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Review

Methods for monitoring autophagy

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Abstract

Autophagy is an intracellular bulk degradation system that is found ubiquitously in eukaryotes. Autophagy is responsible for the degradation of most long-lived proteins and some organelles. Cytoplasmic constituents, including organelles, are sequestered into double-membraned autophagosomes, which subsequently fuse with lysosomes where their contents are degraded. This system has been implicated in various physiological processes including protein and organelle turnover, the starvation response, cellular differentiation, cell death, and pathogenesis. However, methods for monitoring autophagy have been very limited and unsatisfactory. The most standard method is conventional electron microscopy. In addition, some biochemical methods have been utilized to measure autophagic activity. Recently, the molecular basis of autophagosome formation has been extensively studied using yeast cells; these studies have provided useful marker proteins for autophagosomes. Importantly, most of these proteins are conserved in mammals. Using these probes, we can now specifically monitor autophagic activity.

Keywords: Autophagy; Degradation; LC3; MDC

Contents

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1. Introduction

Eukaryotic cells have two major protein degradation systems. One is the ubiquitin-proteasome system, which is responsible for the selective degradation of most short-lived proteins (Hershko & Ciechanover, 1998; Hochstrasser, 1996). The other is the lysosomal system. In this system, proteins from both inside and outside of the cell are delivered to the lytic compartment. There are at least four pathways to the lysosome for degradation. The first pathway is endocytosis/phagocytosis, which mediates the degradation of extracellular materials and plasma membrane proteins. The second pathway is macroautophagy, which is mediated by a unique organelle termed the autophagosome (Blommaart, Luiken, & Meijer, 1997b; Dunn, 1994; Seglen & Bohley, 1992) (Fig. 1). When autophagy is induced, a membrane cisterna called the isolation membrane (also known as the phagophore) encloses a portion of cytoplasm, resulting in the formation of the autophagosome. The sequestration step is generally thought to be non-selective. Finally, the outer membrane of the autophagosome fuses with the lysosomal membrane. Various hydrolytic enzymes are supplied to the autophagosome and the cytoplasm-derived contents are degraded together with the inner membrane of the autophagosome. This degradative structure is termed the autolysosome or the autophagolysosome. The third pathway is microautophagy, in which a small portion of cytoplasm is engulfed by the lysosome membrane itself. The fourth pathway is called chaperone-mediated autophagy and is a type of selective degradation system (Dice, 1990). Cytosolic proteins containing KFERQ-like motifs are recognized by a cytosolic chaperone Hsc73. When the resulting complexes bind to a lysosomal receptor, Lamp2a, the substrates are unfolded and transported into the lysosomal lumen for degradation (Cuervo & Dice, 1996). This review article will focus on macroautophagy (simply referred to as autophagy unless otherwise specified).

Autophagy is thought to be required for normal turnover of cellular components and for the starvation response (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004; Mortimore & Poso, 1987; Tsukada & Ohsumi, 1993). Genetic studies revealed that autophagy also plays important roles in differentiation/development in *Saccharomyces cerevisiae* (Tsukada & Ohsumi, 1993), *Drosophila melanogaster* (Juhasz, Csikos, Sinka, Erdelyi, & Sass, 2003), *Dictyostelium discoideum* (Otto, Wu, Kazgan, Anderson, & Kessin, 2003) and *Caenorhabditis elegans* (Melendez et al., 2003). In addition, autophagy may be involved in a certain type of cell death (Clarke, 1990) and human diseases such as neurodegener-

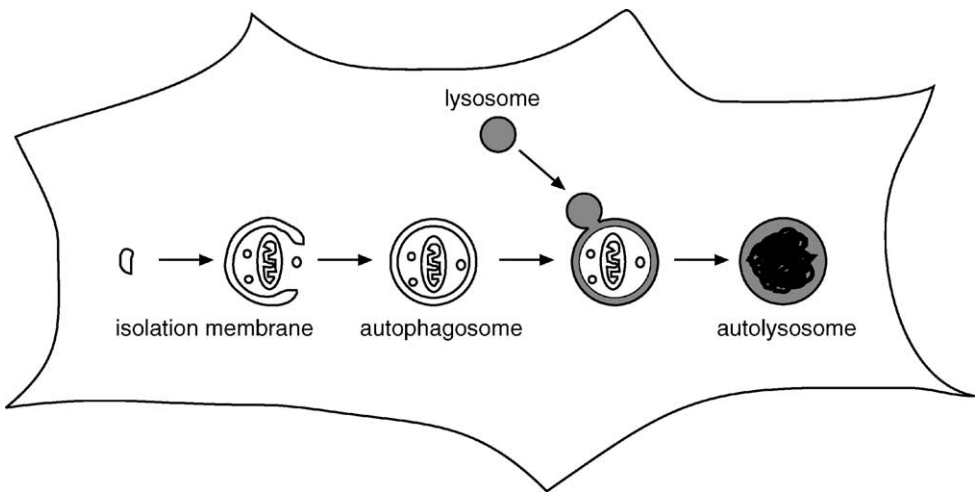

Fig. 1. The process of macroautophagy in mammalian cells. A small volume of cytoplasm is enclosed by the autophagic isolation membrane, which eventually results in the formation of an autophagosome. The outer membrane of the autophagosome then fuses with the lysosome where the cytoplasm-derived materials are degraded (modified from ref. (Mizushima, Yoshimori, et al., 2003b)).

