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# Molecular Mechanisms of Apoptosis Induced by Magnolol in Colon and Liver Cancer Cells

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Magnolol has been reported to have anticancer activity. In this study we found that treatment with 100 μm magnolol induced apoptosis in cultured human hepatoma (Hep G2) and colon cancer (COLO 205) cell lines but not in human untransformed gingival fibroblasts and human umbilical vein endothelial cells. Our investigation of apoptosis in Hep G2 cells showed a sequence of associated intracellular events that included (a) increased cytosolic free $Ca^{2+}$; (b) increased translocation of cytochrome *c* (Cyto *c*) from mitochondria to cytosol; (c) activation of caspase 3, caspase 8, and caspase 9; and (d) downregulation of bcl-2 protein. Pretreatment of the cells with the phospholipase C inhibitor 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1 *H*-pyrrole-2,5-dione (U73122) or the intracellular chelator of $Ca^{2+}$ 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA/AM) inhibited the subsequent magnolol augmentation of $[Ca^{2+}]_i$ and also the activation of caspase-8 and caspase-9, so that the occurrence of apoptosis in those cells was greatly reduced. Pretreatment of the cells with ZB4 (which disrupts the Fas response mechanism) also decreased the subsequent magnolol-induced caspase-8 activation and reduced the occurrence of apoptosis. We interpreted these findings to indicate that the above-listed sequence of intracellular events led to the apoptosis seen in Hep G2 cells and that $[Ca^{2+}]_i$, Cyto *c*, and Fas function as intracellular signals to coordinate those events. 

Key words: caspases; cytochrome *c*; bcl-2; calcium; Fas

## INTRODUCTION

As stated in the recent review by Akriviadis et al. [1], liver and colorectal cancers remain prevalent, deadly, and increasingly costly to patients and to society. Since there are many complex causative factors in these cancers, the ongoing search for effective therapies most often is based on new discoveries about the underlying cellular mechanisms. The focus most recently has been on the mechanisms concerned with cell proliferation (mitosis) and the opposing mechanisms leading to programmed cell death (apoptosis). Concurrently, studies also are aimed at discovering pharmaceutical agents that might interfere with these cellular mechanisms in cancerous cells so as to block the growth of tumors and thereby find use as therapeutic agents.

One such pharmaceutical agent is magnolol, a phenolic compound isolated in pure form from the bark of the tree *Magnolia officinalis*. Magnolol, also known as *Hou p'u* among Chinese herbalists, has been found to be effective in causing the regression of (or blocking of the formation of) skin papilloma tumors in mice treated with the carcinogens 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate [2]. Since so little else is known about the details of magnolol action and the cellular mechanisms involved, we chose to examine this matter in cultures of human cancer cells.

Previously, Wang and Chen [3] reported that magnolol, applied in vitro to rat neutrophil cells, stimulates an increase in intracellular cytosolic free $Ca^{2+}$ in a dose-dependent manner. They observed that this $Ca^{2+}$ derives both from intracellular stores and from increased $Ca^{2+}$ influx across the cell plasma membrane. Elevated cytosolic free $Ca^{2+}$ generally leads to disruption of mitochondrial membrane function, mitochondrial swelling, and the translocation of Cyto *c* (cytochrome *c*) out to the cytoplasm [4–6]. Thereafter, the Cyto *C* evidently

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Abbreviations: HUVEC, human umbilical vein endothelial cells; Cyto *c*, cytochrome *c*; MEM, Eagle's minimal essential medium; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; G3PDH, glycerol-3-phosphate dehydrogenase; PARP, poly(ADP-ribose)polymerase; *p*NA, *p*-nitroaniline; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1 *H*-pyrrole-2,5-dione; S.E., standard error; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester.

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leads to the appearance of caspase-activating proteins, the activation of caspase activity, and eventually the type of cell death known as apoptosis [7,8].

In the present study, we found that 100 μM magnolol, added to cultures of human hepatoma (Hep G2), induced sharp elevations in cytosolic-free $Ca^{2+}$, the activation of Fas-mediated pathways, and the translocation of Cyto *c* from mitochondria to cytoplasm. Thereafter, widespread apoptosis occurred, with marked reduction in cell proliferation. In Hep G2 cells, we noted that apoptosis was associated with activated caspase activity, while the anti-apoptosis protein bcl-2 was downregulated. For comparison, human untransformed cells (gingival fibroblasts and human umbilical vein endothelial cells (HUVEC)) were incubated in culture and treated with 100 μM magnolol; in these noncancerous cells, apoptosis and the associated disruption of intracellular organization did not occur. The details of these experiments are described below and shed new light on the molecular mechanisms involved in magnolol-induced apoptosis.

