Autophagy Inhibition Enhances Apoptosis Induced by Ginsenoside Rk1 in Hepatocellular Carcinoma Cells

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Our previous study indicated that ginsenoside Rk1 has anti-tumor activity and that its mode of action in HepG2 cells treated for 48 h involves coordinated inhibition of telomerase and induction of apoptosis. In the present study, we found that Rk1 induces both G1 phase arrest and autophagy, but not apoptosis, at an earlier stage of treatment. A 24-h incubation of HepG2 cells with Rk1 induced G1 phase arrest. Rk1-induced autophagy was documented by the conversion of microtubule associated protein light chain 3 (LC3)-I to LC3-II, an autophagosome marker, and monodansyl-cadaverine (MDC) incorporation into autolysosomes. Combination of Rk1 with an autophagy inhibitor, such as bafilomycin A1 or beclin 1 siRNA, enhanced the anti-cancer effects of these events on cancer are not known. Most evidence indicates a role for autophagy in sustaining cell survival. Paradoxically, cell death resulting from progressive cellular consumption has been attributed to unrestrained autophagy.9–14 Panax ginseng C. A. Meyer (ginseng) is one of the most popular herbal drugs in the Orient for the treatment of disease. Ginseng saponins (ginsenosides) are the major active compounds in ginseng. They possess anti-inflammatory, anti-cancer, and neuroprotective activities.15–18 In a previous study, we found that ginsenoside Rk1 obtained from heat-processed Sun Ginseng (SG) had an anti-tumor effect on human hepatocellular carcinoma HepG2 cells in vitro when Rk1 was incubated for 48 h.17,19

The aim of the present study was to determine the function of Rk1-induced autophagy in the HepG2 hepatocellular carcinoma cell line during an early stage (24 h of treatment), that led to Rk1-induced apoptosis at 48 h. We focused our attention on a hepatocellular carcinoma-derived cell line due to the poor prognosis for hepatocellular carcinoma, which is largely due to lack of an effective therapy.20 Our results indicate that Rk1 has a novel function as an inducer of autophagy, a defense mechanism against apoptosis, in the early stage of Rk1-induced apoptosis.

Materials and Methods

Cell culture and chemicals. Human hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection

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Abbreviations: CDK4, cyclin dependent kinase 4; FACS, fluorescence-activated cell sorting; LC3, microtubule associated protein light chain 3; MDC, monodansyl-cadaverine; PARP, poly (ADP-ribose) polymerase; PE, phosphatidylethanolamine; Rb, retinoblastoma; siRNA, small interfering RNA; UVRAG, UV irradiation resistance associated tumor suppressor gene
(ATCC, Manassas, VA). Cells were grown in Dulbecco’s modified Eagle medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere with 5% CO2. Rk1 was isolated from SG as previously described.19) The compound was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at a concentration of 20 mM to produce stock solution. For treatment of cells, the compound was diluted in culture medium to the appropriate concentration.

**Analysis of cell viability.** Proliferation of cells was measured with Cell Counting Kit-8 (CCK8) (Dojindo Laboratories, Kumamoto, Japan).21) Briefly, exponentially growing cells were seeded in a 96-well plate at a density of 1.0 × 104 cells/well. The next day, the cells were treated in triplicate with various concentrations of Rk1. After incubation for 24 h, 10 μl of the kit reagent was added to each well, and the cells were incubated for an additional hour. Cell viability was assessed by scanning with a microplate reader at 450 nm. Control cells were exposed to culture media containing 0.5% v/v DMSO.

Western blot analysis. Whole-cell lysates were prepared by incubation of cells in RIPA buffer (Cell Signaling, Beverly, MA) supplemented with 1X protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride (PMSF) according to the manufacturer’s instructions. Proteins (40 μg/lane) were separated by electrophoresis with NuPAGE 4–12% Bis-Tris gels (Invitrogen), blotted onto PVDF transfer membranes, and analyzed with the indicated antibodies. The bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) and LAS-4000 film (Fujifilm, Tokyo).22,23) Monoclonal antibodies to GAPDH and CDK4 and polyclonal antibodies to LC3B, phospho-Rb, and beclin-1 were purchased from Cell Signaling Technology. Antibody to the cleaved form of PARP was obtained from BD Biosciences Pharmingen (BD Biosciences, San Jose, CA).

