Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs

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Abstract Geranylgeranoic acid (GGA; all-*trans* 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenoic acid) has been shown to induce apoptosis in a human hepatoma-derived cell line, HuH-7. We aimed not only to confirm the apoptogenic properties of GGA and its derivatives, but also to search for natural GGA in medicinal herbs. GGA induced apoptosis in human hepatoma-derived cell lines, HuH-7, PLC/PRF-5, and mouse transformed hepatocyte-derived cell line, MLE-10, in a dose- and time-dependent manner, but failed to induce cell death in human hepatoblastoma-derived HepG-2 and mouse primary hepatocytes in the same condition. Besides GGA, 4,5-didehydro GGA, 14,15-dihydro GGA, and 2,3-dihydro GGA were also active to induce cell death in HuH-7 cells, while 4,5-didehydro-10,11,14,15-tetrahydro GGA, 4,5,8,9-tetrahydro GGA, farnesoic acid, and geranylgeraniol were inert. By using liquid chromatography/mass spectrometry, we found natural GGA as a negative ion of *m/z* 303.4 in a Chinese herb, *Schisandra chinensis*, and Schisandra GGA was identified by derivatization with both mild methylation and catalytic hydrogenation. Some other GGAs hydrogenated in the different degrees, including phytanic acid (perhydro GGA), were also found in *S. chinensis*. GGA and phytanic acid were detected in 24 out of 25 herbs tested. The present study is the first report of natural GGA in medicinal herbs.—Shidoji, Y., and H. Ogawa. **Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs. *J. Lipid Res.* 2004.** 45: **1092–1103.**

Supplementary key words apoptosis • cancer chemoprevention • hepatoma

The efficacy of a synthetic 20-carbon polyprenoic acid (all-*trans* 3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid) on prevention of second primary hepatoma has been proven in a double-blinded and randomized phase II clinical trial with postoperative hepatoma patients with few side effects (1), and, recently, it was revealed that the polyprenoic acid significantly increased a 5-year survival rate after a radical therapy of primary hepatoma in these patients (2). We have also shown that the polyprenoic acid binds to cellular retinoic acid binding protein (CRABP) (3) as well as to nuclear retinoid receptors (4), exerts transcriptional activation of some hepatocyte-specific genes in hepatoma cells (5), and has preventive actions in chemical and spontaneous hepatocarcinogenesis (6,7). We named this compound "acyclic retinoid" (8).

Although acyclic retinoid shares characteristics of natural retinoids in vitro and in vivo (6), acyclic retinoid apparently differs from natural retinoids such as all-*trans* and 9-*cis* retinoic acids in the following respects: *1)* acyclic retinoid upregulated the cellular level of albumin mRNA in human hepatoma-derived cell lines, HuH-7 and PLC/PRF-5, while all-*trans* retinoic acid downregulated the expression (5); *2)* acyclic retinoid induced apoptosis in these cell lines, whereas neither all-*trans* nor 9-*cis* retinoic acid did (9); and *3)* acyclic retinoid showed no growth-promoting activity in vitamin A-deficient animals (unpublished observation). Therefore, we have speculated that acyclic retinoid may have a different metabolism from retinoic acid in cells and animals and may mimic other biological components such as acyclic diterpenoids, for example, geranylgeranoic acid (GGA), from which it differs for the additional double bond at a position of C4 and C5.

From a chemical structural point of view, GGA belongs to isoprenoids or terpenoids, which are the most functionally and structurally varied group of plant metabolites. Plants have two distinct biosynthetic routes for the formation of isopentenyl diphosphate (IPP; C5), either in the mevalonic acid pathway or in the methylerythritol phosphate pathway (10) (**Scheme 1**). IPP represents the activated monomer building block for all other isoprenoids.

Abbreviations: Chl, chlorophyll; CHL-P, geranylgeranyl reductase; CRABP, cellular retinoic acid binding protein; GGA, geranylgeranoic acid; GGOH, geranylgeraniol; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; LC/MS, liquid chromatography/mass spectrometry; phytylPP, phytyl diphosphate; PPARα, peroxisome proliferator-activated receptor α; RT, retention time; SIR, selected ion recording.

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Condensation of dimethylallyl diphosphate with one IPP in a head-to-tail fashion generates geranyl diphosphate (C10); addition of a second IPP produces farnesyl diphosphate (C15); a third IPP gives rise to geranylgeranyl diphosphate (GGPP; C20); and so on. From this trunk route, the branched isoprenoid biosynthetic pathway produces a myriad of cellular products and plays a pivotal role in plant life. This pathway supplies primary metabolites for normal growth and development and synthesizes a vast array of secondary metabolites in response to biotic and abiotic environmental stress (11). GGPP is one of the key isoprenoids to be allocated to the synthesis of various end products necessary for plant growth and defense, such as gibberellins, carotenoids, quinones, chlorophylls (Chls), tocopherol, or geranylgeranylated proteins in plants (12).

To our knowledge, GGA (C20) has so far been recognized as only a synthetic substance, and there has been no report of the existence of natural GGA in plants or animals. Although a variety of cyclic diterpenoids have been discovered in nature, there is limited information available on acyclic diterpenoids, except for phytanic acid (3,7,11,15-tetramethyl hexadecanoic acid). Phytanic acid (C20) is an isoprenoid-derived 3-methyl branched-chain fatty acid, originating from the phytol (C20) side chain of plant Chl. Although phytanic acid is easily found in normal human plasma, there has been no report of the existence of phytanic acid in plants except for some dry lichen species from *Lecanorales* (13) and walnuts (14). Microorganisms, which are present in the rumen of ruminants, are thought to release phytol from Chl, after which phytol is converted into phytanic acid (15). Because humans are not capable of releasing phytol from Chl (16), all phytanic acid is considered to enter the human body via the diet. In particular, ruminant fats, fish, and dairy products are rich sources of phytanic acid (17).

Based upon the chemical structural similarity of GGA to phytanic acid, it seems reasonable for us to assume that GGA can be enzymatically derived from geranylgeraniol (GGOH; C20), which is a common precursor of all natural diterpenoids hydrolyzed from GGPP by a phosphatase enzyme (Scheme 1). If this metabolic pathway exists in plant cells, we should be able to find GGA as a natural component of traditional medicinal herbs, especially liver disease-preventive herbs.

In the first part of the present study, we show that exogenous GGA is a micromolar inducer of apoptosis in human hepatoma-derived cells. In the second and main part, we searched for naturally occurring acyclic diterpenoid acids, including GGA in plants. Liquid chromatography/mass spectrometry (LC/MS) analysis was performed with a variety of traditional medicinal plants mainly used as liver tonics to clarify whether these plants contain GGA as natural substances. We carried out a screening of 25 dry herbs and some fresh herbs. The identification of GGA was performed by chemical modification through both methylation and hydrogenation. Here we show a representative LC/MS analysis of GGA in herbal extracts from *Schisandra chinensis*.

MATERIALS AND METHODS

Materials

All-*trans* GGA and 4,5-didehydro GGA were obtained from Nikken Chemicals Co. (Saitama, Japan), and other GGA derivatives were provided by Eisai Co. (Tokyo), and all-*trans* GGA and all-*trans* GGOH for HPLC or LC/MS analysis were prepared by Kuraray Co. (Okayama, Japan). Phytanic acid, arachidonic acid, arachidic acid, 11,14-eicosadienoic acid, 8,11,14-eicosatrienoic acid, 5,8,11-eicosatrienoic acid, and platinum (IV) oxide were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, n-hexane, chloroform (HPLC grade), ethanol, dehydrated diethyl ether, and 5 M sodium hydroxide solution were from Wako Pure Chemical Industries (Osaka, Japan). Ammonium acetate was from Fluka Bio Chemika (Switzerland), and N-methyl-N-nitro-N-nitrosoguanidine was from GL Sciences (Tokyo, Japan). All other chemicals were of reagent grade.

