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Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3

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Various epidemiologic and experimental *in vivo* and *in vitro* studies have suggested that polyphenols derived from fruits, vegetables and beverages might decrease the risk of developing lifestyle diseases, such as cardiovascular disorders and cancer. Apples are a major dietary source of polyphenols. Here we investigated the antitumor activity of apple polyphenols (APs) and procyanidins, namely condensed tannins, both *in vitro* and *in vivo* studies. APs and procyanidins inhibited the growth of transplanted B16 mouse melanoma cells and BALB-MC.E12 mouse mammary tumor cells, and increased the survival rate of the host mice-transplanted B16 cells. Among the APs, the apple procyanidins specifically, rather than other polyphenols such as chlorogenic acid, (−)-epicatechin, phloridzin and procyanidin B2, had a major effect on cell proliferation and induced apoptosis *in vitro*. The apple procyanidins increased mitochondrial membrane permeability and cytochrome *c* release from mitochondria and activated caspase-3 and caspase-9 within the tumor cells. In addition, we separated eight procyanidin fractions according to the degree of polymerization using normal-phase chromatography, and detected strong antitumor activity in the procyanidin pentamer and higher degree fractions. Our results indicate that the oral administration of apple procyanidins inhibits the proliferation of tumor cells by inducing apoptosis through the intrinsic mitochondrial pathway.

Introduction

Previous epidemiologic and cohort studies have suggested that various phytochemicals in the human diets derived from plants (such as fruits and vegetables) might decrease the risk of developing lifestyle diseases, including cardiovascular disease and cancer (1–5). These results have been supported by various *in vivo* animal studies and *in vitro* molecular investigations. Many natural substances in the human diets have been identified as potential chemopreventive agents, including vitamins and carotenoids. Polyphenols, particularly flavonoids, which are found in fruits (e.g. grapes and apples), vegetables (e.g. onions) and beverages (e.g. tea, cocoa and red wine) have attracted a great deal of attention not only because they are strong antioxidants but also because they appear to have various physiological functions, including anti-carcinogenic activity.

Polyphenols are secondary metabolites of plants that are classified according to their structures (e.g. flavan-3-ols and anthocyanins). Some polyphenols are ubiquitous in plants, whereas others (e.g. isoflavones) are restricted to particular families or species. Apples are an important source of flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones and anthocyanins) in the human diet and are widely consumed throughout the world (6,7). Procyanidins, which consist of (+)-catechin and (−)-epicatechin units and linked together through 4 → 8 and 4 → 6 interflavonoid bonds, are particularly abundant in apples. Procyanidins have many isomeric forms, depending on the extent of polymerization and the nature of their constituent units (8). Apple procyanidins, namely apple condensed tannins (ACTs), are a mixture of various oligomers ranging from dimers to pentadecamers (9).

Recently, some polyphenols in the human diets have been reported to suppress the cell proliferation by inducing tumor cell apoptosis (10–13). (−)-Epigallocatechin-3-gallate in green tea has been shown to inhibit the initiation, promotion and progression stage of carcinogenesis in some models via the induction of apoptosis (14–17). Furthermore, activation of the caspase (a family of cysteine proteases) cascade induces numerous cell changes that are markers of apoptosis, including endonucleolytic cleavage of DNA, proteolytic cleavage of nuclear and cellular proteins, cell membrane blotting and production of apoptotic bodies (18–21). Caspase-3 is a notable effecter caspase in apoptosis and represents a convergence point for two different caspase-dependent apoptosis pathways: the mitochondrial pathway and the death receptor (members of the tumor necrosis factor receptor family) pathway. Previous studies have suggested that apple polyphenols (APs) might have anti-proliferative (22,23) and anti-carcinogenic (24–26) activities using various tumor cells. However, owing mainly to the numerous types of polyphenol and the many procyanidin isomers present in apples, the specific compounds responsible for this antitumor activity and the mechanism of apoptosis remain unclear.

The current study investigated the antitumor effects of APs *in vivo*, the mechanism of AP-induced apoptosis and whether ACTs affect caspase activities and mitochondrial membrane potency in the tumor cells *in vitro*. B16 mouse melanoma cells were used for the transplanted test because it was easy to distinguish between the transplanted cells and tissues, which were black color, and normal tissues. And, as whole apple extracts have been reported to inhibit mammary cancer growth in the model (22), we investigated the effects of APs on the growth of tumor tissue using BALB-MC.E12 mouse mammary *in vivo*. To identify the active compounds within ACTs, we investigated the anti-proliferative activities of individual APs and procyanidin fractions separated according to the degree of polymerization *in vitro*.

Materials and methods

Materials

Procyanidin B2 [epicatechin-(4β→8)-epicatechin] was purified using the method described by Shoji *et al.* (8). The purity of procyanidin B2 was detected over 97% by high-performance liquid chromatography. (−)-Epicatechin was supplied by Sigma–Aldrich (St Louis, MO). Chlorogenic acid was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and phloridzin was obtained from Funakoshi Co., Ltd (Tokyo, Japan). Unless otherwise stated, all other reagents and chemicals used were obtained commercially extra-pure grade.

Preparation of AP

The APs were prepared from unripe apples (*Malus pumila* cv. Fuji) by the method of Shoji *et al.* (27). The procyanidin-rich ACT fraction, which accounted for up to 85% of the total procyanidin content, was separated using a preparative column. The ACT fraction comprised dimers (14.1%), trimers (8.4%), tetramers (6.8%), pentamers (3.1%), hexamers (2.9%) and other polymers (40.3%); it also included flavan-3-ols monomers (13.3%), other flavonoids (4.0%) and non-flavonoid phenolcarboxylic acid derivatives (1.7%). In order to fractionate the procyanidins according to the degree of polymerization, we also performed normal-phase chromatography using an Inertsil PREP-SIL packed column (i.d. 30 × 250 mm; GL Science, Tokyo, Japan) with hexane–methanol–ethyl acetate as the mobile phase. Eight procyanidin fractions, ranging from monomer to heptamer, octamers and higher polymers, were concentrated by rotary evaporation at 45°C and lyophilized. Each procyanidin fraction separated by normal-phase chromatography was analyzed by high-performance liquid chromatography-electrospray ionization mass spectrometry and confirmed over 95%.

