	DM/ Norm p-value
ACTN3	actinin, alpha 3	0.10	0.40	0.49	0.015	0.018	0.062
AGL	amylo-1, 6-glucosidase (glycogen debranching enzyme)	0.20	0.64	0.92	0.001	0.192	0.732
MYBPC2	myosin binding protein C, fast type	0.22	0.48	0.44	0.013	0.354	0.329
PGAM2	phosphoglycerate mutase 2 (muscle)	0.35	0.61	0.63	0.001	0.022	0.017
SRL	sarcalumenin	0.45	1.10	0.66	0.140	0.790	0.404
TNNT3	troponin T type 3 (skeletal, fast)	0.47	0.67	0.73	0.002	0.062	0.103
TRIM72	tripartite motif-containing 72	0.48	1.31	0.59	0.001	0.064	0.121
PGM1	phosphoglucumutase 1	0.51	0.63	0.91	0.152	0.012	0.340
MYOZ1	myozenin 1; FAT2; calsarcin-2	0.51	0.82	0.68	0.100	0.156	0.059
MYOM2	myomesin (M-protein) 2, 165kDa	0.52	0.65	0.62	0.005	0.006	0.013
ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast-twitch 1	0.53	0.51	0.95	0.026	0.019	0.745
NEB	nebulin	0.56	0.68	0.77	0.034	0.008	0.133
MYH1	myosin heavy chain type 2b fibers, fast	0.56	0.48	0.76	0.097	0.013	0.164
PFKM	phosphofructokinase, muscle	0.56	0.62	0.78	0.025	0.003	0.029
CASQ1	calsequestrin 1 (fast-twitch, skeletal muscle)	0.58	0.85	0.65	0.112	0.509	0.194
GOT1	glutamic-oxaloacetic transaminase 1	0.59	0.73	0.85	0.003	0.080	0.106
TTN	titin	0.61	0.78	0.75	0.361	0.007	0.062
TNNT1	troponin T type 1 (skeletal, slow)	0.61	0.83	0.52	0.020	0.471	0.107
PYGM	phosphorylase, glycogen	0.62	0.63	0.74	0.204	0.100	0.209
MYOM1	myomesin 1	0.62	0.74	0.86	0.232	0.028	0.251
TNNC2	troponin C type 2 (fast)	0.63	0.86	0.79	0.068	0.246	0.074
TNNI2	troponin I type 2 (skeletal, fast)	0.63	0.90	0.83	0.015	0.358	0.133
AK1	adenylate kinase 1	0.68	0.79	0.78	0.001	0.216	0.201

Symbol	Name	IBM/ Norm Fold	PM/ Norm Fold	DM/ Norm Fold	IBM/ Norm p-value	PM/ Norm p-value	DM/ Norm p-value
PDLIM5	PDZ and LIM domain 5	0.70	0.64	0.77	0.013	0.031	0.020
MYOM3	myomesin 3 like, novel protein	0.71	0.98	0.64	0.001	0.922	0.061
MDH2	malate dehydrogenase 2, NAD (mitochondrial)	0.73	0.88	0.75	0.140	0.121	0.085
LDHA	lactate dehydrogenase A	0.73	0.89	0.80	0.002	0.720	0.491
LDB3	zasp protein; LIM domain binding 3	0.73	0.73	0.79	0.001	0.011	0.033
HADHA	hydroxyacyl-Coenzyme A dehydrogenase	0.73	0.78	0.85	0.152	0.278	0.594
GPI	glucose phosphate isomerase	0.75	1.16	1.05	0.100	0.419	0.740
ENO3	enolase beta	0.75	0.76	0.92	0.005	0.077	0.473

Table 2
Most upregulated proteins detected in inclusion body myositis (IBM)

Columns indicate fold-ratios and p-values of IBM, polymyositis (PM), and dermatomyositis (DM) compared to normal. Proteins were included for which 5 or greater peptides in diseased muscle (either IBM, PM, or DM) were identified, and for which the fold-ratio for IBM compared to normal was 5.0 or greater. * = no fold ratios can be calculated for gelsolin, as no peptides were identified in normal samples. Shaded boxes indicate a p-value of less than 0.05.

Symbol	Name	IBM/Norm Fold	PM/Norm Fold	DM/Norm Fold	IBM/Norm p-value	PM/Norm p-value	DM/Norm p-value
LMNA	lamin A/C	27.32	14.42	18.92	0.0046	0.0921	0.0467
FLNA	filamin A, alpha	22.38	3.40	1.35	0.0485	0.2951	0.7933
IGHM	ig mu heavy chain	20.82	14.81	14.75	0.0023	0.0433	0.1652
MYH9	myosin heavy chain 9, non-muscle	20.63	13.66	3.62	0.0485	0.0317	0.3038
MYH8	myosin heavy chain, neonatal	17.99	30.13	0.58	0.0394	0.0704	0.7414
MYH3	myosin heavy chain, developmental/embryonic	17.56	58.16	1.45	0.0180	0.0912	0.8087
LUM	lumican	17.29	17.16	15.15	0.0019	0.0003	0.0003
MYH11	myosin heavy chain smooth muscle isoform	16.66	4.66	2.74	0.0456	0.0614	0.4593
COL6A2	collagen, type VI, alpha 2	13.81	13.55	8.40	0.0004	0.0016	0.0460
IGHA	ig alpha	12.45	5.49	8.54	0.0002	0.0083	0.0014
AHNAK	AHNAK nucleoprotein (desmoyokin)	11.26	5.15	2.10	0.00001	0.0210	0.1581
CAT	catalase	10.50	1.78	5.28	0.0231	0.0067	0.0024
ANXA2	annexin A2	10.35	8.21	5.55	0.0002	0.5463	0.3298
FBN1	fibrillin 1	9.62	4.48	5.44	0.0302	0.0032	0.0285
HSPA5	heat shock 70kDa protein 5	8.85	7.35	4.65	0.0005	0.0138	0.0104
C3	complement component 3	7.98	3.75	8.10	0.0012	0.0893	0.1542
VIM	vimentin	7.66	5.32	4.15	0.0071	0.0106	0.0322
COL6A3	collagen, type VI, alpha 3	6.38	6.44	4.31	0.0034	0.0005	0.0067
SERPINA1	serpin peptidase inhibitor, alpha-1 antiproteinase	6.20	5.54	6.31	0.0007	0.0095	0.0258
GSN	gelsolin	*	*	*	0.0035	0.0147	0.1096