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Autophagic Punctum

The values and limits of an in vitro model of Pompe disease

The best laid schemes o' mice an' men...[†]

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In Pompe disease, a lysosomal glycogen storage disorder, cardiac and skeletal muscle abnormalities are responsible for premature death and severe weakness. Swollen glycogen-filled lysosomes, the expected pathology, are accompanied in skeletal muscle by a secondary pathology—massive accumulation of autophagic debris—that appears to contribute greatly to the weakness. We have tried to reproduce these defects in murine, Pompe myotubes derived from either primary myoblasts or myoblasts with extended proliferative capacity. The cells accumulated large lysosomes filled with glycogen, but, to our disappointment, did not have autophagic buildup even though basal autophagy was intact. When we suppressed autophagy by knocking down Atg7, we found that glycogen uptake by lysosomes was not affected, suggesting that macroautophagy is not the major pathway for glycogen delivery to lysosomes. But two apparently incidental observations—a peculiar distribution of both microinjected dextran and of small acidic structures adjacent to the interior membrane of large alkalized glycogen-containing lysosomes—raised the possibility that glycogen traffics to the lysosomes by microautophagy or/and by the engulfment of small lysosomes by large ones. The cultured myotubes, therefore, appear to be a useful model for studying the mechanisms involved in glycogen accumulation in Pompe disease and to test substrate deprivation approaches.

[†]From *To a Mouse*, Robert Burns, 1785

Macroautophagy (referred to as autophagy), a major pathway for delivery of proteins and organelles to lysosomes, has been implicated in many cellular and developmental processes and in several human diseases, including Pompe disease, a lysosomal glycogen storage disorder caused by deficiency of acid alpha-glucosidase. Patients with this disorder display a continuum of phenotypes, the most profound of which includes severe muscle wasting and cardiac failure in infancy. In milder, late-onset forms, cardiac muscle is spared, but progressive and debilitating skeletal muscle weakness and wasting lead to premature death due to respiratory insufficiency. While the presence of large glycogen-filled lysosomes is a hallmark of the disorder, we have found that the cellular pathology in the diseased skeletal muscle also involves failure of autophagosomal turnover and massive autophagic buildup.

Enzyme replacement therapy (ERT) with recombinant human enzyme (Myozyme, Genzyme Corporation, Framingham, MA) has recently become available. While its effect on glycogen clearance in cardiac muscle is remarkable, as evidenced by a significantly increased life span beyond infancy, skeletal muscle has remained quite recalcitrant to treatment. A similar skeletal muscle resistance to ERT in our murine Pompe model is associated with the presence of autophagic buildup in myofibers. Our interest in autophagy was driven not only by its relevance to therapy, however, but also because macroautophagy is the presumed mechanism by which glycogen is delivered to the lysosomes. Normally, the bulk of cellular glycogen is processed in the cytoplasm; only a small portion of glycogen is found in the lysosomes. Excessive autophagy leading to destructive autophagic buildup in Pompe muscle might therefore be predicted to increase lysosomal glycogen if indeed macroautophagy is the route of glycogen entry to the lysosomes. If we could manipulate autophagy, we might expect simultaneously to decrease the buildup, diminish or prevent glycogen load in the lysosome, and perhaps enhance the effect of ERT. Studies of this sort are burdensome in mice and beg the development of an in vitro Pompe model.

Such a muscle cell model involves the derivation of primary myoblasts—muscle stem cells, which are capable of forming multinucleated myotubes—structures that mimic muscle fibers. We

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isolated primary myoblasts from satellite cells that had migrated from fast glycolytic single muscle fibers. The choice of fast fibers was dictated by the fact that the autophagic buildup in Pompe mice was observed in fast rather than slow muscle fibers. The problem with the primary myoblasts, however, is that they lose the ability to form myotubes after only a few passages. To address the problem, we transduced primary myoblasts derived from Pompe fibers with a CDK4-containing retrovirus; these transduced myoblasts can undergo multiple passages in culture while retaining their potential to terminally differentiate into myotubes. At this point we figured that we were home free.

The Pompe myotubes contained multiple small and large LAMP-1-positive/cation-independent mannose 6-phosphate receptor (CI-MPR)-negative structures, which identified them as lysosomes (Fig. 1). The average cross-sectional area of the largest lysosomes in Pompe myotubes was $30 \mu\text{m}^2$ as compared to $2.8 \mu\text{m}^2$ in wild type. These strikingly large lysosomes were filled with glycogen, lacked two major lysosomal enzymes, cathepsins B and D, and displayed a non-acidic pH range that measured between 5.2 and 7.5.

Despite these gross lysosomal abnormalities, the autophagic buildup seen in fast muscle was absent. Although the myoblasts were derived from fast muscle fibers, phenotypically they resemble slow fibers, which exhibit lysosomal pathology but not the autophagic buildup. Not only was the buildup missing, there were no visible signs of increased autophagy in the Pompe myotubes. So much for our “best laid schemes”.