Abbreviations: ACT, apple condensed tannin; AP, apple polyphenol; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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Cell lines

B16 mouse melanoma cells (JCRB0202) and BALB-MC.E12 mouse mammary tumor cells (JCRB0233.2) were purchased from Health Science Research Resources Bank (Osaka, Japan). Both types of cells were cultured in Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, 100 μM penicillin and 100 μM streptomycin (all from Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Transplantation and growth of tumor cells

All the animal experiments followed the guidelines for animal experimentation of Hirosaki University, Japan. Female C57BL/6 and BALB/c mice (Charles River, Yokohama, Japan) were used at 5–6 weeks of age and were kept under conventional conditions. They were kept on a cycle consisting of 12 h of light and 12 h of darkness; the lights were turned on at 8:00 a.m. and off at 8:00 p.m. The B16 and BALB-MC.E12 cells, which cultured as described above, were harvested with 0.25% trypsin (Invitrogen) in 0.1 mM ethylenediaminetetraacetic acid solution and washed three times with serum-free, antibiotics-free minimal essential medium. The C57BL/6 and BALB/c mice were injected subcutaneously with 10^6 B16 cells or 10^6 BALB-MC.E12 cells, respectively. The test transplanted mice ($n = 15$) were allowed to drink the sample solution freely, whereas the control tumor-transplanted mice were given access to distilled water only. Tumor sizes (= width × length × height) of the transplanted mice were measured during the test period. The survival rates of the tumor-transplanted mice were compared between the AP-treated, ACT-treated and control groups of both C57BL/6 and BALB/c mice. For the histological studies, the tumors were removed from the mice that received euthanasia by cervical dislocation at 14 days after transplantation, and fixed in cold 4% paraformaldehyde/phosphate-buffered saline (PBS), then embedding in paraffin and subjected to the histological tests described below.

Histology

Sections (4 μm thick) were mounted on Mac-coated slides (Matsunami Trading Co. Ltd, Osaka, Japan). Sections that had been deparaffinized and rehydrated were stained with hematoxylin and eosin. And, apoptotic tumor cells were stained by the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method using an *in situ* cell death detection kit (POD; Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's instructions. The slides were counterstained with hematoxylin. Each stained section was examined under high-power fields (×200), and TUNEL-positive cells identified in the tumor tissue where necrosis did not occur were further evaluated.

Cell proliferation assay

B16 cells or BALB-MC.E12 cells were seeded at the density of 2×10^4 cells per well of a 96-well flat-bottomed microplate and pre-cultivated with 10% fetal calf serum–minimal essential medium for 24 h. The cells were incubated in the absence or presence of various diluted concentrations of the test sample for 24 h. Cells were then reacted with the Tetra Color One kit (Seikagaku Co., Kokyo, Japan), which included a mixture of tetrazolium salt (WST-8; Nacalai Tesque, Kyoto, Japan) and electron mediator, for 2 h at 37°C, and the optical density of each well was observed at an absorbance of 450 nm (with a reference absorbance of 630 nm) by a plate reader.

TUNEL assay

An ApopTag Fluorescein direct *in situ* apoptosis detection kit (Chemicon, Temecula, CA) was used to detect apoptosis cells. Briefly, BALB-MC.E12 cells were seeded at a density of 10^6 cells per 10 cm tissue culture dish. After 48 h, any non-adherent floating cells were removed by washing with PBS, and the culture medium was changed. Cells were then cultured in the absence or presence of 25 μg/ml of AP, ACT or the separated procyanidin monomer-to-hexamer fractions for 24 h. After harvested, cells were fixed with 1% paraformaldehyde in PBS for 1 h on ice and then precipitated and resuspended in cold PBS. Cold ethanol was added to the cell suspension (70% ethanol at the final concentration) and it was preserved at −20°C until use. Fixed cells were washed three times with PBS and centrifuged. The cell pellets were treated with TUNEL in reaction buffer for 1 h at 37°C in the dark. The fluorescence intensity of fluorescein isothiocyanate in the positive cells was analyzed with a fluorescence-activated cell sorting flow cytometer (FACScan; Becton Dickinson, Mountain View, CA).

Caspase-3 activity assay

Caspase-3 activity within the cells was measured using a caspase-3 assay kit (Serotec, Raleigh, NC) according to the manufacturer's protocol. Briefly, BALB-MC.E12 cells were seeded at the density of 5×10^6 cells per 10 cm tissue culture dish and were cultured for 48 h. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm (green) using Fluoroscan Ascent system (Thermo Electron Co., Waltham, MA). To determine the effects of caspase inhibitors, cells were pre-treated with a caspase-3 inhibitor (z-DEVD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk; all from BioVision, Mountain View, CA) for 2 h. Cells were then treated with 25 μg/ml of ACT for 2 h and collected by centrifugation at 200*g* for 3 min.

Mitochondrial membrane depolarization

BALB-MC.E12 cells were treated with 25 μg/ml of ACT for 2 h, and a Mito PT apoptosis detection kit (B-Bridge International, San Jose, CA) was used to measure the mitochondrial membrane permeability according to the manufacturer's protocol. Briefly, cells were incubated with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolo carbocyanin iodide (JC-1) for 30 min at 37°C, then washed twice with cold buffer and resuspended in washing buffer. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 590 nm using Fluoroscan Ascent system (Thermo Electron).

Protein extraction

BALB-MC.E12 cells were seeded at a density of 1×10^5 cells per 10 cm tissue culture dish and cultured for 72 h at 37°C in 5% CO_2. Cells were then cultured in the presence of 25 μg/ml ACT for 2, 4 and 6 h. Non-treated cells were prepared as a control. Cells were then washed three times with PBS, scraped and collected. Centrifugation at 200*g* was carried out to form a cell pellet. To prepare a whole protein for western blotting analysis of caspase, the cell pellet was lysed at 4°C in lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Nonidet-P40, 0.25% sodium deoxycholate and 150 mM NaCl], supplemented with one protease inhibitor cocktail tablet (Complete Mini, Roche, Diagnostics). After sonication, the cell lysates were centrifuged at 105 000*g* for 1 h, and the supernatant was stored at −80°C until required. The cytosolic fraction for the cytochrome *c* analysis was separated using a cytochrome *c* releasing assay kit (BioVision). The cytochrome *c* protein in the resulting cytosolic fraction was analyzed using a cytochrome *c* releasing apoptosis assay kit (BioVision) according to the manufacturer's instructions. The protein determinations were carried on in diluted samples using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Western blotting analysis

