CHAPTER THIRTEEN

AUTOPHAGY PATHWAYS IN GLIOBLASTOMA

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Abstract

Glioma cells are more likely to respond to therapy through autophagy than through apoptosis. The most efficacious cytotoxic drugs employed in glioma therapy, such as temozolomide and rapamycin, induce autophagy. Oncolytic adenoviruses, which will soon be tested in patients with gliomas at the University of Texas M. D. Anderson Cancer Center, also induce autophagy. Autophagy in gliomas thus represents a promising mechanism that may lead to new glioma therapies. In this chapter, we present the methods for studying autophagy in glioma cells, including assessment of in vitro cellular markers acidic vesicle organelles, and green fluorescent protein (GFP)-LC3 punctation; biochemical

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markers LC3-I/II conversion, p62 degradation, Atg12–Atg5 accumulation, and p70S6K dephosphorylation; and ultrastructure of the autophagosomes. In addition, we will address how LC3B and Atg5 up-regulation during autophagy can be examined through immunostaining in treated tumors and the potential of these proteins for use as surrogate markers to monitor therapeutic effects in clinical trials. Finally, we will discuss the challenges of studying autophagy in gliomas and the future directions of such use.

1. Introduction: Autophagy and Gliomas

Malignant astrocytic gliomas such as glioblastoma multiforme (GBM) are the most common and lethal intracranial tumors (Furnari et al., 2007). Despite implementation of intensive therapeutic strategies and supportive care, the median survival duration of patients with GBM has remained at 12 months for the past decade (Furnari et al., 2007). Gliomas are resistant to therapies that induce apoptosis (type I programmed cell death) (Furnari et al., 2007; Lefranc and Kiss, 2006) (Ziegler et al., 2008). However, several lines of evidence indicate that GBM cells seem to be less resistant to therapies that induce autophagy (type II programmed cell death) (Lefranc et al., 2007; Lefranc and Kiss, 2006). For example, rapamycin’s disruption of the pathway controlled by mTOR induces marked autophagic processes in GBM cells (Iwamaru et al., 2007). Temozolomide, currently the most efficacious cytotoxic drug employed to combat glioblastoma, exerts its cytotoxicity by inducing autophagic cell death (Kanzawa et al., 2004). Recent reports show that oncolytic adenoviruses, a promising alternative therapy for gliomas, also cause autophagic cell death in glioma cells and even in brain tumor stem cells (Ito et al., 2006; Jiang et al., 2007). However, the role of autophagy in causing cell death, rather than occurring along with cell death, is still not clear. For example, controversial results have been reported on the effect of inhibiting autophagy on cytotoxicity induced by various treatments (Baird et al., 2008; Ito et al., 2006; Kanzawa et al., 2004).

Autophagy is a dynamic subcellular process that degrades damaged or obsolete organelles and proteins (Rubinsztein et al., 2007; Xie and Klionsky, 2007) and occurs as a cellular response to stresses such as starvation or pathogen infection (Kondo et al., 2005; Levine, 2005). Autophagy has been implicated both in development and immunity (Levine and Deretic, 2007; Levine and Klionsky, 2004), and an abnormality in this autophagic process is related to diseases such as cancer (Kondo et al., 2005; Levine and Kroemer, 2008; Mizushima et al., 2008). Thus, for gliomas, understanding autophagy mechanisms at the molecular level will not only help identify efficacious points of intervention but also help explain the genesis of gliomas.
2. Prioritization of Methods to Characterize Autophagy in Gliomas

