Unequivocal evidence for endogenous geranylgeranoic acid biosynthesized from mevalonate in mammalian cells

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Abstract Geranylgeranoic acid (GGA) has been reported to induce autophagic cell death via upregulation of lipid-induced unfolded protein response in several human hepatoma-derived cell lines, and its 4,5-didehydro derivative has been developed as a preventive agent against second primary hepatoma in clinical trials. We have previously reported that GGA is a natural diterpenoid synthesized in several medicinal herbs. Here, we provide unequivocal evidence for de novo GGA biosynthesis in mammals. First, with normal male Wistar rats, the levels of GGA in liver were found to be far greater than those in other organs analyzed. Second, we demonstrated the metabolic GGA labeling from the 13C-labeled mevalonolactone in the human hepatoma-derived cell line, HuH-7. Isotopomer spectral analysis revealed that approximately 80% of the cellular GGA was newly synthesized from mevalonate (MVA) in 12 h and the acid picked up pre-existing farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), suggesting that GGA is derived from FPP and GGPP through the MVA pathway. Third, zaragozic acid A, a squalene synthase inhibitor, induced dose-dependent upregulation of endogenous GGA content in HuH-7 cells and their concomitant cell death. These results strongly suggest that a cancer-preventive GGA is biosynthesized via the MVA pathway in mammals.—Shidoji, Y., and Y. Tabata. Unequivocal evidence for endogenous geranylgeranoic acid biosynthesized from mevalonate in mammalian cells. J. Lipid Res. 2019, 60: 579–593.

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In 1981, Muto, Moriwaki, and Omori (1) found that a synthetic polypropenoic acid of all-trans,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid could be bound to cellular retinoic acid-binding protein (CRABP). Thereafter, we demonstrated that the polypropenoic acid has preventive actions in both chemical and spontaneous hepatocarcinogenesis in rodents (2, 3) and shows transcriptional activation of some hepatocyte-specific genes, including the albumin (ALB) gene, in human hepatoma cells (4). It also transactivates a reporter gene through either retinoic acid receptor (RAR)-β or retinoid-X receptor (RXR)–response element (5). Hence, Muto and Moriwaki (6) named this compound “acyclic retinoid,” and they initiated a clinical trial to investigate whether acyclic retinoid prevents recurrences and second primary hepatomas after curative surgical resection or the percutaneous injection of ethanol into the initial hepatoma. As a result, the efficacy of acyclic retinoid in preventing second primary hepatoma was first reported in a placebo-controlled double-blinded and randomized phase II clinical trial with postoperative hepatoma patients in 1996 (7). Recently, the findings of Muto et al. (7) have been essentially confirmed by another multicenter clinical trial with larger size cohorts and different doses of acyclic retinoid (8, 9).

Although acyclic retinoid shares some characteristics with natural retinoids in vitro and in vivo (6), acyclic retinoid also shows apparently different characteristics from natural retinoids, such as all-trans and 9-cis retinoic acids, at the following points: 1) Acyclic retinoid upregulates ALB mRNA in the human hepatoma-derived cell lines, HuH-7 and PLC/PRF/5, but all-trans retinoic acid conversely downregulates the expression (4). 2) Acyclic retinoid is extremely less toxic than natural retinoids in a human hepatoma cell line of PLC/PRF/5 (10) as well as in vivo (7–9, 11). 3) Acyclic retinoid shows no growth-promoting activity in vitamin A-deficient animals (M. Omori, personal communication). Therefore, we have speculated that acyclic retinoid may not be just a retinoid, but may be a derivative of geranylgeranoic acid (GGA) because the above-mentioned

Abbreviations: ARA, arachidonic acid; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGA, geranylgeranoic acid; GGal, geranylgeranial; GGOH, geranylgeraniol; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; ISA, isotopomer spectral analysis; MRM, multiple reaction monitoring; MVA, mevalonate; MVL, mevalonolactone; SSE, sum of squared error; UPR, unfolded protein response; ZAA, zaragozic acid A.

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3,7,11,15-tetramethyl-2,4,6,10,14 hexadecapentaenoic acid can be renamed 4,5-didehydroGGA because neither all-trans nor 9-cis retinoic acid caused hepatoma cell death under the same conditions (14).

