intron 14 of *COL6A1*; Patient 5: c.812G>A (p.G271D) in *COL6A2*; Patient 6: c.901G>T (p.G301C) in *COL6A2*. All patients had SSCD. CD of collagen VI: compound heterozygous mutation: c.5692delG (V898fs) and c.8237delG (pA2913fs) in *COL6A3*.

Immunocytochemical Detection and Quantitative Assay for Binding of Mutated Collagen VI. Fibroblasts from six SSCD patients, one CD patient, and one control were cultured as previously reported.[8] C2C12 cells were cultured in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) with 20% fetal bovine serum for 3 days and were differentiated to myotubes in DMEM with 5% horse serum for 5 days. For immunocytochemical observation the culture media from patients' fibroblasts, which had been adjusted to contain relatively the same concentration of collagen VI, were added to the myotubes. For quantitative assay, 2.5 times serial dilution of the media was added. Myotubes were cultured further for 3 days and processed for measurement.

Cell fixation and blocking procedures were similar to published protocol.[8] For immunocytochemical staining, samples were incubated for 1 h in mixtures of rabbit polyclonal antibody against collagen VI (Abcam, Cambridge, UK; ab6588) and rat polyclonal antibody against laminin $\alpha 2$ (Alexis, Laufelfingen, Switzerland) followed by appropriate fluorescent secondary antibodies. For quantitative assay, samples were incubated with anti-collagen VI antibody followed by horseradish peroxidase–labeled secondary antibodies and fluorescent horseradish peroxidase detection system.

Western Blot Analysis. Analysis of collagen VI in the cultured media of fibroblasts was performed according to our recent report.[8]

RESULTS

Collagen VI in the Cultured Media from UCMD Patients' Fibroblasts. Western blot analysis of cultured media of fibroblasts from patients 1–6 with SSCD showed that collagen VI was similarly secreted (Fig. 1A). Collagen VI was not secreted in culture medium of CD fibroblasts (data not shown).

Binding of Collagen VI to ECM Surrounding Myocytes. By adding collagen VI-containing cultured media of patients 1–6 fibroblasts to C2C12 myotubes, we examined the binding of mutated collagen VI to the myotubes. By immunocytochemistry, normal collagen VI in control medium bound to ECM surrounding myotubes showed costaining with laminin $\alpha 2$ (Fig. 1B). The mutated collagen VI in medium from SSCD (patients 1–6) showed marked reduction of binding to ECM (Fig. 1C: p.G284R in *COL6A1*, 1D: p.G271D in *COL6A2*). Myotubes without addition of culture medium showed no staining of collagen VI surrounding the myotubes (Fig. 1E). Quantitative binding assay demonstrated normal collagen VI bound to myocyte cultures with dose-dependence. There was remarkable reduction of binding of mutated collagen VI from SSCD patient cells with that from the CD patient as a negative control (Fig. 1J).

DISCUSSION

Our previous results indicated that many UCMD patients with SSCD have heterozygous mutations in THD of *COL6* genes.[11] Moreover, we have demonstrated that collagen VI with p.G284R mutation is secreted into the extracellular space,[8] but the produced microfilaments show weak binding to the substrate and remarkably reduced cell adhesion of fibroblasts.[8]

In this study we tried to expand the collagen VI interaction into myocyte cultures, because in SSCD, collagen VI is specifically deficient in the sarcolemma. Using cultured myotubes we demonstrated that normal collagen VI can bind to the ECM surrounding myocytes and that mutations in THD of *COL6* genes may cause reduced binding of collagen VI to ECM. This phenomenon may explain the lack of collagen VI in the sarcolemma in SSCD. Despite the relative homogenous phenotype among the mutant cells, however, in vitro binding assays revealed some variation. This suggests that collagen VI binding may not totally explain the pathomechanism in this disease, and other unknown factors may play a role. For example, a recent study suggested that in UCMD the biogenesis and secretion of mutated collagen VI oligomers from fibroblasts in skeletal muscle can be affected.[17] This discrepancy requires careful interpretation of the results and should be addressed in future studies.

Both glycine substitution and deletion of the N-terminal region of THD by mutations in *COL6* genes showed the reduction in binding to ECM surrounding myocytes, suggesting that this region may have important roles for binding to the ECM. Collagen VI microfibrils have been shown to bind to several cell-surface receptors and ECM molecules,[9,12,13,16] but the physiological binding partners that interact with the THD remain unclear. The association with these binding partners may be essential in the anchorage of collagen VI myofibrils on basal lamina.

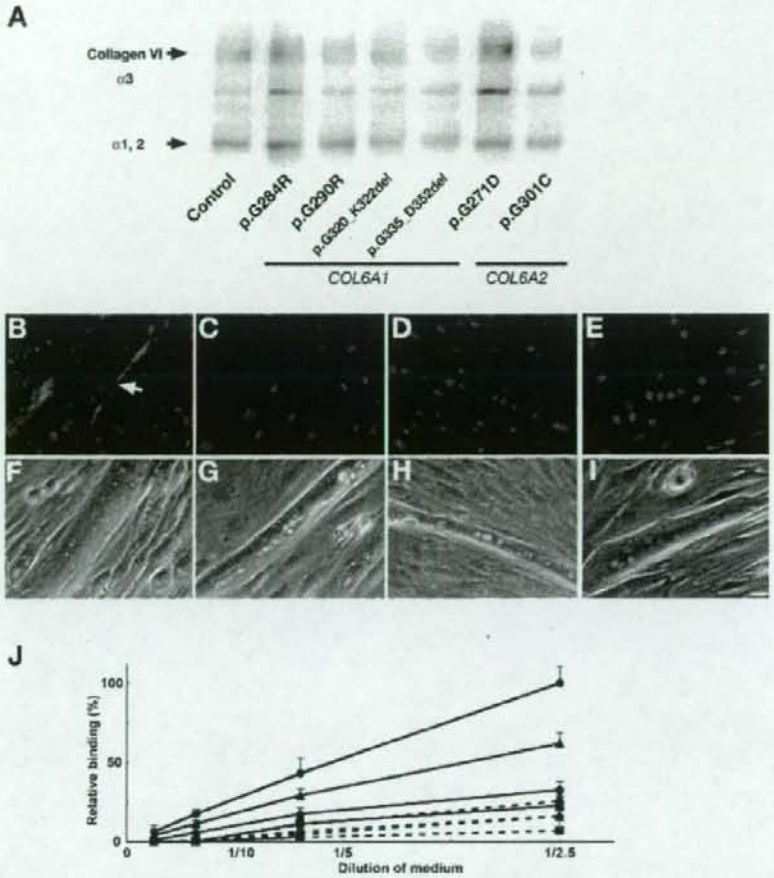

FIGURE 1. (A) Western blot analysis of collagen VI in cultured medium. Collagen VI was present in the cultured medium of fibroblasts from Patients 1–6 (lane 2–7) as well as control (lane 1). **(B–E)** Immunostaining of collagen VI (green) and laminin (red) in C2C12 myocytes after co-culture with media of patients' fibroblasts. **(F–I)** Phase-contrast images. **(B,F)** Myocytes cultured with control medium. **(C,G)** With medium of Patient 1 fibroblasts with p.G284R mutation in *COL6A1*. **(D,H)** With medium of Patient 5 fibroblasts harboring p.G271D mutation in *COL6A2*. **(E,I)** C2C12 myocytes without fibroblast medium. Collagen VI binds to ECM surrounding myotubes specifically stained with anti-laminin α2 antibody (arrow). **(J)** Quantitative binding assay of collagen VI-containing culture media in patient and control fibroblasts. Control fibroblast: circle on solid line; Patient 1: triangle on solid line; Patient 2: diamond on solid line; Patient 3: circle on dashed line; Patient 4: rectangle on solid line; Patient 5: triangle on dashed line; Patient 6: diamond on dashed line; complete deficiency: rectangle on dashed line. Error bars denote standard deviation ($n = 7$).

The clinical findings of SSCD were the same as those of CD.[11] It can be surmised that the primary deficiency of collagen VI in the sarcolemma may potentially be relevant in the pathomechanism of UCMD. Furthermore, our results suggest that anchorage of collagen VI microfibrils to the ECM of myofibers, probably basal lamina, must be extremely important in the maintenance of muscle function.

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Review

Lysosomal myopathies: An excessive build-up in autophagosomes is too much to handle

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Abstract

Lysosomes are membrane-bound acidic organelles that contain hydrolases used for intracellular digestion of various macromolecules in a process generally referred to as autophagy. In normal skeletal and cardiac muscles, lysosomes usually appear morphologically unremarkable and thus are not readily visible on light microscopy. In distinct neuromuscular disorders, however, lysosomes have been shown to be structurally abnormal and functionally impaired, leading to the accumulation of autophagic vacuoles in myofibers. More specifically, there are myopathies in which buildup of these autophagic vacuoles seem to predominate the pathological picture. In such conditions, autophagy is considered not merely a secondary event, but a phenomenon that actually contributes to disease pathomechanism and/or progression. At present, there are two disorders in the muscle which are associated with primary defect in lysosomal proteins, namely Danon disease and Pompe disease. Other myopathies which have prominent autophagy in the skeletal muscle include X-linked myopathy with excessive autophagy (XMEA). In this review, these disorders are briefly characterized, and the role of autophagy in the context of the pathomechanism of these disorders is highlighted.

