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Rapid Loss in the Mitochondrial Membrane Potential during Geranylgeranoic Acid-Induced Apoptosis

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A synthetic geranylgeranoic acid (GGA) induced apoptotic cell death in a human hepatoma cell line, HuH-7, but not in mouse primary cultured hepatocytes. Prior to chromatin condensation, GGA induced a dramatic loss of the mitochondrial membrane potential in 1 hour and in a dose dependent manner in HuH-7 cells, but not in the primary hepatocytes. Pretreatment with synthetic tetrapeptide cysteine protease inhibitor, either acetyl-Tyr-Val-Ala-Asp-chloromethylketone or acetyl-Asp-Glu-Val-Asp-aldehyde, blocked GGA-induced apoptosis without preventing a rapid loss of the mitochondrial membrane potential. α-Tocopherol prevented the cells from GGA-induced apoptosis as well as from a rapid loss of the membrane potential. The present study strongly suggests that GGA induces apoptosis in hepatoma cells through derangement of mitochondrial function and subsequent activation of the cysteine protease cascade. © 1997 Academic Press

We have reported that a synthetic polyprenoic acid of all-*trans* 3,7,11,15-tetramethyl- 2,4,6,10,14-hexadecapentaenoic acid binds to cellular retinoic acid-binding protein (CRABP) (1) as well as to nuclear retinoid receptors (2), shows transcriptional activation of some hepatocyte-specific genes in hepatoma cells (3) and has preventive actions in chemical and spontaneous hepatocarcinogenesis (4,5). Recently, the efficacy of the acid on prevention of second primary hepatoma has been proven in a double-blinded and randomized phase II clinical trial with post-operative hepatoma patients (6). Although the polyprenoic acid was developed initially by screening its ligand activity for CRABP, the acid apparently differs from retinoic acids at the following respects. 1) The polyprenoic acid upregulated albumin mRNA in human hepatoma-derived cell lines, HuH-7 and PLC/PRF-5, while all-*trans* retinoic acid downregulated the expression (3), and 2) the polyprenoic acid induced apoptosis in these cell lines while neither all-*trans* nor 9-*cis* retinoic acid did (7).

Recently, Bansal & Vaidya (8) have found geranylgeranyl pyrophosphatase activity which specifically catalyze dephosphorylation of all-*trans* geranylgeranyl pyrophosphate (GGPP), one of the well-known isoprenoid donors for protein isoprenylations (9). Once geranylgeraniol (GGOH) is produced through dephosphorylation of GGPP by the specific pyrophosphatase, it seems reasonable to assume that the non-specific fatty alcohol dehydrogenase/ fatty aldehyde dehydrogenase system might produce geranylgeranoic acid (GGA). Therefore, we have speculated that the polyprenoic acid may be a derivative of GGA, from which it differs for an additional double bond, on this basis we can call the acid as 4,5-didehydro GGA (or 3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid). In this context, we addressed a question whether or not GGA itself can induce apoptosis.

During apoptotic cell death, cytosolic cysteine protease family including interleukine-1β-converting enzyme (ICE) and cysteine protease precursor 32 (CPP32) have repeatedly been confirmed to be activated and the resultant active CPP32 cleaves poly-ADP-ribosyl polymerase, a nuclear enzyme responsible for DNA repair and integrity of nucleosomes, and sterol response element-binding protein (10, 11). Although a molecular mechanism for activation of ICE family protease cascade remains to be solved at present, dysfunction of mitochondrion is now one of the most likely candidates to trigger the protease cascade (12, 13). Diterpenes such as retinoids are well-established to disturb mitochondrial electron transport and oxidative phosphorylation system (14). Hence, we were very much interested to know whether GGA cause a loss of the mitochondrial membrane potential and activation of ICE family protease cascade.

In the present report, we show that exogenous GGA induced apoptosis in human hepatoma-derived cell line through a dramatic loss of the mitochondrial membrane potential and subsequent activation of ICE family protease cascade.

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MATERIALS AND METHODS

Materials

All-*trans* GGA was provided by Eisai Co. Tokyo, and GGOH was prepared by Kuraray Co. Okayama. Rhodamine 123 (2-[6-amino-3-imino-3H-xanthen-9-yl]-benzoic acid methyl ester) and α-tocopherol was purchased from Sigma Chem. Co., MO. Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (an irreversible inhibitor for ICE) and acetyl-Asp-Glu-Val-Asp-aldehyde (an inhibitor for CPP32) were obtained from Takara Biochemicals, Tokyo.

