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Fine Structure of the Autophagosome

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Summary

This chapter describes the electron microscopic fine structure of early and late autophagic vacuoles in mammalian cells. Detailed instructions are given for the preparation of cells for conventional electron microscopy and for the identification of autophagic vacuoles by morphology. Electron microscopy remains one of the most accurate methods for quantitation of autophagic vacuole accumulation. Therefore, quantitation of autophagic vacuoles by electron microscopy and point counting is also described. Finally, a short description is given for preparation of ultra thin cryosections for immunogold labeling of autophagic vacuoles.

Key Words: Autophagy; electron microscopy; point counting; volume fraction; cryosectioning, immunoelectron microscopy.

1. Introduction

1.1. The Significance of Autophagy

Autophagy is a lysosomal degradation pathway for cytoplasmic material ***(1,2)*** that is important for survival during short-term starvation. By degrading some nonessential components, cells get nutrients for energy production and vital biosynthetic reactions. Autophagy is essential for energy metabolism during starvation ***(3,4)*** and immediately after birth ***(5)*** and for cell homeostasis in muscle, liver, and pancreas ***(6,7)***. Autophagy contributes to growth regulation—impaired autophagy can lead to cancer ***(8,9)***—and to longevity ***(10)***. Further, autophagy has been shown to reduce the toxicity of the protein aggregates in Huntington's disease ***(11)***. In the central nervous system, inhibition of autophagy causes neurodegeneration ***(12,13)***. In addition, autophagy plays a role in innate immunity in defense against viral infection ***(14)*** and intracellular bacteria ***(15)***.

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1.2. The Autophagic Pathway

After an induction signal such as starvation, autophagy starts when a flat membrane cistern wraps around a portion of cytoplasm and forms a closed double-membraned vacuole containing cytosol and/or organelles ***(16)***. The membrane cistern has been called the *phagophore* or the *isolation membrane*. The sealed vacuole is called the *autophagosome* and is devoid of any lysosomal proteins. Autophagosomes mature by fusing with endosomal and lysosomal vesicles, which also deliver lysosomal membrane proteins and enzymes ***(17,18)***. The segregated cytoplasm is then degraded by lysosomal hydrolases, and the degradation products are transported back to cytoplasm. A schematic drawing of autophagy is presented in **Fig. 1**.

The term *amphisome* refers to an autophagosome that has fused with an endosome, and *autolysosome* refers to an autophagosome or amphisome that has fused with a lysosome. The general term *autophagic vacuole* refers to an autophagosome, amphisome, or autolysosome. In electron microscopy, autophagic vacuoles can be identified as membrane-bound vesicles containing cytoplasmic material or organelles ***(1)***. Morphologically, autophagic vacuoles can be further classified into early or initial autophagic vacuoles (AVi),

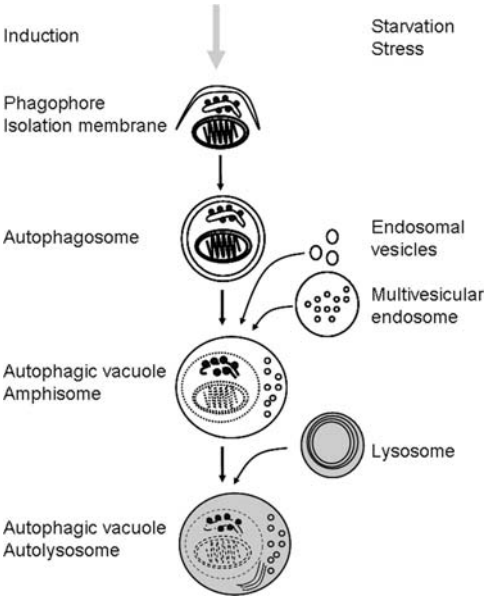

Fig. 1. Schematic presentation of the formation and maturation of autophagosomes in mammalian cells. The nomenclature of the different autophagosome stages is given on the left side of the drawing. Further details are given in the text.

containing morphologically intact cytosol or organelles, and to late or degradative autophagic vacuoles (AVd), containing partially degraded cytoplasmic material ***(17,19,20)***. (The morphology of early autophagic vacuoles is demonstrated in **Figs. 4A,B, 5B**, and **6A,B**. Late autophagic vacuoles are shown in **Figs. 5B and 6A,B**.)

1.3. Identification of Autophagic Vacuoles by Light and Electron Microscopy

Several novel yeast genes essential for autophagy (Atg genes) were recently characterized ***(21)***. Many mammalian homologs have been identified for these yeast genes. The microtubule-associated protein 1 light chain 3, or LC3, was shown to be the mammalian homologue of Atg8 ***(22)***. LC3 is the only known marker protein for autophagic vacuoles. Anti-LC3 has been used as a marker for autophagosomes, especially in light microscopy, but also in electron microscopy ***(23)***. LC3 is present mainly in autophagosomes. Because it is degraded by the incoming lysosomal hydrolases, less LC3 is present in amphisomes and autolysosomes ***(23)***. In electron microscopy, autophagic vacuoles can also be identified by morphology, particularly when using samples embedded in conventional plastic resins. This chapter deals with the fine structure of autophagic vacuoles in plastic embedded samples, with a short description of the quantitation of autophagy. Finally, reference is made to the fine structure of autophagic vacuoles in thin cryosections which are used for immunogold labeling.

