Adenoviruses induce autophagy to promote virus replication and oncolysis

Humberto Rodriguez-Rocha a,1,2, Jorge G. Gomez-Gutierrez a,2, Aracely Garcia-Garcia a,1, Xiao-Mei Rao b, Lan Chen a,1, Kelly M. McMasters a, Heshan Sam Zhou a,b,⁎

a Department of Surgery, University of Louisville School of Medicine, Louisville, KY 40292, USA
b James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY 40292, USA

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ABSTRACT

Adenoviruses with deletion of E1b have been used in clinical trials to treat cancers that are resistant to conventional therapies. The efficacy of viral replication within cancer cells determines the results of oncolytic therapy, which remains poorly understood and requires further improvement. In this report, we show that adenoviruses induce autophagy by increasing the conversion of LC3-I to LC3-II and the formation of the Atg12–Atg5 complex. Inhibition of autophagy with 3-methyladenine (3MA) resulted in a decreased synthesis of adenovirus structural proteins, and thereby a poor viral replication; promotion of autophagy with rapamycin increased adenovirus yield. These results indicate that adenovirus-induced autophagy correlates positively with viral replication and oncolytic cell death, and that autophagy may generate nutrients that can be used for building viral progeny particles. These results further suggest that chemotherapeutic agents that increase cancer cell autophagy may improve the efficacy of oncolytic virotherapy.

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Introduction

Adenoviruses (Ads) with E1b deletion can selectively replicate in cancer cells and cause oncolysis. Oncolytic viruses have been used in clinical trials and cancer treatments (Crompton and Kirn, 2007; Garber, 2006; Koski et al., 2010); however, as single agents, the antitumoral effects of oncolytic Ads have been somewhat disappointing in preclinical and clinical trials (Kirn et al., 2001; McCormick, 2003; Yamamoto and Curiel, 2010; Zhao et al., 2003). We previously reported that intratumoral injection of viruses could efficiently inhibit small human tumor growth in an animal model, but could not efficiently repress large tumors (Zhao et al., 2003). Many clinical studies have shown that the oncolytic approach alone could not efficiently destroy tumors in patients, especially for large tumors (Kirn et al., 2001; McCormick, 2003). Thus, oncolytic virus replication and spread are likely restricted in the larger tumor mass. This restriction may be a major hurdle limiting the efficacy of virotherapy. We believe that increasing the efficacy of viral replication within cancer cells will improve the results of oncolytic therapy.

Autophagy is a regulated process of degradation and recycling of cellular constituents, this process is important in organelle turnover and the bioenergetics management of starvation (Levine and Klionsky, 2004; Ohsumi, 2001). Autophagy starts with formation of double-membrane vesicles containing cytoplasm and organelles, a structure known as autophagosome. Ultimately, autophagosomes fuse with lysosomes where the bulk cytoplasmic content undergoes degradation, resulting in the liberation of amino acids and fatty acids that can be reused by cells (Baehrecke, 2005; Klionsky, 2001; Klionsky and Emr, 2000). During autophagy, several autophagy (Atg) proteins have been implicated in autophagosome formation. Atg5 and Atg12 form a complex that is required to recruit other proteins to the autophagosomal membrane. LC3 is the mammalian equivalent of yeast Atg8. The precursor form of LC3 is post-modified into two forms, LC3-I and LC3-II. LC3-I is localized in the cytosol and LC3-II in autophagosomal membranes. LC3-II can be used to estimate the abundance of autophagosomes before they are destroyed through fusion with lysosomes. Thus, Atg5/Atg12 complex formation, and the LC3-II conversion and integration into autophagosomal membrane, are the major hallmarks of activation of the autophagic process.

Some reports have suggested that autophagy has dual roles, acting as a survival mechanism and as a caspase-independent form of programmed cell death (Gozuacik and Kimchi, 2007; Wang et al., 2006; Yu et al., 2004). Autophagy is also involved in combating infection by breaking down pathogens (Gutierrez et al., 2004). However, some viruses subvert the autophagic pathway to promote their own replication (Jackson et al., 2005). The role of autophagy in Ad-mediated oncolytic cell death has not been well characterized. Kondo's group has reported that hTERT-Ad, a conditionally replicating Ad regulated by the human telomerase reverse transcriptase promoter, killed cancer cells by inducing autophagy instead of apoptosis, and proposed that autophagy is an antitumoral effect, not...
Autophagy may be an intracellular host defensive mechanism against viruses, or it can be used by viruses for their own benefit of efficient replication (Colombo, 2005). We examined the effect of autophagy on Ad replication. Since 3MA inhibits autophagy by...
suppressing the formation of the pre-autophagosomal structure (Kondo et al., 2005; Shintani and Klionsky, 2004), A549 cells were infected with Ad5 or Adhz60 in the absence or presence of 3MA at a concentration of 10 mM. Viruses were harvested after infection and titered on HEK-293 cells. We observed that the autophagy inhibitor 3MA reduced the viral yields about 10-fold for both Ad5 and Adhz60 (Fig. 4A). The differences were statistically significant, as shown by the Student t-test.

