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The First Molecular Evidence That Autophagy Relates Rimmed Vacuole Formation in Chloroquine Myopathy[1]

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Chloroquine myopathy is a drug poisoning disease involving rimmed vacuole formation. By Western blot analysis, we investigated posttranslational modification of LC3 in cultured cells with a high concentration of chloroquine, and found that the autophagosome membrane-bound form of LC3 increased dose-dependently. We also constructed a disease model by excessive chloroquine injection into rats and unusual immunohistochemical alteration was chased using anti-LC3 antibodies. With chloroquine treatment, muscle atrophy occurred predominantly in soleus muscle and unusual autophagosomes were accumulated. Therefore, we concluded that autophagy plays an important role in rimmed vacuole formation in certain muscular atrophies.

Key words: autophagy, chloroquine, LC3, muscle atrophy, rimmed vacuole.

Autophagy is a process involving the bulk degradation of cytoplasmic components in eukaryotic cells *via* the lysosomal/vacuolar system (*1*). Rimmed vacuoles (RVs) are a pathological hallmark of some myopathies (*2*), and are thought to be a by-product of an abnormally induced autophagic process (*3*). But there has been no molecular evidence to support this hypothesis, because no molecules that control the autophagy are known so far.

Chloroquine, an anti-malarial drug, prevents lysosomal function by raising the pH in the lysosomal system. Chronic intoxication with this drug results in myopathic changes with high lysosomal enzymatic activities and the formation of numerous rimmed vacuoles (*4*). Long-term chloroquine injection into rats can also induce myopathic changes in the soleus muscle, which is composed of type 1 (red) fibers (*5*). So chloroquine-treated rats are a good model for analysis of autophagosome formation. Pathological changes in chloroquine-treated rats are similar to those in DMRV (distal myopathy with rimmed vacuole formation). DMRV is an autosomal recessive disorder preferentially involving anterior tibial muscles. In muscle biopsy specimens, rimmed vacuoles are observed in atrophic fibers. Necrotic and regenerating fibers are seen in about half of such specimens, but they are not severe enough to account for the muscle atrophy (*6*). So the formation of rimmed vacuoles is thought to be a clue as to this type of muscle atrophy (*3*).

In the wild type yeast, *Saccharomyces cervisiae*, many autophagosomes are formed in the cytosol, when the cells are under starvation conditions (*7, 8*). Taking advantage of yeast genetics, apg (autophagy-defective) mutants defective in the formation of autophagosomes were isolated (*9, 10*). One of the APG gene products, Apg8p, is covalently attached to autophagosome membrane phosphatidylethanolamine *via* C-terminal glycine of Apg8p, as in the case of ubiquitination (*11*). Apg7p is a multi substrate E1-like enzyme for Apg8p and Apg12p (*12*). Also, Apg3p is likely to be a novel E2-like enzyme and specifically required for the Apg8p membrane targeting (*12*). In mammalian cells, it was recently found that mammalian Apg8p homolog LC3 (microtubule-associated protein light chain3) also conjugates with the autophagosome membrane *via* the C-terminus (*13*). LC3 protein is firstly translated as a precursor form. After its translation it is processed at the C-terminus, becoming an 18kDa protein (LC3-I) (*13*). LC3-I conjugates with phospholipid and becomes a membrane-bound mature form (LC3-II). Due to this dynamic nature, LC3 could be the most useful marker protein for chasing mammalian autophagy.

In this study, we tried to demonstrate the relation between rimmed vacuole and autophagosome formation. For this purpose, we obtained chloroquine-treated rats, and analyzed them immunohistochemically with an anti-LC3 antibody.

The DMRV muscle biopsy specimen was provided by Muscle Bank (National Center of Neurology and Psychiatry). The anti–rat LC3 polyclonal antibodies and pCI-neo-myc-LC3 mammalian expression vector were kindly provided by Ms. Y. Kabeya (National Institute of Basic Biology). The anti-*myc* monoclonal antibodies were purchased from CLONTECH.

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pCI-neo-myc-LC3 was transiently transfected into COS-7 cells using Fugene 6 (Roche) and then incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) and treated in DMEM containing 0.5–10 μM chloroquine diphosphate at 37°C for 48 h. After that, the cells were harvested and lysed in PBS on ice. Also, the lysate was sonicated and centrifuged at 10,000 ×*g* for 20 min at 4°C. The supernatant and pellet were respectively subjected to 15% SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted with anti-rat LC3 antibodies.

