Growth inhibition and apoptosis induction of human melanoma cells by ω-hydroxy fatty acids

Akihisa Abe[a] and Kanji Sugiyama[b]

We examined the anti-tumor activity and structure–activity requirements of ω-hydroxy fatty acids (ω-HFAs) on the human melanoma cell line G361. The ω-hydroxystearic acid (ω-HSA) had strong growth-inhibiting and cytotoxic activity. Although ω-hydroxypalmitic acid (ω-HPA) also had growth-inhibiting and cytotoxic activity, these effects were relatively low. The effects of both these acids were dose and time dependent. Further, DNA laddering, which is an index of apoptosis, was also observed in G361 cells on treatment with these compounds. On the other hand, the ω-HFAs tested in this study, ω-hydroxymyristic acid and ω-hydroxyeicosanoic acid, had no growth-inhibiting or cytotoxic activity. Treatment for 12 h with 100 μM of ω-HPA and ω-HSA resulted in the expression of caspase-3 activity, and then increased upon 24 h, suggesting that the cell death induced by ω-HPA and ω-HSA was apoptosis. Fatty acids and dicarboxylic acids, which are analogs of ω-HFAs, had no cytotoxicity. However, fatty alcohols and diols, which have a 16- to 18-carbon chain length had weak cytotoxicity. From these results, the most effective carbon chain length is 18. Furthermore, the hydroxyl group at one end of the carbon chain and the carboxyl group at the other end seem to be required for the cytotoxic effect. At least one end of the carbon chain must have a hydroxyl group. The carbon chain length of ω-HFAs appears to be closely related to the cytotoxicity. This study revealed the potent cytotoxic actions of ω-HFAs on the human melanoma cell line G361. *Anti-Cancer Drugs* 16:543–549 © 2005 Lippincott Williams & Wilkins.

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[a]Department of Biochemistry, Tokyo Medical University, Shinjuku, Shinjuku-ku, Tokyo, Japan and [b]Second Department of Oral Anatomy, School of Dentistry, Meikai University, Sakado, Saitama, Japan.

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Correspondence to A. Abe, Department of Biochemistry, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan.
Tel/fax: +813-3351-0564;
e-mail: aabe-bc@tokyo-med.ac.jp

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Introduction

Melanoma is the most aggressive form of skin cancer [1,2]. In the initial stage surgery is the preferred treatment, but once it spreads, surgery is ineffective [2–4]. Patients with advanced melanoma, such as lymph node involvement and distant metastases, have 5-year survival rates of 50 and 10–20%, respectively [1]. This poor prognosis largely results from resistance to conventional chemotherapy [1–7]. Furthermore, the overwhelming majority of melanoma is not radioresponsive [8]. Anti-tumor agents are known to have concomitantly serious cytotoxicity not only to malignant cells and tissues, but also to normal tissues. Therefore, there is a great demand for the development of new approaches, including chemotherapy, against melanoma. Recently, many studies on the anti-tumor effects of naturally occurring products have been performed for low-toxic agents. Reports in which natural products, such as polyphenols and others, induced apoptosis in cancer cells are especially increasing in number [9–13]. We reported that ω-hydroxypalmitic acid (ω-HPA), a kind of ω-hydroxy fatty acid (ω-HFA), has anti-tumor effects [14]. It is known that ω-HPA is included in some plant leaves and fruit skin, such as apple skin [15–17]. It is very interesting that such a simple structure compound has anti-tumor action. Furthermore, this compound may have low toxicity in humans and animals because it is naturally contained in apple skin [15–17].

The aim of this study was to investigate the cytotoxic effects of ω-HFAs and the structure–activity relationship of these compounds in human melanoma cells.