As for the molecular and cellular mechanisms of GGA-induced cell death, several studies have so far been conducted. The following are descriptions of cellular and molecular events relating to GGA-induced human hepatoma cell death in chronological order. The earliest is cytoplasmic splicing of X-box binding protein 1 (XBP1) mRNA, a canonical marker for unfolded protein response (UPR), observed 5 min after adding GGA in culture medium (15). Overproduction of superoxide in mitochondria and conversion from LC3-I to LC3-II, a marker for autophagic response, can be detected after 15 min (16); and after 30 min, GFP-LC3 granules appear (16) and cyclin D1 protein disappears (17). Two hours after adding GGA, the XBP1s protein (derived from a spliced form of XBP1 mRNA) is detected in the nucleus (15), the cellular level of phosphorylated p53 at Ser10 is increased (18), and the membrane potential of mitochondria (ΔΨm) is lost (16). Nuclear translocation of the cytoplasmic p53 is induced with a 3 h treatment with GGA (18). The cell death is finally detectable 6-8 h after adding GGA (19). Recently, we reported that GGA at micromolar concentrations upregulated the cellular protein levels of TP53-induced glycosylation and apoptosis regulator (TIGAR) and synthesis of cytochrome c oxidase 2 (SCO2) without their transcriptional upregulation and consequently induced a metabolic shift from aerobic glycolysis to mitochondrial respiration, as revealed by metabolomics analysis in 2 h (20). In guinea pig fibroblast-derived cell lines, GGA induced a transient increase of mitochondrial superoxide production in 15 min and dissipation of ΔΨm in 2 h, and cell death became evident in 6 h (21).

In 1981, all-trans geranylgeranyl diphosphate (GGPP) synthetase, which catalyzes two consecutive isoprenyl-transfer reactions from isopentenyl diphosphate (IPP) to geranyl diphosphate and to farnesyl diphosphate (FPP), was found in pig liver (22); but at that time, these authors were unaware of the metabolic fate or biologic roles of all-trans GGPP. At present, GGPP is well-known as an isoprenoid donor for protein isoprenylation (23). But as Dallner’s group has pointed out, GGPP synthetase activity is almost 100-times higher than protein-geranylgeranyl transferase activity in rat brain (24). GGPP may have other metabolic pathways apart from protein-geranylgeranylation or may be required for other as yet unidentified cellular processes.

In this context, Bansal and Vaidya (25) have made an interesting finding regarding geranylgeranyl diphosphatase activity in rat liver microsomes. The enzyme was the most active at physiologic pH and highly specific for GGPP. In other words, it did not hydrolyze FPP and other allyl diposphates. Once geranylgeraniol (GGOH) is produced through dephosphorylation of GGPP by the specific diphosphatase, it seems reasonable to assume that the nonspecific fatty alcohol dehydrogenase-fatty aldehyde dehydrogenase system might produce GGA through an intermediate of geranylgeraniol (GGal). However, we found that the first oxidation reaction from GGOH to GGal did not require NAD+, but requires molecular oxygen, at least in a cell-free system of either HuH-7 cell lysates (26) or rat liver homogenates (27). At present, the GGA biosynthetic pathway from GGOH is not yet clear, but we have preliminarily reported the natural existence of endogenous GGA in human hepatoma HuH-7 cells (26) and also in blood serum from healthy young volunteers (28) using LC/MS.

In the first part of the present study, we demonstrate unequivocal evidence that endogenous GGA exists in several organs of male rats using LC/MS/MS. The second part of the present study demonstrates the metabolic labeling of endogenous GGA with a stable 13C isotope from 15-g-mevalonolactone (MVL) in a human hepatoma-derived HuH-7 cell line. Finally, we describe that a pharmacological upregulation of endogenous GGA could induce cell death in HuH-7 cells. Further metabolites of GGA, including 2,3-dihydroGGA in HuH-7 cells, are also described.

MATERIALS AND METHODS

Chemicals

All-trans GGA and 2,3-dihydroGGA were prepared by Kuraray Co. (Okayama, Japan) and Kowa Pharmaceutical (Tokyo, Japan). Arachidonic acid (ARA), cis-8,11,14-eicosatrienoic acid (di-homo-γ-linolenic acid), MVL, acetonitrile (LC/MS grade), ethanol, and Dulbecco’s PBS, without calcium chloride and magnesium chloride, suitable for cell culture [PBS (−)] were all purchased from Sigma-Aldrich (St. Louis, MO). Methanol and DMEM (high glucose) were from Wako Pure Chemical Industries (Osaka, Japan). Chloroform was obtained from Kanto Chemical Co. (Tokyo, Japan). FBS was bought from HyClone, Thermo Fisher (Tokyo, Japan). DL-[1,2-13C2]MVL was received from ISOTEC (Miamisburg, OH). Pravastatin was provided by Daiichi Sankyo Co. (Tokyo, Japan). Zaragozic acid A (ZAA) was provided from Merck (Darmstadt, Germany). All chemicals other than those stated above were of reagent grade.

