Anti-tumor Activity of the Ginsenoside Rk1 in Human Hepatocellular Carcinoma Cells through Inhibition of Telomerase Activity and Induction of Apoptosis

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The ginsenoside Rk1 is one of major components of heat-processed Panax ginseng C. A. Meyer, Sun Ginseng (SG). Here, we investigated the mechanisms underlying the anti-tumor activity of Rk1 in human hepatocellular carcinoma HepG2 cells in vitro. Rk1 markedly inhibited telomerase activity and cell growth along with significant morphological change. The expression levels of telomerase reverse transcriptase (hTERT) and c-Myc mRNA were obviously decreased with Rk1 treatment, while that of telomerase RNA (hTR) was not. Furthermore, Rk1 induced apoptosis through activation of caspases-8 and -3. However, Fas-associated death domain (FADD) expression decreased with Rk1 treatment, though it was known that the signaling cascade of FADD was associated with caspase-8 activity. Interestingly, activation of extracellular-regulated kinase (ERK) increased with Rk1 treatment. In conclusion, these results represent the first identification of the biological activity of Rk1 against HepG2 cell growth and show that the mechanism underlying the anti-tumor activity of Rk1 involves coordination between inhibition of telomerase activity and induction of apoptosis.

Key words ginsenoside Rk1; telomerase; apoptosis; extracellular-regulated kinase; c-Myc; human hepatocellular carcinoma

Panax ginseng C. A. Meyer (ginseng) is one of the most common herbal medicines in the Orient used for the treatment of disease. Ginseng saponins (ginsenosides) are the major active components in ginseng and possess anti-inflammatory, anti-cancer, and neuroprotective activity.1–3) Of the ginsenosides, Rk1, Rg3, Rg5, F42, etc. are isolated from heat-processed ginseng, Sun Ginseng (SG), but they are not detected in raw or air-dried ginseng.4,5) The enhanced anti-tumor activity of SG is attributed to the generation of ginsenosides due to heat processing.4,6) The molecular mechanisms underlying the anti-tumor activity of SG-derived ginsenosides have not been comprehensively investigated.

High telomerase activity is found in the vast majority of human malignant tissues and many immortal cell lines. The fundamental components of telomerases are telomerase reverse transcriptase (hTERT), telomerase RNA (hTR), and telomerase-associated proteins.7) Of these, hTR and telomerase-associated proteins are expressed ubiquitously in both normal and cancerous tissues,8) whereas hTERT is expressed at high levels in human tumors and either not at all or at very low levels in normal tissues.7) The rate-limiting component of telomerase is hTERT, and thus, the expression level of hTERT correlates with telomerase activity.

Fas-associated death domain (FADD) interacts with the cytosolic tail of the Fas/APO-1 receptor by binding to the COOH-terminal death domain (DD) to expose the NH2-terminal death effector domain (DED).9,10) Recruitment of caspases-8 and -10 by DED forms the death-inducing signaling complex (DISC), resulting in the oligomerization of these proteases and their subsequent cleavage and activation.11) Phosphorylation of FADD at Ser194 is involved in the G2/M phase arrest in Burkitt lymphoma and breast cancer cells.12,13)

Members of the mitogen-activated protein kinase (MAPK) family represent one type of serine/threonine protein kinase, and are key modulators of cell activation, including apoptosis and cell cycle progression. To date, three major MAPKs, extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, have been identified. ERK is generally activated by growth factors and is involved in the regulation of cell proliferation.14,15) Activation of ERK is often associated with cell cycle arrest.16,17)

In this study, the mechanism underlying the anti-tumor activity of the ginsenoside Rk1 was investigated by examining the effects of Rk1 on cell growth, telomerase activity, and apoptosis in human hepatocellular carcinoma HepG2 cells.

MATERIALS AND METHODS

Chemicals The ginsenoside Rk1 (Fig. 1) was isolated from SG as previously described18) and confirmed by NMR and MS spectroscopic analysis. The compound was dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mM and stored at −20 °C. When required, the compound was diluted with medium to the appropriate concentration.

Cell Culture and Treatment The human hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco’s modified Eagle medium (Gibco, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO2. In this study, the HepG2 cells were incubated in medium with or without Rk1 for 48 h.

Analysis of Cell Viability Cell viability was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Briefly, exponentially growing cells were...
seeded in a 96-well plate at a density of 1.0×10⁴ cells/well in triplicate. The next day, the cells were treated with Rk1 at concentrations ranging from 12.5 to 100 μM. After incubation for 48 h, 10 μL of the kit reagent was added and the cells were incubated for an additional 1 h. Cell viability was measured by scanning with a microplate reader at 450 nm. Control cells were exposed to culture media containing 0.5% (v/v) DMSO.

