Autophagy flux inhibition mediated by celastrol sensitized lung cancer cells to TRAIL-induced apoptosis via regulation of mitochondrial transmembrane potential and reactive oxygen species

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Received April 11, 2018; Accepted November 15, 2018

DOI: 10.3892/mmr.2018.9757

Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is well known as a transmembrane cytokine and has been proposed as one of the most effective anti-cancer therapeutic agents, owing to its efficiency to selectively induce cell death in a variety of tumor cells. Suppression of autophagy flux has been increasingly acknowledged as an effective and novel therapeutic intervention for cancer. The present study demonstrated that the anti-cancer and anti-inflammatory drug celastrol, through its anti-metastatic properties, may initiate TRAIL-mediated apoptotic cell death in lung cancer cells. This sensitization was negatively affected by N-acetyl-l-cysteine, which restored the mitochondrial membrane potential (ΔΨm) and inhibited reactive oxygen species (ROS) generation. Notably, treatment with celastrol caused an increase in microtubule-associated proteins 1A/1B light chain 3B-II and p62 levels, whereas co-treatment of celastrol and TRAIL increased active caspase 3 and 8 levels compared with the control, confirming inhibited autophagy flux. The combined use of TRAIL with celastrol may serve as a safe and adequate therapeutic technique for the treatment of TRAIL-resistant lung cancer, suggesting that celastrol-mediated autophagy flux inhibition sensitized TRAIL-initiated apoptosis via regulation of ROS and ΔΨm.

Introduction

Advanced tumors are occasionally non-responsive to mono-therapies. By contrast, combination chemotherapy may stimulate the regression of specific types of tumors even at progressive stages, and this particularly has served an important role in cancer treatment for a number of years (1,2). Furthermore, it has been suggested that effective and favorable drug combinations should be explored to improve combination chemotherapeutics, which may markedly improve the current outcomes of cancer treatment.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a well-known transmembrane cytokine, and a member of the tumor necrosis factor family that mediates apoptosis in a variety of tumor cell types (3,4). TRAIL has been proposed as one of the most effective anti-cancer therapeutic agents, owing to its efficiency to selectively induce cell death in a variety of tumor cells whilst exhibiting minimal toxicity in the majority of normal cells (5,6). The binding of TRAIL to death receptors may also engage the extrinsic apoptotic pathway and trigger apoptotic signaling (7). TRAIL induces apoptosis by binding to death receptors and activating the Fas-associated death domain protein, ultimately leading to the activation of the effector caspase cascade, involving caspase-8, -9, -10, and -3 (8,9). A previous study has demonstrated that a number of cancer cells, together with lung A549 cells, acquire resistance to the apoptotic effects of TRAIL (10).

Celastrol is a triterpenoid, initially isolated from the Chinese herb Tripterygium wilfordii, or ‘Thunder God Vine’, that has been extensively applied in the treatment of autoimmune and neurodegenerative diseases (11-14). Previous studies have revealed that celastrol possesses multiple biological and pharmacological functions, in vitro and in vivo, including anti-microbial, anti-inflammatory and anti-cancer activities against prostate, breast and pancreatic cancer cells (15,16). It was also identified to have multiple mechanisms or signaling pathways that contribute to its anti-cancer activity, based on its efficiency to selectively initiate apoptosis in numerous tumor cell types (15,17).

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Autophagy is an intracellular catabolic mechanism, whose primary functions are to sustain cellular homeostasis by recycling long-lived unrequired proteins, remove exhausted, redundant and defective cellular elements, and promote cell survival during brief periods of nutrient-starvation and other stressors (18,19). During autophagy, double membrane vesicles develop to form autophagic vacuoles named...
autophagosomes. These fuse with lysosomes to create auto-lysosomes, wherein sequestered segments are degraded by lysosomal enzymes (20,21). The autophagosome formation is mediated by the ubiquitin-like protein ATG12-autophagy protein (Atg5)-Atg16 complex and microtubule-associated proteins 1A/1B light chain 3B (LC3)-II, an LC3-I-phospholipid conjugate widely applied as an autophagy marker (22,23). Sequestosome 1 (p62) is a ubiquitin-binding protein and a well-known autophagy marker. It organizes into autophagosomes by directly interacting with LC3, and is completely degraded by autophagy. Inhibition of autophagy results in low levels of p62 accumulation (19). It was previously revealed that the inhibition of autophagy flux sensitizes cancer cells to conventional radio and chemotherapy treatment (24-26), confirming that autophagy flux inhibition may be an appropriate and effective technique for cancer treatment. The antimalarial drug chloroquine (CQ), which functions as an autophagy inhibitor, prevents lysosome acidification and lysosomal fusion with autophagosomes, and also inhibits the degradation of metabolic stress, thereby inducing apoptosis (27-30).