Cell culture

Human hepatoma-derived cell lines, PLC/PRF-5 and HuH-7, human hepatoblastoma-derived cell line, HepG-2, and mouse c-H-*ras* transformed hepatocyte-derived cell line, MLE-10, which was kindly supplied by Dr. T. Kitagawa (Cancer Institute, Tokyo) (18), were all maintained with Dulbecco's-MEM containing 10% fetal bovine serum (FBS). Hepatocytes were prepared from livers of 8-week-old male Balb/c mice after digestion with collagenase perfusion. The isolated hepatocytes were cultured with Williams' medium E supplemented with 50 ng/ml epidermal growth factor, 10^{-7} M insulin, 10^{-6} M dexamethasone, and 10% FBS.

Treatment of cells with isoprenoids

Twenty four hours before the treatment of cells in 6-well plates with isoprenoids, the media were replaced by FBS-free Dulbecco's-MEM. After addition of the isoprenoids at the indicated concentrations, viable cells were counted by the trypan blue dye-exclusion method at the indicated time points.

DNA fragmentation assay

After the treatment with isoprenoids, the detached and the loosely attached cells were collected by flushing with phosphate buffered saline, and the cell suspension was centrifuged at 400 *g* for 10 min. The pelleted cells were lysed with 0.5% SDS in 10 mM Tris-HCl buffer, pH 8.0, containing 25 mM EDTA and 0.1 M NaCl, and digested with 0.1 mg/ml proteinase K at 50°C overnight. The DNA was extracted twice with a solvent mixture of phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v). After precipitation with ethanol and digestion with 10 μg/ml RNase A, the washed DNA was loaded onto a 1.5% agarose gel in 89 mM Tris, 80 mM boric acid, and 0.2 mM EDTA, pH 8.0. The fragmented DNA was stained with ethidium bromide after electrophoresis (100 V, 1.5 h) with a 100-bp DNA ladder maker (Invitrogen, CA).

Preparation of plant extracts

The dried fruits of *S. chinensis* (Schisandra; China) were homogenized in methanol with Polytron-MR3100 (probe type: 3012/2, KINEMATIKA, Switzerland) and extracted with methanol/chloroform (1:1; v/v) at 4°C overnight. The resultant monophasic extracts were evaporated to dryness and the residues were dissolved in ethanol and then partitioned with H_2O/ethanol/n-hexane (1:2:4; v/v/v). The upper organic phase was evaporated to dryness, and the residues were dissolved in ethanol. The ethanolic solution was analyzed by LC/MS. The other dried herbs were also extracted in the same procedure. *Curcuma longa* (turmeric, rhizome; China), *Glycyrrhiza uralensis* (licorice, root; China), *Rheum palmatum* (rhubarb, root; China), *Saus-*

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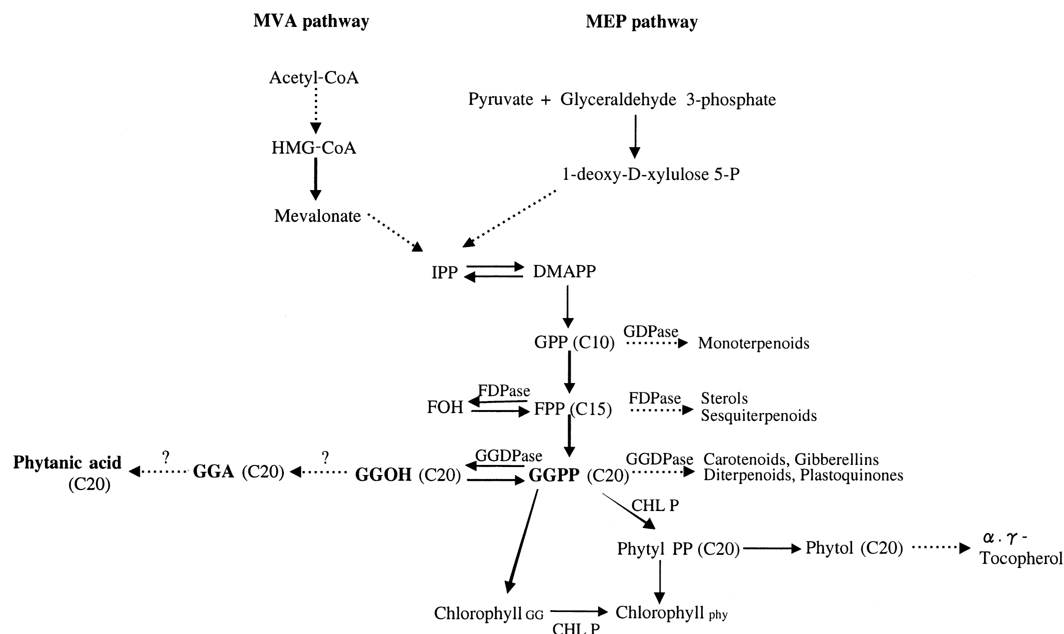

Scheme 1. Schematic diagram of the isoprenoid pathways via the acetate/mevalonate [mevalonic acid (MVA)] or the GAP/pyruvate [methylerythritol phosphate (MEP)] in plants. The representative primary and secondary metabolites are shown. The details are described in the text. CHL-P, geranylgeranyl reductase; DMAPP, dimethylallyl diphosphate; FDPase, farnesyl diphosphate; FOH, farnesol; FPP, farnesyl diphosphate; GDPase, geranyl diphosphatase; GGDPase, geranylgeranyl diphosphatase; GPP, geranyl diphosphate; HMG-CoA, hydroxymethylglutaryl-CoA.

surea lappa (saussurea, root; China), *Gentiana scabra* (gentian, root; China), *Bupleurum falcatum* (bupleurum, root; China), *Astragalus membranaceus* (astragalus, root; China), *Trichosanthes kirilowii* (snake gourd, root; China), *Coptis chinensis* (coptis, root; China), *Scutellaria baicalensis* (skullcap, root; China), *Cornus officinalis* (cornelian cherry, fruit; China), *Cinnamomum cassia* (cinnamon, bark; China), *Magnolia obovata* (magnolia, bark; Japan), *Commiphora abyssinica* (myrrh, resin; China), and *Ganoderma lucidum* (reishi, whole plant; China) were purchased from a local pharmacy. *Rosa canina* (rosehip, fruit; Chile), *Cynara scolymus* (artichoke, stem and leaves; Germany), *Echinacea augustifolia* (echinacea, root; Egypt), *Taraxacum officinale* (dandelion, root; Hungary), and *Citrus aurantium* (orange peel, peel; Egypt) were from a local herb shop. *Picrorrhiza kurroa* (hellebore, rhizome; India), *Bauhinia variegata* (mountain ebony, bark; India), and purified *Commiphora mukul* (Indian bdellium tree, resin; India) were generously provided by Dr. S. P. Sardeshmukh (Ayurveda Hospital and Research Center of Baratiya Sanskriti Darshan Trust, India). *Emblica officinalis* was from the Institute of Traditional Oriental Medicine (Tokyo, Japan). Other plants analyzed in additional experiments are the fresh herbs *Aloe barbadensis* (aloe, gel) and *Vinca rosea* (rose periwinkle, whole plant), which were obtained from a local private garden, and marine algae, Nori (sun-dried laver) and Kajime (dried seaweed), which were purchased from a local seafood store.