Cell Culture

Human hepatoma-derived cell lines, PLC/PRF-5 and HuH-7, human hepatoblastoma-derived HepG-2, and mouse hepatocyte-derived cell line, MLE-10, kindly supplied from Dr. Kitagawa T. (Cancer Institute, Tokyo) (15) were all maintained with Eagle-MEM containing 10 (v/v) % fetal calf serum (FCS). 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% FCS. Two days before the treatment of cells with isoprenoids, the media were replaced by FCS-free Eagle-MEM. After addition of the isoprenoids with or without cysteine protease inhibitor, viable cells were counted by the trypan-blue dye exclusion method at the indicated time points.

Fluorescence Microscopy

Chromatin condensation. The GGA-treated cells were fixed with methanol/acetic acid (1:1) and stained with Hoechst 33258 (Sigma). Fluorescence of the dye bound to DNA was observed on a fluorescence microscope with a Zeiss Axioskop.

Mitochondrial membrane potentials. After GGA treatment, the mitochondrial membrane potential was visualized by vital staining of mitochondria with 10 μM Rhodamine 123. pH-sensitive fluorescence of Rhodamine 123 was observed in Bio-Rad MRC-1024 laser scanning confocal imaging system with Kr-Ar ion laser equipped with Nikon Diaphot 300 inverted fluorescence microscope. The data obtained for microscopic images were processed on NIH Image program (ver 1.61, developed at the U.S National Institutes of Health) to measure the fluorescence intensity.

DNA Fragmentation Assay

After the isoprenoid treatment, genomic DNA was analyzed by a 1.5% agarose gel electrophoresis. The fragmented DNA was stained with ethidium bromide after electrophoresis as described elsewhere (7).

RESULTS

Induction of Apoptosis in Hepatoma Cells by Geranylgeranoic Acid (GGA)

As shown in Fig. 1A, an overnight treatment with GGA (10 μM) gave rise to a dramatic reduction in number of viable HuH-7 cells whereas number of primary hepatocytes remained unchanged in the presence of 10 μM GGA in the medium. The GGA-induced cell death in HuH-7 cells was due to apoptosis as evidenced by chromatin condensation (data not shown) and typical nucleosomal incision of genomic DNA (Fig.1B).

Immediate Loss of Mitochondrial Membrane Potential by GGA in Hepatoma Cells

Vital staining of the cells with Rhodamine 123 revealed the cytoplasmic existence of the functional mitochondria and excluded its fluorescence from nuclei as shown in Figs.2, 3 and 5. The fluorescence of Rhodamine 123 is pH-sensitive so that it depends on alkaline pH in the mitochondrial matrix, which is maintained by the mitochondrial inner membrane potential. Fig.2A clearly shows that HuH-7 cells lost the mitochondrial membrane potential in 1 hour after addition of 10 μM GGA in the medium. The lowering effect of GGA lasted overnight (data not shown). The rapid loss of the membrane potential was dependent on concentrations of GGA in medium (Fig.3A). In a sharp contrast, GGOH (20 μM) was totally inactive to decrease the mitochondrial membrane potential in HuH-7 cells, indicating that a terminal carboxylic group of GGA is important to let the potential down (Fig.3A).

Mouse primary cultured hepatocytes, however, were resistant to GGA treatment up to 40 μM, and kept the mitochondrial membrane potential intact as shown in Figs.2B and 3B.

Involvement of ICE Protease Family Cascade in GGA-Induced Apoptosis

Pretreatment of the cells with 200 μM acetyl-Tyr-Val-Ala-Asp-chloromethylketone, an irreversible inhibitor of ICE completely blocked the GGA-induced apoptosis in HuH-7 cells until the end of experiment (Fig.4A). Acetyl-Asp-Glu-Val-Asp-aldehyde, an inhibitor for CPP 32 was also effective to retard GGA-induced apoptotic cell death. However, the ICE inhibitor at the same concentration did not prevent a rapid loss of the mitochondrial membrane potential at 1 hour after addition of GGA and the inhibitor-treated cells restored it in 24 hours after the GGA treatment (Fig.5).

Inhibitory Effect of α-Tocopherol on GGA-Induced Apoptosis

Anti-oxidant vitamin, α-tocopherol gave no effect on the cell growth by itself. But it rescued the cells from GGA-induced cell death in a dose dependent manner from 25 μM up to 100 μM, 10-fold molar excess over GGA (Fig.4B), and 100 μM α-tocopherol prevented a rapid loss of the mitochondrial membrane potential and even looked to enhance the membrane potential over the control level (Fig.5).