The mitochondria serve a pivotal role in the intrinsic pathway of cell death by transmitting apoptotic signals to the cytosol, leading to the activation of the caspase cascade. Changes in mitochondria appear following the activation of cell death signals, including the formation of reactive oxygen species (ROS), loss of mitochondrial membrane potential ($\Delta \Psi_m$), opening of the permeability alteration pore, and release of apoptotic bodies into the cytosol (31-36). ROS-inducing agents have been applied to kill tumor cells in cancer therapy (37,38).

The present study aimed to explore the use of celastrol as a sensitizing agent to TRAIL-initiated apoptosis in A549 cells. It was revealed that a combined regimen of celastrol and TRAIL resulted in an improved outcome compared with treatment with either celastrol or TRAIL alone.

Materials and methods

Cell culture. Human lung A549, HCC-15 and Calu-3 cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). During experimentation, cells were switched to RPMI containing 1% FBS.

Reagents. Recombinant celastrol and chloroquine (20 $\mu$M) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). TRAIL (200 ng/ml) was purchased from Abfrontier; Young in Frontier, Co., Ltd. (Seoul, South Korea).

Cell viability assay. A549, HCC-15 and Calu-3 cells were plated in 12-well plates and pre-exposed to celastrol (1, 2 and 4 $\mu$M) for 12 h in 37˚C incubator, and were then additionally treated with TRAIL protein (200 ng/ml) for an additional 2 h. Cell morphology was assessed under a light microscope (Nikon Corporation, Tokyo, Japan, magnification, x100), and cell viability was evaluated by a crystal violet staining method. Cells were stained with a staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde) for 10 min at room temperature, washed four times with PBS, and were dried. Cells were then lysed with 1% SDS solution. The absorbance value was then measured at wavelength of 550 nm using a plate reader. Cell viability was expressed as relative dye intensity compared with that of the control.

Trypan blue exclusion assay. Cell viability was evaluated by trypan blue exclusion assay (Sigma-Aldrich; Merck KGaA), using a hemocytometer. Following each treatment, the cells were counted and re-suspended in PBS. Trypan blue dye solution (0.4%) was added to the cell suspension for 5 min at room temperature. Unstained cells were viable and stained cells were dead. The total cell number and trypan blue-positive cells were counted using a light microscope in a blinded manner. The percentage of surviving cells was calculated using the formula: Number of stained cells/number of total cells x100. Each treatment was performed in triplicate.

ROS determination. A549 cells (1.0x10⁴ cells/well) in 12-well plates, were pre-incubated with N-acetyl cysteine (NAC) for 1 h and then incubated with TRAIL alone or combined with CQ (10 mM). The formation of ROS was ascertained through application of the cell permeable fluorescent marker dihydroethidium (DHE). Briefly, cells were treated with 5 $\mu$M DHE for 30 min at 37˚C in the dark. The fluorescence was then measured using a fluorescence plate reader at excitation and emission wavelengths of 518 and 605 nm, respectively.

$\Delta \Psi_m$ analysis. The changes in $\Delta \Psi_m$ were assessed using a cationic fluorescent marker. A549 cells were maintained on cover slips in a 24-well plate, incubated with 10 ml JC-1 at 37˚C for 30 min and then washed with PBS. The cells were then mounted with DakoCytomation fluorescent mounting medium (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and visualized under a fluorescence microscope (magnification, x400).

