Proteasomes in Distal Myopathy with Rimmed Vacuoles

Toshihide Kumamoto, Shin Fujimoto, Shin-ichiro Nagao, Tomoko Masuda, Rie Sugihara, Hidetsugu Ueyama and Tomiyasu Tsuda

In a previous report we suggested that muscle fibers in distal myopathy with rimmed vacuoles (DMRV) were degraded by both lysosomal proteolysis (cathepsins) and Ca\(^{2+}\)-dependent, nonlysosomal proteolysis (calpain). Given recent evidence of abnormal ubiquitin accumulation in rimmed vacuoles, we examined the role of the ATP-ubiquitin-dependent proteolytic pathway (proteasomes) in myofiber degradation in this myopathy. Immunohistochemically, proteasomes (26S) were located in the cytoplasm in normal human muscle, but the staining intensity was weak. Quantitative analysis showed more reactivity for proteasomes in DMRV muscles and, to a lesser extent, in muscles from muscular dystrophy, polymyositis, and amyotrophic lateral sclerosis patients. In DMRV, proteasomes often were located within or on the rim of rimmed vacuoles, and in the cytoplasm of atrophic fibers. Ubiquitin accumulation was marked within rimmed vacuoles and was seen less extensively in the cytoplasm of atrophic fibers. The latter proteins colocalized well. In other diseased muscles, proteasomes and ubiquitin showed a positive reaction in the atrophic or necrotic fibers. The results indicate increased proteasome and ubiquitin in these muscle fibers as well as in other diseased muscle fibers. We suggest that the ATP-ubiquitin-proteasome proteolytic pathway as well as the nonlysosomal calpain and the lysosomal proteolytic pathway may participate in the muscle fiber degradation in DMRV.

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Key words: lysosome, muscle degradation, calpain, cathepsins, ubiquitin

Introduction

In distal myopathy with rimmed vacuoles (DMRV), which may be inherited as an autosomal recessive trait, muscle fibers show numerous rimmed vacuoles with the properties of autophagosomes or autolysosomes (1–3). Our previous ultrastructural and immunohistochemical studies of rimmed vacuole contents have revealed numerous degenerating mitochondria, glycogen granules, and cell membrane fragments, and myofibrillar proteins such as myosin, \(\alpha\)-actinin, and actin (3). Previous authors have found increased immunostaining for cathepsins B and L (lysosomal proteases), and calpain (a nonlysosomal intracellular calcium-dependent protease) in DMRV muscle fibers (3–5). We therefore have speculated that the degenerative process in this myopathy progresses mainly through the lysosomal autophagic process and to some extent also via the calpain system (1, 3–5).

Recently some authors have observed that rimmed vacuoles and some vacuole-free fibers in DMRV and inclusion body myositis contain abnormally high amounts of ubiquitin (6, 7), a 76 amino acid intracellular protein present in all eukaryotic cells which targets abnormal or normal proteins for highly selective breakdown by an ATP-dependent pathway, and which also may be involved in modifying cellular proteins (8, 9). Recent evidence has indicated that the nonlysosomal ATP-ubiquitin-dependent proteolytic protease, as a multicatalytic protease complex (proteasome), participates in muscle wasting during various catabolic states (8–14). Degradation of a protein via this pathway involves two distinct steps: signaling by the covalent attachment of multiple ubiquitin molecules, followed by degradation of the targeted protein by an ATP-dependent protease, the 26S (1,500 kD) proteasome (14), with the release of free, reutilizable ubiquitin. The proteasome is found in both the cytoplasm and nucleus of the muscle cell and may be responsible for muscle fiber degradation including breakdown of myofibrillar proteins such as myosin and actin (15–17). Despite this possibility, the intracellular location of proteasomes...
has not been studied in normal and diseased human muscle, including muscle affected by DMRV.

To elucidate the possible role of the ubiquitin-proteasome dependent proteolytic pathway in the muscle fiber degradation of DMRV, we performed histochemical and immunohistochemical studies on muscle biopsy specimens obtained from patients with this myopathy and other neuromuscular disorders.