Autophagy is accompanied by the progressive development of vesicle structures from autophagosomes (not acidic) to amphisome and autolysosomes (acidic) (Klionsky et al., 2008; Paglin et al., 2001). With acidotropic dye acridine orange staining, the acidic compartments in the cell fluoresce bright red, whereas the nucleus and cytoplasm fluoresce bright green and dim red, respectively (Arvan et al., 1984; Delic et al., 1991; Mains and May, 1988). The intensity of the red fluorescence is proportional to the degree of acidity or the volume of the cellular acidic compartments, or both (Paglin et al., 2001). Thus, it is possible to monitor the development of acidic vesicular organelles during autophagy with acridine orange staining in glioma cells (Ito et al., 2006; Iwamaru et al., 2007; Jiang et al., 2007; Kanzawa et al., 2004). However, it is important to keep in mind that this acidotropic dye can also be retained in other intracellular acidic compartments, such as lysosomes, endocytic vesicles, portions of the trans-Golgi apparatus, and certain secretory vesicles (Anderson and Orci, 1988). Therefore, the intensity of the red fluorescence is not exclusively an indication of autophagic vacuoles. The correlation between the intensity of the red fluorescence and autophagy development will vary by cell type and conditions. Thus, this methodology should be used with other autophagic markers.

The most commonly used biochemical marker to monitor autophagic flux is LC3, which can be monitored by examining GFP-LC3 dots and LC3 lipidation on a Western blot (Klionsky et al., 2008) (also see the chapter by Kimura et al., in volume 452). The LC3 protein is a ubiquitin-like protein that can be conjugated to phosphatidylethanolamine (PE) (Klionsky et al., 2008). LC3 is initially synthesized in an unprocessed form, proLC3, which is cleaved from the C terminus, resulting in LC3-I, and is finally modified into the PE-conjugated form, LC3-II. LC3-II is the only protein marker that is reliably associated with completed autophagosomes (Klionsky et al., 2008). LC3 with a green fluorescent protein (GFP) tag at the N terminus, GFP-LC3, is used to monitor autophagy through direct fluorescence microscopy, which is measured as an increase in punctate GFP-LC3 in glioma cells (Aoki et al., 2007a,b; Aoki et al., 2008; Iwamaru et al., 2007; Kanzawa et al., 2004; Klionsky et al., 2008). Because LC3-II runs faster than LC3-I in sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), autophagy can also be indicated by the increase in the conversion of LC3-I to LC3-II in glioma cells (Aoki et al., 2007a,b; Aoki et al., 2008; Ito et al., 2006; Iwamaru et al., 2007; Jiang et al., 2007; Kanzawa et al., 2004).
One of the hallmark biological aberrations in glioma cells is the alteration of the PI3K/AKT/mTor pathway (Furnari et al., 2007). The activated pathway negatively regulates autophagy (Kondo et al., 2005). Thus, the inhibition of mTOR activity, which is indicated by the dephosphorylation of its substrate p70S6K, is correlated with autophagy in glioma cells (Ito et al., 2006; Iwamaru et al., 2007). Other autophagic markers can also be used to detect autophagy in glioma cells. For example, when glioma cells are infected with oncolytic adenoviruses, the Atg12–Atg5 conjugate is dramatically up-regulated (Jiang et al., 2007), whereas the long-lived protein p62 (Sequestosome 1 protein, SQSTM1), a ubiquitin- and LC3-binding protein (Komatsu et al., 2007), was degraded.

One of the most sensitive and reliable methods used to detect autophagic compartments in mammalian cells is transmission electron microscopy (Eskelinen, 2008; Klionsky et al., 2008) (see also the chapter by Ylä-Anttila in volume 452). This method can be used for both qualitative and quantitative analysis of changes in various autophagic structures (Klionsky et al., 2008). The advantage of this method is that it does not depend on the availability of specific antibodies or probes (Eskelinen, 2008). The method’s drawbacks include requiring an electron microscopy laboratory, taking longer than most other methods, and requiring special expertise that can only be gained by experience to correctly interpret the data (Eskelinen, 2008).

