RESEARCH ARTICLE

Autophagy is a regulator of TRAIL-induced apoptosis in NSCLC A549 cells

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Received: 8 July 2016 / Accepted: 9 November 2016 / Published online: 18 January 2017
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Abstract Autophagy, a catabolic process by which cytoplasmic components are degraded in lysosomes, plays an important role in the maintenance of cellular homeostasis. Dysregulation of autophagy is associated with several diseases. However, few studies have addressed the role of autophagy in the lung, and its role in lung diseases remains unclear. In the present study, we examined the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on autophagy in A549 cells and explored the underlying mechanisms. We showed that TRAIL promoted autophagosome formation, as detected by the levels of LC3-II, and its effect on promoting autophagy was dependent on the expression of the autophagy related genes (ATGs) Atg5, Atg7, and beclin-1. TRAIL-induced ATG expression was attenuated by JNK silencing or treatment with the JNK inhibitor SP600125, indicating the involvement of the JNK pathway. Crosstalk between autophagy and apoptosis was demonstrated by silencing the autophagy related genes Atg5, Atg7, and beclin-1, and the dependence of TRAIL-induced apoptosis on autophagy-related gene expression. Taken together, our results indicate that TRAIL promotes autophagy in A549 cells via a mechanism involving the modulation of ATG expression through the JNK pathway. Inhibition of autophagy enhanced TRAIL-induced cell proliferative inhibition and apoptosis in A549 cells.

Keywords Autophagy · Trail · Autophagy related genes · Apoptosis

Abbreviations

TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
ATGs	autophagy related genes
JNK	c-Jun terminal kinase
FADD	Fas-associated death domain protein
COPD	chronic obstructive lung disease
siRNA	Small interfering RNA
siCon	Small interfering RNA control.

Background

Autophagy is a catabolic process by which cytosolic proteins are degraded in the lysosome. It plays important roles in the degradation of misfolded protein aggregates and the elimination of invading micro-organisms and damaged organelles, and is therefore essential for cellular homeostasis and cellular defense mechanisms (Wang et al. 2009). Autophagy plays a dual role as a pro- and anti-survival process, and dysregulation of autophagy is associated with several diseases including cancer, neurodegeneration, and cardiomyopathy (Komatsu et al. 2006; Maiuri et al. 2007; Levine and Kroemer 2008). Autophagy is enhanced in the epithelium of patients with chronic obstructive lung disease (COPD) and contributes to disease progression; however, it is impaired in the alveolar macrophages of COPD patients (Chen et al. 2008; Monick et al. 2010). In cystic fibrosis, autophagosome formation is impaired by depletion of beclin-1 (Luciani et al. 2010). However, few studies have investigated the role of autophagy in lung diseases.

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The process of autophagy is characterized by the formation of the autophagosome, which is a double-membrane-bound vacuole that engulfs cellular material targeted for degradation and fuses with the lysosome, leading to the degradation of its contents (Liang et al. 1998; Kihara et al. 2001; Wang et al. 2009). Autophagy related genes (ATGs) are essential to the process of autophagy and approximately 30 Atg proteins have been characterized in yeast and 10 in humans. Beclin-1, which was first identified as an interacting partner of the antiapoptotic protein Bcl-2, is an autophagy related protein (its yeast homolog is Atg6) that plays a role in the formation of autophagosomes. During the formation of autophagosomes, another Atg protein, the soluble cytosolic form of microtubule-associated protein 1 light chain 3 (LC3-I), is conjugated to phosphatidylethanolamine to generate LC3-II, which is recruited to the autophagosomal membrane and degraded after the fusion of autophagosomes to lysosomes (Tanida et al. 2008); therefore, the LC3-II/LC3-I ratio is a measure of autophagic activity.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor family, is a selective apoptosis inducer that has been studied as a target for cancer therapy because of its ability to kill tumor cells without significant cytotoxicity to normal cells (Park et al. 2007). TRAIL binds to the death receptors DR4 or DR5, inducing receptor trimerization and the recruitment of Fas-associated death domain protein (FADD), leading to the activation of caspase cascades and cell death (Aggarwal 2003; Bellail et al. 2010). In addition to the induction of apoptotic cell death, TRAIL and its receptors mediate signaling pathways in a caspase-independent manner, such as via c-Jun terminal kinase (JNK) and p38 signaling cascades (Muhlenbeck et al. 1998; Di Pietro and Zauli 2004; Guo et al. 2005). TRAIL also induces autophagic cell death in several cell types, and the shift from TRAIL induced apoptosis to the TRAIL-mediated cytoprotective autophagy has been implicated in the resistance of tumor cells to TRAIL (Han et al. 2008). TRAIL was also shown to induce cell death via necroptosis, a form of programmed cell death, when necrosome assembly is stimulated by p62-dependent recruitment to the autophagosomal membrane, indicating that autophagy related proteins can mediate the formation of a scaffold to regulate the mechanisms of cell death (Goodall et al. 2016).

