Induction of Apoptosis by Garcinol and Curcumin through Cytochrome *c* Release and Activation of Caspases in Human Leukemia HL-60 Cells

Min-Hsiung Pan,[†] Won-Ling Chang,[†] Shoei-Yn Lin-Shiau,[‡] Chi-Tang Ho,[§] and Jen-Kun Lin*,[†]

Institutes of Biochemistry and Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, and Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08901

Garcinol, a polyisoprenylated benzophenone, was purified from *Garcinia indica* fruit rind. The effects of garcinol and curcumin on cell viability in human leukemia HL-60 cells were investigated. Garcinol and curcumin displayed strong growth inhibitory effects against human leukemia HL-60 cells, with estimated IC_{50} values of 9.42 and 19.5 μM, respectively. Garcinol was able to induce apoptosis in a concentration- and time-dependent manner; however, curcumin was less effective. Treatment with garcinol caused induction of caspase-3/CPP32 activity in a dose- and time-dependent manner, but not caspase-1 activity, and induced the degradation of poly(ADP-ribose) polymerase (PARP). Pretreatment with caspase-3 inhibitor inhibited garcinol-induced DNA fragmentation. Treatment with garcinol (20 μM) caused a rapid loss of mitochondrial transmembrane potential, release of mitochondrial cytochrome *c* into cytosol, and subsequent induction of procaspase-9 processing. The cleavage of D4-GDI, an abundant hematopoietic cell GDP dissociation inhibitor for the Ras-related Rho family GTPases, occurred simultaneously with the activation of caspase-3 but preceded DNA fragmentation and the morphological changes associated with apoptotic cell death. Of these, Bcl-2, Bad, and Bax were studied. The level of expression of Bcl-2 slightly decreased, while the levels of Bad and Bax were dramatically increased in cells treated with garcinol. These results indicate that garcinol allows caspase-activated deoxyribonuclease to enter the nucleus and degrade chromosomal DNA and induces DFF-45 (DNA fragmentation factor) degradation. It is suggested that garcinol-induced apoptosis is triggered by the release of cytochrome *c* into the cytosol, procaspase-9 processing, activation of caspase-3 and caspase-2, degradation of PARP, and DNA fragmentation caused by the caspase-activated deoxyribonuclease through the digestion of DFF-45. The induction of apoptosis by garcinol may provide a pivotal mechanism for its cancer chemopreventive action.

Keywords: *Garcinol; curcumin; apoptosis; cytochrome c; caspase-9; caspase-2; caspase-3; poly-(ADP-ribose) polymerase; DNA fragmentation factor; caspase-activated deoxyribonuclease*

INTRODUCTION

During the past decade, a large number of natural products and dietary components have been evaluated as potential chemopreventive agents (*1*). Dietary factors play an important role in human health and in the development of certain chronic diseases including cancer (*2*, *3*). Some foods contain antitumor compounds as well as mutagens and/or carcinogens (*4*). Recent research has also focused on the presence of minor constituents or non-nutrients, which possess antimutagenic and anti-carcinogenic properties, in diets (*5*). Such compounds are candidates for chemopreventive agents against cancer development in humans.

Garcinol is a polyisoprenylated benzophenone derivative from *Garcinia indica* and other species (*6*−*9*). The dried rind of *G. indica* (cv. Kokum) is used as a garnish for curry and in traditional medicine in India. Garcinol is structurally similar to curcumin, which contains both a phenolic moiety and a β-diketone moiety. Although garcinol has been reported to possess antioxidative (*10*) and antibiotic activities (*9*) and suppressed colonic aberrant crypt foci (ACF) formation (*11*), its other biological activities are not well-known.

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa* L., gives the golden yellow color and unique flavor to curry. Recently, curcumin has been considered a potentially important chemopreventive agent against cancer (*12*). Animal studies have demonstrated that curcumin inhibits carcinogenesis in various tissues, including skin (*13*, *14*), colorectal (*15*, *16*), oral (*17*), forestomach (*16*, *18*), and mammary cancers (*19*, *20*). The inhibition of tumor formation by curcumin has been attributed to its anti-initiation (*16*, *21*) and anti-promotion (*14*, *16*) effects. The anti-initiation effect may result from its ability to inhibit the formation of DNA damage (*22*−*24*), whereas the antipromotion effect may be mediated through antiproliferation or apoptosis promotion of the initiated cells (*25*−*28*). In addition, curcumin inhibited the induction of iNOS in activated macrophages and the production of NO by mouse peritoneal macrophages (*29*−*31*). It has been shown that

* Address correspondence to this author at the Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan [telephone (886)-2-2356-2213; fax (886)-2-2391-8944; e-mail jklin@ha.mc.ntu.edu.tw].

[†] Institute of Biochemistry, National Taiwan University.

[‡] Institute of Toxicology, National Taiwan University.

[§] Rutgers University.

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curcumin strongly inhibits both c-Jun and NFκB activation by phorbol 12-myristate 13-acetate (PMA) or tumor necrosis factor α (TNF-α) treatment (*32*, *33*). This inhibitory effect may account for the anti-inflammatory and antioxidant properties of curcumin (*34*, *35*). Previously, we have found that curcumin selectively kills immortalized and transformed cells by apoptosis (*36*).