## MATERIALS AND METHODS

### Cell Culture

Three human malignant cell lines (COLO 205, Hep G2, and HT 29) and two types of human primary cells (gingival fibroblasts and HUVEC) were used in this study. COLO 205 (CCL-222; American Type Culture Collection, Rockville, MD) originated from a poorly differentiated human colon adenocarcinoma. Hep G2 (HB-8065; American Type Culture Collection) was derived from a human hepatoma. HT 29 (HTB-38; American Type Culture Collection) originated from a human colon adenocarcinoma. Human gingival fibroblasts were harvested by enzymatic dissociation. HUVEC were harvested from the human umbilical vein by enzymatic dissociation, as previously described [9]. The cells were grown in RPMI 1640 (for COLO 205 and HT 29), Eagle's minimal essential medium (MEM) (for Hep G2 and human fibroblasts), or M199 (for HUVEC), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 mg/mL), and 0.3 mg/mL glutamine in a humidified incubator (37°C, 5% $CO_2$). Magnolol (Pharmaceutical Industry, Technology and Development Center, Taiwan) was added at the indicated doses in 0.1% dimethyl sulfoxide (DMSO). For control specimens, the same volume of the 0.1% DMSO without magnolol was added.

### Analysis of DNA Fragmentation

As previously described [10], the cells treated with magnolol in 0.1% DMSO or without magnolol (control) cells were seeded onto 100-mm dishes. The cells were harvested, washed twice with ice-cold phosphate-buffered saline, resuspended in Tris–normal saline–EDTA (10 mM Tris-HCl at pH 7.6, 140 mM sodium chloride, and 1 mM EDTA), and lysed in 4 mL of extraction buffer (10 mM Tris-HCl at pH 8.0, 0.1 M EDTA at pH 8.0, 20 mg/mL pancreatic RNase, and 0.5% sodium dodecyl sulfate) at 37°C for 2 h. Proteinase K then was added at a final concentration of 100 mg/mL, and the mixture was incubated for another 3 h at 50°C. The DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamyl alcohol (24:1 vol/vol) and then precipitated with 0.1 vol of sodium acetate at pH 4.8 and with 2.5 vol of ethanol at −20°C overnight. Finally, it was centrifuged at 13,000 $\times g$ for 1 h. Genomic DNA was quantitated, and equal amounts of DNA sample in each lane were electrophoresed in a 2% agarose gel. The DNA was visualized by ethidium bromide staining. Genomic DNA isolated from 10 mM terbinafine-treated HT 29 cells showing DNA fragmentation was included in this experiment to serve as a positive control.

### Flow Cytometry

As previously described [11], the cells were seeded onto 100-mm dishes and grown in MEM supplemented with 10% FCS. After growing to subconfluence, the cells were treated with 100 μM magnolol in the presence or absence of various inhibitors in 1% FCS, harvested at 4 h after release with trypsin-EDTA, washed twice with phosphate-buffered saline/0.1% dextrose, and fixed in 70% ethanol at 4°C. The DNA content of the nuclei was determined by staining nuclear DNA with a solution containing propidium iodine (50 mg/mL) and DNase-free RNase (2 U/mL) and measuring the relative DNA content of nuclei using a fluorescence-activated cell sorter. The proportion of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickinson, San Jose, CA).

### Protein Extraction and Western Blot Analysis

Western blot analysis was performed as previously described [12,13]. The protein samples (50 μg per lane) were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel, and electrophoresis was performed at a constant 150 V for 3–4 h at 16–18°C. After protein separation, each gel was transferred onto an Immobilon-P membrane. Immunodetection was carried out by probing with appropriate dilutions of specific antibodies at room temperature for 2 h. Anti–caspase-3, anti–bcl-2, and anti-bax monoclonal antibodies (Transduction, San Diego, CA); anti–caspase-8 and anti–caspase-9 monoclonal antibodies (PharMingen, San Diego, CA); anti–Cyto *c* monoclonal antibody (Zymed, San Francisco, CA); and anti–glycerol-3-phosphate dehydrogenase (G3PDH) monoclonal antibody (Biogenesis, Kingston, NH) were used at a dilution concentration of

1:1000. Anti–poly(ADP-ribose)polymerase (PARP) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) was used at a concentration of 1:250 dilution. The secondary antibodies, alkaline phosphatase–coupled anti-mouse or anti-rabbit antibody (Jackson, Westgrove, PA), were incubated at room temperature for 1 h at dilution concentrations of 1:5000 or 1:1000, respectively. The specific protein complexes were identified using nitro blue tetrazolium chloride/bromo-chloro-3-indolyl-phosphate (Kirkegaard Perry Laboratory, Gaithersburg, Maryland). In each experiment, membranes also were probed with anti-G3PDH antibody, to correct for differences in protein loading.