HPLC/MS analysis of herbal extracts

HPLC was performed using a Waters 2690 separations module (Waters, MA) equipped with a semimicro Capcell-Pak UG80 reverse-phase C18 column (2.0 mm × 50 mm, 3 μm; Shiseido Fine Chemicals, Tokyo). The mobile phase consisted of methanol 50 mM ammonium acetate aqueous solution (90:10; v/v) at a flow rate of 0.1 ml/min. One microliter of a sample was injected into the column by the automatic injector. Inline MS was performed by using a Waters ZMD 2000 (Micromass, UK) with an electrospray interface in the negative or positive ion mode, specifically with a capillary voltage of 3 kV and a cone voltage of 30 V. Dehydrated nitrogen was used for both cone gas (56 l/h) and desolvation gas (496 l/h), with the source and desolvation temperatures being held at 130°C and 350°C, respectively. The data acquisition program (MassLynx NT, version 3.5, Micromass) was set to scan in the total ion current at m/z 200 to m/z 350 negative ions, or in the selected ion mode at m/z 303.4, m/z 311.4 of negative ion, and m/z 319.5 of positive ion.

Methylation with diazomethane

The methylation with diazomethane was conducted with authentic GGA and herbal extracts in the same procedure. A 100 μl aliquot of samples was dried under nitrogen, and the residues were treated with diazomethane. To this end, diazomethane was released from 130 mg of N-methyl-N-nitro-N-nitrosoguanidine by addition of 0.5 ml of water and 0.6 ml of 5 M NaOH, and the generated gas was trapped in 3.0 ml of ice-cold ether in a diazomethane generator (millimole size, GL Sciences, Tokyo). A sufficient amount (0.5 ml) of the diazomethane-ether solution was added to the dry residues, and the mixture was left for 30 min at room temperature. After the reaction, the solvent was removed under nitrogen, and the methylated residues were redissolved in 0.25 ml of ethanol. The methylated herbal extracts were compared with the behavior of a methylated GGA standard on LC/MS.

Catalytic hydrogenation

The catalytic hydrogenation was conducted with authentic GGA and herbal extracts in the same procedure. A 0.1 ml aliquot of samples was diluted in 5 ml of ethanol and stirred under H_2 gas in the presence of 10 mg of Pt_2O catalyst for 3 h. After the reaction, the spent Pt_2O was removed by centrifugation and the supernatant was evaporated to dryness. The hydrogenated residues were redissolved in 0.1 ml of ethanol. The perhydrogenated

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GGA standard and herbal extracts were compared with the behavior of authentic phytanic acid on LC/MS.

Quantitative measurement of GGA by selected ion recording

The standard curve for quantification of GGA was made from mass peak areas of ion plots in the selected ion recording (SIR) at *m/z* 303.4 of the negative ion using MassLynx NT software, version 3.5. GGA standard solution (0.2 μl) was injected into the reverse-phase column ranging in amounts from 50 pg to 0.6 ng and detected by LC/MS. The concentrations of GGA in herbal extracts were calculated from the mean mass area by SIR-LC/MS chromatogram with triplicate injection based upon the standard curve.

RESULTS

Cytokilling effect of GGA and its derivatives on hepatoma-derived cell lines

We have reported that 4,5-didehydro GGA induced apoptosis in HuH-7 cells via downregulation of autocrine transforming growth factor-α expression (9). Here, we report that the exogenously added all-*trans* GGA induced cell death in HuH-7, PLC/PRF-5, and MLE-10 cells at micromolar concentrations (**Fig. 1A**) and in 16 h (Fig. 1B) but not in HepG-2 cells and mouse primary-cultured hepatocytes. Cell death was due to apoptosis as revealed by DNA stepladder formation (**Fig. 2**). GGA-induced apoptosis was also morphologically evidenced by chromatin condensation stained with Hoechst 33258 (data not shown).

We next searched what structure in GGA was required to induce apoptosis. For this we used several synthetic isoprenoids. As shown in **Fig. 3**, 4,5-didehydration did not reduce the apoptosis-inducing activity of GGA, and either α- or ω-saturation slightly decreased the activity, but neither 4,5,8,9-tetradehydration nor 4,5-didehydro-10,11,14,15-tetrahydration of GGA kept the activity. The reduction of a terminal carboxylic group to an alcohol group abolished the cell killing activity and so did the reduction in the number of isoprene units to farnesoic acid.

LC/MS analysis of herbal extracts

Inasmuch as GGA showed apoptosis-inducing activity in hepatoma cells, it was worthwhile to search for natural GGA in a hepatoma-prevention strategy with GGA-derivatives. A synthetic GGA has a molecular mass of 304 corresponding to four isoprene units with a carboxyl group in terminal. In order to detect GGA in the extracts from *S. chinensis*, a reverse-phase LC/MS analysis was performed in the negative ion mode of electrospray ionization, because authentic GGA gave a proton-deleted molecular ion, $[M-H]^-$ at *m/z* 303.4, without an appreciable amount of positive ion signals. **Figure 4A** shows the LC/MS chromatogram of total negative ions from *m/z* 200 to *m/z* 350 in *S. chinensis* extracts. Four major peaks were eluted, and each peak was relatively pure as evidenced by their mass spectra (Fig. 4B), indicating that the herbal extracts contained free fatty acids such as linolenic acid (*m/z* 277.3 $[M-H]^-$) in peak 1, linoleic acid (*m/z* 279.3 $[M-H]^-$) in peak 2, palmitic acid (*m/z* 255.3 $[M-H]^-$) in peak 3, and oleic acid (*m/z* 281.3 $[M-H]^-$) in peak 4. The prominent peaks of these four fatty acids were observed as a typical chromatogram for all herbal extracts, as described in Materials and Methods.

In total negative ion mode, no peaks were found around at a retention time (RT) of 7.65 min corresponding to authentic GGA. However, as shown in **Fig. 5**, a GGA-like component (Fig. 5B, peak f) was unveiled as a prominent peak with the same RT as authentic GGA (Fig. 5A) in SIR for *m/z* 303.4 $[M-H]^-$, which is a base ion of GGA (one mass unit lower than the molecular mass). Because arachidonic acid has the same molecular mass as GGA, it was possible to mistake this popular fatty acid for GGA in a

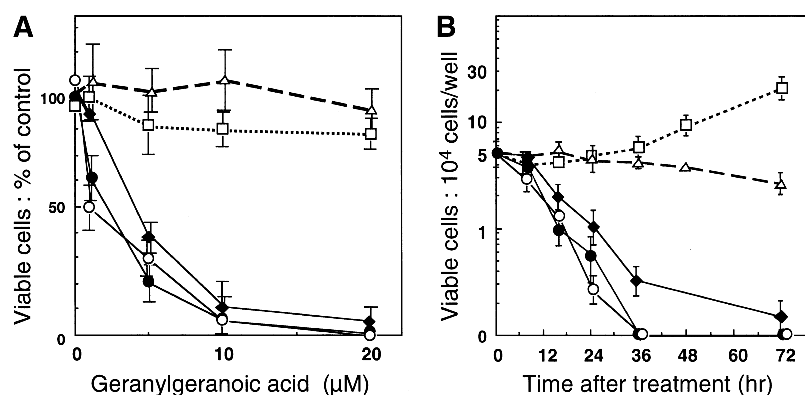

Fig. 1. Induction of cell death in human hepatoma- and mouse-transformed hepatocyte-derived cell lines by synthetic all-*trans* geranylgeranoic acid (GGA). Concentration dependence (A) and time course (B) of the cytokilling effects of GGA were measured in HuH-7 (open circle), PLC/PRF-5 (closed circle), MLE-10 (closed diamond), HepG-2 (open square), and mouse primary hepatocytes (open triangle). Viable cells were counted by trypan blue dye-exclusion method 24 h after GGA treatment (A) or after treatment with 5 μM GGA (B). An average of four determinations was calculated for each well. Means ± SE of triplicate wells are shown for each point.

