Induction of Apoptosis by the Oolong Tea Polyphenol Theasinensin A through Cytochrome *c* Release and Activation of Caspase-9 and Caspase-3 in Human U937 Cells

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This study examined the growth inhibitory effects of theasinensin A (from oolong tea) and black tea polyphenols, including theaflavin (TF-1), a mixture (TF-2) of theaflavin-3-gallate (TF-2a) and theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3) in human cancer cells. Theasinensin A, TF-1, and TF-2 displayed strong growth inhibitory effects against human histolytic lymphoma U937, with estimated IC_{50} values of 12 μM, but were less effective against human acute T cell leukemia Jurkat, whereas TF-3 and (−)-epigallocatechin-3-gallate (EGCG) had lower activities. The molecular mechanisms of tea polyphenol-induced apoptosis as determined by annexin V apoptosis assay, DNA fragmentation, and caspase activation were further investigated. Loss of membrane potential and reactive oxygen species (ROS) generation were also detected by flow cytometry. Treatment with tea polyphenols caused rapid induction of caspase-3, but not caspase-1, activity and stimulated proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). Pretreatment with a potent caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethyl ketone, inhibited theasinensin A induced DNA fragmentation. Furthermore, it was found that theasinensin A induced loss of mitochondrial transmembrane potential, elevation of ROS production, release of mitochondrial cytochrome *c* into the cytosol, and subsequent induction of caspase-9 activity. These results indicate that theasinensin A allows caspase-activated deoxyribonuclease to enter the nucleus and degrade chromosomal DNA and induces DFF-45 (DNA fragmentation factor) degradation. The results suggest that induction of apoptosis by theasinensin A may provide a pivotal mechanism for their cancer chemopreventive function.

Keywords: *Apoptosis; oolong tea; theasinensin A; caspase; U937 cells*

INTRODUCTION

Green tea polyphenols such as (−) epigallocatechin-3-gallate (EGCG) have been demonstrated to have several inhibitory properties on the growth of tumor cell lines (Lin et al., 1996; Lea et al., 1993). The molecular mechanism of antitumor growth might operate through blocking the signal transduction pathway (Lin and Lee, 1995; Lin et al., 1999). However, other tea constituents such as theaflavins from black tea also have antiproliferative or anticarcinogenic activities (Lea et al., 1993; Yang et al., 1997; Valcic et al., 1996). Furthermore, a new group of polyphenols, namely, theasinensins A, B, C, D, and E, have been isolated from oolong tea (Hashimoto et al., 1988). Oolong tea is a partially fermented tea that is consumed heavily in Taiwan, southern China, and most Eastern countries. Oolong tea contains several polyphenols, and their biological activities are not well understood. It is interesting to note that, on the basis of our present studies, theasinensin A and theaflavins are far more potent than EGCG in inhibiting tumor cell growth and in the induction of caspase-3 and cell apoptosis.

Tea (*Camellia sinensis*) is the most widely consumed beverage worldwide; the major tea beverage is black tea, especially in the Western nations. Black tea leaves are produced through extensive enzymatic oxidation of polyphenols to polymerized products, such as theaflavins 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). It has been demonstrated that black tea could be as effective as green tea in cancer chemoprevention. Recently, Lu et al. (1997) reported that black tea significantly inhibits proliferation and enhances apoptosis in mouse skin tumor models. Halder and Bhaduri (1998) reported that theaflavins and thearubigins have antioxidative properties on human red blood cells. Among black tea components, theaflavins are generally considered to be the more effective components for the inhibition of carcinogenesis, but which of these theaflavins is the most effective is unclear. The structures of these compounds are shown in Figure 1. In a series of studies, 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

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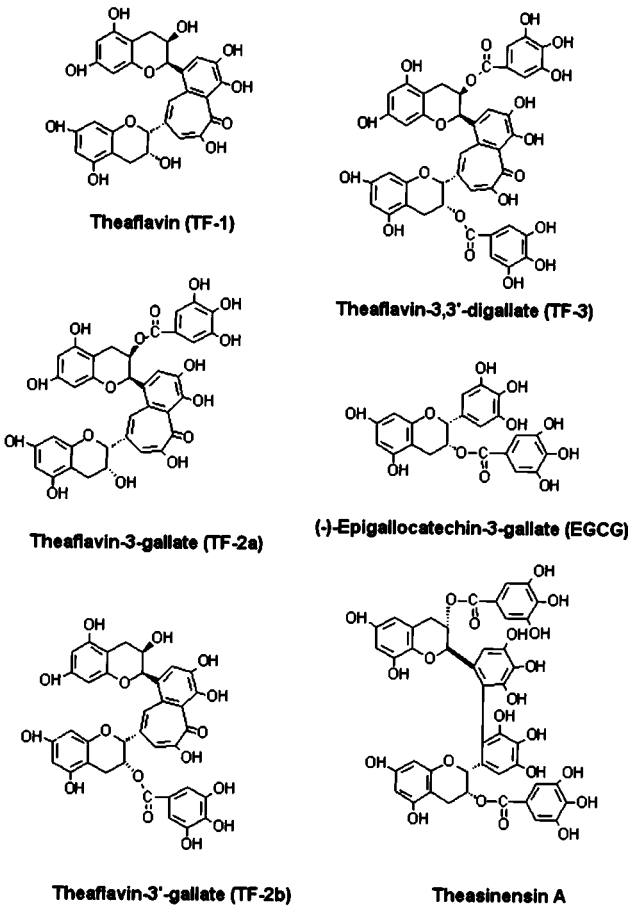

Figure 1. Structures of tea polyphenols, theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-*O*-gallate (TF-2b), (−)-epegallocatechin-3-gallate (EGCG), theaflavin-3,3′-*O*-digallate (TF-3), and theasinensin A. In the present study, TF-2 is a mixture of TF-2a and TF-2b.

(Lin et al., 1999) and inhibits tumor proliferation, epidermal growth factor (EGF), or platelet-derived growth factor (PDGF) receptor kinase activities (Liang et al., 1999) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced protein kinase C and AP-1 (activator protein-1) binding activities in NIH3T3 (Chen et al., 1999).

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 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).

To study the mechanism of the anticancer activities of tea polyphenols, we first examined the antiproliferative effects of these compounds in two human cancer cell lines. Our results clearly demonstrate that theasinensin A can induce apoptosis in a dose-dependent manner in human U937 cells. We further evaluated the molecular mechanisms of the apoptotic effects induced by theasinensin A. The results of the present study will provide a molecular basis for understanding the healthy effect of oolong tea.