### Measurement of Caspase Activity

As previously described [14], caspase activity was measured by using caspase-8 and caspase-9 colorimetric activity assay kits (Chemicon, Temecula, CA). Hep G2 cells were lysed by addition of cell lysis buffer, and the protein concentration was measured. Caspase activity was assayed at 37°C in 100 μL of assay buffer containing 25 μg (for caspase-8) or 30 μg (for caspase-9) of the indicated colorimetric peptide. Caspase activity was measured by the release of *p*-nitroaniline (*p*NA) from the labeled substrates Ac-IETD-*p*NA and Ac-LEHD-*p*NA for caspase-8 and caspase-9, respectively, and the free *p*NA was quantified at 405 nm.

### Preparation of Cytosolic Extracts

For study of Cyto *c* translocation, cytosolic extracts were prepared at various times after the cells were treated with DMSO or magnolol, as previously described [15]. Briefly, the Hep G2 cells grown in MEM containing 10% FCS, with or without 100 μM magnolol, were collected by centrifugation at 200 $\times g$ at 4°C for 5 min, washed once with ice-cold phosphate-buffered saline at pH 7.4, and centrifuged at 200 $\times g$ for 5 min. The cell pellet was resuspended in extraction buffer (200 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH at pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors). After a 30-min incubation period on ice, cells were homogenized with a glass dounce homogenizer (40 strokes) and centrifuged at 14 000 $\times g$ for 15 min; the supernatants were removed and used for immunoblotting to quantify Cyto *c*.

### Measurement of Cytosolic Free $Ca^{2+}$ Concentrations

As previously described [16], the Hep G2 cells were seeded onto coverslips and grown in MEM medium supplemented with 10% FCS. After the cells had grown to 60–70% confluence, the medium was changed to MEM supplemented with 1% FCS for 24 h and then replaced with fresh MEM supplemented with 1% FCS containing 5 μM fura-2/AM (Molecular Probes, Eugene, Oregon) and incubated for 45 min at 37°C. After washing with Phocal buffer (10 mM HEPES, 125 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, 0.5 mM $NaH_2PO_4$, 5 mM $NaHCO_3$, and 1.8 mM $CaCl_2$) three times, the coverslip was put into a quartz cuvette containing 1.8 mL of Phocal buffer with a magnetic stir bar. Fluorescence activity was monitored with a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan) at 510 nm, with excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity by using the equation $[Ca^{2+}]_i = K_d Q[(R - R_{min})]/(R_{max} - R)]$, where R represents the fluorescence intensity ratio Fλ1/Fλ2, in which λ1 (∼340 nm) and λ2 (∼380 nm) are the fluorescence detection wavelengths for the ion-bound and ion-free indicator, respectively.

The values of $F_{max}$, $F_{min}$, $R_{max}$, and $R_{min}$ were obtained at the end of each experiment by the sequential addition of 10 μM ionomycin (BIOMOL Research Laboratories, Plymouth Meeting, PA) and 50 mM EGTA. Q was the ratio of $F_{min}$ to $F_{max}$ at λ2 (∼380 nm). The dissociation constant ($K_d$) was taken as 240 nM. To study the source of the magnolol-induced elevation of cytosolic free $Ca^{2+}$, the Hep G2 cells were pretreated with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA/AM) or 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) for 30 min, followed by treatment with 100 μM magnolol.

### Statistics

All data were expressed as the mean values ± standard error (S.E.). Comparisons were subjected to Student's *t* test or analysis of variance followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

## RESULTS

### Magnolol Induced Apoptosis in Human Malignant Cell Lines

The occurrence of apoptosis, a natural death process in cells, most commonly is associated with arrested mitotic activity, decreased DNA replication, fragmentation of DNA, and activation of caspase-type enzymes. Accordingly, we examined COLO 205 and Hep G2 cells treated with magnolol to determine whether these indications of apoptosis appeared. At concentrations of 0–50 μM magnolol, apoptosis was not noted. When the magnolol concentration was increased to 100 μM, however, apoptosis was seen in COLO 205 (Figure 1a) and Hep G2 cells (Figure 1b) but not in cultured human nontransformed gingival fibroblasts (Figure 1c) and HUVEC (Figure 1d). Hence, magnolol was used at 100 μM in all of the remaining experiments in this study.