A 10 μg of protein sample from the cytosolic fraction or a 30 μg of protein from the whole-protein fraction, prepared as described above, was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 12% gel. Following electrophoresis, the proteins were electrotransferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The membranes were blocked with 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20 and 5% skim milk (Yotsuba, Sapporo, Japan). They were then immunoblotted with primary goat or rabbit polyclonal antibodies (1:1000 dilutions). The β-actin, cytochrome *c*, caspase-3, caspase-8 and caspase-9 proteins were detected using the rabbit polyclonal anti-β-actin antibody (1:5000 dilutions; Abcam, Cambridge, MA), mouse monoclonal anti-cytochrome *c* antibody (1:200 dilution; BioVision), rabbit polyclonal anti-cleaved caspase-3 antibody (1:1000 dilution; Trevigen, Gaithersburg, MD), rabbit polyclonal anti-caspase-8 H-134 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-caspase-9 antibody (1:500 dilution; Calbiochem, Darmstadt, Germany), respectively. Secondary antibodies conjugated to horseradish peroxidase-linked donkey anti-rabbit immunoglobulin G antibody (1:5000 dilution; Amersham Pharmacia Biotech) or sheep anti-mouse immunoglobulin G antibody (1:5000 dilution; Amersham Pharmacia Biotech) were used for the detection, followed by enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech).

Statistics

Survival curves were calculated using the Kaplan–Meier method and were compared using a log-rank test. The tumor size was compared between the control and AP-treated or ACT-treated groups using the Mann–Whitney *U*-test. A one-way analysis of variance using the Tukey's *post hoc* test was used to assess the differences in caspase activity. Comparisons of the mitochondrial membrane potential between the treatment and control groups were conducted using the Student's *t*-test. All graphs were produced using GraphPad Prism® software.

Results

Effect of oral administration of APs on the mice-transplanted tumor cells

Among the mice transplanted with B16 cells, the survival rates in the groups, given APs and ACTs by oral administration, were higher than that of the control group (Figure 1A). Deaths began to occur in the control mice group on day 17 after transplantation, and all the control

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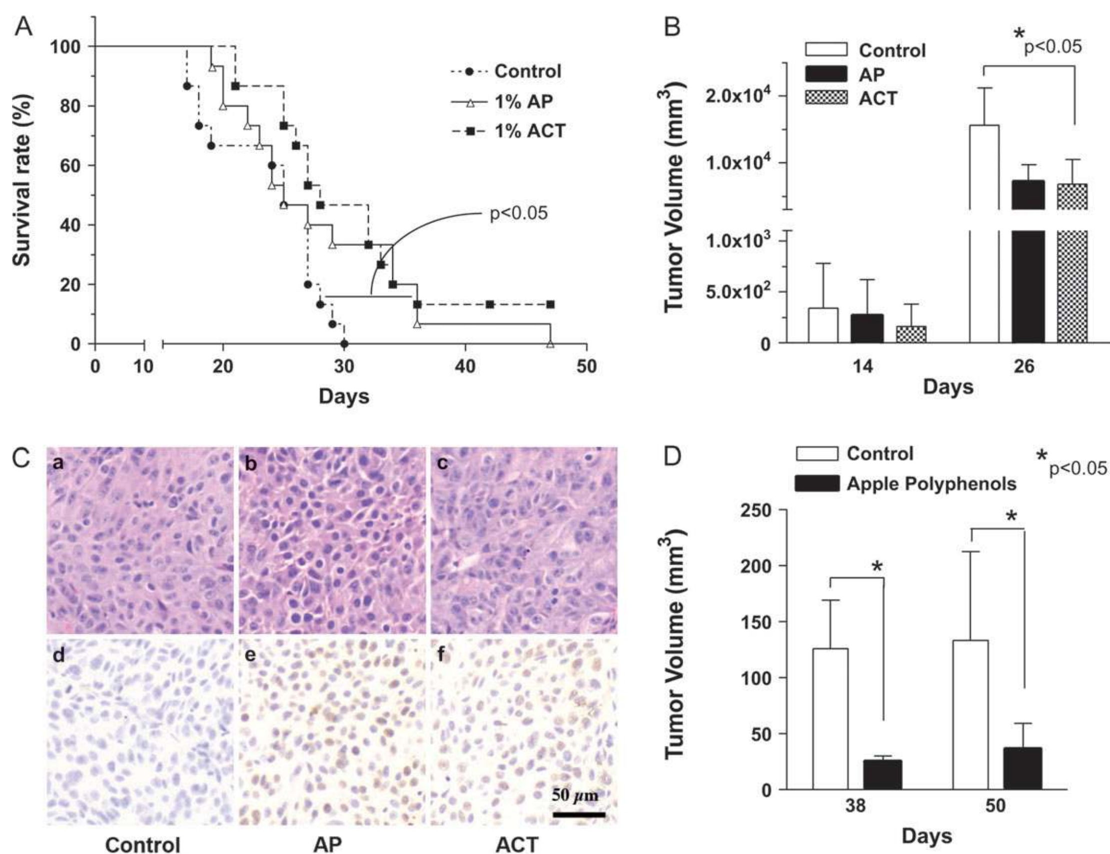

Fig. 1. Effects of AP and ACT on mice with subcutaneous transplants of B16 and BALB-MC.E12 cells. (**A**) Survival curves of mice transplanted with B16 cells. The transplanted mice (n = 15) were treated with distilled water (closed circle), 1% AP solution (open triangle) and 1% ACT solution (closed square), respectively. (**B** and **D**) Tumor size of mice transplanted with B16 and BALB-MC.E12 cells. C57BL/6 and BALB/c mice were transplanted with 1×10^6 B16 and BALB-MC.E12 cells, respectively. Tumor size was measured by vernier calipers and expressed as width (mm) × length (mm) × height (mm) = tumor size (mm^3). All values are the mean ± SD of the surviving mice. An asterisk indicates a significant difference between the ACT or AP and control groups ($P < 0.05$). (**C**) Hematoxilin–eosin (**a**, **b** and **c**) and TUNEL staining (**d**, **e** and **f**) of the tumor tissues in the control (**a** and **d**), AP-treated (**b** and **e**) and ACT-treated groups (**c** and **f**). Scale bar, 50 μm.