**Telomerase Activity Assay** Telomerase activity was measured with the PCR-based telomere repeat amplification protocol (TRAP) using the TRAPEze ELISA Telomerase Detection Kit (Chemicon, CA, U.S.A.) as described by the manufacturer. Cells were homogenized in 1X CHAPS lysis buffer containing 200 units/ml of RNase inhibitor on ice for 30 min. Then, 50 ng of each cell extract was analyzed in the TRAP reaction. The cell extracts were added to the reaction mixture containing biotinylated telomerase substrate (TS) primer, biotinylated reverse primer, internal control primers, dATP, dTTP, dGTP, dinitrophenyl (DNP)-labeled dCTP and Taq polymerase, and PCR was performed (primer elongation at 25 °C, 30 min; amplification for 33 cycles at 94 °C, 30 s; 55 °C, 30 s). The level of generated TRAP product was determined by assessing the level of horseradish peroxidase (HRP) activity using the anti-DNP antibody conjugated HRP according to the manufacturer’s instructions. Whole-cell lysates (50 μg/lane) were separated by NuPAGE 4—12% Bis-Tris gel (Invitrogen), blotted onto a PVDF transfer membrane, and then analyzed with the desired antibodies. The bound antibodies were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.) and LAS-4000 (Fujifilm, Tokyo, Japan). Monoclonal antibodies to GAPDH, caspase-8, and cleaved caspase-8 and polyclonal antibodies to caspase-3, cleaved caspase-3, PARP, FADD, ERK (p44/42 MAP Kinase), phosphorylated ERK (Thr202/Tyr204), caspase-9, Bcl-2, Bcl-xL, Bax, and Puma were purchased from Cell Signaling Technology, Inc. (Cell Signaling, MA, U.S.A.). The antibody to the cleaved form of PARP was obtained from BD Biosciences Pharmingen (BD Biosciences, CA, U.S.A.).

**RESULTS**

**Rk1 Suppresses Cell Growth** To assess the effect of Rk1 on cell viability, HepG2 cells were incubated with Rk1 at 0 to 100 μM. Rk1 suppressed cell growth in a dose-dependent manner (Fig. 2A). Rk1-induced cell death was significant at 75 and 100 μM Rk1, resulting in 55 and 95% cell death, respectively. Morphological changes observed by phase-contrast microscopy (Fig. 2B) showed that cells treated with high concentrations of Rk1 transformed themselves into apoptotic bodies and detached from the culture dish. These findings suggest that Rk1 induces apoptotic cell death through some mechanism.

**Rk1 Inhibits Telomerase Activity** To examine the effect of Rk1 on telomerase activity, we performed the TRAP-ELISA assay using purified telomerase from HepG2 cells treated with different concentrations of Rk1. Figure 3A shows that Rk1 dramatically inhibits telomerase activity in a dose-dependent manner. In cells exposed to 100 μM Rk1, telomerase activity decreased to 27.8% of that of the control. Investigations into whether Rk1-induced inhibition of telomerase activity was due to direct interference by Rk1 showed that Rk1 did not directly interfere with telomerase, telomeric repeats, or Taq polymerase (Fig. 3B). Taken together, these findings support the manufacturer’s recommendations. Briefly, whole cells were collected, rinsed with PBS, incubated with 100 μl of annexin-V-fluor labeling solution containing 2 μl of annexin-V-FITC and 2 μl of PI at RT for 15 min in the dark, and analyzed by a FACSCalibur flow cytometer. At least 2000 events were evaluated in these experiments.

**Western Blot Analysis** The cells were lysed using a NucBuster Protein Extraction Kit (Novagen, WI, U.S.A.) supplemented with 1X protease inhibitor cocktail and 1 mM DTT according to the manufacturer's instructions. Whole-cell lysates (50 μg/lane) were separated by NuPAGE 4—12% Bis-Tris gel (Invitrogen), blotted onto a PVDF transfer membrane, and then analyzed with the desired antibodies. The bound antibodies were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.) and LAS-4000 (Fujifilm, Tokyo, Japan). Monoclonal antibodies to GAPDH, caspase-8, and cleaved caspase-8 and polyclonal antibodies to caspase-3, cleaved caspase-3, PARP, FADD, ERK (p44/42 MAP Kinase), phosphorylated ERK (Thr202/Tyr204), caspase-9, Bcl-2, Bcl-xL, Bax, and Puma were purchased from Cell Signaling Technology, Inc. (Cell Signaling, MA, U.S.A.). The antibody to the cleaved form of PARP was obtained from BD Biosciences Pharmingen (BD Biosciences, CA, U.S.A.).