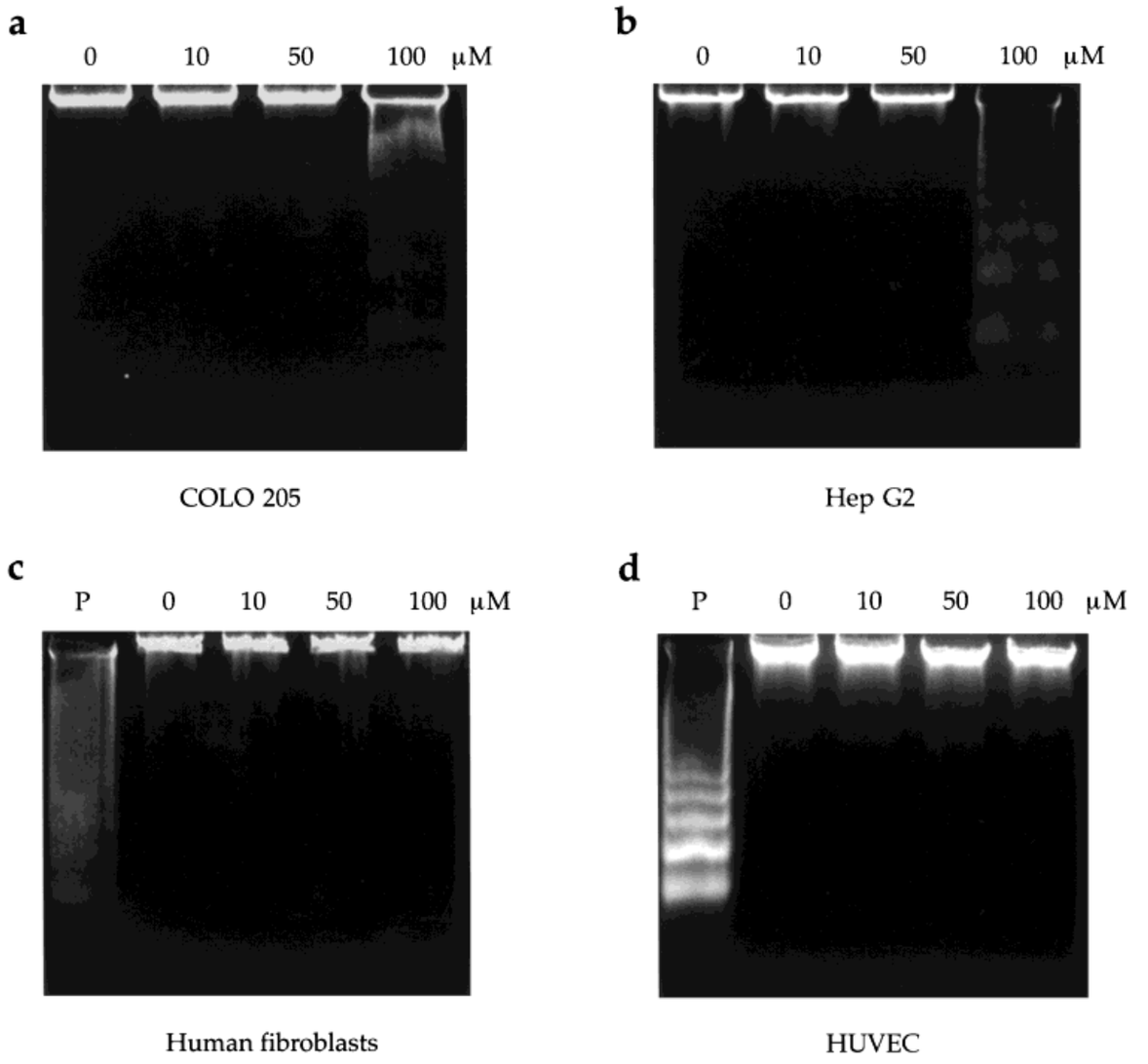


Figure 1. Electrophoresis of genomic DNA from COLO 205 and Hep G2 treated with magnolol. Genomic DNA isolated from 10 μM terbinafine–treated HT 29 cells was included in this experiment to serve as a positive control. A typical DNA ladder pattern associated with apoptosis was seen in COLO 205 (a) and Hep G2 cells (b) but not in human gingival fibroblasts (c) or HUVEC (d) treated with 100 μM magnolol for up to 36 h. P, positive control.