2. Material

1. *Fixation for Plastic Embedding*: 2% glutaraldehyde (electron microscopy grade) in 0.2 *M* hydroxyethyl piperazine ethane sulfonate (HEPES), pH 7.4.
2. *Fixation for Cryosectioning and Immunolabeling*: 4% paraformaldehyde (PFA, use the powder form) in 0.2 *M* HEPES, pH 7.4. This solution may contain 0.05–0.2% glutaraldehyde (electron microscopy grade).
3. *Resin for Flat Embedding*: Agar 100 (Agar Scientific, Stansted, UK) and LX-112 (Ladd Research, Williston, VT, USA) have been tested with the flat embedding protocol. Other Epon equivalent resins may work as well, but may cause problems with cells seeded on plastic. Many resins partially dissolve plastic, and thus it will become very difficult to detach the block from the dish. Therefore, glass cover slips are recommended when other Epon equivalent resins are used for flat embedding.
4. *Resins for Embedding of Cell Pellets*:

   a) Durcupan mixture (Fluka):
   Component A: 10 g
   B: 10 g
   C: 0.3 g
   D: 0.3 g

Mixture can be stored at −20°C for some days. Do not open the container before the mixture has reached room temperature.

b) Agar 100 mixture (Agar Scientific): see manufacturer's instructions. Use the medium formula. Other Epon equivalent embedding media will probably work as well.

5. *1.84 M Sucrose–20% PVP for Cryoprotection*: Prepare 2.3 *M* sucrose in phosphate-buffered saline (PBS) by dissolving 39.365 g sucrose in 50 mL PBS. This takes some hours with constant mixing. You may gently warm the solution (37°C) to enhance dissolving. Put 40 mL of 2.3 *M* sucrose in a 100-mL Erlenmeyer flask. Add 10 g polyvinylpyrrolidone (PVP) (Sigma, Mw 10,000). Mix with a magnetic stirrer overnight to dissolve. Check the pH with a pH paper—if necessary, adjust the pH to 7.4 using 1.1 *M* Na_2CO_3 (usually less than 1 mL is necessary). For 1.7 *M* sucrose–15% PVP, use 2.1 *M* sucrose and add 7.5 g PVP.

3. Methods

3.1. Resin Flat Embedding of Aldehyde-Fixed Animal Cell Cultures

1. Grow cells on plastic dishes or glass cover slips. Incubate cells in serum and amino acid free medium for 1–2 h to induce autophagy. From **step 2** onward, everything is done in a fume hood, with gloves. Do not let the cells dry out at any point during the procedure.
2. Fix the cells by changing the culture medium to 2% glutaraldehyde in 0.2 *M* HEPES, pH 7.4. Incubate at room temperature (RT) for 2 h.
3. Wash the cells in 0.2 *M* HEPES (and storage at 4°C for up to some weeks).
4. Wash the cells in PBS three times.
5. Postfix in 1% Osmium tetroxide in water at RT for 1 h. Osmium is very toxic and volatile; use a good fume cupboard and gloves.
6. Wash the cells in water twice.
7. Stain the cells in 2% uranyl acetate in water at RT, in the dark, for 1 h. This step gives contrast to autophagosome membranes.
8. Dehydration (RT):
   70% ethanol in water 3 times 3 min
   95% ethanol 3 times 3 min
   100% ethanol 3 times 3 min
9. Optional: Dip cover slips in propylene oxide. Proceed quickly to **step 10**, as propylene oxide evaporates very quickly. Do not use propylene oxide on plastic dishes—it dissolves plastic.
10. Infiltration of resin: Aspirate almost all ethanol from the cells. Add a few drops of 100% resin and swirl the dish to spread the resin to the cells. The resin layer should cover the cells, but it should not be thick. Optimally add less than 1 mm layer of resin. Incubate for 1–2 h at RT, until all ethanol has evaporated.
11. Fill gelatin or Beam capsules with resin. Place the capsules upside down to the cells. Make sure no air bubbles stay between the cells and the resin. It is

possible to check the cells with an inverted phase contrast microscope and select the desired area for the capsules.

12. Polymerize the resin at 45°C overnight and then at 60°C for one day.
13. The capsules can be released from the plastic dishes by gentle heating on a hot plate, then gently swirling the capsule. Glass cover slips can be removed by dipping the glass to liquid nitrogen for 5–10 s, and then knocking on the glass to remove it from the block. Make sure all glass is removed before using a diamond knife to cut sections.
14. Cut 70- to 80-nm sections with a diamond knife, and stain the sections with uranyl acetate and lead citrate. *See* **Notes 1–3** on the fine structure of autophagosomes.

Option 1. Use reduced osmium tetroxide instead of osmium tetroxide in water **(step 5)**. This enhances the contrast of autophagosome-limiting membranes, but reduces the contrast of ribosomes. Thus, the later maturation stages of autophagic vacuoles are more difficult to identify. Protocol: Mix 2% osmium tetroxide in water and 0.2 *M* sodium cacodylate, pH 7,4, to give the final concentrations of 1% osmium tetroxide and 0.1 *M* cacodylate, and add 15 mg/mL $K_4Fe(CN)_6$. Incubate at RT for 1 h.