mice had died by day 30 after the transplantation. In contrast, in the AP-treated and ACT-treated groups, deaths began to occur on days 19 and 21 after transplantation, and some of the mice in the ACT group survived until day 47. The difference in the survival rate between the control group and ACT-treated groups was statistically significant ($P < 0.05$). Additionally, there was a statistically significant difference in tumor size between the control [15 635.2 ± 5547.6 mm^3 (average ± SD)] and ACT-treated (6837.4 ± 3700.7 mm^3) groups on day 26 after transplantation ($P < 0.05$; Figure 1B). TUNEL staining of the tumor tissues in mice transplanted with B16 cells was performed 14 days after transplantation. Few TUNEL-positive cells were hardly observed in tissues of the control group. In contrast, TUNEL-positive cells were observed sporadically in both the AP-treated (19.0%) and ACT-treated (13.8%) groups (Figure 1C), suggesting that oral administrations of APs and ACTs induced apoptosis in the tumor cells *in vivo*.

Although the tumor growth in mice transplanted with BALB-MC.E12 cells was slower than that in mice transplanted with B16 cells, APs inhibited the tumor growth in both cell types. The tumors were detected in 2 of the 10 mice in the AP-treated group compared with 9 of the 10 mice in the control group. Additionally, the tumor size in the AP-treated group was 27.2% of that in the control group on day 50 after the transplantation (Figure 1D). Statistically significant difference in the tumor size between the control group and AP-treated groups were detected on days 38 and 50 ($P < 0.05$). However, mice in both the control and AP-treated groups survived until 50 days after transplantation (data not shown).

Anti-proliferative effect of APs on tumor cells in vitro

The effects of APs, the major polyphenols comprising APs (chlorogenic acid, (−)-epicatechin, procyanidin B2 and phloridzin; Figure 2A), and ACTs on the proliferation of B16 and BALB-MC.E12 cells were determined *in vitro*. APs at a concentration of 500 μg/ml had only a minor effect on the cell proliferation of B16 cells (Figure 2B). And, (−)-epicatechin and procyanidin B2, which is (−)-epicatechin dimer, at the same concentration (500 μg/ml) slightly suppressed the B16 cell proliferation. In contrast, the B16 cell proliferation was totally suppressed by ACTs and chlorogenic acid at a concentration of 500 μg/ml.

The proliferation of BALB-MC.E12 cells was notably suppressed by APs at a concentration of 100 μg/ml and by ACTs at a concentration of 25 μg/ml (Figure 2C), whereas chlorogenic acid, (−)-epicatechin and procyanidin B2 at a concentration of 100 μg/ml slightly suppressed the BALB-MC.E12 cell proliferation. However, phloridzin did not affect the proliferation of either B16 or BALB-MC.E12 cells.

These results suggested that ACTs, but not the individual polyphenols tested, had a significant effect on the proliferation of both B16 and BALB-MC.E12 cells. Moreover, ACTs suppressed the proliferation of both types of tumor cells in a dose-dependent manner, despite differences in their effective concentration. The sensitivities of APs and ACTs for BALB-MC.E12 cells were higher than those for B16 cells, and the half-maximal inhibitory concentration (IC_{50}) values for the proliferation of the B16 and BALB-MC.E12 cells were significantly different for APs and ACTs: >500 and 201.0 μg/ml in

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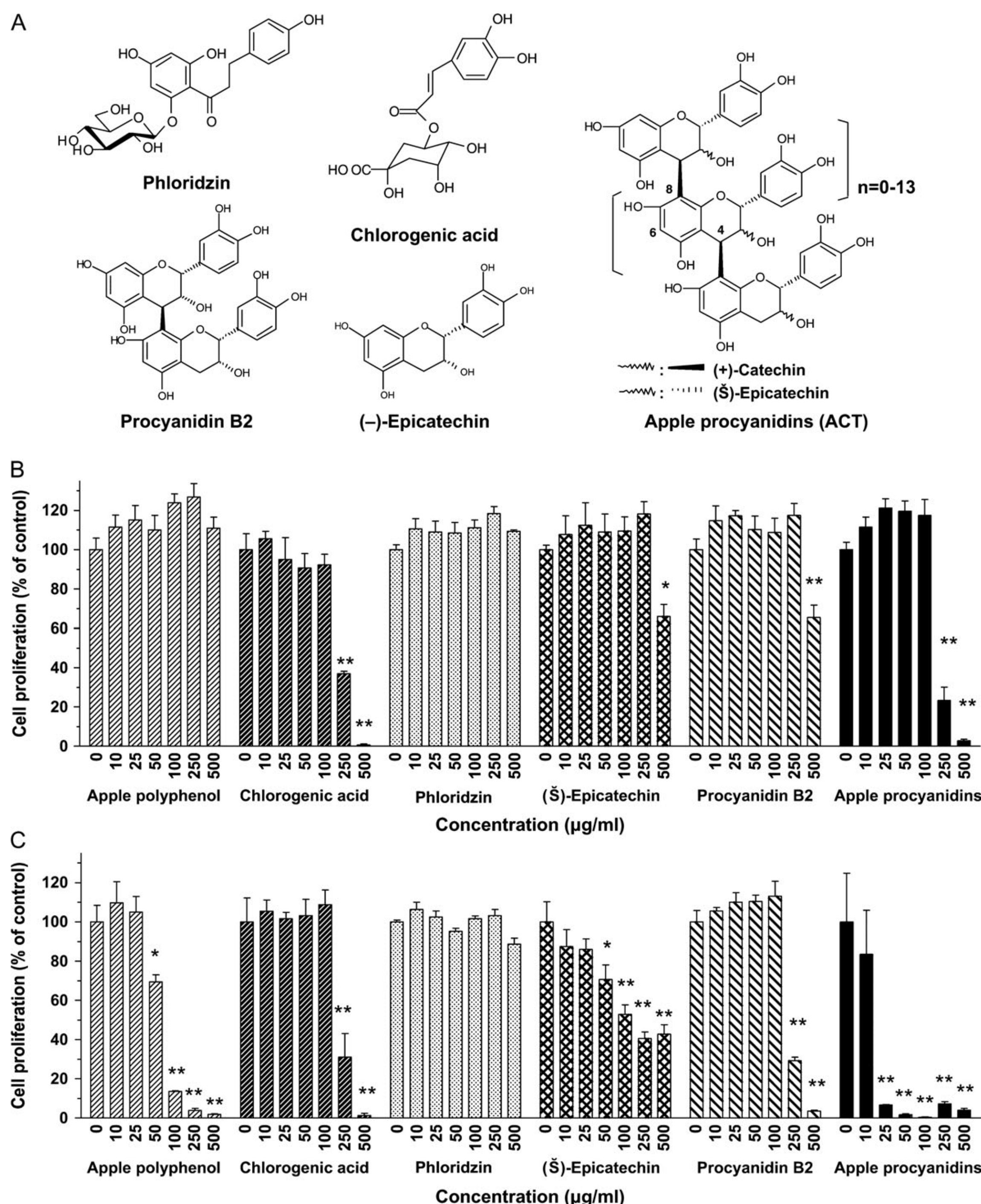