Keywords: Acid maltase; Autophagy; Lysosome; LAMP-2 myopathy

1. Introduction

Lysosomes are organelles found in plant and animal cells containing hydrolytic enzymes that digest macromolecules. These membrane-bound structures represent the final destination for many endocytic (phagic), autophagic and secretory molecules targeted for destruction or recycling [1]. Accordingly, numerous cellular processes are thought to depend on lysosomal function, including the turnover of cellular proteins, inactivation and downregulation of surface receptors, supply of endocytosed nutrients, inactivation of pathogenic organisms, repair of plasma membrane, and loading of processed antigens. Acidity in the lumen of lysosomes is maintained by vacuolar ATPases to achieve effective digestion by acid hydrolases. Lysosomes bud from the Golgi apparatus. When lysosomes fuse with phagosomes, the vesicles produced by endocytosis, they become phagolysosomes. Alternatively when they fuse with autophagosomes, which are the vesicles produced by autophagy, they are called autophagolysosomes. In this essence, lysosomes are dynamically maintained fusing with endosomes in cells.

Autophagy is a highly regulated process in an organized system that can either be involved in the turnover of long-lived proteins and whole organelles (mitochondria and endoplasmic reticulum) or can specifically target distinct organelles, thereby eliminating supernumerary or damaged organelles [2]. In general, autophagy is involved in the bulk degradation of cytoplasmic components within lysosomes. It is a process by which cells adapt their metabolism to starvation, which can be imposed by decreased extracellular

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nutrients or by decreased intracellular metabolites that result from the loss of growth factor signals, including insulin [3]. By the catabolism of macromolecules and bulk degradation of organelles, autophagy generates metabolic substrates and thereby allows for adaptive protein synthesis. Although it has been established that autophagy is regulated by various factors, recent works have demonstrated that autophagy could also occur as a non-regulated spontaneous process for renewal of the molecules and organelles [4].

Skeletal muscles and neuronal tissues are the primary organs where autophagy is physiologically enhanced [5]. In several neuromuscular disorders, the accumulation of autophagic vacuoles is seen in skeletal myofibers [6–11]. Based on this pathological finding, these diseases are called autophagic vacuolar myopathies (AVM). Two disorders belonging to this group have been associated with primary lysosomal protein defects, namely, Danon disease and Pompe disease [12]. Despite the observation that generation of autophagic vacuoles can be remarkable in the skeletal and/or cardiac muscles, their precise relevance in each disorder and the mechanism by which they are formed remain to be clarified. Among AVM, in addition, a select group of myopathies has a peculiar pathological characteristic, called autophagic vacuoles with sarcolemmal features (AVSFs), because on light microscopy the autophagic vacuoles are lined by sarcolemmal proteins [13]. This latter group includes Danon disease and X-linked myopathy with excessive autophagy (XMEA). In this review, we will focus on these primary lysosomal protein deficiencies and related myopathies, highlighting the role of autophagy in the pathomechanism of the diseases.

2. Danon disease

2.1. Clinical and genetic features of Danon disease

Danon disease was originally reported as "lysosomal glycogen storage disease with normal acid maltase" because pathologic features apparently resemble those of acid maltase deficiency [14]. Historically, it has been referred to as "glycogen storage disease IIb (GSDIIb) by some authors. However, Danon disease is not a glycogen storage disease as the disease is caused by the primary deficiency of a lysosomal membrane protein, lysosome-associated membrane protein-2 (LAMP-2), instead of a glycolytic enzyme [15]. Glycogen accumulation, in fact, is not a constant feature, and detailed pathological features are different from those of acid maltase deficiency. Therefore, the disease is aptly called Danon disease, while the use of its original name, GSDIIb, should be discouraged. Danon disease is characterized by an X-linked dominant inheritance pattern, as a result of which males are more severely affected than females, although females develop symptoms at a later onset [16].

The typical clinical picture is characterized by a triad of hypertrophic cardiomyopathy, myopathy, and mental retardation [14]. Myopathy is usually mild and is evident in most male patients (90%), whereas it is seen only in one third of female patients. Weakness and atrophy predominantly affect neck and shoulder-girdle muscles, but distal muscles can also be involved. All male patients have elevated serum creatine kinase (CK) levels, even those without apparent muscle symptoms. In contrast, serum CK is elevated in only 63% of female patients. Mental impairment is variable, but is usually mild in men while it is often not seen in women. Other organs like the liver [17,18] and retina [19,20] can also be involved.

The causative protein, LAMP-2 is a single spanned membranous protein with molecular mass of 95–120 kDa. The large luminal-ectodomain is highly glycosylated with some *O*-glycans and a large number of *N*-glycans, constituting about 60% of the total mass of these proteins and divided into two homologous domains by a hinge region [21]. The transmembrane region is followed by a short C-terminal cytoplasmic tail. This cytoplasmic region has a well-conserved tyrosine residue, which is thought to provide a crucial signal for trafficking of LAMP-2 molecules to lysosomes. LAMP-2 has three isoforms, LAMP-2a, LAMP-2b, and LAMP-2c. LAMP-2a was reported to function as a receptor for chaperon mediated autophagy, in which the certain cytosolic proteins with a consensus sequence containing a sequence motif related to the pentapeptide KFERQ, such as ribonuclease A and glyceraldehyde-phosphate dehydrogenase, are selectively taken up and degraded in the lysosome [22,23]. LAMP-2b and LAMP-2c result from alternative splicing of exon 9.

LAMP-2 is mainly localized in the limiting membranes of lysosomes and late endosomes and is also found in small amounts in early endosomal membranes and the plasma membrane. LAMP-2 is also present on the limiting membrane of late autophagic vacuoles. In addition to the localization in the lysosomal limiting membrane, LAMP-2 is also detected in the lysosomal/endosomal lumen. It has been suggested that the luminal LAMP molecules are soluble, but it is also possible that these are associated with the internal membranes of lysosomes or endosomes [21].

For the transport of lysosomal membrane proteins, the function of adaptor protein complex 3 (AP-3) has been implicated, although the exact transport step mediated by AP-3 has been controversial. AP-3 is one of the four known heterotetrameric adaptor complexes, all of which play a role in the selection of cargo molecules and vesicle budding, and each of which mediates a distinct membrane trafficking step. Loss of functional AP-3 complex leads to defects in the function of lysosomes and lysosome-related organelles. Recent studies [24] demonstrated that after synthesis, lysosomal proteins reach the early endosomes either directly from the trans-Golgi network or indirectly from a pathway via the plasma membrane. These proteins will then enter the tubular extensions that emerge from the endosomal vacuoles. The default pathway from the tubular endosomes is to the plasma membrane (cycling pathway), but there is also a specific path mediated by AP-3. In this

exit, AP-3 containing membranes selectively concentrate lysosomal membrane proteins; hence in other words, this AP-3 pathway mediate transport of lysosomal membrane proteins to late endosomes/lysosomes [24].

2.2. LAMP-2 and autophagy

It has been demonstrated that LAMP-2 is required for the conversion of early autophagic vacuoles to vacuoles, indicating its involvement in the fusion of autophagic vacuoles with endosomes and lysosomes. LAMP-2-deficient mice have been generated by Saftig's group [18]. These mice exhibit elevated mortality after 20 days of age, and show accumulation of autophagic vacuoles in liver, kidney, pancreas, and cardiac and skeletal muscles. Evidence showing the failure in the normal progression of autophagic process in the absence of LAMP-2 have been presented by using cultured hepatocytes, and include: accumulation of early autophagic vacuoles; intracellular mistargeting of lysosomal enzymes and LAMP-1, instead of the elevation of lysosomal enzyme secretion; improper cathepsin D processing; abnormal retention of mannose-6-phosphate receptors in autophagic vacuoles; reduction of degradation of long-lived proteins; and non-significant induction of autophagic protein degradation after starvation.

Quantitative electron microscopy also indicated that the half life of autophagic vacuoles was prolonged suggesting that retarded consumption rather than increased formation of autophagic vacuoles was the cause of their accumulation [25]. From these findings, the group discussed that the accumulation of early autophagic vacuoles in LAMP-2-deficient hepatocytes is due to a defect in their maturation to late autophagic vacuoles that actively degrade their content.

Skeletal muscles from the patients with Danon disease show scattered small basophilic granules in myofibers, in addition to mild to moderate variation in fiber size without necrotic or regenerating process (Fig. 1, first column) [13]. Lysosomal acid phosphatase activity is enriched in these granules, showing accumulation of lysosomal organelles in myofibers. Autophagy-related proteins were also accumulated together with lysosomal proteins (Fig. 2).