It is well known that autophagy degrades intracellular components, such as organelles and long-lived proteins, to liberate amino acids and fatty acids that can be reused within cells, especially under starvation condition (Baehrecke, 2005; Klionsky, 2008; Klionsky and Emr, 2000). Therefore, it is possible that Ads may promote autophagy to provide elements for viral particle production. If so, inhibition of autophagy should decrease or delay viral protein synthesis. We analyzed the production of viral structure proteins in cells treated with the autophagy inhibitor 3MA. The production of viral structural proteins of both Ad5 and Adhz60 at 24 h was strongly inhibited in cells treated with 3MA (Fig. 4B). The decreased viral protein production, caused by 3MA treatment, correlates with inefficient virus replication as shown in Fig. 4A. These results suggest that autophagy plays a critical role in viral structure protein synthesis,

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**Fig. 2.** Autophagy activation and integration of LC3 into autophagosome. A549 cells were transfected with pEGFP-LC3 followed by infection with Ad5, Adhz60, or AdlacZ. (A) Integration of GFP-LC3 into the autophagosome is depicted by punctate structures (indicated by arrows) and was analyzed by fluorescence microscopy at 36 h p.i. Images were taken with Kodak MDS 290 software (magnification ×40). (B) The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-LC3-positive cells. A representative experiment is shown from three performed.

**Fig. 3.** Expression of the classical autophagy hallmarks. A549 cells were infected with Ad5, Adhz60, or AdlacZ. LC3 and Atg5 expression and modification were analyzed in a time-course assay. LC3-I and LC3-II were detected with a pAb anti-LC3. To detect Atg5 or Atg12–Atg5 complex, a pAb anti-Atg5 was used.
likely by degrading intracellular components necessary for building progeny virus particles.

Treatment of Ad5-infected cells with 3MA prevents autophagosome formation and inhibits the conversion of LC3-I to LC3-II

We investigated whether 3MA could prevent the conversion of GFP-LC3-1 to GFP-LC3-II induced by Ad5. A549 cells were transfected with pEGFP-LC3 followed by infection with Ad5. The treated cells were then cultured in the absence or presence of 3MA at a concentration of 10 mM. We found that 3MA repressed the conversion of LC3-I to LC3-II, resulting in a diffuse GFP-LC3 pattern (Fig. 5A).

We further investigated whether 3MA might inhibit conversion of LC3-I to LC3-II at the protein level. We analyzed the effect of 3MA in cells infected with Ad5, because this virus induced significant conversion of LC3-I to LC3-II. A549 cells were infected with Ad5 in presence of 3MA (Fig. 5C). The conversion of LC3-I to LC3-II was significantly inhibited by 3MA in Ad5-infected cells compared to the result in the absence of 3MA (Fig. 3). The results indicate that inhibition of autophagy with 3MA results in decreased viral replication.

Role of autophagy in adenovirus replication and oncolysis

To further investigate the role of autophagy in oncolytic therapy, we compared the effect of 3MA (autophagy inhibitor) and rapamycin (autophagy inducer) on virus replication and cancer cell viability. First, we verified the autophagy induction function of rapamycin in our experimental conditions. Rapamycin induces autophagy by inhibiting the mammalian target of rapamycin (mTOR), an autophagy-associated molecule (Kondo et al., 2005; Shintani and Klionsky, 2004). A549 cells were transfected with pEGFP-LC3 followed by treatment with rapamycin at a concentration of 50 nM. Conversion of the cytoplasm-diffused pEGFP-LC3-I to membrane-associated pEGFP-LC3-II puncta was observed by fluorescence microscopy at 36 h p.i. Images were taken with Kodak MDS 290 software (magnification, ×40). (C) A549 cells were infected with Ad5 in presence of 3MA (10 mM). LC3 expression was analyzed in a time-course assay, LC3-I and LC3-II were detected with a pAb anti-LC3. A representative experiment is shown from three performed.