Animals

Male Wistar rats (5 weeks old) were obtained from CLEA Japan, Inc., Tokyo, Japan. The rats were fed a conventional diet (CE-2; CLEA Japan) for several days, and then the animals were euthanized under isoflurane anesthesia by collecting blood through the vena cava and the 12 organs listed in supplemental Table S1 were immediately excised, weighed, and instantaneously frozen at −25°C until use. These research plans were approved by the animal experiment ethics committee of the University of Nagasaki.

Treatment of HuH-7 cells with pravastatin and ZAA

A human hepatoma-derived cell line of HuH-7, Cell Bank (RIKEN, Wako, Saitama, Japan), was maintained with DMEM.
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(high glucose) containing 5% FBS at 37°C in a humidified atmosphere of 5% CO₂. HuH-7 cells (5 × 10⁵ cells/dish, in a 100 mm diameter dish) were inoculated and cultured with DMEM containing 5% FBS for 24 h; thereafter, the medium was replaced with FBS-free DMEM 1 day before pravastatin and/or ZAA treatment. After 48 h treatment with 120 μM pravastatin and/or 15 μM ZAA, the cells were harvested using a plastic cell lifter (Nunc, Roskilde, Denmark).

Metabolic labeling of GGA with 13C-MVL

HuH-7 cells (5 × 10⁵ cells/dish) were inoculated and cultured with DMEM containing 5% FBS for 24 h; thereafter, the medium was replaced with FBS-free DMEM 1 day before pravastatin treatment. After 24 h treatment with 120 μM of pravastatin, 15 μM ZAA and 2 mM 13C-MVL were both added into the culture medium and incubated for 48 h. The cells were harvested using a cell lifter. To chase the labeled GGA, the cells under the same conditions as above were replenished with fresh FBS-free DMEM containing 120 μM of pravastatin and incubated for 24 h. Then, the cells were incubated for a further 48 h with 15 μM ZAA and 2 mM cold MVL. They were finally harvested using a plastic cell lifter.

Labeling experiments for isotopomer spectral analysis. HuH-7 cells (5 × 10⁵ cells/dish) were preincubated for 12 h with 120 μM of pravastatin and 15 μM of ZAA. Then, 2 mM of 13C-MVL were added and the cells were further incubated. The cells were harvested to extract the cellular lipids at the indicated time points (6, 12, 24, 48, and 72 h after addition of 13C-MVL) and the resultant lipid extracts were analyzed by LC/MS/MS to discriminate and measure each isotopologue. The monomeric precursor for GGA is not mevalonate (MVA), but rather both dimethylallyl di-phosphate (DMAPP) and IPP. However, because it is difficult to accurately quantify DMAPP and IPP, we performed the original isotopomer spectral analysis (ISA) by assuming MVA as a precursor of GGA. A proportion of isotopologues of MVA in tracer 13C-MVA derived from 13C-MVL preparation was calculated according to the supplier’s specification.

Lipid extraction and quantitative measurement of GGA contents

Male Wistar rat organs. Each organ was hand-homogenized using a Potter glass grinder in 10 vol of methanol. Then, the homogenates were transferred to a screw-capped glass tube and were added into 20 vol of chloroform. All sample suspensions were covered with aluminum foil and agitated reciprocally at 4°C overnight. After centrifugation, the supernatant was collected into a new screw-capped glass tube and evaporated to dryness under a nitrogen stream on a Reacti-Therm™ heating module (Pierce, Roskilde, Denmark). After 48 h treatment with 120 μM of pravastatin, 15 μM ZAA and 2 mM cold MVL, they were separated to extract the cellular lipids at the indicated time points (6, 12, 24, 48, and 72 h after addition of 13C-MVL) and the resultant lipid extracts were analyzed by LC/MS/MS to discriminate and measure each isotopologue. The monomeric precursor for GGA is not mevalonate (MVA), but rather both dimethylallyl di-phosphate (DMAPP) and IPP. However, because it is difficult to accurately quantify DMAPP and IPP, we performed the original isotopomer spectral analysis (ISA) by assuming MVA as a precursor of GGA. A proportion of isotopologues of MVA in tracer 13C-MVA derived from 13C-MVL preparation was calculated according to the supplier’s specification.

