Autophagy inhibition augments resveratrol-induced apoptosis in Ishikawa endometrial cancer cells

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Abstract. Resveratrol (RSV), a polyphenolic compound derived from red wine, inhibits the proliferation of various types of cancer. RSV induces apoptosis in cancer cells, while enhancing autophagy. Autophagy promotes cancer cell growth by driving cellular metabolism, which may counteract the effect of RSV. The present study aimed to elucidate the correlation between RSV and autophagy and to examine whether autophagy inhibition may enhance the antitumor effect of RSV in endometrial cancer cells. Cell proliferation, cell cycle progression and apoptosis were examined, following RSV exposure, by performing MTT assays, flow cytometry and annexin V staining, respectively, in an Ishikawa endometrial cancer cell line. Autophagy was evaluated by measuring the expression levels of light chain 3, II (LC3-II; an autophagy marker) by western blotting and immunofluorescence. Chloroquine (CQ) and small interfering RNAs targeting autophagy related (ATG) gene 5 (*ATG5)* or 7 (*ATG7)* were used to inhibit autophagy, and the effects in combination with RSV were assessed using MTT assays. RSV treatment suppressed cell proliferation in a dose-dependent manner in Ishikawa cells. In addition, RSV exposure increased the abundance of the sub-G1 population and induced apoptosis. LC3-II accumulation was observed following RSV treatment, indicating that RSV induced autophagy. Combination treatment with CQ and RSV more robustly suppressed growth inhibition and apoptosis, compared with RSV treatment alone. Knocking down *ATG5* or *ATG7* expression significantly augmented RSV-induced apoptosis. The results of the present study indicated that RSV-induced autophagy may counteract the antitumor effect of RSV in Ishikawa cells. Combination treatment with RSV and an autophagy inhibitor, such as CQ, may be an attractive therapeutic option for treating certain endometrial cancer cells.

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Abbreviations: AMPK, AMP-activated protein kinase; ATG, autophagy-related gene; CQ, chloroquine; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; IC_{50}, half-maximal (50%) inhibitory concentration; LC3, light chain 3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RSV, resveratrol; siRNA, small interfering RNA

Key words: endometrial cancer, resveratrol, autophagy, apoptosis, ATG5, ATG7, chloroquine

Introduction

Endometrial cancer is the most common gynecologic malignancy, and its incidence is increasing worldwide (1). A strong association exists between endometrial cancer and metabolism. Individuals with diabetes mellitus or obesity have 1.8 or 1.5-fold higher relative risks for developing endometrial cancer, respectively (2,3). In addition, metabolic modifiers, including metformin (an oral antidiabetic drug for type-II diabetes mellitus), have been reported to induce antitumor effects in endometrial cancer (4,5).

Resveratrol (RSV) is a natural polyphenol found in a variety of plant-based foods and beverages, such as red wine (6). RSV is able to regulate various physiological functions, such as blocking inflammation and protecting against cardiovascular dysfunctions and obesity (6-8). These activities suggest that RSV may serve as a promising metabolic modifier in endometrial cancer. Indeed, an antitumor role of RSV has been reported in endocrine-associated cancers, including endometrial cancer (9-11). However, the mechanism underlying its antiproliferative effect is debated. The effects of RSV have been suggested to be dependent on estrogen, epidermal growth factor downregulation, protein kinase B (AKT) inactivation, and adenosine monophosphate-activated protein kinase (AMPK) activation (11-14). Loss of AMPK activity can promote oncogenesis (15). Metformin is known to activate AMPK through liver kinase B1 (LKB1) phosphorylation, and this activation is suggested to be involved in its antitumor effect (16). RSV was previously revealed to activate sirtuin 1 (SIRT1) (17). SIRT1 is able to deacetylate certain proteins that regulate longevity and

cellular stress, such as tumor protein p53 (TP53) (18,19). Thus, various factors are associated with the antitumor effects of RSV. In addition, cytostatic and cytotoxic effects have been observed following RSV treatment in cancer cells (20).