MATERIALS AND METHODS

Cell Culture and Chemicals. Human histolytic lymphoma (U937) cell line and acute T cell leukemia (Jurkat) cell line, 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 (10000 units of 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), and TF-3 (theaflavin-3,3′-digallate) were isolated from black tea as described previously.[8] (−)-Epigallocatechin-3-gallate was purified from Chinese tea (longjing tea, *Camellia sinensis*) as described in our previous work (Lin et al., 1996), and its purity was >95%. Theasinensin A was chemically synthesized by radical oxidant of EGCG. The inhibitors of caspase-3 (Z-Asp-Glu-Val-Asp-fluoromethyl ketone, Z-DEVD-FMK) and caspase-1 (acetyl-Tyr-Val-Ala-Asp-aldehyde, Ac-YVAD-CHO) were purchased from Calbiochem (La Jolla, CA). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Moleclar Probes, Eugene, OR) and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO).

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 buffer saline (PBS)]. Green fluorescence was detected between 500 and 525 nm by using an Olympus microscope (Olympus America, Inc., Lake Success, NY). Bright-staining condensed chromatin was detected in apoptotic cells.

Cell Survival Assay. Cells were plated at a density of 1×10^5 cells/100 μL/well into 96-well plates. After overnight growth, cells were pretreated with a series of concentrations of tea polyphenols. The final concentrations of dimethyl sulfoxide (DMSO) in the culture medium were <0.1%. Following 12 h of incubation with these tea polyphenols, the cell viability was assayed with a Luminescent ATP detection assay kit (Packard BioScience B.V.). Briefly, 50 μL of cell lysate was used to assay luminescent ATP. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence counter (Packard 9912V1, Meriden, CT) in single photon counting mode for 0.1 min/well, following 2 min of adaptation in the dark.

Annexin V Flow Cytometric Assay. Perturbations in the cellular membrane occur during the early stages of apoptosis and lead to a redistribution of phosphatidylserine to the external side of the cell membrane. Annexin V selectively binds to phosphatidylserine, and this has enabled the use of fluorescein-labeled annexin V for the identification of cells undergoing apoptosis. FITC-labeled annexin V apoptosis detection kit was obtained from Pharmingen, Becton Dickinson Co. (San Diego, CA). Ten thousand events were collected with a FACScan flow cytometry (Becton Dickinson, San Jose, CA).

DNA Extraction and Electrophoresis Analysis. U937 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(hydroxymethyl)aminomethane (pH 8.0), and 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 Tris-borate/EDTA electrophoresis buffer (TBE). Approximately 20 μg of DNA was loaded in each well and visualized under UV light and photographed.

Western Blotting. The nuclear and cytosolic proteins were isolated from human histolytic lymphoma U937 cells (5×10^5 cells/mL) after treatment with 25 μM theasinensin for 0, 1, 3, 6, and 12 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 phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets

on ice for 30 min, followed by centrifugation at 10000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by bicinchoninic acid assay (BCA; Promega Corp., Madison, WI). 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% bromophenol 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 electrotransferred onto an immobile membrane (PVDF; Millipore Corp., 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, Inc., Lake Placid, NY)], anti-DFF45/ICAD antibody (MBL, Naka-Ku, Nagoya, Japan), at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of the blotting agent (ECL, Amersham Corp., Arlington Heights, IL) after exposure of the filters to Kodak X-Omat films. 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 Inc., Flanders, NJ).

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 phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promega's CaspACE Assay System Corp., Madison, WI). Briefly, 50 μg of total protein, as determined by bicinchoninic acid assay (Promega Corp.), was incubated with 50 μM substrate acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide (Ac-DEVD-MCA), acetyl-Leu-Glu-His-Asp-aminotrifluoromethyl coumarin (Ac-LEHD-AFC), or acetyl-Try-Val-Ala-Asp-methylcoumaryl-7-amide (Ac-YVAD-MCA) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (MCA) and aminotrifluoromethylcoumarin (AFC) was measured by excitation at 360 or 400 nm and emission at 460 or 505 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-4500).

Analysis of Mitochondrial Transmembrane Potential. Change of mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, U937 cells were exposed to theasinensin A (25 μM), and mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes). Fluorescence was measured after the cells had been stained for 15 min at 37 °C.

Flow Cytometry. U937 cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for 12 h. Then cells were harvested, washed with PBS, resuspended in 200 μL of 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 quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

ROS Production Determination. ROS production was monitored by flow cytometry using DCFH-DA. This dye is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cells were treated with theasinensin A (25 μM) for different time periods, and DCFH-DA (30 μM) was added into the medium for a further 30 min at 37 °C.

RESULTS

Treatment with Tea Polyphenols Causes Dose-Dependent Inhibition in Cell Growth. The structures of tea polyphenols are illustrated in Figure 1. We first tested the effect of tea polyphenols on cell growth. Human histolytic lymphoma U937 cells and acute T cell leukemia Jurkat cells were treated with different concentrations of tea polyphenols. After 12 h of treatment, the number of live cells was determined by means of an ATP content test. As shown in Figure 2, the inhibitory activity was dose-dependent with U937 cells. TF-1, TF-2, and theasinensin A displayed approximately the same extents of inhibition on cell growth, showing IC_{50} values of ∼12 μM. The IC_{50} values for TF-3 and EGCG were as low as 19.6 and 26 μM, respectively. The Jurkat cells appeared to be less susceptible to the inhibitory effect of tea polyphenols. Among the five tea polyphenols, namely, TF1, TF2, TF3, EGCG, and theasinensin A, the IC_{50} values were 23.5, 29.6, 35.1, 45.2, and 25.3 μM, respectively.

Induction of Apoptosis by TF-1, TF-2, TF-3, EGCG, and Theasinensin A. 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. To characterize the cell death induced by tea polyphenols, we examined the nuclear morphology of a dying cell with a fluorescent DNA-binding agent, acridine orange, within 12 h of treatment with 25 μM TF-1, TF-2, TF-3, EGCG, and theasinensin A; cells clearly exhibited significant morphological changes and chromosome condensation, which is indicative of apoptotic cell death (Figure 2C). No altered nuclear morphology was evident in either control cells [treated with vehicle (0.05% DMSO)] or cells treated with TF-3 and EGCG. After treatment of U937 cells with 25 μM various tea polyphenols or 0.05% DMSO (as control) for 12 h, the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in ethidium-stained gels (Figure 3A). In contrast, the intact genomic DNA was found in control. At 25 μM theasinensin A, digested genomic DNA was evident at 12 h (Figure 3B). The cell death response was dose dependent. At 25 μM theasinensin A, digested genomic DNA was evident as early as 4 h but not at 0 h (Figure 3C).