Apoptosis, a morphologically distinct form of programmed cell death, is an evolutionary highly conserved phenomenon that plays an important role in the regulation of cellular activities in eukaryotes (*37*). Various stimuli, such as cytokines and anticancer drugs, as well as growth factor deprivation and radiation damage, cause a cell to undergo a rapid, inflammatory-free clearance that is characterized by cell shrinkage, blebbing of plasma membranes, nuclear condensation, and DNA fragmentation (*38*−*40*). Apoptosis consists of two stages: the stage of the commitment, when the target cell is being sentenced to death; and the stage of execution, when proteins, DNA, and other structural components of the cell are destroyed. Death signals originating from the death receptors, such as TNF and Fas, or mitochondria trigger the activation of caspase-8 or caspase-9, respectively. These activated initiator caspases, in turn, activate the downstream executioner caspases-3, -6, and -7 (*41*, *42*). The cross-talk between the two pathways is mediated by BID, which upon cleavage by caspase-8, can activate the mitochondrial pathway of cell death (*43*, *44*). To date, at least 10 distinct caspases in mammalian cells have been identified (*45*). Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular proteins, which are characteristically proteolyzed during apoptosis. For example, caspases-2, -3, -7, and -9 can cleave poly(ADP-ribose) polymerase (PARP) (*39*), and caspase-6 can cleave nuclear lamins (*46*). The active caspase-4 cleaves the precursor form of caspase-1 to generate the mature form (*47*), and activated caspase-1 cleaves zymogen of caspase-3 to produce the active form (*48*). The cellular signaling pathways involved in controlling apoptosis remain poorly defined as well. In particular, little is known about the mechanisms underlying the dramatic cytoskeletal, morphological, and membrane changes that accompany cell death. Intensive studies have clarified the functions of small GTP-binding protein, a protein superfamily that consists of the Ras, Rho, Arf, Rab, and Ran families. It has been proposed that the Rho family of GTPase is involved in the regulation of integrin activity and in the organization of the actin cytoskeleton (*49*, *50*). The activities of the different Rho proteins are regulated by at least three types of regulator protein: GDP/GTP exchange protein, GTPase-activating proteins, and GDP dissociation inhibitors (GDIs) (*51*). The last comprise a family of proteins consisting of three members: Rho-GDI 1, D4-GDI (also named Rho-GDI 2 or Ly-GDI), and Rho-GDI 3. Rho-GDI 1 is expressed ubiquitously, whereas D4-GDI is expressed in hemopoietic tissues and Rho-GDI 3 in brain, lung, kidney, testis, and pancreas (*52*). Recently, several in vivo and in vitro studies have indicated that some potent chemopreventive agents, such as sulindac and other nonsteroidal anti-inflammatory drugs, induce apoptosis in colonic tumor, leading to the prevention of colon cancer (*53*, *54*).

In this study, we first explore the induction of apoptosis signaling pathway by garcinol in human leukemia cell line, HL-60. Our results clearly demonstrate that garcinol more strongly induced apoptosis than curcumin in a dose-dependent manner in HL-60 cells. Garcinol-induced apoptosis can activate caspase-2, caspase-3, and caspase-9, leading to the cleavage of PARP, D4-GDI, and DFF-45. These results suggest that garcinol induced apoptosis through a caspase-dependent mechanism, which may contribute to the chemopreventive functions.

MATERIALS AND METHODS

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 (10000 units of penicillin/mL and 10 mg/mL streptomycin). Medium was normally changed to phenol red-free RPMI 1640 before polyphenol treatment. 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). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO). Garcinol was isolated from *G. indica* dried fruit rind (*10*).

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 12 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, 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 garcinol or curcumin. 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 a 2-min adaptation in the dark.

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(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 promyelocytic leukemia HL-60 cells (5×10^5 cells/mL) after treatment with 20 μM garcinol for 0, 1, 3, 6, 9, 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% 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 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 PARP) (UBI, Inc., Lake Placid, NY), anti-bcl-2 antibody, anti-bad antibody, anti-bax antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-caspase-3 antibody and D4-GDI (Imgenex Corp., San Diego, CA), anti-caspase-9 antibody (Pharmingen, Becton Dickinson Co., San Diego, CA), and 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 Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD-AMC), Ac-Val-Asp-Val-Ala-Asp-AMC (Ac-VDVAD-AMC), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), Ac-Val-Glu-Ile-Asp-AMC (Ac-VEID-AMC), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) at 30 °C for 1 h. The release of methylcoumaryl-7-amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (Hitachi F-4500).

Analysis of Mitochondrial Transmembrane Potential. Change of mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, HL-60 cells were exposed to garcinol (20 μM) and curcumin (20 μM) for 1 h, and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR). Fluorescence was measured after staining of the cells for 15 min at 37 °C.

Flow Cytometry. HL-60 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).

RESULTS

Treatment with Garcinol and Curcumin Causes Dose-Dependent Reduction in Cell Survival. The structures of garcinol and curcumin are illustrated in Figure 1. We first tested the effect of garcinol and curcumin on cell viability. Human leukemia HL-60 cells were treated with different concentrations of garcinol and curcumin. After 12 h of treatment, a Luminescent ATP cell proliferation assay kit as described under Materials and Methods determined the live cells. As shown in Figure 2, garcinol appeared to be a more potent inhibitor of cell viability with an IC_{50} of 9.42 μM than curcumin (IC_{50} of 19.5 μM), and inhibition of the cell viability was found to be dose-dependent.

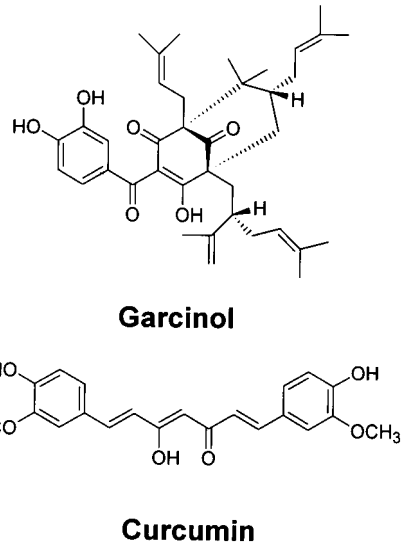

Figure 1. Chemical structures of garcinol and curcumin.

