Antitumor effects of ginsenoside Rg3 on human hepatocellular carcinoma cells

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Abstract. The antitumor effects of ginsenoside Rg3 have been reported in several kinds of human malignant tumors. The purpose of this study was to investigate whether ginsenoside Rg3 can inhibit the growth of human hepatocellular carcinoma cell lines and to discuss the possible molecular mechanism(s). We cultured the human hepatocellular carcinoma cell lines, SMMC-7721 and HepG2. The cells were treated with different concentrations of ginsenoside Rg3 (0, 25, 50, 75 and 100 μg/ml), and the cell proliferation was detected by MTT assay at the 12, 24, 36 and 48 h time-points. Flow cytometry experiments were carried out to investigate the effect of Rg3 on cell apoptosis after the cells had been treated with Rg3 (50 and 100 μg/ml) for 24 and 48 h. The expression levels of *caspase-3*, *bax* and *bcl-2* in Rg3-treated cells (100 μg/ml, 48 h), as well as normal cells were detected through real-time PCR experiments. MTT assay showed that the inhibition rate of cell proliferation in the Rg3 groups was significantly higher compared to the control groups in both the SMMC-7721 and HepG2 cell lines, and the inhibition rate increased with increasing Rg3 concentrations and duration of treatment. Flow cytometry analysis demonstrated that the Rg3 groups had a significantly higher cell apoptotic rate compared to the control groups in both the SMMC-7721 and HepG2 cell lines, and that the effect of Rg3 on cell apoptosis occurred in a concentration- and time-dependent manner, as was also shown by the MTT assay. Real-time PCR analysis showed that the gene expression levels of *caspase-3* and *bax* were significantly enhanced in the Rg3 groups compared to the control groups in both the SMMC-7721 and HepG2 cell lines, but the gene expression level of *bcl-2* was significantly inhibited. These results indicate that ginsenoside Rg3 can effectively inhibit the growth of human hepatocellular carcinoma cell lines by inhibiting cancer cell proliferation and promoting cancer cell apoptosis, and it may promote cancer cell apoptosis via the endogenous mitochondrial-mediated caspase-dependent apoptotic pathway.

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Introduction

One of the most serious complications of chronic liver disease is hepatocellular carcinoma. Worldwide, it is the 4th most common cancer and the 3rd most common cause of mortality from all cancers. Although a global cancer, it is especially prevalent in the Asia Pacific and sub-Saharan Africa (1). Chemotherapy is one of the most extensively investigated methods in anticancer therapies, but its efficacy and safety remain a primary concern, as the toxicity and other side-effects of chemotherapy are severe. In recent years, the search for anticancer drugs has leaned towards natural products; one of the most common natural drugs is ginsenoside Rg3 (2).

Ginsenoside Rg3 is an effective chemical trace component extracted from ginseng. The molecular formula of ginsenoside Rg3 is $C_{42}H_{72}O_{13}$ and its molecular weight is 784.30 Da (3). Studies have found that ginsenoside Rg3 may have antitumor effects in several kinds of cancers, but its role in human hepatocellular carcinoma and the molecular mechanisms involved are not yet well comprehended. In this study, we cultured the human hepatocellular carcinoma cell lines, SMMC-7721 and HepG2, and investigated the effects of Rg3 on cell proliferation and apoptosis. We also studied the possible mechanisms involved by evaluating the expression profiles of apoptotic-related genes.

Materials and methods

Cell culture. The human hepatocellular carcinoma cell lines, SMMC-7721 and HepG2 (ATCC; Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum plus ampicillin and streptomycin routinely, and incubated in 5% CO_2 at 37˚C.

MTT assay. MTT assay was performed to examine the cell proliferation. Cells in logarithmic phase were collected, an amount of $5x10^3$ cells was seeded into 96-well plates with 100 μl medium, and the plates were incubated at 37˚C in a humidified incubator, 5% CO_2 for 24 h. The medium was then removed and 100 μl medium with different concentrations of Rg3 were added to each well. A control group and four Rg3 groups were set up,

Table I. PCR primers.