Interestingly, sarcolemmal proteins (such as dystrophin and its associated proteins, the extracellular matrix proteins, acetylcholine esterase) are recruited into large vacuolar structures surrounding those lysosomal granules, forming AVSFs. On electron microscopy, these larger AVSFs are lined with a layer of basal lamina and contain small autophagic vacuoles, multilamellar bodies, and electron dense materials inside. Furthermore, vacuolar membranes with sarcolemmal features formed a closed space on serial sections [13]. Therefore, the AVSF must be independent from the sarcolemma and the inner portion of AVSF should be topologically equivalent to the extracellular space. The mechanism by which this membrane is generated remains to be clarified; sarcolemmal membrane indentation is unlikely, however, and rather de novo generation is most possible especially in cases by which mistransport of sarcolemmal proteins to intracellular vacuoles occur. Another feature of this AVSF is an increase in its frequency with aging, and this is correlated with the progression of muscle weakness [13]. Thus, AVSF may be a hallmark for progression of disease, at least in the skeletal muscle of Danon disease patients.

In LAMP-2-deficient mice, lysosomal granules in myofibers are very prominent and appear as small basophilic granules (Fig. 1, second column) [18]. In several fibers, these granules are observed to be clustered, and can surround some empty spaces (Fig. 1, second column, double arrows). Some fibers are positive for sarcolemmal proteins (Fig. 2) and acetylcholine esterase activity as the muscle in human patients, but the number of fibers with the AVSF is lower even in the muscles of older LAMP-2-deficient mice, which is probably consistent with their milder skeletal muscle symptoms.

It has been shown that a significant number of patients with hypertrophic cardiomyopathy are associated with LAMP-2 mutation, emphasizing the importance of screening for mutations in this gene among patients with non-established etiology of cardiomyopathy [7]. Expectedly, all Danon disease patients present with severe cardiac symptoms, which include cardiomyopathy with or without dysrhythmia; this parameter this regarded as the most important prognostic factor. All deceased patients suffered from cardiac failure (ages at death: 19 ± 6 years for male; 40 ± 8 years for female). On histological observation, cardiomyocytes show severe vacuolation and degeneration, including myofibrillar disruption and lipofuscin accumulation.

In LAMP-2-deficient mice, the cardiac contractile function has been analyzed in detail [26]. The hearts of these mice are enlarged to 50% of heart/body weight ratio, demonstrating cardiomyopathy. These mice display reduced ejection fraction and reduced cardiac output in heart, inferring poor cardiac function. In vitro force measurement of isolated cardiac trabeculae in LAMP-2-deficient mice showed significantly lower twitch force to half of those in wild type. In pathological observation, variation in fiber size and fibrosis are noted and become more remarkable as the mice age, in addition to the presence of increased lysosomal granules in most fibers. Grouped small basophilic granules surrounding empty spaces, like those seen in the skeletal muscle, are noted with higher frequency. On electron microscopy, large clusters of small autophagic vacuoles are seen, and these contain cytosolic and polymorphic materials. In older mice (19 months), large autophagic vacuoles are observed, which are assumed to be formed by fusion of numerous small vacuoles.

The attempt to clarify the whole functions of LAMP-2 by using LAMP-2-deficient mice has some difficulties due to the presence of LAMP-1 molecule. LAMP-1 is also lysosomal protein with high homology with LAMP-2. Both proteins show 37% sequence homology in amino acid, but the topology of the molecules, the positions of cysteine residues and heavy glycosylation are conserved. It has been

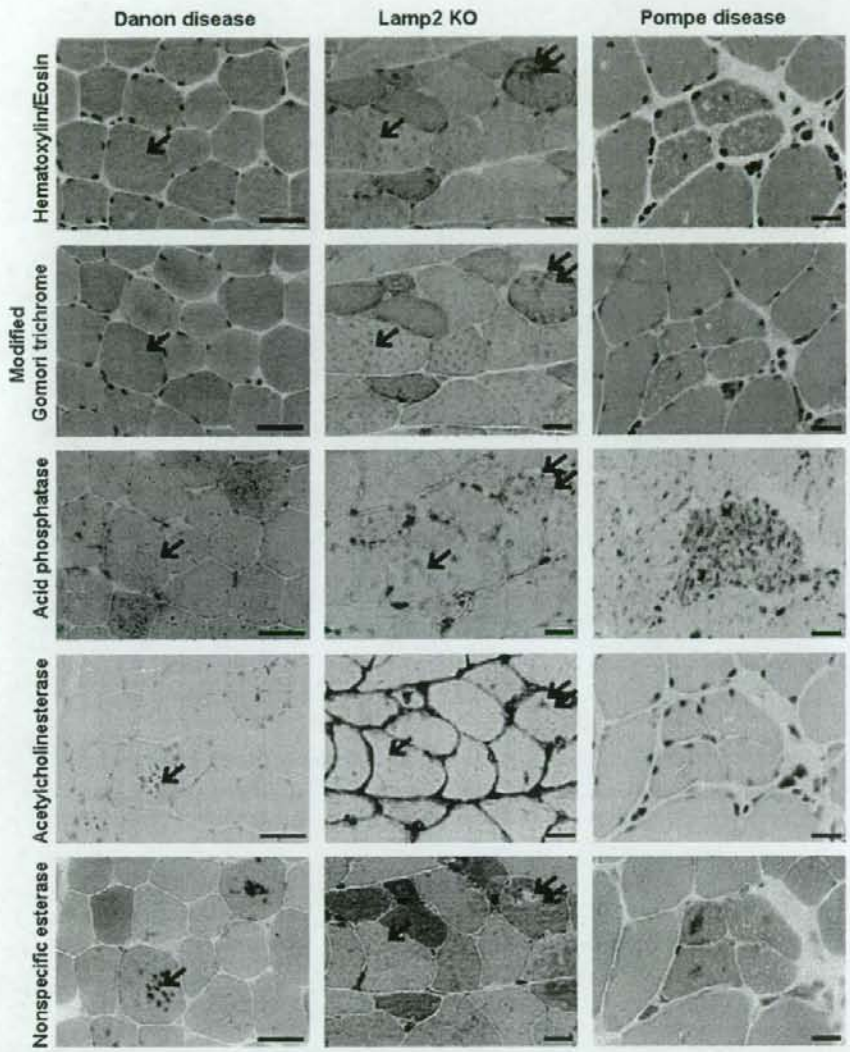

Fig. 1. Routine histochemistry of skeletal muscles from 13-year-old boy with Danon disease (first column), 9 months old LAMP-2-deficient mouse (second column), and 39 y/o woman with Pompe disease (third column). Vacuoles seen in hematoxylin and eosin and modified Gomori trichrome (arrows) are highlighted by acetylcholinesterase and nonspecific esterase. Fibers with such vacuoles have high acid phosphatase activities. In mice, basophilic granules that surround empty spaces are seen in higher frequency (double arrows) compared to Danon patients. Bars represent 20 μm.

thought that these proteins have redundant function and can compensate each other's function. In fact, in LAMP-1 mice [27], upregulation of LAMP-2 protein was observed in the kidney, spleen and heart, which suggests coupling in the regulation between LAMP-1 and LAMP-2 levels. The presence of two homologous proteins will conceal the exact functions of each protein.

Further attempts to analyze the whole function of LAMPs were done by using LAMP-1 and LAMP-2 double-deficient cells from double gene-knocked out embryos [28]. After amino acid starvation, these double-deficient cells accumulate abnormally high amounts of autophagic vacuoles which are positively stained with LC3-II, otherwise known as microtubule-associated protein light chain 3 [29]. Lysosomal vesicles were larger and more peripherally distributed when compared with control cells. However, the lysosomal enzyme activities, cathepsin D processing, and mannose-6-phosphate receptor expression levels were not affected. Surprisingly, LAMP-1 and LAMP-2-deficiencies also did not affect long-lived protein

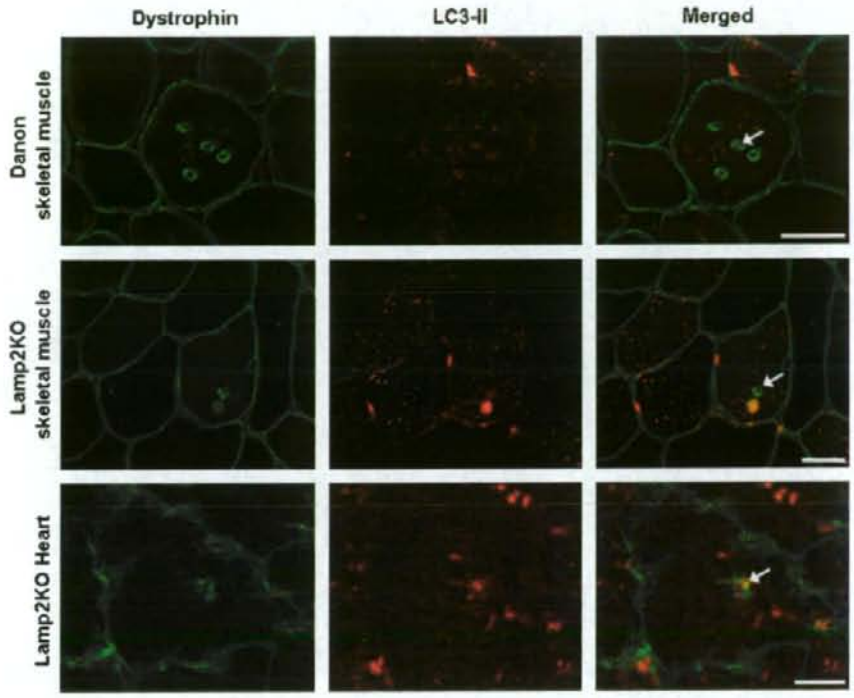

Fig. 2. Autophagic vacuoles with sarcolemmal features (AVSF). Cryosections from Danon patient and LAMP-2-deficient mouse skeletal muscle, and LAMP-2-deficient cardiac muscle are stained with dystrophin (green) and LC3-II antibodies, showing AVSF (arrows). Bars represent 20 μm (skeletal muscles) and 10 μm (cardiac muscles).