Patients and Methods

Patients

Muscle biopsy specimens were taken from the biceps or rectus femoris of 19 patients: 5 with DMRV; 4 with Duchenne or Becker muscular dystrophy (DM/BMD); 1 DMD and 3 BMD; ages 12 to 34 years; 5 with polymyositis (PM); ages 37 to 77 years, and 5 with amyotrophic lateral sclerosis (ALS); ages 44 to 65 years; Table 1). All DMRV patients fulfilled clinical, neurophysiologic, and myopathologic criteria for late-onset DMRV described by previous authors (2, 3, 18). DMRV patients included three women and two men with a mean age of 42.6 years (range, 30 to 58 years; Table 1). Cases 2 and 3 were siblings, probably representing autosomal recessive inheritance; other occurrences were sporadic. The mean age at onset of illness was 32 years (range, 25 to 48), and mean illness duration was 10 years (range, 3 to 20). Serum creatine kinase levels were slightly elevated except in patient 2. Electromyography revealed primarily myopathic changes in all cases, but also included some neuropathic features except in case 5. Biopsies were taken from the biceps brachii (cases 1, 2, 3, and 5) or rectus femoris (case 4) under local anesthesia. Portions of biceps brachii or rectus femoris muscles removed in orthopedic procedures from five patients with no neurologic deficits (age range, 37 to 68 years) were used as control specimens.

Histochemical and immunohistochemical studies

Muscle biopsy specimens have frozen rapidly in isopentane cooled in liquid nitrogen. Serial transverse-sections 10 μm thick were cut using a cryostat. Frozen sections were stained using routine histochemical methods (19).

For the immunohistochemical study, unstained frozen sections were fixed in acetone for 10 minutes. Each section was incubated for 10 minutes in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) containing 10% (w/v) nonfat dry milk and then washed. The specimens then were incubated with polyclonal primary antibodies for 1 hour at room temperature. Polyclonal antibodies specific either for cathepsins B and L or ubiquitin were diluted 1:100 with PBS, while monoclonal antibodies for the 26S proteasome was diluted 1:40 in TBS. After washing, either biotinylated goat anti-mouse immunoglobulin G (IgG) or alkaline phosphatase-conjugated goat anti-rabbit IgG were added respectively for 30 minutes. Respective specimens then were incubated for 30 minutes using a Vectastain ABC Kit or Alkaline Phosphatase Substrate Kit III (Vector Labs., Burlingame, CA). Sites of antibody binding were visualized by staining with 0.02%, 3,3'-diaminobenzidine tetrahydrochloride (DAB) plus 0.01% H₂O₂ for 8 minutes when using the Vectastain ABC Kit.

Immunostaining was shown to be specific, as no staining was obtained when sections were allowed to react without first-layer antibodies or with normal rabbit serum substituted for first-layer antibodies.

Quantitative analysis

The number and diameter of strongly proteasome-positive fibers were counted in all muscle fibers of each muscle specimen, totaling 1,018 muscle fibers for all DMRV specimens, using a Nikon Cosmazon ISA image analyzer (Nikon, Tokyo) attached to an NEC PC-9801 vm computer (NEC, Tokyo). Differences between control and DMRV groups were evaluated