Fig. 2. Anti-proliferative effects of APs, individual polyphenols (chlorogenic acid, phloridzin, (−)-epicatechin and procyanidin B2) and apple procyanidins (ACTs) on B16 and BALB-MC.E12 cells. (**A**) Chemical structures of the major APs and ACTs. (**B** and **C**) Anti-proliferative effects in B16 and BALB-MC.E12 cells. B16 and BALB-MC.E12 cells at densities of 2×10^4 per well were seeded in a 96-well culture plate and cultivated with 10% fetal calf serum–minimal essential medium for 24 h. Cells were then treated with APs, individual polyphenols and ACTs at the indicated concentrations for 24 h. Cell viabilities were measured by Tetra Color One kit (Seikagaku Co.). The results are plotted as the means ± SEs ($n = 4$) of the percentage of viable cells relative to the control. An asterisk indicates a significant difference between each procyanidin fraction-treated and control cells ($^*P < 0.05$; $^{**}P < 0.01$).

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B16 cells and 74.8 and 15.3 µg/ml in the BALB-MC.E12 cells, respectively.

ACT-induced apoptosis in tumor cells

The morphologies of BALB-MC.E12 cells treated with 25 µg/ml of APs and ACTs for 24 h were observed by phase-contrast microscopy (Figure 3A). Few changes were recognized in the AP-treated cells compared with the control cells. However, the ACT-treated cells were, notably, denatured, and these morphologies were observed at higher frequencies among the B16 cells than among the BALB-MC.E12 cells (data not shown). To characterize the proliferation of BALB-MC.E12 cells, which appeared to have a higher sensitivity to APs and ACTs than B16 cells, the frequencies of apoptosis among those treated with 25 µg/ml of APs and ACTs for 24 h were measured by a TUNEL

assay using a flow cytometer. The TUNEL-positive cell rate in the ACT-treated cells was 93.5% of that of the non-treated cells and was higher than that of the AP-treated cells (87.2%; Figure 3B).

ACT-induced caspase activity in apoptotic cells

We further investigated the molecular events underlying the ACT-induced apoptosis in the highly sensitive BALB-MC.E12 cells. Caspases play important roles in denaturing the cellular infrastructure during apoptosis. To assess the involvement of caspase-3, we measured its activities in the ACT-treated and non-treated control BALB-MC.E12 cells. Caspase-3 activity in the ACT-treated cells significantly increased than that in the control cells ($P < 0.01$; Figure 4A). Moreover, caspase-3 activation in the ACT-treated cells was reduced to 61.0% of that in the ACT-treated cells by a caspase-3 inhibitor (z-DEVD-fmk).

The major upstream signal transducers of caspase-3 are the death receptor-related caspase-8 and mitochondria damage-related release of factors (such as cytochrome *c* and Apaf-1) following caspase-9 activation. Then, to clarify the role of the caspases further, we investigated caspase-3 activity in the ACT-treated cells using specific inhibitors of caspase-8 (z-IETD-fmk) and caspase-9 (z-LEHD-fmk). Caspase-3 activity in the ACT-treated cells was significantly inhibited by the caspase-9 inhibitor ($P < 0.01$), whereas the caspase-8 inhibitor had no such effect.

Caspases are produced as a precursor and need to take on an activated form through processing before it executes apoptosis. We then performed western blot analysis to detect activated caspases, in order to confirm their involvement in ACT-induced apoptosis (Figure 4B). In the BALB-MC.E12 cells, the amount of active caspase-3 and caspase-9 present increased at 2, 4 and 6 h after ACT treatment. These results suggested that the activation of caspase-3 and the induction of apoptosis by ACTs were dependent upon the initiator caspase-9.

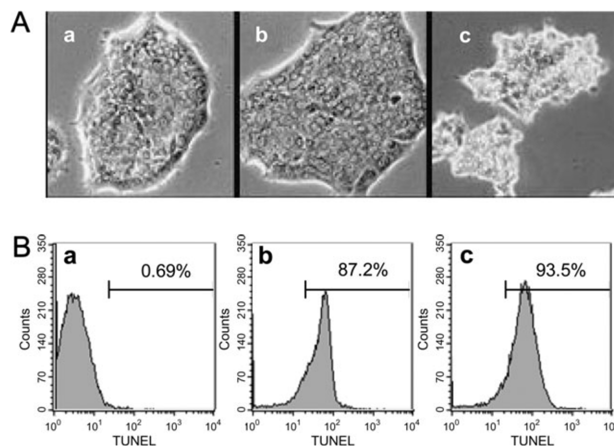

Fig. 3. Morphological changes and apoptosis of BALB-MC.E12 cells induced by treatment with AP and ACT. (**A**) Cell morphologies (×200) by phase-contrast microscopy of control cells (**a**), APs (25 μg/ml) (**b**) and ACTs (25 μg/ml) (**c**) for 24 h. (**B**) TUNEL-positive cells visualized to a FACScan.