degradation rates, including proteolysis, due to chaperone-mediated autophagy. The double-deficient cells and, to a lesser extent, LAMP-2 single-deficient cells, showed an accumulation of unesterified cholesterol in endo/lysosomal compartments as well as reduced amounts of lipid droplets. The cholesterol accumulation in LAMP-1 and LAMP-2 double-deficient cells could be rescued by overexpression of murine LAMP-2a, but not by LAMP-1. Recently Huynh et al. reported that LAMP proteins are required for the maturation of phagosome. In LAMP-1 and LAMP-2 double-deficient cells, the recruitment of RAB7 to phagosomes [30] is delayed. RAB7 has been localized to late endosomes and shown to be important in the late endocytic pathway. Thus, delay in RAB7 recruitment indicates that the progression of autophagic process does not occur smoothly. In addition, the interaction between phagosome and lysosome, and the motility of lysosome and phagosome along microtubules were also affected in these LAMP-1 and LAMP-2 double-deficient cells.

By gathering the information on pathological features of Danon disease, LAMP-2-deficient and double knock-out mice, and by considering the supposed roles of LAMPs, it becomes more difficult to attribute the pathomechanism of this disease on the failure of lysosomal degradation systems. Rather, the structures created during the autophagic process or the autophagic vacuole formation may play a more important contribution to the cardiac dysfunction and muscle pathology in Danon disease. This notion can be supported by the fact that there are more autophagic vacuoles in the cardiac muscles compared to skeletal myofibers in the LAMP-2-deficient mice, whose cardiac symptoms are more severe than the muscle weakness. Moreover, not only the numerous numbers of autophagic vacuoles but also the surrounding AVSFs which occupy the center of myofibers may disturb the function of muscles and could lead to the ultimate destruction of myofibrillar structures.

3. X-linked myopathy with excessive autophagy (XMEA)

XMEA is a rare X-linked recessive AVM originally identified in a Finnish family characterized by a slowly progressive weakness and atrophy of the proximal muscles [31]. Most patients maintain independent ambulation even beyond 60 years of age. In electromyography, myotonic discharges without clinical myotonia are seen.

3.1. Pathologic and genetic features of XMEA

Muscle pathology is similar to the findings seen in Danon disease, whereby AVSFs are characteristic. Never-

theless, there are some features that are different from Danon disease, indicating that XMEA is a distinct myopathy: other characteristic features include deposition of complement C5b-9 and calcium [32] on sarcolemma; electron microscopy (Fig. 3C) shows that the basal lamina appears multi-layered [33] and that numerous exocytosed materials are within the basal lamina [10,31].

Up to this time the causative gene in XMEA has not been identified but mapped to the telomeric region of the long arm of chromosome X (Xq28). Because of the similarity in pathology with Danon, however, it has been hypothesized that the responsible gene probably encodes a lysosomal protein.

4. Pompe disease (acid maltase deficiency)

Pompe disease (or glycogen storage disease type II) is the prototypic lysosomal storage disorder [34,35]. It is an autosomal recessive disease due to primary deficiency of acid α-1,4-glucosidase which is also called acid maltase. Lysosomal acid α-glucosidase (GAA; EC 3.2.1.3) is an exo-1,4- and -1,6-α-glucosidase that specifically hydrolyzes glycogen to glucose. The cDNA for GAA encodes a protein of 952 amino acids with a predicted molecular mass of 110 kDa; furthermore, the newly synthesized precursor undergoes several steps of processing to give the 70- and 76-kDa mature forms. More than 50 mutations have been reported in the gene encoding GAA, leading to a total or partial deficiency of lysosomal GAA. As discussed later, the level of residual enzymatic activity has been correlated with location of mutations, age of disease onset, and severity of disease, although a definite genotype–phenotype correlation cannot be made. Importantly, among all enzymes responsible for glycogen storage disease, GAA is the only enzyme that is localized in the lysosomes while all other enzymes are present in the cytosol. Naturally, lysosomal abnormalities are seen only in Pompe disease among all glycogen storage diseases.

4.1. Clinical, pathologic, and genetic features of Pompe disease

The clinical spectrum of Pompe disease varies greatly [36]. Clinically this disease is classified into two forms: infantile and late-onset [37]. The late-onset form is further divided into childhood, juvenile, and adult types.

Nonsense mutations are more commonly seen in the infantile-onset form of Pompe disease as they result in nearly complete absence of GAA enzyme activity or total inactivity of the enzyme. Missense and splicing mutations may result in either complete or partial absence of GAA enzyme activity and therefore may be seen in both infantile-onset and late-onset Pompe disease [34].

Deficiency of GAA leads to accumulation of lysosomal glycogen in virtually all cells of the body, but the effects are most notable in cardiac and skeletal muscles. Abnormal lysosomal glycogen accumulates in multiple cell types, particularly in the myocytes of skeletal, cardiac and smooth

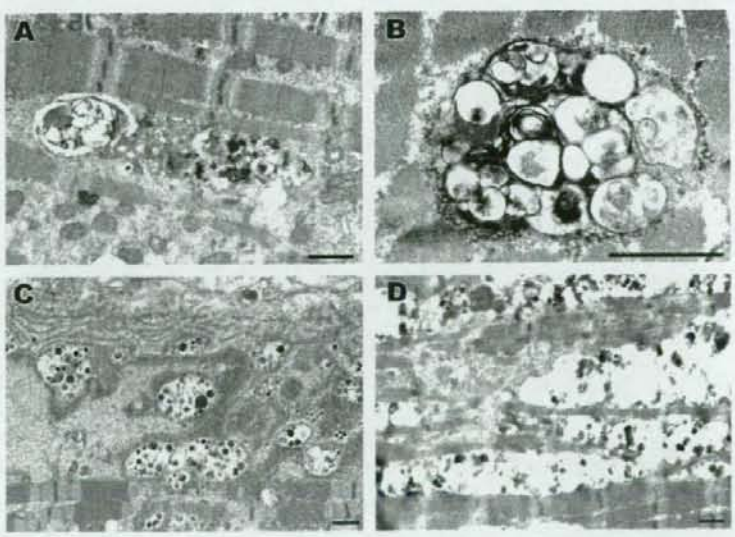

Fig. 3. Electron micrographs featuring evidence of autophagy in lysosomal myopathies. (A and B) were taken from a patient with Danon disease. Note the presence of various intracellular debris surrounded by double-membraned structures and myelin whorls in the myofibers. Autophagy is also a feature of XMEA (C). In addition to the presence of autophagic vacuoles (AVs), other characteristic observations are the duplication of basal lamina and the presence of vacuoles that are seemingly attached to the sarcolemma, indicating exocytotic process. In Pompe disease (D), the number of AVs is remarkably innumerable that they appear to almost replace myofibers. Bar represents 1 μm.

muscle, and has been detected in fetuses as early as 16–18 weeks of gestation.

In the most severe, infantile form of Pompe, disease may be apparent in utero but usually presents in the neonatal period with macroglossia, cardiomyopathy, hypotonia and respiratory insufficiency. In untreated infants, death occurs at around the first year of life due to cardiorespiratory failure. In the late-onset disease, skeletal muscle weakness predominates, and usually no cardiac involvement is seen; however, these patients often show respiratory insufficiency even when they are still ambulant. There are, nonetheless, adult patients who present with very mild symptoms thus they are often misdiagnosed as limb-girdle muscular dystrophy. Overall, there is an inverse correlation between disease severity and the level of residual enzyme activity, with the most severely affected infants having no detectable enzyme activity. Complete deficiency (activity <1% of normal controls) is associated with classic infantile-onset Pompe disease. On the other hand, partial deficiency (activity that is 2–40% of normal controls) is associated with the non-classic infantile-onset and the late-onset forms [38].

Intracytoplasmic vacuoles are prominent in the infantile form of acid maltase deficiency more than in the adult form, which can have a much milder picture in pathological observation. Characteristically, these vacuoles are so large that these occupy most of the space in many muscle fibers, often resulting in a "lace-like" appearance (Fig. 4, first column). Moreover, it has been observed that these vacuoles contain amorphous materials and are strongly stained with periodic acid Schiff staining, indicating that these are glycogen containing.

The lysosomal nature of these vacuoles is demonstrated by high acid phosphatase activity. The striking finding of these prominent vacuoles on pathology makes it less difficult to make a histological diagnosis of infantile acid maltase deficiency. On electron microscopy, the vacuoles contain cytoplasmic debris, electron dense bodies, and myelin figures, in addition to glycogen particles. Glycogen deposition is usually more prominent outside the vacuoles. However, the pathological changes sometimes may appear subtle and thus can easily be overlooked in late-onset cases (Fig. 4, third column) especially when the size of the samples is small and has artifacts.