### Magnolol Activated Caspase-3, Caspase-8, and Caspase-9

Because it has been suggested [17] that apoptosis requires the activation of caspases, we investigated the involvement of caspase activation in magnolol-induced apoptosis in Hep G2 cells by using Western blot analyses. The Hep G2 cells were grown in MEM containing 10% FCS and treated with 100 μM magnolol for various times, as indicated. Figure 2a shows that decreased staining in the procaspase-3 band and degradation of PARP, the substrate for caspase-3, were noted after 36 h of treatment with 100 μM magnolol, indicating that caspase-3 was activated. To elucidate the apoptotic pathways involved in the activation of caspase-3, we examined the changes of caspase-8 and caspase-9 protein levels in the magnolol-treated Hep G2 cells. After treatment of Hep G2 cells with 100 μM magnolol for 36 h, activation of caspase-8 and caspase-9 was evidenced by degradation of the pro-enzymes of caspase-8 and caspase-9 as well as by the appearance of their cleavage products (Figure 2a).

### Magnolol Induced Cyto *c* Release From Mitochondria

It has been shown that activation of caspase-9 occurs during the release of Cyto *c* from

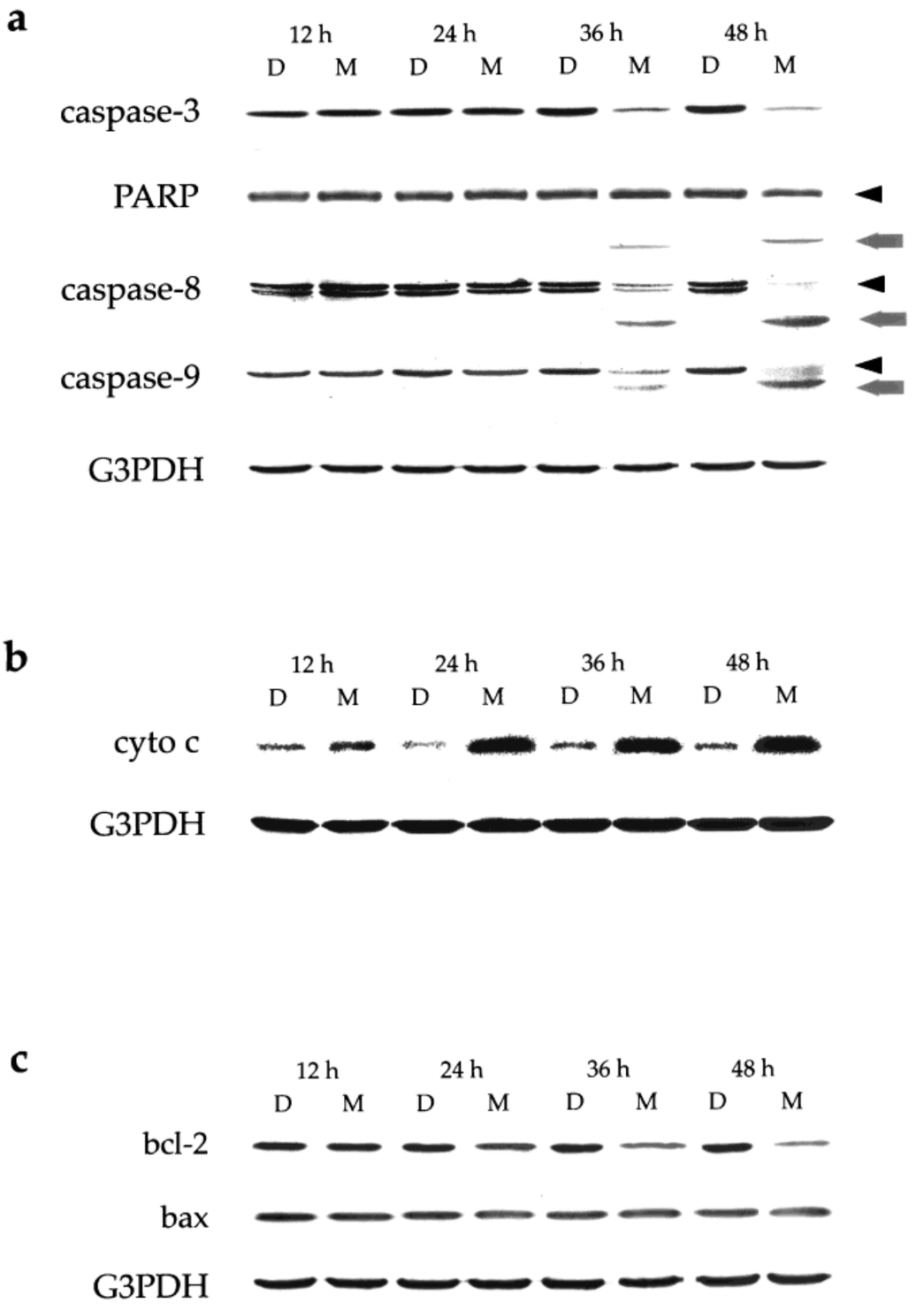


Figure 2. The effect of magnolol on caspase, bcl-2, and bax protein levels and Cyto *c* translocation from the mitochondria to the cytosol. Whole-cell proteins (a and c) or cytosolic proteins (b) were extracted from cultured Hep G2 cells that had been grown in 10% FCS and incubated for the indicated times with 0.1% DMSO or 100 μM magnolol in 0.1% DMSO. After electrophoresis, proteins were transferred onto Immobilon-P membranes and then probed with proper dilutions of specific antibodies. Membranes also were probed with anti-G3PDH antibody to correct for any difference in protein loading. Magnolol time dependently induced the activation of caspases (a), the translocation of Cyto c (b), and bcl-2 down-regulation (c). D, 0.1% DMSO; M, 100 μM magnolol in 0.1% DMSO. The pro-enzymes are indicated by triangles. The cleavage products of the pro-enzymes are indicated by arrows.