Gene	Primer sequence (5'-3')	Product size (bp)
caspase-3	F: TGGTTCATCCAGTCGCTTTG R: ATTCTGTTGCCACCTTTCGG	100
bax	F: TTTGCTTCAGGGTTTCATCC R: GAGTCTGTGTCCACGGCG	186
bcl-2	F: CCCGTTGCTTTTCCTCTG R: ATCTCCCGCATCCCACTC	126
GAPDH	F: GGAAGGTGAAGGTCGGAGT R: TGAGGTCAATGAAGGGGTC	117

F, forward; R, reverse.

and the final concentration of Rg3 in each group was 0, 25, 50, 75 and 100 μg/ml, respectively. Each group had six replicates. A total amount of 20 μl MTT reagent (Sigma, USA) was added to each well at the 12, 24, 36 and 48 h time-points. After culturing the cells for 4 h and removing the medium, 150 μl of DMSO were added into each well, and the cells were incubated for 15 min. Finally, the plates were read at the absorbance of a 490-nm wave length by a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The experiment was repeated three times.

Flow cytometry. The effects of Rg3 on cell apoptosis were studied by flow cytometry (BD FACSAria II Cell Sorter, BD Company, Franklin Lakes, NJ USA). Cells in logarithmic phase were collected, an amount of $2x10^5$ cells was seeded into 6-well plates with 2 ml medium, and the plates were incubated at 37˚C in a humidified incubator, 5% CO_2 for 24 h. The medium was then removed and 2 ml medium with different concentrations of Rg3 were added to the plates. A control and two Rg3 groups were set up, and the final concentration of Rg3 in each group was 0, 50 and 100 μg/ml, respectively. Each group had three triplicates. After 24 and 48 h, the cells were collected, washed twice with PBS (2000 rmp, 5 min, 4˚C) and then the cells were resuspended in 400 μl PBS. After the addition of 5 μl Annexin V-FITC, the plates were gently agitated, and then the cells were incubated for 10 min at room temperature. This was followed by the addition of 10 μl PI (20 μg/ml), and a further incubation for 30 min at 4˚C. Cell apoptosis was then evaluated by flow cytometry. The experiment was repeated three times.

Real-time PCR. Real-time PCR analysis was carried out to detect the expression levels of *caspase-3*, *bax* and *bcl-2* in the SMMC-7721 and HepG2 cell lines. Cells were incubated as those in the flow cytometry experiment, the medium was removed after 24 h, and 2 ml medium with or without Rg3 were added to the plates. Two groups were set up as follows: the control (with no Rg3) and the Rg3 (with 100 μg/ml Rg3). After being incubated for 48 h, total RNA of each group was isolated using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. An amount of 1 μg total RNA was reverse-transcribed into cDNA using the M-MuLV reverse transcriptase kit (Fermentas, Burlington, ON, Canada).

Real-time PCR was performed using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. A total amount of 2 μl cDNA template was used for each PCR reaction. The amplification was performed on the Bio-Rad C1000 real-time thermal cycler (Bio-Rad). The *GAPDH* gene was used as the endogenous control. PCR primers of *caspase-3*, *bax*, *bcl-2* and *GAPDH* (control) are shown in Table I.

Statistical analysis. All values are presented as the means ± standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test with SPSS17.0. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell proliferation. To detect the effects of Rg3 on cell proliferation, we carried out an MTT experiment in the SMMC-7721 and HepG2 cell lines. After the cells had been incubated for 12 h, the cell proliferation of the Rg3 group showed a significantly increased inhibition rate when compared to the control group at each detected time-point (Tables II and III). In addition, the cell proliferation inhibition rate significantly increased in both the SMMC-7721 and HepG2 cells as the Rg3 concentration and treatment duration increased. This result suggests that Rg3 inhibits cancer cell proliferation in a concentration- and time-dependent manner (Fig. 1).

Cell apoptosis. To examine the role of Rg3 in cell apoptosis, we performed flow cytometry in SMMC-7721 and HepG2 cells. After these two types of cells been incubated for 24 and 48 h, the cell apoptotic rate of the Rg3 group was significantly higher than that of the control, and the group of 100 μg/ml showed a significantly increased apoptotic rate when compared to the group of 50 μg/ml. Furthermore, the apoptotic rate in the group

Table II. Effects of Rg3 on cell proliferation in SMMC-7721 cells (A_{490}).