Option 2. Use sucrose in the uranyl acetate en block staining solution **(step 7)**: 1 % uranyl acetate, 0.3 *M* sucrose in water, 1 h, 4°C. This may reduce extraction of some sample components.

3.2. Resin Embedding of Aldehyde-Fixed Animal Cell Pellets for Quantitation of Autophagy

1. Use 6-cm culture dishes. The cells should be semi-confluent. Fix cells in 2% glutaraldehyde in 0.2 *M* HEPES, pH 7.4 at RT for 2 h. Scrape the cells from culture plate using a razor blade after 30 min fixation. Pellet the cells at full speed in an Eppendorf centrifuge for 5 min. Continue fixation as a pellet. Do not resuspend the pellet during the rest of embedding, but use gentle mixing during incubations.
2. Wash the pellets in 0.2 *M* HEPES (and store at 4°C).
3. Wash in PBS three times.
4. Postfix the pellets in 1% osmium tetroxide in water at RT for 1 h.
5. Wash in water twice.
6. Stain the pellets in 2% uranyl acetate in water at RT, in the dark, for 1 h.
7. Dehydration (RT) (mixing is important during dehydration):
   70% ethanol in water 15 min
   95% ethanol 15 min
   100% ethanol twice for 15 min
   propylene oxide 20 min (toxic and volatile)
8. Infiltration of resin:
   resin + propylene oxide (1 + 1) at RT for 2 h
   100% resin overnight, RT

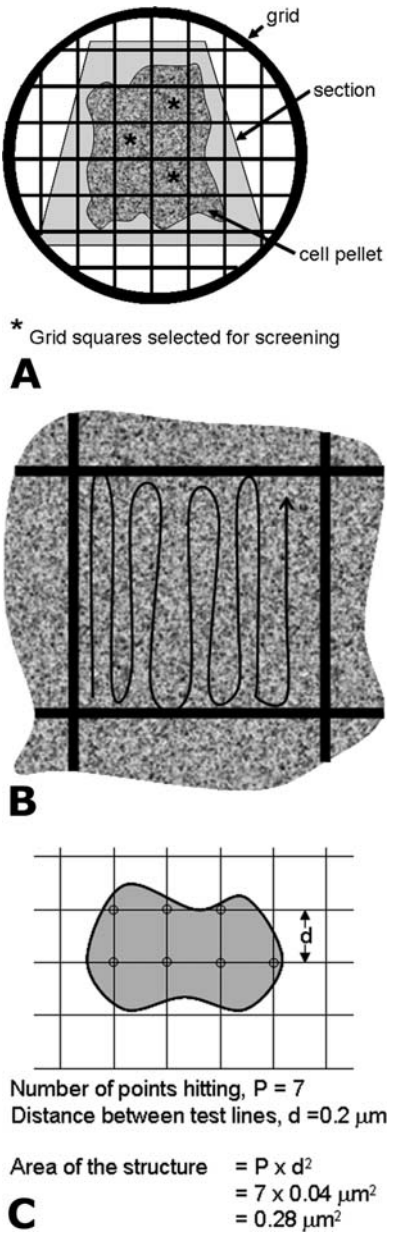

Fig. 2. Principles of autophagic vacuole quantitation and point counting. **(A)** Grid squares can be used as sampling units. It is recommendable to count at least three squares from each sample. The selected squares should be uniformly distributed over the section. When a small magnification (400–600×) is used for this selection, selection bias is easier to avoid because autophagic vacuoles are not visible at low magnification.

9. Transfer the pellets to fresh resin in embedding molds and incubate for 4–6 h, RT.
10. Polymerize the blocks at 60°C for 2 days.
11. Cut 80-nm sections with a diamond knife and stain with uranyl acetate and lead citrate. *See* **Notes 1–3** on the fine structure of autophagosomes.

3.3. Quantitation of Autophagic Vacuoles in Thin Sections

For quantitative estimation of the autophagic accumulation, the orientation of the cells in the sections should be random. This is best achieved when using cell pellets. If flat embedded cells are used, the counting should be done using sections cut from more than one plane, which should locate a few micrometers from each other. This is because it is possible that the structures of interest are more concentrated in a certain region of the cytoplasm (for instance, close to the culture surface or above the nucleus). This problem is most easily avoided when using randomly oriented sections, which can be done using cell pellets.

There are two alternatives for the electron microscopic quantitation. First, the volume fraction, e.g., fraction of the cellular volume occupied by autophagic vacuoles, can be estimated. This can be accurately and efficiently estimated by point counting ***(24)*** **(Fig. 2)**. Second, it is possible to estimate the number of autophagic vacuole profiles per cell area on sections. The advantage of the latter is that is saves a considerable amount of work, since only the cell volume needs to be estimated by point counting.

