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 epidemiologic and experimental studies. Over 97% of the total procyanidin content, was separated using a preparative column 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 blurring and production of apoptotic bodies (18–21). Caspase-3 is a notable effector 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 iso-mers 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)-(4→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 punica 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%), tetromers (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 phenolic carboxylic 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.
Cell lines
B16 mouse melanoma cells (JCRB0202) and BALB-M.CE12 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% CO2 and 95% air.

Transplantation and growth of tumor cells
All the animal experiments followed the guidelines for animal experimentation of Hirofusa 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-M.CE12 cells, which as cultured as described above, were harvested with a 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 10⁶ B16 cells or 10⁶ BALB-M.CE12 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 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 with hematoxylin and eosin. And, apoptotic tumor cells were further evaluated.

Cell proliferation assay
B16 cells or BALB-M.CE12 cells were seeded at the density of 2 × 10⁴ 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., Kodoy, Japan), which included a mixture of tetrazolium salt (WST-8; Nacala 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-M.CE12 cells were seeded at a density of 10⁶ cells per 10 cm tissue culture dish. After 48 h, any non-adhering floating cells were removed with 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 procyonin monomer-to-hexamer fractions for 24 h. After harvested, cells were fixed with 4% 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-M.CE12 cells were seeded at the density of 5 × 10⁵ 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 200g for 3 min.

Mitochondrial membrane depolarization
BALB-M.CE12 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’-tetraethylrhodamine-1,1’,3,3’-tetraethyl-benzamidazolocarbocyanin 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, CA).

Western blotting analysis
A 10 μg 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 mL Tris–HCl (pH 7.4, 125 mM NaCl, 0.2% Tween 20 and 5% skim milk) for 1 h. 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 (1:200 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...
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 ($15,635.2 ± 5,547.6$ mm$^3$ (average ± SD)) and ACT-treated ($6,837.4 ± 3,700.7$ 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
B16 cells and 74.8 and 15.3 ng/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
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).

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 mitochondria, 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 anticytochrome 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,
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 anti-oxidative 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 transition 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 flavonoids (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...
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 \(\mu\)g/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.

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 (x200) 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 \(\mu\)g/ml of each individual fraction for 24 h.
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.

Acknowledgements

We thank N. Hiraka, C. Yamada, C. Tamura, Y. Kubota, A. Kudo, M. Miura and A. Chiba for technical assistance and M. Yasujima of the Clinical Laboratory Medicine, Hirosaki University School of Medicine and Hospital, Japan, for the flow cytometric analysis. We thank S. Masumoto and N. Morichi for carrying out the separation of apple procyanidins according to the degree of polymerization.

Conflict of Interest Statement: None declared.

References

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Received June 11, 2007; revised August 16, 2007; accepted August 18, 2007