Materials and methods

Chemicals

Myristic acid, palmitic acid, stearic acid, eicosanoic acid, myristoyl alcohol, palmitoyl alcohol, stearoyl alcohol, eicosanoyl alcohol and dimethylsulfoxide (DMSO) were purchased from Wako (Osaka, Japan). Tetradecanedioic acid, hexadecanedioic acid and octadecanedioic acid were obtained from GL Science (Tokyo, Japan). Eicosanedioic acid, ω-HPA and hexadecane-1,16-diol were bought from Tokyo Kasei Kogyo (Tokyo, Japan). Tetradecanediol and hexadecanediol were from Aldrich (Milwaukee, WI). ω-hydroxyeicosanoic acid (ω-HEA) was purchased from Matreya (Pleasant Gap, PA, USA). The ω-hydroxymyristic acid (ω-HMA), ω-hydroxystearic acid (ω-HSA) and octadecane-1,18-diol were synthesized chemically according to the method of Ellin *et al.* [18]. The chemical structures of these compounds are shown in Figure 1.

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Fig. 1

$HO-(CH_2)_n-COOH$	ω-Hydroxyfatty acids	
	n : 13	ω-hydroxymyristic acid (ω-HMA)
	: 15	ω-hydroxypalmitic acid (ω-HPA)
	: 17	ω-hydroxystearic acid (ω-HSA)
	: 19	ω-hydroxyeicosanoic acid (ω-HEA)
$CH_3-(CH_2)_n-OH$	Fatty alcohols	
	n : 13	Myristoyl alcohol
	: 15	Palmitoyl alcohol
	: 17	Stearoyl alcohol
	: 19	Eicosanoyl alcohol
$CH_3-(CH_2)_n-COOH$	Fatty acids	
	n : 12	Myristic acid
	: 14	Palmitic acid
	: 16	Stearic acid
	: 18	Eicosanoic acid
$HOOC-(CH_2)_n-COOH$	Dicarboxylic acids	
	n : 12	Tetradecanedioic acid
	: 14	Hexadecanedioic acid
	: 16	Octadecanedioic acid
	: 18	Eicosanedioic acid
$HO-(CH_2)_n-OH$	α,ω-Diols	
	n : 14	Tetradecane-1.14-diol
	: 16	Hexadecane-1.16-diol
	: 18	Octadecane-1.18-diol
	: 20	Eicosane-1.20-diol

Chemical structures of ω-HFAs and analogs.

Cell lines and culture conditions

The human melanoma cell line G361 was obtained from the ATCC (Rockville, MD). G361 cells were cultured in modified Eagle's medium (Sigma, St Louis, MO) with non-essential amino acids, 110 μg/ml sodium pyruvate, 10% fetal calf serum (FCS; Sigma) and 70 μg/ml kanamycin sulfate (Wako) at 37°C in a humidified incubator with 5% CO_2 in air.

Addition of reagents

The ω-HFA and its analogs were dissolved at 100 mM in a DMSO:ethanol (3:1, v/v) solution. Reagent solutions were added to the above culture medium. The cells were incubated with the medium for 12 h to 2 days at 37°C under an atmosphere with 5% CO_2.

Cell growth inhibition

Exponentially growing cells were placed in quadruplicate aliquots 5×10^4 cells/well in a 24-well plate and were cultivated in the presence or absence of agents. After cultivation for 12–24 h, the cell number was counted using a hemocytometer.

Cell viability

Cell viability was assessed by the WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, salt] method, using the Cell

Counting Kit-8 (Dojindo, Kumamoto, Japan) [19]. Briefly, the tumor cells (7×10^4 cells/well) were pre-cultured in a 24-well flat-bottomed microtiter plate for 24 h at 37°C in a 5% CO_2 humidified chamber. Then various concentrations of ω-HFA or its analogs were added and incubated for 1–2 days. After incubation, culture medium was discarded and 1 ml of fresh medium including 40 µl of the WST-8 reagent of the Cell Counting Kit-8 was added to each well and incubation was continued for 1 h. After the incubation, the medium of each well was analyzed using a spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan) at a wavelength of 450 nm. Cell viability was determined by referring to the absorbance of non-treated cells.

Caspase-3 activity

After treatment with ω-HFAs, the assessment of caspase activity was performed by the Caspase-3 Colorimetric Assay Kit (MBL, Nagoya, Japan) using acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) as a caspase-3 substrate [20,21]. Cells were washed twice with phosphate-buffered saline (PBS), resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) for 10 min on ice. After centrifugation for 10 000 *g* at 20 min at 4°C, 50–200 µg of proteins of the resulting supernatant were incubated in buffer assay (100 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, 10 mM dithiothreitol) in the presence of 200 µM substrate. The reaction mixture was incubated at 37°C for 90 min and then the absorbance was read at 405 nm using an ELNX 96 microplate reader (TFB, Tokyo, Japan).