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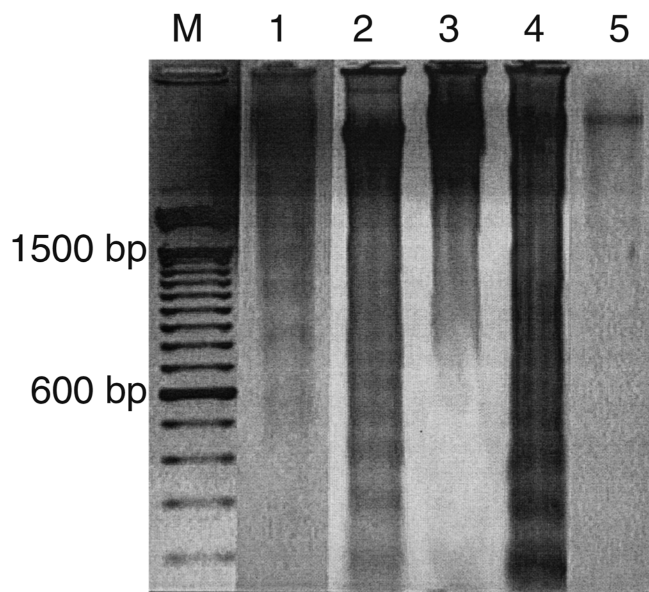

Fig. 2. Induction of DNA fragmentation in human hepatoma- and mouse-transformed hepatocyte-derived cell lines by synthetic all-*trans* GGA. HuH-7 (lane 1), PLC/PRF-5 (lane 2), HepG-2 (lane 3), MLE-10 (lane 4), and mouse primary hepatocytes (lane 5) were treated with 10 μM GGA. The genomic DNA of the detached and loosely attached cells was analyzed on agarose gel electrophoresis with a 100-bp ladder marker (M) after staining with ethidium bromide.

chromatogram of *m/z* 303.4 $[M-H]^-$, although the acid was eluted at an earlier RT of 6.5 min (Fig. 5B, peak e) than that of GGA. In a further analysis, peak f was precisely co-chromatographed with authentic GGA added internally (Fig. 5C). These results indicate the compound in peak f has the identical RT with GGA in the C18 column and may have the same molecular weight as GGA.

Structural characterization of compound in peak f

To confirm the existence of a terminal carboxyl group in the structure of a compound in peak f of Fig. 5B, a further analysis by LC/MS was performed after methylation with diazomethane. LC/MS of authentic GGA after methylation (**Fig. 6A**) revealed a conversion of *m/z* −303.4 $[M-H]^-$ (7.65 min) to *m/z* +319.5 $[M+H]^+$ (22 min). Reduction of GGA (peak I) at 7.65 min by methylation gave a product (peak II) at 22 min in an appreciable amount, suggesting that the carboxyl-end of GGA was esterified with diazomethane in diethyl ether to give methylated GGA (Fig. 6A). The small peak I at 7.65 min after methylation contained GGA that remained underivatized (3%).

As shown in LC/MS of *S. chinensis* extracts after methylation (Fig. 6B), peak f, corresponding to authentic GGA, was reduced, and the new peak was detected and confirmed its identity by showing expected shifts in RT and mass number to those of methylated-GGA standard. The substantial reduction of peak f at 7.65 min of *m/z* 303.4 $[M-H]^-$ by methylation brought about a product at 22 min of *m/z* 319.5 $[M+H]^+$ in a considerable amount. This result indicates that the GGA-like compound in peak f has one free carboxyl group in terminal that was converted to methyl ester.

To obtain inside information about the chemical structure of the compound in peak f of Fig. 5, catalytic hydrogenation of putative isoprene units was carried out, followed by an analysis by reverse-phase LC/MS. As shown in **Fig. 7A**, LC/MS of authentic GGA exposed to catalytic hydrogenation revealed a conversion of *m/z* 303.4 $[M-H]^-$

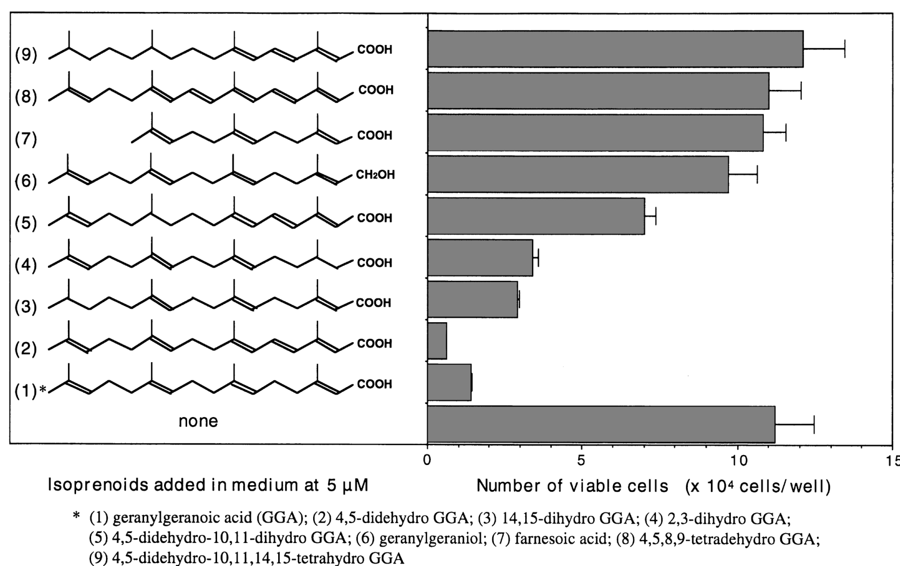

Fig. 3. Cytokilling effects of GGA derivatives in HuH-7 cells. HuH-7 cells in 6-well plates were treated with 5 μM isoprenoids each, shown by their chemical structure. A control experiment was done with an ethanol-vehicle. On the next day, the numbers of viable cells were counted by trypan blue dye-exclusion method. An average of four determinations was measured for each well. Means ± SE of triplicate wells are shown for each compound.

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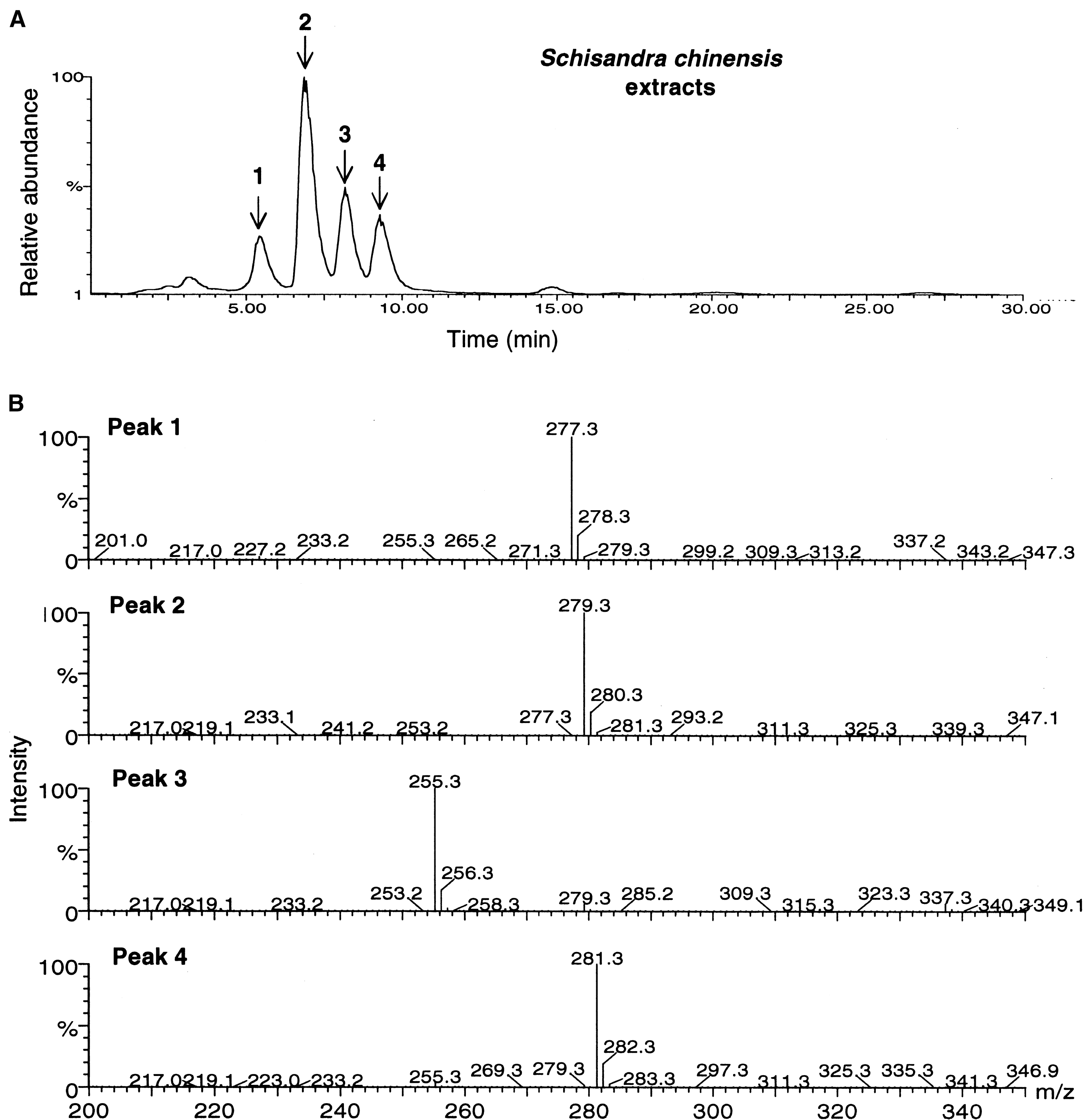