ACT-induced mitochondrial membrane depolarization and cytochrome c release

The abovementioned results suggested that the ACT-induced activation of caspase-3 in BALB-MC.E12 cells involved caspase-9 and the mitochondrial apoptotic pathway. Alterations of the mitochondrial membrane potential of BALB-MC.E12 cells were detected using the lipophilic cation JC-1. This cation forms aggregations in normal polarized mitochondria, resulting in a green–orange emission at a wavelength of 590 nm after excitation at a wavelength of 490 nm. However, in cells depolarized with mitchondria, JC-1 emits only green fluorescence. The fluorescence of the cells treated with 25 μg/ml of ACT for 2 h was significantly decreased compared with that of the control cells ($P < 0.01$; Figure 5A). This finding suggested that the ACTs reduced the mitochondrial membrane potential and stimulated the release of factors such as cytochrome *c* and Apaf-1.

Cytochrome *c* plays an important role in apoptosis (28,29). The protein is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of cytochrome *c* from the mitochondria into the cytosol where it binds to Apaf-1. The cytochrome *c*–Apaf-1 complex activates caspase-9, which in turn activates caspase-3 and other downstream caspases. We therefore investigated cytochrome *c* release from the mitochondria into the cytosol by western blotting analysis using the anti-cytochrome *c* antibody. The presence of cytochrome *c* in the cytosol was detected after 2 h after ACT treatment and continued until 6 h after ACT treatment (Figure 5B).

Effects of degree of polymerization on ACT-induced apoptosis

To determine the specific compounds responsible for the observed anti-proliferative activity, the ACTs were further separated by normal-phase chromatography according to their degree of polymerization,

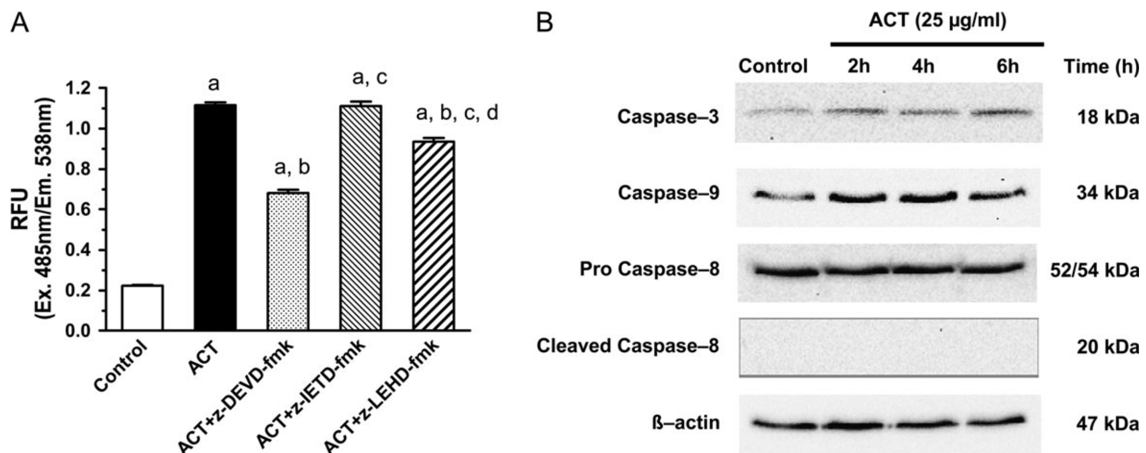

Fig. 4. Apoptotic effects of ACTs related to caspase-3 and caspase-9 in BALB-MC.E12 cells. (**A**) Caspase-3 activities in the BALB-MC.E12 cells seeded at a density of 5×10^6 cells per 10 cm tissue culture dish. After cultivation for 48 h, cells were pre-treated with caspase-3 inhibitor (z-DEVD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) for 2 h, respectively, followed by treatment with 25 μg/ml of ACT for 2 h. Columns show the mean ± SD ($n = 3$). (**a**) Significantly different from the control ($P < 0.01$); (**b**) significantly different from the ACT-treated cell ($P < 0.01$); (**c**) significantly different from the cells treated with caspase-3 inhibitor ($P < 0.01$); (**d**) significantly different from the cells treated with caspase-8 inhibitor ($P < 0.01$). (**B**) Western blotting analysis of caspase-3, caspase-8 and caspase-9 in the BALB-MC.E12 cells treated with ACTs.

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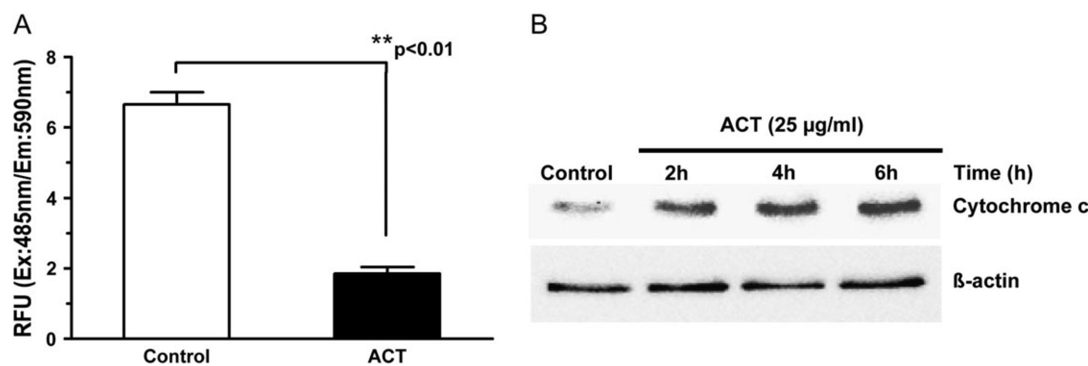

Fig. 5. Effects of ACT on mitochondrial membrane permeability and release of cytochrome *c*. (**A**) Mitochondrial membrane permeability in cells treated with ACTs. Mitochondrial membrane permeability was measured by the method according to the manufacturer's instructions. All values are expressed as the mean ± SE ($n = 8$). An asterisk indicates a significant difference between the ACT-treated and control cells ($P < 0.01$). (**B**) Cytochrome *c* in the cytosolic fraction of the cells treated with ACTs. Cytochrome *c* in the cytosolic fraction was analyzed by western blotting analysis using cytochrome *c* releasing apoptosis assay kit.

