

Anticarcinogenic Effect and Enhancement of Metastatic Potential of BALB/c 3T3 Cells by Ginsenoside Rh₂

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It has been reported that ginsenoside Rh₂, a purified ginseng saponin with a dammarane skeleton, has anticarcinogenic effects on mammalian cells. To determine the significance of these effects on multistage carcinogenesis, we utilized the BALB/c 3T3 cell system. In an *in vitro* two-stage neoplastic transformation assay, the initiating activity of 3-methylcholanthrene (3-MCA) was suppressed by Rh₂ ($\geq 1 \times 10^{-5}$ M) in both BALB/c 3T3 A31-1-1 cells and the more carcinogen-susceptible variant A31-1-13 cells. The suppressive effects in this concentration range were thought to be caused by suppression of DNA replication via indirect Cdk2 inhibition. On the other hand, the promotion steps of both the target cells were not affected by Rh₂ even if the transformation frequency was enhanced by a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). To examine the other effects of Rh₂ on carcinogenesis, we turned our attention to the metastatic phenotype. Using metastatic *src*-transformed A31-1-1 cells, we found that Rh₂ augmented the metastatic potential in an experimental metastasis assay. These data indicate that Rh₂ has diverse effects on the expression of the transformed phenotype in BALB/c 3T3 cells, but support the idea that growth suppression is likely to be a major mechanism of the anticarcinogenic effects of Rh₂.

Key words: Ginseng — Cdk2 — Chemoprevention — Transformation — Metastasis

The discovery of new cancer-preventing agents and elucidation of the basic mechanisms relevant to cancer prevention provide various opportunities for us to design new drugs or to discover micronutrients for suppressing carcinogenesis and/or cancer invasion and metastasis. There are a number of natural and artificial sources for chemical compounds effective in preventing cancer, and many effective agents are being discovered and characterized.¹⁾ The ingredients of medicines used in folk remedies are one such source in the search for chemopreventive agents. Ginsenoside Rh₂ has been purified from the root of *Panax ginseng* C. A. Meyer, an important medicinal resource for thousands of years in China, Korea, and Japan, and has been reported to have anticancer potential based on growth inhibition activity,²⁻⁴⁾ the induction of untransformed phenotype,⁵⁻¹⁰⁾ the induction of apoptosis,¹¹⁻¹⁶⁾ and the modulation of DNA metabolism.¹⁷⁻¹⁹⁾ In this paper, using a two-stage transformation system with BALB/c 3T3 cells,²⁰⁾ we investigated the possibility of utilizing Rh₂ for cancer chemoprevention. Our results indicate that Rh₂ has a chemopreventing activity in the initiation step of *in vitro* carcinogenesis, but does not have any effect on the tumor promotion step. In addition, it caused an enhancement of the metastatic potential of *src*-transformed BALB/c 3T3 cells.

MATERIALS AND METHODS

Cell cultures The BALB/c 3T3 A31 variant cell lines 1-1 (A31-1-1) and 1-13 (A31-1-13) were originally established by Kakunaga and Crow²¹⁾ and have different susceptibilities to radiation- and chemical-induced neoplastic transformation.²⁰⁾ The different susceptibilities were thought to be due to their different potentials for expression of transformed phenotype during the promotion step of carcinogenesis.²²⁻²⁴⁾ For *in vitro* transformation assay, we used cells at an early passage number from the original cell stock. Highly metastatic *v-src*-transformed A31-1-1 cells (1-1src) and poorly metastatic *c-Ha-ras*-transformed A31-1-1 cells (1-1ras1000) were also used in this study.^{25, 26)} All the cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Lot No. SF70521, Bocknek Laboratories, Etobicoke, Ontario, Canada) in 5% CO₂ at 37°C. The transformation experiments were also performed with the same lot of serum.

