Geranylgeraniol, an Intermediate Product in Mevalonate Pathway, Induces Apoptotic Cell Death in Human Hepatoma Cells: Death Receptor-independent Activation of Caspase-8 with Down-regulation of Bcl-xL Expression

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Geranylgeraniol (GGOH), an intermediate of mevalonate metabolism, is known to induce apoptosis in various lines of cancer cells. The present study was undertaken to clarify the signaling pathways of apoptosis induced by GGOH in human hepatoma cells. HuH-7 human hepatoma cells were incubated in the absence or presence of GGOH. Activation of caspase-8/-9/-3 in HuH-7 cells was found after 8 h treatment with GGOH, at which time DNA fragmentation and loss of mitochondrial transmembrane potential ($\Delta\psi_m$) occurred. HuH-7 cells do not express Bcl-2; however, down-regulation of Bcl-xL expression preceded activation of the caspase cascade in GGOH-treated HuH-7 cells, while Bax expression was not changed by GGOH treatment. Addition of caspase inhibitors restored the decreased cell viability of HuH-7 cells by GGOH, including $\Delta\psi_m$, to the baseline level, which indicated that caspase triggers mitochondria-dependent apoptotic pathways in GGOH-treated HuH-7 cells. Similarly, GGOH-mediated apoptosis of HuH-7 cells was clearly prevented by coadministration of ursodeoxycholic acid (UDCA), which led to restoration of the level of Bcl-xL expression. Activation of caspase-8/-9/-3, as well as $\Delta\psi_m$, by GGOH treatment was suppressed by addition of UDCA. Our results indicate that activation of the caspase cascade initiating from caspase-8, which could be accelerated by down-regulation of Bcl-xL expression, plays a key role in an apoptotic process induced by GGOH in human hepatoma cells.

Key words: Geranylgeraniol — Apoptosis — Caspase-8 — Bcl-xL — Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies worldwide, especially in several areas of Asia and Africa. In Japan, it is now the third most common cancer in men and the fourth most common cancer in women, with the annual death rate from HCC exceeding 30,000. Advances in medical technology permit the early recognition and treatment of HCC, but in the most recent reports, the 5-year survival rate barely reached 40%. The low rate of survival after any treatment is probably due to the high incidence of recurrent tumors and second primary tumors; in fact, the incidence is approximately 25% one year after radical surgical reseption and approximately 50% after two years. Since chronic viral hepatitis, particularly cirrhosis, accounts for the large majority of HCC, and since the rates of tumor recurrence and of occurrence of second primary tumors are very high, several approaches to chemoprevention of HCC have been employed in human trials. Among them, polypropenoic acid, given for a defined time postoperatively, has been shown to reduce the formation of metachronous lesions after surgery for resectable HCC. Moreover, the authors have also demonstrated that the chemopreventive effect of polypropenoic acid improves survival of these patients.

Although further research is under way to elucidate the mechanism of action of polypropenoic acid, polypropenoic acid is a derivative of geranylgeranoic acid, and it is likely that, just as geranylgeranoic acid does, it promotes apoptosis of the cells with high transforming potential, leading to the clonal deletion of these cells. Isoprenoids such as farnesol and geranylgeraniol (GGOH) are intermediate products in the mevalonate pathway and function as essential compounds for cell proliferation and differentiation. Indeed, farnesyl and geranylgeranyl pyrophosphate are precursors for prenylation, a posttranslational maturation of diverse proteins involved in cell growth. In addition, recent studies have shown that the orphan nuclear receptor LXRα is positively and negatively regulated by isoprenoids derived from a common precursor, mevalonate. Thus, the regulatory mechanism of isoprenoid biosynthesis is a key event in control of cell growth, although the mode of isoprenoid availability seems to be different in different cell types. Previous studies showed that exposure of cells to GGOH resulted in apoptosis in a variety of cancer cell lines. In human leukemia cell lines HL-60 and U937, GGOH-mediated apoptosis was found to be accompanied by acti-
vation of caspase-3,18,21) which was similar to induction of apoptosis by geranylgeranoic acid in human hepatoma cells.13) Although the upstream molecules triggering the activation of caspase-3 by these agents were not investigated in those studies, identification of the molecules is very important since GGOH and geranylgeranoic acid are not only pharmacological agents, but also are believed to be produced endogenously by eucaryotic cells.13-15)

In the present study, we analyzed the biochemical pathways leading to apoptosis by GGOH in human hepatoma cells. We showed that caspase-8 is a key activator caspase involved in GGOH-mediated apoptosis, and that down-regulation of Bcl-xL expression by GGOH participates in this process.