In many cell types, DNA fragmentation is a late event of apoptosis; this contrasts with the early translocation of phosphatidyserine (PS) from the internal to the external leaflet of the cell membrane. The annexin V assay provides the advantage of quantifying the rate of induction and progression of apoptotic cells. We used the method to examine whether an early PS translocation also occurs during apoptosis induced in U937 cells in the presence of 25 μM theasinensin A. In the experiment, the annexin V-FITC apoptosis detection kit was employed to demonstrate this translocation. As seen in Figure 4A, the means of FITC fluorescence were

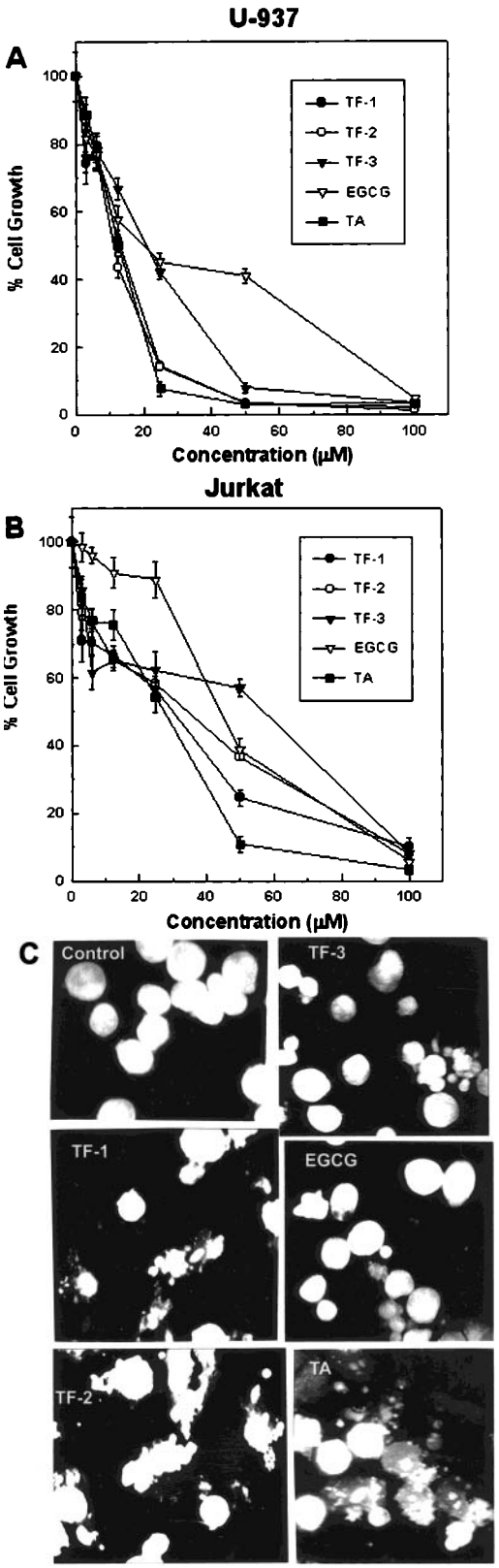

Figure 2. Effects of tea polyphenols on cell growth and morphological changes. (A) U937 and (B) Jurkat cells were treated with tea polyphenols for 12 h, and the cell viabilities were then determined by Luminescent ATP detection assay kit, as described under Materials and Methods. Data were represented as means ± SE for three determinations. (C) U937 cells were either treated with 0.05% DMSO as vehicle control or treated with 25 μM of different tea polyphenols for 12 h. Cells were harvested and washed with ice-cold PBS, followed by staining with acridine orange, and nuclear staining was examined by fluorescence microscopy.

105.92, 129.60, 163.79, 164.88, 162.02, and 170.76 after 0, 0.5, 1, 2, 3, and 6 h of incubation with theasinensin A (25 μM), respectively. To investigate the induction of the sub-G1 cell population, the DNA content of U937 cells treated with theasinensin A for various periods was analyzed by flow cytometry. A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (Telford et al., 1992), was detected in cells that were treated with theasinensin A (25 μM), washed, and stained with propidium iodide. As shown in Figure 4B, the percentages of apoptotic U937 cells (left column) were 3.03, 6.06, 15.34, 21.87, and 52.82% after 0, 1, 3, 6, and 12 h of incubation with theasinensin A (25 μM), respectively. The percentages of apoptotic U937 cells (right column) were 2.81, 2.97, 2.82, 2.50, and 2.98% after 0, 1, 3, 6, and 12 h without theasinensin A (control, 0.05% DMSO), respectively. The peak of apoptosis did not appear until after 3 h of incubation, and this timing is consistent with the appearance of the DNA ladder (Figure 3C).

Activation of Caspase-3 Activity during Theasinensin A Induced Apoptosis. We then asked whether caspases were involved in the cell death response induced by theasinensin A. Caspases are activated in a sequential cascade of cleavages from their inactive forms (Enari et al., 1996). Once activated, caspases can subsequently cleave their substrate at a specific site. 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 theasinensin A induced apoptosis, we used two fluorogenic peptide substrates. Ac-DEVD-MCA is a specific substrate for caspase-3, and Ac-YVAD-MCA detects caspase-1 activity. As illustrated in Figure 5A, theasinensin A (25 μM) induced a dramatic increase in DEVD-specific caspase activity in treated U937 cells. The induction of DEVD-specific activity was rapid. In contrast to the increase in DEVD-specific activity, negligible YVAD-specific activity was observed. As described previously, the cell death response induced by tea polyphenols was strongly dose dependent; we examined the dose dependence of caspase activation as well. U937 cells were treated with different concentrations of theasinensin A for 12 h. Cytosolic proteins were extracted and assayed for caspase activity by incubation with a fluorogenic substrate, DEVD-MCA or YVAD-MCA. The induction of DEVD-specific activity followed the dose-dependent pattern of cell death and was maximal at 25 μM (Figure 5B). Higher theasinensin A concentrations yielded apparently diminished DEVD-specific activities, possibly as a result of loss of cytoplasm by acute necrotic cell lysate. Again, negligible YVAD-specific activity was detectable in these samples. We further studied the activation of caspase-3 proteases by other tea polyphenols. Caspase activity was measured following treatment of cells with different tea polyphenols (at 25 μM) or 0.05% DMSO (control). As shown in Figure 5C, TF-1, TF-2, and theasinensin A induced more DEVD-specific activity than TF-3 and EGCG. Thus, the induction of caspase-3 activity is a specific biochemical event brought about by apoptosis-inducing tea polyphenols.