We addressed, therefore, the question of whether constitutive autophagy was detectable in our *in vitro* Pompe model. LC3-II, a highly specific autophagic marker, was measurable in the cell lysates, and transfection of the myoblasts with GFP-LC3 followed by staining for GFP and LAMP-1 showed that basal autophagy was functional. Thus, the model is suitable for addressing the role of autophagy in the delivery of glycogen to the lysosomes. We suppressed autophagy in Pompe myoblasts by using siRNA to knock down *Atg7*, a critical gene required for the formation of autophagosomes. As expected, *Atg7* siRNA treatment resulted in the absence of LC3-II, but large glycogen-filled lysosomes still

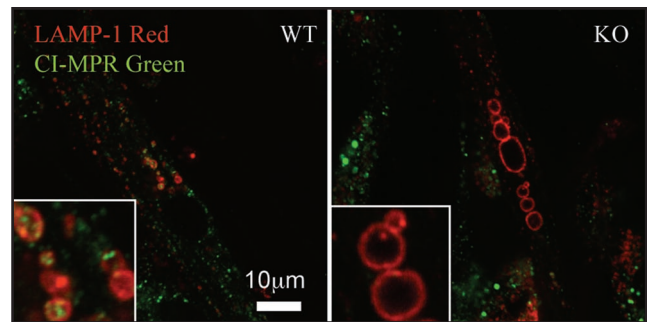


Figure 1. Pompe myotubes (KO) contain extremely large lysosomes never seen in the wild type (WT). Fixed myotubes were double-stained with LAMP-1 and CI-MPR. The large expanded structures in the KO are LAMP-1-positive/CI-MPR-negative, thus validating that they are indeed lysosomes. Small lysosomes and LAMP-1/CI-MPR double-positive late endosomes can be seen in both WT and KO myotubes. The insets show small lysosomes and late endosomes in the WT, and large lysosomes in the KO.

formed in myotubes derived from autophagy-deficient myoblasts, suggesting that macroautophagy is not the major mechanism for delivery of glycogen to the lysosomes.

Although the mechanism by which glycogen enters lysosomes in either normal or Pompe skeletal muscle cells is unknown, the evidence that macroautophagy is not primarily responsible for delivery of glycogen to the very large Pompe lysosomes forces a consideration of alternative mechanisms. Both chaperone-mediated autophagy (assuming that glycogen enters lysosomes bound to a protein with a chaperone-recognition sequence) and microautophagy must be considered as possible mechanisms. Microautophagy as a route for glycogen delivery is supported by experiments with dextran, a close analog of glycogen. When fluorescent-labeled dextran was injected into the cytoplasm of Pompe myotubes (Fig. 2A) it appeared to localize largely to multiple sub-membrane compartments of the large lysosomes; the position of these compartments within the large lysosomes suggests the possibility of glycogen entry by invagination of the lysosomal membrane.

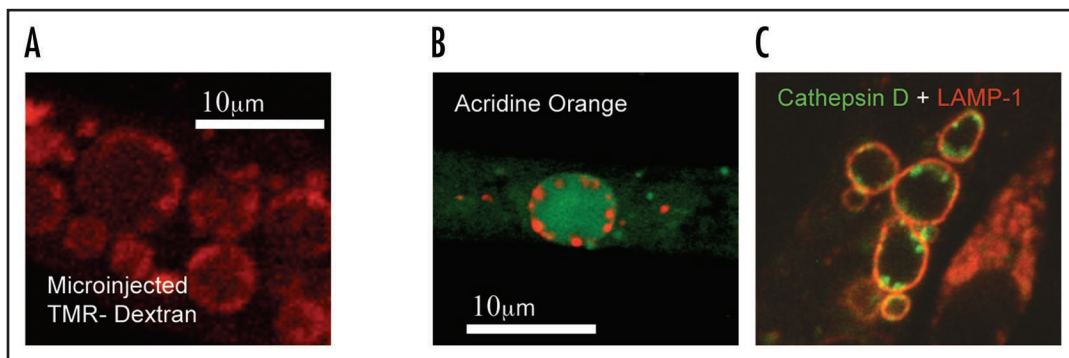


Figure 2. Unusual features of expanded lysosomes. (A) Tetramethylrhodamine (TMR)-labeled dextran was microinjected into KO myotubes, which were analyzed 24 hours later by confocal microscopy. Dextran-positive vesicle-like structures were predominantly found just beneath the lysosomal membrane. (B) Acridine orange staining of a KO myotube. Small acidic (red fluorescence) compartments are found on the interior membrane of a large alkalized (green fluorescence) lysosome. (C) Cathepsin D and LAMP-1 double-staining of a KO myotube. Small cathepsin D-positive compartments are found on the interior membrane of large LAMP-1-positive lysosomes. Their distribution is similar to that of the acidic compartments shown in (B).

Yet another possibility for how the large lysosomes continue to expand and accumulate glycogen is suggested by a peculiar feature of these lysosomes: some of them contain small cathepsin D-positive acidic structures (Fig. 2B and C), perhaps attached to their interior membrane. These small lysosome-like (and thus glycogen-containing) vesicles may eventually rupture once engulfed by the large lysosomes, thus contributing to the glycogen accumulation and expansion of the large lysosomes.

Whatever the mechanism, and despite the absence of autophagic buildup, this *in vitro* Pompe model seems well-suited for studies on trafficking and pharmacological interference of glycogen delivery to the lysosomes.

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