Time-points (h)	Rg3 (μg/ml)				
	0	25	50	75	100
12	0.497±0.016	0.469±0.024[a]	0.432±0.220[a]	0.385±0.029[a]	0.295±0.015[a]
24	0.622±0.027	0.573±0.015[a]	0.512±0.024[a]	0.425±0.017[a]	0.308±0.018[a]
36	0.765±0.028	0.694±0.014[a]	0.587±0.021[a]	0.459±0.030[a]	0.316±0.022[a]
48	0.912±0.027	0.799±0.025[a]	0.585±0.019[a]	0.437±0.020[a]	0.321±0.020[a]

All values represent the means ± SD. [a]$P<0.05$ indicates statistically significant differences vs. the control group.

Table III. Effects of Rg3 on cell proliferation in HepG2 cells (A_{490}).

Time-points (h)	Rg3 (μg/ml)				
	0	25	50	75	100
12	0.456±0.016	0.428±0.016[a]	0.384±0.210[a]	0.336±0.011[a]	0.289±0.017[a]
24	0.609±0.029	0.554±0.019[a]	0.488±0.024[a]	0.421±0.025[a]	0.336±0.018[a]
36	0.753±0.029	0.654±0.017[a]	0.569±0.020[a]	0.466±0.015[a]	0.352±0.018[a]
48	0.908±0.036	0.769±0.025[a]	0.623±0.023[a]	0.457±0.017[a]	0.368±0.021[a]

All values represent the means ± SD. [a]P<0.05 indicates statistically significant differences vs. the control group.

Table IV. Effects of Rg3 on cell apoptosis (%).

Time-points (h)	SMMC-7721 (μg/ml)			HepG2 (μg/ml)		
	0	50	100	0	50	100
24	10.370±2.27	22.33±3.37[a]	37.93±3.37[a]	11.67±2.23	29.3±1.68[a]	36.90±2.00[a]
48	15.737±1.66	37.67±1.88[ab]	46.90±1.45[ab]	17.03±1.05	41.5±1.76[ab]	50.83±3.21[ab]

All values represent the means ± SD. [a]P<0.05 indicates statistically significant differences vs. the control group; [b]P<0.05 indicates statistically significant differences vs. the 24-h group.

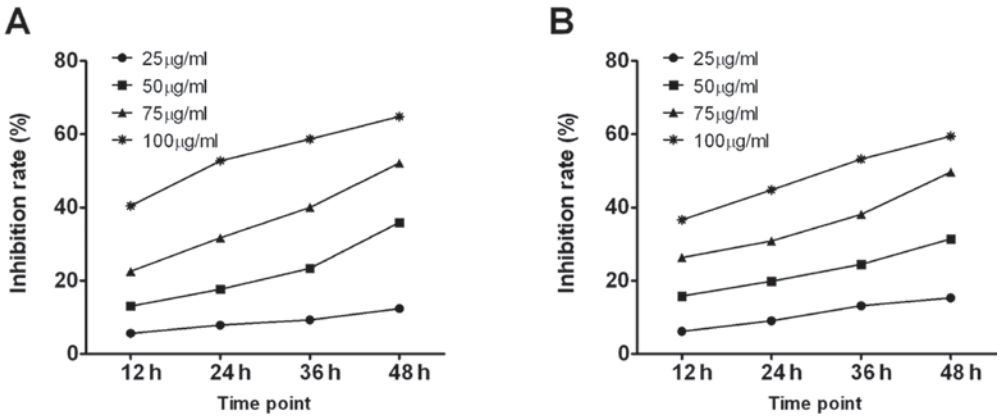

Figure 1. Cell proliferation inhibition rate. (A) SMMC-7721 cell line. (B) HepG2 cell line.