By contrast, RSV may also induce oncogenesis. Notably, RSV is associated with autophagy induction (21-24) and activation of the Raf/MEK/ERK signal transduction cascade (25). Autophagy, which literally means 'self-eating' is a major degradation system that promotes the lysosomal digestion of organelles and cytoplasmic components (26). Autophagic activity is commonly assessed through measuring the expression levels of microtubule-associated protein 1 light chain 3 (LC3). LC3-II is a standard marker of autophagic flux and localizes to autophagosomes. Autophagy-related (ATG) genes 5 (*ATG5)* and 7 (*ATG7)* directly regulate autophagic processes (26). Autophagy has been suggested to promote cancer progression through driving cell metabolism (27). Activation of AMPK and/or extracellular signal-regulated kinase (ERK) signaling was demonstrated to induce autophagy in human cancers (28,29), which may induce the antitumor effect of RSV on cancer cells.

Chloroquine (CQ) is an autophagy inhibitor with an antimalarial effect (30). In addition, CQ and its derivative, hydroxychloroquine, have been used to treat connective tissue diseases, including rheumatoid arthritis, systemic lupus erythematosus and Sjögren's syndrome (31-33). CQ exhibits antitumor effects *in vitro* and *in vivo* by inhibiting autophagy, and various clinical trials have been conducted using CQ in certain types of cancer (34,35). We recently reported that autophagy inhibition by CQ suppressed endometrial cancer cell proliferation, and improved cisplatin sensitivity (36). Therefore, autophagy inhibition may potentiate the antitumorigenic effects of RSV in endometrial cancer cells.

The purpose of the present study was to investigate the effects of RSV on endometrial cancer cell proliferation and autophagy. In addition, the study also addressed whether autophagy inhibition enhances the effect of RSV, which would suggest a potential new treatment strategy for endometrial cancer.

Materials and methods

Chemicals and antibodies. RSV and CQ were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibodies against LC3 (#M152-3) and β-actin (#M177-3) were obtained from MBL International Corporation (Woburn, MA, USA) and Sigma-Aldrich, respectively. Rabbit monoclonal antibodies against SIRT1 (#ab32441) were purchased from Abcam (Cambridge, UK). Antibodies against phospho-AMPKα (p-AMPKα) at Thr172 (#2535), phospho-AKT at Ser473 (#9271P), phospho-Erk1/2 (p44/42 MAPK; #9101), phospho S6 ribosomal protein at Ser240/244 (#2215), LC3β (#2775), and cleaved poly (ADP-ribose) polymerase (PARP) (#9544) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). An Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (Ig)G secondary antibody (#A-11001) was obtained from Invitrogen, Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The Ishikawa endometrial cancer cell line was provided by Dr Masato Nishida (National Hospital Organization Kasumigaura Medical Center, Tsuchiara, Japan). Ishikawa cells were grown at 37˚C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both obtained from Thermo Fisher Scientific, Inc.) in a humidified 5% CO_2 incubator.

MTT assays. Ishikawa cells (3,000 cells/well) were seeded 24 h prior to RSV treatment. Subsequently, the cells were grown for 72 h in DMEM, which contained increasing doses of RSV (0.1-200 μM). At the endpoint, 10 μl of the Cell Counting kit-8 reagent containing the tetrazolium salt WST-8 was added to the wells, according to the protocol of the manufacturer (Dojindo, Molecular Technologies, Inc., Kumamoto, Japan), and absorbance (450 nm) was measured in a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Proliferation was normalized to absorbance measurements observed in control cells treated with dimethyl sulfoxide alone.

Cell cycle analysis. Ishikawa cells ($5x10^5$ cells/60-mm dish) were grown in the presence of RSV (25 μM) for 72 h. Cell cycle analysis was performed as previously described (36) in three independent experiments.

Apoptosis measurements by double staining with annexin V and propidium iodide (PI). Ishikawa cells were plated in 60-mm dishes for 24 h prior to 24 h incubations at 37˚C with the indicated drugs and/or small interfering RNAs (siRNAs), at the indicated doses. As described previously (36), the cells were trypsinized, washed two times with phosphate-buffered saline (PBS), and stained with PI and fluorescein isothiocyanate (FITC)-conjugated annexin V, using the FITC Annexin-V Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA), as directed by the manufacturer. Apoptotic cells were measured as double-positive cells in three independent experiments using a BD FACSCalibur flow cytometer, and expressed on a percentage basis.