4.2. Enzyme replacement therapy for Pompe

Enzyme replacement therapy (ERT), using recombinant human GAA, is now available in clinical practice in the US, Canada, Europe, Middle East, Latin America, and Asia Pacific [39]. As currently Pompe disease is the only hereditary muscle disease for which ERT is available, clinicians and pathologists should always consider the possibility of Pompe disease, especially in patients with late-onset form, even though it may be difficult to make a diagnosis solely based upon clinicopathological features. ERT seems to be highly effective especially in infantile cases.

Preliminary studies showed that ERT can also benefit some patients with late-onset form to some extent; however, the effect in adult cases remains to be established. The efficacy of ERT seems to be better when it is given early in the course of symptom development and before irreversible muscular damage has occurred. This notion is especially relevant for patients with severe pathological changes, where increased cytoplasmic glycogen released from lysosomes is probably inaccessible to the membrane receptor-dependent targeting mechanism [40].

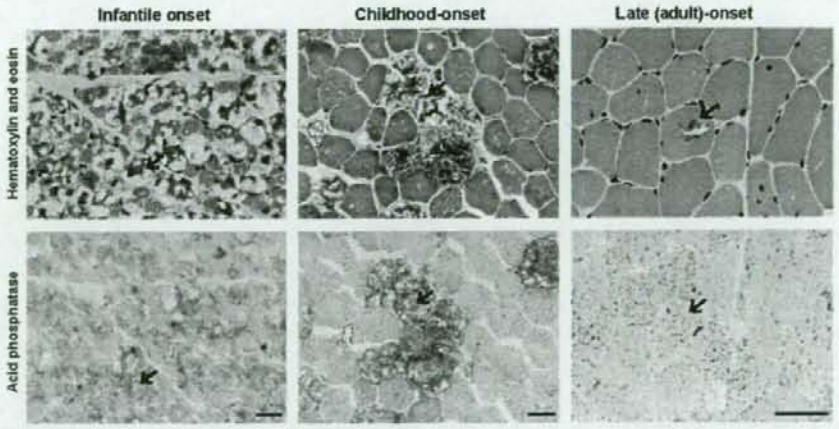

Fig. 4. Clinical forms of Pompe disease. The classic, infantile type shows the most severe pathological changes, with large acid phosphatase-positive vacuoles (arrow) which occupy almost the whole fibers, giving the so-called "lace-like" appearance. The childhood or juvenile-onset form also has these vacuoles in the myofibers, but to a much lesser extent. Very subtle changes are seen in the late-onset form, with only few fibers having intracellular accumulation of small vacuoles. Note that in all cases, the acid phosphatase staining is increased in most fibers, even in those that appear morphologically normal. Bar represents 50 μm.

4.3. Animal models for Pompe disease

The mouse models of Pompe disease have been developed by targeted disruption of the murine GAA gene by Bijvoet et al. [41,42]. In homozygous knock-out mice, glycogen-containing lysosomes are detected soon after birth in liver, heart, and skeletal muscle cells. By 13 weeks of age, large focal deposits of glycogen and lysosomal vacuolar structures were observed. Electron micrography showed lysosomal glycogen storage. Furthermore, the heart is typically enlarged and the electrocardiogram is abnormal.

The pathologic mechanism by which glycogen accumulation eventually causes muscle malfunction is not fully understood, but has been mainly considered to be secondary to the energy crisis in skeletal muscles due to failure in digesting lysosomal glycogen to glucose; as a result, muscle cells should be deprived of a necessary source of energy. With this hypothesis, however, several issues remain blur. For example, it has been shown that ERT effectively cleared glycogen accumulation in type I fibers, despite the increased accumulation of glycogen in muscle predominantly composed of type I fibers. Furthermore, this theory cannot clarify why autophagic buildup is more remarkable in type 2 muscle fibers of the Pompe disease mouse model [43].

An additional pathomechanism for Pompe can be considered, aside from the retarded metabolism of glycogen. Recently, Raben's group has excellently demonstrated the importance of autophagy in skeletal muscles, thus shedding some light in the pathogenesis Pompe disease mouse model [43]. The cellular pathology in this disease affects the pathways involved in endocytic and autophagic processes. They have reported the dramatic expansion of endocytic vesicles, decrease in mobility of late endocytic vesicles, and increase in luminal pH in a subset of late endosomes/lysosomes in GAA knock-out myoblasts. Using isolated single fibers from these mice, they demonstrated that type 2 fibers contain large regions of autophagic buildup spanning the entire length of the fibers. In addition, they found out that type 2 fibers were resistant to ERT, and this phenomenon is probably influenced by the low amount of proteins involved in endocytosis and trafficking of lysosomal enzymes combined with increased autophagy in these fibers.

On electron microscopy, type 1 fibers contained only occasional double-membrane autophagosomes, while only in type 2 fibers was autophagic buildup evident. In type 2 fibers, the autophagic regions contained vesicles with morphological features representative of various stages of the autophagic process. In addition, the intracellular microtubule network is disorganized in the area of this autophagic buildup [44]. These findings clearly indicate that in Pompe disease, failure of the lysosomal degradation of glycogen cause the extensive accumulation of various kinds of autophagic vacuoles leading to dysfunction of cellular trafficking, continuous autophagic buildup, and marked abnormality of cytoskeletal organization in muscle fibers, which may enhance the autophagic process.

5. Conclusion

The pathomechanism of lysosomal autophagic myopathies has been primarily considered to be due to the functional defect of lysosome. Recent data, however, point to another direction.

In Danon disease, mere lysosomal dysfunction cannot provide an adequate explanation by which patients develop muscle weakness. Rather, the increase in autophagic vacuoles within the myofibers could be more responsible for the disruption of myofibrillar structures, and ultimately lead to myofiber breakdown and loss of function. In XMEA, on the other hand, despite the fact that the genetic cause should be distinct from Danon, the presence of similar picture at least in terms of pathology could suggest that similar mechanism leading to muscle fiber loss exists.

Pompe disease can no longer be viewed simply as a glycogen storage disease. A number of evidence now highlights that the primary defect in Pompe disease is not entirely attributed to the accumulation of glycogen in lysosomes, but instead the massive accumulation of autophagic vacuoles which has the most profound effect on the myofibrillar organization. These unwanted and undigested intracellular debris, as a downstream phenomenon to lysosomal dysfunction, can affect endocytic trafficking which can prevent proper delivery of enzyme for therapy.

The collected data from clinical studies and biomolecular researches strongly emphasize the role of autophagic buildup in the pathomechanism of these diseases. The formation of specific structures due to the accumulation of autophagic vacuoles in the center of myofibers, the AVSF in Danon disease and XMEA, and the autophagic buildup in Pompe disease, are indeed critical for the onset of symptoms and the progression of disease.

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Recent advances in distal myopathy with rimmed vacuoles (DMRV) or hIBM: treatment perspectives

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Purpose of review

Distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy is an adult-onset autosomal recessive, slowly progressive and debilitating myopathy due to mutations in the gene that regulates the synthesis of sialic acid. This review aims to update our knowledge of this myopathy and to review studies about pathomechanism and therapeutic strategies.

Recent findings

Owing to the mutated gene, it was expected that the pathomechanism of this myopathy would be based on hyposialylation, a highly controversial phenomenon. This concept has been supported by findings in two recently generated animal models. In addition, the intracellular amyloid-β accumulation in a distal myopathy with rimmed vacuole mouse model is relevant to similar findings in patients.

Summary

Clarifying the role of hyposialylation in distal myopathy with rimmed vacuole/hereditary inclusion body myopathy could potentially lead to a therapeutic strategy for this progressive myopathy. In addition, strategies aimed at preventing amyloid-β deposition or enhancing its clearance could also be beneficial, as this epiphenomenon is now known to occur early in the course of the disease.

Keywords

amyloid, muscle atrophy, sialic acid

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Introduction

Distal myopathy with rimmed vacuoles (DMRV), otherwise known as Nonaka myopathy or hereditary inclusion body myopathy (hIBM) is an early adult-onset, slowly progressive myopathy. In general, patients become non-ambulatory 12 years after the onset [1]. As the name implies, the disease is characterized clinically by the early involvement of distal muscles and pathologically by the so-called rimmed vacuoles, which are immunoreactive to various proteins, by intracellular protein accumulation, scattered angular fibers, and by tubulo-filamentous inclusions both in the nucleus and in the cytoplasm.

DMRV/hIBM is due to mutations in the uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase/*N*-acetylmannosamine (ManNAc) kinase (*GNE*) gene [2–4], which encodes a bifunctional enzyme that catalyzes the two exclusive rate-limiting reactions of sialic acid synthesis in the cytosol [5]. Sialic acids are the most abundant terminal monosaccharides of glycoconjugates in eukaryotic cell surfaces, and – besides conferring stability to glycoproteins – they are involved in a variety of cellular functions [6,7]. Mutations in the gene responsible for DMRV/hIBM can affect the kinase or epimerase domain, occur in homozygous or compound heterozygous mode, and are not correlated to disease phenotype or severity. Mutations decrease the enzymatic activity by 70–90% [8]. The identification of the genetic cause has not explained why mutations in a gene involved in sialic acid synthesis cause a myopathy. Various theories have been proposed, and in recent years there has been increased research to verify them. We will review these theories and highlight recent attempts to clarify the pathophysiology of DMRV/hIBM.