ative diseases (Petersen et al., 2001; Ravikumar, Duden, & Rubinsztein, 2002), α1-antitrypsin deficiency (Teckman & Perlmutter, 2000), myopathy (Nishino et al., 2000) and cancer (Qu et al., 2003).

Although the importance of autophagy has been increasingly recognized, we still have only a limited number of good diagnostic methods to monitor autophagy. This problem apparently impedes the complete understanding of autophagy, and in some cases, it may result in confusion.

During this decade, rapid progress in understanding the molecular mechanisms of autophagy has been made using yeast genetics (Klionsky & Emr, 2000; Ohsumi, 2001; Reggiori & Klionsky, 2002). A number of autophagy-related genes including *APG* and *AUT* genes have been identified (Thumm et al., 1994; Tsukada & Ohsumi, 1993). The nomenclature of these autophagy genes was recently unified and termed *ATG* (Klionsky et al., 2003). Most of the gene products function at the step of autophagosome formation. Since the membrane dynamics of autophagy in yeast are quite similar to those in mammalian cells, these discoveries prompted us to search for mammalian homologues of yeast *ATG* genes. Indeed, several mammalian homologues have been identified and some of their gene products were found in autophagy-related structures (Mizushima, Ohsumi, & Yoshimori, 2002; Mizushima, Yoshimori, & Ohsumi, 2003). These molecules can be used as specific probes for autophagy.

2. Morphological methods

2.1. Electron microscopy

To date, electron microscopy has been the only reliable method for monitoring autophagy. Autophagic phenomena were initially discovered by electron microscopy (Ashford & Porter, 1962). The autophagosome is a double membrane structure containing undigested cytoplasmic material including organelles, while the autolysosome is a single membrane structure containing cytoplasmic components at various stages of degradation (Fig. 1). Since clear differentiation between autophagosomes and autolysosomes is sometimes difficult, these structures are often generalized as “autophagic vacuoles”.

Electron microscopy is a method requiring many skills and much time, and unfortunately it is sometimes difficult to distinguish autophagic vacuoles from other structures just by morphology. In particular, heterophagic vacuoles containing endocytosed or phagocytosed materials need to be carefully distinguished from autophagic vacuoles. It is quite difficult, if not impossible, to separate late stage autophagic vacuoles from heterophagic vacuoles (Pfeifer, 1987). In addition, in stressed or dying cells, other organelles such as the endoplasmic reticulum often swell and these structures might be mistaken for autophagic vacuoles. It should also be noted that a double membrane structures containing cytoplasmic components are not always autophagosomes. For example, when a portion of a cell protrudes into the next cell like a hernia and such a protrusion is cut perpendicular to the axis, it can appear as a double membrane structure. Thus, conventional EM images must be carefully assessed. Immunoelectron microscopy using antibodies against autophagosomal marker proteins is a more specific method (see below) (Kabeya et al., 2000; Mizushima et al., 2001).

Quantification of autophagic activity by EM is possible. The area or volume of autophagic vacuoles is calculated and expressed as the ratio to the total cytoplasmic area or volume. This is the only method that can assess the extent of autophagy revealed by EM in a quantitative manner. Although recent development of computer software allows rapid quantification, a large number of pictures of different cells must be examined for such morphometric analysis.

In yeast, accumulation of undigested autophagic bodies in the vacuole can be seen simply by light microscopy. It is achieved by using vacuolar protease-deficient strains or by treatment with protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF) to inhibit the degradation of autophagic bodies (Takeshige, Baba, Tsuboi, Noda, & Ohsumi, 1992). This method cannot be applied to mammalian cells because the size of the lytic compartment (lysosome) is too small.