3. **In Vitro Cellular Markers**

3.1. **Quantification of acidic vesicular organelles with acridine orange staining**

i. Add acridine orange (Sigma–Aldrich, St. Louis, MO, USA, A8097) to glioma cells (∼5 × 10⁵ cells) cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA, 10437-028, 10378-016) at 1.0 μg/ml. Note that nonadherent cells growing in an aggregated cluster, such as brain tumor stem cells, need to be digested with accutase solution (400-600 U/mL, Sigma-Aldrich, A6964) and dissociated by pipetting before adding acridine orange.

ii. Incubate for 15 min under normal culture conditions.

iii. Remove the medium and wash 1 time with phosphate-buffered saline (PBS).

iv. For adherent cells, detach the cells with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Carlsbad, CA, USA, 15400-054), and separate the cells with pipetting. Do not overdigest the cells,
as this will change the permeability of the cells and cause the cells to lose acridine orange staining.

v. Suspend the cells in 1.5 ml of PBS and pellet in a microcentrifuge at 14,000 rpm for 20 s.

vi. Resuspend the cells in 0.5 ml of PBS, and keep the samples on ice.

vii. Analyze the cells with a flow cytometer. Green (510–530 nm, FL1-H channel) and red (>650 nm, FL3-H channel) fluorescence emissions from $10^4$ cells illuminated with blue (488 nm) excitation light are measured with a FACSCalibur from BD Biosciences using CellQuest software (San Jose, CA, USA). In the dot plot panel, set the bar for FL3-H in the control sample for your treatment so that the AVO-positive cells (the dots above the bar) are approximately 5% of the population (Fig. 13.1). Measure the test samples under the same condition. A significant increase in the percentage of AVO-positive cells may indicate the occurrence of autophagy (Fig. 13.1).

3.2. Assessing a punctate pattern of GFP-LC3 fluorescence (GFP-LC3 dots)

LC3-I is cytosolic; after LC3-I is processed into LC3-II, the latter is associated with the autophagosome membrane (Kabeya et al., 2000). Therefore, the main population of GFP-LC3 is normally diffuse in the cell.
During autophagy, the tagged protein is recruited to the autophagosomal membrane and presents a punctate pattern (Klionsky et al., 2008). When the GFP-LC3 dots are countable, they can be quantified either by the number of dots per cell or the number of cells with GFP-LC3 dots exceeding the average number of dots in the control cells (Aoki et al., 2008; Klionsky et al., 2007). Both transient and stable expression of GFP-LC3 can be used for studies (Aoki et al., 2008; Klionsky et al., 2007). The protocol is as follows:

i. Seed $5 \times 10^4$ cells/well glioma cells in 4-well chamber slides. For stable expression, treat the cells directly with an autophagy-inducing agent (e.g., Delta-24-RGD adenovirus at 50 pfu/cell for 48 h).

ii. For transient expression, transfect the cells with a GFP-LC3 plasmid (Kabeya et al., 2000) using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA, 11814443001) according to the manufacturer’s instructions.

iii. Treat the cells with autophagy-inducing agents. If the treatment lasts longer than 48 h, treat the cells first with the agents, and then transfect the cells with the GFP-LC3 plasmid.

iv. 24–48 h after transfection, examine the cells directly or fix them with 4% paraformaldehyde in PBS for 30 min at 4 °C, and then examine the GFP-LC3 dots under a fluorescence microscope (Fig. 13.2). Determine the number of GFP-LC3 dots per cell in GFP-LC3-positive cells. Count a minimum of 50–100 cells per sample for triplicate samples per condition per experiment (Pattingre et al., 2005).