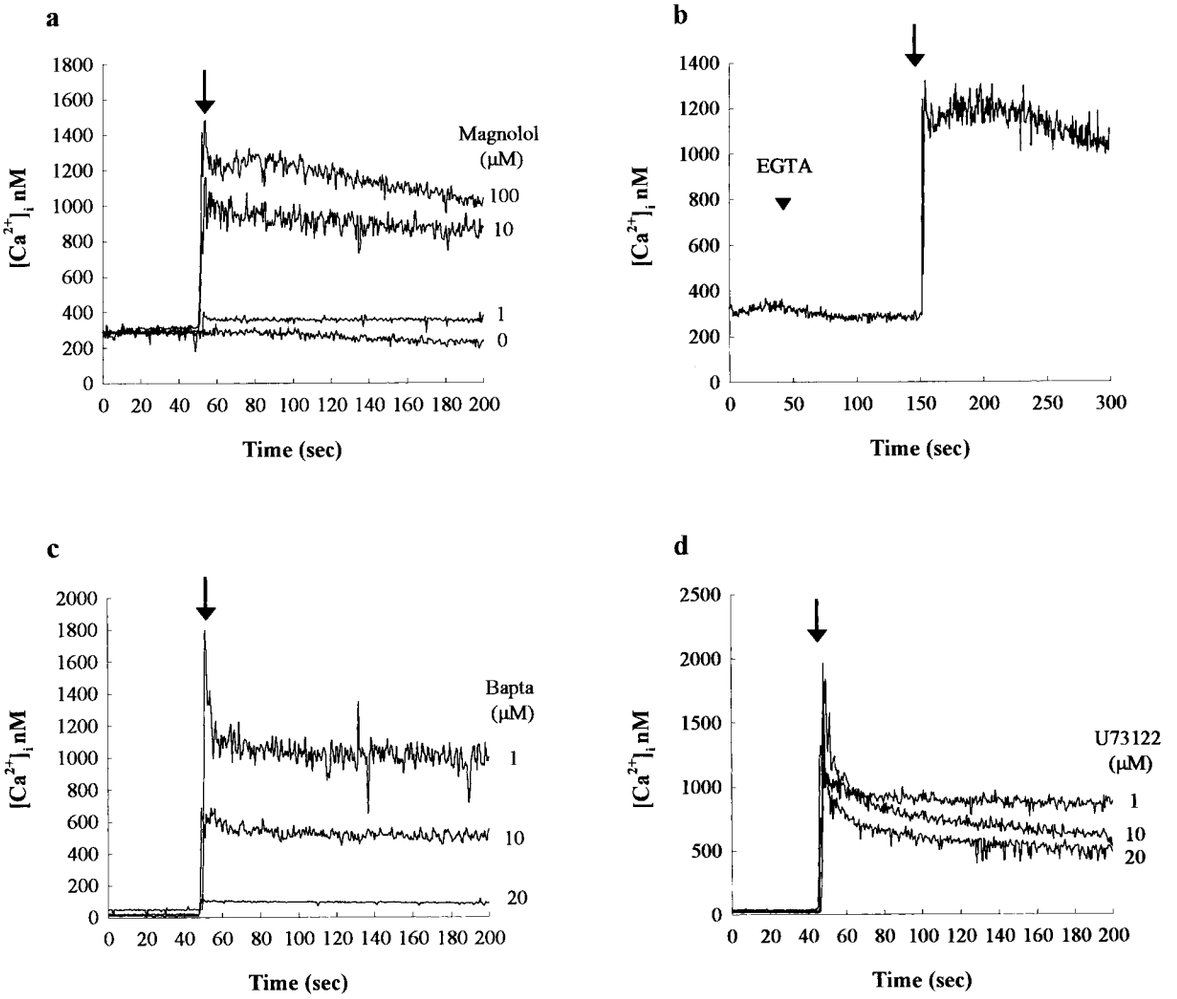


Figure 3. Magnolol-induced elevation of cytosolic free $Ca^{2+}$ in Hep G2. The cells were grown in MEM supplemented with 1% FCS. For the measurement of $[Ca^{2+}]_i$ changes, the fura-2/AM–loaded cells were stimulated with various concentrations (a) or 100 μM (b–d) of magnolol (arrow). (a) Dose-dependent elevation of $[Ca^{2+}]_i$ in Hep G2 cells treated with magnolol. (b) Pretreatment with 2 mM EGTA for 100 s did not affect the magnitude of the magnolol-induced elevation of $[Ca^{2+}]_i$ in Hep G2 cells. (c) Pretreatment of Hep G2 cells with BAPTA/AM for 30 min reduced the magnitude of the magnolol-induced elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. (d) Pretreatment of Hep G2 cells with U73122 for 30 min reduced the magnitude of the magnolol-induced elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. The time points of magnolol and EGTA administration are indicated by arrows and a triangle, respectively.

mitochondria [18]. To examine whether this activation occurs in magnolol-induced apoptosis in Hep G2 cells, Cyto *c* release was monitored at various times after treatment with 100 μM magnolol. Figure 2b shows that magnolol treatment resulted in a significant accumulation of Cyto *c* in the cytosol fraction of cell extracts. This magnolol-induced elevation of cytosolic Cyto *c* was noted at 12 h and peaked at 36 h after magnolol treatment. Under the same conditions, caspase activation was not seen until 36 h after magnolol treatment (Figure 2a). Evidently, translocation of Cyto *c* occurred in the magnolol-treated Hep G2 cells first, and activation of caspase-8 and caspase-9 and DNA fragmentation followed thereafter.

## Magnolol Downregulated Bcl-2 Protein

Proteins of the bcl-2 family also are believed to be involved in the control of apoptosis [19]. Bcl-2 directly or indirectly operates to prevent the release of Cyto *c* from mitochondria. On the other hand, bax can trigger mitochondria to release Cyto *c* from mitochondria and thereby initiate apoptosis. Accordingly, we examined the changes in bcl-2 protein levels in magnolol-treated Hep G2 cells. Treatment of Hep G2 cells with 100 μM magnolol caused a time-dependent downregulation of bcl-2 protein, as shown by its decreased staining on gels (Figure 2c). In contrast, bax protein levels were not changed significantly.

