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The C/EBPbeta Isoform, Liver-Inhibitory Protein (LIP), Induces Autophagy in Breast Cancer Cell Lines

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Abstract

Autophagy is a process involving the bulk degradation of cellular components in the cytoplasm via the lysosomal degradation pathway. Autophagy manifests a protective role in stressful conditions such as nutrient or growth factor depletion; however, extensive degradation of regulatory molecules or organelles essential for survival can lead to the demise of the cell, or autophagy-mediated cell death. The role of autophagy in cancer is complex with roles in both tumor suppression and tumor promotion proposed.

Here we report that an isoform of the C/EBPbeta transcription factor, liver-enriched inhibitory protein (LIP), induces cell death in human breast cancer cells and stimulates autophagy. Overexpression of LIP is incompatible with cell growth and when cell cycle analysis was performed, a DNA profile of cells undergoing apoptosis was not observed. Instead, LIP expressing cells appeared to have large autophagic vesicles when examined via electron microscopy. Autophagy was further assessed in LIP expressing cells by monitoring the development of acidic vesicular organelles and conversion of LC3 from the cytoplasmic form to the membrane-bound form. Our work shows that C/EBPbeta isoform, LIP, is another member of the group of transcription factors, including E2F1 and p53, which are capable of playing a role in autophagy.

Keywords

C/EBPbeta; autophagy; transcription factor; cell death; breast cancer cells

Introduction

Autophagy is an evolutionarily conserved cellular process responsible for self-cannibalization through a lysosomal degradation pathway. It is a multi-step process that is characterized by the formation of double-membrane vesicles called autophagosomes. These autophagosomes engulf bulk cytoplasm which leads to the degradation of long-lived or damaged proteins and the turnover of various cytoplasmic organelles such as mitochondria,

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endoplasmic reticulum (ER), and Golgi. (1,2). Eventually, the outer membrane of autophagosomes fuses with lysosomes generating autolysosomes, where lysosomal hydrolases degrade the cytoplasm-derived contents of the autophagosome together with its inner membrane (1). This process takes place in cells at a basal level in order to reuse the individual components as renewable resources to provide components and energy for cell survival (2,3). In addition, autophagy is essential for maintaining cell survival following a variety of extracellular and intracellular stimuli including: stress, nutrient-starvation, growth factor deprivation, and accumulation of damaged organelles (1). However, this mechanism of cell autonomous survival is inevitably self-limited and the ultimate consequence is autophagy-mediated cell death if the stress imposed on the cell is sustained.

Although the induction of autophagy can contribute to cell survival, extensive autophagy can also lead to “type II programmed cell death” (4). This implies that activation of autophagy may lead to different outcomes depending on the cell genetic background as well as the duration and strength of the stress-inducing stimulus. It has been suggested that in early stages of tumor formation, a defective autophagic system leads to the accumulation of damaged proteins and organelles (5). This increase in genotoxic substances may lead to both failure to constrain cell growth and mutations. Yet, at later stages of tumorigenesis, autophagy may be a means by which tumor cells survive under oxygen and nutrient limiting conditions, providing extra time for adaptation via the induction of angiogenesis and/or motility and invasion (5). The mechanisms by which autophagy may be regulated to provide complete cellular destruction or a survival advantage remains unknown.

One mechanism by which autophagy is regulated is through transcriptional control. This area has become more intricate with recent studies focusing on the roles of different transcription factors and their ability to induce or regulate autophagy in a cell context and stimulus specific manner. An example of this is the activation of E2F1, which upregulates the expression of autophagy genes—LC3, ATG1, and damage-regulated autophagy modulator (DRAM) by directly binding to their promoters (6). Other findings indicate dual roles for the transcription factor p53 in autophagy. Tasdemir et al. reports cytoplasmic p53 negatively regulates autophagosomes formation (7). Others have shown that nuclear p53 directly induces DRAM 1 and autophagy (8). The transcription factor NF- κ B controls the expression of Beclin 1 by interacting with the *BECN1* promoter (38). Also, JNK has been shown to control the expression of Beclin 1 through c-Jun. JNK controls autophagy by both cytoplasmic and nuclear effects (39).

C/EBPbeta is a basic leucine zipper transcription factor transcribed from an intronless gene that gives rise to three protein isoforms from a single mRNA (9,10). This is due to alternative translation initiation at three in-frame methionine initiator codons or regulated proteolysis (10,11). Full length C/EBPbeta, C/EBPbeta-1, is produced from translation initiation at the first in-frame ATG while a second isoform, C/EBPbeta-2, results from translation initiation at the second in-frame ATG 21–23 amino acids downstream. Initiation at the third ATG gives rise to the third isoform, C/EBPbeta-3, which has an apparent molecular weight of 20kDa (10). The structure of C/EBPbeta is such that the transactivation domain resides in the N-terminal region and the protein dimerization and DNA binding domains reside in the C-terminal end. Unlike the first two isoforms, C/EBPbeta-1 and C/EBPbeta-2 (termed LAP* and LAP in rodents), the third isoform, C/EBPbeta-3 (termed LIP in rodents) lacks the entire N-terminal activation domain, while retaining the DNA binding/protein dimerization domain (10). Therefore, this protein acts as a transcriptional repressor since it can occupy the C/EBPbeta consensus DNA elements within promoters of target genes. In this work we show that the transcriptional repressor, C/EBPbeta-3 or LIP induces autophagy and cell death when overexpressed in breast cancer cells.