Treatment with TF-1, TF-2, TF-3, EGCG, and Theasinensin A Causes Degradation of Poly(ADP-ribose) Polymerase, an Endogenous Substrate of Caspase-3. Activation of caspase-3 leads to the cleavage

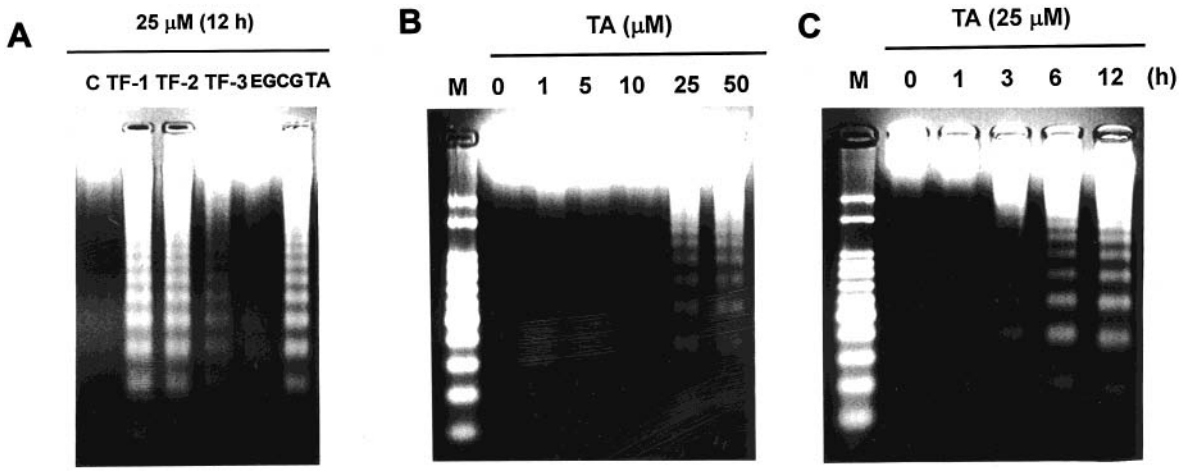

Figure 3. Induction of DNA fragmentation by various tea polyphenols in U937 cells. (A) Cells were treated with various polyphenols (25 μM each) for 12 h, and DNA fragmentation was analyzed by electrophoresis in 1.8% agarose gel. (B) For genome digestion, cells were treated with different concentrations of theasinensin A for 12 h. (C) Cells were treated with 25 μM theasinensin A for indicated time periods. Cellular DNA was extracted and analyzed by agarose electrophoresis. M, marker. Data shown are representative of three independent experiments.

of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis (Tewari et al., 1995). Treatment of U937 cells with 25 μM theasinensin A caused a time-dependent proteolytic cleavage of PARP, with accumulation of M_r 85000 and the concomitant disappearance of the full-size M_r 116000 molecule (Figure 5D). PARP cleavage was apparent within 3 h of theasinensin A treatment, roughly following the appearance of caspase activity (Figure 5A) and preceding DNA fragmentation (Figure 3A). Treatment with other apoptosis-inducing tea polyphenols or 0.05% DMSO as control, TF-1, and TF-2 at 25 μM also caused PARP degradation (Figure 5E). In contrast, PARP cleavage was not apparent in control. Again, TF-3 and EGCG had little effect.

A Caspase-3 Inhibitor, Z-DEVD-FMK, Abolishes Apoptosis Induced by Theasinensin A. The above result clearly indicates that caspase-3 protease is activated in response to the apoptosis induced by theasinensin A. To determine whether the activation of caspase-3 is required for the induction of cell death by theasinensin A, we pretreated U937 cells with caspase inhibitor. As shown in Figure 6, an inhibitor of caspase protease, Z-DEVD-FMK, was able to inhibit theasinensin A stimulated DEVD-specific activity and cell death in a dose-dependent manner. In contrast, Ac-YVAD-CHO, an inhibitor of caspase-1 activity, had little effect at 200 μM, consistent with the high substrate specificity of different caspases.

Treatment with Theasinensin A Caused the Loss of Mitochondrial Transmembrane Potential (Ψ_m) and ROS Generation. There is increasing evidence that altered mitochondrial function is linked to apoptosis and a decreasing mitochondrial transmembrane potential is associated with mitochondrial dysfunction. Thus, we next evaluated the effect of theasinensin A on the mitochondrial transmembrane potential (Ψ_m). We measured $\Delta\Psi_m$ using the fluorescent probe 3,3′-dihexyloxacarbocyanine [DiOC6(3)] and monitored it using flow cytometry. As shown in Figure 7A, a comparison of U937 cells exposed to theasinensin A with control cells, there was a sharp decline in DiOC6(3) fluorescence and the DiOC6(3) fluorescence intensity shifted to left. The intensities were 106.97, 80.0, 42.60, 37.28, 37.50, and 45.12 after 0, 0.5, 1, 2, 3, and 6 h of incubation with theasinensin A (25 μM), respectively.

Furthermore, we studied the loss of mitochondrial transmembrane potential resulting in the generation of ROS by assessing ROS generation using the fluorescent probe DCFH-DA and monitoring by flow cytometry. U937 cells were treated with 25 μM theasinensin A for different time periods, followed by the addition of 30 μM DCFH-DA into the medium for a further 0.5 h (Figure 7B). An increase of intracellular peroxide levels by 25 μM theasinensin A was detected for 0.5 h; after 3 h, the peroxide levels were reduced to the control level. These data indicated that the increment of ROS might play a role as an early mediator in theasinensin A induced apoptosis. These findings point to an effect of theasinensin A on mitochondrial function and accumulation of ROS. These features are cues for the induction of apoptosis.

Theasinensin A Induction of Cytochrome *c* Release and Caspase-9 Activation. The process of cell death may involve the release of cytochrome *c* from the mitochondria, which subsequently causes apoptosis by activation of the caspases. Together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* released from mitochondrial (Li et al., 1997). As shown in Figure 8A, the release of mitochondria cytochrome *c* into cytosol was detected at 1 h in theasinensin A treated U937 cells. Furthermore, we examined the activation of caspase-9 by fluorogenic peptide substrate: Ac-LEHD-AFC is a specific substrate for caspase-9. Theasinensin A induced a rapid activity to an ~4.5-fold increase after the addition of theasinensin A for 3 h (Figure 8B).