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and the apoptosis-inducing activities of the individual fractions were investigated in BALB-MC.E12 cells. Monomer and dimer fractions slightly suppressed the cell BALB-MC.E12 proliferation and had similar effects to (−)-epicatechin and procyanidin B2. However, the inhibitory effects on the cell proliferation were dependent upon the degree of polymerization: tetramer and higher fractions suppressed the proliferation of BALB-MC.E12 cells in a dose-dependent manner, whereas the pentamer and higher degree fractions had inhibitory effects similar to those of the ACTs (Figure 6A).

The morphologies of cells treated with the individual procyanidin fractions were compared (Figure 6B). The morphologies did not change in the cells treated with tetramer and lower fractions. However, cells treated with pentamer, hexamer and higher degree fractions were remarkably denatured to a similar extent as those treated with ACTs. In addition, the TUNEL-positive cell rate was low (<3%) in cells treated with monomer, dimer and trimer fractions, whereas those treated with tetramer, pentamer, hexamer and higher degree fractions had high TUNEL-positive rates (85.4, 96.8 and 98.2%, respectively; Figure 6C). These results suggested that the apoptosis activities of the ACTs were dependent upon the degree of polymerization and corresponded with those of procyanidins of pentamer and higher degree fractions.

Discussion

The human diets contained many phytochemicals, including polyphenols, which are thought to have physiological antioxidative effects. Polyphenols have potential uses as antioxidant and the new seeds of antitumor drug, although the correlation between their antioxidative and anti-carcinogenic activities remains unclear. Apoptosis provides a basic therapies approach to inducing death in cancer cells. Among the polyphenols, (−)-epigallocatechin-3-gallate in green tea (14–17) and resveratrol in wine (30–34) have been reported to possess antioxidative activity and to induce apoptosis in the tumor cells.

APs and ACTs also have strong antioxidative activities (23,35) and reportedly possess various physiological functions, including anti-allergy (36,37) and anti-melanogenesis (38). Previous studies have demonstrated that APs inhibit cell proliferation and induce apoptosis in some tumor cells such as lung, colon and breast cancer (22–26). However, it has remained unclear whether APs induce apoptosis in tumor cells and indicate physiological anti-carcinogenic properties. The current study demonstrated that APs, particularly ACTs, induce apoptosis in mouse melanoma cells and mouse mammary tumor cells *in vitro*, as well as in transplanted tumor tissues in mice *in vivo*.

In the case of the mice-transplanted B16 cells, the growth of tumor tissue after transplantation could be observed until the death of the host. However, the tumor growth in the mice-transplanted BALB-MC.E12 cells was slower than that of B16 cells, and mice in both the control and AP-treated groups survived until 50 days after transplantation. The difference in survival rate is caused by the growth of tumor tissue, though it is unclear whether the growth in the host differs between both tumor cells. We think that main reasons are due to the characterizations of the tumor cells, the stability of the tumor cells to the host mice, the malignancy and the reaction against the host immunity. Further study is needed to examine the differences of the sensitivity against ACTs and the tumor growth in the host in other tumor cells including both cells.

Apoptosis occurs through two main pathways: the first involves the extrinsic or cytoplasmic pathway and is triggered by death receptors; the second involves intrinsic mitochondrial damage, the release of cytochrome *c* and the activation of caspase-9. To establish the mechanism of ACT-induced apoptosis, we investigated the activity of caspase-3 and found it to be significantly increased in the ACT-induced apoptotic cells, which was in agreement with previous findings reported by Gosse *et al.* (26) in human metastatic colon carcinoma cell.

ACT-induced apoptosis was found to be relevant for the activation of caspase-3, not via the death receptor-related caspase-8, but rather the mitochondria damage-related caspase-9. Mitochondrial membrane damage, the releases of cytochrome *c* and Apaf-1 from mitochondria, and the activation of caspase-9 have all been observed during apoptosis induced by various cellular stresses, as well as by (−)-epigallocatechin-3-gallate (17,39) and resveratrol (30). Additionally, proanthocyanidins derived from grape seeds (40) and other plants sources (41,42) have been reported to inhibit cell proliferation in the human cancer cells. To our knowledge, this is the first report to show that ACTs can induce mitochondria membrane permeability transitions and cytochrome *c* release. Anti-apoptotic Bcl-2 and Bcl-xL proteins and pro-apoptotic Bax proteins have been reported to promote and inhibit the release of cytochrome *c* from mitochondria into the cytosol upstream of the caspase-9 pathway (43–45). The ratio of these molecules plays a central role in the regulation of the apoptotic machinery. It is therefore possible that the expression ratios of Bcl-2 family proteins are altered by ACTs. Further research is needed to investigate the mechanism in ACT-induced apoptosis.

Previous epidemiological studies have suggested that the consumption of apples can reduce the risk of cancers, especially lung cancers, and that catechins and flavonols (such as rutin) in APs have cancer chemopreventive activities. However, the identities of the active compounds within the APs have remained unclear. Because ACTs, which are abundant polyphenol in apple (46), are a mixture of various isomers according to the degree of polymerization and the constituent units and have polymerization degree ranging from dimer to pentadecamer, it was difficult to separate procyanidins according to the degree of polymerization and isolate pure standard from the mixture included in other polyphenols. Therefore, the relationship between