Square or hexagonal grid openings can be used as sampling units in the quantitation **(Fig. 2)**. The whole grid square is systematically scanned under the microscope for the presence of early and late autophagic vacuoles **(Fig. 2B)**. When estimating the number of vacuoles per cell area, only the number of vacuoles in the grid square is recorded. When estimating volume fraction, each autophagic vacuole is photographed at 12.000× magnification for point counting of the area

Fig. 2. *(Continued)* **(B)** Screening of the selected grid squares for the presence of autophagic vacuole profiles at higher magnification 12,0000×. **(C)** Point counting. A square lattice is placed on top of a microscopic photograph or photographic negative. The intersections of the lattice lines are used as test points. Points hitting the structure of interest (autophagic vacuole, or cell in a cell pellet) are counted. The distance between points (*d*) is calibrated with the final magnification of the photograph. These two parameters are used to calculate the area of the structure, as indicated below the drawing. This procedure can be done on the computer screen, if the photographs are digital. The lattice is created using the computer program. If the microscope is using a film camera, point counting can be performed using a light box. The lattice is printed on an overhead foil which is then placed on top of the film sheet. A magnifying loop can be used to help counting.

of the vacuoles (point counting 1; **Fig. 2C**). This can be done directly from photographic negatives if a digital camera is not available in the microscope.

The cell area can be measured using about 400× magnification, which allows the whole grid square to fit in one photograph. The area of the cell profiles is then estimated using point counting with a different lattice (point counting 2; **Fig. 2C**). Finally, the number of vacuoles per cell area is simply calculated by dividing the number of vacuoles in the grid square with the cell area in the same grid square. Similarly, the volume fraction is calculated by dividing the area occupied by autophagic vacuoles (from point counting 1) with the cell area in that grid square (from point counting 2). Thus, the ratio of areas directly gives the ratio of volumes ***(25)***. It is recommendable to count at least three grid squares for each sample to get a representative estimate **(Fig. 2A)**. These squares should locate evenly over the whole thin section and should be selected using systematic random sampling ***(25)***.

In most cases, the number of autophagic vacuole profiles per cell area gives a very similar result compared to the stereologically more accurate volume fraction **(Fig. 3A,B)**. Since the former saves work, it is better suited for experiments containing several samples. It should be kept in mind, however, that the number of profiles per cell area is *not* equal to the number of vacuoles per cell area. This is because large vacuoles have a greater probability than small ones to hit the thin section. For more exact discussion, please refer to stereology literature ***(25)***. For a general discussion of quantitation of autophagic vacuoles, *see* **Notes 4** and **5**.

3.4. Preparation of Cultured Cells for Cryosectioning and Immunogold Labeling

Use confluent/subconfluent cultures. There are basically two options for fixation: paraformaldehyde mixed with a low concentration of glutaraldehyde or paraformaldehyde only. Glutaraldehyde gives better ultrastructural preservation, but not all antigens or antibodies tolerate glutaraldehyde treatment. Use 4% paraformaldehyde (PFA) in 0.2 *M* HEPES, pH 7.4, and fix for 1–2 h at room temperature. This solution may contain 0.05–0.2% glutaraldehyde. If you use 4% PFA without glutaraldehyde, incubate the cells in this solution for 2 h, followed by 2% PFA in 0.2 *M* HEPES at 4°C overnight. In both options, transfer the cells to a fresh buffer after fixation. For long-distance shipping, leave the cells in 2% PFA in 0.2 *M* HEPES.

Gelatin embedding helps to preserve the fine structure of cells during sectioning. Gelatin embedding is not recommended if you wish to label lipid antigens. For gelatin embedding, first wash the cells in PBS. Then scrape the cells from the culture dish in PBS and transfer to an Eppendorf tube. Pellet the cells at full speed in an Eppendorf centrifuge. Add 200 µL 10% gelatin in

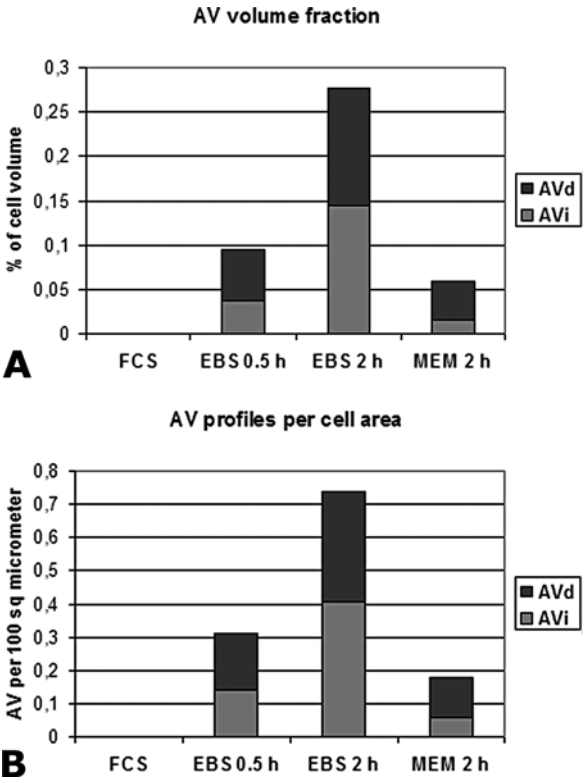

Fig. 3. Estimation of autophagic vacuole accumulation in mouse embryonic fibroblasts. The cells were fixed without treatment (FSC), or incubated in serum and amino-acid–free medium for 30 min (EBS 0.5 h) or 2 h (EBS 2 h), or in serum-free culture medium for 2 h (MEM 2 h). Cells were fixed and prepared for electron microscopy according the protocol given in **Subheading 3.2**. Accumulation of autophagic vacuoles was estimated both as volume fraction **(A)** and as number of profiles per cell area **(B)**. The number of profiles is given per 100 μm^2. AVi, initial autophagic vacuoles containing morphologically intact cytoplasm; AVd, late autophagic vacuoles containing partially degraded, but still identifiable, cytoplasmic material.