HuH-7 cells. HuH-7 cells and the conditioned medium were separately collected in each tube by centrifugation (200 g, 8 min). To extract the total cellular lipid, the cell pellets were added to chloroform/methanol (2:1 v/v; 20-fold volume over cell volume) and sonicated on ice (three times; each 30 s). After standing overnight at room temperature and being centrifuged, the supernatant was transferred to a screw-capped glass tube and evaporated to dryness under a nitrogen stream. The residues were dissolved with 100 μl of ethanol and filtered through a cartridge of Cosmonic Filter S (0.45 μm) prior to LC/MS/MS analysis.

LC/MS/MS analysis

LC was performed by using a Waters Acquity Ultra Performance LC apparatus (Waters, Milford, MA) equipped with an Acquity UPLC-HSS T3 column (100 × 2.1 mm, 1.8 μm (Waters)). A tandem quadrupole mass detector system was operated in multiple reaction monitoring (MRM) mode. Nitrogen was used as a desolvation gas (800 l/h) and a cone gas (50 l/h). The desolvation temperature was 450°C. Argon was used as the collision gas (0.15 ml/min) and the collision energy was 20 eV. The capillary voltage was set at 3.0 kV, and the source temperature was 135°C for ESI. The chromatographic run was operated by linear gradients between solution A (acetonitrile) and solution B (miliQ water). The elution was conducted at a constant flow rate of 0.30 ml/min as follows: 0–9 min, isocratic 74% A; 9–10 min, a linear ascending gradient from 74% A to 100% A; 10–15 min, 100% A; 15–16 min, a linear descending gradient from 100% A to 74% A; 16–19 min, 74% A. All compounds measured in this study were separated in isotropic elution (0–9 min) of 74% acetonitrile in water. For sample analysis, 5 μl of each sample were injected onto the column, and the total data-acquiring time was 11 min.

Concentration-dependent cytotoxicity of ZAA against HuH-7 cells

HuH-7 cells (1.0 × 10⁶ cells/well, 6-well plate) were cultured with DMEM containing 5% FBS for 24 h. Thereafter, the medium was replaced with FBS-free DMEM 1 day before treatment with increasing concentrations (0–60 μM) of ZAA with or without pravastatin (120 μM). After addition of ZAA, the number of viable cells was counted by the trypan blue dye exclusion method at 48 h after addition of ZAA. LD₅₀ was calculated using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA).

Statistical analysis

Statistical comparisons were performed using a t-test or ANOVA with post hoc Scheffe where appropriate. All data, unless specified, are presented as mean ± SE, with a statistically significant difference defined as P ≤ 0.05.

RESULTS

Endogenous GGA in rat tissues

Because we have already reported that endogenous free GGA was identified in the human liver cancer-derived cell line, HuH-7 (26), and blood serum of healthy volunteers (28) using LC/MS, we attempted to detect and quantify endogenous free GGA in each tissue of male Wistar rats. First, we decided to demonstrate endogenous free GGA in livers as compared with free ARA, a structural isomer of GGA using LC/MS/MS, which allowed us to separately quantify GGA and ARA in each organ of the rat. As a result, MRM tracings of each compound in the liver extract clearly indicated the natural existence of free GGA in the liver, as shown in Fig. 1A. Because the standard curves of GGA and ARA were linear (r² = 0.99 for both), at least in the range shown in supplemental Fig. S1, GGA and ARA in each organ of the rat were quantified. Whereas all tissues tested contained endogenous free GGA measured using LC/MS/MS, the hepatic free GGA content was exceptionally high,
Fig. 1. Tissue distribution of endogenous free GGA, ARA, and 2,3-dihydroGGA in male Wistar rats measured using reverse phase LC/MS/MS analysis. A: Representative chromatograms of the rat hepatic lipid extract tracing GGA (m/z 303 → 98) and ARA (m/z 303 → 259), and of the rat thymus lipid extract tracing 2,3-dihydroGGA (m/z 305 → 168). The down arrow represents elution times of di-homo-γ-linolenic acid. The tissue distribution of endogenous GGA (B) and 2,3-dihydroGGA (C) in male Wistar rats and a molar ratio of 2,3-dihydroGGA to GGA in each tissue (D) are shown in bar graphs. All bars represent the mean ± SE (n = 5). ***P< 0.001 versus each other organ (B); *P< 0.05 and ***P< 0.001 versus thymus (C); and *P< 0.05 versus liver (D) (ANOVA with post hoc Scheffe).
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followed by the reproductive organs, such as prostate and testis, and neuronal tissues of cerebrum and cerebellum (Fig. 1B, supplemental Table S1). Blood serum and epididymal adipose tissue contained only a few free GGA. A tissue distribution of free ARA was in the range of previous reports (29, 30), so the molar ratio of free GGA to free ARA was also highest in the liver (supplemental Fig. S2A).