**MATERIALS AND METHODS**

**Chemicals and cell culture** GGOH was a generous gift from Eisai Co. (Tokyo), and ursodeoxycholic acid (UDCA) was obtained from Tokyo Tanabe Co. (Tokyo). HuH-7 and HepG2 human hepatoma cells were maintained in a chemically defined medium, IS-RPMI,22) containing 2% and 5% fetal bovine serum, respectively. For each experiment, the medium was replaced with serum-free IS-RPMI. Cell growth was analyzed using 48-well multiplates, and 2×10^4 cells were placed into each well and incubated at 37°C in 5% CO_2. One day later, the medium was replaced with fresh medium or fresh medium containing GGOH (1–50 μmol/liter). The cells were further incubated at 37°C in 5% CO_2, and the cell viability was determined after incubation using the colorimetric method as described previously.23) In some experiments, caspase-8 inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (IETD-FMK, Enzyme System Products, Livermore, CA), broad caspase inhibitor, Z-Val-Ala-Asp-CH2DCB (Z-VAD-CH2DCB, Phoenix Pharmaceuticals, Inc., Mountain View, CA) or UDCA was added to the cell culture.

**Apoptosis assay** To detect DNA fragmentation on agarose gel electrophoresis, cells were resuspended in lysis buffer containing 10 mM EDTA, 0.5% Triton X-100 and 10 mM Tris-HCl (pH 8.0) at 4°C for 10 min and pelleted by centrifugation at 16 000 rpm for 20 min. The supernatant was treated with RNase A and proteinase K at 37°C for 1 h, then precipitated by addition of isopropanol. The precipitate was resuspended in TE buffer, and electrophoresed in a 2% agarose gel. The fragmented DNA was detected by ethidium bromide staining. DNA fragmentation was also quantified in terms of the percentage of cells with hypodiploid DNA as described previously.24) In brief, cells were fixed with 70% ethanol and treated with RNasea (100 μg/ml, Sigma Chemical Co., St. Louis, MO) and then stained with propidium iodide (100 μg/ml, Sigma) for 30 min on ice. The stained cells were analyzed by a flow cytometer (Epics XL, Beckman Coulter, Hialeah, FL) to detect the presence of cells with hypodiploid DNA. In addition, cells were stained with the potential-sensitive fluorescence dye DiOC6(3), 3,3′-dihexyloxa-carbocyanine iodide (Fluoreszenz Technologie, Grottenehofstr, Austria), to determine the drop of mitochondrial transmembrane potential (ΔΨm) as reported previously.25) Briefly, cells were stained with 40 nmol/liter of DiOC6(3) for 15 min at 37°C. They were washed once with phosphate-buffered saline (PBS), and the ΔΨm, which is an indicator for mitochondrial dysfunction, was measured by flow cytometry (Epics XL). Activated Asp-Glu-Val-Asp ase (DEVase; estimated as a measure of caspase-3) in treated cells was also estimated by flow cytometry as described previously.26) Briefly, the cells were centrifuged to remove the culture medium. DEVD substrate containing rhodamine was then added to the cell pellet and the mixture was incubated in a 5% CO_2 incubator at 37°C for 60 min. After incubation, samples were analyzed with a flow cytometer (Epics XL) to determine the percentage of cells expressing intracellular DEVDase activity.

**Western blotting** Cells were washed three times with PBS, collected and lysed by addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of aprotonin, 1% NP40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14 000 rpm for 30 min at 4°C. The supernatant was collected, and the protein concentration was determined using a Bio-Rad (Melville, NY) protein assay kit. The same amount of protein from each lysate (20 μg/well) was subjected to 15% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween 20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody (mouse monoclonal anti-human caspase-3, Transduction Laboratories, Lexington, KY; mouse monoclonal anti-human caspase-8, MBL, Nagoya; mouse monoclonal anti-human caspase-9, Millennium Biotechnology, Ramona, CA; mouse monoclonal anti-human Bcl-2, DAKO Japan, Kyoto; rabbit polyclonal anti-human Bax, Santa Cruz Biotechnology, Santa Cruz, CA; mouse monoclonal anti-human Bcl-xL, Trevigen, Gaithersburg, MD; mouse monoclonal anti-human XIAP, MBL; mouse monoclonal anti-human β-actin, as an internal control for western blot analysis, Sigma Chemical Co.). The membranes were washed with PBS-T and incubated with a 1:1000 dilution of sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase. The ECL system (Amersham Life Science, Buckinghamshire, England) was used for detection.
RESULTS