DISCUSSION

The present communication clearly shows that geranylgeranoic acid (GGA) induced apoptosis in human hepatoma-derived HuH-7 cells through a rapid loss of the mitochondrial inner membrane potential and sub-

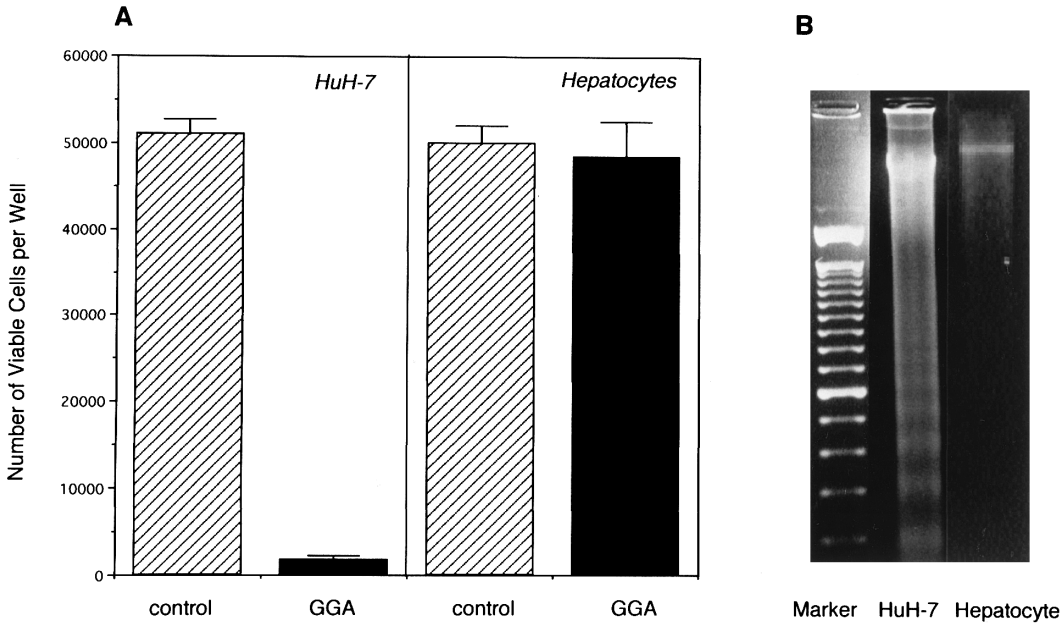

FIG. 1. Induction of apoptosis in HuH-7 cells by geranylgeranoic acid (GGA). (A) Subconfluent cultures of HuH-7 cells or mouse primary hepatocytes were treated with vehicle alone (control, ▨) or 10 μM GGA ethanolic solution (GGA, ■) in a 12-well plastic plate. After overnight treatment, the number of viable cells was counted by trypan-blue dye exclusion method. Mean ± SE in 3 determinations. (B) Eight hours after addition of 10 μM GGA, genomic DNA purified from HuH-7 cells or mouse hepatocytes were applied on agarose gel electrophoresis with 100-bp DNA ladder marker (Gibco BRL).

sequent involvement of ICE family cysteine protease cascade. The concentration dependence, time course and other kinetic analyses of GGA-induced apoptosis have been reported separately (16).

GGA-induced apoptosis is not restricted to HuH-7, but also detected in the most of other tumor-derived cell lines including pulmonary, cervical and mammary tumor-derived cells (17). The GGA-induced apoptosis seems specific for malignant or premalignant cells, because mouse primary cultured hepatocytes were resis-