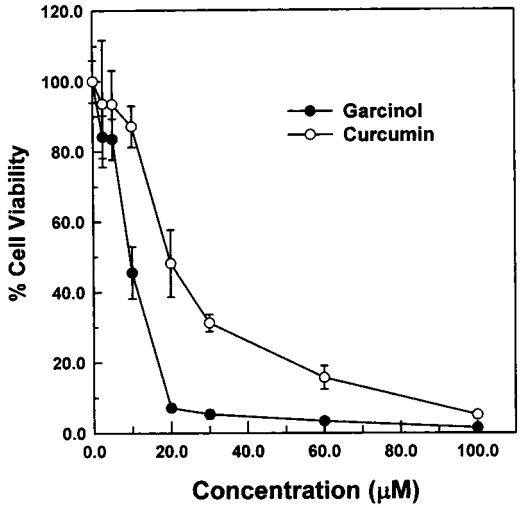

Figure 2. Effects of garcinol and curcumin on cell viability. HL-60 cells were treated with garcinol and curcumin for 12 h. HL-60 cells were either treated with 0.05% DMSO as vehicle control or treated with garcinol (or curcumin) of different concentrations for 12 h. Cell viability then was determined by Luminescent ATP detection assay kit, as described under Materials and Methods. Data were represented as means ± SE for three determinations.

Effect of Garcinol and Curcumin on DNA Fragmentation of HL-60 Cells. The induction of DNA fragmentation was demonstrated by incubating HL-60 cells with different concentrations of garcinol for 12 h (Figure 3A). DNA fragmentation became apparent at 10 μM garcinol treatment, and this DNA fragmentation response was dose-dependent (Figure 3A). In contrast, HL-60 cells showed only slightly induced DNA fragmentation by curcumin treatment. When cells were treated with 20 μM garcinol, DNA ladders were just visible 3 h after treatment, and increasing DNA fragmentation was observed from 3 to 12 h (Figure 3B). Within 3 h of treatment with 20 μM garcinol, cells clearly exhibited significant morphological changes and

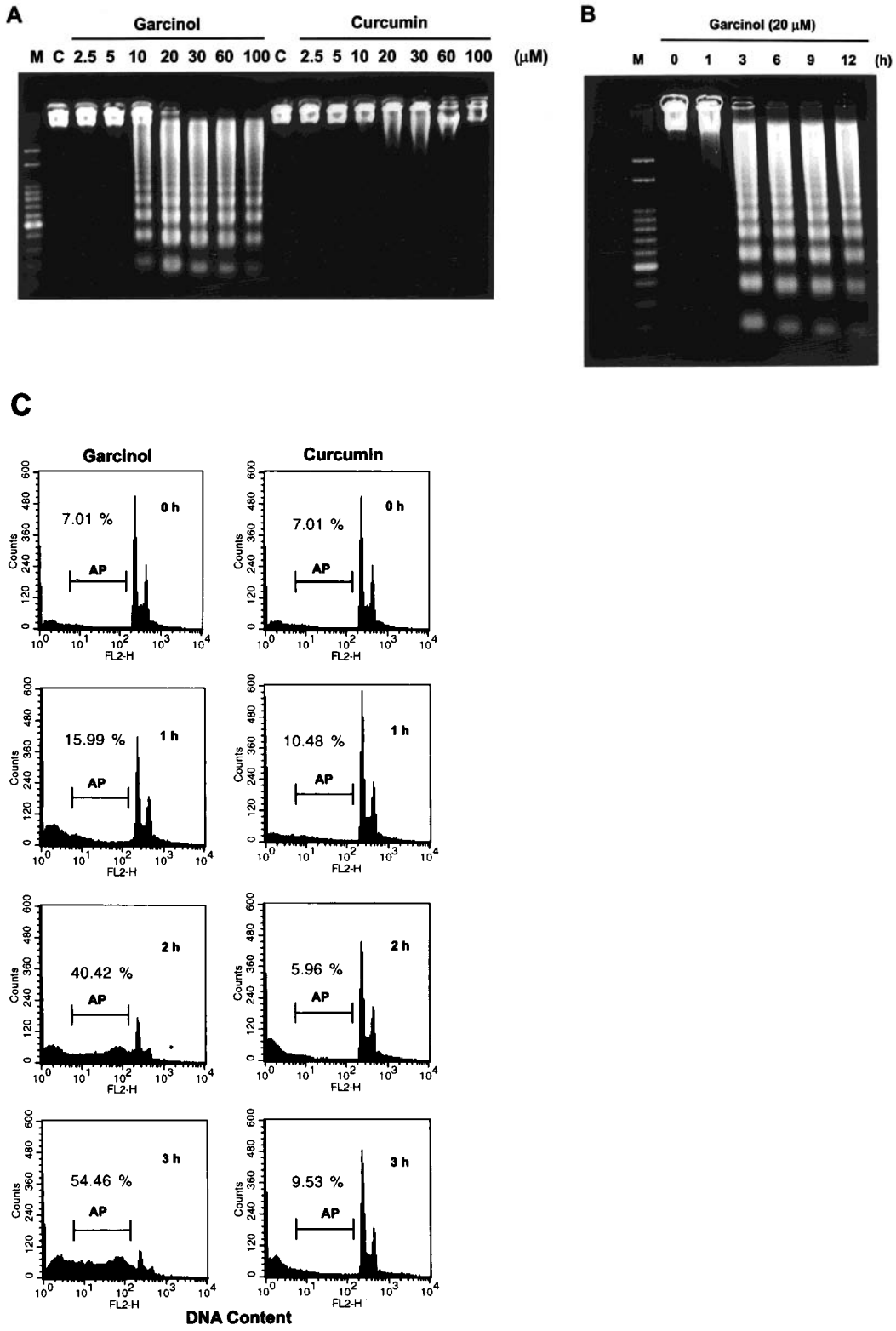

Figure 3. Induction of DNA fragmentation in HL-60 cells by garcinol or curcumin. (A) HL-60 cells treated with increasing doses of garcinol or curcumin for 12 h. (B) Time-dependent increasing doses of fragmentation ladders induced by 20 μM garcinol. Agarose gel analysis of DNA fragmentation was performed. M, DNA ladder marker; C, control. Data shown are representative of three independent experiments. (C) Determination of sub-G1 cells in garcinol- and curcumin-treated HL-60 cells by flow cytometry. HL-60 cells were treated with 20 μM garcinol (left column) or with 20 μM curcumin (right column) for 0, 1, 2, and 3 h. The method of flow cytometry used is described under Materials and Methods. AP (apoptotic peak) represents apoptotic cells with a lower DNA content.

