Research Paper

Deconstructing Pompe Disease by Analyzing Single Muscle Fibers

To See a World in a Grain of Sand…"*

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KEY WORDS

ABSTRACT

Autophagy is a major pathway for delivery of proteins and organelles to lysosomes where they are degraded and recycled. We have previously shown excessive autophagy in a mouse model of Pompe disease (glycogen storage disease type II), a devastating myopathy caused by a deficiency of the glycogen-degrading lysosomal enzyme acid α-glucosidase. The autophagic buildup constituted a major pathological component in skeletal muscle and interfered with delivery of the therapeutic enzyme. To assess the role of autophagy in the pathogenesis of the human disease, we have analyzed vesicles of the lysosomal-degradative pathway in isolated single muscle fibers from Pompe patients. Human myofibers showed abundant autophagosome formation and areas of autophagic buildup of a wide range of sizes. In patients, as in the mouse model, the enormous autophagic buildup causes greater skeletal muscle damage than the enlarged, glycogen-filled lysosomes outside the autophagic regions. Clearing or preventing autophagic buildup seems, therefore, a necessary target of Pompe disease therapy.

INTRODUCTION

Autophagy ("self-eating") is an evolutionarily conserved lysosomal process of cytoplasmic degradation.[1,2] The process normally occurs at basal levels in most tissues and its main role is to provide the routine turnover of cytoplasmic components, thus maintaining a balance between the biogenesis of cell structures and their degradation. Under conditions of starvation and other forms of stress, increased autophagic activity is a critical survival mechanism.[3-5]

Three forms of autophagy are recognized: chaperone-mediated autophagy, microautophagy, and macroautophagy.[6] The central event in macroautophagy (hereafter referred to as autophagy) is the de novo formation and elongation of a membrane which sequesters a region of the cytoplasm and damaged organelles, such as mitochondria, into a double membrane-limited vacuole, the autophagosome.[1,2,4,7-9] Autophagosomes fuse with late endosomes/lysosomes[10,11] resulting in the delivery, degradation, and recycling of the sequestered components.

Autophagy has been implicated in a number of cellular and developmental processes, and in several human diseases,[12-15] including Pompe disease,[16,17] a lysosomal glycogen storage disorder caused by deficiency of acid α-glucosidase. The deficiency of the enzyme results in progressive expansion of glycogen-filled lysosomes in multiple tissues, with cardiac and skeletal muscle being the most severely affected clinically.[18-20] We recently demonstrated a profound failure of productive macroautophagy in skeletal muscle of a mouse model of Pompe disease (KO). Massive autophagic buildup in muscle fibers, rather than the buildup of lysosomal glycogen, appeared to cause progressive muscle destruction. Furthermore, we have shown that autophagic accumulation in muscle fibers was associated with resistance to enzyme replacement therapy.[21-23]

We now demonstrate that the underlying skeletal muscle pathology and the extent of autophagy in Pompe patients are similar to those in mice. Autophagy appears to play a major role in the pathogenesis of the disease, a finding that has important implications for the effectiveness of enzyme replacement therapy with the recombinant human acid α-glucosidase.

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MATERIALS AND METHODS

Subjects. Nine untreated patients with the late-onset form of Pompe disease were included in the study. All had clinical symptoms consistent with Pompe disease. The diagnoses were confirmed histologically, biochemically, and by mutation analysis. Six patients were from Italy and three patients were from Brazil. Three infantile patients were diagnosed through the newborn screening program in Taiwan; two of them (age 7 and 9 months) had been on enzyme replacement therapy with alglucosidase alfa (Genzyme Corp., Framingham, MA) for six months at the time of biopsy. Incisional biopsies were obtained from the quadriceps or the biceps muscle. Local institutional review boards at all sites approved the protocol, and all patients or parents provided written informed consent.