Taken together, these data are consistent with DNA fragmentation, caspase-3 activity, and loss of mitochondrial transmembrane potential. Therefore, these data suggest that mitochondrial dysfunction caused cytochrome *c* to be released into the cytosol and then activation of the cascade between caspase-9 and caspase-3 during theasinensin A inducing U937 cell apoptosis.

Treatment with Theasinensin A Caused Cleavage of DFF-45, an Inhibitor of Endonuclease. We further explored the possibility that activation of caspase-3 may also induce DFF-45 protein degradation. Treatment of U937 cells with 25 μM theasinensin A

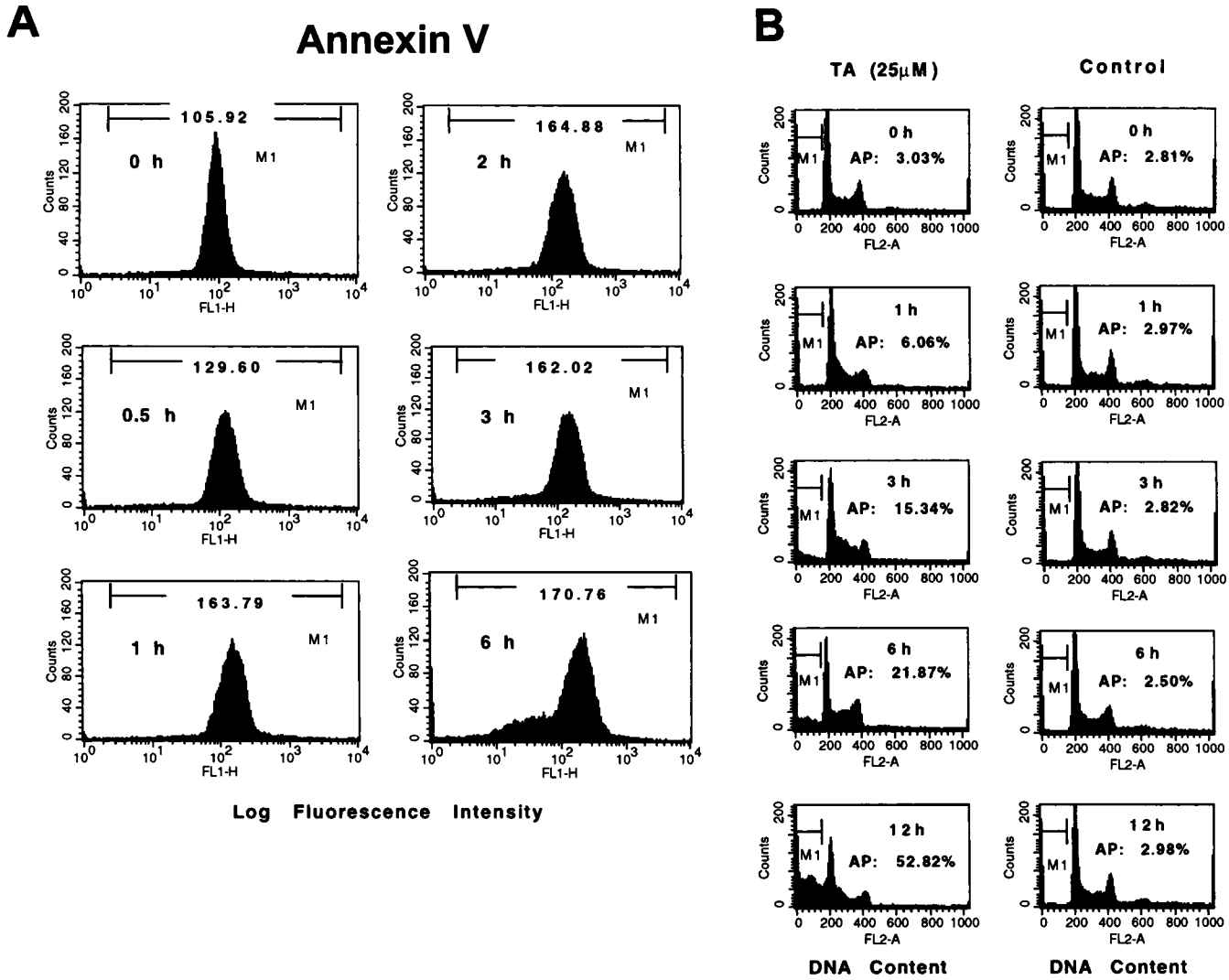

Figure 4. Determination of apoptosis in theasinensin A treated U937 cells by annexin V-FITC staining; the sub-G1 region was quantitated by flow cytometry. (A) U937 cells were treated with 25 μM theasinensin A or treated with 0.05% DMSO as vehicle control for 0, 0.5, 1, 3, and 6 h. The method of flow cytometry used is described under Materials and Methods. Data are presented as log fluorescence intensity. This is representative of three separate experiments. (B) U937 cells were treated with 25 μM theasinensin A or with 0.05% DMSO as vehicle control for 0, 1, 3, 6, and 12 h. The method of flow cytometry used is described under Materials and Methods. AP (apoptotic peak) represents apoptotic cells with a lower DNA content. The data presented are representative of three independent experiments.

caused proteolytic cleavage DFF-45 at 3 h (Figure 8C). The activation of caspase-3 was also observed 3 h later (Figure 5A) with a time course that paralleled the cleavage of DFF-45. As already described, ICAD is a mouse homologue of human DFF-45, caspase-3 cleaves DFF-45, and, once caspase-activated deoxyribonuclease is released, it can enter the nucleus, where it degrades chromosomal DNA (Liu et al., 1997; Sakahira et al., 1998).

DISCUSSION

A variety of stimuli can induce physiological cell death. Here, we have demonstrated that tea polyphenols also are able to induce apoptosis in two human cancer cell lines. Theasinensin A, TF-1, and TF-2 show strong growth inhibition against human cancer cell lines U937 and Jurkat. TF-3 and EGCG have lower inhibitory activities. Jurkat cells appear to be less susceptible to inhibition by these polyphenols.