chromosomal condensation, which is indicative of apoptosis cell death after acridine orange staining (data not shown). A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (*55*), was detected in cells that were treated with garcinol (20 μM), washed, and stained with propidium iodide. As shown in Figure 3C the percentages of apoptotic HL-60 cells (left column) were 7.01, 15.99, 40.42, and 54.46% after 0, 1, 2, and 3 h of incubation with garcinol (20 μM), respectively. The percentages of apoptotic HL-60 cells (right column) were

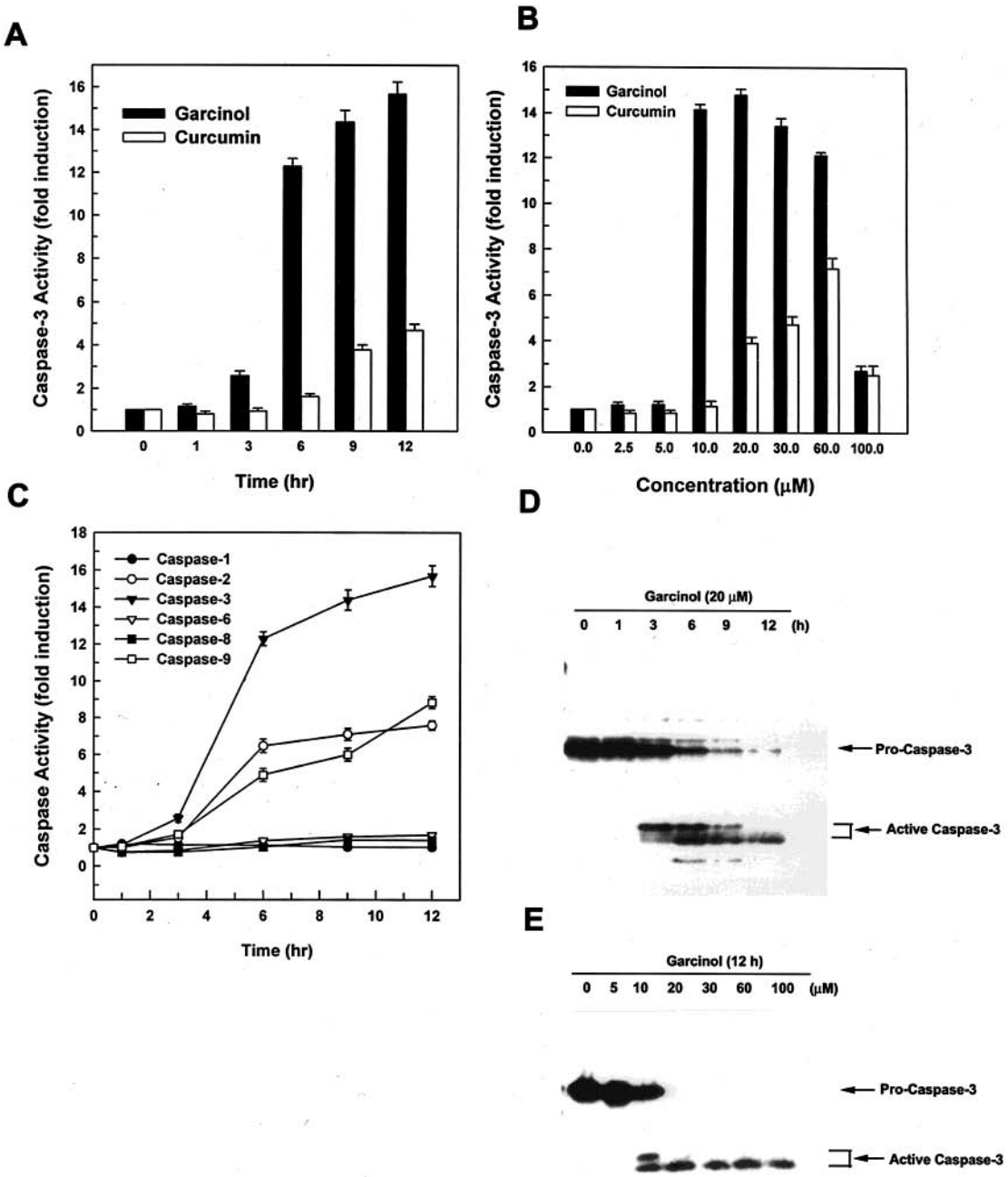

Figure 4. Induction of caspase activities. (A) Kinetics of caspase-3 activation. Cells were treated with 20 μM garcinol and curcumin, respectively, for different time periods or treated with 0.05% DMSO as vehicle control. (B) Dose-dependent activation of caspase-3 by garcinol and curcumin, respectively, following treatment with different concentrations of garcinol or curcumin for 12 h. (C) Kinetics of caspase activation. Cells were treated with 20 μM garcinol for different time periods. Caspase activities were analyzed as described under Materials and Methods. Data represent means ± SE for three determinations. (D) Western analyses of caspase-3 activation by garcinol for different time periods and (E) treatment with different concentrations of garcinol or curcumin for 12 h. Western blot data presented are representative of those obtained in at least three separate experiments.

7.01, 10.48, 5.96, and 9.53% after 0, 1, 2, and 3 h of incubation with curcumin (20 μM), respectively. The peak of apoptosis did not appear until after 2 h of incubation with garcinol. HL-60 cells exposed to 20 μM garcinol for 3 h resulted in 54.46% apoptosis, and this timing is consistent with the appearance of the DNA ladder (Figure 3C). The percentage of apoptotic HL-60 cells remained constant throughout the course of the experiments.