2.2. Monodansylcadaverine (MDC) staining

A fluorescent compound, monodansylcadaverine (MDC) has been proposed as a tracer for autophagic vacuoles (Biederbick, Kern, & Elsasser, 1995). Sub-

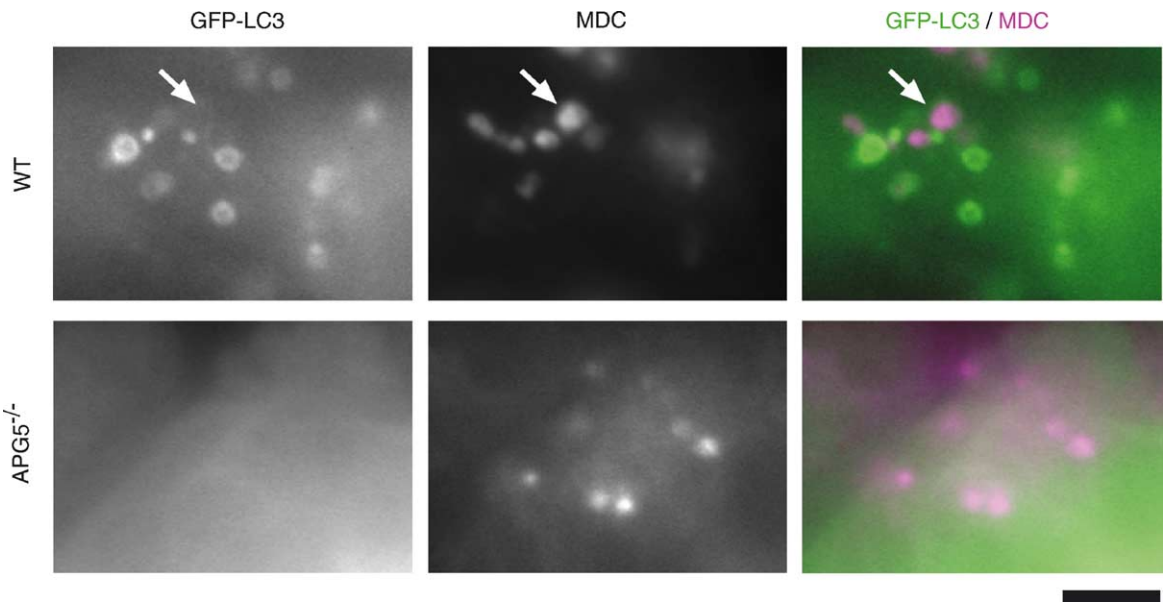

Fig. 2. MDC does not label autophagosomes. Wild-type (upper panels) and *ATG5*$^{-/-}$ ES cells (lower panels) stably expressing GFP-LC3 were starved and stained with MDC. Arrows indicate a MDC dot labeled with GFP-LC3 very weakly, which may represent an autolysosome. Bar, 5 μm.

cellular fractionation analyses demonstrated that MDC-positive structures contained lysosomal enzymes, but not early/late endosomal markers; these results led to a proposal that MDC could be a specific marker for autophagic vacuoles. However, most MDC signals do not co-localize with GFP-LC3 labeling (see below), which mainly represents autophagosomes, suggesting that MDC is not a marker for autophagosomes (Fig. 2). MDC positive structures accumulate primarily at perinuclear regions whereas autophagosomes are detected almost evenly throughout the cytoplasm. These MDC dots co-localize well with staining for late-endosomal and lysosomal markers (Munafo & Colombo, 2001). Although some investigators observed an increase in MDC-staining compartments upon amino acid starvation, staining for lysosomal markers showed that these compartments were virtually indistinguishable from lysosomes (E. Bampton and A. Tolkovsky, personal communication). Moreover, MDC staining is only obtained when the compartments into which it loads are acidic. Neutralisation of these compartments leads to swift loss of MDC staining and/or lack of MDC uptake (E. Bampton and A. Tolkovsky, personal communication). However autophagosomes are not generally acidic. Some MDC positive dots stain weakly for GFP-LC3 (Fig. 2, arrows). These structures probably represent autolysosomes, as LC3 gradually dissociates from the autophagosomal outer membrane after the fusion with lysosome and LC3 on the inner membrane is degraded (Kabeya et al., 2000). Some investigators feel that paraformaldehyde (PFA) fixation increases the specificity of the MDC staining. It is true that PFA fixation greatly reduces the MDC fluorescent signal, but the staining pattern is essentially the same as that in living cells. Therefore this method does not help to distinguish autophagic vacuoles from lysosomes.

We have previously generated an Atg5-deficient mouse embryonic stem (ES) cell clone (Mizushima et al., 2001). This is the first mammalian cell line defective in autophagic activity. Autophagic vacuoles are not generated at all in these cells (Mizushima et al., 2001). However, even in *ATG5*$^{-/-}$ ES cells, a significant number of MDC positive dots were still present (Fig. 2). Therefore, MDC is not a specific marker for autophagic vacuoles even though some MDC positive structures indeed represent au-

tolysosomes and the number of MDC dots correlates with autophagic activity in some cases. Studies reporting increased autophagic activity using MDC as an autophagic marker should therefore be carefully interpreted.