Isolation of fixed single muscle fibers and immunofluorescence microscopy. Fibers were prepared from muscle biopsies obtained from nine late-onset and three infantile-onset patients. Biopsy specimens were removed and pinned to Sylgard-coated dishes for fixation with 2% paraformaldehyde in 0.1 M phosphate buffer for 30 min, followed by PBS rinses and fixation in cold methanol (-20°C) for 6 min. Samples were stored in 50% glycerol in PBS at -20°C. Before use, each sample was transferred first to 25% glycerol/PBS, then to 12% glycerol/PBS, and finally to PBS/0.04% saponin. A portion of each biopsy was used for H&E staining. Single fibers were obtained by manual teasing. The fibers were placed in 24-well plate (~20 fibers/well) in 0.5 ml of blocking buffer (1% BSA, 5% goat serum, and 0.2% triton in PBS) for 1 h. The permeabilized fibers were then incubated with primary antibody overnight at 4°C, washed in PBS, incubated with secondary antibody for 2 h at room temperature, washed again, and mounted in Vectashield (Vector Laboratories, Burlingame, CA) on a glass slide for analysis by confocal microscopy. The following primary antibodies were used: rabbit antiserum to bovine cation-independent MPR (CI-MPR; 1:15,000; a gift from Dr. Stuart Kornfeld, Washington University, St. Louis); anti-LC3 (microtubule-associated protein 1 light chain 3; 1:250; a gift from Dr. T. Ueno, Juntendo University School of Medicine, Japan) and mouse anti-human LAMP2 (lysosomal associated membrane protein 2) monoclonal antibody (1:100; BD Pharmingen, San Diego, CA). Alexa Fluor 488 and 568 secondary antibodies for immunostaining were purchased from Invitrogen (Carlsbad, CA). The fibers were analyzed by confocal microscopy (Zeiss LSM 510 META).

Human myoblast cultures. Human myoblast cultures were obtained from the Muscle Tissue Culture Collection at the Friedrich-Baur Institute (Department of Neurology, Ludwig-Maximilians University, Munich, Germany). These human papillomavirus (HPV16) transformed myoblasts from control and late-onset Pompe patients (diagnosis was confirmed by mutation analysis) were prepared as described.[24] These cells show significantly extended life span compared to primary adult myoblasts due to the expression of HPV16-E6E7 genes.[24] Myoblasts were grown on collagen-coated dishes in proliferation medium containing 20% fetal bovine serum, 10% horse serum, and 1% chick embryo extract in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA). For analysis by confocal microscopy, myoblasts were plated in a chambered coverglass pre-treated with 3.5 mg/cm^2 Cell-Tak (BD Biopsciences, Bedford, MA) and cultured in proliferation medium. For starvation, the cells were cultured in Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA) for 6 h. A fibroblast cell line from a healthy individual served as a control for the starvation experiments (a starvation-induced increase in autophagy was observed
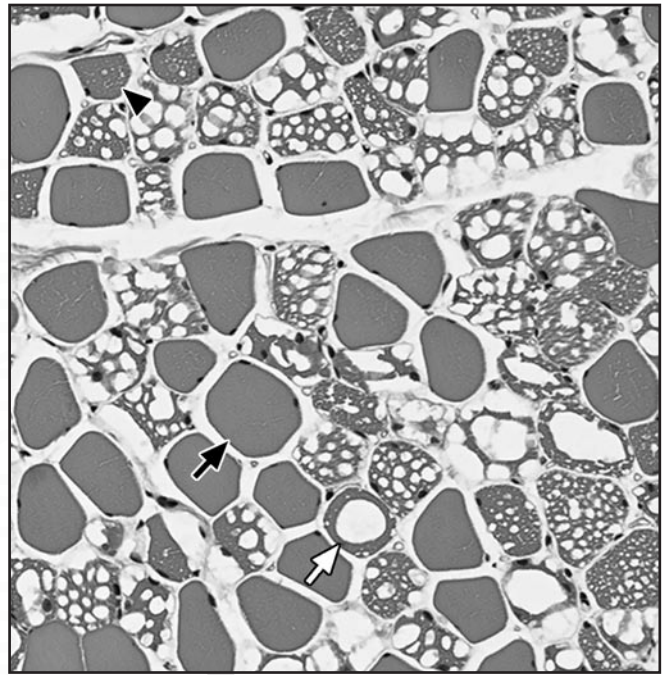

