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Induction of apoptosis by penta-*O*-galloyl-β-D-glucose through activation of caspase-3 in human leukemia HL-60 cells

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Abstract

Penta-*O*-galloyl-β-D-glucose is structurally related to (−)-epigallocatechin gallate and is isolated from hydrolyzed tannin. Penta-*O*-galloyl-β-D-glucose can inhibit tumor promotion by teleocidin. We investigated the effects of penta-*O*-galloyl-β-D-glucose and various tea polyphenols on cell viability in human leukemia HL-60 cells. In this study, we demonstrated that penta-*O*-galloyl-β-D-glucose was able to induce apoptosis in a concentration- and time-dependent manner; however, other polyphenols were less effective. We further investigated the molecular mechanisms of penta-*O*-galloyl-β-D-glucose-induced apoptosis. Treatment with penta-*O*-galloyl-β-D-glucose caused induction of caspase-3/CPP32 activity in dose- and time-dependent manner, but not caspase-1 activity, and induced the degradation of poly-(ADP-ribose) polymerase. Pretreatment with acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) inhibited penta-*O*-galloyl-β-D-glucose-induced DNA fragmentation. Furthermore, treatment with penta-*O*-galloyl-β-D-glucose (50 μM) caused a rapid loss of mitochondrial transmembrane potential, release of mitochondrial cytochrome *c* into cytosol, and subsequent induction of procaspase-9 processing. Our results indicate that penta-*O*-galloyl-β-D-glucose allows caspase-activated deoxyribonuclease to enter the nucleus and degrade chromosomal DNA, and induces DFF-45 (DNA fragmentation factor) degradation. These results lead to a working hypothesis that penta-*O*-galloyl-β-D-glucose-induced apoptosis is triggered by the release of cytochrome *c* into the cytosol, procaspase-9 processing, activation of caspase-3, degradation of poly-(ADP-ribose) polymerase, and DNA fragmentation caused by the caspase-activated deoxyribonuclease through the digestion of DFF-45. The induction of apoptosis by penta-*O*-galloyl-β-D-glucose may provide a pivotal mechanism for its cancer chemopreventive action.

Keywords: Penta-*O*-galloyl-β-D-glucose; Apoptosis; Cytochrome *c*; Caspase-9; Caspase-3; Poly-(ADP-ribose) polymerase; DNA fragmentation factor; Caspase-activated deoxyribonuclease

1. Introduction

Penta-*O*-galloyl-β-D-glucose is structurally similar to (−)-epogallocatechin gallate (Lin et al., 1990) (Fig. 1), which can be obtained in large amounts from hydrolyzed tannin and has anti-tumor promoting activity in two-stage carcinogenesis experiments with mouse skin (Fujiki et al., 1992). It has been reported that the hydrolyzable tannins, caffeic acids, berginin and its derivatives show low anti-tumor activity. During experiments, 3-*O*-tetragalloylquinic acid markedly prolonged the life span of mice and cured one out of six mice, but further galloylation of this compound did not lead to an increase in the anti-tumor activity. Penta-*O*-galloyl-β-D-glucose, in which the glucose core is saturated with galloyl groups, is more active than other galloylglucoses (Miyamoto et al., 1987). Miyamoto et al. indicated that a suitable molecular mass with a high level of galloylation may be essential for the anti-tumor activity of ellagitannins, but the stereochemical composition of free phenolic hydroxyl groups may also be an important factor. Uric acid, which causes gout, is formed from xanthine in the presence of xanthine oxidase. The inhibitory activity of monomeric hydrolyzable tannins on xanthine oxidase increases with the increasing of molecular weight, which is accompanied by an increase in the number of phenolic hydroxyl groups in the molecule (Hatano et al., 1990).

Tea is one of the most popular beverages in the world because of its attractive flavor and aroma, and the major tea beverage is black tea, especially in the western nations.

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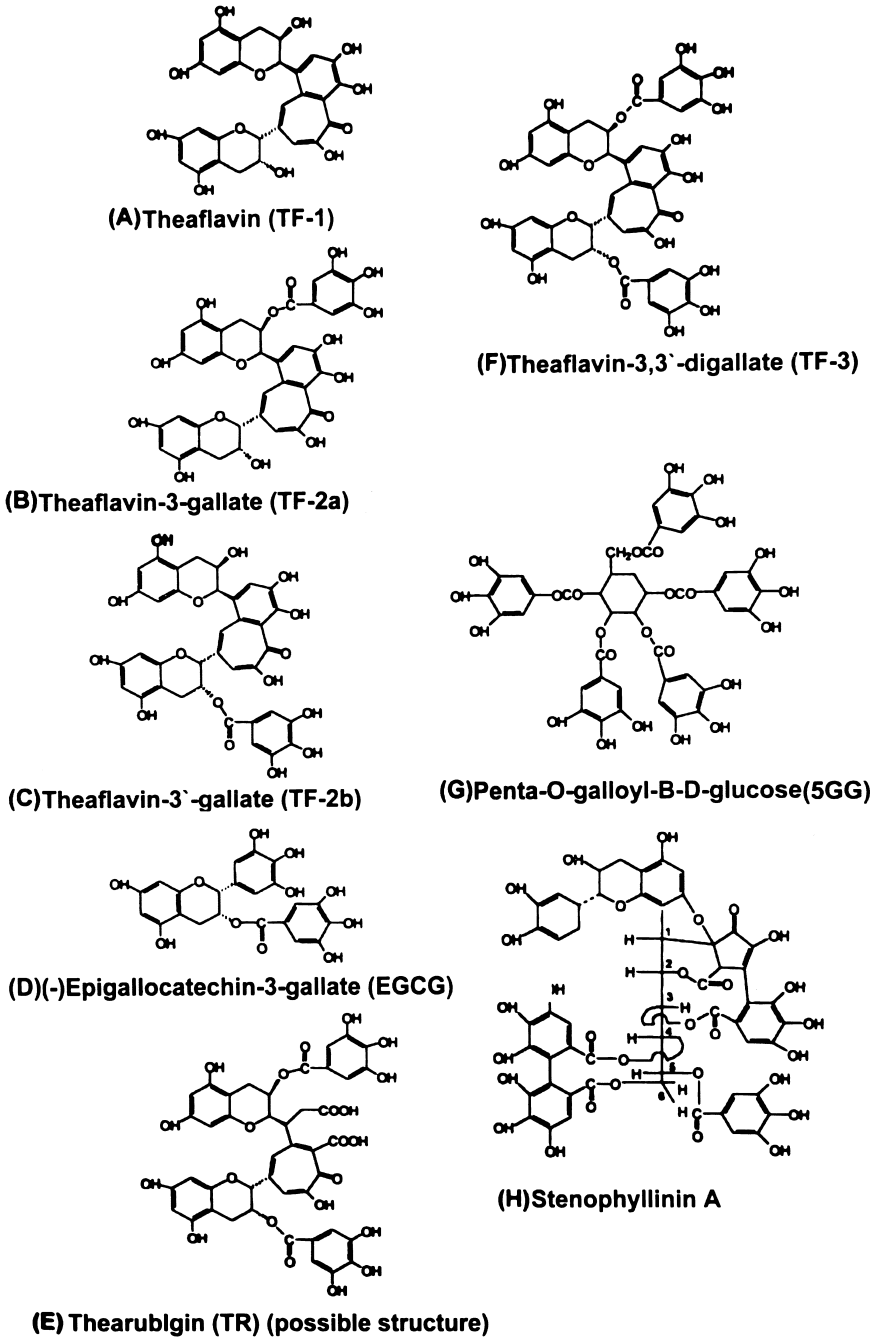

Fig. 1. Structures of penta-*O*-galloyl-β-D-glucose (5GG) and some galloyl derivatives. (A) Theaflavin (TF-1), (B) theaflavin-3-gallate (TF-2A), (C) theaflavin-3′-*O*-gallate (TF-2B), (D) (−)-epegallocatechin-3-gallate (EGCG), (E) Thearubigin (TR), (F) theaflavin-3-3′-*O*-digallate (TF-3), (G) penta-*O*-galloyl-β-D-glucose (5GG), (H) Stenophyllin A. In the present study, TF-2 is a mixture of TF-2A and TF-2B.