Theasinensin A is one of the components of semifermented tea (oolong tea). On the basis of the estimated IC_{50} values for U937 cells, theasinensin A was a more potent inhibitor of cell growth and inducer of apoptosis than EGCG. This induction of apoptosis occurred within several hours, consistent with the view that tea polyphenols induce apoptosis by activating the pre-existing apoptosis machinery. Indeed, treatment with tea polyphenols caused an induction of caspase-3 activity and a degradation of PARP, which precedes the onset of apoptosis. Pretreating cells with the caspase-3 inhibitor Z-DEVD-FMK inhibited theasinensin A induced caspase-3 activation and DNA fragmentation, suggesting that apoptosis induced by theasinensin A involves a caspase-3 mediated mechanism. However, we were unable to detect any significant changes in the activity of caspase-1 during the tea polyphenol treatment, and a specific caspase-1 inhibitor, Ac-YVAD-CHO, had little effect on theasinensin A stimulated apoptosis and DNA fragmentation. These results suggest that apoptosis induced by tea polyphenols involves a caspase-3 mediated mechanism. Questions remain as to how caspase-3 is activated by tea polyphenols. This raises the possibility that factors or proteases other than caspase-1 are

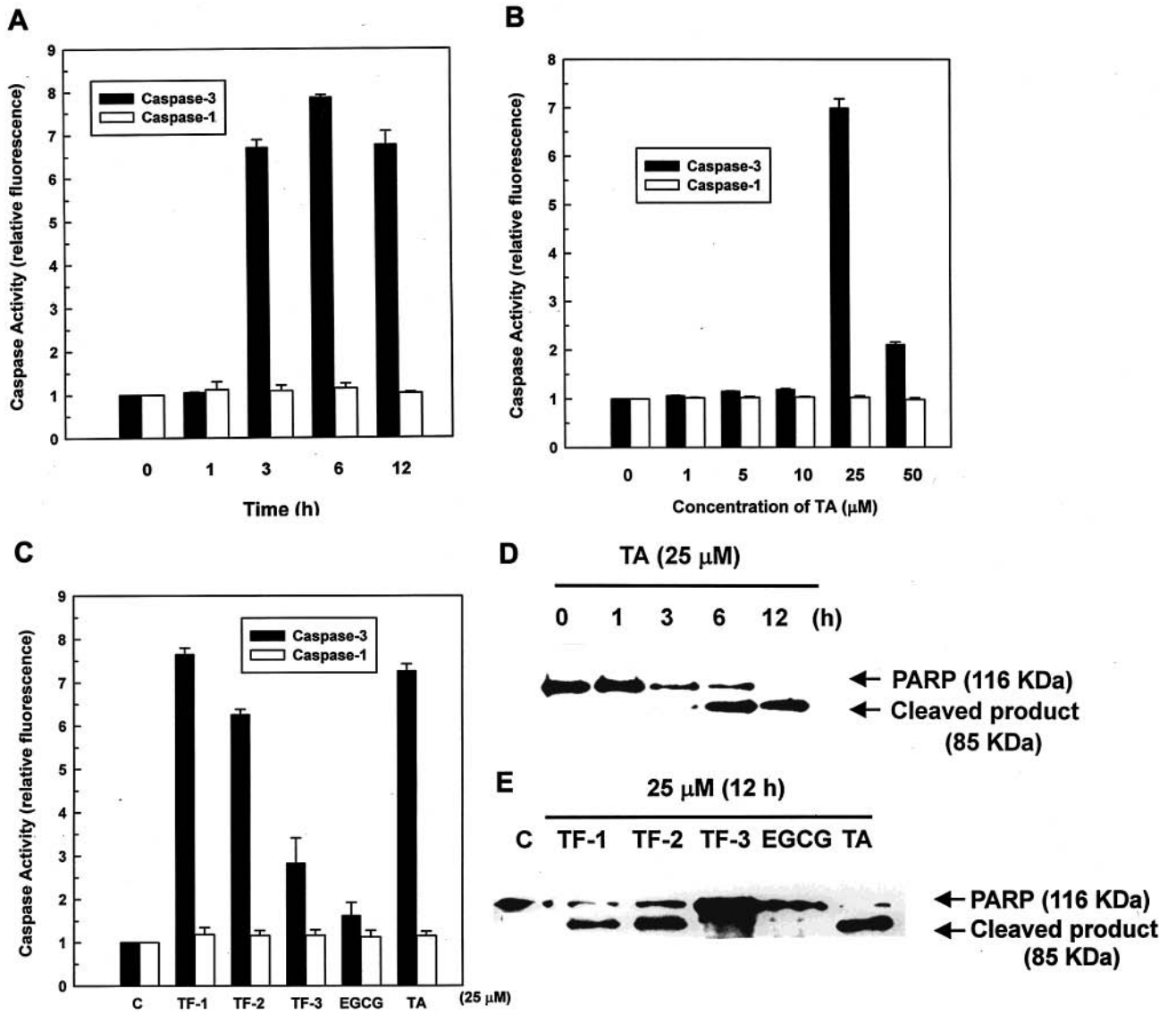

Figure 5. Involvement of caspase-3 activation in PARP degradation in theasinensin A induced apoptosis. (A) Kinetics of caspase-3 activation. U937 cells were treated with 25 μM theasinensin A for different time periods or treated with 0.05% DMSO as vehicle control. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 or caspase-1 was determined by incubation of 50 μg of total protein with fluorogenic substrate, Ac-DEVD-MCA or Ac-YVAD-MCA, respectively, for 1 h at 30 °C. The release of MCA was monitored spectrofluorometrically (excitation = 360 nm; emission = 460 nm). (B) Dose-dependent activation of caspase by theasinensin A. Following treatment with different concentrations of theasinensin A for 12 h, cells were harvested, and caspase activity was determined as described above. (C) Cells were treated with different tea polyphenols (at 25 μM) as indicated, and caspase activity was determined as described in (A). Data represent means ± SE for three determinations. (D) Kinetics of PARP cleavage by theasinensin A. U937 cells were treated as indicated and analysis by western blotting as described under Materials and Methods. (E) Cleavage of PARP by other tea polyphenols. After treatment of cells with 25 μM TF-1, TF-2, TF-3, EGCG, and theasinensin A for 12 h, PARP cleavage was analyzed by Western blotting. The experiment was repeated three times with similar results.

involved in the activation of caspase-3. 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 (Tewari et al., 1997). These data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* release from mitochondria. However, a correlation between onset of apoptosis and a loss of mitochondrial inner transmembrane potential ($\Delta\Psi_m$) was noted, and this may attributed to a mitochondrial permeability transition (PT) (Marchetti et al., 1996). Pharmacologic inhibitors of the PT were reported to inhibit apoptosis, thus supporting a role for this mitochondrial event. A simple model suggested that triggering the PT (which could occur as a consequence of ROS, ceramide, Ca^{2+}, or caspases) would lead to cytochrome *c* release, and this would thus link upstream signals to downstream caspase activation via the apoptosome.

