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Review

How to Interpret LC3 Immunoblotting

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KEY WORDS

autophagy, LC3, immunoblotting, autophagic flux, p62

ABBREVIATIONS

LC3	microtubule-associated protein light chain 3
PE	phosphatidylethanolamine
MEF	mouse embryonic fibroblast

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ABSTRACT

Microtubule-associated protein light chain 3 (LC3) is now widely used to monitor autophagy. One approach is to detect LC3 conversion (LC3-I to LC3-II) by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes. However, LC3-II itself is degraded by autophagy, making interpretation of the results of LC3 immunoblotting problematic. Furthermore, the amount of LC3 at a certain time point does not indicate autophagic flux, and therefore, it is important to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors. Another problem with this method is that LC3-II tends to be much more sensitive to be detected by immunoblotting than LC3-I. Accordingly, simple comparison of LC3-I and LC3-II, or summation of LC3-I and LC3-II for ratio determinations, may not be appropriate, and rather, the amount of LC3-II can be compared between samples.

INTRODUCTION

Recently, the need to test cells and/or protein extracts for autophagic activity has increased, for example, to evaluate the effectiveness of RNAi or newly developed compounds. A variety of different methods are currently available, most of which have been extensively described in recent reviews.[1-3] Among them, LC3 immunoblotting is the most widely used. Although LC3 has several homologs in mammals, LC3B is most commonly used for autophagy assays. Endogenous LC3 is detected as two bands following SDS-PAGE and immunoblotting: one represents LC3-I, which is cytosolic, and the other LC3-II, which is conjugated with phosphatidylethanolamine (PE) and is present on isolation membranes and autophagosomes, and much less on autolysosomes.[4,5] Nascent LC3 (proLC3), which is neither LC3-I nor LC3-II, is not detected under normal conditions because it is processed by Atg4 into LC3-I immediately after synthesis.[4] Although the molecular weight of LC3-II is larger than that of LC3-I due to the addition of PE, LC3-II migrates faster than LC3-I in SDS-PAGE probably because of its extreme hydrophobicity. LC3 conversion from LC3-I to LC3-II therefore represents PE-conjugation, not processing of LC3. LC3-I is usually detected on a gel at a molecular mass around 16 kD, and LC3-II at approximately 14 kD. The amount of LC3-II is closely correlated with the number of autophagosomes, serving as a good indicator of autophagosome formation.[4] However, since LC3-II itself is degraded by autophagy, LC3 immunoblotting is sometimes interpreted inappropriately. In this review, we discuss possible pitfalls and important cautions regarding LC3 immunoblot analysis. Cautions related to LC3 microscopy have been described elsewhere.[6]

DETECTION OF AUTOPHAGIC FLUX

During a short starvation period, the amount of LC3-I decreases and that of LC3-II increases (Fig. 1A). However, if cells are subjected to longer starvation, both LC3-I and LC3-II disappear (Fig. 1A). This is because LC3-II is present both on inner and outer autophagosome membranes, with the former being degraded inside autolysosomes, whereas LC3 on the outer membrane is deconjugated by Atg4 and returns to the cytosol.[7] If cells are treated with lysosomal protease inhibitors such as E64d and pepstatin A, degradation of LC3-II is partially inhibited, whereas that of LC3-I is not affected (Fig. 1A). This can also be achieved using bafilomycin A1, which inhibits autophagosome-lysosome fusion.[8,9] These findings indicate that the amount of LC3-II at a certain time point does

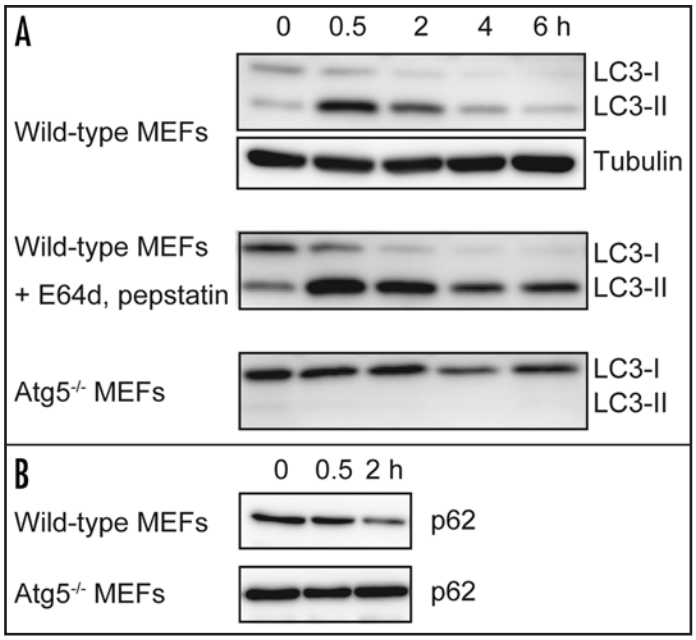

Figure 1. Expression levels of LC3-I, LC3-II and p62 during starvation. (A) *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-LC3 antibody[11] and anti-tubulin antibody. E64d (10 μg/ml) and pepstatin A (10 μg/ml) were added to the medium where indicated. Positions of LC3-I and LC3-II are indicated. (B) Degradation of p62 by autophagy. Cells were cultured as in (A) and p62 expression levels were analyzed using anti-p62 antibody (Progen Biotechnik).