Table 1. Frequency of Strongly Proteasome-Positive Fibers in Muscles of Neuromuscular and Control Patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Case No.</th>
<th>Age</th>
<th>Sex</th>
<th>Biopsy</th>
<th>Rimmed vacuoles</th>
<th>Total number of muscle fibers/case</th>
<th>Proteasome-positive fibers</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMRV</td>
<td>1</td>
<td>30</td>
<td>M</td>
<td>Bi</td>
<td>20.0%</td>
<td>2,502</td>
<td>3.7%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40</td>
<td>M</td>
<td>Bi</td>
<td>14.6%</td>
<td>490</td>
<td>21.2%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31</td>
<td>M</td>
<td>Bi</td>
<td>21.5%</td>
<td>2,721</td>
<td>4.0%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>58</td>
<td>F</td>
<td>Bi</td>
<td>3.5%</td>
<td>1,190</td>
<td>12.4%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>54</td>
<td>F</td>
<td>Bi</td>
<td>5.5%</td>
<td>2,037</td>
<td>10.5%</td>
<td>–</td>
</tr>
<tr>
<td>DMRV, mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.4±7.2%</td>
<td>2,502±7.2%</td>
<td>3.7±0.1%</td>
<td>0.013</td>
</tr>
<tr>
<td>DMD/BMD</td>
<td>(n = 4)</td>
<td>12–34</td>
<td>M (4)</td>
<td>Bi</td>
<td>0</td>
<td>778–1,367</td>
<td>3.4±3.1%</td>
<td>0.044</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>(n = 5)</td>
<td>37–77</td>
<td>M (2)</td>
<td>Bi</td>
<td>0</td>
<td>292–3,884</td>
<td>3.9±3.4%</td>
<td>0.037</td>
</tr>
<tr>
<td>ALS</td>
<td>(n = 5)</td>
<td>44–65</td>
<td>M (2)</td>
<td>Bi</td>
<td>0</td>
<td>883–1,751</td>
<td>5.6±6.1%</td>
<td>NS</td>
</tr>
<tr>
<td>Control</td>
<td>(n = 5)</td>
<td>37–68</td>
<td>M (2)</td>
<td>Bi</td>
<td>0</td>
<td>844–1,171</td>
<td>0.1±0.1%</td>
<td>–</td>
</tr>
</tbody>
</table>

*These values represent a range, 0.1 ±0.1%b each value is the mean ± standard deviation. DMRV: distal myopathy with rimmed vacuoles, DMD/BMD: Duchenne or Becker muscular dystrophy, ALS: amyotrophic lateral sclerosis, Case No.: case number, n: total number of subjects, M: male, F: female, Bi: biceps brachii muscle, Rf: rectus femoris muscle, Rimmed vacuoles: frequency of rimmed vacuoles in 200 muscle fibers, Proteasome-positive fibers: frequency of strongly proteasome-positive fibers among the total number of muscle fibers, P: p value versus controls, NS: not statistically significant.
using an unpaired t test.

**Antibodies**

Polyclonal rabbit antibodies against bovine muscle calpain and against rat liver cathepsins B and L were provided by Prof. D. E. Goll (Muscle Biology Group, The University of Arizona, Tucson, AZ) and from Prof. E. Kominami (Department of Biochemistry, Juntendo University School of Medicine, Tokyo), respectively. These antibodies were used as previously described (3, 23, 24). The polyclonal antibody to calpain reacted with both μ- and m-calpain and with both activated calpain and inactive procalpains. Polyclonal antibody raised against ubiquitin from bovine brain and monoclonal antibody against 26S proteasomes from bovine liver were obtained commercially from Biomeda (Forster City, CA) and Progen Biotechnik GmBH (Heidelberg, Germany), respectively. Both antibodies crossreacted with their human antigens (7, 17). Anti-proteasome antibody was also specific for its respective antigen as determined by Western blotting against crude human muscle extracts.

**Results**

**Histologic, histochemical, and immunohistochemical studies**

Our histologic and histochemical findings in DMRV muscles corresponded to previous reports (1–3, 18). All muscles of DMRV showed mild to moderate accumulations of rimmed vacuoles, predominantly in the atrophic fibers. These occurred in 3.5 to 21.5% of 200 muscle fibers in each specimen of DMRV, without any evidence of inflammation such as cellular response (Table 1) (3). Other diseased muscles showed respective disease-specific pathologic changes but no rimmed vacuoles. The normal control muscles had a normal appearance and no rimmed vacuoles.

Immunohistochemical findings for cathepsins B and L, and calpain were similar to those described elsewhere (3). Most muscle fibers in normal control specimens showed little or no reaction for cathepsins B and L. In DMRV, cathepsins B and L were located inside or on the rim of rimmed vacuoles (Fig. 1A, B). Additionally, positive reactions for both cathepsins occasionally were found in the cytoplasm of atrophic fibers with rimmed vacuoles (Fig. 1C).