Figure 1. H&E stained section of muscle biopsy from a late-onset patient showing a typical vacuolar myopathy and a phenotypic variability among muscle cells. The black arrow points to a normal myofiber; the arrowhead points to a mildly affected myofiber; the white arrow points to a severely affected myofiber.

in these cells). Myoblasts were induced to differentiate in medium containing 2% horse serum and 0.5% chick embryo extract in DMEM for 1–4 weeks. Myoblasts and myotubes were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in pre-warmed DMEM for 30 min at room temperature, followed by fixation in cold methanol as described above for muscle fibers. Cells were blocked for 1 h, incubated with primary antibodies for 24 h at 4°C, washed, and incubated with secondary antibodies. The coverglass was then washed three times with PBS and the cells were analyzed by confocal microscopy.

RESULTS

Nine late-onset patients, ranging in age from 10–56 years, were included in the study. All were compound heterozygous and shared the most common mutation associated with adult-onset disease in Caucasian patients, c.-32-13T → G.[25,26] None had been treated with recombinant enzyme. The clinical spectrum ranged from fatigability without muscle weakness to severe hypotonia, limited mobility, and wheelchair dependence. More severe clinical manifestations were associated with a higher percentage of vacuolated fibers in H&E stained muscle biopsies (data not shown) in all but one patient. In this severely affected patient, the biopsy looked nearly normal. An example of a biopsy from a severely affected patient is shown in Figure 1.

Confocal microscopy was used to analyze single muscle fibers stained for a specific late endosomal/lysosomal marker, LAMP2, and for a specific autophagosomal marker, LC3.[27] Analysis of multiple individual fibers (50–100 fibers from each patient) allowed us to identify the apparent pathological stages of progression—from very small to very large areas of autophagic buildup accompanied by fiber
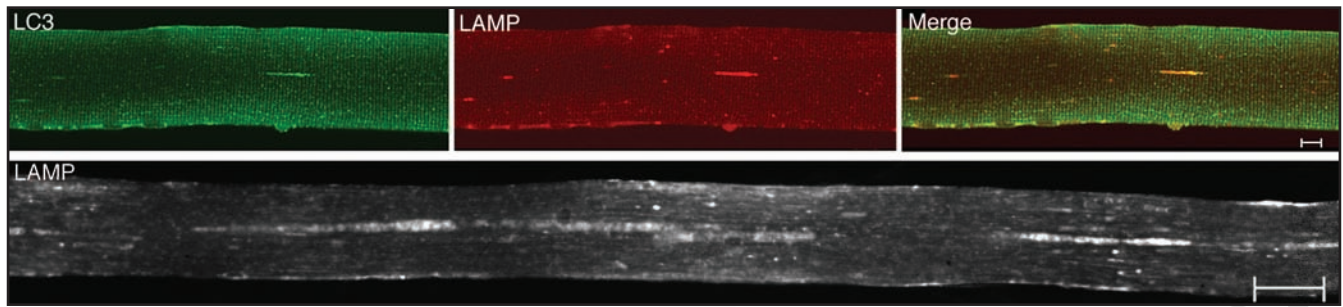
Figure 2. Confocal image of single muscle fibers (at an early stage of disease) immunostained for LC3 and LAMP2. Top: Tiny centrally located area of autophagy. The inability of lysosomes to process LC3-positive autophagosomes is indicated by the presence of double-positive structures (shown in yellow on the merge image). Bar 10 μm. Bottom: The early pathology can be observed at various places along the fiber length. Bar 50 μm.
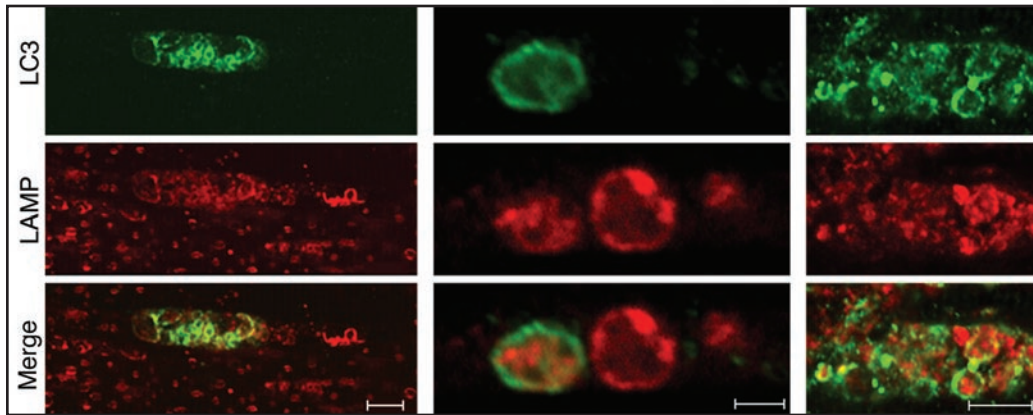