Many biological functions of tea polyphenols have been reported, including anti-oxidative activity (Ho et al., 1992; Osawa, 1992; Katiyar et al., 1993), anti-carcinogenic activity (Nakamura and Kawabata, 1981; Yamane et al., 1995), anti-inflammatory activity, inhibition of protein kinase C (Yoshizaqa et al., 1992), and anti-proliferative effects (Lea et al., 1993; Yang et al., 1998) on various cell lines. Induction of apoptosis in human lymphoid leukemia cells has also been demonstrated (Hibasami et al., 1996). Black tea leaves are produced through extensive enzymatic oxidation of polyphenols to polymerized products, such as theaflavines and thearubigins. The major theaflavins in black tea are theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (Chen and Ho, 1995). The structures of these compounds are shown in Fig. 1. Recently, Lu et al. reported that black tea significantly inhibits proliferation and enhances apoptosis in mouse skin tumor models. In our reports, we found that theaflavin-3,3′-digallate blocks nitric oxide (NO) synthase by down-regulating the activation of nuclear factor κB (NF-κB) in

macrophages (Lin et al., 1999) and inhibits tumor proliferation, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) receptor kinase activity (Liang et al., 1999), and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced protein kinase C and AP-1 (activator protein-1) binding activities in NIH3T3 (Chen et al., 1999). However, the mechanisms for the growth inhibition and apoptosis are not yet known.

Apoptosis is induced by a variety of stimuli, such as genotoxic compounds (Barry et al., 1990), tumor necrosis factor (Laster et al., 1988), Fas ligand (Nagata and Golstein, 1995), and various environmental stresses (Buttke and Sandstrom, 1994). Despite of the diversity of apoptosis-inducing agents, numerous experiments indicate that signals leading to the activation of members of the intracellular cysteine protease family, for instance, the caspases may play a pivotal role in the initiation and execution of apoptosis induced by various stimuli (Faleiro et al., 1997). To date, at least 10 distinct caspases in mammalian cells have been identified (Alnemri et al., 1995).

Caspase-1 or interleukin 1β-converting enzyme was originally identified as a cysteine protease responsible for the processing of interleukin 1β (Kostura et al., 1989). However, caspase-1 knockout mice do not show apparent defects in apoptosis (Li et al., 1995). The most intensively studied apoptotic caspase is caspase-3, previously called CPP32/Yama/Apopain, which is believed to play the role of the executioner most downstream in the apoptotic pathway (Nicholson et al., 1995). In response to certain apoptotic stimuli, the protein cytochrome *c* is released from mitochondria (Kluck et al., 1997; Yang et al., 1997). Quite recently, a critical role of mitochondria in mediating apoptotic signal transduction pathway has been demonstrated (Vander Heiden et al., 1997). The release of cytochrome *c* may trigger the interaction between Apaf1, a mammalian CED-4 homologue, and pro-aspase-9, which in turn results in the conversion of pro-caspase-9 to active caspase-9. Activated caspase-9 then cleaves and activates pro-caspase-3, an event that leads to the cleavage of other death substrates, cellular and nuclear morphological changes, and ultimately cell death (Li et al., 1997; Zou et al., 1997).

In this study, we first examined the anti-proliferative effects of these compounds on human leukemia cells. Our results clearly demonstrate that penta-*O*-galloyl-β-D-glucose can induce apoptosis in a dose-dependent manner in HL-60 cells. We further evaluated the molecular mechanisms of the apoptotic effects induced by penta-*O*-galloyl-β-D-glucose.

2. Materials and methods

2.1. Cell culture and chemicals

Human promyelocytic leukemia (HL-60) cells obtained from American Type Culture Collection (Rockville, MD) were grown in 90% RPMI 1640 and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), supplemented with 2 mM glutamine (GIBCO BRL), 1% penicillin/streptomycin (10,000 U penicillin/ml and 10 mg/ml streptomycin). Medium was normally changed to phenol red-free RPMI 1640 before polyphenol treatment. TF-1 (theaflavin), TF-2 (a mixture of theaflavin-3-gallate and theaflavin-3′-gallate), TF-3 (theaflavin-3,3′-digallate) and TR (thearubigin) were isolated from black tea as described previously (Chen and Ho, 1995). (−)-Epigallocatechin-3-gallate was purified from Chinese tea (Longjing tea, *Camellia sinensis*) as described in our previous report (Lin et al., 1996) and its purity was more than 95%. Penta-*O*-galloyl-β-D-glucose, was isolated from the leaves of *Macaranga tanarins* (L.) as described previously (Lin et al., 1990). Stenophyllinin A was isolated from the roots of *Rosa taiwanensis* Nakai as described previously (Lin and Hung, 1996). The inhibitors of caspase-3 protease (Z-VAD-FMK) and caspase-1 protease (acetyl-Tyr-Val-Ala-Asp-aldehyde, Ac-YVAD-CHO) were purchased from Calbiochem (La Jolla, CA) and Ac-DEVD-CHO was purchased from Pharmingen (Becton Dickinson, San Diego, CA). Propidium iodide was obtained from Sigma (St. Louis, MO).

2.2. Acridine orange staining assay

Cells (5×10^5) were seeded into 60-mm petri dishes and incubated at 37°C for 24 h. The cells were harvested after treatment for 24 h, and 5 μl of cell suspension was mixed on a slide with an equal volume of acridine orange solution [10 μg/ml in phosphate-buffered saline (PBS)]. Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (Olympus America, Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

2.3. Cell survival assay

Cells were plated at a density of 2×10^5 cells/100 μl/well into 96-well plates. After overnight growth, cells were pretreated with a series of concentrations of polyphenols. The final concentrations of dimethyl sulfoxide (DMSO) in the culture medium were less than 0.1%. Following 24 h of incubation with drugs, the cell viability was assayed with CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 20 μl of combined solution of a tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium: inner salt) and an electron-coupling reagent, phenazine methosulfate, was added to each well. After incubation for 2 h at 37°C in a humidified 5% CO_2 atmosphere, $A_{490\ nm}$ was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA).

2.4. Subcellular fractionation

Mitochondrial and cytosolic (S100) fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiolthione, 17 μg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, 2 μg/ml leupeptin [pH 7.4]). Cells were passed through a needle 10 times. Unlysed cells and nuclei were pelleted by centrifugation (10-min, $750 \times g$). The supernatant was spun at $100{,}000 \times g$ for 15 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was spun at $100{,}000 \times g$ for 1 h. The supernatant from this final centrifugation represents the S100 fraction.

2.5. DNA extraction and electrophoresis analysis

HL-60 cells (5×10^5 cells/ml) were harvested, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5% mg/ml proteinase K, 50 mM Tris (hydroxy methyl) aminomethane (pH 8.0), 10 mM EDTA at 56°C for 3 h and treated with RNase A (0.5 μg/ml) for another 2 h at 56°C. The DNA was extracted by phenol/chloroform/isoamyl (25/24/1) before loading and analyzed by 1.8% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in TBE (Tris-borate/EDTA electrophoresis buffer). Approximately 20 μg DNA was loaded in each well and visualized under UV light and photographed.