We injected chloroquine diphosphate (SIGMA), 50 mg/kg per day by means of an osmotic mini-pump (DURECT) intraperitoneally, into male 2 w Wistar rats for 8–12 weeks. After the injection treatment, we dissected the rats, taking out the soleus and other skeletal muscles. After the dissection, the muscles were immediately frozen in liquid nitrogen-cooled isopentane for histochemistry, or fixed with glutalaldehyde and embedded in resin for electron microscopy.

Seven-micrometer-thick serial sections were cut with a cryostat at –25°C. For general histology, sections were stained by the hematoxylin and eosin (H&E), and modified Gomori trichrome methods. Acid phosphatase activity in tissues was detected by biochemical staining (*14*).

Muscles were fixed in 0.5% glutaraldehyde at room temperature for 2 h and then washed in 0.1 M cacodylate buffer for 24 h. After the first fixation, they were post-fixed with 1% osmium tetroxide at room temperature for 30 min. Fixed samples were washed with and dehydrated in an ethanol gradient series, 50 to 100%. After that, they were infiltrated with and embedded in Epoxy resin, and then baked at 70°C for 60 h. Plastic-embedded tissue was thin-sectioned with a microtome, and stained with uranyl acetate and lead citrate. Samples were examined under a transmission electron microscope.

Six-micrometer-thick serial muscle sections were cut with a cryostat at –25°C. Sections were dried up and fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. After fixation, the sections were rinsed in PBS and blocked with 2% bovine serum albumin in PBS containing 1.5% goat serum for 30 min at room temperature. After blocking, the first antibody raised against rat LC3 protein was applied, followed by incubation at 37°C for 30 min. Anti–Rat IgG antibodies labeled with FITC (Jackson Immuno Research Laboratories, Inc.) were diluted 1:150 and used as the second antibodies. After 30 min incubation at 37°C, we observed the sections under a confocal laser microscope.

Firstly, we determined the localization of the *myc*-LC3 fusion protein in chloroquine-treated cells by Western blot analysis. We transfected the *myc*-tagged LC3 construct (pCI-neo-myc-LC3) into COS-7 cells and then incubated the calls with or without chloroquine for 72 h. Cell lysates were sonicated, centrifuged at 10,000 ×*g* and analyzed by Western blot analysis with anti-*myc* antibodies. As shown in Fig. 1 A, lanes 1 and 2, *myc*-LC3-I was detected only in the supernatant fraction, while *myc*-LC3-II was detected in the pellet. There was no significant difference in the expression. The same result was obtained with anti-LC3 antibodies (lanes 3 and 4). On electron microscopy, it was observed that LC3 was associated specifically with autophagosome membranes or the cytoplasmic distribution (*11*). Therefore, we concluded that LC3-II was located in autophagosomes, not other membranes. To reveal the effect of chloroquine on the autophagosome formation by cells, we incubated cells in the chloroquine-containing medium and then investigated the cellular localization of LC3. Since LC3-II was formed only when autophagosomes were generated, the LC3-II/LC3-I ratio represents the density of endogenous autophagosomes in cells. As shown Fig. 1, B and C, we found that only the amount of LC3-II drastically increased in dose-dependent manner. This suggests that chloroquine causes an increase in endogenous autophagosomes, not lysosomal enzyme activities, in mammalian cells (*15*).