Figure 3. Who is eating whom? Left: Single muscle fiber from a late-onset patient shows increased autophagic activity (LC3-positive autophagosomes) in the area where the lysosomes without clear membrane boundaries are grouped. Bar 10 μm. Both the center (infantile patient, bar 2 μm) and right (late-onset patient, bar 10 μm) panels show LAMP2-positive lysosomes without clear membrane boundaries inside LC3-positive autophagosomes.
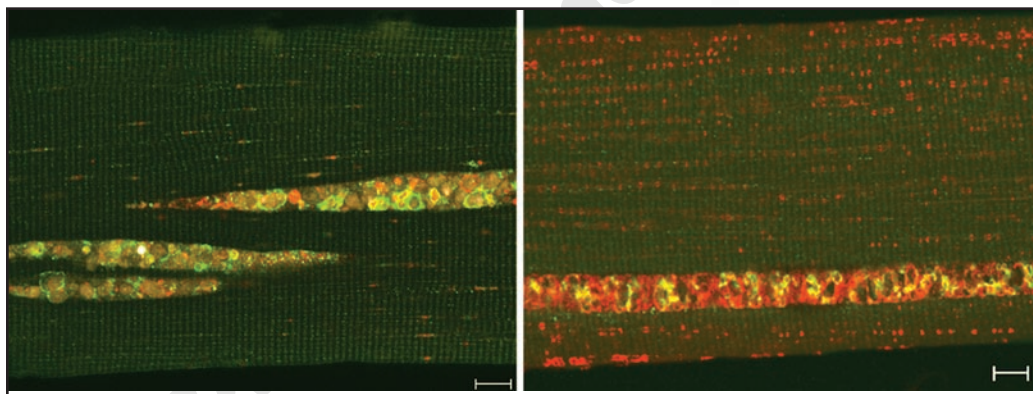

Figure 4. Confocal images of fibers at an intermediate stage immunostained for LC3 (green) and LAMP2 (red) (merged). The two images illustrate different localizations of the autophagic areas in the fibers (the fiber on the right shows a less common peripheral localization). In both fibers, the surrounding cytoplasm contains relatively small or normal lysosomes. These fibers are from late-onset patients. Bar 10 μm.

of LC3-positive autophagosomes abutting the row of lysosomes, a pattern which is not observed in normal fibers (not shown). The appearance of LAMP2/LC3-double positive structures within this area (shown in yellow in Fig. 2 merge image) suggests that the lysosomes, even in a minimally affected fiber, are unable to eliminate the autophagosomes and recycle their contents. The process can be observed at multiple places along the fiber length (Fig. 2, bottom). This early pathology is likely to be missed by transmitted light microscopy.

In more severely affected fibers, both LAMP2-positive and LC3-positive dot-like structures enlarge to ring-shaped vesicles exceeding 2 microns in diameter. The lysosomes within the area of increased autophagic activity are clustered and appear to lack clear membrane boundaries (Fig. 3). An unusual feature of some of the LAMP2-positive structures at this stage is that they are located inside the autophagosomes, rather than the other way around (Fig. 3).