3. Biochemical methods

3.1. Bulk degradation of long-lived proteins

In contrast to the ubiquitin-proteasome system which predominantly degrades short-lived proteins, autophagy is believed to account for the majority of degradation of long-lived proteins (Mortimore & Poso, 1987). Therefore, measurement of bulk degradation of long-lived proteins is often used to monitor autophagic activity. Cells are usually incubated with [^{14}C] (or [^{3}H])-valine or leucine to label all cellular proteins. A subsequent short incubation with an excess of unlabelled valine or leucine washes out the labeling of short-lived proteins, resulting in the specific labeling of long-lived proteins. After washing, cells are incubated again in the presence of cold valine or leucine that prevents reutilization of the radiolabeled amino acids for protein synthesis. Then the trichloroacetic acid (TCA)-soluble radioactivity in the culture supernatant, which corresponds to that of amino acids and peptides, is measured. In addition, the TCA soluble radioactivity within cells can be measured if necessary. The accumulated radioactivity represents degradation of long-lived proteins. One consideration in the use of this method is that excess amount of cold amino acids in the chase medium could inhibit autophagy. Since leucine is the most effective suppressor of autophagy (Mortimore & Poso, 1987), valine may be a preferred tracer.

However, this method is apparently not specific for macroautophagy and is not even specific for lysosomal proteolysis (Fuertes, Martin De Llano, Villarroya, Rivett, & Knecht, 2003). Thus, lysosomal protein degradation is often estimated by the difference between the [^{14}C]-valine released from cells treated with and without lysosomotropic reagents such as chloroquine, ammonium chloride and bafilomycin A_1 (a vacuolar H^+-ATPase inhibitor). However, macroautophagy is not responsible for all such lysosomal proteolysis. For example, bulk degradation of long-lived proteins in *ATG5*$^{-/-}$ ES cells is upregulated during nutrient starvation, accounting for 30–40% of the total degradation in wild-type cells, indicating that some pathway(s) other than macroautophagy is also enhanced during starvation (Mizushima et al., 2001). Other lysosomal protein degradation pathway such as chaperone-mediated autophagy might be responsible for the remaining degradative activity because it is also upregulated by serum starvation (Dice, 1990). However, it is also possible that in the *ATG5*$^{-/-}$ cells, proteolytic pathways other than macroautophagy is up-regulated to compensate the autophagy-negative phenotype.

Likewise, autophagic activity is often presented as the difference between cells treated with and without 3-methyladenine (3-MA). 3-MA is the most commonly used inhibitor of autophagic sequestration (Seglen & Gordon, 1982) and it is now thought to induce its effects via its activity as a PI3 kinase inhibitor (Blommaart, Krause, Schellens, Vreeling-Sindelarova, & Meijer, 1997; Petiot, Ogier-Denis, Blommaart, Meijer, & Codogno, 2000). The target is probably the class III PI3 kinase complex, which contains beclin-1/Atg6/Vps30, mammalian Vps34 and p150 (mammalian Vps15) (Kihara, Kabeya, Ohsumi, & Yoshimori, 2001; Liang et al., 1999). However, the effect of 3-MA, which is used at 10 mM consentration, is not specific for macroautophagy. Indeed, 3-MA has some effects on membrane trafficking including endocytosis (Punnonen, Marjomaki, & Reunanen, 1994), and it also mildly alkalinises the lysosomal lumen (Caro, Plomp, Wolvetang, Kerkhof, & Meijer, 1988). Furthermore, 3-MA treatment can inhibit phosphorylation of JNK and p38, which are key signal transducers of stress-induced apoptosis (Xue, Fletcher, & Tolkovsky, 1999). 10 mM 3MA also inhibits the mitochondrial permeability transition pore in isolated mitochondria (Xue, Borutaite, & Tolkovsky, 2002). Finally, starvation-induced proteolysis in *ATG5*$^{-/-}$ cells can be further suppressed by 3-MA, indicating that 3-MA may affect degradative pathways other than macroautophagy (Mizushima et al., 2001). Therefore, 3-MA-dependent proteolysis does not always indicate macroautophagy. Thus, we should be especially cautious about the usage of 3-MA to implicate autophagy, particularly in cell death studies.

3.2. Delivery of cytoplasmic components to lysosome

An ideal method to assess autophagic activity is measuring the delivery of cytosolic material to lysosomes. This approach is the most successful using yeast cells (Noda, Matsuura, Wada, & Ohsumi, 1995). Pho8p, a vacuolar alkaline phosphatase (ALP), is an integral membrane protein. When Pho8Δ60p, which lacks the membrane spanning region and cytosolic tail, is expressed cells lacking endogenous Pho8p, an inactive proform is detected in the cytosolic fraction. If cytosolic pro-Pho8Δ60p is transported to the vacuole via the autophagic pathway, it is immediately processed by proteinase A and becomes the mature form. The ALP activity of such engineered cells is very low under growing conditions, but it is clearly enhanced during starvation (Noda et al., 1995). This induction is abrogated if autophagy-defective mutants are used. Thus, we can determine the autophagic activity by simple measurement of ALP activity using such genetically modified strains.