not indicate the total autophagic flux. This flux is more accurately represented by differences in the amount of LC3-II between samples in the presence and absence of lysosomal protease inhibitors. If one does not have any other clear evidence of autophagic degradation, this type of analysis will strengthen the data.

An alternative method for detecting the autophagic flux is measuring p62 (SQSTM1/sequestosome 1) degradation.[10] p62 can bind LC3, thus serving as a selective substrate of autophagy. The level of p62 decreases during starvation in wild-type MEFs, but not in Atg5$^{-/-}$ MEFs,[11] suggesting that the reduction is mediated by autophagy. In addition, basal levels of p62 are upregulated in Atg5$^{-/-}$ MEFs, indicating that accumulation of p62 could be a good indicator of autophagy suppression (Fig. 1B).[12,13] However, the expression level of p62 can also be changed independent of autophagy.[14-16] Therefore, presentation of only p62 data may not be sufficient to estimate the autophagic flux, and combination with other methods will be preferred.

IMMUNOREACTIVITY OF LC3-I AND LC3-II

Another problem with the LC3 conversion method is that immunoreactivity of LC3-I and LC3-II differs. The increase in LC3-II is usually much larger than the decrease in LC3-I (Fig. 1A). This is not because the total expression level of LC3 increases during starvation; the level of LC3-I is very stable, or rather decreases during starvation in Atg5$^{-/-}$ MEFs. This indicates that synthesis of LC3 protein is not upregulated during starvation, although several reports have suggested that LC3 mRNA is induced during autophagy induction.[17,18] The apparent increase in total LC3 in wild-type MEFs is believed to be the result of overestimations of LC3-II levels during

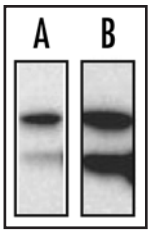

Figure 2. PC12 cells were cultured in the absence of serum and amino acids for 2 hours. Total cell lysates were then prepared and subjected to Immunoblot analysis using monoclonal anti-LC3 antibody [Molecular & Biological Laboratories (MBL)]; Code #M115-3, Clone #51-11) (A) and polyclonal antibody raised against the N-terminal peptide of LC3 (B).[4]

immunoblotting,[5] the sensitivity of detection of LC3-II by anti-LC3 antibody having been shown to be much higher than that of LC3-I in most cases. This effect is particularly apparent if we use antibody generated against the N-terminal peptide of LC3 (Fig. 2). A similar phenomenon has also been observed with yeast Atg8; PE-conjugated Atg8 is more sensitive to detection by anti-Atg8 antibody, particularly when raised against its N terminus.[19] Based on these findings, it is now believed that some conformational change is produced at the N terminus of Atg8/LC3 after PE-conjugation, which allows better detection, presumably due to exposure of an antibody-reactive epitope.

What, therefore, is the best indicator of autophagy? The amount of LC3-II, the LC3-II/LC3-I ratio or LC3-II/(LC3-I + LC3-II) ratio is now used. Since LC3-II tends to be more sensitive than LC3-I in immunoblotting, simple comparison of LC3-I and LC3-II, or summation of LC3-I and LC3-II, is not appropriate. Rather, comparison of the amount of LC3-II among samples is likely to be a more accurate method.

CASE STUDIES (Fig. 3)

Q1. What does this result indicate?

A1. It indicates that the treatment causes either upregulation of autophagosome formation or blockage of autophagic degradation. These two possibilities cannot be distinguished from the results shown in this part of the panel. The increase in LC3-II simply indicates the accumulation of autophagosomes, but does not guarantee autophagic degradation. If, however, the amount of LC3-II further accumulates in the presence of lysosomal protease inhibitors, this would indicate enhancement of the autophagic flux (A1). However, if the LC3-II level were to remain unchanged, it is likely that autophagosome accumulation occurred due to inhibition of autophagic degradation; for example, blockage of autophagosome-lysosome fusion.

Q2. Can it be autophagy defective?