Progression of the pathologic changes is marked by spreading of the autophagic area, accumulation of intralysosomal autofluorescent material (lipofuscin), an increase in vesicle number and size (reaching up to 10 microns in diameter), and fiber atrophy. The average diameter of the affected fibers (48.5 μm

atrophy—in different fibers. These stages were recognized in each patient, but the proportion of fibers at different stages varied among the patients.

The first recognizable sign of lysosomal dysfunction is the formation of a row (20–80 μm in length) of small lysosomes, often centrally localized, in an otherwise healthy-looking fiber (Fig. 2). Increased autophagic activity is already evident, as shown by the presence

± 11.9; n = 25) is less than 50% of that of the unaffected fibers (101.7 μm ± 23.4; n = 23). It is not uncommon to find multiple distinct pathological areas next to each other (Fig. 4, left panel). The autophagic buildup appears to be the main pathology, rather than the expanded lysosomes in the rest of the fiber (Fig. 4). Once recognized, the autophagic areas can readily be seen in single fibers by transmitted light microscopy even without staining (Fig. 5).

The next recognizable pattern involves loss of the vesicular structures within the autophagic area. At this point, the area is totally devoid of the cation-independent mannose 6-phosphate receptor (CI-MPR). The hallmark of this pattern is partial or complete disintegration of muscle structure (Fig. 6). Thus, the autophagic areas, which begin at multiple points along the fiber, eventually enlarge, come together, and may totally replace myofibrils in muscle fibers. The expanded lysosomes outside of the autophagic area seem to have a lesser effect on muscle cell structure (Fig. 7).

In addition to the samples from late-onset patients, biopsies from three infantile patients were analyzed. The patterns described above were also recognizable in muscle fibers from these patients. Despite the fact that two of the infants had been on enzyme replacement therapy at the time of biopsy, some of the fibers (~20%) still contained clearly identifiable areas of autophagic buildup (not shown). The ability of therapy to resolve the buildup remains unclear, since the baseline biopsies were not available.

Subsets of fibers from some patients showed an additional pathological pattern: hugely expanded lysosomes (exceeding 10 microns), which were aligned and perhaps connected, were seen in the absence of significant autophagic activity. The structure and distribution of these lysosomes are reminiscent of those observed in type I Pompe mouse muscle fibers, which also did not have autophagic buildup (Fig. 8).

The recognition of the extent of autophagy and its location within muscle fibers has changed the way we interpret light microscopy of muscle biopsies. Huge, centrally-localized holes are present in virtually all biopsies we have examined (Fig. 9). These holes have previously been interpreted as processing artifacts, but in retrospect, they are most likely regions of autophagic buildup. As we have seen in isolated muscle fibers, glycogen-filled lysosomes (PAS-positive structures in Fig. 9) in the surrounding areas are much less prominent.

In an attempt to create an in vitro model of the autophagic buildup, we analyzed myoblasts derived from normal individuals and late-onset patients. Unexpectedly, control myoblasts, unlike unaffected muscle fibers, have a high basal level of autophagic activity as shown by the presence of punctate and vesicular LC3 positive staining (Fig. 10A). No difference was observed between control and Pompe myoblasts (Fig. 10B). Neither showed a significant response to starvation (not shown), but autophagy was significantly induced when the myoblasts differentiated into myotubes (shown for Pompe myotube in Fig. 10C).

DISCUSSION

The main purpose of this work was to determine whether the data on the pathological autophagosome accumulation observed in skeletal muscle of a KO mouse model is applicable to human disease. In mice, age-dependent autophagic accumulation was evident very
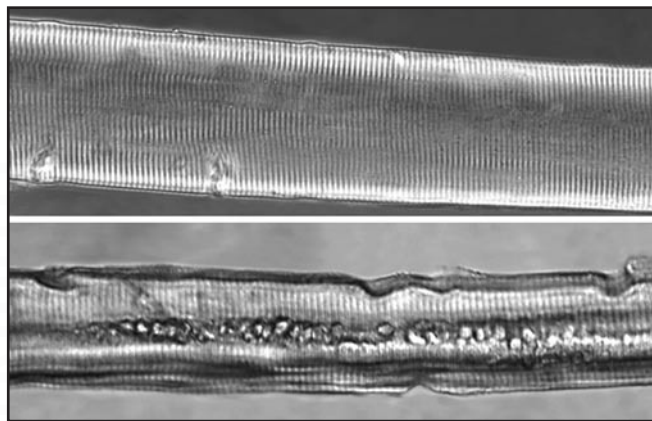