Induction of apoptosis in human hepatoma cells by GGOH
HuH-7 cells or HepG2 cells were incubated in the absence or presence of varying concentrations of GGOH (1–50 µmol/liter). When numbers of viable cells were counted 16 h after the incubation, the cell viability was reduced by GGOH in a dose-dependent manner (Fig. 1A). To clarify whether GGOH induced apoptosis in human hepatoma cells, a kinetic study on DNA fragmentation in response to 20 µmol/liter of GGOH was performed. DNA ladder formation, an indicator of inter-nucleosomal DNA cleavage, began to be detected on agarose gel electrophoresis 8 h after incubation, followed by an increasing trend at 16 h (Fig. 1B). DNA fragmentation was also quantified by flow cytometer, demonstrating a clear induction of hypodiploid* HuH-7 cells by GGOH treatment (Fig. 1C). DNA fragmentation in GGOH-treated HepG2 cells in proportion to the reduced cell viability was also demonstrated (data not shown).

Activation of the caspase cascade including caspase-8, caspase-9 and caspase-3 during GGOH-mediated apoptosis
To ensure the involvement of caspases in GGOH-mediated apoptosis, the expression of pro-caspase-3, pro-caspase-8 and pro-caspase-9 was evaluated by western blotting (Fig. 2). These forms are converted into the active subunits during the course of activation.27–30 Thus, decreases in densities of pro-caspase-3,-8 or -9 band by western blotting indicate activation of the respective caspase. The mouse monoclonal anti-human caspase-3, caspase-8 and caspase-9 used in the current experiment recognize 32 kDa pro-caspase-3, 55/54 kDa pro-caspase-8, and 46 kDa pro-caspase-9, respectively. The level of expression of pro-caspase-8 was clearly decreased 8 h after treatment with 20 µmol/liter of GGOH and the molecule was hardly detected at 16 h, indicating that caspase-8 was fully activated. The level of expression of pro-caspase-9 was decreased 8 h after GGOH treatment and was almost undetectable at 16 h. Similarly, pro-caspase-3 expression was decreased 8 h after GGOH treatment, at which time DNA fragmentation was detectable (Fig. 1B), and the decrement was increased at 16 h. Activation of caspase-3 was also estimated by flow cytometric analysis, which demonstrated the increase of intracellular DEVDase+ HuH-7 cells by GGOH treatment (Fig. 3). The involvement of caspase in GGOH-induced apoptosis was further studied by means of the following experiments.

Figs. 3 and 4 showed that the addition of IETD-FMK as well as Z-VAD-CH2DCB almost completely inhibited apoptotic cell death of HuH-7 cells by GGOH with restoration...
of ΔΨm. These results are consistent with recent reports suggesting that the sequential activation of caspases initiating from caspase-8 is one of the major pathways for the activation of caspase-3 in response to apoptotic stimuli, and furthermore, activated caspase(s), probably caspase-8, appeared to be a principal factor triggering mitochondrial dysfunction in GGOH-treated human hepatoma cells.

Fig. 2. Western blot analysis of activation of the caspase cascade and alterations in expression of Bax, Bcl-xl, and XIAP during GGOH treatment in HuH-7 cells. HuH-7 cells were incubated with 20 µmol/liter of GGOH for the indicated periods (lanes 1–5), and western blotting was performed as described in “Materials and Methods.” Results shown are from a representative experiment from a total of 4 performed.

Fig. 3. DEVDase activity in HuH-7 cells induced by GGOH. HuH-7 cells were incubated with 20 µmol/liter of GGOH for 8 h, then DEVDase activity in the cells was examined as described in “Materials and Methods.” Note that DEVDase activity in HuH-7 cells was clearly induced by GGOH, which was completely inhibited by the addition of Z-VAD-CH2DCB (200 µmol/liter). The percentage is that of intracellular active DEVDase+ cells. Results shown are from a representative experiment from a total of 4 performed.

Down-regulation of expression in Bcl-xl and X-chromosome-linked inhibitor of apoptosis protein (XIAP) but not Bax by GGOH during apoptosis The process of apoptosis is regulated by Bcl-2-related proteins and mem-