**Cell cycle analysis.** To detect changes in the cell cycle, cells were collected, washed with cold phosphate buffered saline (PBS), and fixed in 70% ethanol at 4 °C for 30 min. They were then washed twice with PBS, resuspended in 500 μl of propidium iodide (PI) staining solution containing 40 ng/ml PI and 20 μg/ml RNase A in PBS, incubated at room temperature (RT) for 30 min in the dark, and then analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and a ModFit LT V2.0 computer program. At least 20,000 events were assessed in this experiment.

**Confocal microscopy analysis.** To detect expression of LC3, cells were seeded on sterile coverslips placed on 12-well plates. The next day, they were treated with Rk1. At 24 h post-treatment, they were fixed in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at a concentration of 20 mM to produce stock solution. For treatment of cells, the compound was diluted in culture medium to the appropriate concentration.

**Results**

Rk1 induced an antiproliferative response prior to observation of apoptosis

To evaluate the effect of Rk1 on cell proliferation at a stage earlier than Rk1-induced apoptosis (48 h incubation with Rk1), HepG2 cells were treated with a compound at concentrations of 0, 50, 75, and 100 μM for 24 h. Rk1 slightly reduced the cell growth rate, in a dose-dependent manner (Fig. 1A), as compared with previous data, which that cell growth was more substantially suppressed when Rk1 treatment proceeded for 48 h.17) At a dose of 100 μM, Rk1 inhibited HepG2 cell proliferation by about 40%, as compared with the vehicle control (0.01% v/v DMSO). Next we examined whether Rk1-induced antiproliferation was due to apoptosis. Western blot analysis was used to detect cleaved forms of PARP that are involved in the apoptotic response. Rk1 treatment did not trigger cleavage of PARP. Taxol, which was used as a positive control to induce apoptosis in HepG2 cells, induced apoptosis (Fig. 1A, B). In addition, we found that Rk1 treatment did not increase the population of cells in the sub-G1 phase, which indicates sub-diploid DNA content indicative of apoptotic DNA fragmentation (Fig. 2A). These findings suggest that Rk1 inhibits cell proliferation in the early stage of Rk1-induced apoptosis, although it does not induce apoptosis.

Rk1 caused G1 phase arrest

To determine whether Rk1 would induce cell cycle arrest, we analyzed the effect of Rk1 on the cell cycle distribution using PI staining and flow cytometry. When HepG2 cells were treated for 24 h with various Rk1 concentrations, the population of cells in the G1 phase increased significantly, from 53.3% to 91.9%, as the concentration of Rk1 was increased (Fig. 2A). Western blot analysis of molecular markers related to G1 phase arrest showed significant changes, including suppressed levels of CDK4, cyclin D1, and phosphorylated-Rb (Fig. 2B). The western blot data are consistent with the G1 arrest phenomenon observed in the flow cytometry analysis. These results suggest that Rk1 markedly induces G1 phase arrest.

Rk1 induced autophagy

We verified that Rk1 triggers G1 phase arrest and does not induce apoptosis in the early stage of Rk1-induced apoptosis. Recent investigations indicate that chemotherapeutic drugs, or any of several other anticancer stimulii, such as aromatic amine p-anilinoaniline, induce autophagy but not apoptosis in various cancer cells.3) Furthermore, the cytotoxicity of many anticancer agents is mediated by autophagy activation, which is associated with G1 arrest.8,24,25) In order to determine whether Rk1 causes autophagy in HepG2 cells, we measured the incorporation of MDC, a marker for the acidic compartment of autolysosomes.25,26) As shown in Fig. 3A, increased production of autophagic vacuoles was detected as the concentration of Rk1 was increased. Treatment of HepG2 cells with bafilomycin A1 markedly reduced the accumulation of the vacuoles induced by Rk1 treatment. Bafilomycin A1, an inhibitor of vacuolar H+ ATPase, prevents the transition of auto-
phagosomes to autophagolysosomes by disrupting the fusion of autophagosomes to lysosomes. 5,26,27)

To obtain better insight into the mechanism of Rk1-induced autophagy, next we analyzed the effects of treatment with Rk1 on LC3-II protein, the lipidated form of the mammalian microtubule-associated protein 1 light chain, LC3-I. LC3-II is produced during autophagosome formation. The amount of LC3 protein, particularly LC3-II, has been found to correlate with the extent of autophagy.28) As shown in Fig. 3B, confocal imaging analysis showed that LC3 expression was remarkably up-regulated in a dose-dependent manner by Rk1 treatment. In this regard, the number of LC3+ dots or vacuoles increased from 0.25/cell to 3.4/cell after a 24-h treatment with 100 µM Rk1 (Fig. 3C). In addition, increased amounts of LC3 proteins, particularly LC3-II, were detected by western blot analysis (Fig. 3D and E).