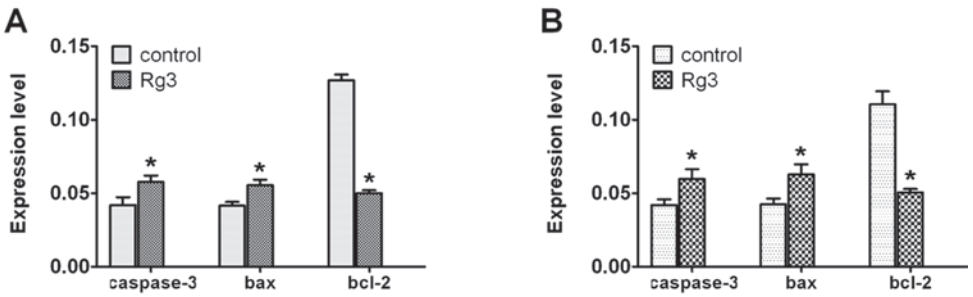

Figure 2. Expression levels of *caspase-3*, *bax* and *bcl-2*. (A) SMMC-7721 cell line. (B) HepG2 cell line. All values represent the means ± SD. *P<0.05 denotes statistically significant differences vs. the control group.

of 48 h was higher than the group of 24 h (Table IV). This results was statistically significant and showed that Rg3 promoted cancer cell apoptosis in a concentration- and time-dependent manner which was consistent with the results of the MTT assay.

Expression levels of caspase-3, bax and bcl-2. In an effort to determine the effects of Rg3 on the expression profile of apoptotic-related genes, we used real-time PCR technology to detect the mRNA expression levels of *caspase-3*, *bax* and *bcl-2* genes in the Rg3 and control groups. Real-time PCR analysis demonstrated that the expression levels of *caspase-3* and *bax* were upregulated in the Rg3 groups when compared to the control group in both the SMMC-7721 and HepG2 cell lines, but the expression level of *bcl-2* was downregulated in the Rg3 group when compared to the control (Fig. 2).

Discussion

Ginseng is one of the most commonly used Chinese medicines in China, Asia and Western countries. The beneficial effects of ginseng have been attributed to the biological activities of its constituents, the ginsenosides (4). To date, more than 150 naturally occurring ginsenosides have been isolated from roots, leaves, stems, fruits and flower heads of ginseng. It has been reported that the potential health effects of ginsenosides include anticarcinogenic, immunomodulatory, anti-inflammatory, anti-allergic, antiatherosclerotic, antihypertensive, antidiabetic and antistress activities and effects on the central nervous system (5). Amongst all ginsenosides isolated from ginseng, ginsenoside Rg3 has gained much attention for its antitumor properties (6). Studies have found that Rg3 has significant anticarcinogenic effects in several kinds of malignant tumors, such as melanoma (7), colon (8-10), ovarian (11,12), prostate (13), breast (14) and lung cancer (15,16), but its role in human hepatocellular carcinoma is not yet well comprehended.

In the present study, we investigated the role of Rg3 in the human hepatocellular carcinoma cell lines, SMMC-7721 and HepG2, by detecting cell proliferation and apoptosis. The results demonstrate that Rg3 inhibits cancer cell proliferation and promotes cancer cell apoptosis in a concentration- and time-dependent manner. We speculate that Rg3 may have antitumor effects in human hepatocellular carcinoma by inhibiting cancer cell growth. This result is consistent with previous studies on the anticarcinogenic effects Rg3 in other malignant tumors (7-16).

In order to study the molecular mechanism of Rg3 in human hepatocellular carcinoma, we carried out real-time PCR analysis to detect the expression levels of apoptotic-related genes before and after liver cancer cells being were treated with Rg3. Our results showed that Rg3 upregulated *caspase-3* expression. *Caspase-3* is a final executive molecule in the caspase-dependent apoptotic signal transduction pathway. This suggests that Rg3 may significantly promote liver cancer cell apoptosis through the caspase-dependent pathway. Furthermore, we found that Rg3 may upregulate *bax* and downregulate *bcl-2* expressions. *Bax* is a pro-apoptotic gene and *bcl-2* is an anti-apoptotic gene from the *bcl-2* family. *Bcl-2* family is an important regulator in the endogenous mitochondrial apoptotic pathway, which can determine whether the mitochondria stimulate cell apoptosis and release pro-apoptotic factors, such as cytochrome c (17,18). This indicates that Rg3 may promote liver cancer cell apoptosis in an endogenous mitochondrial apoptotic pathway through a caspase-dependent mechanism.

