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Original Articles

Cytoprotective role of autophagy against BH3 mimetic gossypol in ATG5 knockout cells generated by CRISPR-Cas9 endonuclease

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ABSTRACT

Previously, we demonstrated the association between autophagy and gossypol-induced growth inhibition of mutant BRAF melanoma cells. Here, we investigate the role of autophagy in ATG5 knockout cell lines generated by the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas-mediated genome editing. The MTT assay revealed that the inhibitory effect of gossypol was weaker on ATG5 knockout cells than that on the wild type (WT) cells. The conversion of non-autophagic LC3-I to autophagic LC3-II and RT-PCR confirmed the functional gene knockout. However, Cyto-ID autophagy assay revealed that gossypol induced ATG5- and LC3-independent autophagy in ATG5 knockout cells. Moreover, gossypol acts as an autophagy inducer in ATG5 knockout cells while blocking the later stages of the autophagy process in WT cells, which was determined by measuring autophagic flux after co-treatment of gossypol with chloroquine (late-stage autophagy inhibitor). On the other hand, inhibition of autophagy with 3-MA or Beclin-1 siRNA caused a partial increase in the sensitivity to gossypol in ATG5 knockout cells, but not in the WT cells. Together, our findings suggest that the resistance to gossypol in ATG5 knockout cells is associated with increased cytoprotective autophagy, independent of ATG5.

Introduction

BRAF somatic missense mutations have been identified in almost 50% of all malignant melanoma cases [1]. Frequent BRAF mutations led to the development of mutated BRAF inhibitors for the treatment of melanomas [2,3]. However, the emergence of acquired resistance to BRAF inhibitors limited their effectiveness in the treatment of melanoma [4]. Our findings showed that gossypol retained its efficacy even after long-term treatment. Recently, we demonstrated that gossypol suppresses BRAF mutant A375P melanoma growth through a mechanism independent of MEK-ERK inhibition [5].

Gossypol is a BH3-mimetic small-molecule inhibitor of Bcl-2 with potent anticancer activity in several types of malignancies [6,7]. We found that Ras-NIH 3T3/Mdr cells, which exhibit a strong cross-resistance to many chemotherapeutic agents, exhibited high sensitivity to gossypol [8]. Lian et al. [9] showed that gossypol induces autophagy by blocking Bcl-2–Beclin 1 interaction in the endoplasmic reticulum (ER).

The autophagic process is a type of cellular catabolic degradation mechanism responsible for the removal of long-lived proteins and damaged organelles by the lysosome [10]. However, there is an ongoing debate whether autophagy induction is cytoprotective or cytotoxic in cancer [11]. Autophagic cell death is responsible for the death of some cancer cells with increased autophagic flux [12]. Our previous results also showed high levels of gossypol-induced autophagy in the gossypol-sensitive cells [5,8,13]. In particular, we found that a functional $p21^{Cip1}$ is a prerequisite for gossypol-induced autophagic cell death in melanoma cells [5]. Conversely, it has been reported that gossypol induces both Beclin 1-dependent and Beclin 1-independent cytoprotective autophagy in cancer cells [14].

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated (Cas) system was first discovered in bacteria, which degrades foreign nucleic acid via RNA-programmable host defense mechanism [15–18]. The CRISPR-associated protein Cas9 is an RNA-guided DNA endonuclease that uses RNA–DNA complementarity to identify target sites for sequence-specific double-stranded DNA (dsDNA) cleavage. Recently, the CRISPR/Cas9 system has been suggested as an efficient way to selectively inactivate genes.

To further investigate the role of autophagy in gossypol-induced growth inhibition of A375P melanoma cells, we established ATG5 knockout cell line with CRISPR/Cas9 system. Autophagy protein 5 (ATG5) is an E3 ubiquitin ligase necessary for autophagy because of its role in autophagosome elongation [19–21]. The ATG5 complex is necessary for LC3-1 and phosphatidylethanolamine conjugation to form LC3-II. We found that gossypol inhibited wild type (WT) cell proliferation more potently than that of ATG5 knockout cells. Furthermore, ATG5 gene knockout in A375P cells by CRISPR/Cas9

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system still exhibited autophagy induction after treatment with gossypol, indicating that gossypol was capable of mediating ATG5-independent autophagy.