Immunofluorescent staining. A549 cells (5.0x10⁴ cells/well) were cultured on poly-L-lysine coated coverslips. Following differentiation and specific treatment, the cells were fixed with 4% paraformaldehyde at room temperature 15 min and permeabilized with 0.1% Triton X-100. The cells were then incubated for 60 min at room temperature with blocking solution (5% FBS in Tris-buffered saline) followed by overnight incubation at 4˚C with anti-p62 (1:250; cat. no. PA5-20839; Invitrogen; Thermo Fisher Scientific, Inc.) and cleaved caspase-3 (1:250; cat. no. 9665; Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies. Subsequent to washing with PBS, the cells were incubated with secondary antibody (Alexa Fluor® 488-conjugated donkey polyclonal anti-rabbit; 1:500; cat. no. A-21206; Thermo Fisher Scientific, Inc.; and Texas Red-X-conjugated goat polyclonal anti-mouse; 1:500; cat. no. T-6390; Thermo Fisher Scientific, Inc.) for 2 h in the dark. Finally, immunostaining was visualized under a fluorescence microscope (magnification, x400).

Western blot analysis. Western blot analysis was performed as described previously (39). Briefly, immunoprecipitation assay buffer (Qiagen, Inc, Valencia, CA, USA) was used...
to extract total protein in A549 cells. The supernatant was collected by centrifugation (13,282 x g; 4°C; 10 min). The protein concentration was determined using the Pierce Bicinchoninic Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (30 µg) were separated on 10% SDS-PAGE gels and blotted onto polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat dried milk at 25°C for 1 h, followed by incubation with primary antibodies overnight at 4°C. Antibodies against β-actin were purchased from Sigma-Aldrich; Merck KGaA, and those against cleaved caspase-8 were obtained from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA). Cleaved caspase-3 and LC3 antibodies were purchased from Cell Signaling Technology, Inc., and p62 antibodies were purchased from EMD Millipore (Billerica, MA, USA). Membranes were incubated with horseradish peroxidase conjugated secondary antibody (cat. no. 4410; 1:2,000; Cell Signaling Technology, Inc.) at 25°C for 1 h. The immune-reactive protein bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK). Densitometry of the signal bands was conducted using the Bio-1D densitometer (Vilber Lourmat, Eberhardzell, Germany).

Figure 1. Cela sensitizes TRAIL-initiated apoptosis in A549 cells. A549 cells were treated with different dose of cela (1, 2 and 4 µM) for 12 h and then exposed to TRAIL (200 ng/ml) for 2 h. (A) The morphology of the cells was evaluated by interference light microscopy (magnification, x100). (B) Cellular viability was assessed with crystal violet staining. (C) Quantification data for the crystal violet staining cell viability. (D) Cellular viability was evaluated with trypan blue dye exclusion assays. *P<0.05 and **P<0.01 vs. TRAIL untreated group and only TRAIL treatment group. Cela, celastrol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
Statistical analysis. Statistical analysis was performed using GraphPad Prim (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were performed in triplicate, and the data are expressed as the mean ± standard error. Significant differences between control and treated samples were analyzed using one-way analysis of variance followed by Duncan's post hoc test.
Results

Celastrol sensitizes TRAIL-initiated apoptosis in A549 cells. Alterations in cell morphologies were examined under a light microscope. Treatment with either celastrol or TRAIL alone did not or only marginally induced cell death (Fig. 1), and no morphological changes were observed. However, a combined regimen of TRAIL and different concentrations of celastrol markedly increased the number of apoptotic cell deaths compared with celastrol or TRAIL alone (Fig. 1A-D). These data indicate that celastrol promoted TRAIL-initiated apoptosis in A549 cells.

Autophagy flux is inhibited by celastrol in A549 cells. LC3-II and p62 expression increased following celastrol treatment (Fig. 2A-C). Immunofluorescence staining also demonstrated that varying doses of celastrol increased p62 protein levels (Fig. 2D). The combined regimen of TRAIL and celastrol increased active (Ac)-caspase 3 and Ac-caspase 8 levels (Fig. 2E-G). Immunofluorescent staining results also suggested that Ac-caspase 3 levels of combined celastrol and TRAIL treatment were increased than only TRAIL treatment (Fig. 2H), indicating that autophagy flux was inhibited by celastrol.

Celastrol increases TRAIL-mediated cell death by attenuating autophagy flux. The cell morphology results indicated that increased apoptosis was achieved by co-treatment of TRAIL and celastrol or CQ (Fig. 3A). The combined regimen of CQ and TRAIL markedly increased cell death and attenuated cell viability (Fig. 3B and C). These results suggested that celastrol sensitized TRAIL-initiated cell death by attenuating autophagy flux.