Amyloid deposition ... another Alzheimer's disease phenotype in muscle?

Intracellular amyloid-β deposition in myofibers has been documented only in few muscle diseases, namely sporadic inclusion body myositis (sIBM) and DMRV/hIBM [9]. Although the causative gene for DMRV/hIBM has been identified, the gene or specific factor responsible for IBM remains elusive. Pathologically, the features of DMRV/hIBM are very similar to those of sIBM, except for the presence of inflammation in sIBM. Both diseases are vacuolar myopathies, and, more strikingly, both have

similar intracellular deposits that are congophilic and immunoreactive to amyloid-β precursor protein (AβPP), amyloid-β, phosphorylated tau, presinilin-1, α-synuclein, proteins related to oxidative stress, and others. Therefore, it has been proposed that, in spite of different causes, both diseases may have similar pathogenic mechanisms, at least as far as muscle is concerned.

Several studies have attempted to clarify the mechanism of amyloid deposition in IBM, comparing it with the pathomechanistic theories proposed in Alzheimer's disease, including increased mRNA expression of AβPP, mitochondrial abnormalities, and neuromuscular junction anomaly [10,11]. The role of amyloid deposition in the pathogenesis of muscle diseases has been highlighted in a sIBM mouse model, in which a correlation of intracellular amyloid levels and motor weakness was seen [11]. Increased intracellular amyloid-β can cause abnormal signal transduction, modulation of other genes, induction of mitochondrial dysfunction and oxidative stress, proteosomal activation, alteration of calcium homeostasis, and hyperphosphorylation of tau. The intracellular accumulation of amyloid-β has been difficult to conceptualize and has been controversial even in Alzheimer's disease, though recent studies have made this concept more acceptable in the field [12,13]. As a prerequisite to discuss this problem, it is important to know where amyloid-β is produced from AβPP, its precursor protein. AβPP is localized at the plasma membrane, but recently it has been shown to localize also at the trans-Golgi network, endolysosomal, lysosomal, and mitochondrial membranes, and endoplasmic reticulum. Thus, the liberation, and consequent deposition, of amyloid-β can occur wherever AβPP and the β- and γ-secretases involved in its processing are located [14•,15–18,19•]. However, the mechanism by which amyloid-β remains intracellular is still controversial. It has been proposed that newly generated amyloid-β is not secreted and remains intracellular [20,21], or that secreted amyloid-β is taken up again by the cell, as amyloid-β can bind to various biomolecules and be transported back into the intracellular pool [22–26].

As of now, however, little is known about the role of this amyloid epiphenomenon in DMRV/hIBM. It has been originally proposed that the genetic defect could somehow predispose the muscle to enter a premature state of 'ageing milieu' [9], but the mechanism by which *GNE* mutations could lead to such state is far from understood. Despite the lack of evidence connecting directly amyloidogenesis and DMRV/hIBM, the fact that amyloid deposition is an early pathological finding makes it an attractive therapeutic target.

One of the endopeptidases implicated in AβPP processing is neprilysin. This type II membrane metalloendopeptidase has an active domain containing a zinc binding motif, which is capable of degrading the monomeric and oligomeric forms of amyloid-β peptide [27]. An inverse relationship between the levels of neprilysin and amyloid-β peptide has been observed, and neprilysin has been shown to catabolize amyloid-β peptides and reduce toxicity in the brain [28]. As in muscle monomeric, oligomeric, and fibrillar forms of amyloid-β have been detected [29], neprilysin has been considered a candidate molecule that could contribute to our understanding of amyloidogenesis in DMRV/hIBM. In hereditary myopathies, Broccolini *et al.* [30] demonstrated that neprilysin expression was directly associated with the degree of muscle fiber regeneration and that in IBM neprilysin colocalized with intracellular amyloid-β deposits. Furthermore, they showed that neprilysin participates in muscle cell differentiation and regeneration, and its expression is regulated at the posttranscriptional level, showing a rapid increase in the early stage of myoblast differentiation followed by a gradual reduction thereafter. Inhibition of neprilysin activity resulted in impaired muscle differentiation that was mainly associated with an abnormal regulation of Akt activation. They suggested that neprilysin is capable of cleaving the insulin growth factor binding protein 5, thus modulating the activation of insulin growth factor-I (IGF-I)/Akt pathways within muscle fibers. These data suggest that enhancing neprilysin activity in muscle may reduce the steady-state level of amyloid-β in vulnerable fibers and promote the regeneration capacity of myofibers through the modulation of IGF-I dependent pathways.

To date, many strategies have been aimed at clearing amyloid deposition, but a discussion of these therapeutic attempts is beyond the scope of this review.

Hyposialylation: a central role in the pathomechanism of distal myopathy with rimmed vacuole/hereditary inclusion body myopathy?

As GNE is involved in sialic acid biosynthesis, it follows that decreased sialic acid, or hyposialylation, should be considered to play a role in the symptomatology or disease progression in DMRV/hIBM. *GNE* mutations reduce the UDP-GlcNAc 2-epimerase and ManNAc kinase activities, and the extent of the reduction is mutation dependent [8,31,32]. Presumably, these reduced enzymatic activities would reduce sialic acid concentration because the two enzymes are rate limiting in sialic acid biosynthesis.

Lessons from in-vitro studies

Studies analyzing the sialylation of glycoproteins have had varied results. α-Dystroglycan, a functional protein of the sarcolemma, is highly sialylated but a defect in its sialylation, albeit controversial [8,33–35], does not seem to contribute to the pathomechanism of DMRV/hIBM. This is

understandable, because a defect in the sugar chains of α-dystroglycan would result in muscle necrosis, a situation that has not been seen in DMRV/hIBM patients, except for anecdotal reports [36]. Another glycoprotein that was implicated in DMRV/hIBM is the neural cell adhesion molecule (NCAM), the most abundant polysialylated protein in mammalian cells. The presence of polysialic acid (PSA) on NCAM has been shown to decrease cell adhesion and is critical for a variety of processes, including brain development, synaptic plasticity, axon guidance and pathfinding, neurite outgrowth, and general cell migration. The expression of NCAM and PSA-NCAM in muscle is a good index of muscle regeneration. In DMRV/hIBM, NCAM has been shown to be hyposialylated [37] and its protein expression is enhanced in regenerating fibers. The upregulation of NCAM in DMRV/hIBM could be a secondary response to the presence of degenerating fibers, though it may not be effective in promoting muscle regeneration because of hyposialylation.

Neprilysin, which was mentioned earlier in this review, also showed altered sialylation. Broccolini *et al.* [38••] showed that in various inflammatory and hereditary myopathies, including hIBM, the immunoreactivity to neprilysin was increased in rare vacuolated fibers and colocalized with the amyloid-β (Aβ140) signals, besides being upregulated in regenerating fibers. Intriguingly, the amount of neprilysin is lower in hIBM than in control muscles, in contrast to the increased protein expression in inflammatory myopathies (polymyositis and dermatomyositis) and in muscular dystrophies (both Duchenne and Becker). These findings were corroborated by enzymatic analysis of neprilysin. As neprilysin is characterized by the presence of several *N*-glycosylation sites and contains large amounts of sialic acid, changes in these sugar moieties affect its stability and enzymatic activity. Interestingly, in glycoprotein-enriched hIBM muscles, the affinity of neprilysin to *Macckia amurensis* lectin (MAL), which binds to glycoproteins through specific sialylated structures, is remarkably reduced, a sign of hyposialylation. This finding was supported by further in-vitro studies, whereby differentiated myotubes from hIBM patients and normal controls treated with neuraminidase (an enzyme that cleaves sialic acid from cell surface) showed reduced neprilysin enzymatic activity and reduced binding to MAL. In addition, myotubes experimentally de-sialylated with neuraminidase showed deposition of amyloid-β, which colocalized with neprilysin immunosignal. This work has clearly shown that neprilysin is indeed hyposialylated, and this is accompanied by reduction of enzymatic activity. It is, however, not clear why this phenomenon (at least in experimental de-sialylation of cell membrane in myotubes) should lead to increased intracellular amyloid-β accumulation, because, though neprilysin can cleave AβPP, its physiologic location in the plasma membrane does not explain the intracellular cleavage of AβPP. This raises the possibility that experimental de-sialylation might have triggered other mechanisms capable of inducing intracellular accumulation of amyloid-β.