***In vitro* transformation assay** Exponentially growing cells (10⁴) were seeded onto 60-mm culture dishes (Nalge Nunc International, Tokyo), with 4 ml of culture medium in the presence of 10% fetal bovine serum. Transformation was initiated 24 h later with 2.5 μg/ml 3-methylcholanthrene (3-MCA) for A31-1-1 cells or with 1 μg/ml 3-MCA for A31-1-13 cells. After the initiation period with 3-MCA for 24 h, the cells were treated according to the

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following protocols. To test the effect of Rh₂ on the initiation step of cell transformation, the initiated cells were treated with Rh₂ in the presence of 2% fetal bovine serum. Two days later, the Rh₂-containing medium was replaced with fresh medium containing 10% fetal bovine serum and then culture was continued by replacing the fresh medium twice weekly. To test the effect of Rh₂ on the promotion step of cell transformation, the initiated cells were cultured in fresh medium containing 10% fetal bovine serum by replacing the fresh medium once. Seven days later, the culture medium was replaced by fresh medium containing phorbol ester and/or Rh₂. Promotion was continued by replacing the medium containing these factors twice weekly. Two weeks later, the culture medium was replaced by fresh medium containing 10% fetal bovine serum and then culture was continued by replacing the fresh medium twice weekly. Thirty to 35 days after the initiation of the experiment, all cultures were fixed in methanol and stained with 2% Giemsa solution. Morphological transformation was determined under a dissecting microscope. Only densely stained multilayer foci with criss-crossing of the cells at their periphery were scored.²⁰⁾

Assay of Cdk2 activity The immune complex kinase assay was carried out as described by Matsushime *et al.*²⁷⁾ The 3-MCA-initiated cells treated with Rh₂ to test the effect of Rh₂ on the initiation step of cell transformation were collected, suspended in immunoprecipitation buffer and sonicated. Lysates (200 mg protein) were precipitated with protein A-Sepharose beads precoated with anti-Cdk2 antibody (Santa Cruz BioTechnology, Santa Cruz, CA). The immunoprecipitated proteins on the beads were washed and suspended in kinase buffer containing the substrate, histone H1 (1 mg, Roche Diagnostics, Tokyo), cold adenosine 5'-triphosphate (ATP) (50 mM) and [γ -³²P]ATP (5 mCi, Amersham Pharmacia Biotech, Tokyo, ~3000 Ci/mmol). To determine the *in vitro* suppression effect, Rh₂ (2×10^{-5} M) or olomoucine (2×10^{-5} M, Promega, Madison, WI) was added to the reaction mixture. Olomoucine is a chemically synthesized Cdk2 inhibitor that is competitive for ATP binding.²⁸⁾ After incubation for the kination, the samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The phosphorylated protein was autoradiographically visualized and the incorporated ³²P was measured with a Bio-Imaging Analyzer (BAS 2000, Fuji, Tokyo).

Experimental metastasis assay Male BALB/c nude mice were obtained at 6–7 weeks of age. Exponentially growing 1-src cells or 1-tras1000 cells were treated with or without Rh₂ (2×10^{-5} M for 24 h). The cells were collected by trypsinization and washed with phosphate-buffered saline. For the experimental metastasis assay, 5×10^5 cells were injected intravenously into the tail vein of 7- to 8-week-old nude mice. The mice injected with the cells were killed after 2 weeks and autopsied to establish the

extent of metastasis.²⁵⁾ Metastatic lung nodules were counted after insufflation with 15% India ink.²⁹⁾

RESULTS

To establish whether Rh₂ has anticarcinogenic effects on cell transformation, we used a two-stage transformation system with BALB/c 3T3 cells. In this system, variant cell lines with different susceptibilities to chemical and physical carcinogens are available as target cells for the estimation of initiation and promotion effects of agents.²⁰⁾ Using this system, therefore, it is possible to evaluate the chemoprevention effects of Rh₂ on the two distinct stages of carcinogenesis, 'initiation' and 'promotion,' in cells with different levels of carcinogen susceptibility.^{20, 21, 30, 31)}

It was reported previously that Rh₂ had a suppressive activity on both spontaneous and carcinogen-induced formation of sister chromatid exchanges at Rh₂ concentrations lower than 1×10^{-10} M.¹⁹⁾ To assess this chemoprevention activity, we first applied Rh₂ during the initiation period (48 h; 2nd–3rd day of BALB/c 3T3 cell transformation protocol) at a wide range of concentrations (2×10^{-5} – 1×10^{-12} M) to 3-MCA-initiated cells (transformation-susceptible A31-1-1 cells treated with 2.5 μ g/ml 3-MCA for 24 h and highly transformation-susceptible A31-1-13 cells treated with 1 μ g/ml 3-MCA for 24 h). In control culture without 3-MCA treatment, Rh₂ showed no effect on BALB/c 3T3 cell transformation at any concentration tested (data not shown). This is consistent with the report that Rh₂ is not mutagenic in the Ames test³²⁾ using *Salmonella typhimurium* (unpublished data). In initiated cells, the transformation frequencies of both A31-1-1 and A31-1-13 cells were significantly suppressed by Rh₂ only at a high concentration range ($\geq 1 \times 10^{-5}$ M), while no effect was found at a lower concentration range (1×10^{-7} – 1×10^{-12} M) (Fig. 1). The inhibition of DNA replication after carcinogen treatment reduces the frequency of transformation.^{33, 34)} Rh₂ induces G₁ block at the high concentration range via indirect Cdk2-inhibition ($\geq 1 \times 10^{-5}$ M).²⁾ Thus, the suppressive effects of Rh₂ on the initiation of transformation are thought to be due to this mechanism. Indeed, the dose-response of Cdk2 inhibition by Rh₂ treatment was correlated closely with the Rh₂-induced suppression of cell transformation when measured by the immune complex kinase assay described previously,²⁾ although Rh₂ did not directly inhibit the Cdk2 activity *in vitro* (Fig. 2).