Table 1. Oligonucleotide Primer Sequences Used for RT-PCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3')</th>
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<tr>
<td>hTERT</td>
<td>Forward primer CggAagAgTgTCTTggAgCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer ggATgAAGggATCTTggA</td>
</tr>
<tr>
<td>hTR</td>
<td>Forward primer TCTAACCCTAATgAgAaggCCgTAg</td>
</tr>
<tr>
<td></td>
<td>Reverse primer ggTTTgCTCATggAgTggAg</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Forward primer AAgACTCCAgCgCCTTCTTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer gTTTCCAACTCggATCTg</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer CggAgTGACggATTgTgCgTAT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer AgCCTTCCATgTgAgAC</td>
</tr>
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**Flow Cytometric Analysis** Apoptotic cell death was assessed by annexin-V/PI double staining using an Annexin-V-FLUOS Staining Kit (Roche, Penzberg, Germany) according to the manufacturer’s recommendations. Briefly, whole cells were collected, rinsed with PBS, incubated with 100 μl of annexin-V-fluor labeling solution containing 2 μl of annexin-V-FITC and 2 μl of PI at RT for 15 min in the dark, and analyzed by a FACSCalibur flow cytometer. At least 2000 events were evaluated in these experiments.
Rk1 inhibits telomerase activity in HepG2 cells through an indirect mechanism. Rk1 Decreases hTERT and c-Myc mRNA Expression

The mechanism of telomerase inhibition by Rk1 was investigated by assessing the levels of hTR, hTERT, and c-Myc mRNA by RT-PCR (Fig. 4). hTR and hTERT are the essential components of telomerase, and c-Myc is a regulator of hTERT gene expression.\(^\text{20}\) For this analysis, HepG2 cells were treated with 100 \(\mu\)M Rk1, because significant inhibition of telomerase activity was observed at this concentration.

Fig. 2. Effect of Rk1 on HepG2 Cell Viability
(A) Cells were treated with Rk1 at concentrations ranging from 12.5 to 100 \(\mu\)M for 48 h or with 0.5% (v/v) DMSO as a control. Relative cell viability was measured by the CCK-8 assay. Data are shown as the mean±S.D. of three independent experiments. (B) Morphological changes were confirmed using phase-contrast microscopy.

Fig. 4. Rk1 Causes Down-Regulation of hTERT and c-Myc mRNA in HepG2 Cells
The cells were treated without (control) or with 100 \(\mu\)M Rk1 for 48 h. One microgram of total RNA was used for RT-PCR. GAPDH was used as a loading control.

Fig. 5. Rk1 Induces Apoptosis in HepG2 Cells
The cells were incubated in the absence (control) or presence of 100 \(\mu\)M Rk1 for 48 h, and stained with FITC-conjugated Annexin V and PI for flow cytometry. The cell populations shown at the lower right (Annexin V+/PI-) represent early apoptotic cells.

Fig. 6. Rk1 Activates Caspase-8 and Induces Apoptosis in HepG2 Cells
Western blots showing the levels of phosphorylated ERK (42, 44 kDa), ERK (42, 44 kDa), FADD (28 kDa), procaspase-8 (57 kDa), cleaved caspase-8 (18 kDa), procaspase-3 (35 kDa), cleaved caspase-3 (17, 19 kDa), PARP (316 kDa), cleaved PARP (85 kDa) (A), procaspase-9 (47 kDa), and cleaved caspase-9 (35, 37 kDa) (B) in HepG2 cells treated without (control) or with 100 \(\mu\)M Rk1 for 48 h. Fifty micrograms of each protein were separated by SDS-PAGE. GAPDH (37 kDa) was used as an internal control.
Myc mRNA expression.