![Mock Delta-24-RGD](image.png)

**Figure 13.2** GFP-LC3 punctation induced by Delta-24-RGD adenovirus in U-87 MG-GFP-LC3 cells. The cells were infected with the virus at 50 pfu/cell. Forty-eight hours later, the green fluorescence was observed under a fluorescence microscope. Note the punctation of GFP-LC3 and rounding up of the cells showing the cytopathic effect caused by the virus.
4. **In Vitro Biochemical Markers**

4.1. Immunoblotting to determine the increase in LC3-II

LC3 expression in glioma cells is usually relatively abundant. An increase in LC3-II during autophagy has been confirmed in a panel of glioma cells and treatments (Aoki *et al*., 2007a,b; Aoki *et al*., 2008; Ito *et al*., 2006; Iwamaru *et al*., 2007; Jiang *et al*., 2007; Kanzawa *et al*., 2004). The protocol is as follows:

i. Wash and collect cells ($\approx 5 \times 10^6$ cells) in 4 ml of PBS.
ii. Pellet the cells with centrifugation at 500g for 5 min.
iii. Remove PBS, and resuspend the cells in approximately 4 cell-pellet volumes (typically approximately 80 $\mu$l) of PBS plus protease inhibitor cocktail (Sigma-Aldrich, P9599).
iv. Add an equal volume of 2 $\times$ SDS sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% glycerol) to the cell suspension. Mix the sample with pipetting until the sample becomes viscous and clear.
v. Heat the samples at 95°C for 10 min.
vi. Quantify the protein concentration of the samples with Bradford dye (Bio-Rad, Hercules, CA, USA, 500-0006) according to the manufacturer’s instructions.
vii. Separate the proteins in a 12% SDS-PAGE gel at 50 V for stacking and 100 V for resolving in a Bio-Rad Mini-PROTEAN Electrophoresis System. Load approximately 40–60 $\mu$g of protein per lane.
viii. Transfer the proteins from the gel to a nitrocellulose or PVDF membrane.
ix. Block the membrane with 10% nonfat milk in Tris-Buffered Saline Tween 20 (TBST) (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) at room temperature for 1 h.
x. Incubate with rabbit polyclonal anti-LC3B antibody (1:3000 dilution, Novus Biologicals, Littleton, CO, USA, NB600-1384) diluted with 5% nonfat milk in TBST at 4°C overnight.
xii. Remove the primary antibody. Rinse the membrane once with TBST, and wash the membrane with TBST for 5 min.
xiii. Incubate the membrane in HRP-labeled secondary antibody goat antirabbit immunoglobulin G (IgG) (1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-2004) diluted with 2% nonfat milk in TBST at room temperature for 1 h.
xiv. Remove the antibody, and rinse the membrane once with TBST. Wash the membrane with TBST three times for 15, 5, and 5 min at room temperature.
xiv. Visualize the protein-antibody complexes with the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). LC3-I and LC3-II are detected at a molecular mass of approximately 16 and 14 kDa respectively (Mizushima and Yoshimori, 2007) (Fig. 13.3).

4.2. Immunoblotting to examine changes in the Atg12–Atg5 conjugate, p62, and p70S6K dephosphorylation

During adenovirus-induced autophagy, we observe a dramatic increase of the Atg12–Atg5 conjugate (Jiang et al., 2007) and a significant decrease in the p62 (SQSTM1/sequestosome 1) protein (Fig. 13.3). The procedure for the immunoblotting analysis of these proteins is similar to the procedure for the LC3 protein. Because the Atg12–Atg5 conjugate is approximately 55 kDa, p62 is approximately 62 kDa, and p70S6K is approximately 70 kDa, it is best to separate the proteins in a 10% SDS-PAGE gel. The primary antibody for Atg5 can be obtained from Cosmo Bio (Japan, CAC-TMD-PH-AT5), and the antibody for p62 is available from Santa Cruz Biotechnology (sc-25575). While autophagy occurs in glioma cells, the substrate of mTOR (p70S6K) is dephosphorylated as demonstrated by immunoblotting analysis (Ito et al., 2006; Iwamaru et al., 2007). Rabbit polyclonal anti-p70S6K and anti-phospho-p70S6K (Thr389) antibodies are available from Cell Signaling Technology (Beverly, MA, USA, 9202, 9205).