Although the epididymis, another reproductive organ, was not listed in supplemental Table S1, the tissue concentrations of GGA in each part of the epididymis were calibrated as 28.82 ± 2.13 pmol/g in the caput (n = 5), 8.75 ± 3.07 pmol/g in the cauda (n = 5), and 5.96 ± 1.13 pmol/g in the corpus (n = 5). The epididymal caput was, after the liver, a tissue with a high GGA concentration.
Endogenous 2,3-dihydroGGA in rat tissues

Because Sagami’s group (31) reported that rat thymocytes produce 2,3-dihydroGGA from GGal or GGOH through GGA and Dulaney’s group (32, 33) found 2,3-dihydroGGA with phytanic acid in the serum and urine of patients with Refsum disease, we also tried to detect and quantify endogenous 2,3-dihydroGGA in male Wistar rat tissues using an MRm mode of LC/MS/MS (Fig. 1C, supplemental Fig. S1B). Unlike the tissue distribution of GGA, the 2,3-dihydroGGA content was highest in thymocytes. Furthermore, the hepatic 2,3-dihydroGGA content was the second lowest value after epididymal fat, excluding serum (Fig. 1C). When a molar ratio of 2,3-dihydroGGA to GGA in each organ was calculated, it was highest in the thymus (141.2 ± 34.3), second highest in the seminal vesicle (106.0 ± 41.4), and lowest in liver (5.1 ± 1.5); in other organs, it was in the range of 30–75, excluding serum (15.4 ± 1.9), as shown in Fig. 1D and supplemental Table S1.

Metabolic labeling of GGA using 13C-MVL in human hepatoma HuH-7 cells

Because the liver was the main organ that contained endogenous free GGA, we attempted to observe the biosynthesis of GGA using human liver cancer-derived HuH-7 cells. Given our previous observations that HuH-7 cells contain endogenous GGA, and that GGA can be produced from GGOH in the HuH-7 cell lysates (26), we examined whether cellular GGA is metabolically labeled with a stable isotope of 13C in the cells after adding 13C-labeled MVL to the culture medium.

Prior to the labeling experiment, we confirmed that endogenous GGA was sensitive to pravastatin treatment. After 48 h treatment with 120 μM of pravastatin, endogenous GGA became undetectable in HuH-7 cells (Fig. 2A, B). Squalene synthesis is a major flow of the mammalian MVA pathway, so we tried to block the major flow by using ZAA (squalstatin) to augment a metabolic flow from FPP to GGPP. As a result, the cellular GGA content was increased by 15–20 times after 48 h treatment with 15 μM of ZAA (Fig. 2C), and most of the content was depleted by cotreatment with pravastatin and ZAA (Fig. 2D).

After complete depletion of endogenous GGA, we successfully detected 13C-labeled GGA in HuH-7 cells using LC/MS/MS with 2 mM of 13C-MVL in the culture medium (Fig. 3A). Then, the labeled GGA could be completely chased by another 48 h treatment with the same concentration of cold MVL (Fig. 3B).

ISA with 13C-MVL in HuH-7 cells

We performed ISA using a stable isotope-labeled 13C-MVL to test for the presence of gradients of precursor enrichment, as illustrated by in vivo sterol synthesis (34).
When both ZAA and pravastatin were simultaneously added 24 h prior to addition of 13C-MVL, 13C was incorporated to different extents into newly synthesized GGA in 6 h, which showed all the varieties of mass isotopomers and isotopologues of GGA. As shown in Fig. 4A and B, we detected all eight MS/MS peaks at a retention time corresponding to GGA, which had been expected in advance from combinations of five isotopic molecular ions (m/z 303–307) and two isotopic fragment ions (m/z 98 and 99). The peak area of each isotopologue with fragment ion of 98 was 13 for M (a molecular ion of 303), 12 for M+1, 7 for M+2, and 5 for M+3, arranged in descending order (Fig. 4C). However, the peak area of each GGA isotopologue with fragment ion of 99 was 12 for M+1, 13 for M+2, 15 for M+3, and 18 for M+4, each of which was aligned in ascending order, as shown in Fig. 4C.

We analyzed the data using the ISA framework as measured mass isotopologue distributions of GGA. To elucidate the biosynthetic flow and pathway of GGA from MVA, a simple metabolic model, as shown in Fig. 5A, was first constructed and ISA was performed with this model. Here, D is a factor described as the MVA pool dilution with the 13C-MVA; therefore, D should have been constant during the experiment because we used pravastatin to prevent further dilution of the MVA pool by endogenous MVA synthesis. Indeed, D values were constant from 0.766 at 12 h to 0.768 at 72 h. However, g(t) is a time-dependent parameter. When g(t) was plotted along with incubation time after adding 13C-MVL (Fig. 5D), it increased from 0.56 at 6 h to 0.88 at 12 h and reached a plateau of 0.9 at 24 h.