2.6. Western blotting

The nuclear and cytosolic proteins were isolated from human promyelocytic leukemia HL-60 cells (5×10^5 cells/ml) after treatment with 50 μM penta-*O*-galloyl-β-D-glucose for 0, 3, 6, 12, 18 and 24 h. The total proteins were extracted by adding 200 μl of cold lysis buffer (50 mM Tris–HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl floride; 1% NP-40; and 10 μg/ml leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at $10{,}000 \times g$ for 30 min at 4°C. The cytosolic fraction (supernatant) proteins were measured by Bicinchoninic Acid Assay (BCA; Promega). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol and 0.1% bromphenol blue. The mixtures were boiled at 95°C for 5 min and subjected to 12.5% SDS-polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was ordinarily carried out on SDS-polyacrylamide gels (SDS-PAGE). Following electrophoresis, proteins on the gel were electro-transferred onto an immobile membrane (PVDF; Millipore, Bedford, MA) with transfer buffer composed of 25 mM Tris–HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide. The membranes were then immunoblotted with primary antibodies (1:1000 of rabbit polyclonal antibodies to human poly-(ADP-ribose) polymerase (UBI, Lake Placid, NY), anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase-9 antibody (Pharmingen, Becton Dickinson, San Diego, CA), at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of the blotting agent (ECL, Amersham, Arlington Heights, IL), after exposure of the filters to Kodak X-Omat films. The anti-DFF45/ICAD antibody (MBL, Naka-Ku, Nagoya, Japan) was visualized incubation with coloregenic with substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as suggested by the manufacturer (Sigma). The mitochondria and cytosolic fractions isolated from cells were used for immunoblot analysis of cytochrome *c* as described. The cytochrome *c* protein was detected by using anti-cytochrome *c* antibody (Research Diagnostic, Flanders, NJ).

2.7. Activity of caspase

Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin after treatment. Cell lysates were clarified by centrifugation at $12{,}000 \times g$ for 20 min at 4°C. Caspase activity in the supernatant was determined by a fluorogenic assay

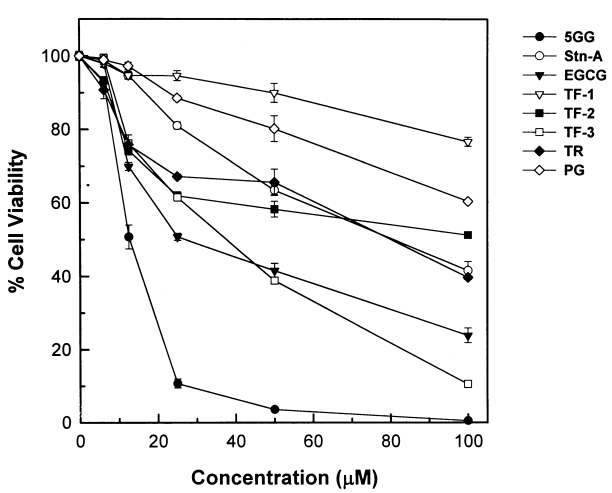

Fig. 2. Effects of different polyphenols on cell viability. HL-60 cells were treated with polyphenols for 24 h. Cell viability then was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay as described. The percentage of cell viability was calculated as a ratio of A_{490} of treated cells and control cells (treated with 0.1% DMSO vehicle). Data represent means ± SE for three determinations.

(Promega's CaspACE™ Assay System, Madison, WI). Briefly, 50 μg of total protein, as determined by bicinchoninic acid assay (Promega), was incubated with 50 μM substrate acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide (Ac-DEVD-MCA) or acetyl-Tyr-Val-Ala-Asp-methylcoumaryl-7-amide (Ac-YVAD-AMC) at 30°C for 1 h. The release of MCA was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Hitachi F-4500).

2.8. Analysis of mitochondrial transmembrane potential

Loss of mitochondrial transmembrane potential was monitored by flow cytometery. Briefly, HL-60 cells were exposed to penta-*O*-galloyl-β-D-glucose (50 μM) and mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6(3)); (Molecular Probes, Eugene, OR). Fluorescence was measured after staining the cells for 15 min at 37°C.

2.9. Flow cytometry

HL-60 cells ($2x10^5$) were cultured in 60-mm petri dishes and incubated for 24 h. Then cells were harvested, washed with PBS, resuspended in 200 μl PBS, and fixed in 800 μl of iced 100% ethanol at −20°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at 37°C for 30 min. Then 1 ml of propidium iodide solution (50 μg/ml) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was

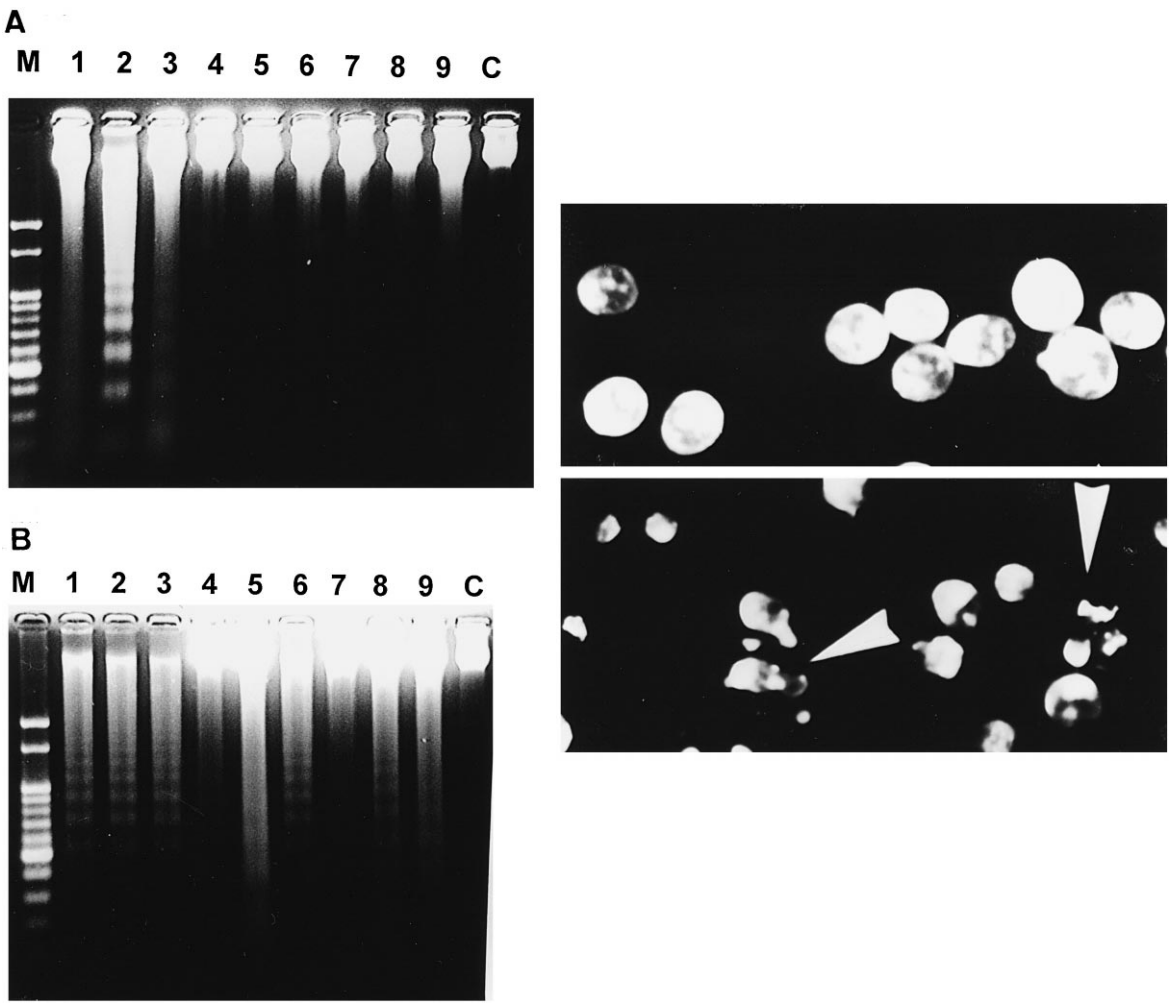

Fig. 3. Induction of DNA fragmentation by various polyphenols in HL-60 cells and apoptosis induced by penta-*O*-galloyl-β-D-glucose. (A) DNA fragments were separated by electrophoresis in 1.8% agarose gel. Cells were treated with various polyphenols (50 μM each) for 24 h and (B) for 48 h including line 1, (−)-epegallocatechin-3-gallate; line 2, penta-*O*-galloyl-β-D-glucose; line 3, stenophyllin A; line 4, Theaflavin; line 5, TF-2; line 6, theaflavin-3-3′-*O*-digallate; line 7, Thearubigin; line 8, propyl gallate; line 9, gallic acid, and C, control. (C) The chromatin condensation of apoptotic cells induced by penta-*O*-galloyl-β-D-glucose for 24 h. The condensed chromosomes are seen as spots in the nucleus by acridine orange staining; apoptotic cells are shown as white arrowheads.

quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickenson, San Jose, CA).