![Image of immunoblot results](image_url)

**Figure 13.3** Expression of molecular markers of autophagy in malignant glioma U-87 MG cells after adenovirus infection. The cells were infected with replication-deficient adenovirus AdGFP or wild-type adenovirus (Adwt) at 10 pfu/cell. Seventy-two hours later, the cells were collected; the proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting with rabbit polyclonal anti-LC3 (1:3000 dilution; Novus Biologicals), rabbit polyclonal anti-Atg5 (1:2000 dilution, Cosmo Bio), mouse monoclonal anti-p62 (1:400 dilution), and goat polyclonal antiactin (1:1000 dilution, Santa Cruz Biotechnology). AdGFP was used as the control for viral infection, and actin was used as a loading control.
5. **Electron Microscopy to Monitor the Autophagic Vacuoles**

i. Fix the cells (≈1 × 10^6 cells) with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h.

ii. Wash the cells with 0.1% Millipore-filtered cacodylate buffered tannic acid (Sigma-Aldrich, 403040).

iii. Postfix the cells with 1% phosphate buffered osmium tetroxide for 1 h.

iv. Stain the cells with 1% Millipore-filtered uranyl acetate.

v. Dehydrate the samples in increasing concentrations of ethanol (50%, 15 min; 70%, 15 min; 95%, 15 min; 100%, twice for 15 min).

vi. Infiltrate and embed the samples directly in Spurr’s low-viscosity medium.

vii. Polymerize the samples in a 70 °C oven for 2 days.

viii. Cut the samples in ultrathin (50–100 nm) sections with a Leica Ultracut microtome (Leica, Deerfield, IL, USA).

ix. Stain the sections with 5% uranyl acetate solution for 15 min, rinse with distilled water, and then stain with Reynold’s lead citrate Solution for 3–5 min, and rinse with distilled water in a Leica EM Stainer.

x. Examine the images in a JEM 1010 transmission electron microscope (JEOL, USA, Peabody, MA, USA) at an accelerating voltage of 80 kV. Digital images are obtained using AMT Imaging System (Advanced Microscopy Techniques, Danvers, MA, USA). Membrane-bound compartments accumulate in the cytoplasm (Fig. 13.4).

6. **In Vivo Analysis of Biochemical Markers**

There are very limited approaches to monitor autophagy in vivo. Atg5 and LC3B are successfully detected in the mouse brain in intracranially implanted gliomas treated with autophagy-inducing agents (Aoki et al., 2008; Jiang et al., 2007) (Fig. 13.5). The procedures to detect the Atg5 protein in the tumor xenografts through immunofluorescence are listed here.

i. Deparaffinize paraffin-embedded 5–µm sections of mouse brain tumor in xylene using 3 changes of 5 min each time.

ii. Hydrate the sections with 100% ethanol 2 times for 5 min each time.

iii. Hydrate the sections with 95% ethanol 2 times for 5 min each time.

iv. Rinse the sections in PBS 3 times for 5 min each time.

v. Block the samples with 3% normal goat serum (Santa Cruz Biotechnology, sc-2043) in PBS at room temperature for 30 min.
vi. Incubate the samples overnight at 4°C with anti-Atg5 antibody (1:200 dilution, Cosmo Bio, Japan, CAC-TMD-PH-AT5).

vii. Wash the samples 3 times with PBS plus 0.1% Tween 20 for 5 min each time.

viii. Incubate the samples with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, A-11008) for 50 min at room temperature.

ix. Wash the samples 3 times with PBS plus 0.1% Tween 20 for 5 min each time.