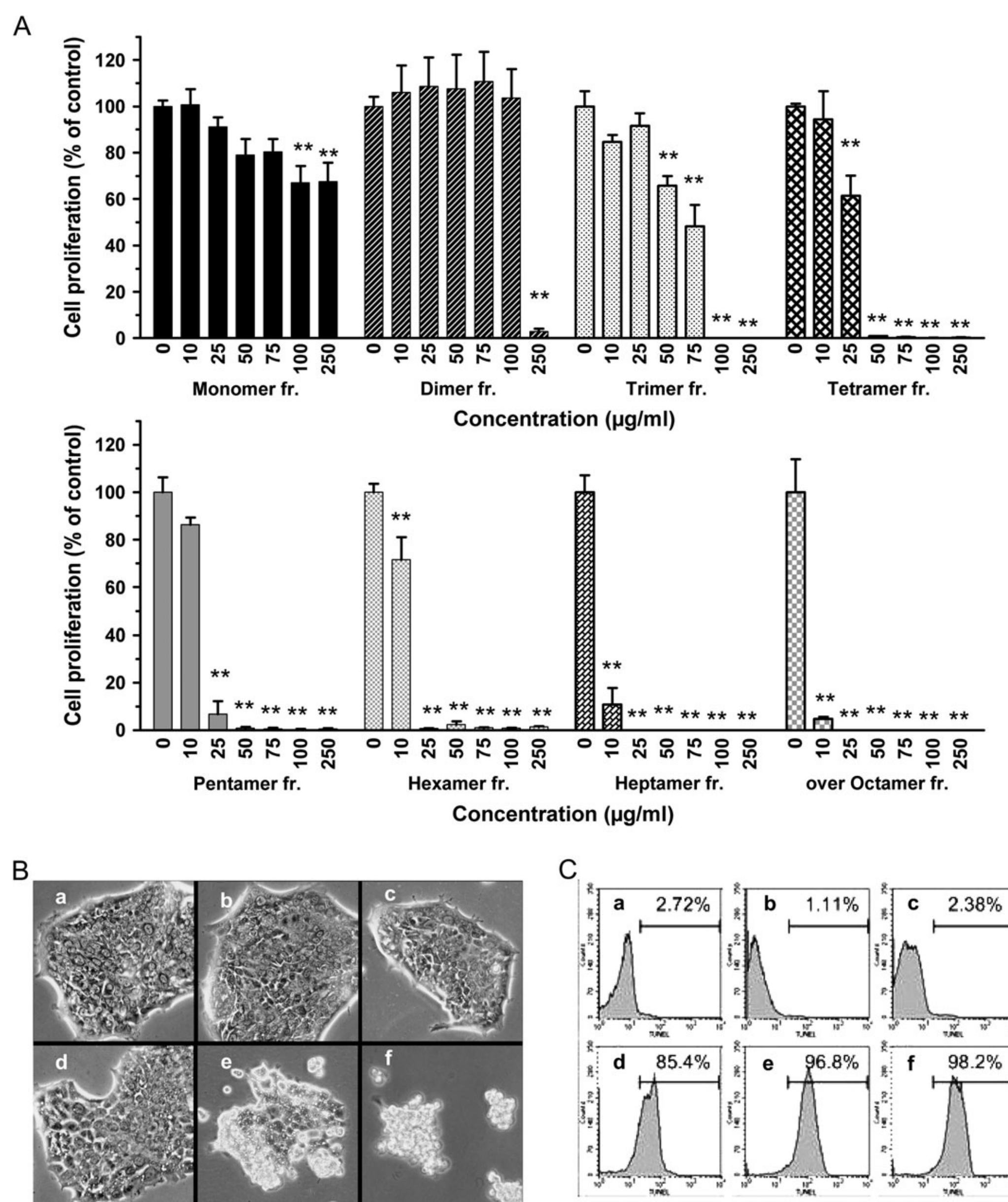

Fig. 6. Anti-proliferative effects of the degree of polymerization of ACTs on BALB-MC.E12 cells. (**A**) Anti-proliferative effects in BALB-MC.E12 cells. The results are plotted as the means ± SEs ($n = 4$) of the percentage of viable cells relative to the controls. An asterisk indicates a significant difference between each procyanidin fraction-treated and control cells ($^{*}P < 0.05$; $^{**}P < 0.01$). (**B**) Morphological changes (×200) of BALB-MC.E12 cells induced by treatment with the monomer fraction (**a**), dimer fraction (**b**), trimer fraction (**c**), tetramer fraction (**d**), pentamer fraction (**e**) and hexamer and higher degree fractions (**f**). (**C**) TUNEL-positive cells according to a FACScan. Cells were treated with 25 μg/ml of each individual fraction for 24 h.

procyanidins structure (degree of polymerization and constituent unit) and anti-carcinogenic activity has thus remained ambiguous. However, the development of the improved preparative chromatography techniques has allowed us to separate fractions up to octamer according to the degree of polymerization using normal-phase chromatography (27).

Our *in vitro* study demonstrated that ACTs, especially pentamers and higher degree fractions, in APs, but not other components, such as (−)-epicatechin and chlorogenic acid, inhibited the proliferation of B16 and BALB-MC.E12 cells, although the sensitivity against ACT differed considerably between the two cell types. Moreover, the inhibitory activity of ACTs was dependent on the degree of polymerization. Previously, Kozikowski *et al.* (41) reported that procyanidin pentamers from cacao induced apoptosis of human breast cancer cells *in vitro*, although the active concentration of the cacao procyanidins (100 μg/ml) was higher than those in the current study. Proanthocyanidins have been reported to bind non-specifically to proteins and enzymes (47). However, recent reports suggested that proanthocyanidins react specifically with proteins and enzymes, and the cell membrane according to the something to restrict such as angiotensin I-converting enzyme (48) and pancreatic lipase (49), by the something to be restricted. We reported previously that procyanidin trimer-to-pentamer fractions strongly suppressed the melanin production by B16 melanoma cell *in vitro* (38). Together with other reports, our findings indicate that the various biological activities of procyanidins are strongly dependent upon their stereochemistry, structures and polymerization degree, although the precise effect of the degree of polymerization remains unclear. Further research is needed to examine the relationships between cell functions and enzyme inhibition, the degree of polymerization and structure of procyanidins and the sensitivity of other tumor cells against ACTs as it has been known that the sensitivities of cancer cells against the drugs differ considerably according to the species and the original.

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In conclusion, our study shows that ACTs inhibited tumor growth and induce apoptosis into the tumor cells *in vivo*. Furthermore, the ACT-induced apoptosis was related to the activation of caspase-3 through the mitochondrial pathway. In particular, the way in which apoptosis is induced by the activity of pentamer and higher degree procyanidins, which is dependent upon the degree of polymerization, remains of particular interest because the bioavailability of procyanidins has not been sufficiently investigated, and it remains unclear whether procyanidin pentamers can be absorbed and transferred to tumor tissue. Recently, we reported that apple procyanidin concentrations in the rat plasma reach a maximum at 2 h after oral administration and that procyanidin pentamers could be detected in rat plasma (50). These results suggest that procyanidin pentamers with antitumor activity can directly affect tumor cells *in vivo* even after oral administration. Further investigations of bioavailability such as metabolites and distribution are needed to clarify the antitumor activities of procyanidin polymers higher than pentamers after oral administration *in vivo*. Such researches will be helpful in identifying effective new cancer chemopreventive agents.

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