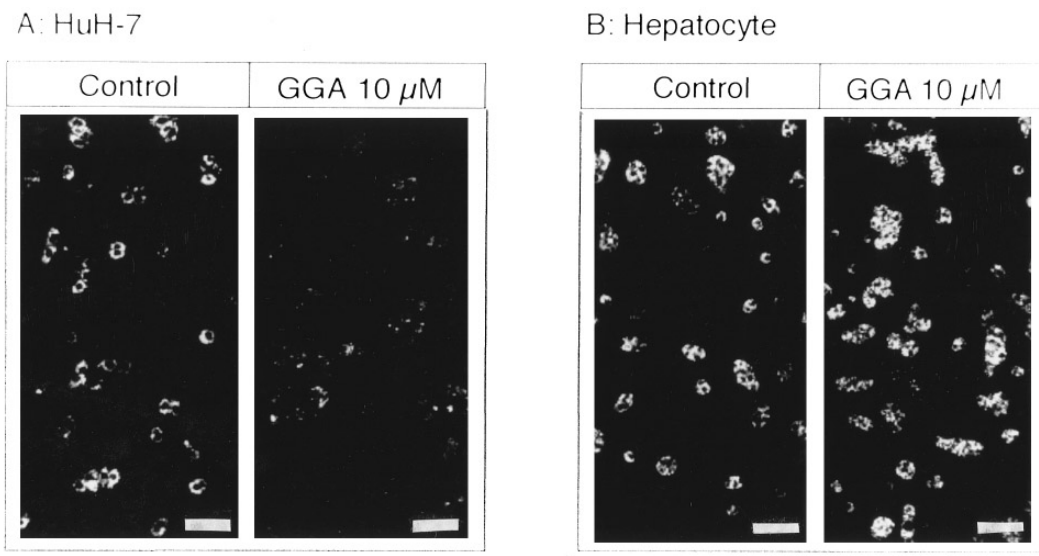

FIG. 2. A rapid loss of mitochondrial membrane potential revealed by Rhodamine 123 staining. HuH-7 cells (A) or mouse primary cultured hepatocytes (B) were treated with vehicle alone (*Control*) or 10 μM GGA (*GGA 10 μM*) for 1 hour and the cells were stained with 10 μM Rhodamine 123 in the culture medium. The mitochondrial membrane potential-dependent fluorescence was observed with laser scanning confocal imaging system. White bars represent 50 μm-length.

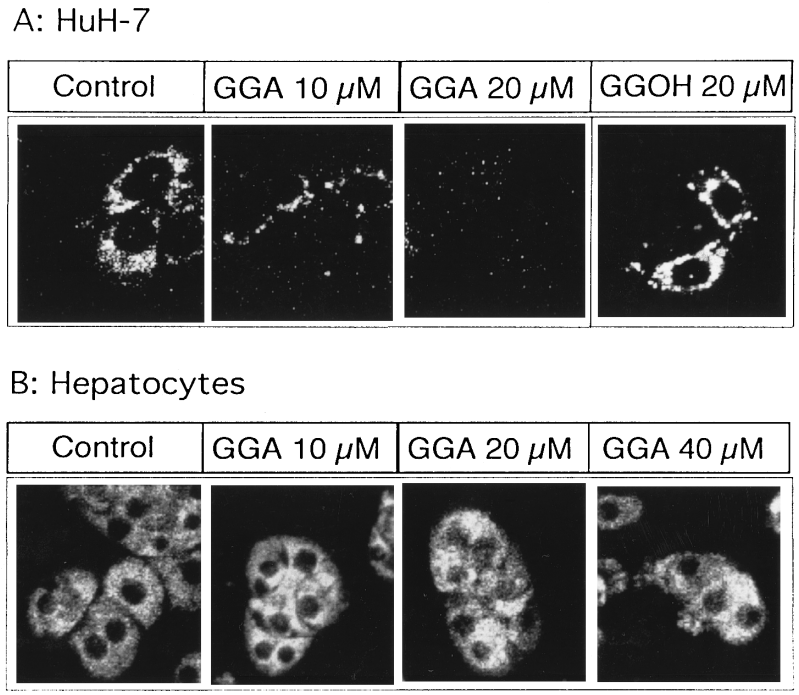

FIG. 3. Cellular changes in mitochondrial membrane potential revealed by Rhodamine 123 staining. Experimental procedures were essentially the same as in the legend to FIG.2. (A) Concentration dependence of GGA effect on the mitochondrial membrane potential and inactivity of geranylgeraniol (GGOH). HuH-7 cells were treated for 1 hour with 10 μM GGA, 20 μM GGA, 20 μM GGOH or ethanol alone as control. (B) Insensibility of mouse primary cultured hepatocytes to GGA treatment. Mouse primary hepatocytes were exposed to GGA up to 40 μM for 1 hour.

tant to GGA treatment as shown in the present study. This insensibility of the hepatocytes can be explained by unresponsiveness of the mitochondrial membrane potential in 1 hour after addition of GGA (Figs. 2, 3). As mentioned previously, GGA is a potent ligand for CRABP which is thought to be involved in intracellular transport of retinoids. CRABP is known as a sort of onco-fetal protein (18), in fact, the protein was present