Materials and methods

Antibodies and reagents

Rabbit polyclonal anti-LC3 was obtained from Sigma (St. Louis, MO, USA). For the apoptosis assay, the FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). For the flow cytometric autophagy assay, Cyto-ID Green dye was purchased from ENZO Life Sciences, Inc. (Farmingdale, NY, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Life Technologies (Carlsbad, CA, USA). The reagents for SDS–polyacrylamide gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). Gossypol and 3-methyladenine (3-MA) were obtained from Sigma (St. Louis, MO, USA).

Cell lines and cell culture

The A375P melanoma cell line was obtained from YOUAI Co., Ltd. (Suwon-Si, Gyeonggi-Do, Korea). The A375P cells were maintained at 37 °C in DMEM supplemented with 10% FCS, penicillin-streptomycin, and glutamine. For experimental purposes, cells were cultured in 60-mm tissue culture dishes until they reached ~80% confluence. Gossypol was dissolved in dimethyl sulfoxide (DMSO) and freshly diluted for each experiment. The DMSO concentration was less than 0.1% in all experiments.

CRISPR-Cas9 plasmid

The optimized sgRNA construct, targeting ATG5, and the Cas9 expression construct, pRGEN-Cas9-CMV, were obtained from ToolGen (Seoul, Korea). The ATG5 exon located at the second coding exon was selected for guide RNA design (Fig. 1A). The sgRNA was located behind an U6 promoter and contained the following target sequence 5′-GTGCTTCGAGATGTGTGGTTTGG-3′ or 5′-AAGATGTGCTTCGAGATGTGTGG-3′. For the establishment of ATG5 knockout cell lines, pHRS_HumanATG5_CMV containing hygromycin resistance gene (ToolGen) was used. This plasmid expresses a hygromycin-resistance protein when the target sequences are cleaved by nuclease.

DNA mismatch-specific (T7E1) endonuclease assay

Cells in 24-well plates were cultured to 50–60% confluence. The cells were co-transfected with 0.4 μg of ATG5 single-guide (sg) RNA plasmid and 0.4 μg of Cas9 expression plasmid using Lipofectamine 2000. The cells were then harvested and genomic DNA was extracted using QuickExtract DNA extraction solution (Epicentre, Madison, WI, USA), 24 h post transfection. A region of exon 2 of the *ATG5* gene was amplified with genomic DNA-specific primers (forward primer, 5′-GGCTTGAAAGACTGATGCAGA-3′; reverse primer, 5′-GTCAAGAAGGCACCATAGCTG-3′). Homoduplex PCR products were denatured and rehybridized using stepdown annealing conditions to generate homo- and heteroduplexes. The mixture of duplexes was treated with T7E1 nuclease for 20 min at 37 °C. The products were electrophoresed on a 2% agarose gel.

Establishment of Atg5 knockout cell line with the CRISPR/Cas9-mediated genome editing

A375P cells were cultured in six-well dishes to 70–80% confluence. Cells were cotransfected with 1 μg of ATG5 sgRNA plasmid, 1 μg of pRGEN-Cas9-CMV, and 1 μg of pHRS_HumanATG5_CMV using Lipofectamine 2000 (Life Technologies). After transfection, these cells were treated with 150 μg/mL of hygromycin for 2 days. Surviving cells were reseeded at 0.4 cell/well of a 96-well plate for isolation of single cell clones. ATG5 knockout in the expanded colonies was confirmed by RT-PCR using the following primer sets: 5′-GCTTCGAGATGTGTGGTTTG-3′ and 5′-CAGTGGTGTGCCTTCATATT-3′. The human β-actin primers were used as control primers for amplification. The specificity of the PCR products was confirmed by 2% agarose gel electrophoresis.

Beclin-1 siRNA transfection

For Beclin-1 knockdown, a pool of three target-specific Beclin-1 siRNAs was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The targeted sequences were as follows: CAGCUCAACGUCACUGAAAtt, GAGAUCUUAGAGCAAAUGAtt, and GGAUGACAGUGAACAGUUAtt. A non-targeting siRNA (Santa Cruz Biotechnology, CA, USA) was used as a control. Where indicated, the cells were transiently transfected with Beclin-1 siRNA or a non-targeting control siRNA for 24 h using Lipofectamine RNAiMAX in Opti-minimal essential medium I (Life Technologies).