PBS at 37°C and mix gently with the pipet tip. Incubate at 37°C for 5 min. Pellet the cells again at room temperature (suitable speed needs to be tested for each cell type). Remove the gelatin from the tube, and add a fresh 200 µL of 10% gelatin, and mix with the tip. Pellet again at room temperature to get a firm pellet. Keep the tube in ice for 30 min to set the gelatin. Take a small spatula and dip it to sucrose-PVP (see below). Then use the wet spatula to transfer the gelatin and cells to a Petri dish on ice, and cut the cell pellet to small cubes/sticks (maximum side length about 2 mm).

Before freezing, the cells need to be incubated in a cryoprotectant. 2.1–2.3 *M* sucrose in PBS or a mixture of sucrose and PVP in PBS (1.84 *M* sucrose–20% PVP, or 1.7 *M* sucrose–15% PVP) are mostly used. PVP gives plasticity to the block and thus helps cryosectioning. Incubate the cell cubes in sucrose-PVP at 4°C, with occasional mixing, for several hours to overnight, until the cubes sink to the bottom.

Trim the cubes under a stereo microscope and mount on cryoultramicrotome holders at 4°C (in a cold room, or using an ice bath). For optimal freezing, the maximum size of the cubes should be about 1 mm. Freeze the mounted cubes by punching into liquid nitrogen. Store the samples in liquid nitrogen until sectioning.

Cut 70- to 80-nm sections at −100°C using a cryoultramicrotome and a cryodiamond knife. Use a mixture of sucrose and 1.5% methyl cellulose (1 + 1–4 + 1) to pick up the sections. Compared to picking up sections with sucrose only, this greatly improves the ultrastructure of the cells ***(26)***. Transfer sections on carbon-coated Formwar grids and leave the extra sucrose–methyl cellulose on the grids. The grids can be stored section side up, at 4°C, for several months. To remove the sucrose–methyl cellulose, incubate the grids on PBS twice for 5 min.

Immunogold label the sections using standard procedures for Tokuyasu sections ***(24)***. Finally embed the sections in methyl cellulose–uranyl acetate. **Notes 6** and **7** deal with the use of cryosections to study autophagy.

4. Notes

4.1. Fine Structure of Autophagosomes and Autophagic Vacuoles in Plastic Sections

1. By definition, autophagosomes are double-membrane limited vacuoles that contain undegraded cytoplasm and no lysosomal proteins. In plastic sections, the two limiting membranes are often so close to each other that it is not possible to see them as separate membranes (**Fig. 4A**, large arrowheads). Sometimes the limiting membrane may appear to contain multiple layers (**Fig. 4B**, arrow). It is possible that this is an artifact caused by the chemical fixation. On the other

Fig. 4. Fine structure of autophagosomes (early/initial autophagic vacuoles) in plastic embedded hepatocytes isolated from mouse liver. The cells were incubated in serum and amino acid free medium to induce autophagy. **(A)** The autophagosome on top of the panel contains a mitochondrion, endoplasmic reticulum, and ribosomes. The limiting membrane (large arrowheads) is visible only partially as a double membrane (arrows). Below the autophagosome, a U-shaped membrane cistern, a putative phagophore (small arrowhead), seems to be in the process of wrapping around a peroxisome (p). **(B)** Rough endoplasmic reticulum