In yeast, the cytosolic proform of aminopeptidase I (API) is selectively enclosed by autophagosome-like structures called Cvt vesicles (Klionsky & Ohsumi, 1999). These Cvt vesicles then fuse with the vacuole, and API is released into the vacuolar lumen and processed into the mature form. These two forms can be separated by SDS-PAGE. Although this cytoplasm to vacuole targeting (Cvt) pathway is a biosynthetic pathway, most of the genes required for the Cvt pathway overlap with the original *APG* genes. Furthermore, API is delivered to the vacuole via the autophagic pathway during starvation. Therefore, API maturation is often used as an indicator for the autophagic ability of strains of interest. Since the Cvt pathway is a constitutive process, induction of autophagy cannot be assessed by this method.

In contrast, similar approaches have not been successful using mammalian cells. Cytosolic enzymes such as lactate dehydrogenase (LDH) were used as endogenous probes (Seglen & Bohley, 1992). The accumulation of these enzymes in sedimented vacuoles in the presence of lysosomal proteinase inhibitors was measured. This procedure has been successfully applied to many studies. Instead of endogenous tracer, radiolabled exogenous probes such as [^{14}C]-lactose and [^{3}H]-raffinose were used, but electroinjection is required to introduce the probes into the cytosol, which is a significant limitation. No Cvt-like pathways have been discovered in higher eukaryotes.

4. Specific markers for autophagy

4.1. GFP-LC3 localization

Yeast genetics have revealed that two ubiquitylation-like conjugation systems are required for autophagosome formation (Ohsumi, 2001). One system mediates the conjugation of Atg12–Atg5 (Mizushima, Noda, et al., 1998a), and the second system produces covalent linkage between Atg8 and phosphatidylethanolamine (PE) (Ichimura et al., 2000). These two conjugation systems are highly conserved in mammals (Kabeya et al., 2000; Mizushima, Kuma, et al., 2003a; Mizushima, Sugita, Yoshimori, & Ohsumi, 1998b; Mizushima, Yoshimori, & Ohsumi, 2002b; Tanida, Tanida-Miyake, Komatsu, Ueno, & Kominami, 2002; Tanida, Tanida-Miyake, Ueno, & Kominami, 2001). The majority of the mammalian Atg12–Atg5 conjugate exists in the cytosol as a complex with Atg16L (Kuma, Mizushima, Ishihara, & Ohsumi, 2002; Mizushima, Kuma, et al., 2003a; Mizushima, Noda, & Ohsumi, 1999). Only a small fraction of the Atg12–Atg5·Atg16L complex localizes to the autophagic isolation membrane throughout its elongation process, and the complex dissociates from the membrane when autophagosome formation is complete (Mizushima et al., 2001; Mizushima, Kuma, et al., 2003a) (Fig. 3A). Using gene-targeting methods, we have demonstrated that the mouse Atg12–Atg5 conjugate is essential for elongation of the isolation membrane (Mizushima et al., 2001).

There are three Atg8 families in mammals. Among them, localization of the LC3 protein has been analyzed in detail (Kabeya et al., 2000). LC3 associates with the isolation membrane in an Atg5-dependent manner and remains on the membrane even after spherical autophagosomes are completely formed (Kabeya et al., 2000; Mizushima et al., 2001) (Fig. 4).

These molecules are very good marker proteins for autophagic membranes. Atg12–Atg5 and Atg16L are specific markers for the isolation membrane, and LC3 is a general marker for autophagic membranes. These localizations are easily examined by generat-