Celastrol-mediated autophagy flux inhibition promotes TRAIL-initiated apoptosis. LC3-II and p62 expression were markedly increased in A549 cells treated with celastrol or CQ alone, confirming that celastrol inhibits autophagy flux (Fig. 4A-C). Immunofluorescence staining results also demonstrated that p62 protein levels were increased (Fig. 4D). The combined regimen of TRAIL and CQ increased Ac-caspase 3 and Ac-caspase 8 protein levels (Fig. 4E-G). The immunofluorescent staining results also demonstrated that Ac-caspase 3 expression was increased (Fig. 4H). These results also indicated that the promotion of the TRAIL-initiated apoptotic mechanism by celastrol was due to the attenuation of autophagy flux.

Attenuation of autophagy stimulates ROS generation and changes in ΔΨm. As demonstrated in Fig. 5A, ROS levels were increased in cells treated with celastrol combined with TRAIL or CQ. These increases were effectively attenuated when the cells were pre-incubated with N-acetyl cysteine (NAC) for 1 h. Green fluorescence observed in the cells treated with celastrol

Figure 3. Cela improves TRAIL-induced cell death by attenuating autophagy flux. A549 cells were pretreated with CQ prior to exposure to cela (4 µM) for 12 h and then exposed to TRAIL for an additional 2 h. (A) The morphology of the cells was evaluated by interference light microscopy (magnification, x100). (B) Cellular viability was assessed with crystal violet staining. (C) Bar diagram demonstrating the average density of crystal violet staining. *P<0.05 and **P<0.01 vs. TRAIL untreated group and only TRAIL treatment group. Cela, celastrol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CQ, chloroquine.
combined with TRAIL or CQ suggested decreased ΔΨm values compared with that from the TRAIL alone treatment, but treatment with NAC restored these ΔΨm values (Fig. 5B). These data suggest that celastrol-mediated autophagy flux attenuation increased TRAIL-initiated apoptosis via increases in ROS generation and decreasing ΔΨm.

Figure 4. Cela induces a promotion of the TRAIL-initiated apoptotic mechanism by attenuating autophagy flux. A549 cells were pretreated with CQ prior to exposure to cela (4 µM) for 12 h and then exposure to TRAIL for an additional 1 h. (A) LC3-II and p62 protein levels were assessed by western blot analysis. Bar charts indicate the (B) LC3-II/LC3-I and (C) p62/β-actin ratios. (D) Cells were immunostained with p62 (red) and evaluated using fluorescence microscopy. Scale bar=50 µm. (E) Ac-cas3 and Ac-cas8 protein levels were assessed by western blot analysis. Bar charts indicate the (F) Ac-cas8/β-actin and (G) Ac-cas3/β-actin ratios. (H) Cells were immunostained with Ac-cas3 (green) antibody and evaluated using fluorescence microscopy. Scale bar=50 µm. β-actin was used as a control. *P<0.01 vs. TRAIL untreated group and only TRAIL treatment group. Cela, celastrol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CQ, chloroquine; LC3, microtubule-associated proteins 1A/1B light chain 3B; p62, Sequestosome 1; Ac-cas, active caspase.
Figure 5. Attenuation of autophagy stimulates ROS generation and changes of mitochondrial membrane potential. A549 cells were pretreated with NAC (10 mM) and CQ prior to exposure to cela (4 µM) for 12 h and then treatment with TRAIL for an additional 2 h. (A) The ROS level was assessed using a spectrophotometer. (B) JC-1 aggregate forms (red) and mono forms (green) were assessed by confocal microscopy (magnification, x400). *P<0.05 and **P<0.01 vs. TRAIL untreated group and only TRAIL treatment group. Cela, celastrol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CQ, chloroquine; NAC, N-acetyl cysteine; ROS, reactive oxygen species.