Fig. 4. Reverse-phase liquid chromatography/tandem mass spectrometry (LC/MS) analysis of *Schisandra chinensis* extracts. A: LC/MS of the extracts was performed by electrospray ionization and monitoring of total negative ions from *m/z* 200 to *m/z* 350. B: The mass spectra of peaks 1 to 4 in panel (A) are shown: *m/z* 277.3 corresponding to 18:3 ($C_{18}H_{29}O_2^-$, linolenoate ion); *m/z* 279.3 corresponding to 18:2 ($C_{18}H_{31}O_2^-$, linoleate ion); *m/z* 255.3 corresponding to 16:0 ($C_{16}H_{31}O_2^-$, palmitate ion); and *m/z* 281.3 corresponding to 18:1 ($C_{18}H_{33}O_2^-$, oleate ion).

(7.65 min) to 311.4 *m/z* [M-H]$^-$ (13.9 min). Reduction of GGA (peak I) at 7.65 min by hydrogenation gave rise to a product (peak III) at 13.9 min in an appreciable amount at the same RT as authentic phytanic acid, suggesting that four double-bonds in GGA were fully saturated through incorporation of eight hydrogen atoms. No partially hydrogenated GGA was detected. The small peak I at 7.65 min after hydrogenation contained GGA that remained underivatized (1.8%). Further, an additional experiment of arachidonic acid with catalytic hydrogenation confirmed that perhydro-arachidonic acid eluted approximately 5 min later than perhydro GGA at the same RT as arachidic acid (20:0n-6) at *m/z* 311.4 [M-H]$^-$ (results not shown).

LC/MS of *S. chinensis* extracts after hydrogenation (Fig. 7B) showed the substantial reduction in peak f at 7.65 min

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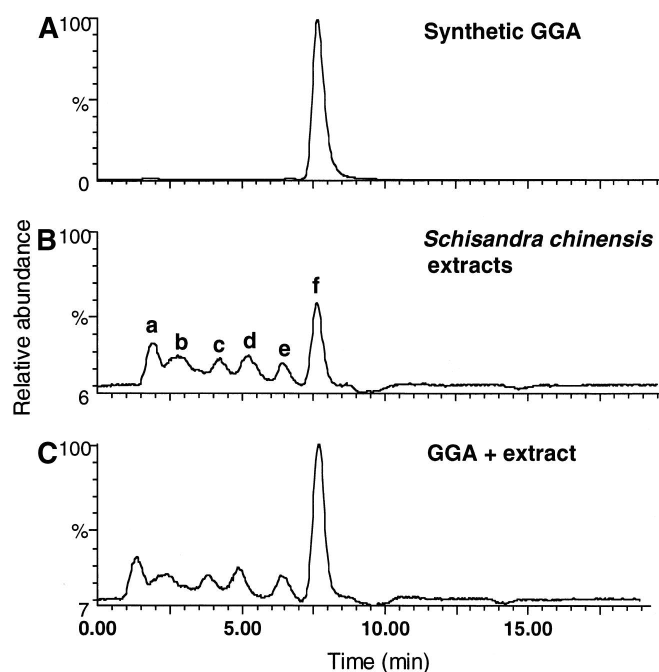

Fig. 5. Selected ion chromatograph (negative ions at *m/z* 303.4) of authentic GGA and *S. chinensis* extracts by LC/MS. A: Authentic GGA was eluted at the retention time (RT) of 7.65 min. B: A prominent component (peak f) in the herbal extracts was eluted at the same RT as authentic GGA. C: Cochromatography of peak f with authentic GGA.

of *m/z* 303.4 [M-H]$^-$ and the significant enhancement of 13.9-min peak at *m/z* 311.4 [M-H]$^-$. Prior to hydrogenation (Fig. 7B, upper panel), the herbal extracts were suggested to contain endogenous phytanic acid. These results indicate that the GGA-like compound in peak f had four isoprenyl double-bonds, as evidenced by incorporation of eight hydrogen atoms after hydrogenation, and the hydrogenated peak f was identical to phytanic acid.

However, the calculated amount of the 13.9-min peak (phytanic acid) produced after hydrogenation was more than the expected area of the reduced peak f. To explore the presence of possible sources for phytanic acid other than GGA or peak f, GGA derivatives saturated to the different extents, such as di- (*m/z* 305.4), tetra- (*m/z* 307.4), and hexa- (*m/z* 309.4) hydrogenation, were surveyed on LC/MS before and after hydrogenation. As shown in **Fig. 8**, the prominent components eluted at *m/z* 303.4, 305.4, 307.4, and 309.4 prior to hydrogenation decreased or disappeared after being subjected to hydrogenation, whereas peak j corresponding to phytanic acid significantly rose. These components were demonstrated to elute at clearly different RTs from common straight-chain fatty acids with the same molecular weight, such as 20:4n-6 (arachidonic acid, *m/z* 303.4), 20:3n-6 (dihomo-γ-linolenic acid, *m/z* 305.4), 20:3n-9 (mead acid, *m/z* 305.4), 20:2n-6 (11,14-eicosadienoic acid, *m/z* 307.4), and 20:0 (arachidic acid, *m/z* 311.4), respectively, except that an authentic standard corresponding to 20:1 at *m/z* 309.4 was not available (Fig. 8). Therefore, it is strongly suggested that peaks g, h, and i might correspond to di-, tetra- and hexa-hydro GGAs, respectively. These results indicate that the significant amount of peak j was produced during the hydrogenation, possibly arising from partially saturated compounds in peaks f, g, h, and i. The similar elution profile of putative GGA derivatives, including phytanic acid, was observed in all herbal extracts as described in Materials and Methods.