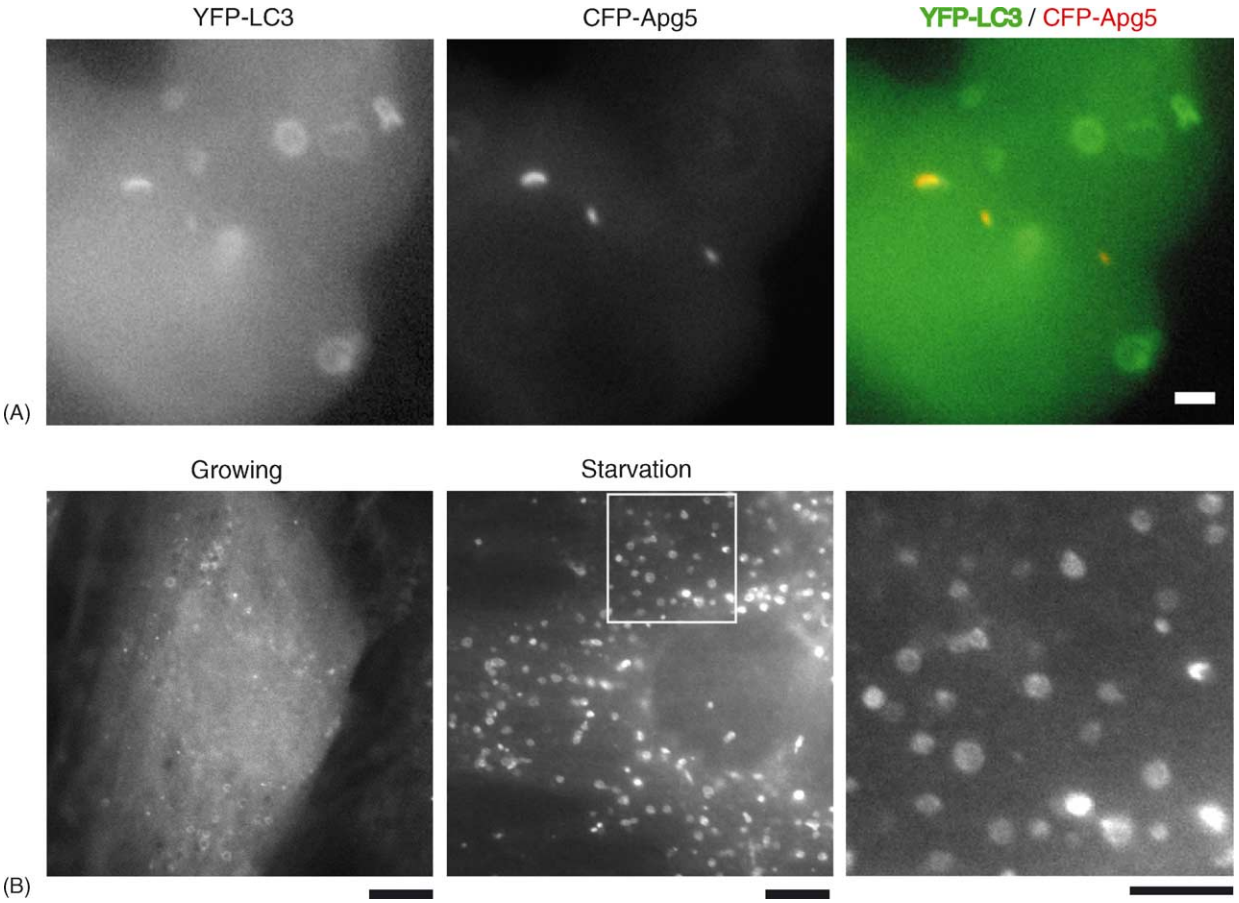

Fig. 3. Localization of autophagic marker proteins. (A) Localization of LC3 and Atg5. ES cells stably expressing YFP-LC3 and CFP-Atg5 were starved for 2 h. Living cells were directly observed with a DeltaVision microscope system (cited from ref. Mizushima, Kuma, et al., 2003a). Bar, 2 μm. (B) Embryonic fibroblasts expressing GFP-LC3 before (left) and after (middle) 2 h starvation. A higher magnification image of the indicated area in the middle panel is shown in the right panel. Bar, 2 μm.

ing chimeric proteins fused with green fluorescent protein (GFP) or its derivatives. Atg12–Atg5 and Atg16L are detected only on cup-shaped structures whereas LC3 is detected on both cup- and ring-shaped structures (Fig. 3A). Since autolysosomes have less membrane-bound LC3 than autophagosomes, the fluorescent signal of LC3 on autolysosomes is weaker or absent (Kabeya et al., 2000). Autophagosomes can be recognized as ring-shaped structures by fluorescence microscopy if their diameters are larger than 1 μm. The size of autophagosome depends on cell type. ES cells and fibroblasts have large autophagosomes that are easily recognized as ring-shaped structures (Fig. 3A and B). In contrast, HeLa cells generate relatively small autophagosomes, which are simply observed as dots. In addition, the basal levels of autophagy seem to be high in HeLa cells. Examination of GFP-LC3 localization is a very simple method, which requires only a high-resolution fluorescence microscope. In addition, real-time observation in living cells is feasible. For example, we have successfully observed a mitochondrion labeled with MitoTracker Red being enclosed by an isolation membrane in an ES cell (unpublished observation). We have also confirmed that over-expression of GFP-LC3 does not affect endogenous autophagy (Mizushima et al., 2004).