Materials and Methods

Adenoviral Constructs and Cell lines

The adenoviral constructs used in these experiments were previously constructed and described by Duong et al (12). The human breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF-7 were obtained from the ATCC (Manassas, VA). MDA-MB-231 cells and MDA-MB-468 cells were maintained in Iscove's Modified Eagle media supplemented with 10% fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT, USA), 10 µg/ml bovine insulin, 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). MCF-7 cells were grown and maintained in Dulbecco's Modified Eagles's medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10 µg/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Inc.) and 10% heat inactivated fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Cell growth and proliferation assays

MDA-MB-231, MCF-7, or MDA-MB-468 cells were grown to subconfluency (60–70%) on 100mm dishes. Cells were either uninfected (NV) or adenovirally infected with Ad-GFP or Ad-LIP at a multiplicity of infection (MOI): 5–10. After 24 hours, cells were trypsinized and collected in normal growth media. Cells were counted with a hemocytometer and plated at a density of 1×10^5 cells/mL for the MDA-MB-231 cell line or 2×10^5 cells/mL for the MDA-MB-468 cell line. Cells were counted every day for seven to nine days. Cells were replenished with normal growth media every third day. Some assays were performed by plating 1×10^6 cells/mL and cells were counted every other day.

The MTS assay was used to monitor cell proliferation. Control (NV and Ad-GFP) MDA-MB-468 cells and Ad-LIP MDA-MB-468 cells were plated 24 hrs post infection in a 96-well plate at a density of 4×10^3 cells per well. Assays were performed at 2–5 days post infection by adding a small amount of the CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI) directly to culture wells as recommended by manufacturer. This reagent contains an electron coupling reagent (phenazine methosulfate) PMS and a tetrazolium compound (inner salt, MTS). MTS is converted by dehydrogenase enzymes, found in metabolically active cells, into a formazan product. After a four hour incubation period, absorbance at 490nm was measured with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

Colony formation assays

Control (NV or Ad-GFP) and Ad-LIP cells were plated 24 hrs post infection. Cells were seeded at different densities (800, 1600, and 3200 cells per 100mm tissue culture dish) for each condition. Cells were grown in normal growth media and replenished every three days. After 12–20 days in culture, plates were fixed with 95% ethanol and stained with Gill No. 3 hematoxylin solution. Plates were then repeatedly rinsed and colonies counted by visual inspection for each condition.

Cell Cycle Analysis

DNA cell cycle profiles of sub-confluent (60–70%) cultures were determined by flow cytometry using a BD FACScan (Becton Dickinson, San Jose, CA). All cultures were harvested at 24, 72, and 96 hrs post infection by trypsinization and pelleting in the presence of 20% fetal bovine serum at $500 \times g$ for 7 minutes. Cells were then counted using a hemocytometer.

Approximately 2×10^6 cells were washed twice in cold phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol overnight. The samples were pelleted at $500 \times g$ for 7 minutes and washed twice with ice-cold PBS. Lastly, the cells were incubated in a staining solution containing 2.5 mg/ml RnaseA, 2.0 mg/ml propidium iodide, 0.1% (v/v) Triton X-100, 1 μ M EDTA in $1 \times$ PBS for 30–60 minutes at 4°C in the dark. Data was collected using BD Cellquest software (BD Biosciences Immunocytometry Systems, San Jose, CA), and cell cycle modeling was performed using Modfit software (Verity Software House, Topsham, ME). The cell cycle profile of each population was generated from DNA content data collected from between 15,000 to 25,000 separate events (13).

Electron Microscopy

MDA-MB-231 cells infected with Ad-GFP or Ad-LIP were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 hour. The samples were then postfixed in 1% OsO₄ in the same buffer for 1 hour. Samples were then dehydrated in a graded alcohol series and embedded in epoxy resin. Representative areas were chosen for thin sectioning and viewed with an electron microscope (JEM 1010 transmission electron microscope; JEOL, Peabody, MA).