Western blot analysis. Soluble proteins from Ishikawa cell lysates were extracted as described previously (36), followed by western blot analysis with the aforementioned primary antibodies (1:1,000) at 4˚C overnight. Bands were detected using the BioRad Blotting system (BioRad Laboratories, Inc., Hercules, CA, USA) with the ECL Select Detection Reagent (GE Healthcare, Little Chalfont, UK).

Immunofluorescence. Ishikawa cells were cultured in DMEM in 6-well plates, on glass coverslips coated with PBS containing 0.1% gelatin. After 24-h incubation at 37˚C, the medium was replaced with DMEM alone (control cells) or DMEM supplemented with 25 μM RSV. The cells were then incubated for an additional 48-h. Subsequently, the cells were washed in PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 prior to blocking in 6% bovine serum albumin (Thermo Fisher Scientific, Inc.). The cells were then incubated overnight at 4˚C with a primary anti-LC3 antibody (diluted 1:200). On the following day, the cells were incubated for 1 h at room temperature with a secondary Alexa Fluor 488-conjugated goat, anti-mouse IgG antibody (1:200). Nuclei were counterstained with Hoechst 33342 dye at a 1:1,000 dilution. The slides were analyzed by confocal fluorescence microscopy (BX50; Olympus Corporation, Tokyo, Japan).

Gene silencing. Ishikawa cells were grown in culture for 24 h prior to gene-silencing experiments conducted with Stealth RNAi siRNAs against *ATG5* or *ATG7* (Invitrogen; Thermo Fisher Scientific, Inc.), using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). A negative control siRNA was used as a control (Invitrogen; Thermo Fisher Scientific, Inc.). siRNA transfections were performed as described previously (36).

Statistical analysis. The data were presented as the mean ± standard error from at least three independent determinations. The significance of differences between ≥3 samples were analyzed by one-way analysis of variance and *post-hoc* testing, whereas the significance between two samples were analyzed by a Mann-Whitney U test, using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

RSV suppresses the proliferation of Ishikawa cells by apoptosis induction. MTT assays were performed in Ishikawa endometrial cancer cells to assess the antitumor activity of RSV. RSV inhibited the proliferation of Ishikawa cells in a dose-dependent manner (Fig. 1A). The half-maximal (50%) inhibitory concentration IC_{50} value was 20 μM. Cell cycle analysis was also performed to elucidate whether growth inhibition by RSV was attributable to cell cycle arrest or cell death. Cell cycle analysis demonstrated that RSV caused a significant increase in the abundance of the sub-G1 population of Ishikawa cells (Fig. 1B). In addition, annexin V-PI double staining showed a significant accumulation of double-positive cells following RSV treatment in Ishikawa cells (Fig. 1C), indicating that RSV induced apoptosis in Ishikawa cells. These results suggested that RSV inhibits the growth of Ishikawa cells, mainly via its cytotoxic effect.

RSV induces autophagy in Ishikawa cells. To elucidate which proteins are associated with growth inhibition by RSV, immunoblotting was performed against cell growth-associated proteins expressed in Ishikawa cells. RSV markedly increased the expression of p-AMPKα and p-ERK (Fig. 2A). However, RSV did not increase SIRT1 expression, or decrease the expression of p-AKT (Fig. 2A). RSV induced LC3-II expression, and LC3-immunofluorescence experiments revealed autophagosome accumulation in the cytosol of Ishikawa cells following 20 μM RSV treatment (Fig. 2A and B). These data strongly suggest that RSV activates AMPK and ERK signaling in Ishikawa cells, with an induction of autophagy.

Pharmacologic autophagy inhibition by CQ augments RSV-inducible apoptosis in Ishikawa cells. Next, we addressed whether RSV-mediated autophagy affects the RSV antitumor effect in Ishikawa cells, by adding CQ in combination with RSV. Cell viability was significantly suppressed by combination treatment (25 μM RSV and 5 μM CQ), compared with RSV treatment alone at 25 μM (Fig. 3A). Combination treatment induced significant cleaved PARP accumulation, compared with RSV treatment alone, as determined by western blot analysis (Fig. 3B). In addition, combination treatment showed a trend towards an increased population of double-positive (apoptotic) cells in the annexin V-PI double staining assays (Fig. 3C). These data indicated that combination treatment with RSV and CQ may induce greater cytotoxicity in Ishikawa cells, as compared with RSV treatment alone.