Garcinol Stimulated Caspase-3 Activity in a Time- and Dose-Dependent Manner. Caspases are believed to play a central role in mediating various apoptotic responses. To monitor the enzymatic activity of caspases during garcinol-induced apoptosis, we used a fluorogenic peptide substrate, Ac-DEVD-AMC, specific for caspase-3. Caspase activities were measured following treatment of HL-60 cells with different concentrations of garcinol and curcumin for 12 h. As shown in Figure 4A, the induction of caspase-3 activity by garcinol paralleled the dose-dependent pattern of apoptosis and was maximal at 20 μM. Higher garcinol concentrations yielded apparently diminished caspase-3 activity, possibly as a result of loss of cytoplasm by acute necrotic cell lysis. However, curcumin induced lower caspase-3 activity than garcinol. We further studied the time course of caspase-3 activation of garcinol and curcumin in HL-60 cells. Caspase-3 activation was determined by treatment with garcinol and curcumin (20 μM). Garcinol

induced a rapid rise in caspase-3 activity to approximately a 16-fold increase after the addition of garcinol for 12 h (Figure 4B). In contrast, curcumin induced a slow and slightly time-dependent caspase-3 activation.

We then asked whether caspases were involved in the cell death response induced by garcinol. Caspases are activated in a sequential cascade of cleavages from their inactive forms (*56*). Once activated, caspases can subsequently cleave their substrate at specific site. For example, caspase-3 cleaves preferentially after a DXXD↓X, whereas caspase-1 cleaves at YXXD↓X. We also monitored the activation of caspase-2, caspase-6, caspase-8, and caspase-9, respectively, during garcinol-induced apoptosis. As illustrated in Figure 4C, garcinol (20 μM) induced a dramatic increase in caspase-2 and caspase-9 activities in treated HL-60 cells. In contrast to the increase in DEVD-specific activity, negligible caspase-1, caspase-6, and caspase-8 activities were observed. Caspase-3 is time- (Figure 4D) and dose-dependently (Figure 4E) activated by garcinol and further shows the cleavage form of caspase-3 by Western blotting. Results presented in Figure 4D show that a detectable activation of caspase-3 (appearance of cleavage form) can first be seen 3 h after the addition of garcinol. As shown in Figure 4E, the induction of caspase-3 activity by garcinol for 12 h paralleled the dose-dependent pattern of apoptosis.

Caspase-3 Inhibitor, Z-DEVD-FMK, Abolishes Apoptosis Induced by Garcinol. The above results clearly indicate that caspase-3 protease is activated in response to the apoptosis induced by garcinol. To determine whether the activation of caspase-3 is required for the induction of cell death by garcinol, we pretreated HL-60 cells with caspase inhibitor. As shown in Figure 5, an inhibitor of caspase protease, Z-DEVD-FMK, was able to inhibit garcinol-stimulated DNA fragmentation (Figure 5A) and caspase-3 activity (Figure 5B). In contrast, Ac-YVAD-CMK, an inhibitor of caspase-1 activity, had little effect at similar concentrations (Figure 5A), consistent with the high substrate specificity of different caspases. Thus, these data suggest that the induction of caspase-3 activity is a specific biochemical event brought about by apoptosis-inducing garcinol.

Treatment with Garcinol and Curcumin Causes Degradation of PARP, an Endogenous Substrate of Caspase-3. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. The cleavage of PARP is the hallmark of apoptosis. PARP (116 kDa) is cleaved to produce an 85-kDa fragment during apoptosis (*48*). We examined the cleavage of PARP in garcinol- and curcumin-induced apoptosis. Treatment of HL-60 cells with 20 μM garcinol caused a time-dependent proteolytic cleavage of PARP, with accumulation of the 85-kDa species and concomitant disappearance of the full-size 116-kDa protein (Figure 6A). PARP cleavage was apparent within 3 h of garcinol treatment, roughly following the appearance of caspase activity (Figure 4A) and preceding DNA fragmentation (Figure 3B). In addition, we determined the cleavage of PARP with different concentrations of garcinol and curcumin for 12 h. Garcinol at 10 μM caused complete PARP degradation, but curcumin was less active than garcinol (Figure 6B,C).

Garcinol-Induced Cytochrome *c* Release and Caspase-9 Activation. 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 garcinol and curcumin on the mitochondrial transmembrane potential (Ψm). We measured $\Delta\Psi$m using the fluorescent probe DiOC6(3) and monitored it using flow cytometry. As shown in Figure 7A, HL-60 cells exposed to 20 μM garcinol for only 1 h showed a sharp decline in DiOC6-(3) fluorescence (mean = 41.48; Ma). In contrast, the mean of DiOC6(3) fluorescence intensity of cells exposed to 20 μM curcumin was 96.31 (Mb). Here, the fluorescence intensity of control cell was 100.01 (Mc).

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 (*57*). Treatment of HL-60 cells was performed with different concentrations of garcinol for 3 h. As shown in Figure 7B, the release of cytochrome *c* into the cytosol