Clues from animal models

The importance of the *GNE* gene in growth and development was highlighted by the discovery that inactivation of the *Gne* gene is embryonically lethal in mice [39]. Although various strategies had been used to generate an animal model for this disease, to date only few animal models have been published. In the knock-in mice carrying M712T, the most common *GNE* mutation among Iranian Jews, homozygous pups (harboring the mutation in both alleles) did not survive beyond the third postnatal day [40••]. Despite the reduced epimerase enzymatic activity, no obvious myopathic phenotype was noted, at least by morphological analysis of muscle. Instead, and rather surprisingly, these mice developed a severe renal phenotype characterized by proteinuria, severe glomerular disease, and podocytopathy that led to their early demise. Interestingly, the mice also exhibited reduced sialylation of podocalyxin, a major podocyte sialoprotein. Administration of the sialic acid precursor ManNAc to pregnant mice increased the survival rate of pups beyond P3 and corrected the hyposialylation of podocalyxin. The role of sialic acid in glomerular disease has been attributed to the reduction of sialic acid and not the loss of sialoglycoproteins [41]; however, no kidney abnormality has ever been reported in DMRV/hIBM patients. Other factors may contribute to the M712T phenotype, as GNE also enhances the activity of the sialyltransferases, GM3 synthase, and GD3 synthase, thereby increasing the synthesis of gangliosides GM3 and GD3 [42], which are also expressed in podocytes and contribute to the charge characteristics of the filtration barrier. Thus, knock-in mice carrying the M712T mutation showed the importance of sialic acid in maintaining the glomerular filtration barrier. It would be interesting to know if organs other than the kidneys were involved in these mice and if such involvement could have contributed to their early demise.

Yet another mouse model showed how hyposialylation may play an important role in the pathogenesis of DMRV/hIBM. This mouse harbored the *GNE* D176V mutation on a *Gne* knockout background (GNE D176V transgenic in *Gne−/−* mouse; DMRV/hIBM mouse) and developed myopathic features similar to those of the DMRV/hIBM phenotype in humans [43••], with hyposialylation of serum and other organs, lower body mass than control littermates, and clinical weakness. At a younger age, these mice had unremarkable findings in muscle pathology, but with time they developed the pathological hallmarks of DMRV/hIBM, including intracellular deposition of amyloid-β and formation of rimmed vacuoles.

Similar to the situation in humans, muscle bulk is reduced even before pathological changes become manifest; thus it would be informative to document if these animals indeed have muscle atrophy.

In contrast to the M712T mice, the DMRV/hIBM mice do not show gross kidney abnormalities, at least at the light microscopy level. Another peculiar finding in these DMRV/hIBM mice is that intracellular amyloid-β deposition seemingly predates rimmed vacuole formation and myofibrillar degeneration [43••]. These findings are in agreement with the observation cited above that in cultured hIBM myocytes, AβPP overexpression precedes IBM-like abnormalities [44].

These DMRV mice also raise several questions. First, some animals had reduced survival rate, suggesting that we should pay closer attention at other organs. More importantly, correcting the hyposialylation in these mice could be a relevant therapeutic strategy to be ultimately used in patients.

The very different phenotypes expressed by the two mouse models need to be explained. In humans, the phenotype associated with the D176V mutations is similar to that associated with the M712T mutation. It is likely that mutation of the endogenous *GNE* gene in the M712T knock-in mouse is more severe, whereas in the DMRV/hIBM mouse, higher expression of the transgenic mutant GNE rescues the severe phenotype and allows the mice to develop milder progression and a myopathic phenotype. This notion is bolstered by the observation that mouse tissues may require a higher level of sialic acid than human tissues, as the normal serum level of sialic acids in mouse is 1.5 times higher than in humans. This concept clearly needs further investigation.

A step closer to developing therapy for distal myopathy with rimmed vacuole/hereditary inclusion body myopathy

Although the concept of hyposialylation in DMRV is still controversial, the data from animal models and various in-vitro analyses cannot be disregarded and have led to the development of various attempts to treat this crippling disorder. Noguchi *et al.* [8] showed that exposing DMRV cells to sialic acid and its precursor or both restored the sialylation status of the cells to a remarkable extent. Similarly, administration of sialic acids to DMRV/hIBM patients could help normalize the sialylation status of muscle glycoproteins and provide clinical benefit.

A pilot study employing one method to deliver sialic acid to the cells was carried out in four patients with DMRV/hIBM [45•], who were given intravenous (i.v.) immunoglobulin G (IVIG), a glycoprotein that contains 8 μmoles of sialic acid per gram. The authors claimed that the administration of a single loading dose (1 g/kg) of IVIG by continuous i.v. infusion for 2 days led to some improvement in quantitative muscle testing, qualitative improvements in activities of daily living, improved muscle strength and endurance in all four patients. However, this so-called improvement was not correlated to the degree of sialylation of the glycoproteins NCAM, transferrin, and α-dystroglycan, raising the possibilities that short-term therapy may not change the sialylation of glycoproteins. Alternatively, glycoproteins other than those included in their study need to be analyzed. Nevertheless, the possibility that the subjective improvement after therapy could be attributed to other effects of IVIG should still be considered. After this initial pilot study, no other DMRV/hIBM patients were treated with IVIG, thus the results in this study remain to be verified.

In the M712T mice, though it is remarkable that administration of ManNAc improved survival and rescued the severe renal phenotype, it is not conceivable to use this as a basis of therapy for DMRV/hIBM patients, because the efficacy of such an agent has never been demonstrated *in vivo* due to the lack of a disease phenotype in the M712T mice. Administration of ManNAc or sialic acid itself to DMRV/hIBM mice is expected to clarify issues surrounding the therapy in this disabling disease.

Conclusion

From these recent studies, it is clear that hyposialylation has a central role in the pathogenesis of this myopathy, though specific details are lacking as to how this phenomenon causes disease. Of greater practical importance, this phenomenon should not be disregarded because it opens an avenue to therapy, and trials addressing this step in the DMRV/hIBM mouse are much anticipated. Another issue that may be worth exploring is the early epiphenomenon of amyloidogenesis, which can be targeted for therapy.

Acknowledgements

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ABSTRACT: Causative genes have been identified only in four types of lipid storage myopathies (LSMs): *SLC22A5* for primary carnitine deficiency (PCD); *ETFA*, *ETFB*, and *ETFDH* for multiple acyl-coenzyme A dehydrogenation deficiency (MADD); *PNPLA2* for neutral lipid storage disease with myopathy (NLSDM); and *ABHD5* for neutral lipid storage disease with ichthyosis. However, the frequency of these LSMs has not been determined. We found mutations in only 9 of 37 LSM patients (24%): 3 in *SLC22A5*; 4 in MADD-associated genes; and 2 in *PNPLA2*. This low frequency suggests the existence of other causative genes. Muscle coenzyme Q_{10} levels were normal or only mildly reduced in two MADD patients, indicating that ETFDH mutations may not always be associated with CoQ_{10} deficiency. The 2 patients with *PNPLA2* mutations had progressive, non-episodic muscle disease with rimmed vacuoles. This suggests there is a different pathomechanism from other LSMs.

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CLINICAL AND GENETIC ANALYSIS OF LIPID STORAGE MYOPATHIES

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Defects in muscle lipid metabolism are due to a heterogeneous group of metabolic conditions. They are caused by problems in transport of fatty acids and carnitine, mitochondrial matrix β-oxidation enzymes, or endogenous triglyceride synthesis. The clinical spectrum of these disorders is variable. Patients often present with hypotonia, muscle weakness, recurrent rhabdomyolysis, and peripheral neuropathy.[21] Lipid storage myopathies (LSMs), which are categorized under the broad category of disorders of lipid metabolism, are invariably characterized by accumulation of lipid droplets in muscle fibers. Among LSMs, genetic causes have been identified in only four disorders: primary carnitine deficiency (PCD); multiple acyl-coenzyme A (acyl-CoA) dehydrogenation deficiency (MADD); neutral lipid storage disease with myopathy (NLDSM); and neutral lipid storage disease with ichthyosis (NLDSI).[1,10,21]

PCD is an autosomal-recessive disorder caused by mutations of the *SLC22A5* gene, which encodes an integral plasma membrane protein, organic cation transporter 2 (OCTN2). It functions to transport extracellular carnitine into cells.[17,22] OCTN2 mutations lead to defective renal reabsorption and reduced tissue storage of carnitine and impairment of long fatty acid metabolism, as carnitine is necessary to incorporate long-chain fatty acids into the mitochondrial matrix for β-oxidation. Clinical features of PCD include severe hypoglycemia and dilated cardiomyopathy in addition to skeletal muscle involvement.[21]

Abbreviations: ABHD5, abhydrolase domain–containing 5; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification 58; CPT II, carnitine palmitoyltransferase type II; Cho, cholesterol; CoA, coenzyme A; CoQ_{10}, coenzyme Q_{10}; ECG, electrocardiogram; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; FFA, free fatty acids; HSL, hormone-sensitive lipase; LSM, lipid storage myopathies; MADD, multiple acyl-coenzyme A dehydrogenation deficiency; NLSDI, neutral lipid storage with ichthyosis; NLSDM, neutral lipid storage disease with myopathy; OCTN2, organic cation transporter 2; PCD, primary carnitine deficiency; PL, phospholipids; PNPLA2, patatin-like phospholipase domain–containing protein 2; RT-PCR, reverse transcript–polymerase chain reaction; SDH, succinate dehydrogenase; TG, triglyceride; VLCAD, very-long-chain acyl-CoA dehydrogenase