In summary, our study shows that ginsenoside Rg3 inhibits the growth of human hepatocellular carcinoma cells, possibly via the endogenous mitochondrial-mediated caspase-dependent apoptotic pathway, thus laying the foundation for further studies on Rg3 in human liver cancer. Nonetheless, its role in animal experiments and clinical trials still requires further investigation.

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References

1. Lui HF: Screening for hepatocellular carcinoma. Int J Hepatol: 363151, 2011.
2. Tan W, Lu J, Chen M, *et al*: Anti-cancer natural products isolated from Chinese medicinal herbs. Chin Med 6: 27, 2011.
3. Kitagawa I, Yoshikawa M, Yoshihara M, Hayashi T and Taniyama T: Chemical studies of crude drugs (1). Constituents of Ginseng radix rubra. Yakugaku Zasshi 103: 612-622, 1983 (In Japanese).
4. Lee DC and Lau AS: Effects of Panax ginseng on tumor necrosis factor-α-mediated inflammation: a mini-review. Molecules 16: 2802-2816, 2011.
5. Christensen LP: Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 55: 1-99, 2009.
6. Jia L, Zhao Y and Liang XJ: Current evaluation of the millennium phytomedicine-ginseng (II): Collected chemical entities, modern pharmacology, and clinical applications emanated from traditional Chinese medicine. Curr Med Chem 16: 2924-2942, 2009.
7. Chen J, Peng H, Ou-Yang X and He X: Research on the antitumor effect of ginsenoside Rg3 in B16 melanoma cells. Melanoma Res 18: 322-329, 2008.
8. Luo X, Wang CZ, Chen J, *et al*: Characterization of gene expression regulated by American ginseng and ginsenoside Rg3 in human colorectal cancer cells. Int J Oncol 32: 975-983, 2008.
9. Kim SM, Lee SY, Yuk DY, *et al*: Inhibition of NF-κB by ginsenoside Rg3 enhances the susceptibility of colon cancer cells to docetaxel. Arch Pharm Res 32: 755-765, 2009.
10. Lee SY, Kim GT, Roh SH, *et al*: Proteomic analysis of the anticancer effect of 20S-ginsenoside Rg3 in human colon cancer cell lines. Biosci Biotechnol Biochem 73: 811-816, 2009.
11. Xu TM, Cui MH, Xin Y, *et al*: Inhibitory effect of ginsenoside Rg3 on ovarian cancer metastasis. Chin Med J (Engl) 121: 1394-1397, 2008.
12. Xu TM, Xin Y, Cui MH, Jiang X and Gu LP: Inhibitory effect of ginsenoside Rg3 combined with cyclophosphamide on growth and angiogenesis of ovarian cancer. Chin Med J (Engl) 120: 584-588, 2007.
13. Kim HS, Lee EH, Ko SR, Choi KJ, Park JH and Im DS: Effects of ginsenosides Rg3 and Rh2 on the proliferation of prostate cancer cells. Arch Pharm Res 27: 429-435, 2004.
14. Zhang Q, Kang X, Yang B, Wang J and Yang F: Antiangiogenic effect of capecitabine combined with ginsenoside Rg3 on breast cancer in mice. Cancer Biother Radiopharm 23: 647-653, 2008.
15. Lu P, Su W, Miao ZH, Niu HR, Liu J and Hua QL: Effect and mechanism of ginsenoside Rg3 on postoperative life span of patients with non-small cell lung cancer. Chin J Integr Med 14: 33-36, 2008.
16. Liu TG, Huang Y, Cui DD, Huang XB, Mao SH, Ji LL, Song HB and Yi C: Inhibitory effect of ginsenoside Rg3 combined with gemcitabine on angiogenesis and growth of lung cancer in mice. BMC Cancer 9: 250, 2009.
17. Gustafsson AB and Gottlieb RA: Bcl-2 family members and apoptosis, taken to heart. Am J Physiol Cell Physiol 292: C45-C51, 2007.
18. Danial NN and Korsmeyer SJ: Cell death: critical control points. Cell 116: 205-219, 2004.