Analysis of DNA fragmentation

The melanoma cells were treated with 100 µM of ω-HFA for 12 h to 2 days. After treatment, the detached cells were collected. These cells were centrifuged at 600 *g* for 5 min at 4°C and washed twice with PBS. Then, 100 µl of lysis buffer containing 10 mM Tris–HCl buffer (pH 7.4), 10 mM EDTA and 0.5% Triton X-100 was added to the pellet and kept on ice for 10 min. Then the samples were centrifuged at 12 000 *g* for 15 min at 4°C. After the centrifugation, the supernatant was incubated at 37°C with 40 µg RNase A (Wako) for 1 h, followed by further incubation for 1 h with 40 µg proteinase K (Wako). Then, DNA was precipitated with 20 µl of 5 M NaCl and 600 µl of 2-propanol and kept overnight at −30°C. After centrifugation for 15 min, DNA was analyzed using 1.5% agarose gel electrophoresis.

Results

Growth inhibition of ω-HFAs

Table 1 shows the growth inhibition effect of ω-HFAs on G361 cells. While ω-HTA and ω-HEA had no growth-inhibiting effect, ω-HPA and ω-HSA did show a growth-inhibiting effect that was both time and dose dependent.

Table 1 The growth-inhibiting effect of ω-HFAs on G361 cells

Agent	Concentration (µM)	Growth inhibition (%)		
		11 h	15 h	24 h
ω-HTA (C14)	50	–	–	–
	100	–	–	–
ω-HPA (C16)	50	–	–	–
	100	8.7	29.8	56.8
ω-HSA (C18)	50	18.6	36.5	59.3
	100	94.6	100	100
ω-HEA (C20)	50	–	–	–
	100	–	–	–

The values are means of quadruplicate experiments.

The growth inhibition effect appeared after 12 h after treatment with ω-HSA, at a concentration of 50 µM. The inhibition rate was 18.6% and then increased to 59.3% at 24 h of treatment. In the presence of 100 µM ω-HPA and ω-HSA for 12 h of treatment, growth inhibition rates were 8.7 and 94.6%, respectively, and then increased to 56.8 and 100% for 24 h of treatment, respectively.

Cytotoxic effects of ω-HFAs

The effects of ω-HFAs on the viability of G361 cells were examined after 1–2 days in culture (Fig. 2A and B). The cytotoxic effects of ω-HPA and ω-HSA were also time and dose dependent. Cytotoxic effects of ω-HSA were greater than those of ω-HPA, but ω-HSA had no significant cytotoxic effect at less than 25 µM for 2 days. The viabilities after treatment with 75 µM of ω-HPA and ω-HSA for 1 day were 95.5 and 68.6%, respectively, decreasing after 2 days of treatment to 80.0 and 39.0%, respectively. After treatment with 100 µM ω-HPA or ω-HSA for 1 day, cell viabilities were 83.3 and 47.1%, respectively, and after 2 days of treatment decreased to 57.6 and 0%, respectively. ω-HTA had slight cytotoxicity since the viability after treatment with 100 µM ω-HTA for 2 days was 94.5%. However, ω-HEA had negligible cytotoxicity.