Figure 5. Transmitted light microscopy of fixed muscle fibers, both isolated from a muscle biopsy of a late-onset patient. The area of autophagy can be easily seen without any staining in the affected fiber.
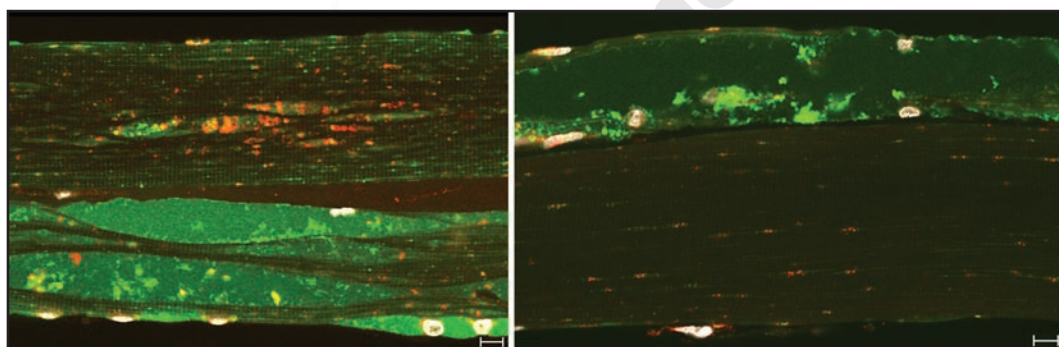

Figure 6. Confocal images of end-stage fibers from late-onset patients immunostained for LAMP2 (green) and CI-MPR (red) (merged). Left: The top fiber shows an apparently earlier stage in which the autophagic areas resemble those in Figure 4. The bottom fiber no longer contains identifiable vesicular structures, and has little, if any CI-MPR. Right: The profoundly affected atrophic top fiber no longer contains any identifiable muscle structure. The bright white structures are nuclei stained with Hoechst. Bar 10 μm.
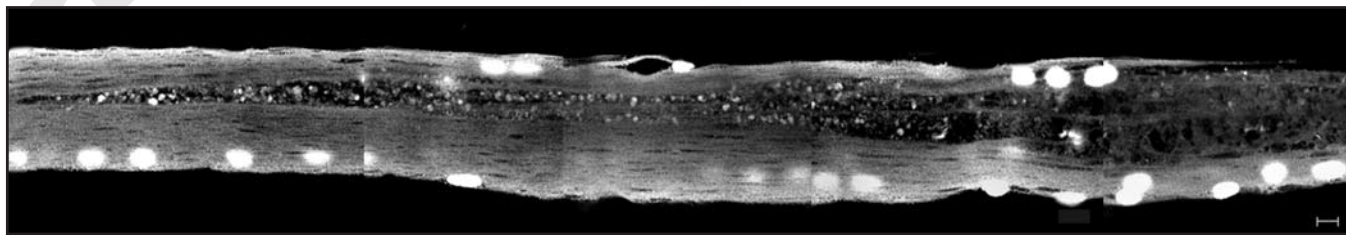