Fig. 4. Caspase inhibitors abolished GGOH-mediated apoptosis of HuH-7 cells. (A) HuH-7 cells were incubated with 20 µmol/liter of GGOH for 8 h (solid bar) or 16 h (blank bar) under the following conditions, and the cell viability was determined by the colorimetric method. Lane 1, cultured without treatment; lane 2, cultured with a caspase-8 inhibitor (IETD-FMK, 200 µmol/liter); lane 3, cultured with GGOH (20 µmol/liter); lane 4, cultured with GGOH in the presence of a caspase-8 inhibitor. Results are expressed as a percentage of untreated controls (lane 1). Data are the mean±SD value (n=4) of a representative experiment. HuH-7 cells were incubated with or without GGOH (20 µmol/liter) in the presence or absence of Z-VAD-CH2DCB (200 µmol/liter) for 8 h, then DNA fragmentation (B) and ΔΨm (C) of the cells were determined by flow cytometry. Note that Z-VAD-CH2DCB almost completely suppressed both DNA fragmentation (B) and ΔΨm (C) in HuH-7 cells induced by GGOH. The percentage is that of hypodiploid DNA+ cells (B) or ΔΨm+ (C) cells. Results shown are from a representative experiment from a total of 3 performed.
bers of the inhibitor of apoptosis (IAP) family of gene products. Thus, the expression of Bcl-2-related proteins and XIAP was analyzed by western blotting (Fig. 2). As previously described, Bcl-2 was not expressed in HuH-7 cells (data not shown). Expression of Bcl-xL, an inhibitor of apoptosis, was reduced 2 h after GGOH treatment and reached very low levels in a time-dependent manner. In contrast, expression of Bax, an inducer of apoptosis, was not affected by GGOH treatment throughout the study. XIAP expression was reduced by GGOH treatment, in which the time course pattern paralleled that of activation of the caspase cascade by GGOH.

**Inhibitory effects of UDCA on GGOH-mediated apoptosis** Recent studies suggest a unique role of UDCA in regulating apoptosis by modulating mitochondrial function and membrane stability. In the present study, the ability of UDCA to inhibit apoptosis induced by GGOH was investigated. The HuH-7 cells were incubated in the absence or presence of 20 µmol/liter of GGOH, 100 µmol/liter of UDCA or both. In comparison with non-treated cells, the number of viable cells was decreased to approximately 40% by GGOH, while the value returned to approximately 80% in the case of coinubation with UDCA (Fig. 5A). Under the same conditions, flow cytometric analysis revealed a marked reduction of ΔΨm by GGOH. However, coadministration of UDCA was associ-

![Fig. 5](image_url) 
**Fig. 5.** Inhibitory effects of UDCA on GGOH-mediated apoptosis of HuH-7 cells. (A) The HuH-7 cells were incubated for 8 h (solid bar) or 16 h (blank bar) in the absence (lane 1) or presence of UDCA (100 mmol/liter, lane 2), GGOH (20 µmol/liter, lane 3) or both (lane 4), and the cell viability was determined by the colorimetric method. Results are expressed as a percentage of untreated controls (lane 1). Data are the mean ± SD values (n=4) of a representative experiment. (B) The HuH-7 cells were incubated for 8 h under the same conditions as in (A). The drop in mitochondrial transmembrane potential (ΔΨm) was determined by staining cells with DiOC6(3) and analyzed by flow cytometry as described in “Materials and Methods.” The percentage in representative plots reflects the reduction in ΔΨm during apoptosis induced by GGOH, and the inhibition by UDCA. The mean ± SD value of 3 different experiments is indicated at the upper right of each plot.

![Fig. 6](image_url) 
**Fig. 6.** Inhibitory effects of UDCA on GGOH-induced activation of the caspase cascade and GGOH-induced reduction of Bcl-xL expression in HuH-7 cells. The HuH-7 cells were incubated for 8 h without treatment (lane 1), with GGOH (20 µmol/liter, lane 2) or with GGOH and UDCA (100 µmol/liter, lane 3). Western blotting was performed as described in “Materials and Methods.” Results shown are from a representative experiment of a total of 4 performed.
ated with approximately 50% inhibition of $\Delta \Psi_m$ induced by GGOH (Fig. 5B). As detected by western blot analysis, reduced Bcl-xL expression in HuH-7 cells by GGOH was restored by UDCA. Furthermore, GGOH-induced activation of the caspase cascade was repressed by UDCA (Fig. 6).

**DISCUSSION**

In the present study, GGOH, an endogenous product in the mevalonate pathway, induced apoptotic cell death of human hepatoma cells by stimulating caspase-3 activity. We have also established that activation of both caspase-8 and caspase-9 is involved in GGOH-mediated HuH-7 cell apoptosis. Since a drop of mitochondrial transmembrane potential was found in GGOH-treated HuH-7 cells, mitochondrial dysfunction induced by GGOH seems to trigger a sequential activation of caspase-9 and caspase-3.