3. Results

3.1. Treatment with polyphenols causes dose-dependent reduction in cell survival

Previous study has shown the inhibitory effect of penta-*O*-galloyl-β-D-glucose on tumor promotion by teleocidin, and penta-*O*-galloyl-β-D-glucose is as effective as (−)-epigallocatechin-3-gallate (Fujiki et al., 1992). The structures of penta-*O*-galloyl-β-D-glucose and other polyphenols are illustrated in Fig. 1. We first tested the effect of these polyphenols on cell viability. Human leukemia HL-60 cells were treated with different concentrations of polyphenols. After 24 h of treatment, the number of live cells was determined by means of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium (MTS) test. As shown in Fig. 2, the viability of HL-60 cells was less than 10% after exposure to penta-*O*-galloyl-β-D-glucose (50 μM) for 24 h. Penta-*O*-galloyl-β-D-glucose appeared to be most potent, with an IC_{50} less than 15 μM. Besides penta-*O*-galloyl-β-D-glucose, theaflavin-3,3′-*O*-digallate and (−)-epigallocatechin-3-gallate were stronger than thearubigin, stenophyllin A, and TF-2. The IC_{50} for cell viability of theaflavin-3,3′-*O*-digal-

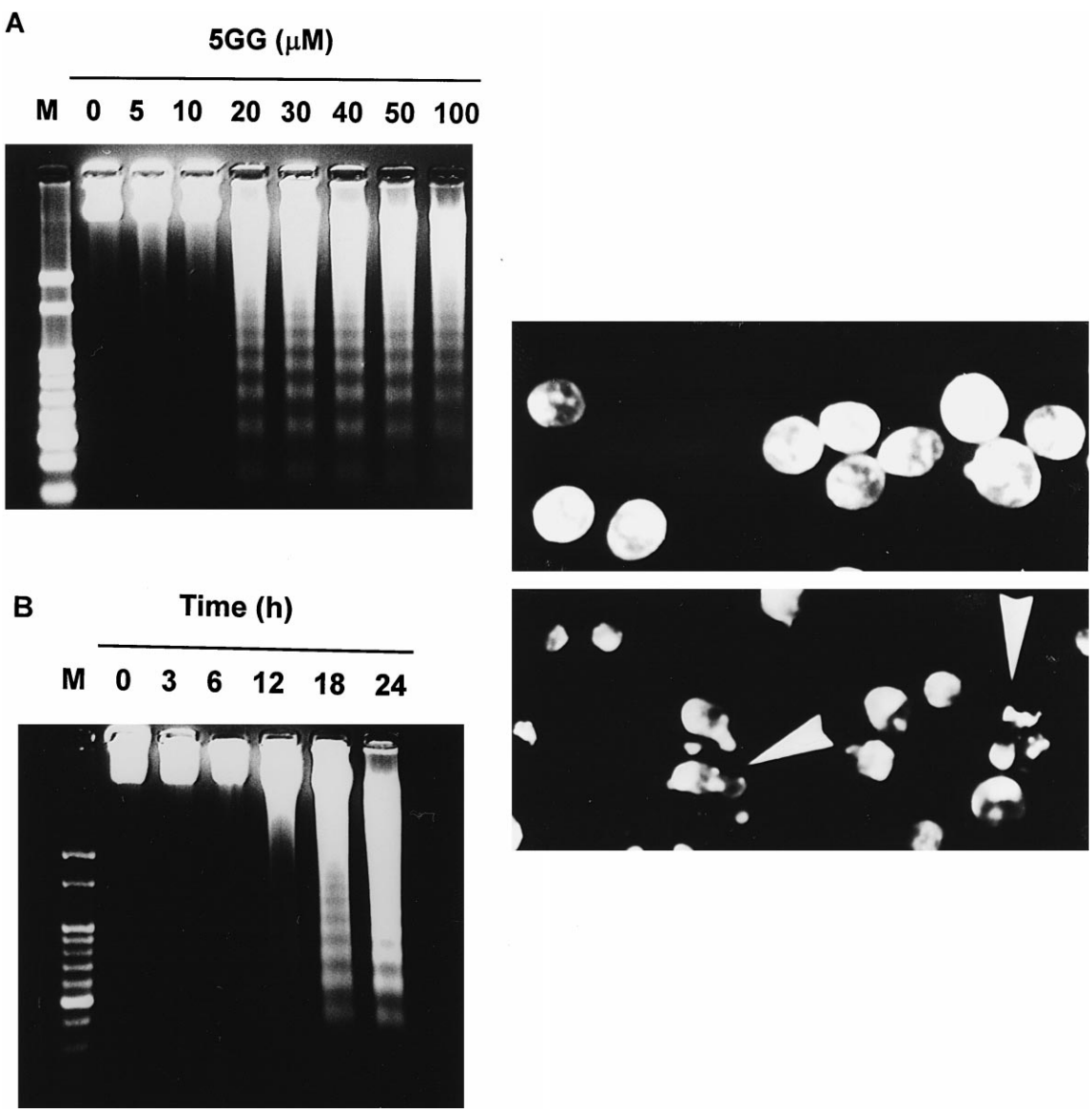

Fig. 4. Induction of the internucleosomeal DNA fragmentation ladder in penta-*O*-galloyl-β-D-glucose-treated HL-60 cells. (A) HL-60 cells were incubated with different doses (5, 10, 20, 30, 40, 50, and 100 μM) of penta-*O*-galloyl-β-D-glucose for 24 h, and agarose gel analysis of DNA fragmentation was performed. (B) Time-dependent increase of DNA ladder were analyzed at different time periods. M, marker.

late and (−)-epigallocatechin-3-gallate was as low as 28 and 38 μM respectively. However, theaflavin and propyl gallate affected cell viability only slightly.

3.2. Induction of apoptosis by penta-*O*-galloyl-β-D-glucose

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and apoptotic body formation. In each case, nucleosomal DNA ladders, which are typical of apoptosis, were visible on agarose gel after staining with ethidium bromide. After treatment of HL-60 cells with 50 μM various polyphenols for 24 h, the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in ethidium-stained gels (Fig. 3A, lane 2). In contrast, for those cells treated with (−)-epigallocatechin-3-gallate and stenophyllinin A, the DNA fragmentation was less obvious (Fig. 3A, lines 1 and 3). Of the polyphenols tested, penta-*O*-galloyl-β-D-glucose had the most potent effect on cell viability and resulted in internuclesomal DNA fragmentation in cells; the other drugs were less effective. When cells were exposed to 50 μM polyphenols for 48 h, digested genomic DNA was clearly visible (Fig. 3B, lines 1, 2, 3, and 6). Fragmentation was also visible in cells treated with propyl gallate for 48 h. These data indicate that (−)-epigallocatechin-3-gallate, stenophyllinin A, and theaflavin-3,3′-*O*-digallate induce genome digestion with increasing treatment time.