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2,3-DihydroGGA as a metabolite of GGA

ISA was performed on 2,3-dihydroGGA using the same samples. Because the fragment ions of 2,3-dihydroGGA were composed of diterpenoic acid containing α- and
Fig. 4. LC/MS/MS chromatograms of mass isotopomers and mass isotopologues of GGA metabolically labeled with $^{13}$C-MVL. A, B: The eight expected combinations of five molecular ions, m/z 303, 304, 305, 306, and 307, and two fragment ions [m/z 98 (A) and 99 (B)], were detected at the same retention time in a single run after being metabolically labeled with $^{13}$C-MVL for 6 h in HuH-7 cells. The peak areas of GGA mass isotopomers found in A and B were plotted in a bar graph (C). In A and B, four isoprene units in GGA are simply illustrated by four circles. Carbon isotopes incorporated in position 1 of each isoprene unit in GGA are indicated by open circles ($^{12}$C) and closed circles ($^{13}$C).
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We detected nine MS/MS combinations (305 → 168 (M+0); 306 → 168 (M+1); 306 → 169 (M+1); 307 → 168 (M+2); 307 → 169 (M+2); 307 → 170 (M+2); 308 → 169 (M+3); 308 → 170 (M+3); and 309 → 170 (M+4)) of 2,3-dihydroGGA (Fig. 5C). ISA on 2,3-dihydroGGA also calculated a D value of 0.788, which is similar to the value calculated using ISA on GGA, and it was constant during the experiment. However, g(t) increased from 0.68 at 12 h, and it gradually caught up with the D value of GGA at 72 h, suggesting that GGA may be metabolized to 2,3-dihydroGGA.

ZAA induced upregulation of GGA and cell death in HuH-7 cells

We have repeatedly reported that HuH-7 cells are sensitive to treatment with exogenous GGA (13, 14), which induces UPR-mediated cell death (15) with an incomplete autophagic response (16), as mentioned in the Introduction. In this study, we revealed that HuH-7 cells are capable of producing endogenous GGA from MVA. In this context, we hypothesized that if upregulation of endogenous GGA can be inducible, it is possible to induce cell death in HuH-7 cells by the resultant upregulated GGA without using exogenous GGA.

We chose ZAA, which prevents a major flow of MVA into the steroid synthesis pathway and augments GGPP synthesis, to upregulate endogenous GGA synthesis from MVA. As a result, 15 μM of ZAA time-dependently increased endogenous GGA from 111 to 1,372 pmol/g in 72 h, and endogenous 2,3-dihydroGGA from 154 to 4,571 pmol/g (Fig. 6A). As expected, ZAA dose-dependently induced cell death of HuH-7 cells in 48 h, as shown in Fig. 6B. The LD50 of ZAA was 22.0 ± 0.07 μM against HuH-7 cells. Because ZAA is an inhibitor of squalene synthase, ZAA-treated cells are expected to become deficient in cholesterol. Hence, we performed a rescue experiment of ZAA-induced cell death with exogenous cholesterol. As a result, the exogenously added cholesterol was unable to rescue the cells from ZAA-induced death (see supplemental Fig. S3). Therefore, to demonstrate that ZAA-induced cell death is due to an increase in GGA synthesis, another rescue experiment was conducted to eradicate endogenous GGA synthesis in the presence of ZAA. When pravastatin was supplied to the culture medium during ZAA treatment, the dose-dependent effect of ZAA on cell death was completely attenuated (Fig. 6B).

**DISCUSSION**

In the present study, we demonstrated unequivocal evidence for mammalian GGA, a hepatoma-preventive isoprenoid. Endogenous free GGA was definitely present in various tissues of 5-week-old male Wistar rats, in especially high concentration in the liver. Another important finding is that GGA is synthesized from MVA in human hepatoma-derived HuH-7 cells through metabolic labeling of endogenous GGA by using 13C-labeled MVL.

GGA is a novel, natural, and biologically active acyclic diterpenoid metabolite, not listed in the LIPID MAPS database (http://www.lipidmaps.org). Initially GGA was noted as a chemically synthesized acyclic retinoid to be used as a preventive drug for second primary hepatoma together with peretinoin or 4,5-didehydroGGA (7). Later, we reported natural occurrence of GGA in several medicinal herbs (19). In the present study, we found that endogenous GGA exists not only in plants but also in mammalian cells. And, at least in male Wistar rats, it is coincidentally distributed at high concentrations specifically in the liver.