The immunoreaction for calpain was diffusely positive in the cytoplasm of normal specimens, but its intensity was weak (Fig. 2A). Increased staining of calpain was observed mainly in small atrophic fibers with rimmed vacuoles in DMRV muscles (Fig. 2B). Occasionally the rim or the surrounding area of the rimmed vacuoles showed intense calpain staining (Fig. 2C).

The cytoplasm of normal control muscle exhibited only a faint immunohistochemical reaction for proteasomes (Fig. 3A). No difference was noted in the staining activity for proteasome between type 1 fibers and type 2 fibers.

Generally, proteasome-positive fibers were more abundant in DMRV than in either normal control specimens or other diseased muscles (Fig. 3B). Positive reactions for proteasomes varied in distribution; in some fibers with rimmed vacuoles, proteasomes were located within these vacuoles (Fig. 3C). In others it appeared randomly in the cytoplasm, primarily in small atrophic fibers (Fig. 3B, E).

In specimens from patients with DMD/BMD, PM, or ALS, proteasome-positive fibers were observed more frequently than in normal controls. Proteasome-positive fibers in DMD/BMD
Proteasomes in Distal Myopathy

Figure 2. Frozen sections of normal human muscle and muscle from a distal myopathy (DMRV) patient. Immunostaining for calpain is intracellular and diffuse in the cytoplasm of normal muscle, but the intensity is weak (A). In DMRV muscle, increased staining of calpain is found in muscle fibers, especially in small atrophic fibers with rimmed vacuoles (B). Occasionally the rim and area surrounding rimmed vacuoles showed intense calpain activity (small arrowheads; C). Bar = 50 μm.

Proteasomes, representing multicatalytic protease complexes, are believed to play a central role in the nonlysosomal ATP-ubiquitin-dependent proteolytic pathway for intracellular protein degradation (8-14). At least two forms of proteasomes are known: a 26S complex responsible for ubiquitin-conjugate degradation and requiring ATP; and a 20S proteasome (700 kD) which represents the proteolytic core of the 26S proteasome (8). Recent evidence has indicated that proteasomes may be involved in the breakdown of skeletal muscle myofibrillar proteins including actin and myosin, and in physiologic and pathologic conditions such as denervation, starvation, metabolic acidosis, cancer and sepsis (8-11, 14, 20). Significant elevation in enzyme activity, protein amount, or protease mRNA levels, as well as elevations of ubiquitinated proteins and ubiquitin, have been observed in those muscles (8, 10, 11, 14, 20). To our knowledge, however, localization of proteasomes in normal and diseased human muscle has not been studied until now, although some animal studies have been reported (15-17).

Proteasomes are distributed both in the cytoplasm and in the nuclei of skeletal muscle fibers in various species (15, 16). In the present immunohistochemical study, their distribution in normal human muscles was similar to that seen in animal muscle, although the intensity was very weak. No differences in staining activity for proteasomes were evident between type 1 and type 2 fibers.

The numbers of strongly proteasome-positive fibers were significantly greater in DMRV than in control muscle. Immunoreactivity for proteasomes was concentrated particularly in the cytoplasm of atrophic fibers. Quantitative studies confirmed that diameters of strongly positive fibers were significantly smaller than for weakly positive fibers. The result suggests that in DMRV, proteasomes increase mainly in atrophic fibers, a
Figure 3. Frozen sections of normal human muscle and muscle from a distal myopathy (DMRV) patient. The cytoplasm of normal control muscle exhibits only faint immunoreactivity for proteasomes (A). Increased staining for proteasomes is found in the cytoplasm of muscle fibers, primarily in small atrophic fibers of DMRV muscle (B). The proteasomes occasionally are located within rimmed vacuoles (large arrowheads; C, E). Ubiquitin immunostaining also is positive occasionally inside or surrounding rimmed vacuole (small arrowheads; D, F), and to some extent in the cytoplasm of atrophic fibers with or without vacuoles (F) in DMRV muscle. Proteasomes and ubiquitin often colocalize in the positive fibers (E, F: serial sections). Bar = 50 µm.
In DMD/BMD, PM and ALS, proteasome-positive fibers were also observed more than in normal controls. Most of these fibers were atrophic or necrotic fibers; these results suggest that the up-regulation of proteasome is not disease specific, and the ubiquitin-proteasome system may be involved in the muscle fiber degradation in other diseased muscles as well as DMRV muscles.