To characterize the cell death induced by penta-*O*-galloyl-β-D-glucose, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding agent, acridine orange. Within 24 h of treatment with 50 μM penta-*O*-galloyl-β-D-glucose, cells clearly exhibited significant morphological changes and chromosomal condensation, which is indicative of apoptotic cell death (Fig. 3C). At 20 μM penta-*O*-galloyl-β-D-glucose, digested genomic DNA was evident at 24 h (Fig. 4A). This cell death response was dose dependent. At 50 μM penta-*O*-galloyl-β-D-glucose, digested genomic DNA was evident at 18 h (Fig. 4B). To investigate the induction of a sub-G1 cell population, the DNA content of HL-60 cells treated with penta-*O*-galloyl-β-D-glucose for various periods was analyzed by flow cytometry (Fig. 5). A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (Telford et al., 1992), was detected. Cells were treated with penta-*O*-galloyl-β-D-glucose (50 μM), washed and stained with propidium iodide. As seen in Fig. 5, the percentage of apoptotic HL-60 cells (left column) was 2.83%, 1.87%, 1.50%, 1.68%, 1.55%, and 1.27% after 0, 3, 6, 12, 18, and 24 h without penta-*O*-galloyl-β-D-glucose, respectively. The percentage of apoptotic HL-60 cells (right column) was 2.89%, 2.47%, 2.86%, 8.25%, 46.99%, and 64.08% after 0, 3, 6, 12, 18, and 24 h of incubation with penta-*O*-galloyl-β-D-glucose (50 μM), respectively. The peak of apoptosis did not appear until after 18 h of incubation and

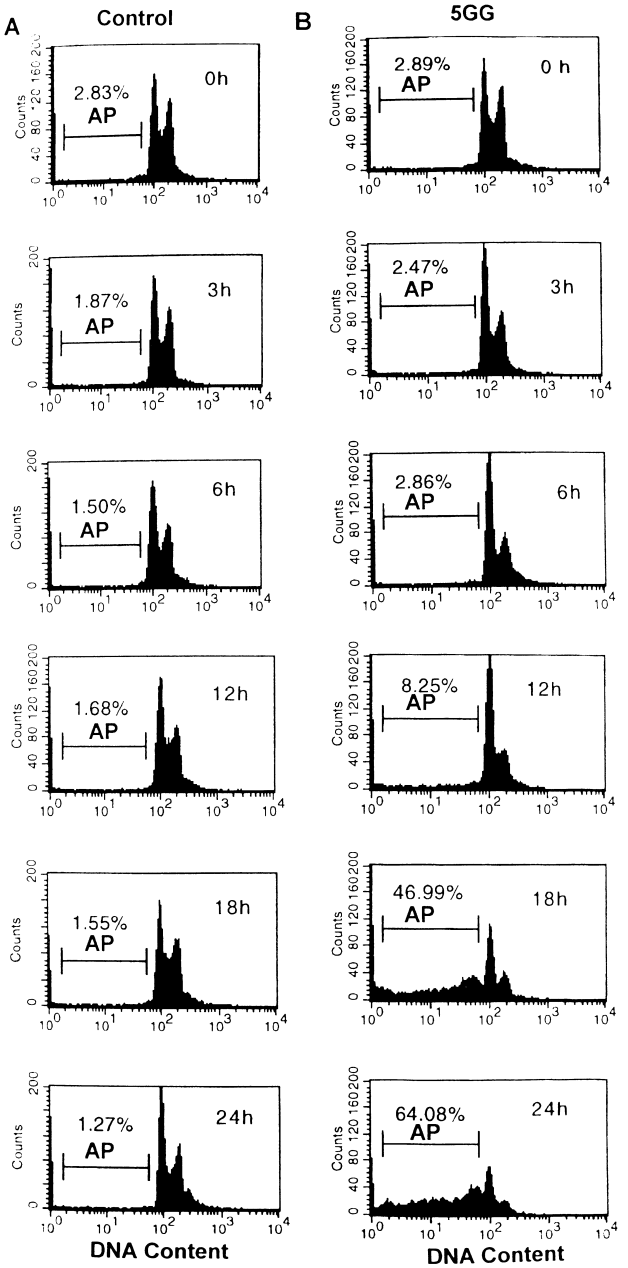

Fig. 5. Determination of sub-G1 cells in control and penta-*O*-galloyl-β-D-glucose treated HL-60 cells by flow cytometry. HL-60 cells were treated with DMSO only as control (A) or treated with 50 μM (B) for 3, 6, 12, 18, and 24 h. The method of flow cytometry used is described in Materials and methods. AP (apoptotic peak) represents apoptotic cells with a lower DNA content.

this timing is consistent with the appearance of the DNA ladder (Fig. 4B). HL-60 cells exposed to 50 μM penta-*O*-galloyl-β-D-glucose for 18 and 24 h resulted in 46.99% and 64.08% apoptosis, respectively.

3.3. Stimulation of caspase-3 activity during penta-*O*-galloyl-β-D-glucose induced apoptosis

We then asked whether caspases were involved in the cell death response induced by penta-*O*-galloyl-β-D-glu-

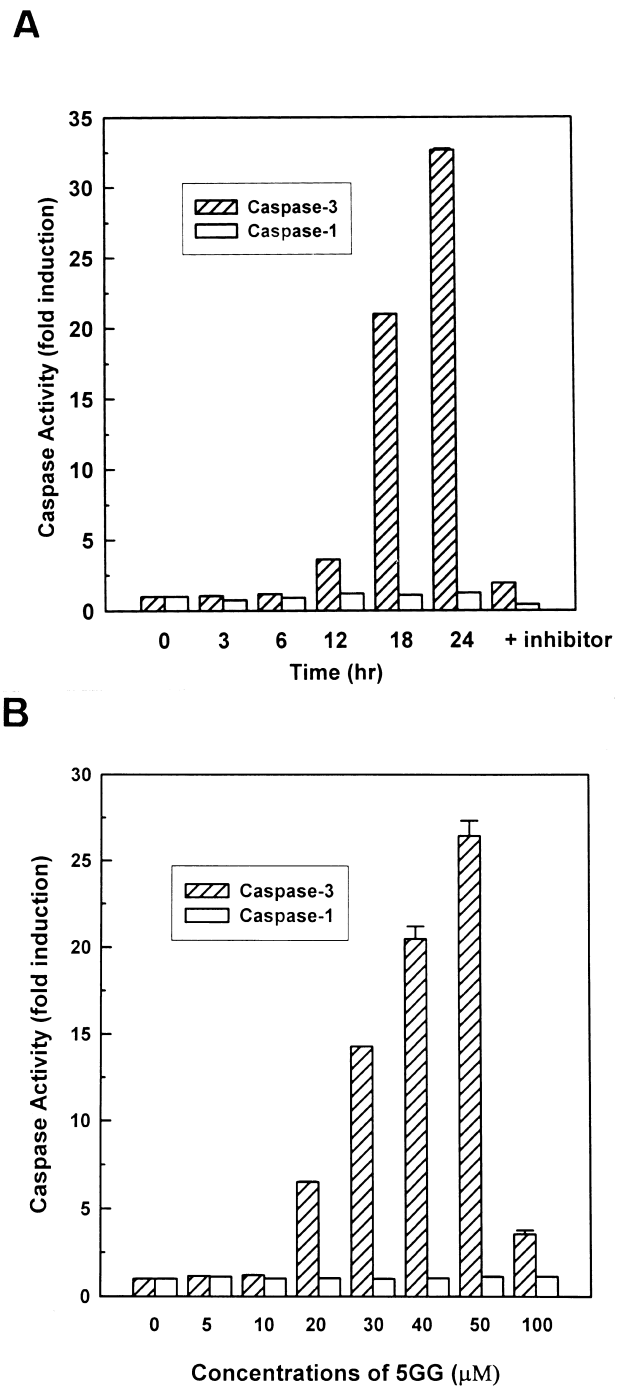

Fig. 6. Induction of caspase-3 activity. (A) Kinetics of caspase-3 activation, cells were treated with 50 μM penta-*O*-galloyl-β-D-glucose for different time periods or treated with 0.05% DMSO as vehicle control. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 and caspase-1 proteases was determined by incubation of 50 μg of total protein with fluorogenic substrates, Ac-DEVD-MCA or Ac-YVAD-MCA, respectively, for 1 h for 30°C. The release of AMC was monitored spectrofluometrically (excitation = 360 nm; emission = 460 nm). (B) Dose-dependent activation of caspase by penta-*O*-galloyl-β-D-glucose. Following treatment with different concentrations of penta-*O*-galloyl-β-D-glucose for 24 h, cells were harvested, and caspase activity was determined as described above. Data represent means ± SE for three determinations.