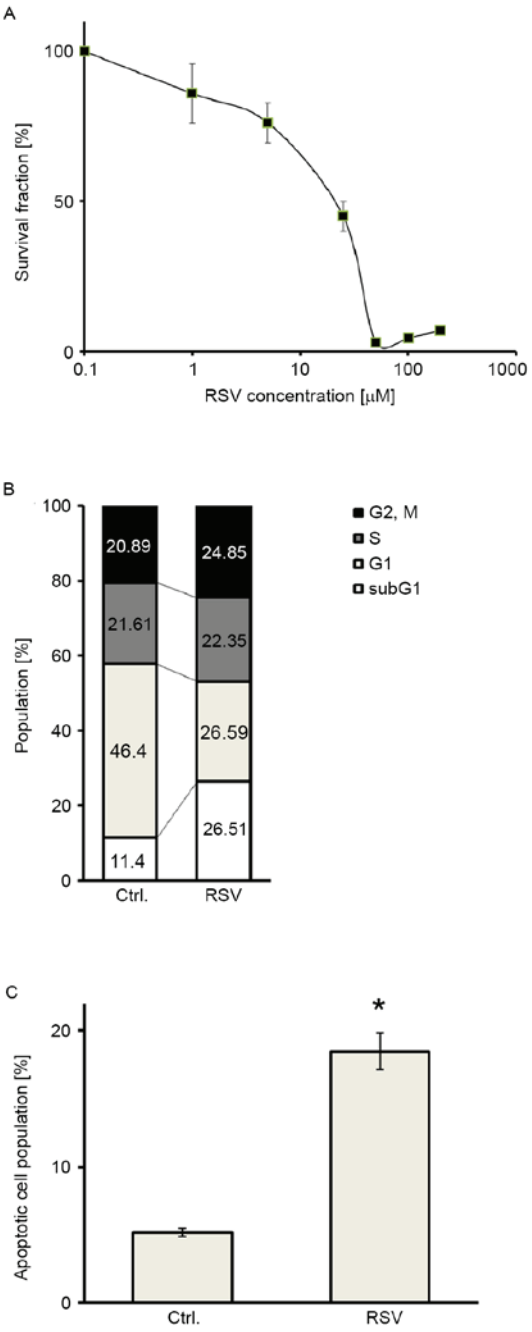

Figure 1. RSV suppresses the proliferation of Ishikawa cells via apoptosis induction. (A) MTT assays for RSV treatment (0.1-200 μM) in Ishikawa cells. The data are presented as the mean ± SE of quadruplicate samples. (B) Cell cycle analysis in Ishikawa cells given no treatment (Ctrl.; left), or treated with 25 μM RSV (right). The data are presented as the mean ± SE of three independent experiments. (C) Annexin V-PI double staining in untreated Ishikawa control cells (left), or cells treated with 25 μM RSV (right). This panel shows the percentage of double-positive (apoptotic) cells. *$P<0.05$. The results are presented as the mean ± SE of three independent experiments. RSV, resveratrol; PI, propidium iodide; SE, standard error.

