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Geranylgeranylacetone inhibits ovarian cancer progression *in vitro* and *in vivo* ☆

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Abstract

Geranylgeranylacetone (GGA), an isoprenoid compound, is an anti-ulcer drug developed in Japan. In our previous study, GGA was shown to inhibit ovarian cancer invasion by attenuating Rho activation [K. Hashimoto, K. Morishige, K. Sawada, M. Tahara, S. Shimizu, M. Sakata, K. Tasaka, Y. Murata, Geranylgeranylacetone inhibits lysophosphatidic acid-induced invasion of human ovarian carcinoma cells in vitro. Cancer 103 (2005) 1529–1536]. In the present study, GGA treatment inhibited ovarian cancer progression *in vitro* and suppressed the tumor growth and ascites in the *in vivo* ovarian cancer model. *In vitro* analysis, treatment of cancer cells by GGA resulted in the inhibition of cancer cell proliferation, the inactivation of Ras, and the suppression of tyrosine phosphorylation of mitogen-activated protein kinase (MAPK). In conclusion, this is the first report that GGA inhibited ovarian cancer progression and the antitumor effect by GGA is, at least in part, derived not only from the suppression of Rho activation but also Ras–MAPK activation.

Keywords: Geranylgeranylacetone; Lysophosphatidic acid; Rho; Ras; Geranylgeranylation; Ovarian cancer

Among many growth-promoting factors known to be present in ovarian cancer ascites, lysophosphatidic acid (LPA) is found at significant levels (~10 μM) and may play an important role in the development or progression of ovarian cancer [1]. LPA has been reported to induce many cellular effects, including mitogenesis, the secretion of proteolytic enzymes [2] and migration activity [3].

Protein isoprenylation, such as geranylgeranylation and farnesylation, is a post-translational modification that is essential for the membrane localization and full function of small GTP-binding proteins (G proteins), such as Rho, Rac, and Ras [4,5]. Since small G proteins play crucial roles in signal transduction, isoprenoids are of fundamental importance in the control of various cellular functions [6]. Recently, several isoprenyl compounds such as farnesol (FOH), geranylgeraniol (GGOH), and geranylgeranoic acid have been shown to induce apoptotic cell death [7,8] and modulate cell motility [9,10]. Accordingly, isoprenyl compounds might influence cancer cell activity.

Geranylgeranylacetone (GGA), an isoprenoid compound developed in Japan, has been used orally as an anti-ulcer drug. GGA protects the gastric mucosa from various stresses without affecting the gastric acid secretion [11]. Moreover, the chemical structure of GGA is similar to that of geranylgeranylpyrophosphate, which is a component in the metabolic pathway of Rho and is essential for geranylgeranylation of Rho [12].

Abbreviations: LPA, lysophosphatidic acid; GGA, geranylgeranylacetone; GGOH, geranylgeraniol; FOH, farnesol; ROCK, Rho-associated kinase.

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In our previous study [13] GGA inhibited Rho activity, cell motility and the invasion of cancer cells, in the same way as do HMG-CoA reductase inhibitors [9,14,15] and bisphosphonates [10,16]. GGA has also been demonstrated to suppress cell growth and induce differentiation or apoptosis by modulating small G protein activation in leukemia cell lines [17,18].

The Ras–Raf–MAP kinase/Erk kinase (MEK)-extracellular signal regulated kinase (Erk) cascade has a central role in regulating tumor cell growth, survival, and has been targeted for therapeutic intervention in the past [19]. LPA is also the prototypic receptor agonist that stimulates Ras through pertussis toxin-sensitive G*i* [20,21]. As such, LPA acts like epidermal growth factor with respect to down stream Ras signaling, particularly MAP kinase. In this study, we analyzed the effects of GGA on Ras/MEK/Erk signaling *in vitro*, and showed that GGA markedly inhibited LPA-induced proliferation of human ovarian cancer cells by attenuating the activation of Ras signaling. Thus, GGA inhibited not only the cell motility [13], but also the proliferation of ovarian cancer cells.

Advanced ovarian cancer cases respond well to platinum-containing chemotherapy after debulking surgery [22]. However, most cases (60–80%) relapse after the remission status [23,24]. Therefore, preventive or consolidation therapy after complete remission is clinically important to avoid recurrence. Here, we tested GGA in the preventive protocol using an intraperitoneal dissemination model [25] of ovarian cancer.