Immunoblot analysis

Whole cell lysates were prepared from 100 mm dishes of 50–90% confluent MDA-MB-231 or MDA-MB-468 cells by scraping the cells at 4°C into STE (10 mM Tris pH 8, 1 mM EDTA, 100 mM NaCl) in the presence of the following protease/phosphatase inhibitors: (10 μ M Na vanadate, 10 mM Na molybdate, 10 mM beta-glycerolphosphate, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). An equal volume of 2X SDS sample buffer and BME was added and samples were boiled for 5 minutes. Relative protein concentrations were determined using Protein Assay Reagent (BioRad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Equal amounts of protein were loaded onto a 10% or an 18% SDS – PAGE and separated by electrophoresis. The 18% SDS-PAGE running gel is prepared using 24mL of 30% acrylamide and 0.15% Bis N,N'-methyleneethylene-bis acrylamide, 10mL of 3M Tris, pH8.8, 0.4mL of 10% SDS and 0.4mL of 10% ammonium persulfate in a final volume of 40mL. The stacker gel is prepared using 6mL of 10% acrylamide and 0.15% N,N'-methyleneethylene-bis acrylamide, 5mL of 1M Tris pH6.8, 0.2mL of 10% SDS and 0.2mL of 10% ammonium persulfate in a final volume of 20mL. The proteins were transferred to an Immobilon-P or Immobilon-FL filter and the blots were processed as described previously (14). Briefly, the nonspecific binding sites were blocked with 5% nonfat dried milk (NFDM) in Tris Buffered Saline (TBS-T: 100 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween-20). Then blots were incubated with primary antibodies: MAP1-LC3 at a 1:1,000 dilution overnight (MBL), Caspase-3 at a 1:1,000 dilution overnight (sc-9662 Cell Signaling), T7-tag at a 1 : 20,000 dilution (EMD Bioscience) in TBS-T containing 0.5% NFDM for 1 hour at room temperature with gentle agitation. The blots were detected with an HRP (horse radish peroxidase)-conjugated goat anti-mouse or anti-rabbit antibody (Cell Signaling). Signal was detected by chemiluminescence using SuperSignal West Pico reagent (Pierce, Rockford, IL, USA). Alternatively, the LI-COR ODYSSEY infrared imaging system (Lincoln, Nebraska) was used for immunoblot analysis and quantitation as per manufacturer's instruction.

Indirect immunostaining and image acquisition

Endogenous HMGB1 and LC3 were detected in control (NV, Ad-GFP) and Ad-LIP MDA-MB-468 cells. All MDA-MB-468 cell cultures were grown in 35 mm dishes fitted with collagen-coated glass coverslips (MatTek Corp, Ashland, MA, USA). Cultures were washed three times in PBS, fixed in 3.7% formalin in phosphate buffered saline (PBS) for 30 min at RT, washed an additional three times, and processed for indirect immunofluorescence. The

cells were washed at least three times in PBS after each treatment. First, the cells were permeabilized in PBS containing 0.1% Triton X-100 for 20 min at RT. The cells were washed and nonspecific binding sites were blocked in PBS containing 5% BSA (Fraction V, Sigma) at 4°C for 24 hours. Immediately following aspiration of the blocking solution, the cells were incubated with HMGB1 polyclonal antibody (Proteintech Group) at a dilution of 1:75 or LC3B polyclonal antibody (Novus Biologicals) at a dilution of 1 : 150 in PBS containing 2% BSA and 0.1% Triton X-100 for 2 hours at RT. The cells were washed as described above. Cells were then incubated for an additional hour at RT in the dark with fluorescent-conjugated Alexa 594 goat-anti-rabbit secondary antibody diluted to a final concentration of 2 µg/ml in PBS containing 2% BSA and 0.1% Triton X-100. The cells were then washed three times in PBS containing 0.1% Triton X-100 and a few final rinses with double-distilled water. In some cases, Hoechst 33342 (Sigma-Aldrich Co., St. Louis, MO) was used to label the nucleus. The cells were visualized on a Leica DM IRB inverted fluorescence microscope equipped with a Nikon DMX 1200C digital camera.

Quantification of Acidic Vesicles by Acridine Orange using Flow Cytometry

Control (NV, Ad-GFP) MDA-MB-231 cells and Ad-LIP MDA-MB-231 cells were stained with acridine orange 96 hours post infection. Cells were trypsinized and prepared from 100 mm dishes. Acridine orange (Polyscience, Warrington, PA) was added at a final concentration of 1µg/mL for a period of 15 minutes at room temperature. Cells were washed and collected in 1 × PBS. Following quantification on a hemocytometer, approximately 1 × 10⁶ cells were stained and then analyzed by flow cytometry. Data was collected using BD Cellquest software (BD Biosciences Immunocytometry Systems, San Jose, CA). Red (>650nm) fluorescence emission from 10⁶ cells illuminated with blue (488nm) excitation light was measured with a BD FACScan (Becton Dickinson, San Jose, CA). Winlist software (Verity Software House, Topsham, ME) was used to determine the fluorescence means and make the overlays. The profile of each population was generated from data collected from a representative sample of 25,000 events. Five separate experiments were performed.

Statistical analysis

Quantitative data are expressed as means ± SD. For comparisons between multiple groups, ANOVA followed by the Student-Newman-Keuls multiple comparisons test was used. Prism 5.0 (GraphPad, La Jolla, CA) was used for all analyses.