A2. It is possible that these cells are autophagy defective even if LC3-II is detected under any conditions. It should be noted that in yeast, significant PE-conjugation of Atg8 occurs even in *atg* mutants other than the *atg8* and *atg12* conjugation mutants.[20] For example, in *vps30/atg6* (*beclin-1* counterpart)-disrupted cells, Atg8-PE is detected even though pre-autophagosomal structure (the site where Atg8-PE is primarily localized in wild-type cells) is not generated. This probably occurs because PE-conjugation can take place on ectopic membranes if the autophagic pathway is blocked. Therefore, the presence of LC3-PE does not always rule out the possibility that these cells are autophagy defective, and thus, one should determine the autophagic flux using lysosomal protease inhibitors to confirm

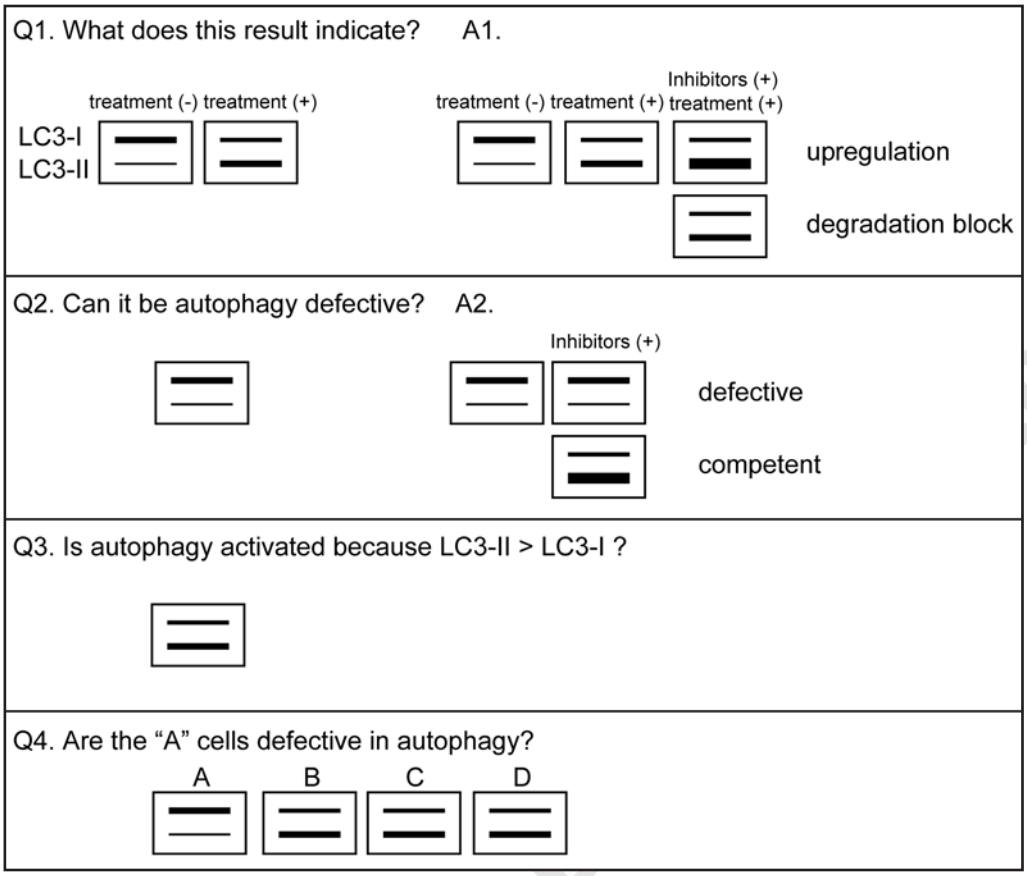

Figure 3. How to interpret LC3 immunoblotting data. See text for details.

whether the cells are autophagy-defective or -competent (A2). Lysosomal protease inhibitor treatment should also be considered even if no LC3-II is detected. A very high degradation capacity can lead to quick disappearance of LC3-II, which might be misunderstood as an autophagy defect.

Q3. Is autophagy activated because LC3-II > LC3-I?

A3. No. As discussed above, LC3-II tends to be much more sensitive in immunoblotting than LC3-I, further depending on the antibody used as shown in Figure 2. As a result, we cannot predict absolute autophagic activity simply by measuring the apparent amount of LC3-II on blots.

Q4. Are the "A" cells defective in autophagy?

A4. If the four cell groups are independently cultured, one should be very careful in drawing conclusions, since there are many factors that affect autophagic activity and LC3 conversion. The amount of LC3-II can fluctuate greatly even if the same cell line is cultured in the same medium with identical equipment. Thus, it is very difficult to compare LC3-II amounts between cells that are independently cultured for long period of time, and, accordingly, even if a single cell line is used, cells should be split shortly (ideally no more than one day) prior to processing the samples for the LC3 conversion assay. To compare autophagic capacity, it would, however, be better to analyze the autophagic flux for each cell line.

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