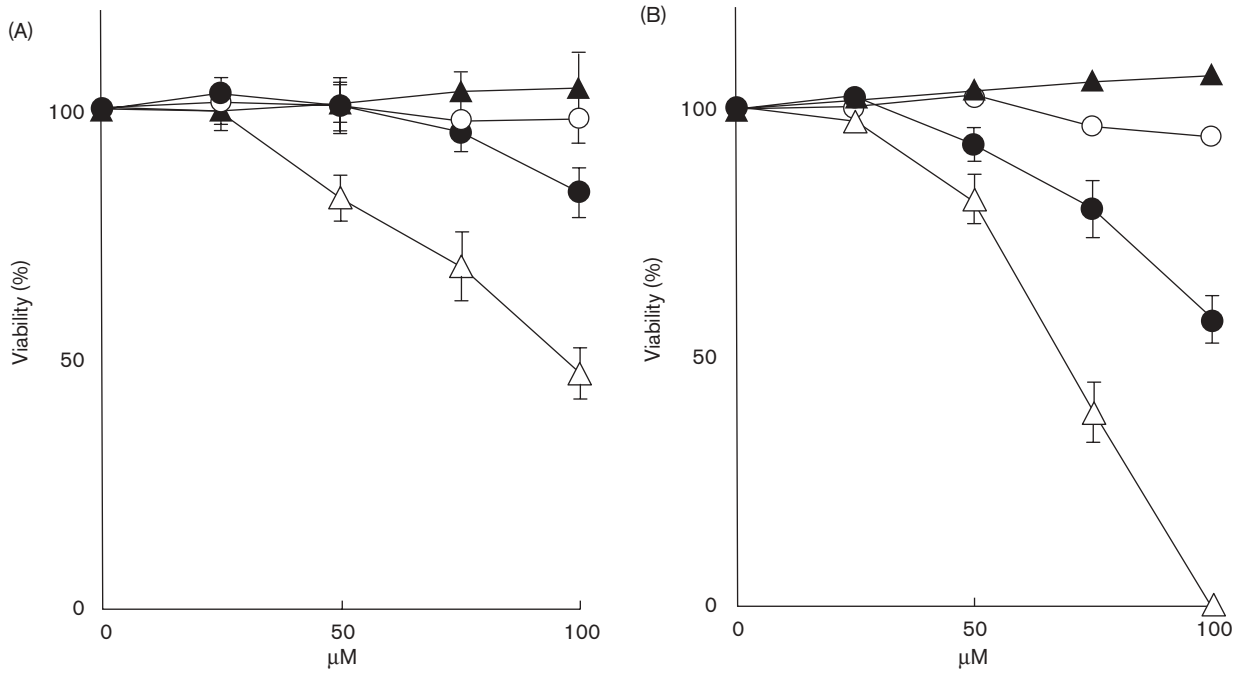
Detection of ω-HFAs induced DNA fragmentation on G361 cells

DNA fragmentation is a characteristic feature of apoptosis [22,23]. A typical experimental result of agarose gel electrophoresis is shown in Fig. 3(A and B). In G361 cells, apoptosis was induced time dependently by ω-HPA and ω-HSA treatment. The DNA ladder fragments were clearly detected after 24 h of treatment with 100 µM of ω-HSA (Fig. 3B); however, in G361 cells treated with 100 µM ω-HPA, clear DNA fragments were detected after 48 h treatment (Fig. 3A).

Activation of caspase on G361 cells by ω-HFAs

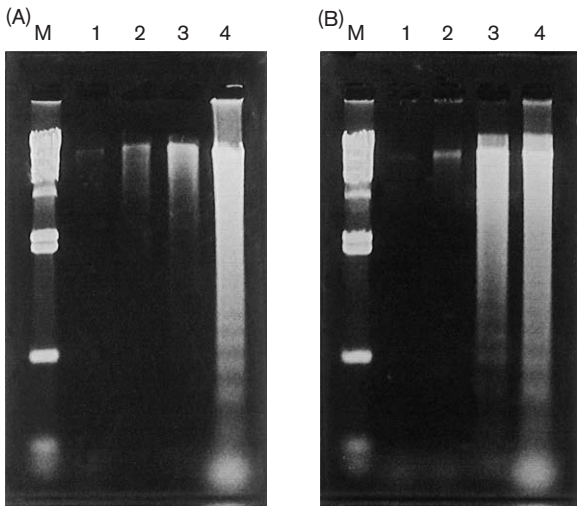
Caspases are cysteine proteases and are the central regulators of apoptosis [24]. Therefore, measurement of caspase activity is often used as a marker of apoptosis. Treatment of G361 cells for 12 h with 100 µM of ω-HPA

Fig. 2



Effects of treatment with several ω-HFAs on G361 cells in terms of period and concentration: (A) 24 and (B) 48 h of treatment. Each ω-HFA is indicated by different markers. (○) ω-HTA, (●) ω-HPA, (Δ) ω-HSA and (▲) ω-HEA. The results of ω-HFA treatment assays are shown as means ± SE from three independent experiments.