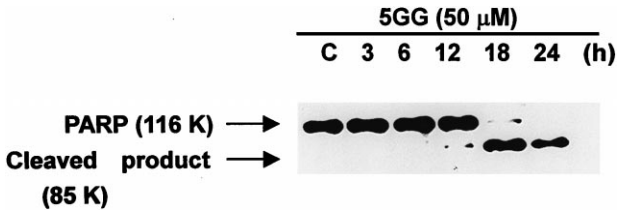

Fig. 7. Time course of poly-(ADP-ribose) polymerase cleavage by penta-*O*-galloyl-β-D-glucose. HL-60 cells were treated as indicated and analysis by western blotting as described in Materials and methods. This experiment was repeated three times with similar results.

cose. Caspases are activated in a sequential cascade of cleavages from their inactive forms (Enari et al., 1996). Once activated, caspases can subsequently cleave their substrates at specific sites. For example, caspase-3 cleaves preferentially after a DXXD↓X, whereas caspase-1 cleaves at YXXD↓X. To monitor the enzymatic activity of caspases during penta-*O*-galloyl-β-D-glucose-induced apoptosis, we used two fluorogenic peptide substrates. Ac-DEVD-MCA is a specific substrate for caspase-3, while Ac-YVAD-MCA detects caspase-1 activity. As illustrated in Fig. 6A, penta-*O*-galloyl-β-D-glucose (50 μM) induced a dramatic increase in DEVD-specific caspase activity in treated HL-60 cells. The induction of DEVD-specific activity was approximately 32 folds compared to the control group at 24 h. In contrast to the increase in DEVD-specific

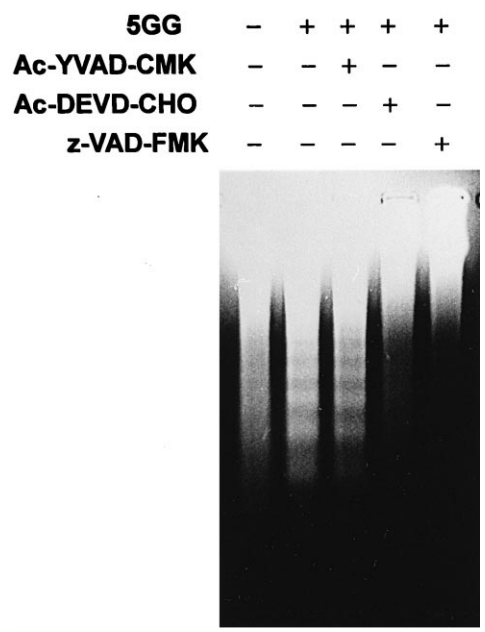

Fig. 8. Effects of caspase inhibitors on DNA fragmentation induced by penta-*O*-galloyl-β-D-glucose. Suppression of penta-*O*-galloyl-β-D-glucose-induced DNA fragmentation by caspase-3 inhibitor. HL-60 cells were pretreated for 1 h with caspase-3 protease inhibitors (Ac-DEVD-CHO and Z-VAD-FMK) or with caspase-1 protease inhibitor (Ac-YVAD-CHO) and then stimulated with 50 μM penta-*O*-galloyl-β-D-glucose for 24 h, and agarose gel analysis of DNA fragmentation was performed. This experiment was repeated three times with similar results.

activity, negligible YVAD-specific activity was observed. Addition of DEVD-CHO (a caspase-3 inhibitor) for 30 min completely inhibited penta-*O*-galloyl-β-D-glucose-induced caspase activity (Fig. 6A). The induction of DEVD-specific activity followed the dose-dependent pattern of cell death and was maximal at 50 μM (Fig. 6B). Higher penta-*O*-galloyl-β-D-glucose concentrations yielded apparently diminished DEVD-specific activity, possibly as a result of loss of cytoplasm due to acute necrotic cell lysis. Again, negligible YVAD-specific activity was detectable in these samples.

3.4. Treatment with penta-O-galloyl-β-D-glucose causes degradation of poly-(ADP-ribose) polymerase, an endogenous substrate of caspase-3

Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is poly-(ADP-ribose)