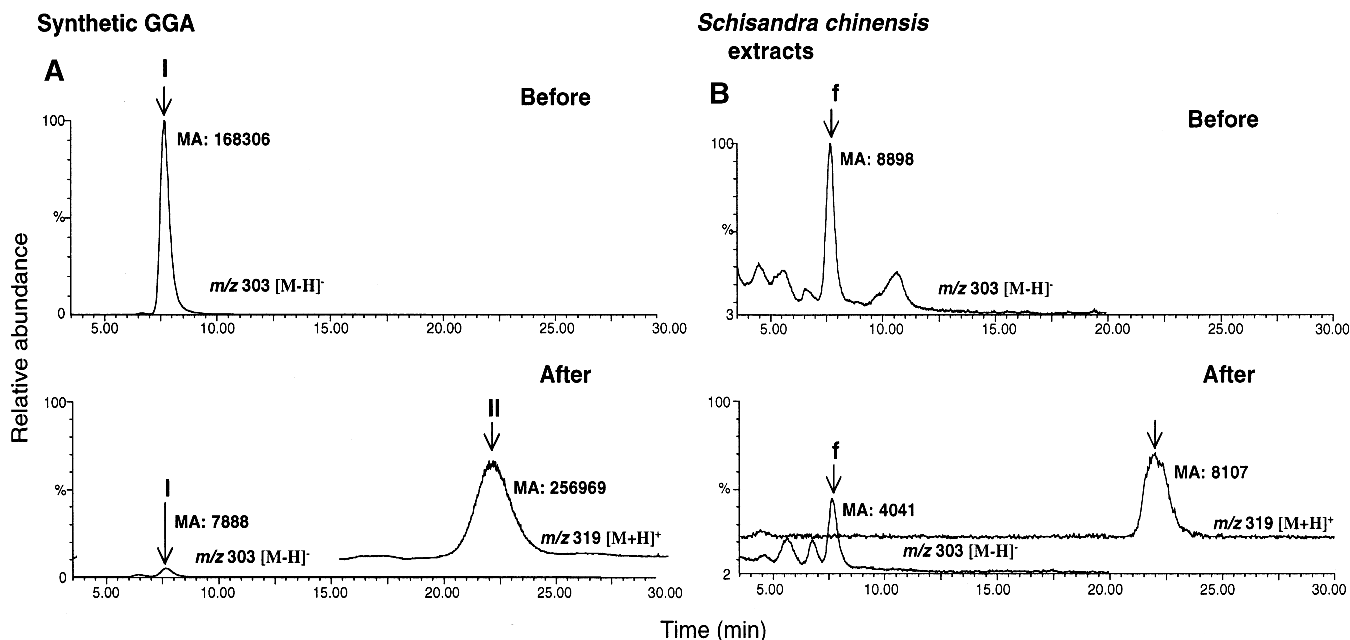

Fig. 6. LC/MS elution profiles of authentic GGA and *S. chinensis* extracts before and after methylation with diazomethane. A: Selected ion tracings of authentic GGA (peak I) for *m/z* 303.4 [M-H]$^-$ and methylated GGA (peak II) for *m/z* 319.5 [M+H]$^+$. B: Selected ion tracings of the herbal extracts for *m/z* 303.4 [M-H]$^-$ and *m/z* 319.5 [M+H]$^+$ are shown. MA, mass area.

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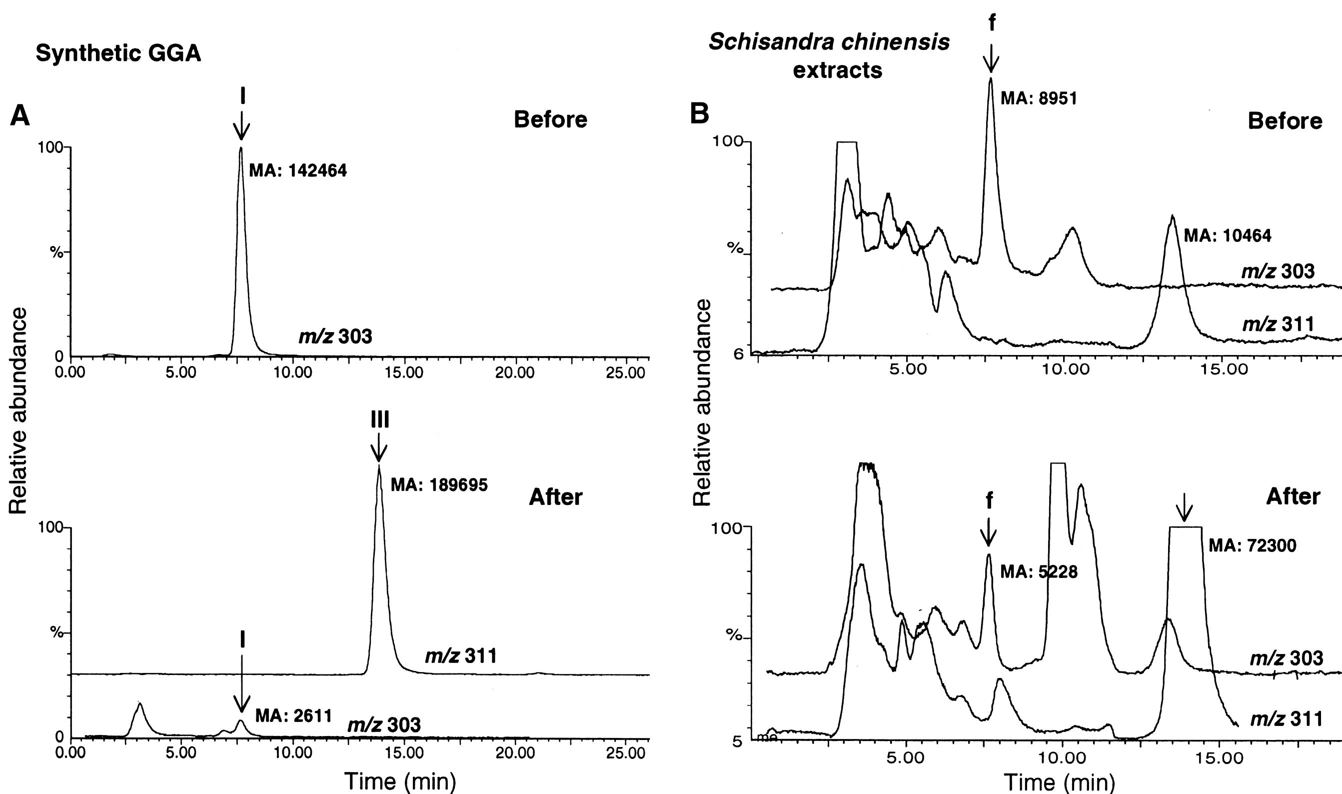

Fig. 7. LC/MS elution profiles of authentic GGA and *S. chinensis* extracts before and after catalytic hydrogenation. Catalytic hydrogenation was performed with PtO_2 and H_2 as described in the Materials and Methods section. A: Selected ion tracings of authentic GGA (peak I) for *m/z* 303.4 $[M-H]^-$ and perhydro GGA (peak III) for *m/z* 311.4 $[M-H]^-$ after being subjected to hydrogenation. Perhydro GGA was eluted at the same RT of 13.9 min as authentic phytanic acid. B: Selected ion tracings of the herbal extracts for *m/z* 303.4 and *m/z* 311.4 $[M-H]^-$ before and after being subjected to hydrogenation.

Amounts of GGA in herbs

As a practical example, the standard curve was established in order to quantify GGA contents in herbs using LC/MS under conditions as described in Materials and Methods. A linear relationship between the amount of injected GGA and the peak area was confirmed, ranging in amounts from 50 pg to 0.6 ng, with the regression values for $R^2 = 0.9834$. The amounts of unesterified GGA in herbal extracts were calculated from the mean mass area with triplicate injection by LC/MS chromatogram based upon the standard curve. The recovery of GGA in the extract was checked using the method of extraction as described in Materials and Methods by addition of known amounts of the GGA standard to herbal homogenates and was greater than 98% throughout the process.