In the present study, we investigated the role of TRAIL-induced autophagy in A549 cells and the involvement of the JNK pathway. The role of the crosstalk between autophagy and apoptosis in response to TRAIL stimulation was also examined.

Materials and methods

Cell lines and culture

The A549 lung adenocarcinoma cell line was obtained from the Type Culture Collection of the Chinese Academy of

Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and 100 U/ml of penicillin and 100 mg/l of streptomycin at 37°C in a 5% CO₂ humidified atmosphere. Logarithmic phase cells were used in the experiments. In some cases, 10% fetal calf serum was changed to serum-free medium depending on the experiment.

Antibodies and reagents

Human recombinant TRAIL was from R&D Systems (Minneapolis, MN, USA); 3-methyladenine (3-MA), bafilomycin A1 (Baf A1), and β -actin antibody were purchased from Sigma. Antibodies against BECN1, ATG5 and ATG7 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against P-JNK1/2, JNK1/2, P-c-Jun, c-Jun, caspase-3, and PARP were purchased from Cell Signaling Technology (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM)/F12 and Fetal Bovine Serum were purchased from Gibco-BRL (Grand Island, NY, USA). The RT-PCR kit and TRIZOL reagent were purchased from Invitrogen (San Diego, CA, USA).

Small interfering RNA and oligonucleotides

Transfection of small interfering RNAs (siRNA) was performed using the HiPerfect reagent (Qiagen). SiRNAs targeting BECLIN1 (sc-29,797), ATG5 (sc-41,445), ATG7 (sc-41,447) and Control siRNA (sc-37,007) were from Santa Cruz Biotechnology. The transfected cells were used for subsequent experiments after 24–48 h.

Cell proliferation and apoptosis assay.

Cell viability was detected by the MTT assay. A549 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, A549 alveolar epithelial cells were treated with increasing concentrations of TRAIL (0, 50, 100, and 200 ng/ml). The cell morphology was photographed under a microscope (inverted microscope; Nikon, Japan). Metabolic activity was determined by adding MTT (5 mg/ml) and further incubating the cells for 4 h, then the precipitate was solubilized by the addition of 150 μ l/well DMSO (Sigma) and shaken for 10 min. Absorbance at a wavelength of 490 nm in each well was measured with a microplate reader (Bio-Tek ELX800, USA). All experiments were performed in triplicate and the experiment was repeated on at least three separate occasions. TdT-mediated dUTP nick end labeling (TUNEL) assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The total number of cells in a given area was determined by using DAPI staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted $\times 100$.

GFP-LC3 fluorescence imaging

For GFP-LC3 transfection, GFP-LC3 cDNA was transferred to a lentiviral vector (CSH-CMV-RfA) and introduced using a lentiviral infection system. Cells lentivirally transduced with GFP-LC3 were visualized using a fluorescence microscope (Olympus IX70) and the percentage of cells with more than five GFP-LC3 dots and the number of GFP-LC3 dots per cell was quantified.

Western blot analysis

A549 cells treated as indicated were lysed using RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. Proteins were separated using 4–20% Tris-glycine SDS-PAGE gels (BioRad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were exposed to the indicated primary antibodies for 8 h at 4°C, followed by the appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence detection system (GE Biosciences).

Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using the RNAiso Plus kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Reverse transcription was performed with high capacity cDNA reverse transcription kits (Takara Bio) according to the manufacturer's instructions. RT-PCR (98°C, 2 min; 36 × 98°C,

5 s; 55°C, 30 s) was carried out using 2× PCR Solution Premix Taq (R004A; Takara Bio). The primer sequences were as follows:

BECLIN1: forward 5'-acagtggacagtttgccaca-3'; reverse 5'-cggcagctccttagattgt-3'

ATG5: forward 5'-ggccatcaatcgaaactcat-3'; reverse 5'-agccacaggacgaaacagctt-3'

ATG7: forward 5'-tcgaaagccatgatgtcgtctt-3'; reverse 5'-ccaagcagcattgatgacca-3'

JNK: forward 5'-ggaaacgaccttctatgacga-3'; reverse 5'-gaaccctcctgctcatctgtcac-3'

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Two-way ANOVA was applied to interpret the differences between treatment groups. Differences were considered statistically significant at $P < 0.05$.