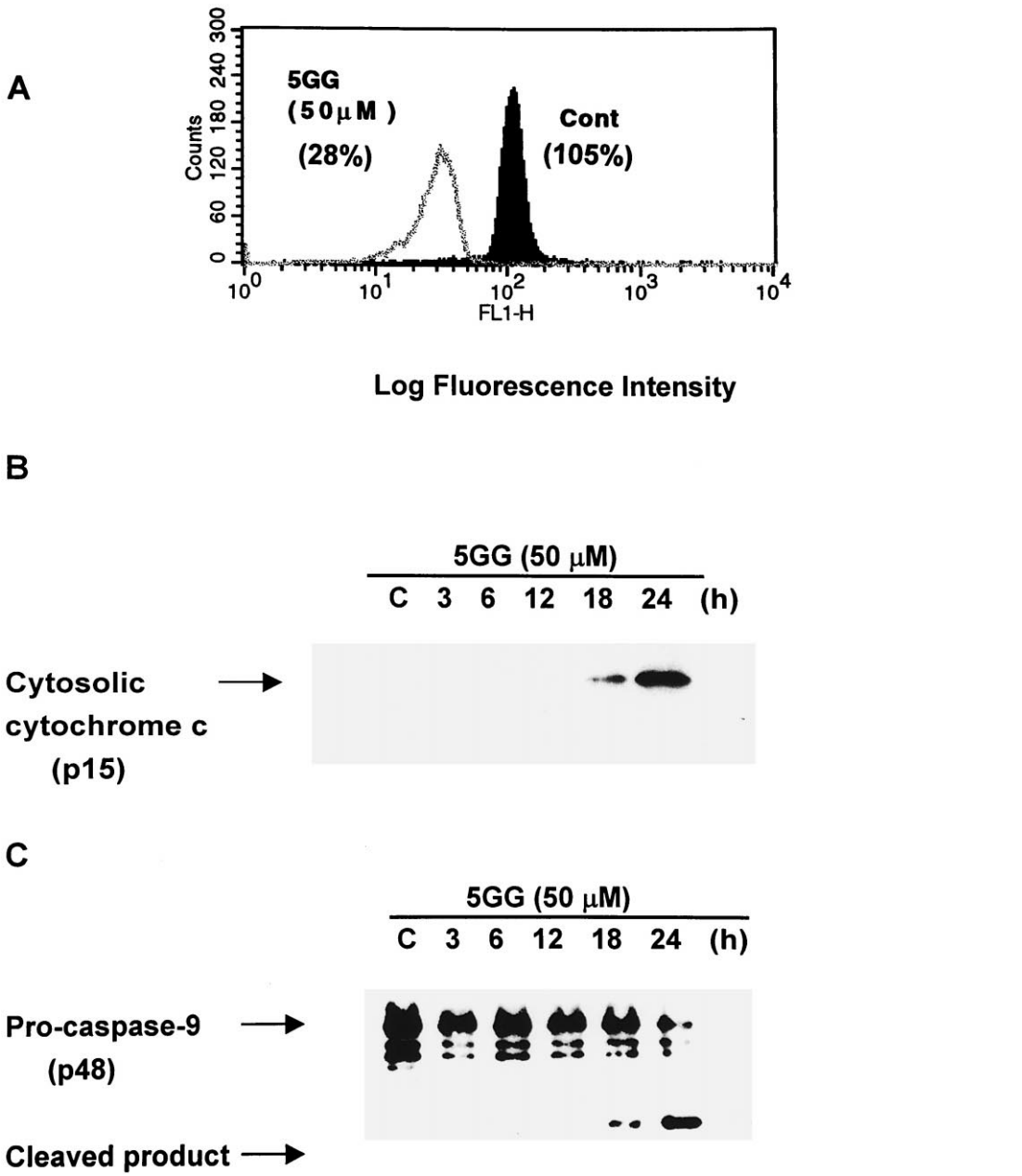

Fig. 9. Induction of mitochondrial dysfunction and the release of cytochrome *c* from mitochondria into the cytosol following the cleavage of caspase-9 in penta-*O*-galloyl-β-D-glucose-induced apoptosis. (A) HL-60 cells were treated with 50 μM penta-*O*-galloyl-β-D-glucose for 1h and were then incubated with 40 nM 3,3′-dihexyloxacarbocyanine and analyzed by flow cytometry. The percentages reflect the reduction of $\Delta\psi_m$ 3,3′-dihexyloxacarbocyanine. (B) Cytochrome *c* release from mitochondria into cytosol. HL-60 cells were treated with 50 μM penta-*O*-galloyl-β-D-glucose and cytochrome *c* was detected by cytochrome *c* antibody. (C) Penta-*O*-galloyl-β-D-glucose induced caspase-9 processing. Total cell lystaes were prepared from HL-60 cells treated with 50 μM in a time-dependent manner and then analyzed by Western blotting.

polymerase. Although poly-(ADP-ribose) polymerase is not essential for cell death, the cleavage of poly-(ADP-ribose) polymerase is another hallmark of apoptosis (Tewari et al., 1995). Treatment of HL-60 cells with 50 μM penta-*O*-galloyl-β-D-glucose caused a time-dependent proteolytic cleavage of poly-(ADP-ribose) polymerase, with accumulation of the *Mr*. 85,000 and the concomitant disappearance of the full-size *Mr*. 116,000 molecule (Fig. 7). Poly-(ADP-ribose) polymerase cleavage was apparent with 18 h of penta-*O*-galloyl-β-D-glucose treatment, roughly following the appearance of caspase activity (Fig. 6A) and preceding DNA fragmentation (Fig. 4B).

3.5. A caspase-3 inhibitor, Ac-DEVD-CHO, abolishes apoptosis induced by penta-O-galloyl-β-D-glucose

The above-results clearly indicate that caspase-3 protease is activated in response to the apoptosis induced by penta-*O*-galloyl-β-D-glucose. To determine whether the activation of caspase-3 is required for the induction of cell death by penta-*O*-galloyl-β-D-glucose, we pretreated HL-60 cells with caspase inhibitor. As shown in Fig. 8, an inhibitor of caspase-3 protease, Ac-DEVD-CHO, and Z-VAD-FMK were able to inhibit penta-*O*-galloyl-β-D-glucose-stimulated DEVD-specific activity and cell death. In contrast, Ac-YVAD-CMK, an inhibitor of caspase-1 activity, had little effect at similar concentrations, consistent with the high substrate specificity of different caspases.

3.6. Penta-O-galloyl-β-D-glucose-induced cytochrome c release and the cleavage of caspase-9

Recently, it has become clear that apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell death process. We next evaluated the effects of penta-*O*-galloyl-β-D-glucose on the mitochondrial transmembrane potential (ψ_m) and the release of mitochondrial cytochrome *c* into the cytosol. We measured $\Delta\psi_m$ using the fluorescent probe 3,3′-dihexyloxacarbocyanine. As shown in Fig. 9A, which compares HL-60 cells exposed to penta-*O*-galloyl-β-D-glucose to control cells, the 3,3′-dihexyloxacarbocyanine fluorescence intensity shifted to the left from 105% to 28% in penta-*O*-galloyl-β-D-glucose-induced apoptotic HL-60 cells at 1 h. These results demonstrate that penta-*O*-galloyl-β-D-glucose causes a decrease in mitochondrial transmembrane potential in HL-60 cells. Caspase-9 binds to Apaf-1 in a cytochrome *c*- and dATP-dependent fashion to become active, and in turn cleaves and activates caspase-3 (Li et al., 1997). As shown in Fig. 9B, the release of mitochondrial cytochrome *c* into the cytosol was detected at 18 h in penta-*O*-galloyl-β-D-glucose-treated HL-60 cells. As indicated in Fig. 9C, the activation of caspase-9 brought about by its cleavage, represented here as a increase in the cleavage product on the western blot, was associated with the release of cytochrome *c* from mitochondria into the cytosol.

Taken together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* released from the mitochondria during penta-*O*-galloyl-β-D-glucose-induced HL-60 cell apoptosis.

3.7. Treatment with penta-O-galloyl-β-D-glucose causes the cleavage of DFF-45, an inhibitor of endonuclease

DFF, a DNA fragmentation factor, has been identified as a heterodimeric protein that triggers DNA fragmentation during apoptosis although DFF has no nuclease activity (Liu et al., 1997). Caspases activated by apoptotic signals cleave DFF-45 to release caspase-activated deoxyribonuclease (Enari et al., 1998; Sakahira et al., 1998). We

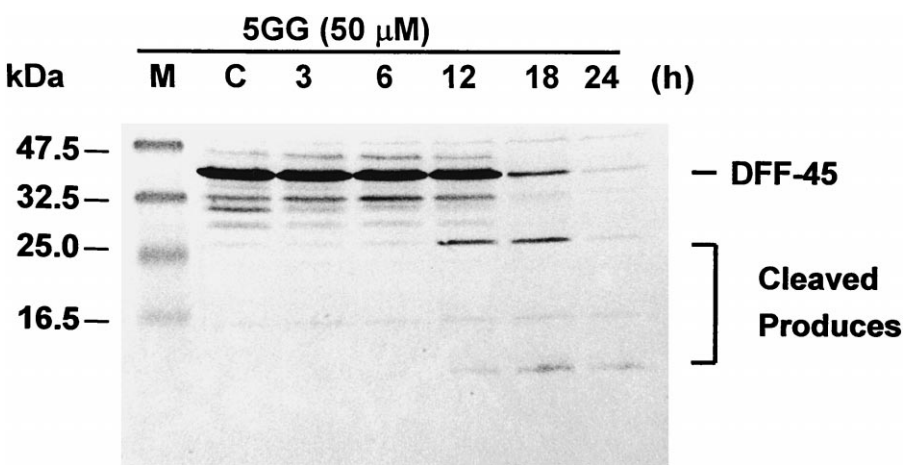

Fig. 10. Cleavage of DFF-45 by penta-*O*-galloyl-β-D-glucose. Kinetics of DFF-45 cleavage by penta-*O*-galloyl-β-D-glucose. Aliquots of 50 μg of HL-60 cell extracts prepared from cells treated with penta-*O*-galloyl-β-D-glucose at indicated times were subjected to 15% SDS-PAGE, and were transferred onto a nitrocellulose filter. The filter was probed by using mouse anti-DFF-45 monoclonal antibody, and the antigen–antibody complexes were visualized with the colorigenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

further explored the possibility that activation of caspase-3 may also induce DFF-45 protein degradation. Treatment of HL-60 cells with 50 μM penta-*O*-galloyl-β-D-glucose caused proteolytic cleavage of DFF-45 at 18 h. With apoptosis, nearly all of the DFF-45 was degraded at 24 h (Fig. 10). The activation of caspase-3 was also observed 18 h later (Fig. 6A), with a time course that paralleled the cleavage of DFF-45 observed when cells were treated with penta-*O*-galloyl-β-D-glucose. As already described, ICAD is a mouse homologue of human DFF-45. Caspase-3 cleaves DFF-45 and, once caspase-activated deoxyribonuclease released, it can enter the nucleus where it degrades chromosomal DNA. (Liu et al., 1997; Sakahira et al., 1998).