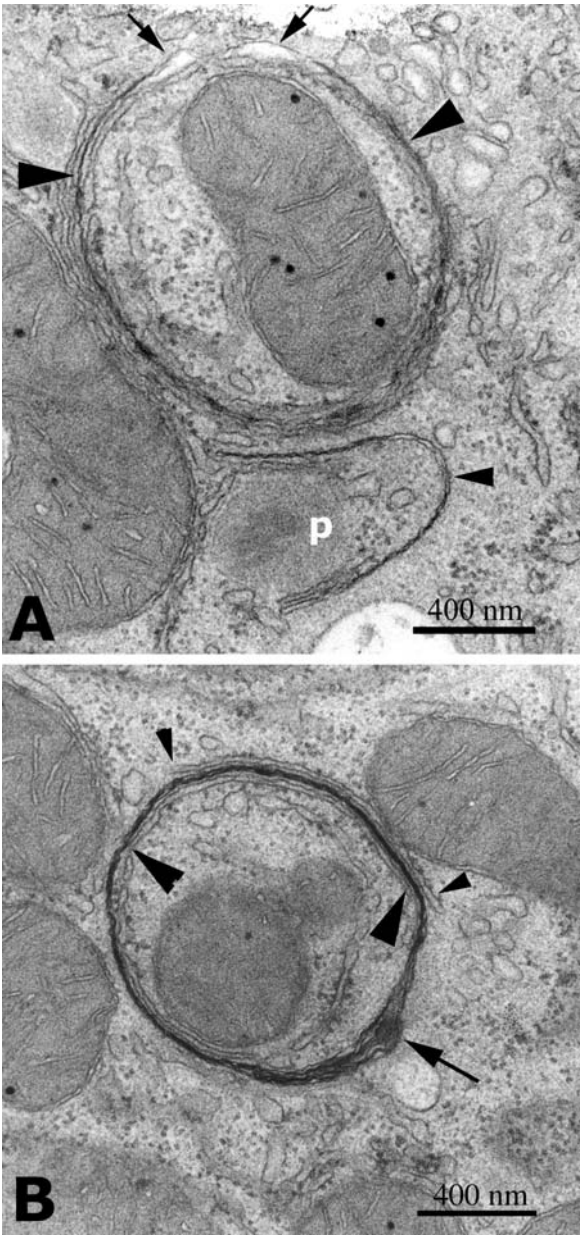

Fig. 4. *(Continued)* tightly follows part of the limiting membrane of the autophagosome (small arrowheads). Another rough cistern is located inside the autophagosome, on the other side of the limiting membrane (large arrowheads). The arrow indicates a region where the autophagosome limiting membrane seems to consist of multiple layers. This may be an artifact caused by aldehyde fixation. (From **ref. *1***.)

hand, it is also possible to see the two limiting membranes in plastic sections. In these cases, there is a narrow empty (electron lucent) space between the two membranes **(Figs. 5B, 6A,B)**. Sometimes the autophagosome limiting membrane does not have any contrast in thin sections. This is probably caused by extraction of lipids during sample preparation. Occasionally, it is possible to observe putative phagophores, U-shaped membrane cisterns which seem to be engulfing portions of cytoplasm (**Fig. 4A**, small arrowhead, and **Fig. 5A**, asterisks). In these cisterns the two separate membranes are usually clearly visible. Typically, a cistern of rough endoplasmic reticulum (ER) is located close to the autophagosome or phagophore. Another rough cistern is often located on the other side of the autophagosome or phagophore limiting membrane (**Figs. 4B and 5A**, small and large arrowheads). Thus, the limiting membrane runs in the space between these two rough ER cisterns. The role of this autophagosome-associated ER, if any, is currently unknown.

2. The cytoplasmic contents of autophagosomes include organelles, such as ER membranes and mitochondria **(Fig. 4A,B)**. Ribosomes are frequently seen inside autophagosomes (**Figs. 4A,B, 5B, and 6A,B**) and serve as a good marker structure for the cytoplasmic contents. The diameter of autophagosome profiles varies between 300 and 400 nm and several micrometers. In many cultured cells the average diameter is about 600 nm. Autophagosomes are frequently observed in fusion profiles with endosomal or lysosomal vesicles ***(18)***. Autophagosomes can also fuse with each other, or several autophagosomes can fuse with a single endo/lysosomal vesicle or late autophagic vacuole **(Fig. 5B)**. In the fusion event, the outer limiting membrane fuses with the endo/lysosome limiting membrane. The contents, still surrounded by the inner limiting membrane, are delivered to the endo/lysosome lumen. This membrane must then be degraded, or at least permeabilized, to allow degradation of the cytoplasmic contents. In late autophagic vacuoles, partially degraded electron-dense ribosomes serve as a good criterion for identification of the cytoplasmic contents (**Fig. 5B**, asterisks, and **Fig. 6A,B**,

Fig. 5. Fine structure of putative phagophores and autophagic vacuoles in plastic embedded mouse embryonic fibroblasts. The cells were incubated in serum and amino acid free medium for 2 h to induce autophagy. **(A)** Two putative phagophores. Rough endoplasmic reticulum surrounds both phagophores (large arrowheads). Another rough cistern is located on the other side of the phagophore membrane sack in both phagophores (small arrowheads). The asterisks (*) indicate the empty space in the lumen of the phagophore, between the two limiting membranes of the coming autophagosome. **(B)** Three autophagosomes (arrows) in the process of fusion with a late autophagic vacuole. Asterisks indicate so-called autophagic bodies, portions of partially degraded cytoplasm still partially surrounded by the autophagosome inner limiting membrane. These can be identified by their contents of electron-dense ribosomes. Ribosomes become more electron dense as a consequence of degradation.