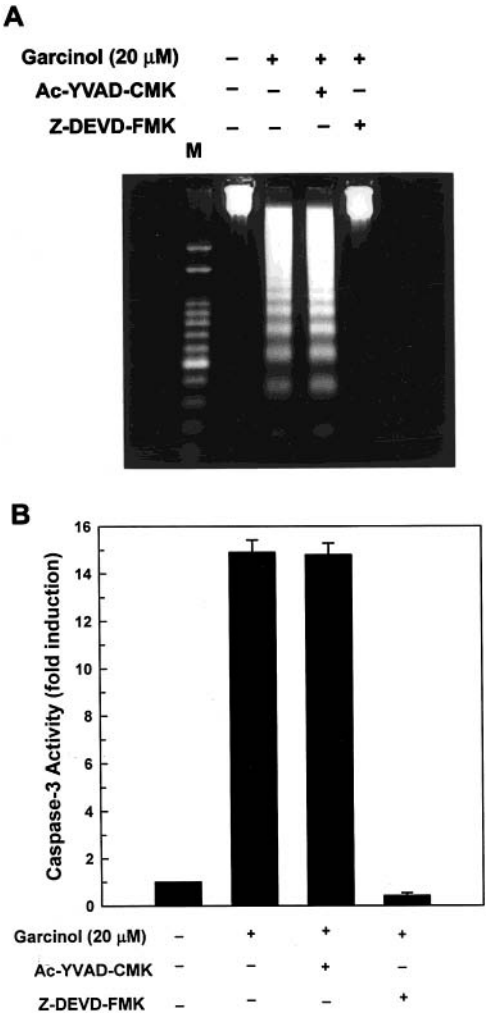

Figure 5. Effects of caspase-3 inhibitors on DNA fragmentation and caspase-3 activity induced by garcinols. (A) Suppression of garcinol-induced DNA fragmentation by caspase-3 inhibitors. M, DNA ladder marker. (B) Inhibition of garcinol-induced caspase-3 activity by caspase-3 inhibitor. HL-60 cells were pretreated for 1 h with caspase inhibitors (Z-DEVD-FMK or Ac-YVAD-CMK) prior to the addition of 20 μM garcinol for 12 h. The caspase-3 activity was detected as decribed under Materials and Methods. Columns represent means for three independent experiments.

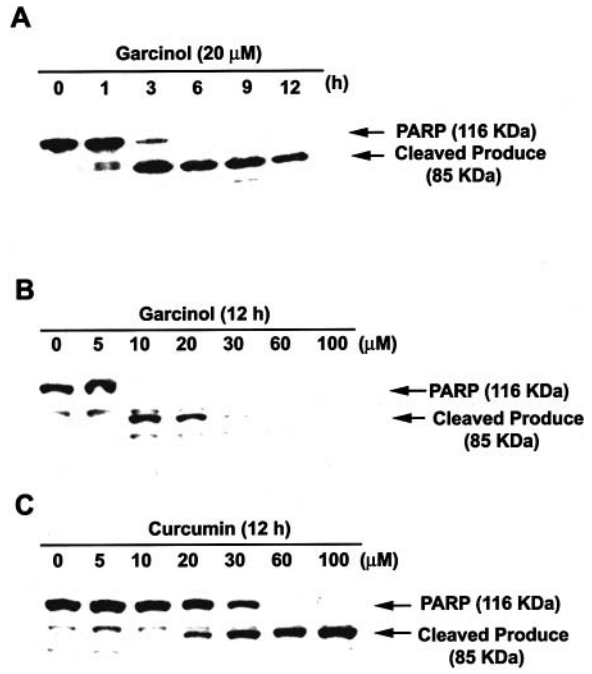

Figure 6. Cleavage of PARP during garcinol- and curcumin-induced apoptosis, respectively. (A) Time-dependent and (B) dose-dependent increases in the cleavage of PARP by garcinol. (C) HL-60 cells treated with increasing doses of curcumin for 12 h. HL-60 cells were treated as indicated and analysis by western blotting as described under Materials and Methods. This experiment was repeated three times with similar results.

by garcinol was highly significant and dose-dependent. In contrast, curcumin had little effect at similar concentrations. Furthermore, we examined the activity and cleavage of caspase-9 during treatment of cells with different concentrations of garcinol and curcumin for 3 h, respectively (Figure 7C). A dose-dependent proteolytic cleavage of procaspase-9, with an increase of the cleaved fragment, was associated with the activity of caspase-9. Again, curcumin had little effect (Figure 7D). These data are consistent with DNA fragmentation, caspase-3 activity cleavage of PARP, and loss of mitochondrial transmembrane potential. Therefore, these results suggest that mitochondrial dysfunction caused cytochrome *c* to be released into the cytosol and then the activation of the cascade between caspase-9, caspase-2, and caspase-3.

Treatment with Garcinol 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 HL-60 cells with 20 μM garcinol caused proteolytic cleavage DFF-45 at 3 h (Figure 8A). The activation of caspase-3 was also observed 3 h later (Figure 4A) with a time course that paralleled with the cleavage of DFF-45. DFF-45 cleavage was apparent within 3 h of garcinol treatment, roughly following the appearance of caspase activity and preceding DNA fragmentation. With apoptosis, nearly all of the DFF-45 was degraded at 6 h. In contrast, curcumin had little effect at similar concentrations (Figure 8B). 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 (*58, 59*).

D4-GDI, Bcl-2, Bad, and Bax Expression during Garcinol Treatment. Several gene products are known to be important in controlling the apoptotic process. To determine if garcinol has an effect on the expression levels of these gene products, a similar time course experiment was performed and cellular lysates were analyzed by Western blotting. These Western blots were probed with antibodies against the 23-kDa fragment of D4-GDI, Bcl-2, Bad, and Bax proteins (Figure 9). When HL-60 cells were exposed to 20 μM garcinol, the cleavage product of D4-GDI began to increase after 1 h of incubation, reaching a maximum level between 3 and 6 h (Figure 9A). The expression levels of Bcl-2 slightly changed after 1 h of garcinol exposure and remained slightly decreased after 6 h of exposure (Figure 9B). In contrast, the Bad protein began to increase after 6 h of incubation (Figure 9C). The expression levels of Bax protein began to increase after 1 h of incubation, reached a maximum level between 3 and 6 h, and thereafter decreased (Figure 9D).

DISCUSSION

Although the yellow pigment garcinol has antioxidant and free radical scavenging effects (*10*) and antibacterial/antifungal activities (*9*), its other biological activities are not well-known. The present results demonstrate for the first time that garcinol can strongly induce apoptosis in human leukemia cells. Curcumin, being a hydrophobic molecule (*60*), passes easily through the plasma membrane into the cytosol. Recently curcumin was shown to cause phosphatidylserine exposure, increased plasma membrane permeability, decreased mitochondrial membrane permeability, and decreased mitochondrial membrane potential and cell shrinkage (*61*), which are typical features of apoptotic cells (*62*). Curcumin is an interesting molecule because of the variety of biological effects it possesses in addition to its potent anticancer activity. However, its exact mechanism of action is not very clear. We have recently shown the inhibition of IκB kinases (IKKs) by curcumin (*63*). During our present comparison studies, we found that garcinol was more effective than curcumin in inhibiting in cell viability and inducing DNA fragmentation.