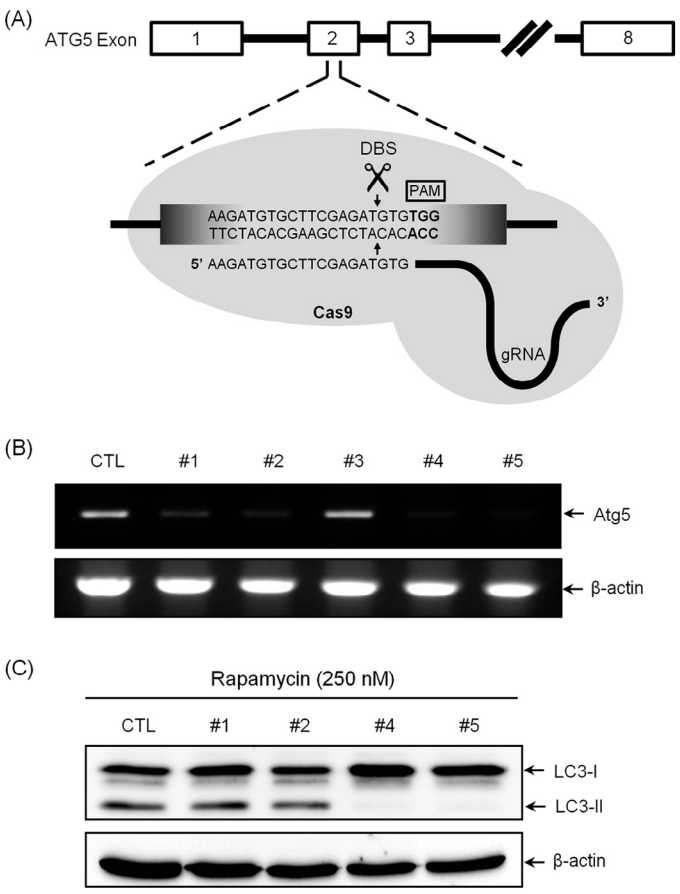

Fig. 1. Generation and validation of ATG5 knockout (KO) cell lines. (A) ATG5 single-guide (sg) RNA and target site. The sgRNA target site is located at exon 2. The 20-nt guide sequence comprising the 5′-end of the chimeric sgRNA is shown. The Cas9 enzyme introduces a double stranded break (DSB; scissors and arrow) near the 5′ side of PAM (NGG; bold), triggering imperfect nonhomologous end joining. (B) RT-PCR was performed to confirm the knockout of *ATG5* gene. The RNA extracted from each clone was examined for the presence of ATG5 RNA transcripts by RT-PCR. ATG5 cDNA was subjected to PCR analysis with specific primers to determine the expression of ATG5 and the human β-actin gene. The PCR-amplified products were subjected to electrophoresis with 2% agarose gel. (C) To confirm the lack of autophagy in the ATG5-knockout cells treated with an autophagy inducer rapamycin (250 nM), the change in the electrophoretic mobility of LC3 from a non-autophagic (LC3-I) form to an autophagic membrane-recruited (LC3-II) form was determined by immunoblotting. β-Actin expression was assessed as protein loading control. The presented results are representative of at least three independent experiments.

Cell growth assay

The cells were plated in quadruplicates in 96-well microliter plates (Costar, Cambridge, MA, USA) at a density of 5×10^3 cells/well and then treated with gossypol at 37 °C in a humidified 5% CO_2/95% air incubator. On day 3, the cells were incubated with MTT at 37 °C for 3 h. The absorbance of the samples against a background control (medium alone), which was used as a blank, was measured at 450 nm using a microliter plate (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

Autophagy monitoring assay by LC3 conversion

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping the cells into RIPA lysis buffer. The lysate protein concentrations were determined with a BCA protein assay reagent kit. For immunoblotting, the whole cell lysates were denatured in Laemmli sample buffer and resolved by SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with a polyclonal anti-LC3 antibody. Detection was accomplished using the ECL-Plus chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized using KODAK Image Station 4000R (Carestream Health, Inc., Rochester, NY, USA).