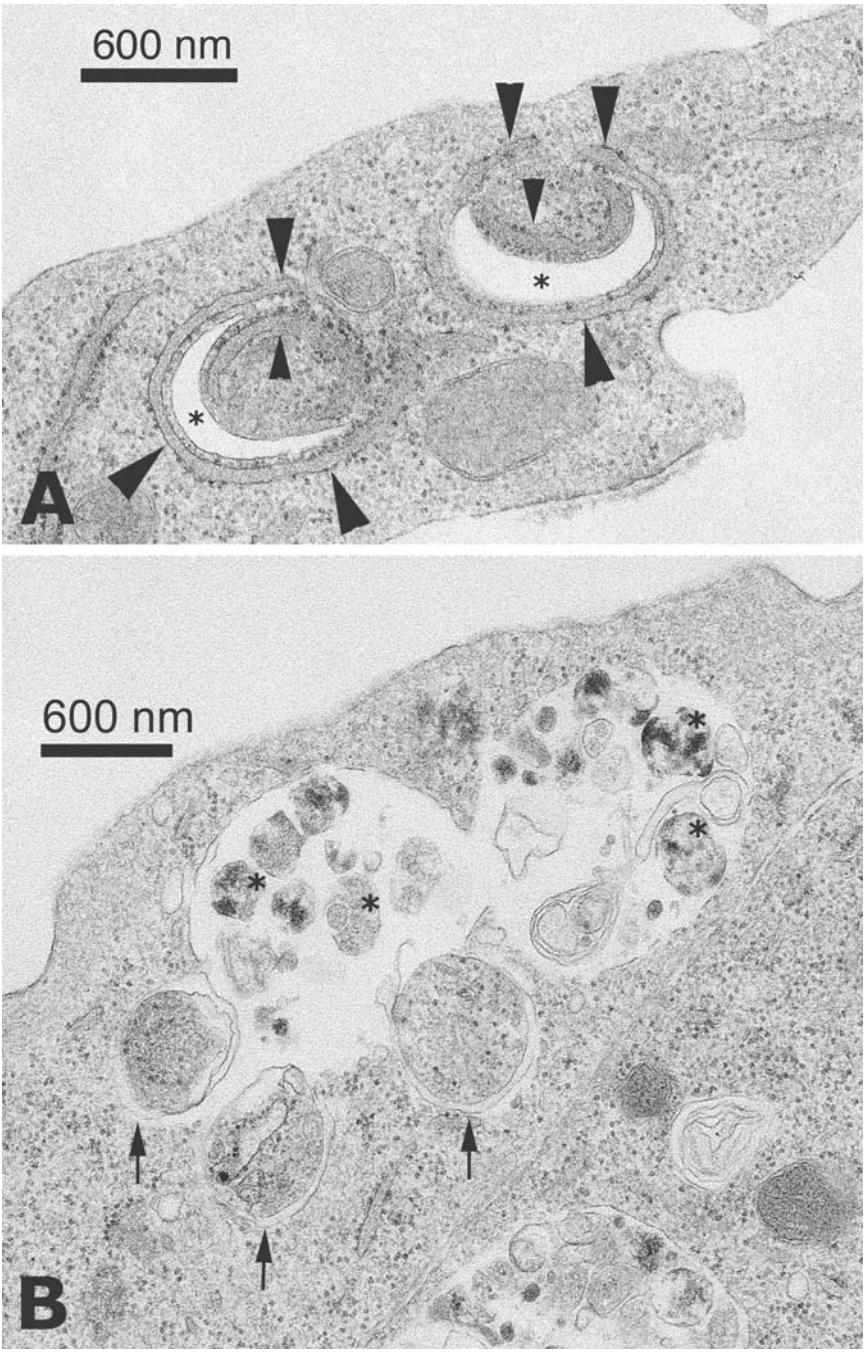

Fig. 5. *(Continued)*

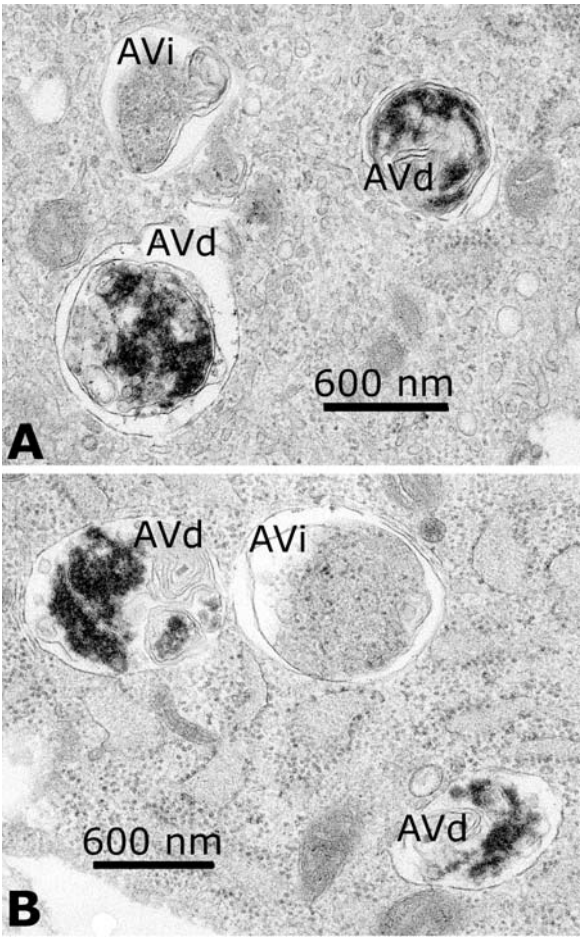

Fig. 6. Fine structure of early and late autophagic vacuoles in plastic embedded mouse embryonic fibroblasts. The cells were incubated in serum and amino-acid–free medium for 2 h to induce autophagy. Early autophagic vacuoles (AVi) contain morphologically intact cytoplasm, which looks identical to the cytoplasm surrounding the vacuoles. Late autophagic vacuoles (AVd) contain material which can still be recognized as cytoplasmic, in this case ribosomes, and looks partially degraded, i.e., more electron dense than the cytoplasm surrounding the vacuole.