Key words: *ABHD5*; *ETF*; *ETFDH*; lipid storage myopathy; *PNPLA2*; *SLC22A5*

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MADD, also known as glutaric aciduria type II, is an autosomal-recessive disorder of fatty and amino acid metabolism[6] caused by defects in electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH). ETF is a heterodimeric protein consisting of two subunits, α and β, that are encoded by different genes, *ETFA* and *ETFB*. ETF receives electrons from mitochondrial flavin-containing dehydrogenases to ETFDH in the inner mitochondrial membrane. ETDFH, in turn, transfers electrons to coenzyme Q. The MADD phenotype varies widely from a fatal neonatal-onset form[19,20] to a much milder late-onset form, which is often associated with a lipid storage myopathy that manifests with muscle weakness and pain. Recently, patients with ETDFH mutations were shown to have secondary coenzyme Q_{10} (CoQ_{10}) deficiency.[7]

Neutral lipid storage disease is characterized by systemic accumulation of triglycerides (TG) in the cytoplasm and includes two distinct diseases: NLSDM and NLSDI (also called Chanarin–Dorfman syndrome). NLSDM is caused by mutations in a gene that encodes adipose triglyceride lipase (ATGL), which is also referred to as patatin-like phospholipase domain-containing protein 2 (PNPLA2).[1,9,23] This protein catalyzes the initial step in TG hydrolysis. On the other hand, NLSDI is due to defects in the gene that encodes the coactivator of ATGL, comparative gene identification-58 (CGI-58), which is also known as abhydrolase domain–containing 5 (ABHD5).[10]

Although the pathological characteristics of LSM are rather uniform, the phenotypic manifestations are remarkably heterogeneous, possibly due to different genetic backgrounds. Thus, genetic analysis has always posed a challenge. In this study, we analyzed all known causative genes for LSM (*SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, and *ETFDH*), as well as *LIPE*, which encodes hormone-sensitive lipase (HSL),[8] among patients who had pathological confirmation of LSM. Our aim was to determine the actual frequency of identifiable mutations and to look for genotype–phenotype correlations that could be helpful for diagnosis.

METHODS

Patients. We retrospectively recruited cases diagnosed with LSM at the National Center of Neurology and Psychiatry (NCNP) from a total of 9639 muscle biopsies obtained between 1978 and 2006. The diagnosis of LSM was made based on characteristic muscle pathology findings: small clear vacuoles on hematoxylin and eosin staining and intramyofiber accumulation of lipid droplets on oil-red-O staining. We excluded cases with obvious mitochondrial abnormalities such as ragged-red fibers, strongly succinate dehydrogenase (SDH)-reactive vessels, and cytochrome *c* oxidase deficiency. Detailed retrospective review of the clinical and pathological findings was performed. Informed consent was obtained from the patients using a form approved by the NCNP ethics board committee.

Mutation Analysis. We sequenced all exons and their flanking regions of all the known causative genes for LSM: *SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, and *ETFDH* in genomic DNA of patients with LSM.

Genomic DNA was extracted from the muscle biopsies using a standard method.[16] We sequenced all exons and their flanking regions of *SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, *ETFDH*, and *LIPE*. Primers were designed from the genomic sequences reported in GenBank (Gene IDs: 6584 for *SLC22A5*, 51099 for *ABHD5*, 57104 for *PNPLA2*, 2108 for *ETFA*, 2109 for *ETFB*, 2110 for *ETFDH*, and 3991 for *LIPE*). We performed direct sequencing of amplified fragments using an automated 3100 DNA sequencer (Applied Biosystems, Foster City, California) with the BigDye Terminator cycle sequencing system, and analyzed DNA sequences with the SeqScape program (Applied Biosystems).

We performed quantitative reverse transcript–polymerase chain reaction (RT-PCR) in RNA obtained from muscle using the QuantiTect SYBR-Green PCR Kit (Qiagen GmbH, Hilden, Germany) and iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, California). We analyzed the amount of transcript for *ETFDH* relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA.

Biochemical Analyses. We measured CoQ_{10} in frozen muscles from patients with *ETF* mutations using a high-performance liquid chromatography (HPLC) method described previously[7] in 2 cases with enough sample size for analysis (patients 6 and 7). For muscle lipid analysis, total lipid was extracted from muscles according to the methods of Folch et al.[5] Extracted lipids were adopted to TLC with petroleum ether/diethyl ether/acetic acid (60:40:1) as a developing solvent to separate TG, cholesterol (Cho), and free fatty acids (FFA) from phospholipid (PL). The lipids were visualized with 50% sulfuric acids/methanol vapor. Band intensities were measured with Quantity One software (Bio-Rad Laboratories). We measured the levels of TG, PL, and FFA relative to Cho amount (TG/Cho, PL/Cho, FFA/Cho). Muscle

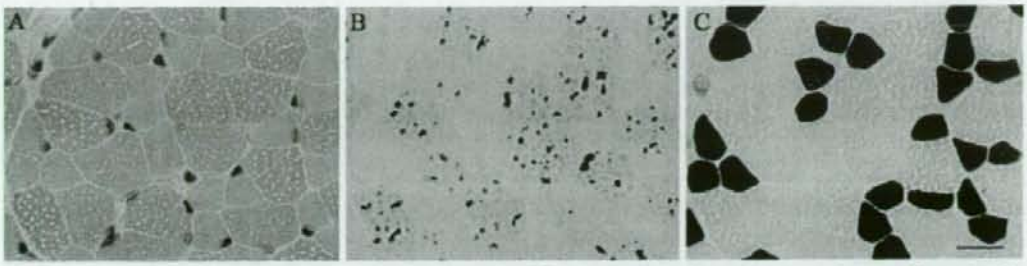

FIGURE 1. Muscle pathology in patient 1 with the SLC22A5 mutation (PCD). Numerous small vacuoles seen on hematoxylin–eosin stain **(A)** are actually lipid droplets, as shown on oil-red-O **(B)**. These vacuoles are seen predominantly in type 1 fibers **(C)**. Bar = 20 μm.

carnitine palmitoyltransferase type II (CPT II) activity was measured using a method described previously.[2]

RESULTS

Pathological and Clinical Features of LSM. Of 9639 frozen muscle biopsies that we had examined pathologically, 47 (0.5%) had LSM. In all 47 patients, there were numerous small vacuoles that were filled with lipid droplets in scattered type 1 and 2 muscle fibers (Fig. 1A, B). Measurement of the width of these vacuoles, indirectly representing the amount of lipid, did not reveal any significant differences among patients (data not shown). In addition, these lipid droplets were found predominantly in type 1 fibers (Table 1 and Fig. 1C), except in patients 10 and 11, who exhibited lipid droplets predominantly in type 2 fibers (Fig. 2).

The clinical features of the 47 patients (23 males and 24 females) are summarized in Table 1. Age at onset varied from 37 days to 75 years. Eight patients had a positive family history. The majority of the patients (55%) had muscle weakness, and all except 1 had generalized or proximal dominant muscle weakness. No correlation was found between the clinical/pathological phenotype and genotype of patients (data not shown).

Genetic Analysis of LSM. DNA was available for only 37 patients. We identified mutations in 9 (24%) patients: 3 in *SLC22A5*; 3 in *ETFDH*; 1 in *ETFA*; and 2 in *PNPLA2* (Table 2). In patient 4, we identified a heterozygous c.1519T>G *ETFDH* mutation in genomic DNA; however, by RT-PCR, only the transcript with this mutation was detected, indicating absence of transcript from the other allele. All mutations were novel except in patients 2 and 3.[11,12] We did not find similar mutations in 100 control chromosomes of Japanese individuals. In addition, we did not find any mutations in *ABHD5* or *LIPE*.

Biochemical Analysis. CoQ_{10} levels were normal in patient 7 and mildly decreased in patient 6, who had *ETFDH* and *ETFA* mutations, respectively (Table 3). The size of the samples permitted lipid analysis in only 14 patients, including 2 patients with mutations: patient 1 with PCD, and patient 6 with MADD. The amount of TG was significantly elevated in all LSM patients (TG/Cho: 12.5 ± 2.26 [mean ± standard error of mean]) when compared with control individuals (5.95 ± 1.72). In contrast, FFA were not increased, and PI, were not significantly different (data not shown). In all 10 patients tested, CPT II activity was normal.

PCD Patients. Patients 1, 2, and 3 harbored mutations in *SLC22A5*. Patient 1 exhibited normal early motor development and appeared healthy until age 8 months when she developed hepatomegaly, coma, hyperammonemia, and non-ketotic dicarboxylic aciduria. On liver biopsy, numerous lipid droplets were seen. Clinical improvement was seen with L-carnitine supplementation, but she eventually succumbed to heart failure when she had an infection. Patients 2 and 3, who are siblings, have been reported previously.[2,12] Briefly, they had slowly progressive muscle weakness and hypertrophic cardiomyopathy, and their developmental milestones were normal until 3 years of age, when mild weakness in the lower limbs became evident. Laboratory examination showed transient high creatine kinase (CK) levels and hyperammonemia. Carnitine levels were decreased in skeletal muscles of these 3 patients (data not shown). Serum carnitine was likewise reduced in patients 2 and 3. Total and free carnitine levels (in μmol/L), respectively, were: 36.1 (normal: 67.6 ± 11.3) and 12.3 (normal: 52.2 ± 10.4) in patient 2; and 35.7 and 11.4 in patient 3. L-carnitine treatment in both cases resulted in marked clinical improvement.

On muscle pathology, both number and size of mitochondria were mildly increased (Fig. 4A). Lipid-