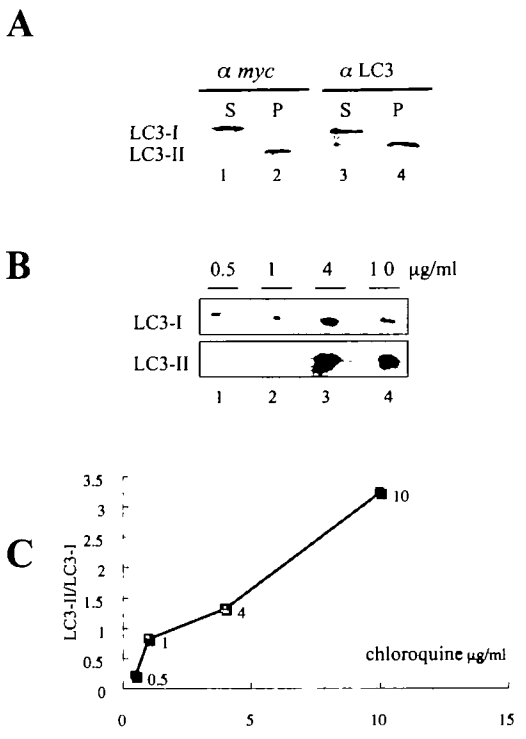

Fig. 1. **Autophagosome detection in COS-7 cells treated with chloroquine.** (A) Western blot analysis with anti-*myc* or anti-LC3 antibodies to detect LC3-I and LC3-II in COS-7 cells. pCI-neo-*myc*-LC3 transfected COS-7 cell supernatants (lanes 1 and 3) were analyzed with anti-*myc* and anti-LC3, respectively. Pellets (lanes 2 and 4) were also analyzed with anti-*myc* and anti-LC3, respectively. (B) LC3-I and LC3-II visualized by Western blot analysis with anti-*myc* antibodies in a pCI-neo-*myc*-LC3 transfected COS-7 cell supernatant (S) and pellet (P). The cells were treated with chloroquine for 48 h. (C) The LC3-II/LC3-I ratio in each lane in (B) was determined with Scion Image (Scion Inc.) and plotted.

To understand the relation between autophagosomal up-regulation in chloroquine-treated cells and the vacuolar atrophy seen in chloroquine myopathy, we tried to construct a disease model of rat by injecting chloroquine with an osmotic mini-pump. At 8 weeks after the first drug injection, we examined the muscle histology under a light microscope, and observed variation in fiber size and atrophic fibers with large rimmed vacuoles predominantly in the soleus muscle (Fig. 2A). Large numbers of basophilic granules and high lysosomal enzyme activity were marked around rimmed vacuoles (Fig. 2B). These are typical pathological features of chloroquine myopathy and other human

muscle atrophies with rimmed vacuoles. So we concluded that chloroquine treatment with an osmotic pump caused myopathy like daily manual injections (*14*). For more detailed analysis, ultrastructural components were investigated under an electron microscope. Multi-membrane components were observed in the space of myofibril arrays (Fig. 2C). The membranes looked unstable. High electron dense materials were marked in the lumen, and the contents