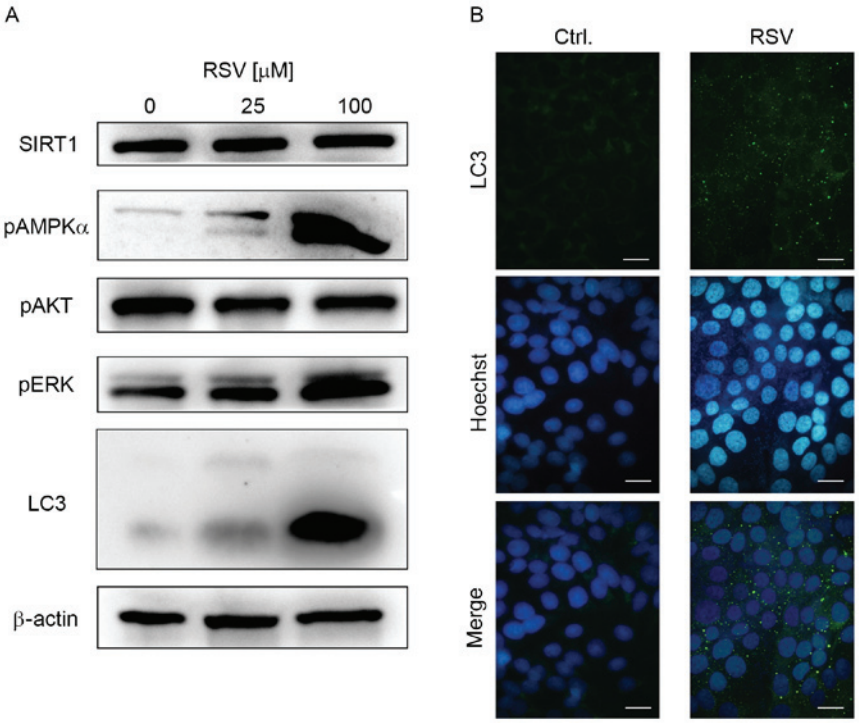

Figure 2. RSV induces autophagy by modulating various cell growth-associated proteins in Ishikawa cells. (A) Immunoblotting of cell growth-associated proteins following RSV treatment at three concentrations (0, 25 or 100 μM) in Ishikawa cells. LC3 was separated on the basis of molecular weight. The upper band was LC3-I (16 kDa) and the lower band was LC3-II (14 kDa), which is a marker of autophagosomes. β-actin was used as a loading control. (B) Detection of autophagosomes by LC3 immunofluorescence in Ishikawa cells. Immunofluorescence in untreated cells (left) and in cells treated with 25 μM RSV (right). These cells were counterstained with Hoechst 33342. Small green dots indicate autophagosome formation. Scale bar, 20 μm. RSV, resveratrol; LC3, light chain 3; SIRT1, sirtuin 1; pAMPKα, phosphorylated AMP-activated protein kinase α; pAKT, phosphorylated protein kinase B; pERK, phosphorylated extracellular signal regulated kinase.

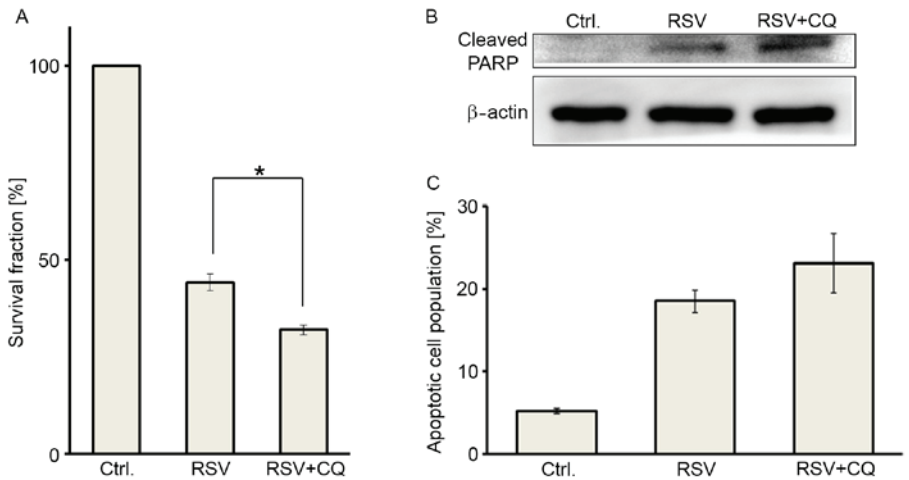

Figure 3. Pharmacologic autophagy inhibition by chloroquine augments RSV-induced apoptosis in Ishikawa cells. (A) Cell viabilities were assessed by performing MTT assays in three groups, including: Untreated control cells (left), cells treated with 25 μM RSV (middle), and cells administered a combination treatment with 25 μM RSV and 5 μM CQ (right). Treated cell survival fraction (%) was compared with the non-treated group (set as 100%). The results are presented as the mean ± SE of three independent experiments. *P<0.05. (B) Immunoblotting of cleaved PARP following each treatment, as described above. β-actin was used as a loading control. (C) Apoptosis was measured by annexin V-PI double staining following each treatment, using the aforementioned RSV and CQ concentrations. The results are presented as the mean ± SE of three independent experiments. RSV, resveratrol; CQ, chloroquine; SE, standard error; PARP, poly ADP ribose polymerase; PI, propidium iodide.