Results

TRAIL promotes autophagosome formation in alveolar epithelial cells

To examine the effect of TRAIL on cell viability, A549 alveolar epithelial cells were treated with increasing concentrations of TRAIL and the effect was analyzed by MTT assay. The results showed that TRAIL induced cell death at high doses (Fig. 1a). To investigate whether TRAIL has an effect on A549 cell apoptosis, cells were treated with 200 ng/mL

Fig. 1 Effect of TRAIL on A549 cell viability and autophagosome formation. **a**, A549 cells were treated with 0–200 ng/ml of TRAIL protein for 24 h. The anti-proliferative effects of TRAIL on A549 cells were evaluated by MTT assay. Data are expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, vs. 0 ng/ml. **b**, A549 cells were treated with 200 ng/mL TRAIL for 24 h. The apoptosis effects of TRAIL on A549 cells were evaluated by TUNEL assay. **c**, A549 cells stably expressing GFP-LC3 were treated with 100 ng/mL TRAIL for 24 h. GFP-LC3 puncta formation was observed by fluorescence microscopy. **d**, The fold change of GFP-LC3 dots per cell and the percentage of cells with more than five GFP-LC3 puncta were quantified

TRAIL. The results showed that A549 cells were moderately sensitive to TRAIL alone (Fig. 1b). The effect of TRAIL on autophagy was examined by analyzing LC3 puncta, which indicate the formation of autophagosomes, in A549 alveolar epithelial cells stably expressing GFP-LC3 and treated with or without 100 ng/mL of TRAIL for 24 h. Quantification of LC3 puncta in fluorescence microscopy images showed that TRAIL significantly increased the percentage of LC3 positive cells (>5 GFP-LC3 puncta) (Fig. 1c) and the fold change of the number of GFP-LC3 puncta per cell, indicating that TRAIL induced the formation of autophagosomes (Fig. 1d). To confirm the effect of TRAIL on A549 cells, the levels of LC3-I and II and those of GFP-LC3 I and II were measured by western blotting in cells treated with or without 100 ng/mL TRAIL for different times or with different concentrations of TRAIL (0–200 ng/mL) for 12 h (Fig. 2a and b). The results showed a time and dose dependent increase in the levels of LC3-II, indicating that TRAIL promotes autophagosome formation and therefore autophagy. To characterize TRAIL-induced autophagy, we performed analyses of “autophagic flux” by using Baf A1 to intentionally prevent

autophagosome-lysosome fusion and degradation to better determine the extent to which the complete autophagic process occurred in A549 cells (Fig. 2c). Figure 2d shows that 3-MA decreased TRAIL-induced LC3-II levels compared with TRAIL treatment alone.

Role of autophagy related genes in TRAIL induced autophagy

The effect of TRAIL on autophagy was further examined by measuring the expression of different ATGs in response to treatment with 100 ng/mL TRAIL. The results showed that TRAIL time-dependently and significantly upregulated beclin-1, Atg5 and Atg7 with approximately 1.5 to 2 fold increases in the expression of ATGs after 24 h of treatment (Fig. 3a). qRT-PCR analysis showed that TRAIL significantly increased the mRNA levels of ATGs by approximately 2–3.5 fold after 24 h of treatment (Fig. 3b) and this effect was reversed by transfection of A549 cells with specific siRNAs against the different ATGs (Fig. 3c). Silencing of beclin-1, Atg5, and Atg7 significantly suppressed the TRAIL induced

Fig. 2 TRAIL induces autophagy in A549 cells. **a** and **b**, Western blot analysis of the dose and time-dependent effects of TRAIL on endogenous LC3-II and GFP-LC3 formation in A549 cells treated with 100 ng/mL TRAIL for 0–24 h or 0–200 ng/mL TRAIL for 12 h. **c** and **d**, Western blot analysis of the effect of Baf A1 (5 nM) and 3-MA (5 mM) on TRAIL-induced LC3-II expression. β -tubulin was used as the endogenous control. The LC3-II/tubulin ratio was calculated with ImageJ 1.44b. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$, compared with the 0 h respective groups. β -tubulin was used as the endogenous control

formation of LC3-II, confirming that TRAIL induced autophagy is dependent on the expression of ATGs (Fig. 3d).

