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Inhibition of human ovarian cancer cell proliferation *in vitro* by ginsenoside Rh_2 and adjuvant effects to cisplatin *in vivo*

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***In vitro* and *in vivo* effects of ginsenoside Rh_2 on human ovarian tumor growth were examined by using a cell line (HRA) derived from ascites of a patient with serous cystadenocarcinoma of the ovary. The HRA cell proliferation *in vitro* was inhibited in a dose-dependent manner with dosages of 10–100 μM of ginsenoside RH_2. DNA, RNA and protein synthesis by the HRA cells was inhibited in a dose-dependent manner at more than 15 μM of ginsenoside RH_2. However, the growth of HRA cells transplanted in nude mice was not significantly inhibited by ginsenoside RH_2. On the contrary, when cisplatin was administered together with 10 μM (but not 1 μM or 100 μM) ginsenoside RH_2, the tumor growth was significantly inhibited 31 days after inoculation and the survival was also significantly prolonged, compared with not only the untreated group but also the groups given cisplatin alone or ginsenoside RH_2 alone. This indicates synergistic effects between cisplatin and ginsenoside RH_2. From monitoring of body weight and hematocrit, concentrations of ginsenoside RH_2 used in this study did not seem to cause any adverse effect.**

Key words: Cisplatin, ginsenoside RH_2, human ovarian cancer cells, nude mice, tumor growth inhibition.

Introduction

Most patients with advanced ovarian carcinoma are treated with multiple drug chemotherapy containing cisplatin after surgery to reduce tumor volume as much as possible. Such a cisplatin-based combination chemotherapy has improved survival from advanced ovarian carcinoma.[1,3] However, most patients with ovarian carcinoma who respond to such therapy subsequently relapse and become resistant to drug therapy.[4] Therefore, in such cases it is of great importance to find ways to enhance the antitumor effect of cisplatin.

Recently, two new compounds, ginsenoside Rh_1 and Rh_2 have been isolated from the methanol extract of the processed root of *Panax ginseng* CA, and Rh_2 (but not Rh_1) caused growth inhibition of cultured B16 melanoma cells.[5] Thus, we decided to investigate the effects of Rh_2 on the growth of cultured human ovarian cancer cells (designated HRA) and its adjuvant effects to cisplatin on the growth of HRA cells inoculated into nude mice. In the present study, we report that Rh_2 inhibits human ovarian cancer cell proliferation *in vitro* (but not *in vivo*) in a dose-dependent maner. Rh_2 also had a significant adjuvant effect to cisplatin on the tumor growth in nude mice.

Materials and methods

Agent

Ginsenoside Rh_2 (Rh_2) was kindly supplied by Korea Ginseng and Tobacco Research Institute, Korea, and Japan Korea Red Ginseng Co. Ltd, Japan. The Rh_2 was dissolved in ethanol and stored at 4°C.

Cell line

The cell line (HRA) used in this study was derived from ascites of a patient with serous cystadenocarcinoma of the ovary.[6] The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin (100 units/ml and 100 μg/ml respectively; Grand

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Island Biological Co.) in 5% CO_2 at 37°C. The medium was changed every 3 days, and the cells were passed when confluency was achieved. The passage number of the HRA cells used in this study was 216 and the doubling time was about 18 h.

In vitro treatment

To determine the effects of Rh_2 on the HRA cell proliferation *in vitro*, viable cells (10^4) were seeded into 24-well Nunc multidishes, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h of culture, various concentrations of Rh_2 were added to the medium. After 48 h of additional culture, cells in each well were harvested and counted using a hematocytometer. All counts were carried out in quadruplicate and the viability was assessed by trypan blue dye exclusion. The numbers of cells in wells containing fresh medium were used as controls. Concentrations of ethanol in each well were less than 0.1% and did not affect cell proliferation. Results were expressed as percentage of control.

Uptake of radiolabeled precursors by tumor cells

To determine the effect of Rh_2 on synthesis of DNA, RNA, and protein by the HRA cells, 10^5 cells/ml were seeded into 24-well Nunc multidishes. After 24 h of culture, various concentrations of Rh_2 were added into the medium. The DNA, RNA, and protein syntheses by the tumor cells were assayed by adding 37 kBq of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-valine to each well during the last 4 h of the additional 48-h incubation period. The cells in each well were harvested with 10% trypsin and collected by centrifugation. The collected cells were precipitated with 15% trichloroacetic acid (TCA) and the precipitate was collected by centrifugation. The amount of radioactivity in the TCA-insoluble material solubilized by 8% sodium dodecyl sulfate was determined by a liquid scintillation counter. Assays were performed in quadruplicate.