It was recently shown that loss of phospholipid asymmetry of the plasma membrane and the $\Delta\Psi_m$ (mitochondrial transmembrane potential) disruption are early features 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 theasinensin A induced the loss of plasma membrane asymmetry and loss of mitochondrial transmembrane potential, the release of cytochrome *c*, activation of caspase-9, and

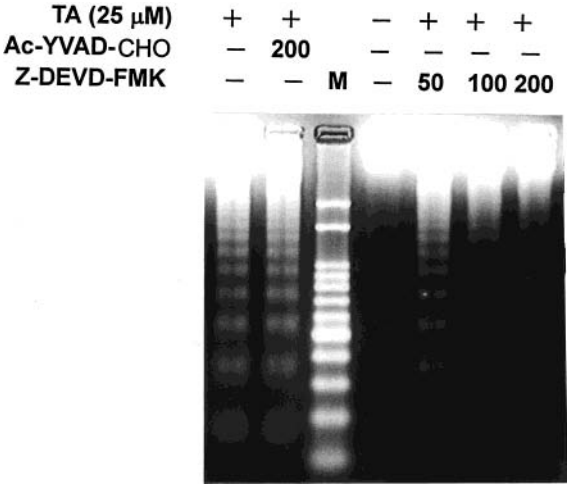

Figure 6. Suppression of theasinensin A induced DNA fragmentation by caspase-3 inhibitor. U937 cells were pretreated for 1 h with caspase-3 protease inhibitors (Ac-DEVD-FMK) or with caspase-1 protease inhibitor (Ac-YVAD-CHO) and then treated with 25 μM theasinensin A for 12 h, and agarose gel analysis of DNA fragmentation was performed. This experiment was repeated three times with similar results. M, DNA ladder marker.

promotion of ROS generation. The increase in ROS was probably due to the affected mitochondria cycling dioxygen through the electron transport assembly and generating ROS by one-electron transfer.

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 (Enari et al., 1998; Sakahira et al., 1998). In our study, we also found that apoptosis induced by theasinensin A caused the cleavage of DFF-45 and activated caspase-activated deoxyribonuclease. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of tea polyphenols.

Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may be triggered either 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 (Thompson, 1995). In human cancer, the most compelling links between apoptosis and treatment sensitivity occur in patients with leukemia or lymphoma. Thus, treatment with tea polyphenols might preferentially cause apoptosis in those abnormal cells, ultimately leading to the prevention of cancer. It should also be noted that theasinensin A (condensation products of two EGCG molecules) showed much higher activity than EGCG. However, theaflavin digallate, with the same number of phenolic hydroxyl groups as theasinensin A, has much less activity than theasinensin A. These data indicate that the three-dimensional

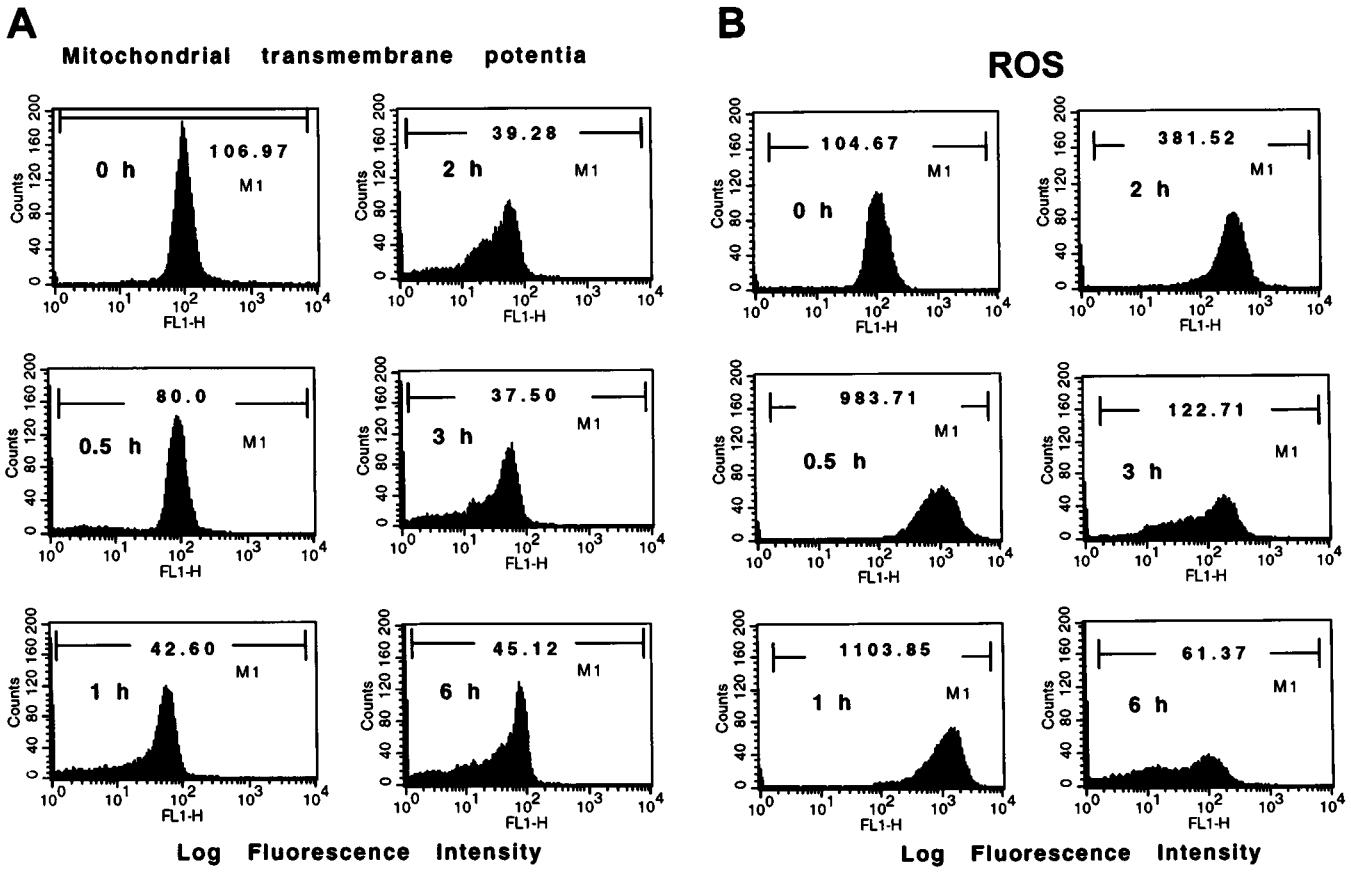

Figure 7. Induction of mitochondrial dysfunction and reactive oxygen species (ROS) generation in U937 cells by theasinensin A. (A) U937 cells were treated with 25 μM theasinensin A for different times (0.5, 1, 2, 3, and 6 h) and then incubated with 40 nM 3,3′-dihexyloxacarbocyanine and analyzed by flow cytometry. Data are presented as log fluorescence intensity. (B) U937 cells were treated with theasinensin A (25 μM) for different times (0.5, 1, 2, 3, and 6 h) and with DCFH-DA for a further 0.5 h, and the fluorescence in the cells was immediately assayed using flow cytometry. Data are presented as long green fluorescence intensity. This is representative of three separate experiments.