GGA was detected for almost all dry herbs in this screening. GGA concentration in herbs ranged from 0.1 μg/g to 2.9 μg/g (**Table 1**), in decreasing order (>1 μg/g): turmeric (root), schisandra (fruit), licorice (root), Indian gooseberry (fruit), rhubarb root (root), hellebore (root), saussurea (root), cornelian cherry (fruit), and rosehip (fruit). There was no significant difference in GGA content according to the plant part used.

In addition to a discovery of natural GGA, the existence of free phytanic acid, fully saturated GGA, was confirmed in LC/MS chromatograms for all dry herbs as shown in an example of *S. chinensis* (Figs. 7, 8). Without quantitative determination of phytanic acid, a simple peak area ratio of GGA to phytanic acid in total negative ion chromatogram was listed in Table 1. The ratio ranged widely from 0.1 to 6.0, and in a higher ratio of GGA (>2.0): turmeric (root) 6.0, schisandra (fruit) 4.4, hellebore (bark) 3.5, snake gourd root (root) 2.9, rhubarb root (root) 2.8, and artichoke (stem, leaves) 2.3; inversely, in a higher ratio of phytanic acid (<0.5): magnolia (bark) 0.1, Indian bdellium tree (resin) 0.2, licorice (root) 0.4, coptis (root) 0.5, Indian gooseberry (fruit) 0.5, and echinacea (root) 0.5. The relationship between the amounts of GGA and phytanic acid significantly correlated in Chinese herbs, but not in Ayurvedic herbs.

Aside from dry herbs, the same LC/MS analysis was performed with fresh herbs, including aloe (*A. barbadensis;* gel) and rose periwinkle (*V. rosea*; whole plant), and marine algae, including Nori (sun-dried laver) and Kajime (dried seaweed), which had no significant amount of GGA (data not shown).

DISCUSSION

In the present study, first we showed that GGA was a micromolar inducer of apoptosis in human hepatoma- and mouse-transformed hepatocyte-derived cell lines, but not

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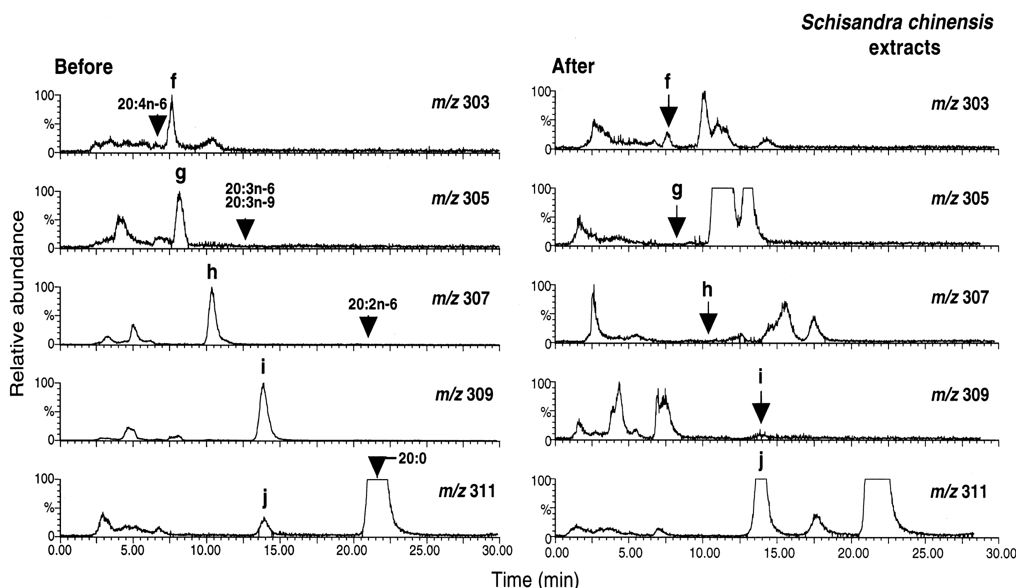

Fig. 8. Selected negative ion tracings for *m/z* 303.4, 305.4, 307.4, 309.4, and 311.4 in *S. chinensis* extracts before and after hydrogenation. Before hydrogenation, prominent components f, g, h, and j eluted well separately from fatty acids such as 20:4n-6 (arachidonic acid), 20:3n-6 (dihomo-γ-linolenic acid), 20:3n-9 (mead acid), 20:2n-6 [11,14-eicosadienoic acid (EDA)], and 20:0 (arachidic acid), respectively.

in human hepatoblastoma-derived cell line and mouse primary hepatocytes. Second, and most importantly in this paper, a natural occurrence of cancer-preventive GGA in medicinal herbs was demonstrated by LC/MS analysis, and we also suggested the existence of some other GGA derivatives, including phytanic acid, in herbal extracts.

Previous studies from our group showed that GGA and 4,5-didehydro GGA were both potent ligands for CRABP (3) and nuclear retinoid receptors (4), so we called these acids "acyclic retinoids." Consistent with our findings and definition of acyclic retinoids, some other acyclic compounds have been reported, such as methoprene acid

TABLE 1. Concentrations of geranylgeranoic acid and its relative amounts to phytanic acid in herbs

Herbs					
Common Name	Latin Name	Part Used	Origin	GGA	GGA/Phytanic Acid
				μg/g dry wt	*mass area ratio*
Turmeric	*Curcuma longa*	rhizome	China	2.9	6.0
Schisandra	*Schisandra chinensis*	fruit	China	2.4	4.4
Licorice	*Glycyrrhiza uralensis*	root	China	2.3	0.4
Indian gooseberry	*Emblica officinalis*	fruit	India	2.1	0.5
Rhubarb root	*Rheum palmatum*	root	China	1.7	2.8
Hellebore	*Picrorrhiza kurroa*	rhizome	China	1.4	3.5
Saussurea	*Saussurea lappa*	root	China	1.1	1.8
Cornelian cherry	*Cornus officinalis*	fruit	China	1.0	0.9
Myrrh	*Commiphora abyssinica*	resin	China	1.0	0.7
Rosehip	*Rosa canina*	fruit	China	1.0	1.6
Cinnamon	*Cinnamomum cassia*	bark	China	0.9	1.3
Artichoke	*Cynara scolymus*	stem, leaves	Germany	0.8	2.3
Gentian	*Gentiana scabra*	root	China	0.7	1.1
Bupleurum	*Bupleurum falcatum*	root	China	0.7	1.8
Orange peel	*Citrus aurantium*	peel	Egypt	0.7	0.8
Echinacea	*Echinacea augustifolia*	root	Egypt	0.7	0.5
Astragalus	*Astragalus membranaceus*	root	China	0.5	1.4
Indian bdellium tree	*Commiphora mukul*	resin	India	0.5	0.2
Mountain ebony	*Bauhinia variegata*	bark	India	0.5	1.5
Magnolia	*Magnolia obovata*	bark	Japan	0.4	0.1
Snake gourd root	*Trichosanthes kirilowii*	root	China	0.4	2.9
Reishi	*Ganoderma lucidum*	whole	China	0.4	0.6
Dandelion	*Taraxacum officinale*	root	Hungary	0.3	0.8
Coptis	*Coptis chinensis*	root	China	0.1	0.5
Skullcap	*Scutellaria baicalensis*	root	China	<0.01	<0.01

GGA, geranylgeranoic acid.