Nude mice

Six-week-old female BALB/c nude mice were obtained from Japan Clea Laboratories, Tokyo, Japan, and maintained in a pathogen-free environment. When necessary, the animals were killed and dissected. The tumor tissues were fixed in formalin for histological examination and stained with hematoxylin and eosine.

In vivo treatment

To determine the adjuvant effects of Rh_2 to cisplatin on HRA tumor growth. 9×10^5 HRA cells were inoculated sc into the right flank of nude mice. After 7 days of tumor inoculation, 2 mg/kg cisplatin (*cis*-diamminedichloroplatinum (II); CDDP), 1 μM Rh_2, 10 μM Rh_2, or 100 μM Rh_2 was administered ip once a week for 5 weeks. The mice were divided into the following groups; 10 mice treated with 0.1 ml medium alone containing 0.1% ethanol; 10 mice treated with 2 mg/kg CDDP alone; 10 mice treated with 1 μM Rh_2 alone; 10 mice treated with 2 mg/kg CDDP and 1 μM Rh_2; 10 mice treated with 10 μM Rh_2 alone; 10 mice treated with 2 mg/kg CDDP and 10 μM Rh_2; 10 mice treated with 100 μM Rh_2 alone; 10 mice treated with 2 mg/kg CDDP and 100 μM Rh_2. The tumor growth was determined by the measurement of diameters in two dimensions of the tumor nodule with a caliper once a week. Tumor volume (cm^3) was calculated according to the following formula: $(4\pi/3)\ (r_1 + r_2)^3/8$, where r_1 is the longitudinal radius and r_2 is the transverse radius. Blood from a tail vein was collected into hematocrit tubes every week, and hematocrit values and body weight were recorded to monitor the side-effects of drugs.

Statistical analysis

Results are presented as the mean $\pm$ SD. The results were analyzed by non-parametric methods.

Results

As shown in Figure 1, proliferation of HRA cells cultured *in vitro* was inhibited in a dose-dependent manner at dosages between 10 and 100 μM Rh_2, while no significant inhibition was observed at less than 10 μM Rh_2. We attempted to elucidate the inhibition mechanism on the HRA cell proliferation by Rh_2 by examining the effect on ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-valine uptakes by the cells (Table 1). Although uptakes of radiolabeled precursors were not significantly inhibited at the IC_{50} concentration (30 μM) of Rh_2, the addition of 60 μM Rh_2 reduced the uptake of ^{3}H-thymidine and

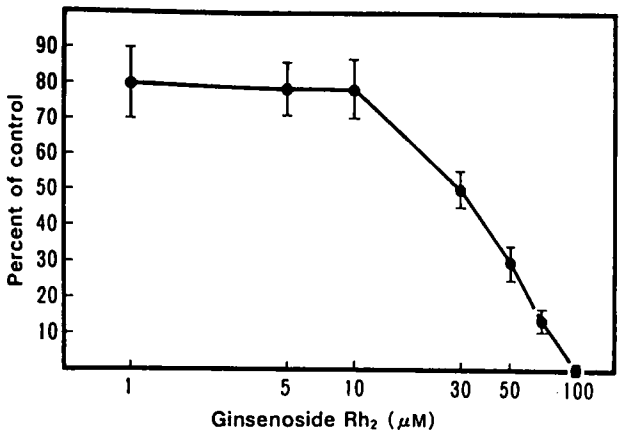

Figure 1. Inhibitory effects of Rh_2 on the HRA cell proliferation. The 10^4 viable cells were seeded into 24-well Nunc multidishes. After 24 h of culture, various concentrations of Rh_2 were added to the medium. After 48 h of additional culture, cells in each well were harvested and counted. Vertical bars show the mean ± SD.

^{3}H-uridine by about 50% of that by 30 μM Rh_2. Inhibition of the uptake of ^{3}H-valine by 60 μM Rh_2 was more marked and the uptake was decreased to about one-fifth of that by 30 μM Rh_2. In the presence of 100 μM Rh_2 the uptake of radiolabeled precursors used in this study was completely inhibited.