RESULTS

LIP expression attenuates proliferation of the MDA-MB-468 breast cancer cell line

We have previously demonstrated that overexpression of LIP in the MCF10A cell line is incompatible with cell growth (13). When attempting to generate a population of LIP-expressing cells by infection with the chimeric retrovirus, LZRS-HisLIP-IRES-GFP, followed by cell sorting, we found we could not establish a cell population stably expressing elevated LIP. We reasoned adenovirally (Ad) infecting the cells would eliminate the problematic expansion of sorted cells when LIP is growth inhibitory. To analyze the effect of LIP expression, we used an adenoviral vector to transduce the breast cancer cell line, MDA-MB-468, with the truncated C/EBPβ gene comprising the C-terminal half encoding a T7-epitope tagged 20kDa LIP protein. The adenoviral vector also encodes a GFP marker protein so that infected cells are easily detected by their green fluorescence; as such control adenoviral vector expressing GFP only was also used. Cells were plated at the same density and adenovirally infected with control Ad-GFP or Ad-LIP on day 0. Uninfected or no virus (NV) control cells are used in our experiments to compare to adenoviral infected cells. Adenoviral infection efficiency was approximately 90% for the GFP only infected cells and

>95% for the LIP-GFP infected cells (Fig. 1A panels a–c). Photomicrographs of the MDA-MB-468 cells in culture three days post infection are shown in Figure 1A. The NV MDA-MB-468 cells and Ad-GFP MDA-MB-468 cells continue to proliferate (Note the control cell cultures are >90% confluent three days after plating). LIP-expressing cells, however, are unable to proliferate in culture leading to a decrease in cell density in comparison to the control cells (Fig. 1A, panel c and f). To quantitate and monitor the decrease in cell density, cell counts were performed daily for 9 days post infection. LIP expression in MDA-MB-468 cells leads to a decrease in cell number (Fig. 1B) after several days post infection, demonstrating that LIP overexpression results in cell death. Next, we performed colony formation assays with MDA-MB-468 cells. These assays are based on the principle that stable expression of certain proteins cause either cell cycle arrest or cell death, thus there is a reduction in colony number. Control MDA-MB-468 cells (NV and Ad-GFP) were able to produce significantly more colonies than the LIP-expressing MDA-MB-468 cells (Fig. 1C). We further examined the proliferation of control (NV, Ad-GFP) and (Ad-LIP) MDA-MB-468 cells by performing MTS assays. LIP expression results in inhibition of the proliferative activity of MDA-MB-468 cells to 20–40% of control cells at days 4 and 5 post infection (Fig. 1D). To confirm LIP expression, we conducted immunoblot analysis of whole cell lysates using anti-T7 antibody. As shown in Figure 1E, we initially detect a high level of the T7-tagged 20kDa LIP protein at day 2 post infection in LIP expressing cells that remain attached to the plate. However, LIP levels decline at day 4 post infection and are undetectable by immunoblot analysis at six days post infection. These results show that cells expressing LIP do not proliferate and are lost from the population.

LIP expression attenuates proliferation of the MDA-MB-231 breast cancer cell line

To extend our findings in other breast cancer cell lines, LIP was introduced into MDA-MB-231 and MCF-7 and cell growth was monitored. Photomicrographs of the MDA-MB-231 cells in culture three days post infection are shown in Figure 2A. In the same way, the Ad-LIP MDA-MB-231 cells are dying or unable to proliferate in culture leading to a decrease in cell density in comparison to the control cells (Fig. 2A, panel c and f). Cell density was monitored by plating cells at the same density post infection and counted every day (MDA-MB-231) or every other day (MCF-7 cells). LIP expression inhibited cell proliferation in MDA-MB-231 (Fig. 2B) and MCF-7 cells (data not shown). Colony formation assays were also performed with the MDA-MB-231 cells. Likewise, the LIP-expressing MDA-MB-231 cells formed fewer colonies than the control (NV and Ad-GFP) MDA-MB-231 cells (Fig. 2C). LIP expression in the MDA-MB-231 was confirmed by immunoblot analysis using the anti-T7 antibody. We detect LIP expression at day 2 post infection; however, LIP levels rapidly decline and are undetectable after four days post infection (Fig. 2D). Interestingly, at day 6 this cell population is beginning to recover, coinciding with the loss of overexpressed LIP protein.

LIP does not block cell cycle progression or induce apoptosis

We considered that LIP expression might inhibit proliferation by blocking the cell cycle or by inducing apoptosis, since we observe a decrease in cell number in the LIP-expressing cell population and lower to almost no colony formation. To address these possibilities, cell cycle analysis and caspase-3 activation were performed on the MDA-MB-231 cell line. FACS analysis was performed at various time points (days 1, 3, and 6 post infection) and revealed no significant differences in the cell cycle phases (Fig. 3A and B). Ad-LIP infected cells did not show a G1 phase arrest and LIP-expressing cells are present in the G2/M phase as well as the S-phase (Fig. 3A panel c). We can conclude that Ad-LIP does not block cell cycle progress. Interestingly, a sub-G1 phase (arrow in Fig. 3A panel a–c), a characteristic of apoptosis, was not detected in Ad-LIP infected cells. To confirm the absence of apoptosis, we examined caspase-3 activation in the Ad-LIP infected cells. Activation of caspase-3 is

key in mediating apoptosis (15). Activation requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments (15). Caspase-3 activation was screened in MDA-MB-231 cells by western blotting (Fig. 3C). LIP-expressing cells did not activate caspase-3 at a low or higher MOI as indicated by the absence of the cleaved caspase fragments (Fig. 3C lanes 4–9) that are present in the positive control (Fig. 3C Lane 3). Activation of caspase-3 was also screened in MDA-MB-468 cells (data not shown) and caspase-3 activation was not detected. Furthermore, chromatin condensation and nuclear breakdown was not observed in the ultrastructural analysis of these cells performed in Figure 5. Collectively, these results indicate that Ad-LIP does not induce apoptosis.