Caspase-8 is an activator caspase known to stimulate processing of the mitochondria-dependent caspase-9 activation in the death receptor-mediated apoptotic process, including Fas-mediated apoptosis and tumor necrosis factor $\alpha$ (TNF-$\alpha$)-mediated apoptosis. In the current study, the use of caspase inhibitors including caspase-8 inhibitor, IETD-FMK, almost completely restored GGOH-induced cytotoxicity in HuH-7 cells and inhibited the reduction of $\Delta \Psi_m$ by GGOH. This suggests that caspase-8 is a key regulatory caspase modulating the mitochondrial function in human hepatoma cell apoptosis induced by GGOH. Fas expression is nearly absent in HuH-7 cells and HuH-7 cells are resistant to TNF-$\alpha$-mediated cytotoxicity (data not shown). Several lines of evidence indicate that caspase-8 is activated irrespective of death receptor, which is induced by Sendai virus infection, adenovirus E1A protein, transforming growth factor-$\beta$ and some anticancer drugs. Although the precise mode of caspase-8 activation remains to be determined, it is conceivable that GGOH induces the activation of caspase-8 without death receptor-elicited signaling.

Bcl-2-related proteins such as Bcl-2, Bcl-xL and Bax are believed to be indispensable molecules regulating mitochondrial function. The former two (Bcl-2 and Bcl-xL) inhibit the release of cytochrome $c$ from mitochondria, while the latter (Bax) accelerates the process, and thus, the balance of expression of Bcl-2/Bcl-xL toward Bax is thought to regulate caspase-9-dependent activation of caspase-3. HuH-7 cells express Bcl-xL and Bax, but do not express Bcl-2, as previously reported. The apparent reduction of Bcl-xL expression without the alteration in Bax expression by GGOH treatment is therefore likely to play an important role in inducing mitochondrial dysfunction in HuH-7 cells during the apoptotic process. Interestingly, inhibition of Bcl-xL expression by GGOH preceded the full activation of caspase-8, although it remains to be elucidated whether this inhibition is due to decreased transcription of the Bcl-xL gene or not. Insufficient activation of caspase-8, which probably failed to be detected by western blotting during the early time course of GGOH treatment, may not be enough to cause the mitochondria to induce the activation of caspase-9. However, the reduced Bcl-xL expression by GGOH can sensitize the mitochondria to processing by caspase-8. Under such conditions, a small but efficient activation of caspase-8 relays the signals to the mitochondria, followed by the efficient activation of caspase-9, and caspase-3 is finally activated. The use of caspase inhibitor almost completely suppressed GGOH-mediated $\Delta \Psi_m$ in the present study, and the previous experimental results that mitochondrial dysfunction can act as an amplifier for activating the caspase cascade and ensuring an apoptotic process in type II Fas signaling pathway support our speculation. Expression of XIAP, which acts downstream of cytochrome $c$ release and inhibits the activation of caspase-9 and caspase-3, was reduced in GGOH-treated HuH-7 cells. This could also be involved in GGOH-mediated apoptosis of human hepatoma cells. However, in contrast to the alterations in Bcl-xL expression by GGOH, the time course analysis showed that reduction of XIAP expression by GGOH occurred in parallel with activation of the caspase cascade. A recent investigation revealed that caspase-3 leads to cleavage of XIAP and produces N-terminal baculoviral inhibitory repeat (BIR) 1 and 2 domain fragments in conjunction with reduced ability of XIAP to inhibit the activation of caspase-3 during Fas-mediated apoptosis. Although we could not analyze BIR 1 and 2 domain fragments of XIAP in the present study, caspase-3 activated by GGOH possibly degrades XIAP, which may further facilitate the activation of caspase-3 and DNA fragmentation.

Recent studies have shown that UDCA can modulate the apoptotic threshold and inhibit apoptosis by protecting the mitochondrial function from several apoptotic stimuli. In our study, coadministration of UDCA clearly inhibited GGOH-mediated HuH-7 cell apoptosis. In fact, addition of UDCA prevented the mitochondrial membrane alteration induced by GGOH. Moreover, activation of caspase-8/-9/-3 by GGOH was suppressed by UDCA, which was, at least in part, associated with restoration of Bcl-xL expression by UDCA. These results again strongly suggest a close relationship between activation of the caspase cascade and Bcl-xL expression during the process of GGOH-mediated HuH-7 cell apoptosis. Since Bcl-xL does not affect the activation process of caspase-8, other molecules involved in the process, such as FLICE-inhibitory protein, may also be important in the inhibitory effect of UDCA toward GGOH-induced human hepatoma cell apoptosis.

The present study showed that GGOH can induce apoptosis in human hepatoma cells through activation of the
caspase cascade. Caspase-8 appears to be primarily activated by GGOH, and reduced Bcl-xL expression facilitates the proteolytic cascade. This apoptogenic activity of GGOH would account for the anti-tumor or chemoprevention effects of isoprenoid compounds on hepatocarcinogenesis.

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