Autophagy inhibition by ATG5 and ATG7 siRNAs augments RSV-induced apoptosis in Ishikawa cells. To elucidate whether RSV-inducible autophagy renders the antiproliferative effect of RSV, the core ATGs, *ATG5* or *ATG7*, were knocked down in Ishikawa cells using two independent siRNAs for each gene. The efficacy of gene silencing and autophagy inhibition by these siRNAs was already confirmed in our previous report (36). MTT assay revealed that the cells were more sensitive to RSV when either *ATG5* or *ATG7* was knocked down (Fig. 4A). Moreover, annexin V-PI double

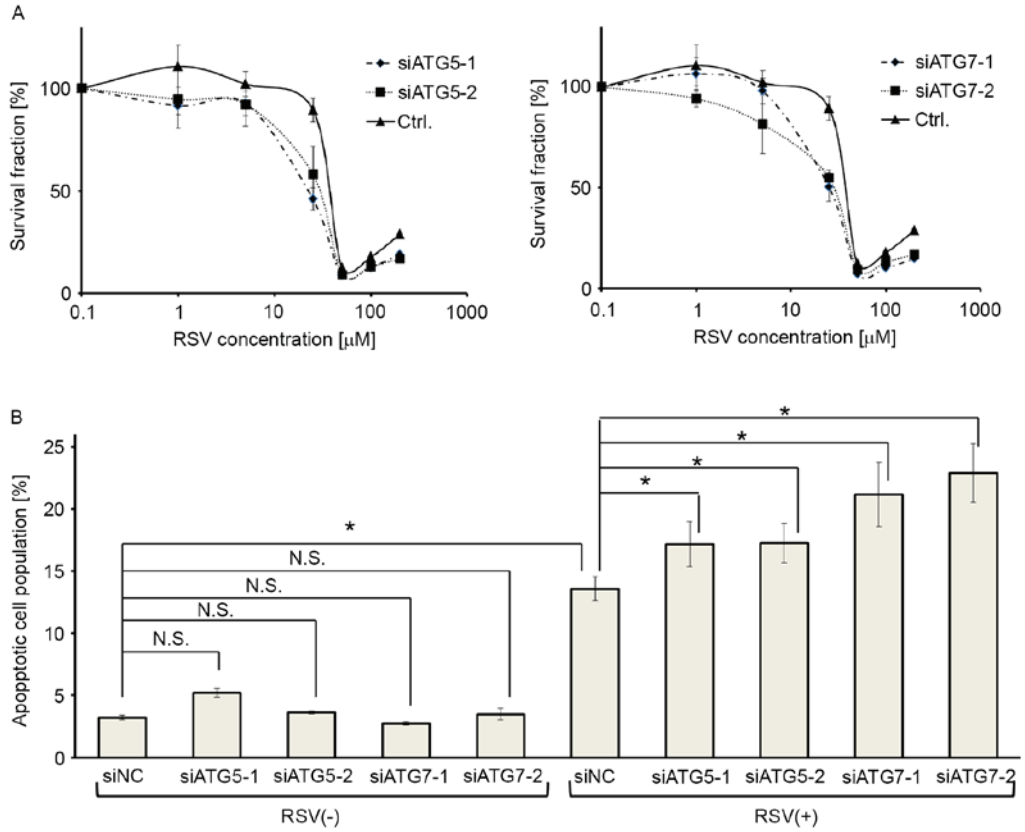

Figure 4. Autophagy inhibition by *ATG5* and *ATG7* siRNA augments RSV-induced apoptosis in Ishikawa cells. (A) MTT assays following RSV treatment (0.1-200 μM) following gene knockdown in Ishikawa cells. Two siRNAs targeting *ATG5* (siATG5-1, siATG5-2; left panel) or *ATG7* mRNA (siATG7-1, siATG7-2; right panel), and a negative control siRNA were used for this assay. The results are presented as the mean ± SE of quadruplicate samples. (B) Annexin V-PI double staining following *ATG5* or *ATG7* knockdown, with and without 25 μM RSV treatment in Ishikawa cells. Four siRNAs (siATG5-1, siATG5-2, siATG7-1, and siATG7-2) and a negative control siRNA (siNC) were used, as described above. Three independent experiments were performed. These results show the percentage of double-positive cells following each treatment. The results are presented as the mean ± SE of three independent experiments. *P<0.05. ATG, autophagy related gene; RSV, resveratrol; si, small interfering; PI, propidium iodide; SE, standard error; CQ, chloroquine.

staining revealed that RSV-induced apoptosis was enhanced by silencing *ATG5* or *ATG7*, whereas the knockdown of *ATG5*, or *ATG7*, alone did not affect apoptosis in cells without RSV treatment (Fig. 4B).