LIP does not induce necrosis

In contrast to apoptosis, necrotic cell death is not a developmentally programmed type of cell death. Necrosis takes place when cells are exposed to extreme stress conditions, which leads to a deregulation of normal cellular activities. High mobility group box 1 (HMGB1) is a non-histone nuclear protein participating in chromatin architecture and transcriptional regulation (40). However, it has also been shown to be secreted from damaged or necrotic cells (40). In order to test whether LIP expression was inducing necrotic cell death, we assayed the intracellular localization of HMGB1 in the MDA-MB-468 cells by immunostaining. MDA-MB-468 cells were forced into necrosis by treatment with deoxyglucose and azide. HMGB1 is no longer localized to the nucleus of these cells as shown in Figure 4 (left panel). Under normal conditions, HMGB1 is localized to the nucleus as seen in control (NV and Ad-GFP) MDA-MB-468 cells. More importantly, LIP expression does not lead to secretion of HMGB1. We detect HMGB1 in the nucleus of the LIP-expressing MDA-MB-468 cells. We repeated these experiments at days 3, 4, and 6 post infection and did not detect any changes in the HMGB1 localization.

Ultrastructure of autophagic vesicles (AV) formed during LIP-induced autophagy

Interestingly, when examining the LIP-expressing cells under the light microscope, the dying/dead cells exhibited a change in cell morphology with increased intracellular vacuoles. Given the vacuolated appearance of the MDA-MB-231 cells infected with Ad-LIP, we wanted to confirm if autophagy was occurring using electron microscopy. Autophagy can be observed through ultrastructural analysis using transmission electron microscopy (TEM) (16). This method is one of the most indispensable methods to detect autophagic compartments in mammalian cells (16). LIP-expressing MDA-MB-231 cells, displayed in Figure 5A (panel c), show a dramatic amount of autophagic vesicles (AV) not seen in the control (NV and Ad-GFP) MDA-MB-231 cells (Fig. 5A panel a and b, respectively). The magnified images illustrate autophagosomes with characteristic double or multiple membranes (Fig. 3B panel a, b). These autophagosomes contain engulfed materials, including degraded cytoplasmic areas, as well as organelles. In addition, there are many multi-lamellar structures observed (Fig. 5B panel b). Empty vacuoles were also identified (Fig. 5B panel c) that are not likely to be autophagic, although their significance is unknown. In addition, EM micrographs reveal “myelin” bodies (Fig 5B, panel d), which is the end result of extensive autophagy. These EM micrographs provide strong evidence that LIP induces autophagy in MDA-MB-231 cells.

LIP overexpression leads to increase acidic vesicles

Autophagy can also be monitored by the development of acidic vesicular organelles (16). Acidification of autophagic vesicles is mediated by the vacuolar H⁺-ATPase in yeast or lysosomal hydrolases in mammalian cells. The lysosomotropic agent acridine orange is a weak base that moves freely across biological membranes when uncharged. Its protonated form accumulates in acidic compartments (17). Therefore, it can be used to quantify acidic AV accumulation. When compared to control uninfected MDA-MB-231 cells, the Ad-LIP

cells show a shift or increase in the red fluorescence. Interestingly, we also detect a side plateau indicative of dying cells (arrow in Fig. 6A panel a). There are no changes detected in the control (NV and Ad-GFP) MDA-MB-231 cells (Fig. 6A panel b). The results of five separate experiments were quantitated as shown in Figure 6B. In comparison to the controls, NV and Ad-GFP MDA-MB-231 cell populations, LIP-expressing cells were determined to have a statistically significant ($p < 0.01$) increase in acidic vesicles (Fig. 6B). There was no statistical difference between the NV and Ad-GFP MDA-MB-231 cells. After performing these experiments at various time points, we detect the greatest changes in acridine orange staining four days post infection.

LIP-induced activation of LC3

Induction of autophagy by LIP was also confirmed by LC3 activation. LC3 is the first mammalian protein known to specifically associate with the autophagosomes (18). LC3 binds the elongating preautophagosomal membrane after activation by proteolytic cleavage and conjugation of a phosphatidylethanolamine molecule to the exposed C-terminus (18,19). The proteolytic cleavage converts LC3 from an 18kD (LC3-I) to a 16kD (LC3-II) protein (18). The conversion of soluble form of LC3 (LC3-I) to the autophagosome-associated (LC3-II) is a well-accepted method for monitoring the onset of autophagy (16). To improve separation of these two small proteins we performed immunoblotting after analyzing protein extracts on an 18% non-Laemmli acrylamide gel. We detect an increase in the membrane-bound form (LC3-II) in LIP-expressing MDA-MB-468 cells beginning at day 3 post infection (Figure 7A lane 5). LC3-II levels greatly increase at days 4 and 5 post infection in LIP-expressing cells (Figure 7A lanes 8 and 11) in comparison to the control (NV and GFP) MDA-MB-468 cells (Figure 7B). In addition to immunoblot analysis, we assayed the intracellular localization of endogenous LC3 in MDA-MB-468 cells using indirect immunostaining and fluorescence microscopy. LC3 has been reported to localize to punctate-structures when the process of autophagy is occurring. The NV and Ad-GFP MDA-MB-468 cells show dispersed LC3 staining (Figure 7C). Even under nutrient rich conditions, we detect punctate LC3 staining in 43% of LIP-expressing cells (Figure 7C). Our results indicate that LIP expression leads to an increase in endogenous LC3-II levels and an increase in punctate LC3 staining reflective of an increase in autophagosomes or autolysosomes.