Next, we attempted to determine whether the inhibitory effect of Rh_2 on tumor growth *in vitro* can be observed in tumors grown in nude mice. As shown in Figure 2, no significant inhibition of the tumor growth was observed at any day after tumor inoculation by Rh_2 alone. By contrast, when 10 μM Rh_2 (but not 1 μM or 100 μM Rh_2) was combined with CDDP, the tumor growth in nude mice was significantly ($P < 0.05$) inhibited 31 days after tumor inoculation, compared with that of the untreated group. When the survival in each treatment group was investigated (Figure 3), the 50% survival of nude mice in the group treated with

Table 1. Effects of ginsenoside Rh_2 on uptakes of radiolabeled precursors by HRA cells

Concentrations (μM)	Radiolabeled precursors		
	^{3}H-thymidine	^{3}H-uridine	^{3}H-valine
0	6481[a]	5091	43834
15	4628	4181	56892
30	4844	3849	50591
60	2426	1897	9513
100	111	21	64

[a] Average from three experiments (cpm).

CDDP + Rh_2 (10 μM) was found to be longest (70 days), followed by 60 days survival of the group treated with CDDP + Rh_2 (100 μM) and 58 days of the groups treated with only CDDP, only Rh_2 (1 μM) or only Rh_2 (100 μM). Furthermore, the survival time of nude mice treated with 2 mg/kg CDDP and 10 μM Rh_2 was significantly prolonged, compared with not only the untreated group but also the groups treated only with CDDP or only with Rh_2.

Discussion

Rh_2 is a plant glycoside with a dammarane skeleton resembling a steroid skeleton such as an aglycone. In the present study, we have demonstrated that Rh_2 inhibited HRA cell proliferation *in vitro* in a dose-dependent manner with dosages between 10 and 100 μM, and that the IC_{50} was 30 μM. Odashima *et al.*[5] have also reported inhibition of B16 melanoma cell growth by Rh_2. With regard to the IC_{50}, the degree of inhibition by Rh_2 of B16 melanoma cell proliferation was 3-fold higher than that of HRA cell proliferation. In experiments on the uptake of radiolabeled precursors by the HRA cells, uptakes of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-valine were not inhibited by 30 μM of Rh_2 while 60 μM of Rh_2 decreased the uptakes of ^{3}H-thymidine and ^{3}H-uridine to about one-half. The uptake of ^{3}H-valine was further decreased to about one-fifth, and when treated with 100 μM Rh_2 the uptake of all radiolabeled precursors used in this study was completely inhibited. These results suggest that Rh_2 inhibits not only DNA or RNA synthesis but also preferentially protein synthesis. However, even if Rh_2 was administered ip to nude mice bearing the HRA cells the tumor growth was not inhibited with Rh_2 alone (Figure 2). When 10 μM of Rh_2 was combined with CDDP, the tumor growth was significantly inhibited, 31 days after tumor inoculation. These results suggest that Rh_2 has adjuvant effects to CDDP with regard to inhibition of the tumor growth *in vivo*. We therefore examined the effects of Rh_2 on the survival of tumor-bearing nude mice. The survival time of nude mice treated with 10 μM Rh_2 and CDDP was longest and significantly longer than that of nude mice in not only untreated groups but also groups treated with single agents. This indicates synergistic effects on the survival by a combination of 10 μM Rh_2 and CDDP (Figure 3). In addition, it is noteworthy that concentrations of Rh_2 and combinations with CDDP used in this study did