4. Discussion

Penta-*O*-galloyl-β-D-glucose, which can be obtained in large amounts from hydrolyzed tannin, had anti-tumor promoting activity in a two-stage carcinogenesis experiment with mouse skin (Fujiki et al., 1992). The present results demonstrate for the first time that penta-*O*-galloyl-β-D-glucose can induce apoptosis in human leukemia cells. Of various polyphenols tested, we found that penta-*O*-galloyl-β-D-glucose was the most potent inhibitor of cell viability and could induce DNA fragmentation.

Black tea is one of the most popular beverages worldwide. Several reports have indicated that black tea can inhibit tumor cell proliferation in animal models (Lea et al., 1993; Lu et al., 1997). Recently, in our laboratory, we have demonstrated that theaflavin-3,3′-digallate and (−)-peigallocatechin-3-gallate strongly inhibit the inducible NO synthase in a murine macrophage cell line, RAW 264.7. The inhibition of inducible NO synthase protein is in the following order: theaflavin-3,3′-digallate > (−)-epigallocatechin-3-gallate > a mixture of theaflavin-3-gallate and theaflavin-3-′gallate > thearubigin > theaflavin (Lin et al., 1999). Among theaflavins, theaflavin-3,3′-digallate, which has gallic acid moieties, exhibited the strongest anti-inflammatory activity, as judged by its suppression of inducible NO synthase induction. Theaflavin, which has no gallic acid moiety, exhibited the least inhibitory effects. Our laboratory also found that theaflavin-3-3′-*O*-digallate, which has two gallic acid moieties, exhibited the strongest inhibition of TPA-induced effects and anti-proliferative activity on tumor cells (Chen et al., 1999; Liang et al., 1999). In our study, various polyphenolic compounds were tested for their ability to cause to apoptosis. Here, we demonstrated that the cancer chemopreventive agent penta-*O*-galloyl-β-D-glucose isolated from hydrolyzed tannin, which has five gallic acids, was the most potent apoptosis inducer among these polyphenols. (−)-epigallocatechin-3-gallate and stenophyllinin A (50 μM) induced apoptosis slightly at 24 h. We conclude that gallic acid moieties are important for the exhibited apoptosis-inducing potency of these polyphenols.

This induction of apoptosis occurred within hours, consistent with the view that penta-*O*-galloyl-β-D-glucose induces apoptosis by activating preexisting apoptosis machinery. Indeed, treatment with penta-*O*-galloyl-β-D-glucose caused an induction of caspase-3 activity and the degradation of poly-(ADP-ribose) polymerase, which preceded the onset of apoptosis. Pretreatment with the caspase-3 inhibitors Ac-DEVE-CHO or Z-VAD-FMK inhibited penta-*O*-galloyl-β-D-glucose-induced caspase activity and apoptosis, suggesting that apoptosis induced by penta-*O*-galloyl-β-D-glucose involves a caspase-3 mediated mechanism. Questions remain as to how caspase-3 is activated by penta-*O*-galloyl-β-D-glucose. In different types of cell death, caspase-1/interleukin 1β-converting-like activity has been found to increase prior to the activation of CPP32-like protease, suggesting the potential role of interleukin 1β-converting enzyme in the activation of CPP32-like protease (Enari et al., 1996). We were unable to detect any significant changes in the activity of caspase-1 during penta-*O*-galloyl-β-D-glucose-induced apoptosis, and a specific interleukin 1β-converting enzyme inhibitor, Ac-YVAD-CHO, had no effect on penta-*O*-galloyl-β-D-glucose-stimulated caspase-3 activity. This raises the possibility that factors or proteases other than caspase-1/interleukin 1β-converting enzyme are involved in the activation of caspase-3. Caspase-3 is activated by two sequential proteolytic events that cleave the 32 kDa precursor at aspartic acid residues to generate an active heterodimer of 20 and 12 kDa subunits (Nicholson et al., 1995). In fact, in vitro studies have previously identified Apaf1, cytochrome *c*, and caspase-9 as participants in a complex important for caspase-3 activation. In vitro depletion of caspase-9 from cytosolic fractions resulted in the failure of caspase-3 activation (Li et al., 1997). Taken together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* released from the mitochondria. Release of cytochrome *c* from the mitochondria has been shown to be an almost universal phenomenon during apoptosis although it is unclear whether the cytochrome *c*-mediated caspase cascade is triggered only by a few apoptotic stimuli, or serves as a general amplification mechanism to accelerate cell death (Reed, 1997). The $\Delta\psi_m$ (mitochondrial transmembrane potential) disruption is an early feature of apoptosis and can be detected in cells that still lack obvious morphological signs of apoptosis. It precedes nuclear DNA fragmentation, exposure of phosphatidylserine residues on the cell surface, and major changes in cellular redox potentials (Kroemer et al., 1997). In this study, we found that penta-*O*-galloyl-β-D-glucose induced the loss of mitochondrial transmembrane potential, the release of cytochrome *c*, and the cleavage of caspase-9.

A number of recent studies indicate that caspase-9, caspase-3, and caspase-activated DNA fragmentation factors may all be a linear, nonredundant pathway during acute apoptosis (Liu et al., 1997; Enari et al., 1998;

Sakahira et al., 1998). In our study, we also found that apoptosis induced by penta-*O*-galloyl-β-D-glucose caused the cleavage of DFF-45 and activated caspase-activated deoxyribonuclease.

The apoptosis-inducing potency of penta-*O*-galloyl-β-D-glucose seems to be correlated to its cancer chemopreventive efficacy in animal models (Fujiki et al., 1992). For example, penta-*O*-galloyl-β-D-glucose treatment reduced the percentage of mice bearing tumors from 100% to 50% in week 20, and the average number of tumors per mouse from 3.3 to 0.9. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of penta-*O*-galloyl-β-D-glucose. Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may either be triggered to undergo apoptosis or to proliferate with genomic damage, leading to the formation of cancerous cells that usually exhibit cell cycle abnormalities and which are more susceptible to various apoptosis-inducing agents (Steller, 1995; Thompson, 1995). Thus, treatment with penta-*O*-galloyl-β-D-glucose may preferentially cause apoptosis in those abnormal cells, ultimately leading to the prevention of cancer. Furthermore, the ability of penta-*O*-galloyl-β-D-glucose alone to induce apoptosis suggests its potential use as chemotherapeutic agent since many anti-cancer drugs are known to achieve their anti-tumor function by inducing apoptosis in the target cells (Barry et al., 1990).

In summary, we have demonstrated that the cancer-chemopreventive agent penta-*O*-galloyl-β-D-glucose is able to induce apoptosis in a dose-dependent manner. These results show that penta-*O*-galloyl-β-D-glucose is able to induce a loss of mitochondrial transmembrane potential and the release of mitochondrial cytochrome *c* into the cytosol. It also induces procaspase-9 processing, activates caspase-3, and produces the cleavage of poly-(ADP-ribose) polymerase and DFF-45, and activation of endonuclease.

Acknowledgements

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