Given that liver is an active organ for cholesterol synthesis in mammals and GGA can be biosynthesized from MVA through FPP and GGPP, it can be reasonably considered that the endogenous GGA concentration must be high in the rat liver. Indeed, the highest concentration of endogenous GGA was observed in the liver of male Wistar rats; its concentration is about 80 nM, which is relatively higher than the level of that in human hepatoma-derived HuH-7 cells [10–15 nM (26)], but is much lower than the concentrations [5–20 μM (13)] inducing cell death in human hepatoma-derived HuH-7 cells.
Fig. 5. ISA on GGA biosynthesis in HuH-7 cells. A: To perform ISA, a simple metabolic model was assumed for the biosynthetic flow and pathway of GGA from MVA. D is a factor of the precursor MVA pool dilution by $^{13}$C-MVL, and g(t) is a time-dependent parameter of the endogenous GGA pool dilution by newly synthesized GGA. B, C: Best fit results found with nonlinear approximation of measured values of GGA or 2,3-dihydroGGA mass isotopologues with their estimated values using ISA. Carbon isotopes incorporated in position 1 of each isoprene unit in GGA are indicated by open circles ($^{12}$C) and closed circles ($^{13}$C). D: Time course changes in g(t) of GGA (closed circles) and 2,3-diGGA (open circles) in HuH-7 cells by ISA are plotted along with the incubation time (0–72 h).

Regarding tissues tested herein other than liver, it is worth mentioning that the GGA concentrations in the testis, prostate, and epididymis (especially in the caput) of male Wistar rats were relatively high among all tissues tested in the present study. Before the observations of this study, we have coincidentally found that, when compared...
with a commercially available breeding feed relatively rich in DHA and EPA, a GGA-supplemented diet increased the reproductive index (the number of weaning mice divided by the number of mated couples) of senescence-accelerated SAM mice (35). This reproduction index promotion activity of dietary GGA was recently reproduced in another C3H/HeN strain of mice (Y. Tabata, S. Uematsu, and Y. Shidoji, unpublished observations). Hence, we speculate that endogenously synthesized GGA may play an important role, at least in male reproductive organs. Neural tissues, such as cerebellum and cerebrum, also contained relatively high concentrations of GGA. Kotti and colleagues reported that GGOH acts specifically and quickly to affect long-term potentiation in the Schaffer collaterals of the hippocampus without affecting protein geranylgeranylation (36, 37). GGA metabolized from GGOH may function to affect
long-term potentiation. As the concentration of endogenous GGA in blood and adipose tissue was extremely low, we can exclude the possibility that the GGA observed in the above-mentioned organs (hepatic, reproductive, and neural tissues) might be contaminated with blood GGA.

Another noteworthy point about tissue distribution is that 2,3-dihydroGGA, which is considered to be a further metabolite of GGA, is distributed at the highest level in the thymus gland. As mentioned above, Kodaira et al. (31) reported that 2,3-dihydroGGA was a major metabolite from exogenous GGOH or GGal, presumably via GGA in the primary culture of rat thymocytes. In the present study, we also found that a molar ratio of 2,3-dihydroGGA to GGA was extremely high in the liver, whereas it was the lowest in the liver (Fig. 1D, supplemental Table S1). This suggested that the conversion rate from GGA to 2,3-dihydroGGA is very fast and that 2,3-dihydroGGA may play a specific role in thymocytes, which should also be explored in the future.

Because GGA was found to be a hepatic lipid in this study, we next analyzed GGA biosynthesis using HuH-7 cells. After confirmation of metabolic incorporation of four atoms of the stable isotope from $^{13}$C-labeled MVL into one molecular GGA in the presence of pravastatin, we performed ISA. ISA was initially introduced as a general method for modeling polymerization biosynthesis reactions (38). GGA is a simple tail-to-head tetramer of an isoprene unit derived from consecutive phosphorylation and decarboxylation of MVA. Therefore, the biosynthetic pathway of GGA is an ideal model to which ISA can be applied. In ISA, it is generally assumed that D should be constant, so we performed ISA in the presence of pravastatin to prevent MVA pool dilution with newly synthesized nonlabeled MVA. To prevent the bulk escape of $^{13}$C-MVA from FPP to the cholesterol pathway via squalene, we added ZAA, an inhibitor of squalene synthase, into the culture medium during the ISA experiment. When we used 2 mM $^{13}$C-MVL, D became 0.77, which means a pool size of endogenous cold MVA was calculated to 0.59 mM at 12 h after pravastatin addition. Because the MVA pool size in the liver has been reported as 2.55 mM (39), we can conclude that the...
Geranylgeranoic acid synthesized from mevalonate in mammals