Oncolytic adenovirus induces activation of autophagy in different types of cancer cells

Since the ability of oncolytic Ads to induce autophagy in A549 lung cancer cells was demonstrated, we studied whether these Ads could

Fig. 4. Effect of 3-methyladenine (3MA) on adenovirus yield and structural protein production. A549 cells were infected with Ad5 or Adhz60 in absence or presence of 3MA. Cells were harvested at indicated time points. (A) For titering, supernatants were serially diluted to determine the titers, plaque forming unit (PFU), in HEK-293 cells. Each point represents the mean of three independent experiments ± standard deviation (SD; bars; P < 0.05). (B) Ad structural proteins were detected with rabbit-antihuman Ad protein viron. One experiment is shown from three performed.

Fig. 5. Inhibition or induction of autophagosome formation and inhibition of LC3-I to LC3-II conversion. (A) A549 cells were transfected with pEGFP-LC3 following infection with Ad5 (MOI = 4) in absence or presence of 3MA (10 mM). (B) A549 cells were transfected with pEGFP-LC3 followed by no treatment or treatment with rapamycin (50 nM). Integration of GFP-LC3 into the autophagosome is depicted by punctate structures (indicated by arrows) and was analyzed by fluorescence microscopy at 36 h p.i. Images were taken with Kodak MDS 290 software (magnification, ×40). (C) A549 cells were infected with Ad5 in presence of 3MA (10 mM). LC3 expression was analyzed in a time-course assay, LC3-I and LC3-II were detected with a pAb anti-LC3. A representative experiment is shown from three performed.
induce the activation of autophagy in other types of cancer cell lines. Therefore, Hela cervical cancer cells, HCT116, and RKO colon cancer cells, or H1299 lung cancer cells were transfected with pEAK12-Actin-LC3-dNGLUC followed by no infection (mock) or infection with Ad5 or Adhz60 (MOI = 4). Secretion of LC3/GLUC was monitored at 36 h after infection with the Renilla Luciferase kit (Promega). Each point represents the mean of three independent experiments ± standard deviation (SD; bars).

Discussion

Ads have been genetically modified for selectively replicating within cancer cells and causing oncolysis. The advantage of virotherapy is that the released progeny viruses further spread infection until all cancer cells are destroyed. This is true for cancer cells in tissue culture. Even when less than 1 percent of the cancer cells are initially infected, all cancer cells are eventually killed in a few days. However, animal experiments and clinical trials have indicated that virus replication and spread in larger tumor masses are restricted, and that the restricted replication and spread may be major hurdles limiting the efficacy of virotherapy (Kirn, 2001; McCormick, 2003; Yamamoto and Curiel, 2010; Zhao et al., 2003). Recent studies suggest that Ads have the ability to induce autophagy (Baird et al., 2008; Ito et al., 2006; Jiang et al., 2007, 2008). Thus, autophagy may be used to promote virus replication and improve oncolysis.

In this study, we have applied wild-type (wt) Ad5, Adhz60 (with deletion of E1b) (Rao et al., 2004), and AdlacZ (with deletion of both E1a and E1b) to study Ad-induced autophagy. We observed that autophagy is induced by replication-competent Ad5 and Adhz60, but not by AdlacZ. We also found that Ad5 and Adhz60 promote the conversion of LC3-I to LC3-II and the formation of the Atg12–Atg5 complex—two hallmarks of autophagy. These processes were more pronounced in cells infected with Ad5, which expresses both E1a and E1b, than in cells infected with the E1b-deleted Adhz60. Thus, autophagy induction appears to be dependent on the E1a gene and viral replication, and the E1b gene may be involved in enhancing autophagy. We do not know the exact mechanisms by which Ad infection activates autophagy. Ad infection may initially trigger cellular autophagic responses that can be taken over by Ads for the benefit of viral replication. It is also possible that a virus-associated or activated protease activity may directly contribute to the conversion of LC3-I to LC3-II and activate the autophagy pathway.