TRAIL induces autophagy via the JNK pathway

To confirm the involvement of the JNK pathway in TRAIL induced autophagy, we first tested the effect of TRAIL treatment on JNK activation (Fig. 4a). The expression of ATGs and the levels of LC3-II were measured in cells transfected with siRNA against JNK. The results showed that knockdown of JNK significantly suppressed the TRAIL induced upregulation of beclin-1, Atg5, Atg7 and the formation of LC3-II (Fig. 4b). The induction of the autophagy marker LC3-II by TRAIL was also reversed by the JNK pathway inhibitor SP600125 (Fig. 4c). GFP-LC3 dot formation induced by TRAIL was suppressed by siJNK or SP600125 (Fig. 4d and e), suggesting that TRAIL induced autophagy via the JNK pathway. Taken together, these results suggest that TRAIL-induced autophagy is mediated by the modulation of ATG

expression via a mechanism involving the JNK pathway in A549 cells.

Inhibition of autophagy increases TRAIL-induced proliferative inhibition and apoptosis in A549 cells

A549 cell viability was examined by the MTT assay and apoptosis was examined by the TUNEL assay. To examine the roles of autophagy and apoptosis in response to TRAIL treatment in A549 cells, cell viability and apoptosis were examined after inhibition of autophagy by silencing of ATGs or treatment with 3-MA. The results showed that TRAIL significantly inhibited cell viability and induced apoptosis, and these effects were enhanced by treatment with 3-MA or silencing of ATGs (Fig. 5a and B). Consistently, inhibition of autophagy by 3-MA or silencing of ATGs also increased TRAIL-induced caspase 3 cleavage and PARP cleavage in A549 cells (Fig. 5c and d). These results indicated that TRAIL induced apoptosis by the inhibition of autophagy.

Fig. 3 Effect of TRAIL on the expression of ATG genes and their role in TRAIL-mediated autophagy activation. **a**, Western blot analysis of the expression of beclin-1, Atg5 and Atg7 in A549 cells treated with TRAIL (100 ng/mL) for different times. β -tubulin was used as the endogenous control. Fold change in expression was calculated. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$. **b**, Quantitative reverse transcription-PCR analysis of beclin-1, Atg5 and Atg7 in A549 cells treated with TRAIL (100 ng/mL) for 24 h. **c**,

Effects of beclin-1, Atg5, or Atg7 knockdown on GFP-LC3 kinetics in A549 cells. Following siRNA transfection, GFP-LC3 puncta formation was analyzed after 24 h of treatment with TRAIL. **, $P < 0.01$ compared with the siRNA control groups respectively. ## $P < 0.01$ compared with the untreated TRAIL groups. **d**, Effects of beclin-1, Atg5, Atg7 knockdown by siRNA transfection on TRAIL induced formation of LC3-II. **, $P < 0.01$

Fig. 4 Knockdown of JNK decreases TRAIL-induced autophagy in A549. **a**, Western blot analyses of the activation of the JNK pathway including P-JNK1/2, JNK1/2, P-c-Jun, and c-Jun. **b**, Effects of JNK knockdown on the TRAIL induced expression of beclin-1, Atg5, Atg7, and LC3-II formation. **, $P < 0.01$ compared with siRNA control transfection on TRAIL treated groups. **c**, A549 cells were treated with TRAIL (100 ng/mL) with or without the JNK pathway inhibitor

SP600125 (10 μ M) for 24 h. Western blot assay was used to detect changes in LC3-I and LC3-II expression. **d** and **e**, A549 cells were treated with TRAIL (100 ng/mL) for 24 h after transfection with siControl or siJNK (**D**) or with or without the JNK pathway inhibitor SP600125 (10 μ M, 1 h) (**E**). GFP-LC3 puncta formation was analyzed. **, $P < 0.01$

Discussion

Autophagy plays a dual role as a cell survival and death mechanism, functioning primarily to maintain homeostasis by recycling long lived proteins, defective organelles and cytoplasmic bacteria (Nishida et al. 2008). However, if autophagic activity is insufficient, the accumulation of proteins and defective organelles triggers apoptosis. Furthermore, excessive autophagy can also lead to cell death, in particular when apoptosis is insufficient. Therefore, the common involvement of several signaling pathways in the regulation of both autophagy and apoptosis is not surprising, and many pathways that are essential for autophagy function through the regulation of ATG activity. For example, Atg5 promotes autophagy as a cytoprotective effect but also has proapoptotic effects, playing a role in the crosstalk between autophagy and apoptosis. Atg5 knockdown inhibits autophagy leading to the induction of apoptosis, and Atg5 overexpression sensitizes cells to apoptotic stimuli (Boya et al. 2005; Yousefi et al. 2006). In the present study, we showed that TRAIL induces autophagy in A549 cells through the JNK mediated modulation of ATG expression and examined the role of the crosstalk between autophagy and apoptosis.