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**Figure 13.4** Representative electron micrographs showing the ultrastructure of the mock-infected (A) and Delta-24-RGD–infected (B) MDNSC11 cells. Note the vacuoles in the virus-infected cells but not in the untreated cells. Close-ups of Delta-24-RGD-infected cell illustrated in B show the cluster of the progenies of Delta-24-RGD (white arrow) in the nucleus (C) and complex autophagic multivacuolar bodies in the cytoplasm (D). This figure has been modified from a previous publication (Jiang et al., 2007) and has been reproduced with permission from Oxford Journals.
Mount the slides with ProLong Antifade Gold Reagent with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes, P-36931) to visualize the cell nuclei.

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**Figure 13.5** Immunofluorescence analysis of viral fiber and Atg5 protein expression in the brain of a mouse that was treated with Delta-24-RGD. The paraffin-embedded section of the mouse brain was double-immunostained with mouse monoclonal antibodies specific for adenoviral fiber protein (4D2, 1:500 dilution; Lab Vision, Fremont, CA, USA, MS-1027-P0) (A) or rabbit polyclonal Atg5 (1:200 dilution) (B) and then with Texas Red– (fiber) or Alexa Fluor 488– (ATG5) conjugated secondary antibodies (1:500 dilution; Molecular Probes, Eugene, OR, USA). Fluorescence for fiber and Atg5 were merged, and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining was used to visualize the cell nuclei (C). Expression of both proteins was positive double headed arrow) within the tumor (T) surrounding necrotic areas (N). Note that, in a close-up of C, the viral and cellular proteins are localized in the same cells around the cells that exhibit virally induced necrosis (D). This figure has been modified from a previous publication (Jiang et al., 2007) and has been reproduced with permission from Oxford Journals.
7. **Autophagy Indicators as Surrogate Markers of Treatment Effect in Clinical Trials**

Research indicates that autophagy is involved in the cell death induced by therapeutic agents for glioma, such as temozolomide, rapamycin, irradiation, and oncolytic adenoviruses (Ito *et al.*, 2006; Ito *et al.*, 2005; Iwamaru *et al.*, 2007; Jiang *et al.*, 2007; Kanzawa *et al.*, 2004). Thus, autophagy indicators can be used to monitor the cellular response to glioma treatment. As mentioned previously, Atg5 and LC3B can be detected in tumor tissues by immunostaining. After treating mice with temozolomide, an increased amount of LC3B is detected using immunofluorescence and immunohistochemistry in gliomas intracranially implanted in mouse brains (Aoki *et al.*, 2008). In the intracranial gliomas treated with oncolytic adenoviruses, Atg5 is readily detected with immunofluorescence, however, Atg5 is not detected in the untreated tumors (Jiang *et al.*, 2007) (Fig. 13.5). These markers show promise for evaluating how patients respond to glioma therapy and for helping predict whether the therapy is efficacious. This information will guide future clinical trials to optimize the therapeutic regimen to achieve the best results for patients with gliomas.

8. **Future Directions**

Intervention via autophagy is a promising approach for glioma therapy, as glioma cells are more sensitive to autophagy-inducing agents than to apoptosis-inducing agents (Lefranc *et al.*, 2007). Further studies dissecting autophagic pathways and molecules that are essential for autophagy in glioma cells, especially those involved in autophagic cell death, will help researchers identify potential targets for glioma therapies. To facilitate these studies, it is critical to develop reliable, specific, quantitative, and simple assays to examine the occurrence of autophagy in glioma cells. At the present time, multiple assays are needed because no single assay satisfies all the criteria for success (Klionsky *et al.*, 2008). Some assays are impossible or difficult to quantify for clinical samples. For example, LC3 punctation is impossible to be visualized by GFP tag in patient samples. In addition, quantification of immunostaining for autophagic markers, such as LC3B or Atg5, will be very difficult, if not impossible. Besides, we are not sure whether the markers can be applied in glioma treatments other than in the scenario reported in this chapter (Aoki *et al.*, 2008; Jiang *et al.*, 2007). In light of these challenges, we feel certain that our progressing knowledge of autophagy and new technologies in this area will help in the development of more optimal assays to be used in glioma research and treatment.
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