Fig. 3



Time course of ω-HFA-induced DNA fragmentation in G361 cells. Cells were treated with 100 μM of ω-HPA (A) and ω-HSA (B). M, λHind III marker; lane 1, untreated cells; lane 2, 12 h treatment; lane 3, 24 h treatment; lane 4, 48 h treatment (in both figures).

or ω-HSA resulted in the expression of caspase-3 activity (Fig. 4), which increased at 24 h, indicating that they were undergoing apoptosis.

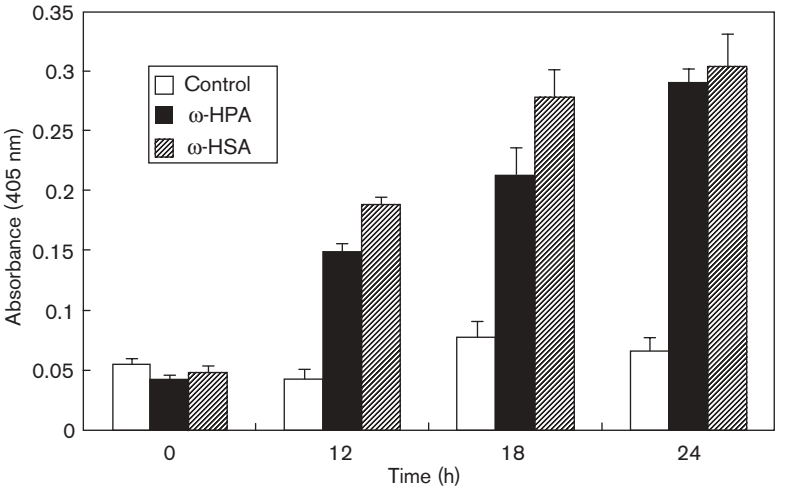
Cytotoxicity of ω-HFA analogs

G361 cells were exposed to fatty acids, fatty alcohols, dicarboxylic acids and diols for 2 days. Fatty acids and dicarboxylic acids had no cytotoxicity (Fig. 5B and D). Treatment with fatty alcohols, myristoyl alcohol and eicosanoyl alcohol also caused no cytotoxicity (Fig. 5A). However, palmitoyl alcohol and stearoyl alcohol had weak cytotoxicity; the viabilities of G361 cells were 84.4 and 82.6%, respectively, after exposures to 100 μM for 2 days (Fig. 5A). Further, hexadecane-1,16-diol and octadecane-1,18-diol also had weak cytotoxicity; the viability of G361 cells being 83.1 and 91.0%, respectively, after treatment with 100 μM. However, tetradecane-1,14-diol had no cytotoxicity (Fig. 5C).

Discussion

This study revealed potent cytotoxic effects of ω-HFAs on human melanoma cell line G361. Those effects of ω-HPA and ω-HSA were dose and time dependent. Furthermore, ω-HPA and ω-HSA were found to induce both internucleosomal DNA fragmentation and caspase-3 activation in G361 cells. Therefore, it is assumed that the cell deaths induced by these two ω-HFAs were through apoptosis. However, in spite of the similarity of the chemical structures and physical properties of these two compounds [25], the degrees of growth-inhibiting and cell death-inducing action between ω-HPA and ω-HSA

Fig. 4



Time course for caspase-3 activity in G361 cells treated with none (outlined columns), 100 μM ω-HPA (solid columns) and 100 μM ω-HSA (cross-hatched columns). Each value represents the mean ± SEM of at least three determinations.

were different. In addition, apart from this result, both ω-HMA, which has a chain length four carbons shorter than ω-HSA, and ω-HEA, which has a chain length two carbon longer than the ω-HSA, had no cytotoxicity. From these results, it is thought that the carbon chain length of ω-HFAs is closely related to the cytotoxicity. However, since the reasons for these results are unclear, further work is needed to clarify this mechanism.