In the present study, we showed that TRAIL promoted LC3-II formation, indicating the induction of autophagy, and its effect on promoting autophagy was mediated by the modulation of Atg5, Atg7 and beclin-1 via the JNK pathway. The TRAIL induced upregulation of Atg5, Atg7 and beclin-1 was attenuated by silencing of JNK, suggesting the involvement of the JNK pathway in the induction of autophagy by TRAIL in A549 cells. Although TRAIL has mostly been studied for its capacity to induce apoptotic cell death through the activation of caspases, TRAIL and its receptors were shown to transmit death signals in a caspase-independent manner (Park et al. 2007). TRAIL was shown to induce cell death in normal epithelial cells via a combined autophagy and apoptosis pathway (Thorburn et al. 2005). TRAIL-induced apoptosis is mediated by binding of FADD and the subsequent activation of caspase-8. However, Thorburn et al. showed that TRAIL activates a FADD-death domain (DD) pathway mediated by both autophagy and apoptosis that is selectively inactivated at the early stages of epithelial cancer development. In breast cancer cells, treatment with exogenous TRAIL induced autophagy in monolayer and 3D cultures, and inhibition of TRAIL signaling blocked the formation of autophagic vacuoles (Mills et al. 2004). TRAIL was shown to induce cell death via two

Fig. 5 Inhibition of autophagy enhances TRAIL-induced cell proliferative inhibition and apoptosis in A549 cells. The anti-proliferative effects on A549 cells were evaluated by MTT assay. **a**, A549 cells were treated with or without TRAIL (200 ng/mL), and the effects of 3-MA, or knockdown of beclin-1, Atg5, or Atg7 on cell viability were assessed by the MTT assay. **b**, Cell apoptosis was

measured by TUNEL assay. **c** and **d**, Caspase 3 cleavage and PARP cleavage detected by western blotting and quantification by densitometry. The ratios of cleaved Caspase3 and cleaved PARP to β -actin are indicated. Data are expressed as the mean $\pm$ SD. *, $P < 0.05$, **, $P < 0.01$ compared with the siRNA control groups

independent pathways, namely FADD-dependent and -independent pathways, both leading to JNK activation in a cell-type specific manner (Muhlenbeck et al. 1998). TRAIL binding to its receptors triggers the formation of a secondary complex that requires FADD and recruits different factors such as RIP1 and TRAF2, leading to the activation of JNK and p38 mitogen activated protein kinase pathways (Varfolomeev et al. 2005), supporting the existence of a TRAIL-mediated non-apoptotic cell death pathway.

The relation between autophagy and apoptosis has been studied extensively. The two processes share common signaling pathways, and which pathway is triggered by a specific

stimulus is partly dependent on threshold values (Gonzalez-Polo et al. 2005). In the present study, TRAIL induced apoptosis in A549 cells at high doses. The pro-apoptotic effect of TRAIL was increased by silencing of Atg5, Atg7, and beclin-1, suggesting that autophagy inhibition is necessary for the pro-apoptotic effects of TRAIL. Since the crosstalk between autophagy and apoptosis is known to depend on the threshold of stimulation, it would be of interest to further examine these effects using different doses and exposure times, as well as other stimuli.

In conclusion, we examined the effects of TRAIL on autophagy in A549 cells and explored the underlying

mechanisms. Our results indicated that TRAIL promoted cell death and induced autophagy, and these effects were suppressed by inhibition or silencing of JNK, suggesting that TRAIL-induced autophagy occurs via a mechanism involving the modulation of JNK pathway activity. Suppression of autophagy enhanced TRAIL induced apoptosis, suggesting that the effects of TRAIL on A549 lung cancer cells involve crosstalk between the autophagy and apoptosis pathways. Given the role of autophagy dysregulation in several human diseases including lung diseases, future studies should be aimed at clarifying the exact role of each pathway and their crosstalk in alveolar cells, which may provide novel targets for the treatment of lung diseases.

Acknowledgments This work was financially supported by National Nature Science Foundation of China (no. 81300005).

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