Figure 6. Cela sensitizes TRAIL-initiated apoptosis in different lung cell lines. (A) Calu-3 morphology was evaluated by interference light microscopy (magnification, x100). (B) Calu-3 viability as assessed with crystal violet staining and (C) percentage of crystal violet positive cells. (D) HCC-15 morphology was evaluated by interference light microscopy (magnification, x100). (E) HCC-15 viability as assessed with crystal violet staining and (F) percentage of crystal violet positive cells. *P<0.05 and **P<0.01 vs. TRAIL untreated group and only TRAIL treatment group. Cela, celastrol; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
Celastrol sensitizes TRAIL-initiated apoptosis in different lung cells. Celastrol or TRAIL treatment alone did not, or only marginally affected, the cell viability of Calu-3 and HCC-15 cells (Fig. 6). Nevertheless, the combined TRAIL and celastrol regimen significantly decreased the cell viability of Calu-3 and HCC-15 cells (Fig. 6A-F). These results suggested that celastrol significantly increased TRAIL-initiated apoptosis in Calu-3 and HCC-15 cells.

Discussion

The aim of the present study was to determine the function of celastrol with or without TRAIL, in lung cancer cells. The results revealed that celastrol-mediated autophagy flux inhibition sensitized TRAIL-mediated apoptosis via regulation of ROS and ΔΨm.

TRAIL is considered one of the most promising anti-cancer agents identified in the previous 25 years, owing to the specificity of its action, initiating apoptosis in specific cell types (5,40-44) and prompting the destruction of cancer cells without causing toxicity to normal cells. Previous studies have confirmed that the repeated administration of TRAIL proteins adequately attenuated tumor growth without causing serious side effects (6,45). In this manner, considerable attention has been paid to TRAIL as a anti-cancer therapy for human cancer. The antitumor activities of celastrol are not only limited to in vitro systems, as this triterpenoid was also identified to inhibit the uncontrolled growth and metastasis of melanoma in xenograft mouse models (46). Previous studies have demonstrated that multiple mechanisms or signaling pathways lead to celastrol-induced cancer cell death (15,17,47). Autophagy flux is a complete system of autophagy, starting with the amalgamation of the autophagosome with the lysosome, resulting in the degradation and recycling of their cargo (18,48). ROS serves an important role in response to cancer therapeutics, considering that ROS formation is associated with cancer initiation and progression.

A previous study have attempted to examine TRAIL sensitizers that are capable of overcoming TRAIL resistance in cancer cells (49). The present study revealed that a combined regimen of TRAIL and different doses of celastrol significantly increased the level of apoptotic cell death compared with treatment with celastrol or TRAIL alone. A previous study has suggested that celastrol treatment attenuated cell proliferation and the induction of apoptosis and autophagy in gastric cancer cells (50). However, the results from the present study indicated that LC3-II and p62 expression increased following celastrol treatment, where co-treatment of celastrol and TRAIL increased Ac-cas3 and Ac-cas8 levels. It has been demonstrated previously that treatment of celastrol in MCF-7 cells induces apoptosis through mitochondrial caspase-dependent and independent signaling pathways (51). Furthermore, Kannaiyan et al (52) identified that celastrol treatment inhibits proliferation and stimulates apoptosis in RPMI-8226 cells, alongside the activation of c-Jun N-terminal kinase and attenuation of the phosphoinositide 3-kinase/protein kinase B signaling pathways. The present study also demonstrated that the combined regimen of TRAIL and celastrol or chloroquine significantly attenuated cell viability and increased cell death. Previous studies have suggested that the inhibition of autophagy stimulates dysfunctional mitochondria and ROS generation in prostate cancer cells (53,54). The data from the present study suggest that celastrol-mediated autophagy flux inhibition increased TRAIL-induced apoptosis via ROS generation and decreases in ΔΨm. In conclusion, the present study demonstrated that celastrol improved TRAIL-initiated apoptosis via inhibiting autophagy flux. In addition, the use of TRAIL in combination with celastrol may provide an adequate therapeutic strategy for safe treatment of TRAIL-resistant lung cancer, suggesting that celastrol-mediated autophagy flux inhibition sensitized TRAIL-initiated apoptosis via the regulation of ROS and the ΔΨm.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the National Research Foundation of Korea, funded by the Korean Government (Grant no. 2016R1A2B2009293).

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

UMN and SYP designed the study. UMN and HHY performed the experiments. UMN and SYP analyzed the data and wrote the manuscript. UMN, HHY and SYP revised and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent of publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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