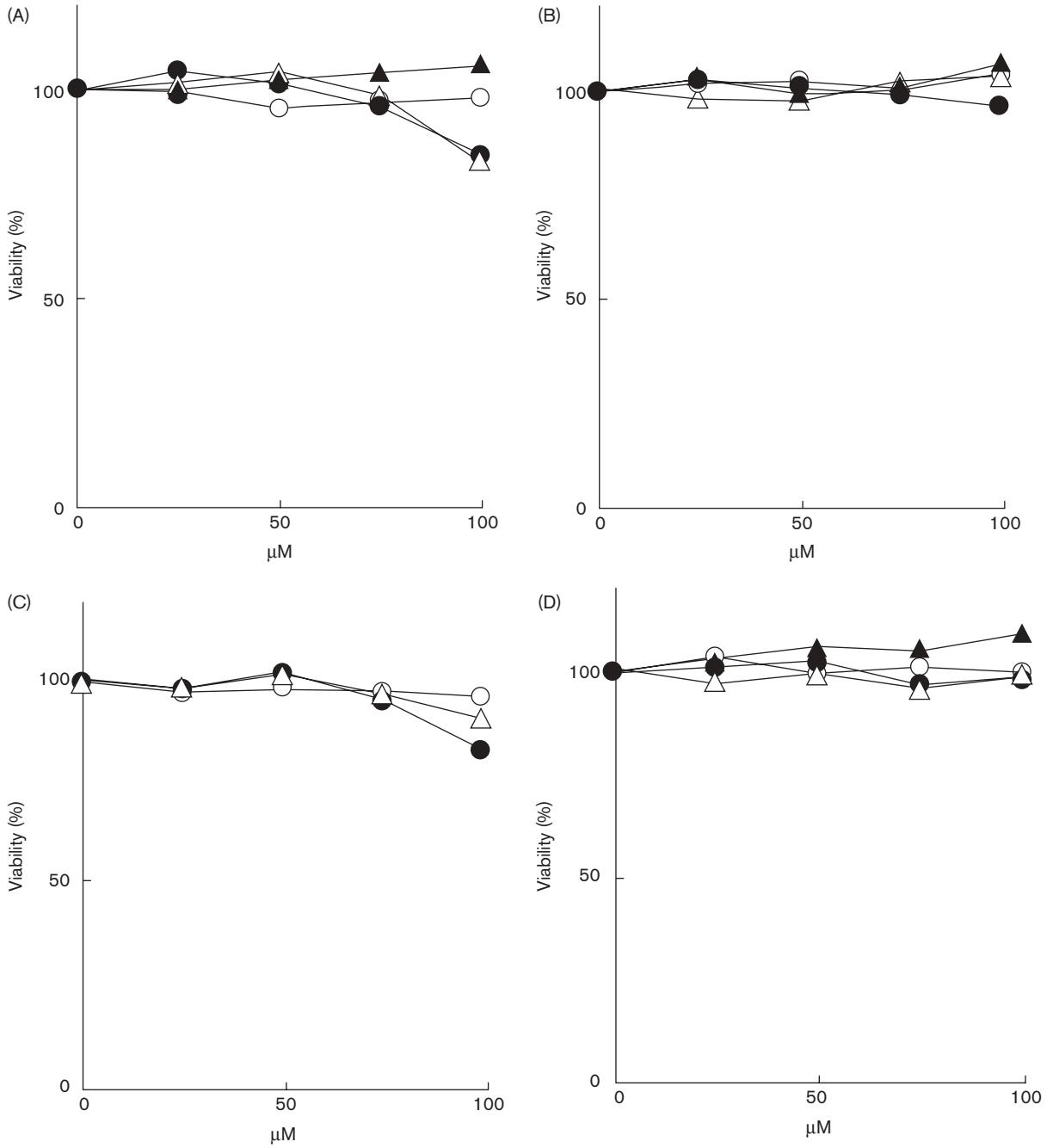
The structures of ω-HFAs are similar to those fatty acids (Fig. 1). A number of investigators have suggested that the cytotoxic activity of fatty acids involves lipid peroxidation [26–29]. In particular, several studies on the biological effects of lipid peroxidation products, lipid hydroperoxides and aldehydes have provided evidence that they can mediate cell death and cell growth arrest, including apoptosis [30–35]. However, in our experiments, fatty acids had no apoptosis-inducing effect (Fig. 5B). Furthermore, although we tried to expose cells to ω-HFAs together with α-tocopherol, as a hydrophobic anti-oxidant, cell death was not inhibited (data not shown). Therefore, in this study, it is thought that cell death by treatment with ω-HFAs did not result from lipid peroxidation products.