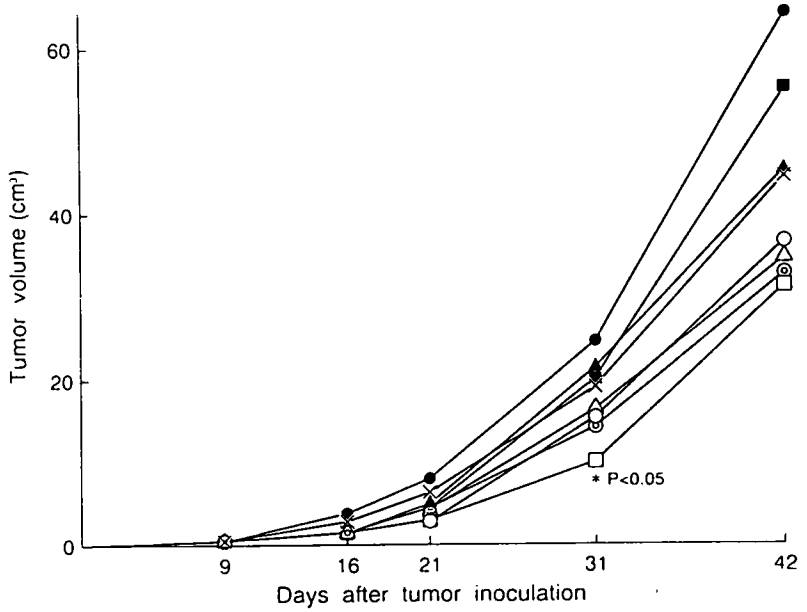

Figure 2. Effects of Rh_2 alone or combinations of Rh_2 and CDDP on growth of the HRA tumor inoculated in nude mice. The 9 × 10^5 HRA cells were inoculated sc into the right flank of nude mice. After 7 days of tumor inoculation, 2 mg/kg CDDP, 1 μM Rh_2, 10 μM_2 Rh_2, or 100 μM Rh_2 was administered ip once a week for 5 weeks. The mice were divided as described in 'Materials and methods.' ×, untreated; ○, CDDP alone; ●, Rh_2 (1 μM); △, CDDP + Rh_2 (1 μM); ▲, Rh_2 (10 μM); □, CDDP + Rh_2 (10 μM); ■, Rh_2 (100 μM); ◎, CDDP + Rh_2 (100 μM); $*P < 0.05$, compared with the untreated group.

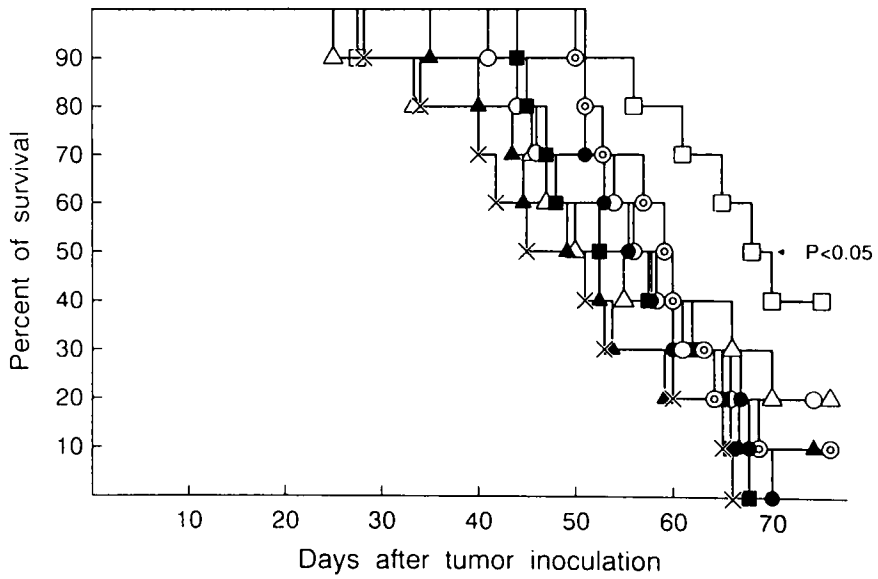

Figure 3. Effects of Rh_2 alone or combinations of Rh_2 and CDDP on the survival time of nude mice with HRA cell tumor. Nude mice in each group were treated as described in 'Materials and methods'. The 50% survival was 51 days for the untreated group (×), 52 days for the group treated with Rh_2 (10 μM) alone (▲), 55 days for the group treated with CDDP + Rh_2 (1 μM) (△), 58 days for the groups treated with CDDP alone (○), Rh_2 (1 μM) alone (●) and Rh_2 (100 μM) alone (■), 60 days for the group treated with CDDP + Rh_2 (100 μM) (◎), and 70 days for the group treated with CDDP + Rh_2 (10 μM) (□). $P < 0.05$, compared with not only the untreated group but also the groups treated with CDDP alone or Rh_2 alone.

not cause loss of body weight and lowering of hematocrit (data not shown). Accordingly, we conclude that a combination of Rh_2 with CDDP may be used for treatment of refractory ovarian carcinoma.

Conclusion

Ginsenoside Rh_2 (Rh_2) inhibited the human ovarian cancer cell (HRA) proliferation *in vitro* in a dose-dependent manner with dosages between 10 and 100 μM. Results of uptakes of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-valine indicated that dosages of more than 15 μM of Rh_2 also inhibited all DNA, RNA and protein syntheses by the HRA cells in a dose-dependent manner. In experiments *in vivo*, the concentrations of Rh_2 used in this study did not inhibit growth of HRA tumor cells inoculated in nude mice. On the contrary, when CDDP was administered together with 10 μM (but not 1 and 100 μM) Rh_2, the tumor growth was significantly inhibited 31 days after tumor inoculation. The survival time of the mice was also significantly prolonged, compared not only with the untreated mice but also with the mice treated with CDDP alone or Rh_2 alone. The concentrations of Rh_2 used in this study did not cause any adverse effect. These results suggest that a combination of Rh_2 and CDDP may be of clinical use for ovarian carcinoma.

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