In contrast, the expression of beclin-1, another protein involved in autophagy, 29,30) was not altered by Rk1 treatment.

Rk1-induced autophagy inhibited cell viability and apoptosis

As mentioned above, we found that Rk1 induced autophagy in the early stage of the Rk1-induced apoptosis. Next we investigated to determine whether Rg5, another compound of ginsenoside, would have an antiproliferative effect and autophagy as well as on Rk1 in HepG2 cells. We found that Rg5 showed a weaker antiproliferative effect than Rk1 and that it did not induce autophagy in HepG2 cells (Supplemental Fig. 1; see Biosci. Biotechnol. Biochem. Web site). Hence, we investigated the function of Rk1-induced autophagy in HepG2 cells. Recent research has demonstrated that autophagy can play either a pro-survival or a pro-death role following treatment with anticancer drugs, by several pathways.14) In agreement with the above data, although Rk1-treated cells were arrested at G1 phase, they did not undergo apoptosis at 24 h. To elucidate the involvement of protective autophagy when HepG2 cells

**Fig. 1.** Rk1 Showed an Antiproliferative Effect in HepG2 Cells after a 24-h Incubation.

The effect was not induced by apoptosis. A, CCK8 assay for antiproliferative (left) and inhibitory (right) effects of Rk1 in HepG2 cells (Taxol, positive control, 0.1 µM). Data represent mean ± S.D. of three independent experiments. *p < 0.05. B, Rk1 did not induce apoptosis. After the cells were treated with various concentrations of Rk1 for 24 h, total cell extracts were probed with antibodies against cleaved PARP (Taxol, positive control, 0.1 µM). This is representative of three independent experiments.

**Fig. 2.** Rk1 Induced G1 Arrest in HepG2 Cells Treated for 24 h.

A, Cell cycle distribution was assessed by flow cytometry. This is representative of three independent experiments. B, Immunoblot analysis of cell cycle markers associated with the G1 phase. Total cell extracts were probed with antibodies against CDK4 and phospho-Rb. This is representative of three independent experiments.

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Fig. 3. Rk1 Induced Autophagy in HepG2 Cells.

Analysis of autophagic vacuoles (A), LC3 aggregation (B), and enumeration of LC3+ dots (C) by confocal microscopy. The expression level of the autophagy marker, LC3, by immunoblot analysis (D) and by densitometric analysis (E). A. HepG2 cells were treated with various concentrations of Rk1 for 24 h and stained with MDC in the presence and the absence of co-treatment with bafilomycin A1. Under MDC staining, mature autophagic vacuoles, such as autophagolysosomes, were observed (white arrow). This is representative of three independent experiments. B. HepG2 cells were labeled with 4',6-diamidino-2-phenylindole (DAPI, blue), and Alexa fluor 488 secondary antibody against LC3B (green). This is representative of three independent experiments. C. LC3+ dots per cell were counted; the data represent the mean ± S.D. of three independent experiments. *p < 0.05. D. Total cell extracts were assayed by immunoblot analysis for expression of LC3-I and LC3-II. This is representative of three independent experiments. E. The expression levels of LC3-I and LC3-II were quantified by densitometric analysis. Data represent the mean ± S.D. for three independent experiments. *p < 0.05, **p < 0.01.
underwent Rk1-induced autophagy, we examined to determine whether Rk1-induced autophagy would inhibit cell viability and apoptosis. By 24 h, co-treatment of HepG2 cells with Rk1 (100 µM) and bafilomycin A1 resulted in markedly increased cell death as compared to that observed when cells were treated with 100 µM Rk1 alone (Fig. 4A and B). To rule out the possibility that bafilomycin A1 is toxic, we measured the cytotoxicity of bafilomycin A1. As shown in Fig. 4C and D, no cytotoxicity caused by bafilomycin A1 was detected. To confirm the role of autophagy in Rk1-treated HepG2 cells, we used siRNA to inhibit beclin 1 expression, since beclin 1 protein is essential for autophagosome production.29,30) Silencing of beclin 1 (Fig. 4E) caused more cell death than treatment with Rk1 alone (Fig. 4F). These data indicate that apoptosis was induced in Rk1-treated HepG2 cells when autophagy was inhibited by bafilomycin A1 or beclin 1 siRNA. Taken together, these results indicate that HepG2 cells respond to Rk1 by activating autophagy to resist apoptosis.
Discussion

We have reported that ginsenoside Rk1 obtained from heat-processed ginseng, Sun Ginseng (SG), exhibited an anti-tumor effect against human hepatocellular carcinoma HepG2 cells in vitro. In the present study, we found that Rk1 exhibited antiproliferative effects characterized by G1 phase arrest and autophagy at an earlier stage that precedes apoptosis. Furthermore, we found that inhibition of Rk1-induced autophagy enhanced sensitization to the Rk1-induced anticancer effect.