**Rk1 Induces Apoptosis through Activation of Caspases-8 and -3, Independent of FADD Expression** Annexin-V/PI double staining was used to investigate Rk1-induced apoptotic cell death in HepG2 cells. As shown in Fig. 5, treatment of HepG2 cells with 100 μM Rk1 induced an increase in the fraction of early apoptotic cells from 0.46 to 16.23%. Western blot analysis was then used to investigate the expression of proteins involved in the apoptotic response to determine if apoptosis occurs via the intrinsic or extrinsic pathway (Fig. 6A). Both activation of caspase-3 and cleavage of PARP were clearly detected after Rk1 treatment. At the same time, the expression of both procaspase-3 and full-length PARP decreased. The 18 kDa active form of caspase-8 was also detected on Rk1-treated cells along with a decrease in the expression of procaspase-8. The expression of FADD, upstream of caspase-8, was reduced following Rk1 treatment. Activation of ERK, which occurred through phosphorylation of Thr202 and Tyr204, was significantly enhanced in Rk1-treated cells.23) The expression levels of Bcl-2, Bcl-xL, Bax, and Puma were also examined, although these proteins are not involved in the apoptotic response (data not shown). In addition, the active form of caspase-9 was not detected after Rk1 treatment (Fig. 6B). These results suggest that Rk1 induces mitochondria-independent apoptosis through activation of caspase-8, the signaling cascade of which was not associated with FADD expression.

**DISCUSSION**

Cancer cells are typically distinguished from normal cells by their resistance to apoptosis and the maintenance of telomerase integrity through telomerase reactivation. Thus, telomerase is an outstanding cancer marker and a key target for anti-tumor therapy.22) In particular, hTERT expression is closely associated with telomerase activity. In the liver, hTERT mRNA expression is progressively increased during hepatocarcinogenesis, and a great induction is found in dysplastic nodules.23) In this study, we found that Rk1 is associated with inhibition of telomerase activity through reduction of hTERT mRNA expression in human hepatocellular carcinoma HepG2 cells.

Caspase-8 activation has been investigated in association with tumor necrosis factor-receptor (TNF-R) family-mediated apoptosis. Oligomerization of caspase-8 is an essential step in the activation process of caspase-8, which generally requires FADD in Fas-dependent apoptosis.24,25) However, in the apoptotic response of anticancer drugs, such as mitomycin C and staurosporine, caspase-8 can be activated in the absence of a FADD-containing receptor signaling complex, suggesting that in this case, apoptosis does not require death receptor signaling.26) Similarly, in Burkitt lymphoma B cells, caspase-8 cleavage and apoptosis are regulated by TGFβ-mediated p38 activation and appear to be independent of FADD.27) Another possible mechanism by which downstream caspase-8 is activated is through Raf/ERK signaling, which is independent of FADD expression.28) Caspase-8-mediated activation of caspase-3 can occur via mitochondria-independent or -dependent pathways.29) Consistent with previous studies, the results of our study in HepG2 cells demonstrate that Rk1 activates caspase-8 through a FADD-independent apoptotic pathway, which then leads to activation of caspase-3.

The transcription factor c-Myc, a member of the Myc family of b/HLH/LZ proteins, regulates the expression of a number of genes associated with apoptosis, although the mechanism by which this occurs is unclear. c-Myc is one of the direct regulatory elements in the promoter region of hTERT, E-boxes; thus, hTERT is a direct target of c-Myc.30) Also, down-regulation of c-Myc appears to induce cell cycle arrest and apoptosis in Jurkat T lymphocytes and acute myeloid leukemia.31,32) Interestingly, it has recently been reported that MAP kinase phosphatase-1 (MKP-1) overexpression might block gene transcription of c-fos, c-myc, and cyclin D1, leading to impaired epithelial proliferation.33) Brondello et al. showed that MKP-1 was transcriptionally induced through ERK activation.34) For these reasons, we postulate that Rk1 activates ERK and up-regulates MKP-1, resulting in activation of caspase-8 and down-regulation of c-Myc, respectively. However, further study is needed to characterize the molecular mechanisms underlying the caspase-8 adaptor whose expression might be controlled by ERK activation.

In order to achieve anti-tumor response, it is important to inhibit telomerase activity and to induce apoptosis. An interesting correlation between the inhibition of telomerase activity and c-Myc expression in apoptotic cells has recently been established. Human melanoma cells have been shown to respond to the suppression of c-Myc expression by entering a crisis phase that involves inhibition of telomerase activity, telomere erosion, and apoptosis.35) In addition, a positive correlation has been reported between inhibition of telomerase activity and apoptosis by curcumin in cancer cells,36) although the signal activating apoptosis in the telomerase-inhibited cells has not been completely characterized. In this study, it appears that phosphorylation of ERK might be related to inhibition of telomerase and induction of apoptosis, though the connection between telomerase and apoptosis is not clear.

In summary, although the exact mechanism by which activation of ERK induces apoptosis is unclear, the anti-tumor activity of the ginsenoside Rk1 appears to involve inhibition of telomerase activity and the induction of apoptosis. These results highlight Rk1 as a promising therapeutic agent for hepatocellular carcinoma.

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**REFERENCES**