Paumen *et al.* reported that palmitic acid and stearic acid induced *de novo* synthesis of ceramide and apoptosis in LyD9 cells [36]. However, in our experiments, fatty acids had no cell death-inducing effects (Fig. 5B). Therefore, it is considered that the mechanism of apoptosis induced by ω-HFAs appears to be different from the mechanism of apoptosis induced by fatty acids.

Interestingly, palmitoyl alcohol and stearoyl alcohol had moderately cytotoxic effects. However, myristoyl alcohol and eicasanoyl alcohol had no cytotoxicity (Fig. 5A). Furthermore, although hexadecane-1,16-diol and octadecane-1,18-diol also had moderate cytotoxicity, tetradecane-1,14-diol had no cytotoxicity (Fig. 5C). On the other hand, dicarboxylic acids, which have two carboxyl groups at both ends of the carbon chain, had negligible cytotoxicity (Fig. 5D). These findings indicate that the most effective carbon length is 18, and that the hydroxyl group at one end of the chain and the carboxyl group at the other seem to be required for the cytotoxic effect. At least one end of this carbon chain must have a hydroxyl group.

Caspase family proteinases are noted as general mediators of the apoptotic cell death signal. In this study, caspase-3 was shown to be activated by treatment with ω-HPA and ω-HSA by 12 h of treatment at a concentration of 100 μM, and then increased at 24 h of exposure (Fig. 4). These results indicate that ω-HPA- and ω-HSA-induced apoptosis is triggered by the activation of caspase-3. Several studies have revealed the late steps of the apoptotic pathway from caspase-3 activation to considerable DNA fragmentation [37–39]. Both the DNA fragmentation factor and caspase-activated deoxyribonuclease (DNase) are specifically activated by caspase-3, and then these activated components can induce DNA fragmentation. Our results also show that marked activation of caspase-3 was observed by treatment with ω-HPA and ω-HSA by 12 h after incubation (Fig. 4) followed by the activation of DNase involved in DNA fragmentation (Fig. 3).

Fig. 5



Cytotoxic effects of ω-HFA analogs on G361 cell lines. The G361 cells were treated with fatty alcohols (A), fatty acids (B), α,ω-diols (C) and dicarboxylic acids (D). (A) Myristoyl alcohol (○), palmitoyl alcohol (●), stearyl alcohol (Δ) and eicosanoyl alcohol (▲). (B) Myristic acid (○), palmitic acid (●), stearic acid (Δ) and eicosanoic acid (▲). (C) Tetradecane-1,14-diol (○), hexadecane-1,16-diol (●) and octadecane-1,18-diol (Δ). (D) Tetradecanedioic acid (○), hexadecanedioic acid (●), octadecanedioic acid (Δ) and eicosanedioic acid (▲).

Melanoma is the most aggressive form of skin cancer. Complete responses to chemotherapy are rare, immunotherapy is virtually ineffective [1,2] and the overwhelming majority of melanomas are not radioresponsive [8]. Patients with advanced disease, such as with lymph node involvement and distant metastases, have 5-year survival rates of 50 and 10–20%, respectively [1,2]. Therefore, there is a great demand for the development of new approaches including chemotherapy against melanoma. A strategy to selectively induce apoptosis of tumor cells without altering healthy cells is a major goal for the future development of new therapeutic

techniques. The ω-HFAs, especially ω-HPA and ω-HSA used in this study, showed strong growth-inhibiting and cell death-inducing effects against the human melanoma cell line G361. Furthermore, since ω-HPA and ω-HSA are natural products contained in apple skin [17], it is thought that ω-HPA and ω-HSA have low toxicity with regard to humans and animals. Only one of several human melanoma cell lines was studied in this paper. Other cell lines should be examined in further studies. Although there have been no epidemiological studies on ω-HPA and ω-HSA, it is considered that they are anti-proliferative and apoptosis-inducing agents, and may be good candidates as chemopreventive and anti-tumor drugs.

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