We applied this method also to in vivo analysis, by generating GFP-LC3 transgenic mice (Mizushima

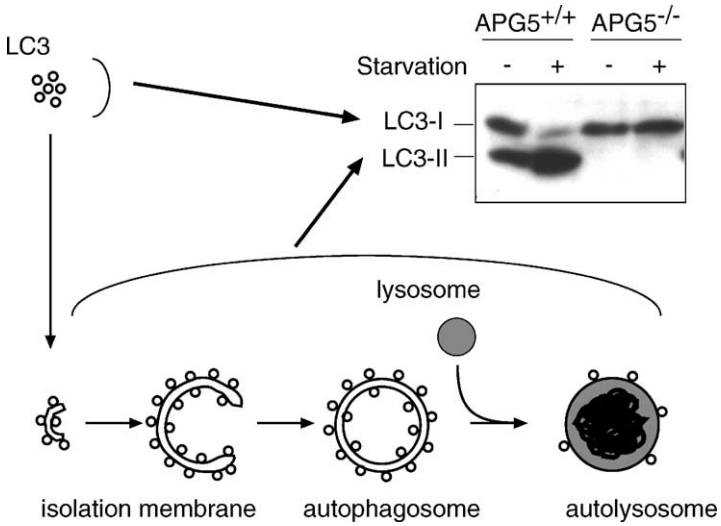

Fig. 4. LC3 as a novel marker for autophagy. Newly synthesized LC3 is immediately processed by mammalian Atg4s and is present in the cytosol as LC3-I. When autophagy is induced, some LC3-I is converted into LC3-II, which is tightly bound to the membrane. LC3-II is most likely a PE-conjugated form. LC3-II migrates faster than LC3-I on SDS-PAGE. The conversion of LC3-I to LC3-II requires Atg12–Atg5.

et al., 2004). Using this transgenic mouse model, occurrence of autophagy in mouse tissues can be directly monitored by simply creating cryosections and subsequent fluorescence microscopic analysis (Fig. 5). Indeed, we observed that autophagy is induced by nutrient starvation in almost all tissues, but pattern of induction differs among tissues. In some tissues, autophagy even occurs actively without starvation treatments (Mizushima et al., 2004).

One of the limitations of this method is the requirement for GFP-LC3 gene transfer. Since transient transfection with lipofection reagents sometimes affects the autophagic status, we routinely use stable transformants. Another difficulty is quantification of the number of GFP-LC3 structures. To extract the dot signals from cytosolic background, “Top hat” algorithm of the Meta Morph Version 6 (Molecular Devise) is useful. It should be also noted that most GFP-LC3 dots represent isolation membranes and autophagosomes, but not autolysosomes. In the strict sense, appearance of GFP-LC3 dots does not guarantee autophagic “degradation”. Therefore, in certain cases, it may be important to see the number and localization of lysosomes. The number of GFP-LC3 dots may depend on the half-life of autophagosomes in each tissue.

It is important that autofluorescent signals must be carefully distinguished, particularly when tissues are examined. Such artifacts may be avoided by the following two methods. First, it is particularly important to compare samples expressing GFP-LC3 with non-expressing control samples. Second, specific GFP-LC3 signals are not detected using other fluorescence filter sets such as rhodamine, Cy5 and UV. True GFP-LC3 signals should be detected specifically by the GFP or FITC filter set.

4.2. Conversion of LC3-I to LC3-II

LC3 undergoes ubiquitin-like post-translational modifications that target it to the isolation membrane; these modifications are essentially similar to those carried out by the yeast Atg8 modification system (Ichimura et al., 2000; Kirisako et al., 2000). Immediately after synthesis, the C-terminal region of LC3 (22 and 5 amino acids in rat and human LC3, respectively) is cleaved (Kabeya et al., 2000). This cleavage is catalyzed by mammalian Atg4 homologues (Hemelaar, Lelyveld, Kessler, & Ploegh, 2003; Marino et al., 2003; Scherz-Shouval, Sagiv, Shorer, & Elazar, 2003) (Kabeya et al., 2004). The processed form, which has a glycine residue at the C-terminal end and is called LC3-I, resides in the cytosol (Kabeya et al., 2000) (Fig. 4). After activation by mammalian Atg7 (Tanida et al., 2001),