AVd). The cytoplasmic contents often appear partly degraded, even when the inner limiting membrane still seems to be intact (**Fig. 6A**, AVd).

3. It should be noted that special attention must be paid when identifying autophagic vacuoles in transmission electron microscopy. There are several examples in the current literature of different organelles, including multilamellar or multi-

vesicular endosomes, even mitochondria, being claimed as autophagic vacuoles. Only vacuoles containing cytoplasmic material, in most cases ribosomes, can be claimed as autophagic.

4.2. Quantitation of Autophagy by Electron Microscopy

4. Before discovery of the Atg proteins, autophagy could only be detected using electron microscopy or biochemical methods ***(1)***. Even today, quantitative electron microscopy is one of the most sensitive methods to detect the accumulation of autophagic vacuoles. In addition, it is possible to detect the nature of the accumulating vacuoles, e.g., whether early autophagic vacuoles (AVi) or more matured late autophagic vacuoles (AVd) accumulate ***(1)***. The ratio of AVi/AVd gives clues as to the cause of the accumulation. If AVi predominate, there is probably a defect in the maturation of autophagosomes into degradative autophagic vacuoles. On the other hand, large accumulation of AVd suggests that there may be a defect in autolysosome formation. It should be noted that most of the AVd are probably amphisomes, since autolysosomes are likely short lived and thus less frequently observed by microscopy.
5. In cultured animal cells, the accumulation of autophagic vacuoles becomes detectable 15–30 min after the cells have been switched to starvation medium. The accumulation increases until 2 h starvation, but longer starvation times do not seem to increase the net accumulation, unless proteinase inhibitors or microtubule drugs are added to the incubation medium to prevent degradation in or formation of autolysosomes ***(18)***.

4.3. Autophagic Vacuoles in Thin Cryosections

6. Postembedding immunogold labeling with thin cryosections is one of the most sensitive methods to immunolabel antigens for electron microscopy ***(24)***. Concerning autophagy detection, the drawback of cryosections is that ribosomes have no contrast in these sections. This makes identification of autophagosomes more demanding, because without ribosomes it is difficult to identify the cytoplasmic contents. Also, classification of AVi and AVd is difficult in cryosections. Immunolabeling of the autophagosome marker LC3 can be used to help identification ***(23)***, but good antibodies are not always available. The level of endogenous LC3 is also relatively low for clear-cut immunogold labeling. Identification of autophagic vacuoles can still be achieved by morphology, at least in cases where clearly identifiable organelles such as mitochondria are seen among the contents (**Fig. 7**). Immunolabeling of lysosomal membrane proteins and enzymes can be used to detect fusion with endosomal or lysosomal vesicles (**Fig. 7**).
7. Some cell types are more suitable for cryosectioning than others. Isolated hepatocytes, for instance, give a good morphology in cryosections even after fixation in 4% paraformaldehyde only (**Fig. 7**). Cells and tissues with high glycogen content are not suitable for this kind of approach because glycogen is washed away during the sectioning and labeling procedure, leaving behind large empty areas

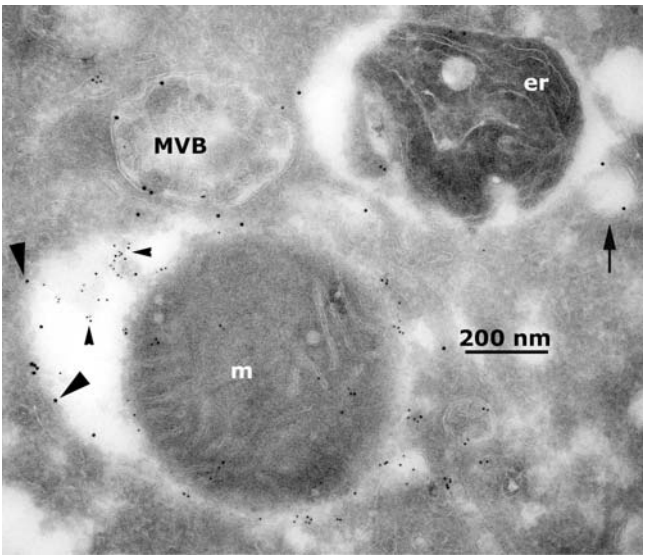

Fig. 7. Fine structure of autophagic vacuoles in a thin cryosection. Mouse hepatocytes were isolated and cultured overnight. Autophagy was induced in serum- and amino-acid–free medium. Thin cryosections were prepared as described in **Subheading 3.4.** and immunogold labeled with anti-cathepsin D (5 nm gold, small arrowheads) and anti-LAMP-2 (10 nm gold, large arrowheads). Autophagic vacuoles were identified by their morphology, using cytoplasmic contents as a criterion. Two autophagic vacuoles are shown. One of them contains a mitochondrion (m), the other partially condensed ER membranes (er). The arrow indicates a small vesicle, possibly about to fuse with the latter autophagic vacuole. MVB, multivesicular body/endosome.

in the sections. Therefore, the animals should be fasted before tissue preparation if glycogen-containing tissues such as liver or muscle are to be cryosectioned for electron microscopy.

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