Materials and methods

Materials. GGA was supplied by Eisai Co. (Tokyo, Japan). Bovine serum albumin (BSA), collagen (type I) and LPA were purchased from Sigma (St. Louis, MO). Anti-phospho-p44/p42 MAPK (Thr 202/Tyr 204) (Erk) polyclonal antibody and anti-p44/p42 MAPK (Erk) polyclonal antibody were purchased from Cell Signaling Technology (Beberly, MA). Anti-MEK1 monoclonal antibody and the Ras activation assay Kit were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-MEK-1/2 (Ser 218/Ser 222) polyclonal antibody, horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride membrane (Hybond-P) and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham (Arlington Heights, IL). The Cell Titer 96AQ kit to monitor cell proliferation was purchased from Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco-BRL (Gaithersburg, MD).

Cell culture. The human ovarian cancer cell lines, Caov-3 and SKOV-3 were purchased from American Type Culture Collection (Rockville, MD). Both cell lines were grown in DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (10 U/ml)-streptomycin (10 U/ml) in 95% air, and 5% CO_2 at 37 °C, and were used within 15 passages after the initiation of culture.

Cell proliferation assessment. Cell proliferation was assessed by the MTT assay using a Cell Titer 96AQ kit according to the manufacturer's instructions. Briefly, the cells (3×10^3/well) were plated in 96-well plates and allowed to attach for 4.5 h, and then cultured with 25 μM LPA and various concentrations of GGA for 24–72 h in DMEM supplemented with 2% FBS. The number of surviving cells was determined by measuring the absorbance at 590 nm (A 590 nm) of the dissolved formazan product formed after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt for 1 h as described by the manufacturer. All of the experiments were carried out in quadruplicate, and proliferation was expressed as the ratio of the absorbance of GGA treated and control cells to the absorbance before treatment.

Ras pull-down assay. The Ras pull-down assay was performed using Ras activation assay kit according to the manufacturer's instructions. Briefly, cells were cultured under serum-free conditions for 24 h, and then pretreated with GGA and GGOH for 24 h. After pretreatment, cells were stimulated with 25 μM LPA for 2 min, washed with cold PBS, and lysed. Cell lysates were clarified by centrifugation, and equal volumes of lysates were incubated with Raf-1 RBD-agarose beads. Bound Ras proteins were detected by Western blotting using a monoclonal antibody against Ras. Western blotting to estimate the total amount of Ras in cell lysates was performed for the comparison of Ras activity (level of GTP-bound Ras) in the same samples.

Western blot analysis. The cells were allowed to attach and cultured in serum-free conditions with or without GGA and GGOH for 24 h. After the treatment, cells were stimulated with 25 μM LPA for 30 min, washed and lysed in sample buffer. Equal amounts of samples were resolved by SDS–PAGE and transferred to Hybond-P membranes. The transferred samples were incubated with the antibody indicated in the text and then incubated with HRP-conjugated IgG. The immunoblotted proteins were visualized with ECL reagents.

Animal study. Six-week-old female nude mice (BALB-c nu/nu, Japan SLC, Inc., Hamamatsu, Japan) were housed in filtered-air laminar-flow cabinets and were manipulated using aseptic procedures. Procedures involving animals and their care were conducted in conformity with the guidelines of our university. The *in vivo* ovarian cancer models using Caov-3 cells were prepared as previously described [26]. Caov-3 cells were injected i.p. as a cell suspension into 10 nude mice for each experimental group. Mice were assigned to one of three treatment regimens, which started the day after tumor inoculation and continued for 7 weeks. GGA as an emulsion with 5% gum arabic was given orally every 2 days. The daily doses of GGA used were as follows: 0, 50, and 100 mg/kg/2 days. The daily dosage levels were determined, according to the GGA doses of previous experiments [27]. At the end of the treatment period, the mice were sacrificed. The volume of ascites was measured, and tumor tissue was weighed.

Statistical analysis. Results are presented as means ± SD. Data were analyzed using one-way ANOVA followed by an unpaired Student's *t*-test for comparison between groups. Differences between groups were considered statistically significant at $p < 0.05$.

Results

LPA-induced cancer cell proliferation was inhibited by GGA

We recently demonstrated that GGA inhibited LPA-induced ovarian cancer cell migration *in vitro* [13]. We also examined the effect of GGA on cancer cell proliferation, based on the findings of previous studies [17,18]. The effects of GGA on the LPA-induced proliferation of Caov-3 cells were studied by performing the MTT assay in the absence or presence of various concentrations of GGA for 3 days incubation. Although GGA did not alter the proliferation or apoptosis after 24 h of treatment [13], the effect of longer treatments (48 or 72 h) with GGA were examined in Caov-3 cells *in vitro*. LPA stimulated Caov-3 cell proliferation by ~30% compared with non-stimulated cells after 48–72 h incubation. GGA significantly inhibited LPA-induced cancer cell proliferation at more than 30 μM and exhibited a dose-dependent inhibition (Fig. 1). Higher concentration (more than 50 μM) of GGA inhibited cell proliferation