This induction of apoptosis occurred within several hours, consistent with the view that garcinol induces apoptosis by activating the pre-existing apoptosis machinery. Indeed, treatment with garcinol caused an induction of caspase-3 activity and degradation of PARP, which precedes the onset of apoptosis. Pretreatment with the caspase-3 inhibitor, Z-DEVD-FMK, inhibited garcinol-induced caspase-3 activation and DNA fragmentation, suggesting that apoptosis induced by garcinol involves a caspase-3-mediated mechanism. The results also indicate that lower caspase-3 activity was found during curcumin treatment than during garcinol treatment. However, we were unable to detect any significant changes in the activity of caspase-1 during garcinol treatment, and a specific caspase-1 inhibitor, Ac-YVAD-CHO, had little effect on garcinol-stimulated apoptosis and DNA fragmentation. This raises the possibility that factors or proteases other than caspase-1 are involved in the activation of caspase-3. Caspase-3 is activated by two sequential proteolytic events that cleave the a 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits (*64*). 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

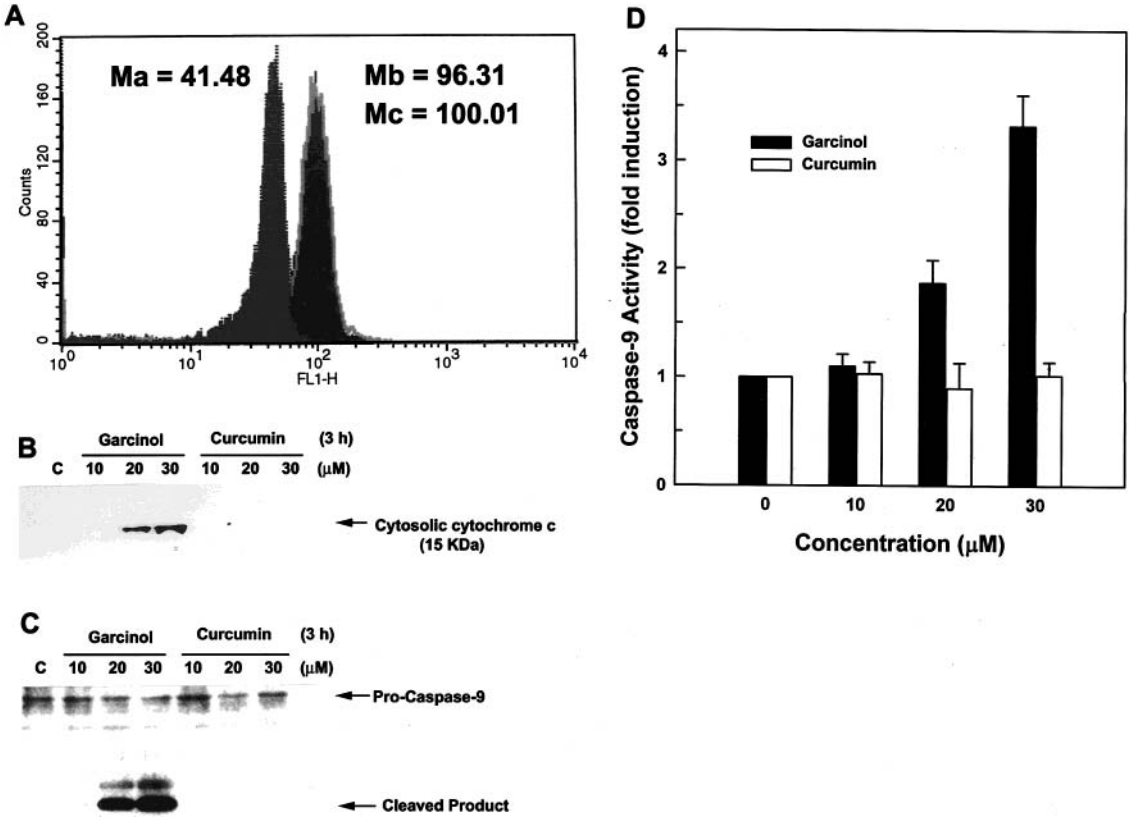

Figure 7. Induction of mitochondrial dysfunction and the release of cytochrome *c* from mitochondria into the cytosol following caspase-9 activation in garcinol-induced apoptosis. (A) HL-60 cells were treated with 20 μM garcinol and curcumin for 1 h and were then incubated with 40 nM DiOC6(3) and analyzed by flow cytometry, respectively. The percentages reflect the reduction of $\Delta\psi$m DiOC6(3). Ma, garcinol; Mb, curcumin; Mc, control. (B) Cytochrome *c* release from mitochondria into cytosol. HL-60 cells were treated with 20 μM garcinol and curcumin, and cytochrome *c* was detected by cytochrome *c* antibody, respectively. (C) Capsase-9 processing was induced by garcinol and curcumin, respectively. (D) Caspase-9 activity was induced by garcinol and curcumin for 3 h, respectively, and detected as described under Materials and Methods. This experiment was repeated three times with similar results.