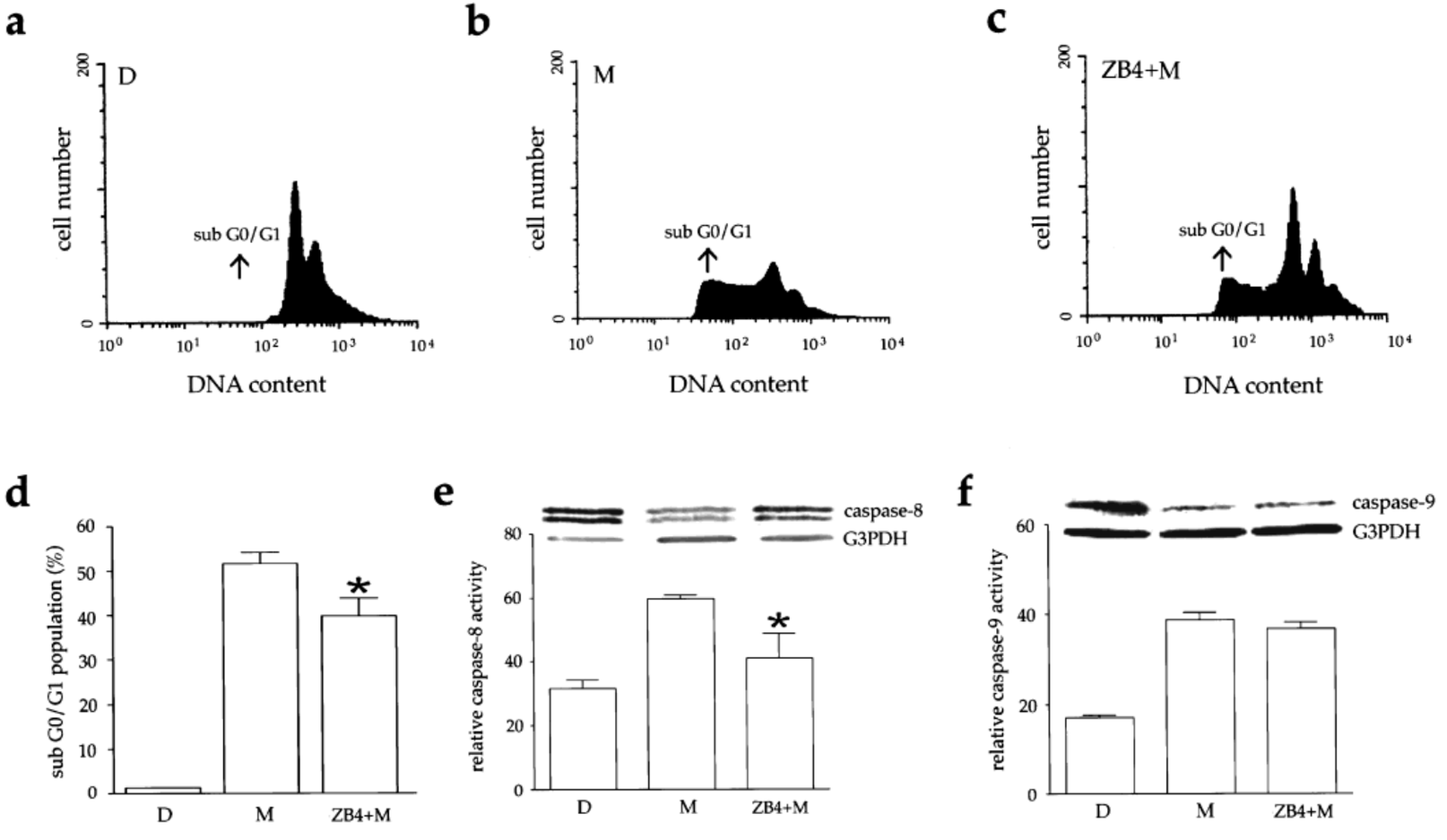


Figure 4. The effect of ZB4 on magnolol-induced apoptosis and caspase activity in Hep G2 cells. The cells were grown in MEM supplemented with 1% FCS. Shown are representative DNA content frequency histograms of Hep G2 cells treated with 0.1% DMSO (a), 100 μM magnolol (b), or 250 ng/mL ZB4 + 100 μM mangolol (c). (d) The average $G_0/G_1$ subpopulation of 0.1% DMSO–treated versus magnolol-treated versus magnolol + ZB4–treated Hep G2 cells. Treatment with ZB4 for 4 h suppressed the magnolol-induced activation of caspase-8 (e) but not caspase-9 (f). The activation of caspase-8 and caspase-9 was evidenced by the degradation of pro-enzymes of caspase-8 and caspase-9, as detected by Western blot analysis; increased caspase activity was examined by a caspase activity assay. Values are means ± S.E. (n = 3). Comparisons were subjected to the Student's *t* test. Significance was accepted at $P < 0.05$. Asterisk, magnolol-treated group different from ZB4 + magnolol-treated group; D, 0.1% DMSO; M, 100 μM magnolol in 0.1% DMSO.