The antiproliferative effects of Rk1 correlated with induction of G1 arrest in a dose-dependent manner. This G1 arrest might have been the result of decreases in G1 arrest-associated proteins caused by Rk1 (Figs. 1 and 2). In recent research, it was shown that cell cycle progression depends on proliferation to such an extent that inhibition of proliferation by nutrient or growth factor deprivation results in cell cycle arrest. Additionally, it was found that mitotic cells arrest their autophagic protein degradation pathway while their organelles and chromosomes are dividing, and that the autophagic pathway restarts during the G1 phase.

Here we report a novel function of Rk1, induction of autophagy, as shown by increased formation of the autophagosomal marker LC3 and the incorporation of MDC in the autolysosomes. We aimed to determine the function of Rk1-induced autophagy. Autophagy has conflicting roles under anti-cancer conditions in various cancer cells. According to a previous article, autophagy has been regarded as a tumor repression mechanism. In contrast, recent studies report that inhibition of autophagy enhanced apoptosis, indicating that autophagy contributes to tumor progression as a protective function against anticancer agents.

Anticancer treatments, including drugs, activate autophagy to kill cancer cells that are resistant to apoptosis. However, we found that inhibition of autophagy by bafilomycin A1 or beclin 1 siRNA sensitized cells to Rk1-induced cell death (Fig. 4). In previous studies, it was established that beclin 1 is a tumor suppressor protein that mediates autophagy and then represses the growth of tumor cells. In a recent study, however, it was found that beclin 1 haploinsufficiency may have an impact on cells that also possess an apoptotic defect, which prevents an apoptotic response to starvation, allowing survival by autophagy. Here, we found shown that silencing of beclin 1 sensitizes cells to Rk1-mediated cell death (Fig. 4E and F). When HepG2 cells were treated with low concentrations of Rk1 (50 and 75 μM) combined with autophagy inhibitors for 24 h, the viability of the cells somewhat decreased relative to the cells treated with Rk1 alone. However, the viability of cells treated with a higher concentration of Rk1 (100 μM) and autophagy inhibitors for 24 h significantly decreased, by 2.4-fold and 3.3-fold, relative to the cells treated with Rk1 alone (Fig. 4A and F). Hence, we speculate that Rk1-induced autophagy, which occurs after exposure to high concentrations of Rk1 for 24 h, might have a function in sustaining cell survival following the onset of Rk1-induced apoptosis. However, after exposure for 24 h to a high concentration of Rk1 and autophagy inhibitors, autophagy was inhibited and apoptosis occurred. Briefly, this result suggests that autophagy provides a protective mechanism against Rk1-induced apoptosis. This result is confirmed by several reports that autophagy inhibition enhances the anticancer effect of arsenic trioxide, hyperthermia, and p53 or alkylating drugs, but, it was established that soybean B-group triterpenoid saponine can suppress colon cancer cell proliferation through S phase cell cycle delay, and can induce autophagic cell death, the hallmark of type-II programmed cell death. Though Rk1 is also a kind of saponine, unlike Rk1, dammarane glycoside, soybean B-group triterpenoid saponins, oleane glycoside, show very different chemical structures. Therefore it can reasonably be inferred that this S phase cell cycle delay and autophagic cell death by soybean B-group triterpenoid saponins might be caused by the difference in chemical structures.

In conclusion, we found that Rk1 produces in vitro growth inhibition, G1 phase cell cycle arrest, and autophagy in the early stage of Rk1-induced apoptosis. We also found that autophagy protects cancer cells from the anticancer activity of Rk1 in HepG2 cells, and that a combination of Rk1 with autophagy inhibitors strengthens the efficiency of proapoptotic chemotherapeutic strategies. Collectively, this study suggests that inhibition of autophagy can increase the therapeutic efficacy of Rk1 in the treatment of hepatocellular carcinoma.

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References