Figure 7. This composite panoramic confocal image of a Hoechst-stained single fiber from a late-onset patient shows varying degrees of autophagic buildup within the same fiber. The lysosomes, the small dark structures outside the autophagic area, look like innocent bystanders compared to the autophagy. Bar 10 μm.
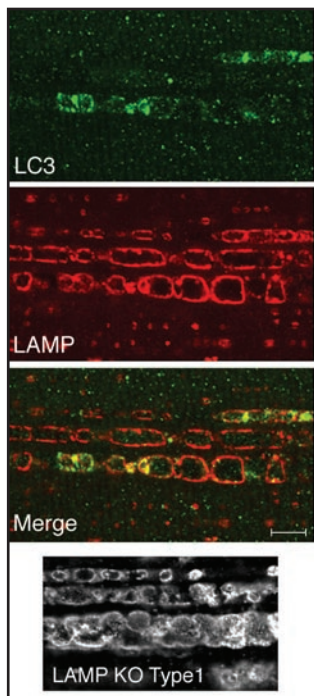
Figure 8. Confocal image of a single fiber from a late-onset patient immunostained for LC3 (green) and LAMP2 (red) shows hugely expanded lysosomes without autophagic buildup. This pattern was observed in type I fibers of the KO mouse model (shown below).
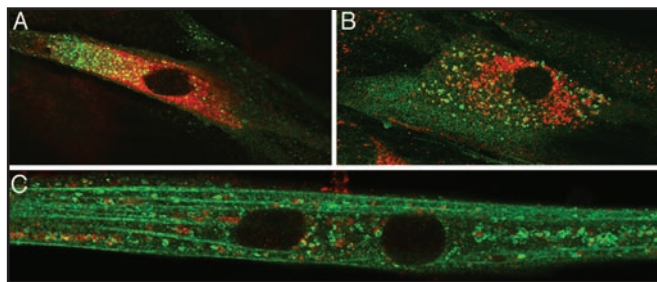

Figure 10. Confocal images of cultured muscle cells immunostained for LC3 (green) and LAMP2 (red) (merged images). (A) Myoblast from normal control individual. (B and C) Myoblast and myotube, respectively, from a late-onset patient.
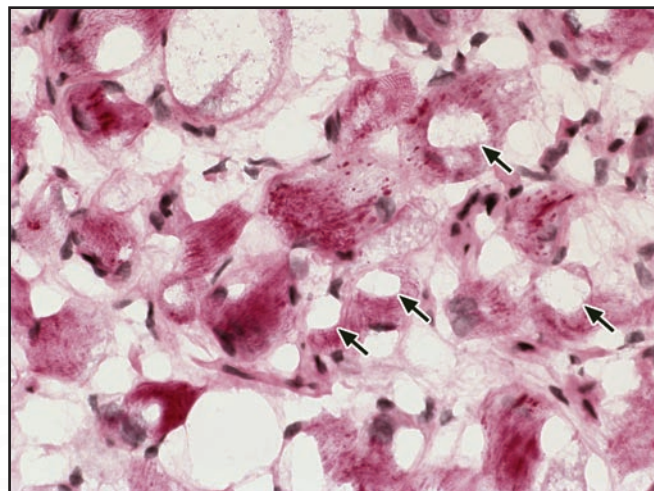

Figure 9. PAS-stained section of muscle biopsy from an additional infantile patient. Huge holes are now interpreted as autophagic areas.

from a single biopsy because muscle cells in late-onset patients show remarkable phenotypic variability.

The data from both human studies and the mouse model led us to reconsider the view of the pathogenesis of the disease and the mechanisms of skeletal muscle damage. The accepted view of the pathology of Pompe disease—the accumulation of glycogen within lysosomes followed by lysosomal rupture—has not changed since the earliest studies.[29,30] The data presented here strongly indicate that this simple view of the pathophysiology is inadequate, since both autophagy and lysosomal enlargement occur to varying extents in different cell populations. In skeletal muscle of both humans and the animal model, the autophagic buildup, sometimes referred to as "biological garbage",[31] appears to have a greater effect on muscle architecture than the expanded lysosomes outside the area.