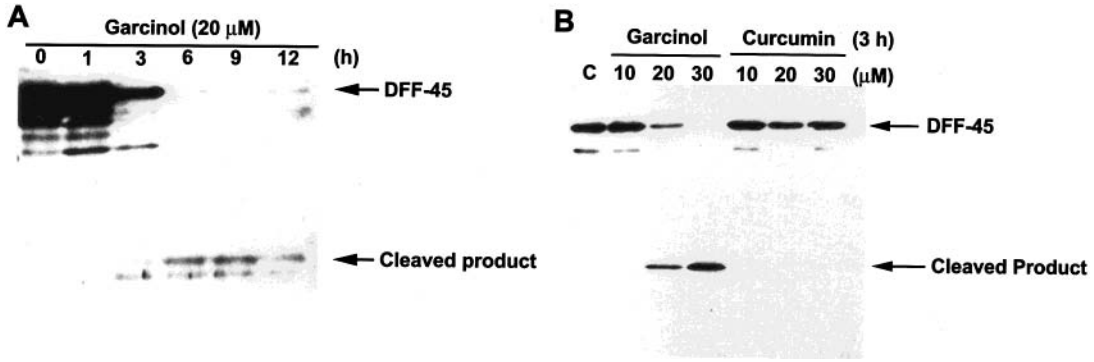

Figure 8. Cleavage of DFF-45 during garcinol-induced apoptosis. (A) Kinetics of DFF-45 cleavage by garcinol. (B) HL-60 cells treated with increasing doses of garcinol and curcumin for 3 h, respectively. This experiment was repeated three times with similar results.

fractions resulted in the failure of caspase-3 activation (*57*). Taken together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* release from 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 (*65*). 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 (*66*). In this study, we found that garcinol strongly induced the loss of mitochondrial transmembrane potential, the release of cytochrome *c*, and the cleavage of caspase-9. However, the induction abilities of curcumin were lower.

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 (*58*, *67*, *59*). In our study, we also found that apoptosis induced by garcinol caused the cleavage of DFF-45 and activated caspase-activated deoxyribonuclease.

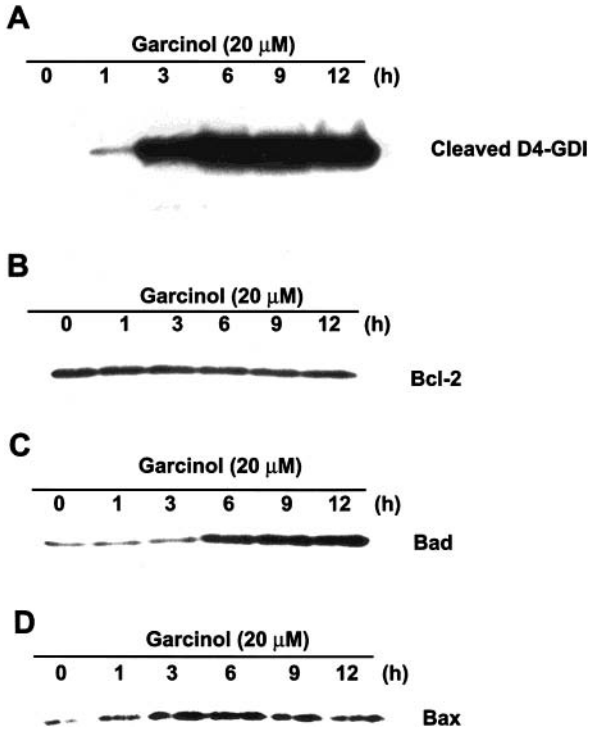

Figure 9. Immunoblot analysis of the cleavage of D4-GDI and the expression of Bcl-2, Bad, and Bax in garcinol-treated HL-60 cells. Cells from each time point following 20 μM garcinol treatment were analyzed. (A) Cleavage of D4-GDI. (B) Expression of Bcl-2, (C) Bad, and (D) Bax, respectively. This experiment was repeated three times with similar results.

The apoptosis-inducing potency of garcinol seems to be correlated to its cancer chemopreventive efficacy in animal models (*11*). For example, dietary administration of garcinol significantly inhibited AOM-induced ACF formation in male F344 rats without causing any adverse effects. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of garcinol. 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 (*68*, *69*). Thus, treatment with garcinol may preferentially cause apoptosis in those abnormal cells, ultimately leading to the prevention of cancer. Furthermore, the ability of garcinol alone to induce apoptosis suggests its potential use as a chemotherapeutic agent because many anticancer drugs are known to achieve their antitumor function by inducing apoptosis in the target cells (*70*).

D4-GDI, a hematopoietic cell-abundant regulator of the Rho family GTPases (*71*), is a substrate for caspase-3 and is cleaved during garcinol-induced apoptosis. Owing to its crucial role as a modulator of Rho GTPase, this might in turn have a significant impact on the mechanisms that induce the cytoskeletal and morphological changes in apoptotic cells. An important area of future research is identification of the genes that are involved in the apoptotic program of cell death. In fact, the finding that cell death occurs at a certain time and at certain locations during precise stages of normal development or metamorphosis implies that there are genes responsible for the occurrence of cell death. The best example of a cell death-associated gene is probably the bcl-2 gene (*72*). In our study, we found that the expression of the Bcl-2 showed slight change. However, we found the amount of Bax and Bad protein increased under the same conditions. The ratio between Bcl-2 and Bax determines cell survival or death. The results presented herein might account for oncogenes that can facilitate cytochrome *c* release independently of p53, although it is worth noting that p53 can induce Bax and proteins that affect mitochondrial function (*73*).

We previously reported that curcumin is unstable at neutral and basic pH values and is degraded to ferulic acid (4-hydroxy-3-methoxycinnamic acid) and feruloylmethane (4-hydroxy-3-methoxycinnamoylmethane) (*74*). In the course of our investigations, we found that >90% of curcumin decomposes rapidly in buffer systems at neutral basic pH conditions. Moreover, tetrahydrocurcumin, one of the major metabolites of curcumin, was more stable than curcumin in 0.1 M phosphate buffer (*75*). We suggested that curcumin was a weaker inductor of apoptosis than garcinol.

In summary, we have demonstrated that the cancer-chemopreventive agent garcinol is able to induce apoptosis in a dose-dependent manner. These results show that garcinol 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 PARP and DFF-45 and activation of endonuclease. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of garcinol.

ABBREVIATIONS USED

DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; Z-DEVD-FMK, Z-Asp-Glu-Val-Asp-fluoromethyl ketone; GDI, GDP dissociation inhibitor.

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