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(19) and phytanic acid (20), as ligands for retinoid-X receptor. At the initial point of our study, we thought that apoptosis-inducing activity of 4,5-didehydro GGA was attributable to the retinoidal function; however, natural retinoids such as all-*trans* and 9-*cis* retinoic acids were unexpectedly unable to induce apoptosis in HuH-7 cells (9). Henceforth, 4,5-didehydro GGA was expected to mimic other natural isoprenoids such as GGA rather than retinoids in terms of apoptosis induction in hepatoma cells. In fact, GGA itself actively induced apoptosis in human hepatoma- (21) and mouse hepatocyte-derived cell lines (Figs. 1, 2). Furthermore, 2,3-dihydro GGA possessed this activity (Fig. 3), even though the α-saturation of GGA totally destroyed the ligand activity for retinoid receptors (4), suggesting that the induction of apoptosis by GGA may not be mediated through retinoid receptors. Recently, it was reported that phytanic acid transactivated peroxisome proliferator-activated receptor α (PPARα), as well as polyunsaturated fatty acids (22). GGA may well be a potent ligand for PPARα and for some other orphan receptors. We cannot even exclude the possibility that GGA may induce apoptosis through a hypothetical GGA-specific receptor, which is now under investigation in our laboratory by using a technique of RNAi with a whole genomic siRNA expression library.

Inasmuch as GGA and its derivatives were shown to induce apoptosis in human hepatoma cell lines (Fig. 3), our next question was whether GGA is present in nature. Isoprenoids are synthesized in all organisms but are especially abundant and diverse in plants, with tens of thousands of compounds reported to date (23). To our knowledge, however, there has been no report of the existence of the acyclic diterpenoid GGA in the isoprenoid biosynthetic pathway in plants or animals.

The present characterization of a GGA-like compound in herbal extracts revealed its structure by LC/MS analysis. The compound, coeluted with authentic GGA, was converted to a product corresponding to GGA methylester after its methylation with diazomethane and was converted to a product incorporated with eight hydrogen atoms corresponding to phytanic acid by catalytic hydrogenation. These results strongly support a conclusion that the GGA-like compound is structurally identical with GGA, which has four isoprenyl double-bonds and a carboxyl group in terminal with a molecular mass of 304, although further isolation analysis is required to warrant the existence of natural GGA.

The amounts of GGA in herbs were in the range of micrograms/grams (Table 1), suggesting that GGA may be relevant to micronutrients such as vitamins. GGA and its derivatives are active to induce apoptosis in hepatoma-derived HuH-7 cells at concentrations of 1∼10 μM (Fig. 1). On the supposition that all of the ingested GGA will appear in blood, several hundred grams of herbs will be required to reach the effective concentrations. It is also worthwhile to mention that the ingested GGA may be selectively taken up and accumulated in tumor cells, because tumor cells show the enhanced expression of CRABP, to which GGA may be bound (3,4). Ratios of GGA to phytanic acid contents varied by 60-fold in our samples of herbs. What does determine a proportion of GGA to phytanic acid in the dry herbs? Chl, the main constituent of the photosynthetic apparatus in plants, consists of two moieties, chlorophyllide and phytol. The hydrogenation of GGPP is catalyzed to phytyl diphosphate (phytylPP) by a geranylgeranyl reductase (CHL-P), as indicated in Scheme 1. It is reported that CHL-P uses GGPP and geranylgeranylated chlorophyllide as substrates and directs phytylPP to the tocopherol- and Chl-synthesizing pathway (24). CHL-P catalyzes a stepwise reduction of geranylgeranyl derivatives to phytyl derivatives. This reductase broad specific for geranylgeranyl moiety could catalyze GGA to phytanic acid conversion in a stepwise mode. Therefore, once GGA is produced from GGOH in plants, a proportion of phytanic acid to GGA contents would be attributable to CHL-P activity.

Furthermore, the finding of other GGA derivatives with only subtle differences on numbers of double bonds in herbal extracts is informative for supporting the existence of GGA and CHL-P activity. We could demonstrate that these peaks did not represent the well-known straight-chain fatty acids, and that these were all converted to phytanic acid by hydrogenation. In the present study, the exact positions of the double bonds for *m/z* 305.4, 307.4, and 309.4 $[H\text{-}M]^-$ were not assigned because authentic standards were not available. However, based upon a mode of CHL-P enzyme action (25), it is reasonable to speculate that these may be 6,7-dihydro (for *m/z* 305.4), 6,7,10,11-tetrahydro (for *m/z* 307.4), and 6,7,10,11,14,15-hexahydro (for *m/z* 309.4) GGAs. Obviously, further structural analysis is required of each isolated compound and additional examination of biological activity for these GGA derivatives found in herbs.

In terms of its distribution, interestingly, GGA and phytanic acid were observed in almost all dry herbs examined in this screening. In additional experiments of other plants, however, fresh herbs, including aloe (*A. barbadensis*; gel) and rose periwinkle (*V. rosea*; whole plant), used as liver tonics had no detectable GGA and its derivatives. Marine algae, including Nori (sun-dried laver) and Kajime (dried seaweed), contained only GGA derivatives, including phytanic acid, with no significant amount of GGA. Although little is known about the presence of phytanic acid in higher plants (14), most of the medicinal dry herbs used in the present study contained both GGA and phytanic acid as a characteristic common to all, independent of their own specific effects, such as liver tonics. The accumulation of these compounds is supposed to be involved with a certain type of plants, such as "dried" plants, because it has been reported that phytanic acid was not found in any food consisting of nondried vegetable (26).

The information that dry herbs, not fresh plants, contain GGA and its derivatives could provide a new vista on the biological significance of GGA in plants. A large variety of products are derived from isoprenoids in plants for their growth and response to environmental stress, which is closely associated with plant defense mechanisms (11). For example, Nah, Song, and Back (27) have reported

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that farnesyl disphosphatase and geranylgeranyl disphosphatase (Scheme 1) were induced in rice seedlings when they were exposed to UVC. Unlike mammals, the isoprenoid metabolism in plants is a cell autonomous process (28). It can, therefore, be presumed that the reactions to environmental stress, such as drying or UV rays, occur in the plant body as plant defense mechanisms even after collecting herbs off the soil. In this context, the secondary metabolites in the isoprenoid pathway, such as GGOH-derived diterpenoids in response to environmental stress, can be synthesized and accumulated during preparation of dry herbs.

The drying process in plants may be a sort of "dying" process that results in cell death. Though it can be a broad interpretation, it is intriguing for us to speculate that GGA may be involved in apoptosis as a defense mechanism in plants. Apoptosis has now been recognized as an indispensable facet of development, defense responses, and tissue sculpturing in both animal and plant, though little is known about the molecular mechanisms of apoptosis in plant cells (29). As the present study demonstrates the possibility that GGA is a natural component of dry herbs, it may also be one of the putative intracrine regulators for apoptosis in plant cells. Traditional medicine has utilized dry herbs for thousands of years, which is not only because of preservation, but may be because of naturally occurring substances induced by a defense mechanism for drying, which may be taken into account empirically in order to obtain the optimal efficacy.

It is interesting that we found the same substance in nature as the one that has been chemically synthesized for cancer chemoprevention of hepatoma. GGA and its 4,5-didehydro derivative were already reported to both inhibit experimental hepato-carcinogenesis and induce differentiation and apoptosis in human hepatoma-derived cell lines. Further, a one-year intake of 4,5-didehydro GGA already has been proven effective for prevention of second primary hepatoma and increased the 5-year survival rate by a phase II double-blinded, placebo-controlled clinical trial with relatively low toxicity (1,2).

Our research suggests that cancer-preventive GGA and its derivatives could be synthesized in the isoprenoid biosynthetic pathway in plants. With regard to the practical use of GGA contained in herbs in order to prevent second primary hepatoma, we have not obtained yet a clear interpretation of the relation between the amount of natural GGA and its effect from the perspective of cancer prevention. However, the presence of natural GGA in herbs is informative for supporting the therapeutic efficacy of 4,5-didehydro GGA in the phase II clinical trial (1) and also will provide concrete information on whether natural GGA and its derivatives can be expected to play an important role in improving primary health care besides cancer prevention (30).

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