To test the chemopreventive effects of Rh₂ on the promotion step of cell transformation, we next applied Rh₂ during the promotion period (2 weeks; 8th–22nd day of BALB/c 3T3 cell transformation protocol) to 3-MCA-initiated A31-1-1 and A31-1-13 cells (A31-1-1 cells treated with 2.5 μ g/ml 3-MCA for 24 h and A31-1-13 cells treated with 1 μ g/ml 3-MCA for 24 h) and also to 3-MCA-initiated 12-O-tetradecanoylphorbol-13-acetate

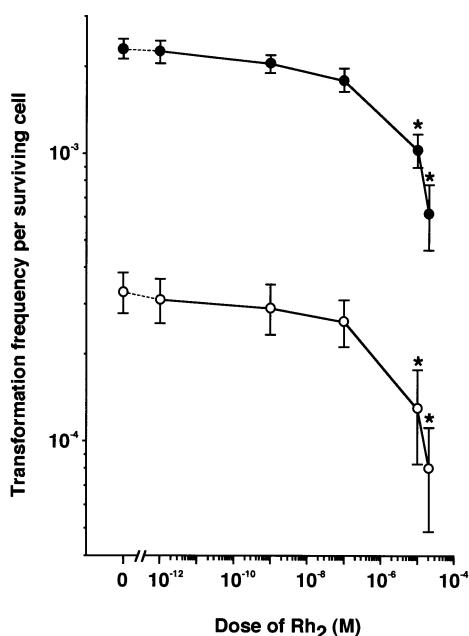


Fig. 1. Effect of Rh₂ on the initiation step of cell transformation using BALB/c 3T3 A31-1-1 cells (○) and A31-1-13 cells (●). The target cells (1 × 10⁴) were treated with or without 3-MCA (2.5 μg/ml for A31-1-1 cells and 1 μg/ml for A31-1-13 cells) for 24 h, and then the initiated cells were treated with Rh₂ for 48 h. The results were confirmed by two independent experiments. The transformation frequencies for non-initiated groups were at a basal level at all Rh₂ concentrations. *, Result significantly different from initiated cells without Rh₂ (P < 0.01 by two tailed Mann-Whitney U test). Data points, means of one representative experiment with over eight dishes. Bars, SE.

(TPA)-promoted A-31-1-1 cells (10⁻¹ μg/ml TPA for 2 weeks during 8th–22nd day). The data clearly indicated that Rh₂ had no effect at any concentration range (2 × 10⁻⁵–1 × 10⁻¹² M) on the tumor promotion step in the BALB/c 3T3 cell transformation system (Fig. 3).

Rh₂ is known to have some biological effects on cell differentiation, such as the stimulation of melanogenesis in melanoma cells,⁷ glucocorticoid-like action in teratocarcinoma cells,⁹ and the modulation of protein kinase C activity in HL-60 cells.¹⁰ Thus, Rh₂ may modulate certain cancerous features in transformed cells. To examine this possibility in the BALB/c 3T3 cell system, we turned our attention to the effects on the metastatic phenotype in *src*-transformed A31-1-1 cells (1-1src cells). 1-1src is metastatic to the lung, and the metastatic potential can be quantitatively detected by intravenous injection of the cells into BALB/c nude mice.²⁵ When 1-1src cells were treated with Rh₂ (2 × 10⁻⁵ M for 48 h), the cells were growth-suppressed and their Cdk2 activity was reduced. This Cdk2 reduction induced by Rh₂ was similar to that observed in