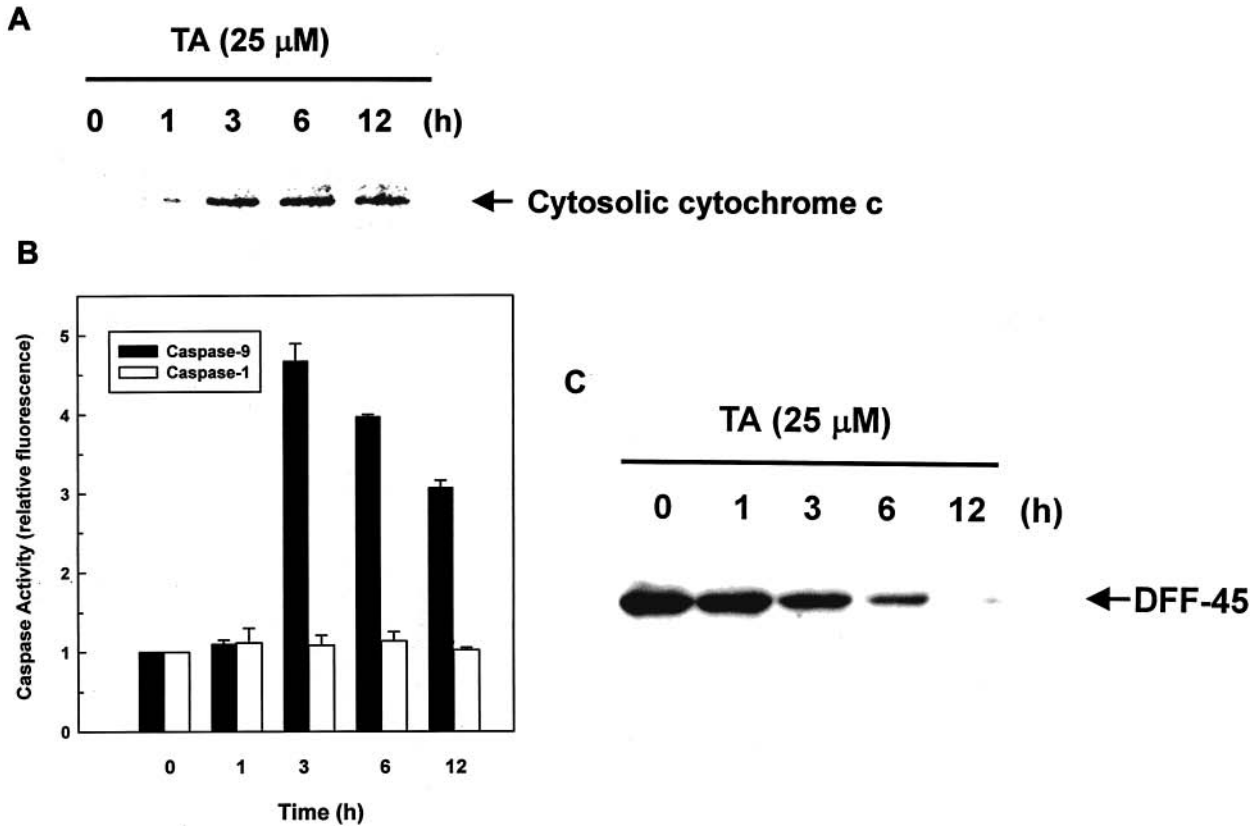

Figure 8. Induction of cytochrome *c* release, caspase-9 activation, and DFF-45 degradation in theasinensin A induced apoptosis. (A) Cytochrome *c* was released from mitochondria into the cytosol. U937 cells were treated with 25 μM theasinensin A, and cytochrome *c* was detected by cytochrome *c* antibody. (B) To determine the kinetics of caspase-9 activation, cells were treated with 25 μM theasinensin A 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-9 and caspase-1 proteases was determined by incubation of 50 μg of total protein with fluorogenic substrate, Ac-LEHD-AFC or Ac-YVAD-MCA, respectively, for 1 h for 30 °C. The release of AFC or MCA was monitored spectrofluorometrically (excitation = 400 nm, emission = 505 nm; or excitation = 360, emission = 460). Data represent means ± SE for three determinations. (C) The determine the kinetics of DFF-45 cleavage by theasinensin A, aliquots of 50 μg of U937 cell extracts prepared from cells treated with theasinensin A 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 then analyzed by Western blotting.

position of phenolic hydroxyl groups is a very important factor in determining the apoptosis-inducing activity. Furthermore, these catechin-related polyphenolic compounds may be useful as cancer chemopreventive and chemotherapeutic agents because many anticancer drugs are known to achieve their antitumor function by inducing apoptosis in the target cells.

In summary, we have demonstrated that the cancer-chemopreventive agents tea polyphenols theasinensin A, TF-1, and TF-2 are able to induce apoptosis in a dose-dependent manner; however, TF-3 and EGCG showed little apoptosis-inducing activity. A previous study (Yang et al., 1998) showed that tea polyphenol-induced production of H_2O_2 may mediate apoptosis and that this may contribute to the growth inhibitory activities of tea polyphenols in vitro. In our study, we also found that ROS were involved in theasinensin A induced apoptosis. These results first show that theasinensin A is able to induce the loss of plasma membrane asymmetry and mitochondrial transmembrane potential and the release of mitochondrial cytochrome *c* into the cytosol. It also induces procaspase-9 processing, activates caspase-3, produces the cleavage of PARP and DFF-45, and activates the endonuclease. Future studies focusing on cell signaling and the biological significance of theasinensin A induced apoptosis would greatly extend our understanding of the mechanisms of chemopreventive functions of oolong tea polyphenols.

ABBREVIATIONS USED

EGCG, (−)-epigallocatechin-3-gallate; TF-1, theaflavin; TF-2, mixture of theaflavin-3-gallate and theaflavin-3′-gallate; TF-3, theaflavin-3,3′-digalate; TA, theasinensin A; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species.

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