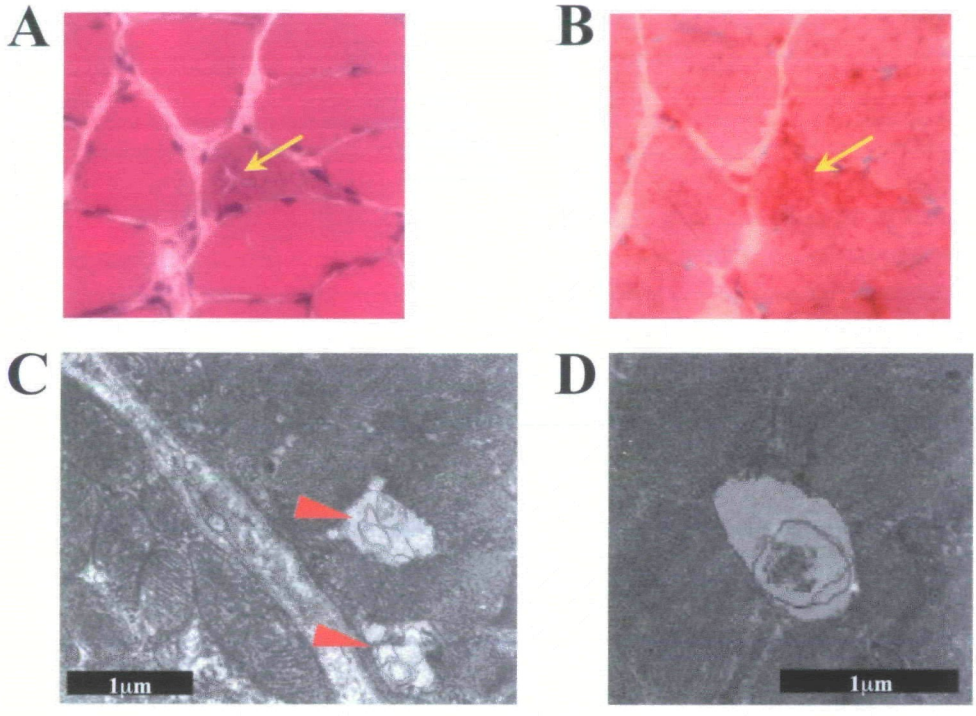

Fig. 2. **Rimmed vacuole formation after long-term injection of chloroquine into rats.** We injected chloroquine diphosphate into male rats, 50 mg/kg per day for 8 weeks. Their soleus muscles were sectioned and stained with H&E (A). Acid phosphatase activity in a serial section of A (B). There were many rimmed vacuoles (arrows) in atrophic fibers. Ultra-thin sections were examined under an electron microscope. Autophagosome-like components (arrowheads) were observed within the myofibrils (C). Cytoplasmic materials were observed within an autophagosome-like component (D).

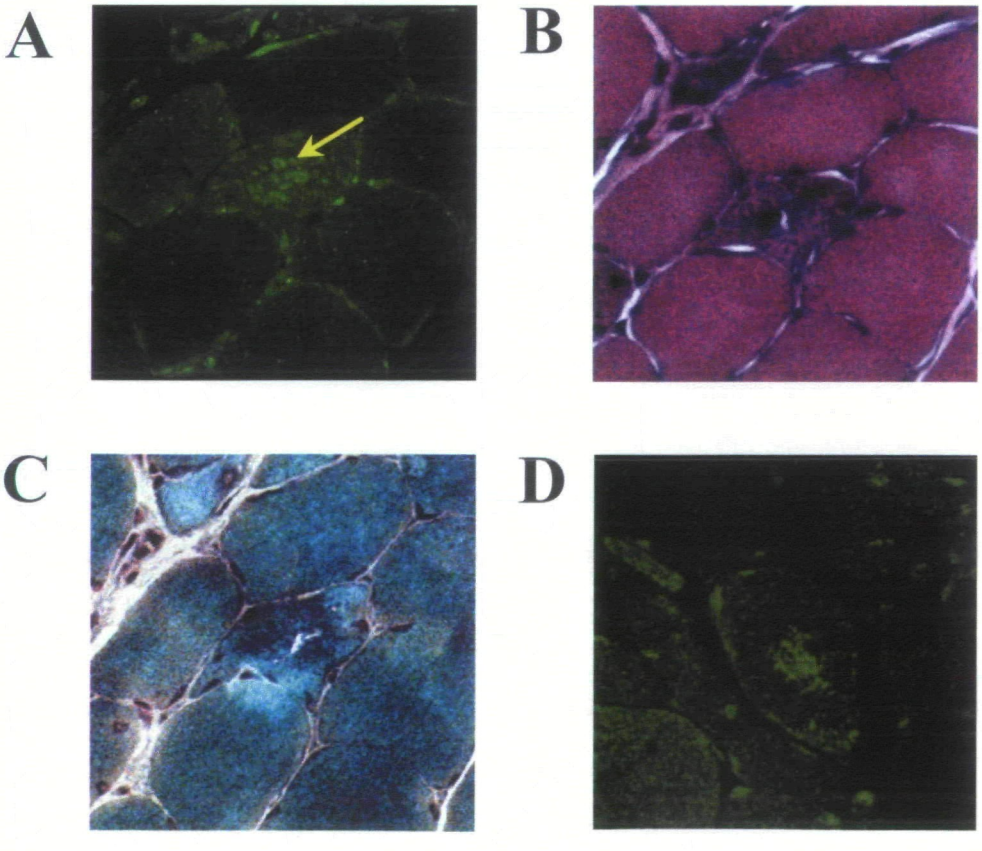

Fig. 3. **Autophagosome accumulation in chloroquine-treated rat soleus and human vacuolar myopathied muscles.** (A) Immunohistochemistry with anti-LC3 antibodies in chloroquine-treated rat soleus muscle. The anti-LC3 antibodies were detected with second antibodies labeled with FITC. Autophagosome accumulation was observed (arrow). Serial sections of A stained with H&E (B) and Gomori trichrome (C). (D) Immunohistochemistry with anti-LC3 antibodies in a DMRV muscle biopsy specimen.

appeared to have been transported from the cytoplasm (Fig. 2D). Because these membrane features were pathologically similar to yeast autophagosomes (*16*), it may be proper to speculate that they are autophagosome phenotypes generated on chloroquine treatment.