Discussion

RSV is an active compound in foods that can prevent cell proliferation of various types of cancer cells. However, RSV also induces autophagy, which can promote stress tolerance and cell survival by maintaining energy production. Therefore, RSV-associated autophagy may hamper its antitumor effect. In this study, we focused on i) antitumor activity and apoptosis induction by RSV, ii) autophagy induction by RSV, and iii) the efficacy of combined autophagy inhibition and RSV treatment in Ishikawa endometrial cancer cells.

Initially, the results demonstrated that RSV suppressed the proliferation of Ishikawa cells. The IC_{50} value of 20 μM for RSV in the Ishikawa endometrial cancer cells was lower than those of cervical, bladder, breast and liver cancer cells (37-39). This result implies that at least certain endometrial cancer cells may be more sensitive to RSV treatment than other types of cancer cells. The antiproliferative effect of RSV on the tumor cells was revealed to be primarily cytotoxic, not cytostatic. Although the mechanism underlying RSV induction of apoptosis remains unclear, AMPK-dependent signaling pathways may be associated with its ability to induce apoptosis (40). Indeed, RSV markedly increased the expression of p-AMPKα in this study. Although a previous report indicated that RSV attenuated cancer cell proliferation in a SIRT1-dependent manner (41), SIRT1 did not accumulate following RSV treatment in Ishikawa cells. Therefore, RSV-induced apoptosis may be independent from SIRT1. Further investigation is warranted to elucidate the mechanism underlying apoptosis induction by RSV.

In addition, autophagy was induced by RSV treatment in Ishikawa cells, results which were concordant with previous findings in ovarian and cervical cancer cells (21,23). To our knowledge, this is the first report of RSV-mediated autophagy in endometrial cancer cells. Activation of either AMPK or ERK has also been reported to induce autophagy (29,42). AMPK Activation inhibits the mammalian target of the rapamycin (mTOR) signaling pathway, which is frequently activated via phosphatase and tensin homolog mutations in endometrial cancers, including Ishikawa cells (43,44). As activation of mTOR signaling is associated with autophagy inhibition (45), AMPK activation by RSV may counteract mTOR-dependent autophagy inhibition (thereby promoting autophagy) in Ishikawa cells. ERK activation is also associated with autophagy induction, as well as cell proliferation (29).

Although the effect of RSV-mediated autophagy on cancer cells is thought to be cancer-type specific (i.e., tumor suppressive in glioma and esophageal cancer (46-48), or tumor-promoting in ovarian and cervical cancer cells (21,23), the results of the present study suggest that RSV-mediated autophagy may serve a protective role against apoptosis in endometrial cancer cells.

Finally, autophagy inhibition by CQ augmented RSV-induced apoptosis in Ishikawa cells. Moreover, specific autophagy inhibition by siRNAs against either *ATG5* or *ATG7* significantly enhanced apoptotic cell death by RSV. We previously reported that CQ treatment alone caused apoptosis in endometrial cancer cells (36). The results indicate that combined RSV and CQ treatment may be a promising therapeutic strategy through autophagy inhibition and apoptosis induction.

This study has several limitations. The precise mechanism underlying RSV-induced apoptosis and autophagy remains unclear. Autophagy induction may also be mediated by other factors that are independent of AMPK and ERK signaling. Biomarkers for predicting sensitivity to RSV or combined treatment (RSV+CQ) should be identified for clinical applications. In addition, the safety and efficacy of combination RSV and CQ therapy should be examined in *in vivo* studies.

In conclusion, the results of the present study revealed that RSV increased apoptosis, and that RSV-mediated autophagy rendered its apoptotic function in Ishikawa cells. Combined autophagy inhibition with RSV treatment significantly augmented apoptosis. Considering that CQ is widely used in clinical settings, combination RSV/CQ therapy may be a viable option for treating endometrial cancer.

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