DISCUSSION

In this study, we demonstrate that C/EBP β -3 or LIP induces cell death and stimulates autophagy in breast cancer cell lines. We had previously found that exogenous LIP expression is incompatible with MCF10A cell proliferation, since the initially LIP-expressing, GFP positive cells rapidly disappear from a mixed population of LIP-expressing and non-expressing cells (13). Cell cycle profiling, ultrastructural analysis, and caspase-3 immunoblot analysis performed here reveal that LIP expression did not lead to a cell cycle block and apoptotic cells are not observed (Figure 3). We examined whether LIP expression leads to necrotic cell death and determined this is not the case (Figure 4). In contrast, ultrastructural analysis using electron microscopy showed a marked induction of autophagy in LIP-expressing cells with the accumulation of autophagic vesicles (Figure 5). The induction of autophagy was also confirmed by quantification of lysosomal induction as well as the analysis of endogenous LC3 by Western blotting and immunofluorescence (Figures 6 and 7). Overall, the data presented show that LIP overexpression leads to the induction of autophagy and cell death.

C/EBP transcription factors are involved in a variety of physiological processes, such as metabolic regulation, cellular differentiation, and stress responses. The expression of the C/EBP β gene has been shown to increase during endoplasmic reticulum (ER) stress. In

recent years, emerging data indicate that ER stress is a strong inducer of autophagy (20). Interestingly, Li et al. describe increased LIP levels during the late phase of ER stress. Recently, Meir et al. examined the distinct roles that the LAP and LIP isoforms play in ER stress (37). They find that LIP augments ER stress-induced cell death in mouse B16 melanoma cells. In addition, LIP inhibited B16 melanoma tumor progression (37). Although both of these studies did not examine any markers of autophagy, elevated LIP may be one way of linking ER stress and the induction of autophagy. It is interesting that LC3(II) levels are highest at 4–5 days postinfection with Ad-LIP while the highest levels of LIP are present at 2–3 days postinfection. It is possible that LIP is repressing or downregulating genes whose products inhibit autophagy. Thus, the half life of such proteins will determine the kinetics of when elevated LC3(II) is observed.

Some studies have described elevated levels of C/EBPbeta-3, or LIP, in both murine mammary hyperplasia and in human breast cancer (22,23). It has been argued that the transcriptional inhibitor LIP isoform is predominantly expressed during proliferative cellular responses and is associated with aggressive tumors. Zahnow et al. have reported that LIP is overexpressed in 23% of infiltrating ductal carcinomas specimens (23). However, our own study on primary breast tumor samples found that high grade, invasive mammary carcinomas showed significant C/EBP-2 expression, but no LIP was detected in any of the samples (14). LIP is known to be easily generated by artifactual proteolysis of the larger isoforms (11), which could explain this discrepancy. It has also been reported that expression of LIP under the control of the whey acidic promoter (WAP) in the mouse mammary gland results in the formation of hyperplastic tissue and carcinomas (24). However, because the LIP transgene was not epitope-tagged in these mice it is not possible to ascertain transgene expression distinguished from any endogenous LIP expression. Moreover, the level of LIP expression (transgene or endogenous) in the mammary tumors was not actually examined (24).

Despite these limitations, our finding that LIP expression induces autophagy may nonetheless help to resolve the paradox over whether LIP is pro- or anti-tumorigenic. This is because autophagy is generally thought to play dual roles in cancer development. For instance, allelic loss of beclin1, an essential autophagy gene, is found with high frequency in human ovarian, prostate, and breast cancers (25,26). The BECN+/- mice and autophagy-related 4C (Atg4C)-deficient mice have an increase in tumor incidence (26,27). Autophagy plays an important role in sustaining organelle and protein quality control, working alongside with the ubiquitin degradation pathway to prevent the accumulation of polyubiquitinated and aggregated proteins (28). By doing so, it limits the accumulation of genome damage and suppresses the mutation rate of tumor cells in which the cell-cycle checkpoints have been inactivated (29). Yet at later stages of tumor development, autophagy may be a means by which tumor cells survive in response to metabolic stress and in hypoxic tumor regions, providing extra time for the recruitment of a blood supply via induction of angiogenesis and/or motility and invasion (30). Thus, it is possible that some expression of LIP could promote cancer by increasing survival of certain cells under stress, explaining the slightly increased incidence (9%) of tumors observed in WAP-LIP transgenic mice (24).