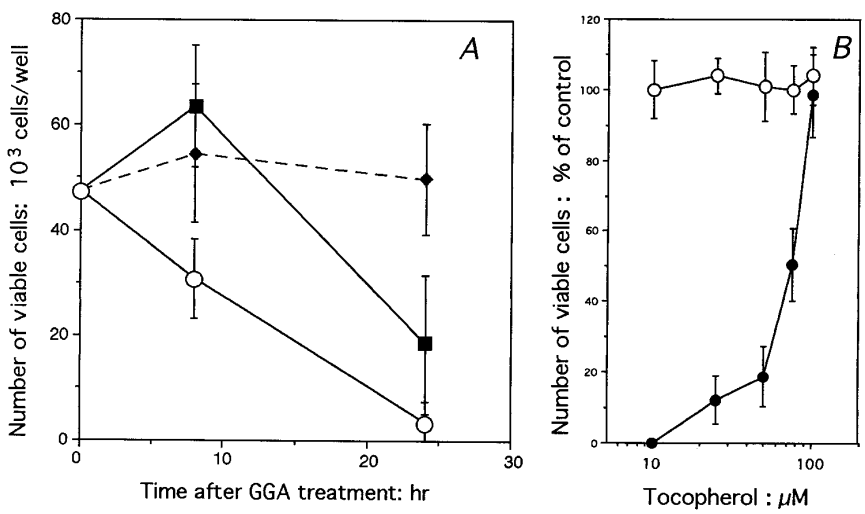

FIG. 4. Inhibitions of GGA-induced cell death. (A) Inhibition by cysteine protease inhibitors. Prior to addition of 10 μM GGA, HuH-7 cells were pretreated for 1 hour with 200 μM acetyl-Tyr-Val-Ala-Asp-chloromethylketone (◆), 200 μM acetyl-Asp-Glu-Val-Asp-aldehyde (■), or phosphate buffered saline (○). At the indicated time points, the viable cells were counted by trypan-blue dye exclusion method. M ± SE (n=3). (B) Inhibition by α-tocopherol. The increasing concentrations (10 - 100 μM) of α-tocopherol were added to HuH-7 cell cultures with (●) or without (○) 10 μM GGA. After overnight incubation, number of the viable cells was counted. M ± S.E. (n = 3).