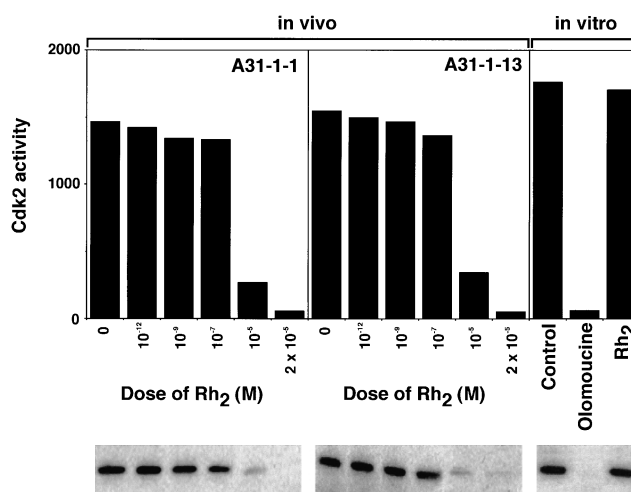


Fig. 2. Effect of Rh₂ on Cdk2 activity in BALB/c 3T3 A31-1-1 cells and A31-1-13 cells. The 3-MCA-initiated and Rh₂-treated cells indicated in Fig. 1 were collected, suspended in immunoprecipitation buffer and sonicated. Lysates were immunoprecipitated with anti-Cdk2 antibody and suspended in kinase buffer. To determine an *in vitro* effect on the suppression of Cdk2, Rh₂ (2 × 10⁻⁵ M) or olomoucine (2 × 10⁻⁵ M, positive control for Cdk2 suppression) was added to the immunoprecipitated samples from control A31-1-13 cells. After incubation for kination, the samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and phosphorylated proteins were autoradiographically visualized. The incorporated ³²P was measured by a Bio-Imaging Analyzer. Columns, means of data from two independent experiments.

Rh₂-treated A31-1-1 and A31-1-13 cells. When the Rh₂-treated 1-1src cells were intravenously injected into the mice, we found that the metastatic potential of the treated cells was significantly increased (Fig. 4A). Quantification was performed by counting pulmonary metastasis nodules, and the data are presented in Fig. 4B. A similar enhancement effect on metastatic potential was observed when *ras*-transformed A31-1-1 cells (1-1ras1000 cells) were treated with Rh₂ (2 × 10⁻⁵ M for 48 h) (Fig. 4B). Such an enhancement effect was not observed at a lower Rh₂ concentration range (≤ 1 × 10⁻⁷ M) (data not shown).

DISCUSSION

Here, we have shown that Rh₂ has an anticarcinogenic effect on the BALB/c 3T3 cell transformation system during the initiation step and also has an enhancing effect on the metastatic potential of *src*-transformed BALB/c 3T3 cells. The transformation frequency is known to be reduced by suppression of DNA replication and to be enhanced by stimulation of DNA replication after carcinogen treatment.^{33, 34} Rh₂ is likely to affect the transforma-

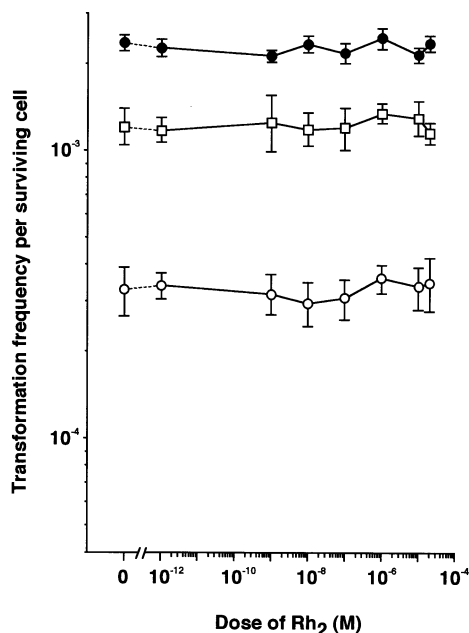


Fig. 3. Effect of Rh₂ on the promotion step of cell transformation using BALB/c 3T3 A31-1-1 cells (○, □) and A31-1-13 cells (●). The target cells (1×10^4) were treated with or without 3-MCA ($2.5 \mu\text{g}/\text{ml}$ for A31-1-1 cells and $1 \mu\text{g}/\text{ml}$ for A31-1-13 cells) for 24 h, and after a week the initiated cells were treated with Rh₂ (○, ●) or with Rh₂ plus $10^{-1} \mu\text{g}/\text{ml}$ TPA (□) for 2 weeks. The results were confirmed by two independent experiments. The transformation frequencies for non-initiated groups were at a basal level at all Rh₂ concentrations. Data points, means of one representative experiment with over eight dishes. Bars, SE.