To gain insight into the mechanism by which LIP expression could induce autophagy in mammary cells we have undertaken preliminary Affymetrix genomic profiling on MDA-MB-231 LIP or GFP expressing cells and NV controls. Remarkably few statistically significant changes in gene transcripts were observed, although a comprehensive analysis will need to include profiling miRNAs which is currently underway. C/EBPbeta plays an essential role in the development of the mammary gland, a process that involves growth and proliferation of the epithelial ductal tree during puberty and early pregnancy, followed by differentiation into secretory epithelial cells at late pregnancy or lactation and finally

involution to return to the virgin-like state (31,32). While many studies have previously focused on apoptosis as the main type of cell death occurring in the mammary gland during involution, accumulating evidence suggests that autophagic cell death also occurs during the involution process (33,34). It is therefore possible that one of the physiological roles for the production of LIP is inducing autophagic cell death during involution of the mammary gland. However, determining whether involution is dependent on LIP expression is complicated by the fact that mammary epithelial cell proliferation and differentiation are severely impaired in C/EBPbeta-null mice (35,36). Consequently, C/EBPbeta-null mice do not lactate and hence there is no postlactational involution. Although it may be difficult to define the physiological role of LIP's ability to induce autophagy, further understanding of the mechanism(s) by which LIP expression stimulates autophagy and leads to cell death may provide new avenues for inducing this alternative death pathway, especially in tumor cells that are resistant to apoptosis.

Abbreviations

LC3	microtubule-associated protein 1 light chain 3
ATG1	autophagy-related gene 1
C/EBPbeta	CCAAT/Enhancer Binding Protein beta
LAP	liver-enriched transcriptional activator protein
NV	no virus or uninfected
Ad	adenoviral
EM	electron microscopy
AV	autophagic vesicles
AVO	acidic vesicular organelles
MEC	mammary epithelial cells
BECN1	Beclin 1
Atg4C	autophagy-related 4C

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Fig. 1.

LIP expression attenuates proliferation of the MDA-MB-468 breast cancer cell line. **A**, Ad-LIP or Ad-GFP were used at an MOI of 10 to infect MDA-MB-468 cells. Fluorescent micrographs are shown for control uninfected (NV) (panel a), Ad-GFP (panel b), and Ad-LIP (panel c) cells. Corresponding brightfield micrographs of control (NV) cells or Ad-GFP cells are shown in panel d and e, respectively. Brightfield micrographs of Ad-LIP cells are shown in panel f. Cells were plated on day 1 post infection at the same density (2×10^6 per 100 mm dish) and images were taken three days post infection. **B**, MDA-MB-468 cells were infected with Ad-LIP (▲) or Ad-GFP (■) and compared to NV (●) control cells. Cells were plated on day 1 post infection at 200,000 cells per 100 mm dish and counted every day. Results are shown as the mean of three experiments. Error bars represent the standard deviation (SD). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing the Ad-LIP to the control (NV and Ad-GFP) MDA-MB-468 cells. # $p < 0.05$; ## $p < 0.01$ comparing the controls (NV and Ad-GFP) MDA-MB-468 cells. **C**, Representative plates with visible colonies from colony formation assays of control (NV, Ad-GFP) and Ad-LIP MDA-MB-468 cells. **D**, The proliferative activity of MDA-MB-468 cells infected with Ad-LIP (▲), Ad-GFP (■), and NV (●) control cells was assessed by the MTS assay. Cells were plated on day 1 post infection at 4×10^3 cells per well. Data are the mean $\pm$ SD of three separate experiments. * $p < 0.05$; ** $p < 0.01$ comparing the Ad-LIP to the control (NV and Ad-GFP) MDA-MB-468 cells. **E**, Cell extracts were prepared from MDA-MB-468 NV cells (lanes 1–3), Ad-GFP cells (lanes 4–6), and Ad-LIP cells (lanes 7–9) at days 2, 4, and 6 post infection. Expression

of T7-tagged LIP was determined by immunoblot analysis using anti-T7 epitope tag antibody. GAPDH is shown as a loading control.

Fig. 2.

LIP expression attenuates proliferation of the MDA-MB-231 breast cancer cell line. **A**, Fluorescent micrographs are shown for control uninfected (NV) (panel a), Ad-GFP (panel b), and Ad-LIP (panel c) cells. Corresponding brightfield micrographs of control (NV) cells or Ad-GFP cells and Ad-LIP cells are shown in panel d–f, respectively. MDA-MB-231 cells were adenovirally infected at an MOI of 10. Cells were plated on day 1 post infection at the same density (1×10^6 per 100 mm dish) and images were taken three days post infection. **B**, MDA-MB-231 cells were infected with Ad-LIP ($\blacktriangle$) or Ad-GFP ($\blacksquare$) and compared to NV ($\bullet$) control cells. Cells were plated on day 1 post infection at 100,000 cells per 100 mm dish and counted every day. Results are shown as the mean of three experiments. Error bars represent the standard deviation (SD). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing the Ad-LIP to the control (NV and Ad-GFP) MDA-MB-468 cells. # $p < 0.05$; # $p < 0.01$ comparing the controls (NV and Ad-GFP) MDA-MB-468 cells. **C**, MDA-MB-231 cells (NV, Ad-GFP, or Ad-LIP) were plated into 100mm dishes at a density of 3200 cells per dish. Colonies were scored by counting visible colonies after 12 days and are presented as the mean value $\pm$ standard deviation from three individual experiments. * $p < 0.05$; ** $p < 0.01$ comparing Ad-LIP to the control (NV and Ad-GFP) MDA-MB-231 cells. **D**, Cell extracts were prepared from MDA-MB-231 Ad-LIP cells (lanes 1–3), Ad-GFP cells (lanes 4–6), and NV cells (lanes 7–9) at days 2, 4, and 6 post infection. Expression of T7-tagged LIP was determined by immunoblot analysis using anti-T7 epitope tag antibody. GAPDH is shown as a loading control.