Like other tissues, muscle contains at least three different pathways for protein breakdown including the lysosomal, calcium-dependent, and ATP-ubiquitin-dependent proteolytic systems (8-11, 14). Both cathepsins B and L, involved in the lysosomal proteolytic pathway, were strongly positive inside and at the rim of rimmed vacuoles, and sometimes were stained in the cytoplasm of small atrophic myofibers with or without vacuoles as well as in some fibers of normal size, as described previously (3–5). Lysosomal proteases are considered to participate in muscle fiber degradation. We previously reported that rimmed vacuoles have properties of autophagosomes or autolysosomes (1–3). Many authors have observed that rimmed vacuoles contain various intracellular organelles or proteins such as mitochondrial remnants, glycogen, cell membrane, tau, ubiquitin, cytochrome-c-oxidase, amyloid-β-protein, and others (6, 7). These organelles or proteins may be degraded through a lysosomal autophagic process in which cathepsins B and L are activated.

Calpain is also known to be involved in myofibril or myofibrillar protein degradation, especially in the early stages, in normal (21) and in pathologic muscle, such as specimens involved by DMD/BMD (22, 23), PM, or dermatomyositis (24). The present immunohistochemical findings for calpain showed a strongly positive reaction mainly in atrophic fibers with or without rimmed vacuoles, in contrast to control muscle, suggesting an increase of calpain activity in these muscles (3). In our previous report, we hypothesized that the myofibrils as well as mitochondria, glycogen, or cell membranes in this myopathy were degraded finally through a lysosomal autophagic process. However, the breakdown of the myofibrils may be not initiated by lysosomal activation; rather it may be the result of

 change that may be responsible for intracellular proteolysis or muscle fiber degradation in this myopathy.
extralysosomal processes such as the calpain system (3).

Thus our data indicate that proteasome activity as well as cathepsins and calpain may be increased in DMRV muscle, especially in atrophic fibers, and suggest that in this myopathy muscle fibers seem to be degraded by at least these three respective pathways. However, the proteasome system may not be involved in the initial steps of myofibril disassembly (25), while nonlysosomal calpain may be responsible for these early degradative steps in DMRV (21, 23).

Accumulations of ubiquitin were observed inside and at the rim of rimmed vacuoles, and to some extent in the cytoplasm, of vacuolated or vacuole-free fibers. This protein often colocalized with proteasomes, suggesting increased activity of the ATP-ubiquitin-dependent proteolytic pathway in this myopathy (8–11, 14). Proteins, including myofibrillar proteins, degraded by this proteolytic pathway are first conjugated to the ubiquitin. In ATP-dependent reactions, ubiquitin-conjugated proteins are degraded by the proteasomes (9).

In DMRV, some authors have identified abnormal accumulations of prion protein and several proteins found in brains of patients with Alzheimer’s disease, such as β-amyloid protein and β-amyloid precursor protein, hyperphosphorylated tau, and α1-antichymotripsin, in addition to ubiquitin (6, 7). Such accumulations, then, may not be disease-specific. Strikingly, in the present work ubiquitin and proteasomes often were both present within or surrounding rimmed vacuoles. Most likely, these proteins are taken up into rimmed vacuoles by nonselective autophagy, suggesting that the most intracellular degradation of proteasomes and ubiquitin occurs in the lysosomal system (26).

Our data clearly support the major involvement of the lysosomal proteolytic pathway in muscle fiber degradation in DMRV, as described previously (3–5). However, concomitant activation of the nonlysosomal calpain and ATP-ubiquitin-proteasome proteolytic systems seems to occur.

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References