tion frequency by this mechanism through Cdk2 inhibition in the higher concentration range ($\geq 1 \times 10^{-5} M$), but to have no effect on the tumor promotion step after the fixation of the transformation initiation. Zhu *et al.*¹⁹⁾ reported that the formation of sister chromatid exchanges in human lymphocytes was suppressed by Rh₂ treatment even at an ultra low concentration of Rh₂ ($1 \times 10^{-13} M$). However, in this study, no significant effect on BALB/c 3T3 cell transformation was detected at such a low concentration of Rh₂.

On the other hand, during the late stages of carcinogenesis in the BALB/c 3T3 cell system, Rh₂ has an enhancing effect on the metastatic potential of *src*-transformed 1-1src cells at the same concentration range as that which is effective for the inhibition of Cdk2. In this concentration range, the Src kinase activity of the cells was not altered by Rh₂ treatment (data not shown). One possible explanation of the enhancing effect on metastatic potential is that Rh₂-induced Cdk2 inhibition contributes to the prevention of apoptosis of the intravenously injected cells in the blood and lung. Indeed, Rh₂-treated 1-1src cells are resis-

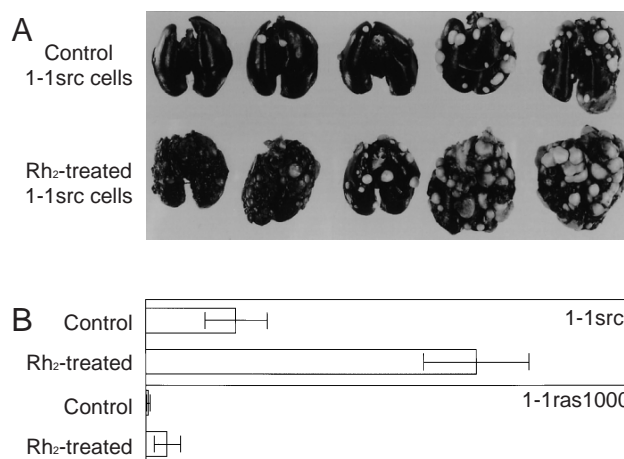


Fig. 4. Enhancement of metastatic capability by Rh₂ in BALB/c 3T3 A31-1-1-derived *v-src*-transformed cells (1-1src) and *c-Ha-ras*-transformed cells (1-1ras1000). Exponentially growing cells were treated with or without Rh₂ ($2 \times 10^{-5} M$ for 24 h), and then the cells (5×10^5 cells per animal) were injected intravenously into the tail veins of 7- to 8-week-old nude mice. The mice were killed after 2 weeks and autopsied to examine them for metastases. Metastasized nodules were observed only in the lung, and typical lung nodules in mice injected with 1-1src cells are shown (A). The nodules were counted after insufflation with 15% India ink (B). The number of nodules was significantly different between control and Rh₂-treated cells ($P < 0.05$ by two tailed Mann-Whitney *U* test). Bars, SE.

tant to X-ray-induced apoptosis (unpublished data). Moreover, the forced overexpression of a dominant negative form of Cdk2 was found to have a suppressive effect on apoptosis in doxorubicin-treated colorectal tumor cells.³⁵⁾ Thus, Cdk2 inhibition is likely to produce an apoptosis-resistant state resulting in an enhanced metastatic potential in transformed cells. Another possible explanation is that the modulation of cellular surfaces by Rh₂, such as increment of the binding sites for peanut agglutinin,³⁶⁾ results in the enhancement of metastatic potential caused by adhesion to blood vessel endothelial cells or between the Rh₂-treated cells. Although further studies are required to elucidate the molecular mechanism of Rh₂ action on the metastatic potential in tumor cells, such an action suggests that possible chemopreventive and chemotherapeutic agents should be carefully evaluated for their effectiveness and side effects. To our knowledge, this paper is the first report to convincingly show side effects on a transformed phenotype by a component of ginseng. As not only shown by us in this report, but also pointed out in a review article,³⁷⁾ the diverse effects of natural components should be understood carefully and accurately in order to discover, develop, and apply them to cancer therapy and for health promotion.

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