To study the role of autophagy in Ad replication, we have applied 3MA to inhibit autophagy and rapamycin to promote the process. Both 3MA and rapamycin have been broadly used in autophagy studies (Kondo et al., 2005; Shintani and Klionsky, 2004). It is known that 3MA inhibits autophagy by suppressing the formation of the pre-autophagosomal structure. Rapamycin inhibits the mammalian target of rapamycin (mTOR), an autophagy-associated molecule, and is used in model studies to induce autophagy. Although we cannot exclude the possibility that 3MA and rapamycin may also affect other processes in the Ad-infected cells, we have shown that 3MA partially represses Ad-induced autophagy, and rapamycin alone can induce autophagy in treated cancer cells in our experimental set (Fig. 5). We observed that 3MA significantly reduced Ad5 and Adhz60 yields in

Fig. 6. Effect of pharmacological inhibition and stimulation of autophagy on adenovirus replication and oncolysis. (A) A549 cells were infected with Ad5 or Adhz60 in the presence or absence of 3MA (10 mM) or rapamycin (50 nM). Seventy-two hours later, titers were determined by serial dilutions of supernatants and titers were measured by standard infection units. Cytotoxicity was analyzed by a MTT assay at 72 h after 3MA (B) or rapamycin (C) treatment. Each point represents the mean of three independent experiments ± (SD; bars).

Fig. 7. Activation of autophagy in different types of cancer cells. Hela, HCT116, RKO, or H1299 cancer cell lines were transfected with pEAK12-Actin-LC3-dNGLUC followed by no infection (mock) or infection with Ad5 or Adhz60 (MOI = 4). Secretion of LC3/GLUC was monitored at 36 h after infection with the Renilla Luciferase kit (Promega). Each point represents the mean of three independent experiments ± standard deviation (SD; bars).
A549 cancer cells and partially prevented Ad-caused cell death (Fig. 6A and B). Rapamycin increased Ad5 and Adhz60 titers 2- to 3-fold (Fig. 6A). Since Ad oncolytic replication is restricted in large tumor masses, promotion of autophagy with chemotherapeutic agents may have the potential to improve the efficacy of virotherapy.

To the best of our knowledge, this study shows, for the first time, that 3MA represses Ad structure protein synthesis (Fig. 4). Autophagy is a catabolic process involving the degradation of cellular components through the lysosomal machinery. Autophagy is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more-essential processes, although this process also plays a number of other roles in normal cell growth, development, and homeostasis (Levine and Klionsky, 2004; Ohsumi, 2001). We have shown that replication-competent Ads strongly induce autophagy in cancer cells; inhibition of autophagy with 3MA partially represses viral protein production and decreases virus titers, and the autophagy inducer, rapamycin, increases virus replication. These results have indicated that virus-induced autophagy correlates positively with virus replication. Since autophagy has a major function in the generation of nutrients for starving cells, and inhibition of autophagy specifically represses viral structural protein synthesis (Fig. 4), we hypothesize that autophagy may play a critical role in viral replication by degrading intracellular components for building progeny virus particles.

We also observed that replicative Ads can activate autophagy in other cancer cell lines, Hela, HT116, RKO, and H1299, but the levels are different from cell to cell. This may reflect the different sensitivities of cancer cells to Ad-induced autophagy or that optimal conditions are not selected for each of the cell lines, such as transfection efficacy of plasmid and autophagy peaking time points. A new publication has shown that human adenovirus type 5 induces autophagy in glioma cells (Jiang et al., 2011). Thus, it is likely that oncolytic Ads are generally able to induce the activation of autophagy.

In summary, we found that wt Ad5 and E1b-deleted oncolytic Adhz60 induced autophagy in cancer cells, the conversion of LC3-I to LC3-II and the complex formation of Atg12–Atg5. Autophagy correlated positively with virus replication and oncolytic cell death. Based on these findings, we suggest that chemotherapeutic agents that increase cancer cell autophagy may promote the efficacy of oncolytic virotherapy. These findings may have clinical relevance in the development of oncolytic virotherapy.

Material and methods

Cell line and culture conditions

Human lung cancer cell lines A549 and H1299 were cultured in MEM-Alpha medium; human cervical cancer cell line Hela and human colon cancer cell line RKO were cultured in RPMI medium; and human colon cancer cell line HCT116 was cultured in McCoy's medium at 37 °C in a humid incubator with 5% CO2. All media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). All cell culture reagents were obtained from Gibco BRL (Bethesda, MD). Adherent cells were collected after trypsinization, as well as GFP-LC3 plasmid, which was generously donated by Dr. Tamotsu Yoshimori (Kabeya et al., 2000). Cells grown to 70% confluency were transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen, 11668-019), according to the manufacturer's protocol. For the luciferase assay and the GFP-LC3 puncta assay, cells were seeded at a density of 1.5 × 10^5 cells in 12-well tissue culture plates.