Massive accumulation of autophagic material may pose a problem for enzyme replacement therapy because autophagosomes communicate extensively with vesicles of the endocytic pathway,[10,11] the route by which the therapeutic enzyme is delivered to the lysosome via a CI-MPR mediated process.[32] In fact, autophagy-related pathology, observed in type II but not in type I-rich muscles in KO mice, was associated with resistance to enzyme replacement therapy. The autophagic buildup persisted in type II KO fibers after months of therapy and served as a sink for most of the endocytosed recombinant enzyme.[22]

Early results from clinical trials indicate that skeletal muscle is a recalcitrant target in humans as well; only a subset of infantile patients achieved significant improvement in muscle function, even at very high enzyme levels,[33,34] raising the possibility that autophagic buildup may be one of the factors responsible for poor muscle response to therapy. Furthermore, analysis of muscle biopsies from infantile patients before and after therapy suggested that poor responders had fewer type I fibers in the biopsies sampled.[30]

Massive amounts of cellular autophagy in skeletal muscle of Pompe patients suggest an induction of autophagy and/or failure of the lysosome to degrade the autophagosomal content. The specific early pathologic events that result in abnormal macroautophagy are not known, but in both patients and KO mice, it is not uncommon to find small, irregularly-shaped lysosomes inside autophagosomes. Since one of the functions of autophagy is the elimination of damaged organelles, it is possible that lysosomes themselves trigger the increase

early, in one-month old animals, well before symptomatic disease. As the disease progressed, the autophagic buildup expanded enormously and occupied a significant volume within the fiber (Supplemental Data Video 1). Eventually, in old mice, the autophagic mass replaced most of the muscle tissue.[20,21] Although the presence of autophagic vacuoles with cytoplasmic degradation products in muscle biopsies from patients with Pompe disease was noted many years ago,[16] the phenomenon has received little attention until recently.[28] We have now established that the abnormal autophagy in skeletal muscle is a major feature in the pathology of the disease in humans.

The different patterns of autophagic accumulation in human fibers correspond to the age-related changes seen in mice. From this, we infer that these patterns reflect the progression of pathology. We observed a wide and continuous range of pathological changes in fibers from eight patients' samples, but we could actually have deconstructed the progression of the disease by analyzing multiple fibers

in autophagy, thus initiating a critical event in the pathogenesis of the disease. We suggest that this series of events be called lysophagy.

Oxidative stress is likely to play a role in autophagy induction, as evidenced by the significant early accumulation of lipofuscin —a product of oxidative damage.[22,31,35,36] Starvation is yet another attractive hypothesis to explain the induction of autophagy. We have previously suggested that in KO mice, the decrease in the amount of available glucose may trigger local autophagic response in glycolytic type II fibers.[21]

Indeed, multiple factors may be at play at different stages of Pompe disease. It is well known that the basal levels of autophagy and the regulation of autophagy are age and organ dependent. For example, skeletal muscle (in particular, fast-twitch type II fibers) is the primary site of increased autophagy in response to starvation in adult mice.[37] In contrast, in neonates, massive starvation-induced autophagy was observed in the heart, diaphragm, alveolar cells, and skin.[38] The regulation of autophagy and its initiating events in vivo are generally not well understood. Hypoglycemia has long been thought to induce lysosomal degradation of glycogen in liver in the immediate postnatal period of starvation.[39,40] On the other hand, the neonatal pattern of starvation-induced autophagy does not include liver,[38] and the role for hepatic glycogen autophagy in the early postnatal period has been challenged by data from autophagy deficient mice: neonates appear to depend on the amino acids produced by autophagy rather than on glucose supply for energy homeostasis and survival.[37]

In this study, undifferentiated human myoblasts, multinucleated myotubes, and muscle fibers showed very different levels of basal autophagic activity. LC3-positive autophagosomes are barely detectable in normal fibers, indicating a low level of activity. In contrast, normal human myoblasts have an active pattern of constitutive autophagy as shown by the presence of multiple LC3-positive vesicular structures. The autophagy was not appreciably enhanced by starvation, but was induced in differentiated myotubes, suggesting a possible role of autophagy in the generation of substrates for cellular remodeling during muscle differentiation. This question, however, is beyond the scope of this paper.

Thus, we have demonstrated a profound abnormality in the autophagic pathway—the presumed route of glycogen delivery to lysosomes—in skeletal muscle in patients with Pompe disease, which can no longer be viewed as simply a lysosomal storage disorder.

Acknowledgements

Note

Supplemental Data Video 1 can be found at:
www.landesbioscience.com/supplement/rabenAUTO3-6-vid.mov.

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