Experimental conditions we performed are restored to the physiological MVA pool size by adding the labeled MVL. However, $g(t)$ is a time-dependent parameter, which means a fractional ratio of newly synthesized GGA after adding $^{13}$C-MVL. Surprisingly, very rapid turnover of the cellular GGA was suggested by a time course of the $g(t)$ parameter. In other words, 12 h after adding $^{13}$C-MVL, more than 80% of the intracellular GGA was replaced by newly synthesized GGA.

Of note, ISA on 2,3-dihydroGGA also revealed that the dihydro-derivative is a metabolite of GGA, consistent with a previous report (31). The metabolite of GGA has the ability to induce lipid droplets in HL-60 cells, whereas GGA has no ability to induce lipid droplets (40). The difference in

Fig. 7. Proposed metabolic flow from MVA to GGA in HuH-7 cells.
this biological activity between GGA and 2,3-dihydroGGA is confirmed with HuH-7 cells (Y. Shidoji, K. Okamoto, and H. Sekiguchi, unpublished observations).

When the labeled isoprene unit is used for GGA synthesis in the isoprene synthesis pathway, theoretically, possible preexisting pools of DMAPP, geranyl diphasphate, and FPP are all available, as shown in supplemental Fig. S3. However, the data indicate that the observed fractional abundance of the M+1 isotopomer labeled with the α isoprene unit was much larger than the estimated value (Fig. 5B), which was calculated using the ISA model fitting assumption that the isoprene unit is randomly incorporated into a GGA consisting of four isoprene units (supplemental Fig. S4). The discrepancy between the observed and estimated values for the M+1 isotopomer labeled with the $^{13}$Cα isoprene unit suggests that the pool size of FPP is larger than those of other two intermediates in HuH-7 cells (supplemental Fig. S4D). Consistently, Holstein et al. (41) reported that the FPP levels were higher than the geranyl diphasphate levels in human myeloma cell lines. Taken together with our previous reports (26, 27), we propose a possible metabolic flow from MVA to GGA in Fig. 7, where GGOH, a dephosphorylated form of GGPP by GGPP pyrophosphatase (25), is enzymatically oxidized by using molecular oxygen to GGAl (26), which is further oxidized to GGA in a NAD-dependent manner (26, 27).

Finally, we should discuss the biological significance of GGA de novo synthesized in mammalian hepatocytes. We have found and reported GGA as a micromolar inducer of cell death in human hepatoma-derived cell lines through both UPR (15) and an incomplete response of autophagy (16). Therefore, in the present study, we tried to induce cell death in HuH-7 cells by upregulating endogenous GGA with ZAA. As a result, we were able to upregulate free endogenous GGA from 0.1 to 1.3 μM and 2,3-dihydroGGA from 0.15 to 4.5 μM in cells by a 3 day treatment with 15 μM ZAA (Fig. 6A). Both GGA and 2,3-dihydroGGA actively induce cell death in HuH-7 cells at their micromolar concentration (19). Consistently, ZAA induced cell death in a concentration-dependent manner (Fig. 6B), which strongly suggests that the intracellular endogenous GGA and 2,3-dihydroGGA induce cell death. Furthermore, when we canceled MVA synthesis by cotreatment with pravastatin, ZAA-dependent cell death was also canceled. This strongly indicates that the cellular toxicity of ZAA for hepatoma cells is not mediated by deficiency of cholesterol and/or other steroid metabolites, but may be conveyed through upregulation of GGA and 2,3-dihydroGGA.

In conclusion, we demonstrated unequivocal evidence for biologically active endogenous free GGA in mammalian tissues. Regarding the metabolic synthesis of GGA and/or 2,3-dihydroGGA from GGGOH in animal cells, we must mention the pioneering studies by Fliesler and Schröepfer (42) and Foster et al. (43). More than a quarter century ago, they commonly found GGA and 2,3-dihydroGGA in bovine retina and the blood fluke, respectively, without discussion on their biological functions. In particular, it is worth noting that Fliesler and Schröepfer (44) originally observed the metabolic labeling of GGA from $^3$H-MVA using the tissue culture system of bovine retina, which strongly supports our idea of endogenous GGA in mammalian cells. The present study paves a road for exploring the metabolism and biological activity of these compounds from the viewpoint of inducing cell death in tumor cells and other biological activities in reproductive and neural tissues.

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