In order to prove this hypothesis, we performed immunohistochemical analysis using anti-LC3 antibodies in wild type and chloroquine-treated rats. We sectioned rat muscle tissues with a cryostat and fixed them in 4% paraformaldehyde in PBS for 20 min. As shown in Fig. 3, LC3-positive fibers were observed in both wild type (data not shown) and chloroquine-treated rat soleus muscle (Fig. 3A). LC3 seemed to be localized in the cytosol and its quantity seemed to be very low. Also, more notably, in the diseased soleus muscle, LC3 expression seemed to be slightly elevated and variable-shaped autophagosomes were observed in degenerative fibers (Fig. 3A, arrow). We thought that these autophagosomes would correspond to the membrane inclusion observed in muscle on electron microscopy. This curious type of autophagosome was often accumulated in the central area of specific fibers. When serial sections were subjected to with histological staining (Fig. 3, A, B, and C), unstable autophagosome aggregations were frequently observed in rimmed vacuoles. These results suggested that rimmed vacuoles were involved in the autophagosome formation. To confirm this type of abnormal autophagosome in human muscle disease, we performed LC3 immunohistochemistry using a human muscle biopsy specimen. As shown in Fig. 3D, autophagosome accumulation in degenerative fibers was also observed in DMRV muscle.

Although many myopathies have been described, chloroquine myopathy, distal myopathy with rimmed vacuole formation (DMRV), oculopharyngeal muscular dystrophy (OPMD), hereditary inclusion body myositis (HIBM), and some other myopathies have been established to be clinically distinct entities. Even with the diversity of their genetic backgrounds (*17–19*), these myopathies shared common pathological features of myopathic changes with rimmed vacuoles. The pathologic changes are somewhat similar to those seen in chronic muscular dystrophy (*20, 21*), but necrotic and regenerative processes are less prominent, and the level of blood creatine kinase, a major diagnostic marker of most muscular dystrophies, is either normal or only mildly elevated. To further characterize this wondering type of atrophy, a study of rimmed vacuoles to determine what role they play in the pathogenesis of muscle fiber atrophy is necessary.

In this study, we confirmed the hypothesis that autophagosomes are abnormally accumulated around rimmed vacuoles in chloroquine myopathy. Also, we found that chloroquine treatment concurrently induces autophagosome accumulation and morphological rimmed vacuole formation in rat skeletal muscles. But the effects of the drug were not the same for each muscle fiber type. That is, chloroquine injection caused degeneration and abnormality in autophagosome formation only in soleus predominant type 1 fibers.

So, determination of where differences in chloroquine sensitivity were derived from was important understanding vacuolar myopathy. In type 1 fibers, LC3 mRNA expression was found to be higher than type 2 ones on Northern blot analysis (data not shown), suggesting autophagy activity itself is higher in type 1 fibers than in type 2 ones. In fact, it is known that cellular lysosome, which is indispensable for autophagy, predominantly exists in type 1 fibers. Therefore, the high autophagy activity in type fibers 1 may be key to their chloroquine sensitivity.

In the disease model, the lysosome function in type 1 fibers is damaged by chloroquine and autophagosome maturation controlled through fusion of the lysosome would be obstructed. Judging from this, the uncovered genetic backgrounds of differnt myopathies may also prevent lysosomal activity. In such a state, cells cannot satisfy the requirements for protein degradation *via* autophagy, and the undegraded proteins and a lot of immature autophagosomes could result in cytoplasmic aggregation. Recently, it was said that protein aggregation within the cytosol itself could be toxic for cells and prevent proteasome activation (*22*). Unstable cytoplasmic membranes and the contents observed in chloroquine-treated rats (Fig. 2B) could possibly be such harmful aggregation.

Autophagy is an extremely basic and widely conserved function throughout eukaryotes from yeast to mammals. So trouble in autophagic processes may lead to a serious dysfunction of protein metabolism within cells. In this sense, research on autophagy is indispensable for revealing the pathogeneses of many neuromuscular disorders.

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