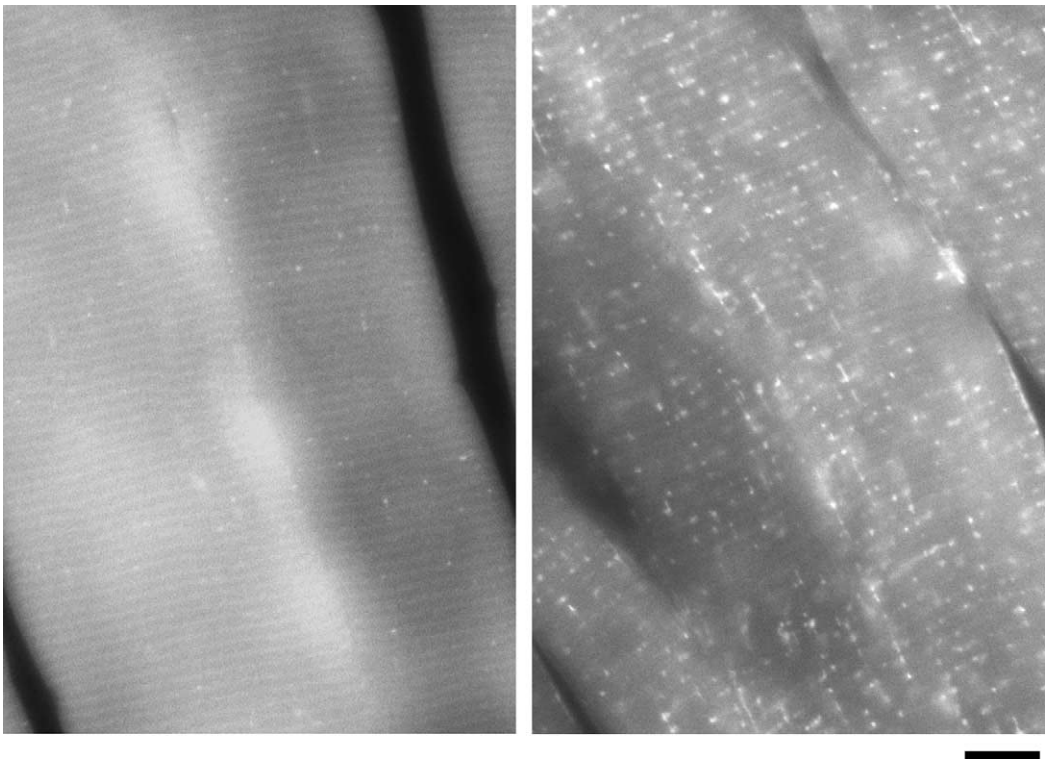

Fig. 5. In vivo analysis of autophagy using GFP-LC3 transgenic mice. Gastrocnemius muscle samples were prepared from GFP-LC3 transgenic mice before (left) or after 24 h starvation (right) and fixed with 4% paraformaldehyde. Cryosections were analyzed by fluorescence microscopy. GFP-LC3 dots represent autophagosomes. Bar 10 μm.

which also functions as an Atg12-activating enzyme, LC3 is transferred to a specific E2, Atg3 (Tanida et al., 2002). Finally, LC3 is likely conjugated to PE as by yeast Atg8 (Kabeya et al., 2004). This final form of LC3, designated LC3-II, associates tightly with the autophagosomal membrane. On SDS-PAGE, LC3-II migrates faster than LC3-I. Consequently, immunoblotting of LC3 usually gives two bands: LC3-I (apparent mobility, 18 kD) and LC3-II (apparent mobility, 16 kD) (Kabeya et al., 2000) (Fig. 4). The amount of LC3-II or the LC3-II/LC3-I ratio correlates with the number of autophagosomes (Kabeya et al., 2000). Formation of LC3-II depends on the Atg12–Atg5 conjugate: LC3-II is not observed at all in either *ATG5*$^{-/-}$ ES cells or these cells engineered to express the conjugation-defective Atg5 mutant (Atg5^{K130R}) (Mizushima et al., 2001) (Fig. 4).

Since the autophagosome is a transient structure, the lifetime of LC3-II is relatively short. Therefore, the level of LC3-II represents the autophagic activity at that moment. It does not indicate the magnitude of the flux through the autophagic pathway. Under conditions that induce autophagy, inhibition of lysyomal activity by protease inhibitors such as E64d and pepstatin A results in enhancement of the amount of LC3-II (Asanuma et al., 2003). It would represent the cumulative autophagic activity during the protease inhibitor treatments.

One possible problem with this method is that LC3-II might be more sensitive than LC3-I in immunoblot analysis (Kabeya et al., 2004). Thus, the amount of LC3-II could be relatively over-estimated. Sometimes the band intensity of LC3-II is stronger than that of LC3-I even in nutrient-rich conditions. Conformational changes or extreme hydrophobicity of LC3-II may account for this phenomenon. Despite this possible limitation, the measurement of LC3-II by immunoblotting is a simple and quantitative method

for determining the autophagic activity of mammalian cells.

5. Concluding remarks

Recently, much attention has been paid to the physiological roles of autophagy in mammals. There is no doubt that autophagy has many roles other than its role in the starvation response; for example, autophagy has been implicated in the process of morphogenesis, homeostasis and pathogenesis. However, these hypotheses are largely based on the observation of autophagy-like structures in cultured cells or tissues by conventional electron microscopy. To obtain more convincing data, specific and quantitative methods should be used in addition to conventional morphological examinations. Once the occurrence of autophagy has been confirmed by these methods, one of the next steps is to determine the role of autophagy. Gene-targeting approaches and development of specific inhibitors may help answer these questions.

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