Fig. 3.

LIP does not block cell cycle progression or induce apoptosis. **A**, Cell cycle profiles of uninfected (NV) MDA-MB-231 cells (a), Ad-GFP cells (b), and Ad-LIP cells (c). Results from a representative experiment are shown. **B**, Quantification of cell cycle analysis of MDA-MB-231 cells at day 6 post infection. Solid black bars indicate the percentage of cells in G0/G1, gray-filled bars the percentage in S-phase, and white-filled bars the percentage in G2-M. **C**, Whole cell lysates were prepared from control uninfected (NV) (lane 1) and Ad-GFP MDA-MB-231 cells (lane 2) at day 3 post infection. Whole cell lysates were also prepared from MDA-MB-231 cells infected with Ad-LIP at a MOI of 10 or 30 (lanes 4–9) at different time points (days 3, 5, and 7) post infection. RKO colon cancer cell line treated with 5-fluorouracil (lane 3) is included as a positive control. Samples were analyzed by 12% SDS-PAGE and immunoblot analysis for caspase-3 activation was performed. Equal amounts of total protein were loaded in each lane as determined by Ponceau S staining.

Fig. 4.

LIP does not induce necrosis. MDA-MB-468 cells were induced into necrosis by adding 6mM deoxyglucose plus 10mM sodium azide for 18h and used as a positive control. Immunofluorescence studies were performed on MDA-MB-468 cells (NV, Ad-GFP, or Ad-LIP) at day 4 post infection. Cells were fixed and stained for DNA (Hoechst top panel) and HMG1 (middle panel).

Fig. 5. Ultrastructure of autophagic vacuoles (AV) formed during LIP-induced autophagy. **A**, Representative electron micrographs shown of uninfected (NV) MDA-MB-231 cells (a), Ad-GFP MDA-MB-231 cells (b), and Ad-LIP MDA-MB 231 cells (c) 2 days post infection. **B**, Electron micrographs of LIP-expressing MDA-MB-231 cells at later stages (six days post infection) of autophagic process. Scale bar: **A**, 2 micron (a–c); **B**, 500nm (a,b); 2 micron (c); 100nm (d).

Fig. 6.

LIP overexpression leads to increase of autophagic vacuoles (AV). LIP-induced appearance of AV was detected by staining with the lysosomotropic agent, acridine orange. Cells were stained and processed for flow cytometric analysis. Mean red fluorescence was determined as described in “Materials and Methods”. **A**, Panel a shows an overlay comparing the control (NV) and Ad-LIP MDA-MB 231 cells. Panel b shows an overlay comparing the control (NV) and Ad-GFP MDA-MB-231 cells. **B**, Quantitation of red fluorescence (>650nm) for control (NV) MDA-MB-231 cells, Ad-GFP, or Ad-LIP MDA-MB-231 cells four days post infection. Results are shown as the mean of 5 separate experiments. Error bars indicate $\pm$ standard error of the mean (SEM). (*p-value<0.01).

Fig. 7.

LIP-induced activation of LC3. **A**, Whole cell lysates were prepared from control uninfected (NV) MDA-MB-468 cells (lanes 3,6,9, and 12), Ad-GFP MDA-MB-468 cells (lanes 1,4,7, and 10), and Ad-LIP MDA-MB-468 cells (lanes 2,5,8, and 11) at different time points (days 2–5) post infection and analyzed by 18% SDS-PAGE. Immunoblot analysis with anti-LC3 antibody and a B-tubulin antibody (used as a loading control) is shown. LC3-I, soluble form of LC3; LC3-II, membrane-bound form of LC3. **B**, Densitometric analysis of LC3-II in MDA-MB-468 cells using the ODYSSEY infrared imaging system. Data shown represents the mean $\pm$ SEM. White-filled bars represent the uninfected (NV) MDA-MB-468 cells, solid black bars the Ad-LIP MDA-MB-468 cells, and shaded bars the Ad-GFP MDA-MB-468 cells. **C**, LC3 immunostaining (red) of control (NV), Ad-GFP, or Ad-LIP MDA-MB-468 cells are shown. The percentage of cells with LC3 punctate staining relative to the total cell number at day 4 post infection is shown in the corner of each panel (mean $\pm$ SD).