Adenoviral vectors

AdCMV-lacZ (AdlacZ) has E1 deletion and has the lacZ gene as a reporter. Adhz60 is an Ad vector with deletion of E1b gene (Rao et al., 2004). Ad5 (Adwt) was obtained from ATCC and does not have any deletions. Cells were infected with wt Ad5, Adhz60 (at an MOI of 4) or AdlacZ (at an MOI of 10) in FBS-free medium for 1 h. Infected cells were then untreated or treated with 3MA at 10 mM or rapamycin at 50 nM. We used a higher MOI of AdlacZ for compensation of its lack of replication ability. The autophagy results are the same when AdlacZ was used at MOIs of 4, 10, or 20. The cultures were collected at indicated time points. Ad titers were determined as follow: cells went through three cycles of freezing and thawing to release viruses from the cells. Lysates were serially diluted to determine the titers in HEK-293 cells by standard infective unit (IFU) measurement.

Gaussia luciferase assay

After transfection with the pEAK12-Actin-LC3-dNGLUC, supernatant samples were collected at indicated time points. GLUC activity was determined using the Renilla Luciferase kit (Promega, E2810). To avoid harvesting luciferase activity from detached cells, supernatants were spun at 14,000 rpm for 5 min. Ten microliters of supernatant was diluted (1:10) in 100 μl 1× renilla lysis buffer. Twenty microliters of this mixture was added to 100 μl of renilla substrate prior to analysis in a luminescence plate reader.

GFP-LC3 puncta

Plasmid vector containing green fluorescent protein (GFP) linked to microtubule-associated protein 1 light chain 3 (LC3) (pGFP-LC3), which was used to detect autophagosome formation in A549 cells caused by Ad5 or Adhz60. After infection with Ad5 or Adhz60, no infected or Ad-infected cells were cultured in the presence or absence of 3MA (10 mM) or rapamycin (50 nM). 36 h later, cells were fixed with 4% formaldehyde and then observed under a fluorescence microscope. Cells were classified as having a predominantly diffuse GFP stain or having numerous punctate structures representing autophagosomes. Images were taken with Kodak MDS 290 software with the 40× objective. The percentage of cells with GFP punctuate pattern was calculated as punctuate GFP cell per total GFP cell percentage.

Western blot analysis

After respective treatment, the cells were harvested and lysed in a radio immunoprecipitation assay (RIPA) buffer. Cell lysates were centrifuged, and protein concentration was determined by Biorad DC protein assay (Bio-Rad, Hercules, CA). Equal amounts (50 μg) of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to Hybond-Polyvinylidene Difluoride (PVDF) membranes (Amersham, Arlington Heights, IL). The membranes were blocked with 5% dry milk in Tris-buffered saline–Tween 20 (TBST), probed with the following primary antibodies: rabbit polyclonal anti-LC3 (Novus Biologicals, NB100-2331); rabbit polyclonal anti-Atg5 (C-terminal) (Sigma-Aldrich, A0713); rabbit anti-human Ad proteins virion (abcam, ab6982); and rabbit-antihuman-α-actin pAb to ensure equal loading of proteins. Next, the membranes were incubated with anti-mouse immunoglobulin (lg) or anti-rabbit lg, peroxidase-linked, species-specific whole antibody (Amersham, Piscataway, NJ). Chemiluminescent detection was performed with ECL reagents, according to the manufacturer's instructions (Amersham).

Plasmids

The pEAK12-Actin-LC3-dNGLUC (autophagy sensor) was kindly provided by Dr. Brian Seed (Kettler and Seed, 2008) and was used for transfection, as well as GFP-LC3 plasmid, which was generously donated by Dr. Tamotsu Yoshimori (Kabeya et al., 2000). Cells grown to 70% confluency were transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen, 11668-019), according to the manufacturer's protocol. For the luciferase assay and the GFP-LC3
**MTT assay**

Cell proliferation was assessed at three days after respective treatments by measuring the conversion of the tetrazolium salt (MTT) to formazan, according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). The supernatant from each plate was collected for measurement of absorbance at a wavelength of 570 nm. The results were expressed as the percentage of live cells.

**Statistical analysis**

The results of the in vitro assays were analyzed with the Student t-test for unpaired data using a one-way ordinary parametric analysis of variance. \( P < 0.05 \) was considered statistically significant.

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**References**