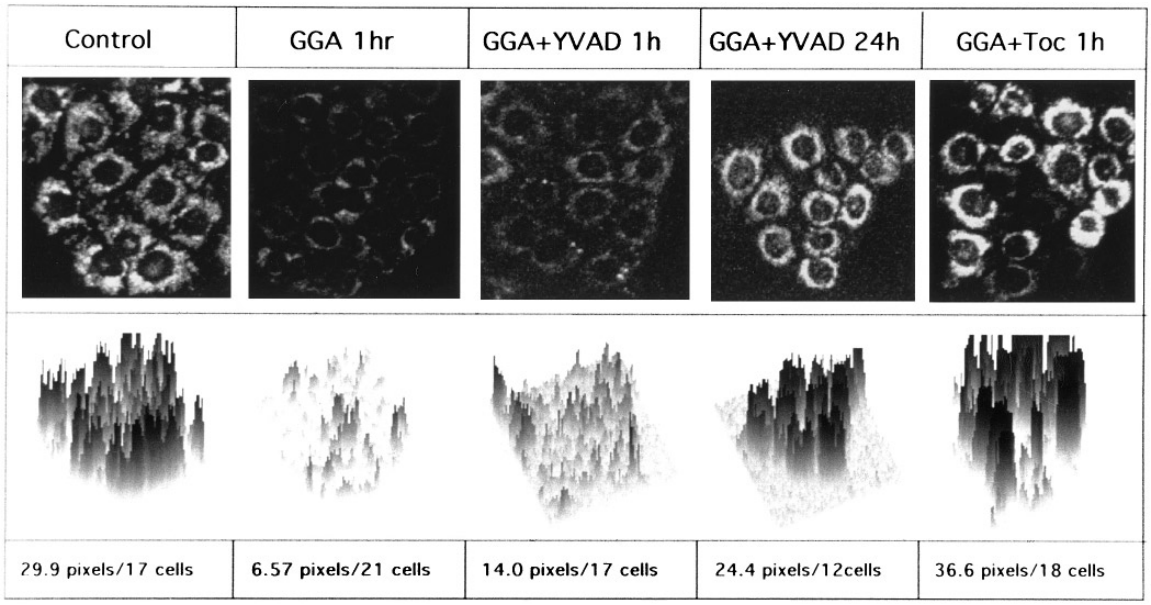

FIG. 5. Effects of cysteine protease inhibitor and α-tocopherol on GGA-induced loss of mitochondrial membrane potential. Experimental procedures were essentially the same as in the legend to Fig.2. HuH-7 cells were treated for 1 hour with vehicle alone (*Control*), 10 μM GGA (*GGA 1 hr*), 10 μM GGA + 200 μM acetyl-Tyr-Val-Ala-Asp-chloromethylketone (*GGA+YVAD 1 hr*) or 10 μM GGA + 100 μM α-tocopherol (*GGA+ Toc 1 hr*) and treated for 24 hours with 10 μM GGA + 200 μM acetyl-Tyr-Val-Ala-Asp-chloromethylketone (*GGA+YVAD 24 hr*). Each fluorescence micrograph was taken at excitation parameter of laser intensity 10% and detection parameters of fluorescence emission filter of 522 nm, Iris 4.0 mm, Gain 1285 V, and Blev 0. The saved images (top) were processed and their surface plots (bottom) were conducted with NIH Image, version 1.61. The fluorescence intensity was measured by NIH image and the mean particle sizes (pixels) per field were shown with number of cells in the same field in the lowest line.

in fetal liver and hepatoma tissues but not in adult hepatocytes (19). Therefore, one can raise a possibility that mouse hepatocytes may be impaired to uptake GGA from the medium, however, we would rather speculate that CRABP in hepatoma cells may accumulate the exogenous GGA into mitochondrial membranes while the hepatocytes lacking CRABP may be incompetent to convey the acid there. This may be the reason why the hepatocytes did not lose the mitochondrial membrane potential and subsequently failed to conduct apoptosis after GGA treatment.

Although a rapid loss of the mitochondrial membrane potential appears indispensable to the GGA-induced apoptosis, the synthetic tetrapeptides of cysteine protease inhibitors blocked the GGA-induced apoptosis without interfering the inhibitory effect of GGA on the mitochondrial function. Recently, a loss of cytochrome c activity has been reported as a potentially fatal component of apoptosis (20) and the released cytochrome c from the mitochondria was involved in activation of ICE family protease cascade with dATP (13). In this context, the derangement of mitochondrion may be a primary but not a final target of GGA to induce apoptosis, and a subsequent activation of ICE family protease cascade is prerequisite to the GGA-induced apoptotic cell death. A massive and continuous derangement of mitochondrial function is expected to be lethal or result in cell death by either apoptosis or necrosis because of depletion of cellular energy. After their overnight survival, however, the GGA-treated cells restored the mitochondrial membrane potential in the presence of an irreversible ICE inhibitor of acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Fig.5), indicating that the GGA-induced dysfunction of mitochondria is reversible. We still have no explanation why the effect of GGA on the mitochondria was transient only in the inhibitor-treated cells whereas the effect of GGA lasted overnight in the absence of the ICE inhibitor.

We reported previously that exogenously added transforming growth factor-α or epidermal growth factor blocked the GGA-induced apoptosis in HuH-7 cells (21). Taking account of the present study, we are now speculating that these growth factors may induce putative endogenous inhibitor(s) for ICE family cysteine proteases to block the GGA-induced apoptosis. In any event, a transient decrease in the mitochondrial membrane potential is not lethal by itself, but subesequent activation of ICE family protease cascade is an essential component of the GGA-induced apoptosis.

As for a molecular mechanism of GGA action on mitochondria, it may be noteworthy to mention that a synthetic retinoid of arotinoid mofarotene, non-ligand for retinoid receptors, down-regulated the gene expression of mitochondrial NADH dehydrogenase (ND-1) consisting of complex I in electron transfer system, prior to its induction of cell death (22).

In conclusion, geranylgeranoic acid (GGA) is a micromolar inducer of apoptosis in malignant cells via inducing a rapid loss of the mitochondrial membrane potential. Recently, we have confirmed that GGA is *de novo* synthesized through mevalonate pathway (16). The previous appraisal of isoprenoic acid for catabolic metabolite or elusive molecule from mevalonate pathway should change into evaluation of the acid as a signalling molecule in determining of cell survival state.

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