### Magnolol Elevated the Cytosolic Free $Ca^{2+}$ Concentration

Green and Reed [7] have proposed that the abnormal elevation of cytosolic free $Ca^{2+}$ is one of the major occurrences in apoptosis. Previously, Wang and Chen [3] reported that magnolol, applied in vitro to rat neutrophil cells, stimulates an increase in cytosolic free $Ca^{2+}$ in a dose-dependent manner. To examine whether magnolol-induced apoptosis is associated with an increase in cytosolic-free $Ca^{2+}$, we compared intracellular fura-2/AM fluorescence activity (an indicator of cytosolic free $Ca^{2+}$ concentration) in the Hep G2 cells treated with or without 100 μM magnolol. Figure 3a shows an initial rapid spike, with a sustained high $[Ca^{2+}]_i$ curve in the magnolol-treated Hep G2 cells, which occurred rapidly and in a dose-dependent manner. Since elevation of $[Ca^{2+}]_i$ can be caused by either $Ca^{2+}$ influx from the external medium or by $Ca^{2+}$ released from internal stores, we treated the cells with 2 mM EGTA to chelate the extracellular free $Ca^{2+}$ and prevent its entry into cells. Figure 3b shows that pretreatment with 2 mM EGTA for 100 s did not affect the magnitude of magnolol-induced elevation of cytosolic free $Ca^{2+}$; hence, the increase of $[Ca^{2+}]_i$ is not due to an influx of extracellular $Ca^{2+}$. In contrast, pretreatment of Hep G2 cells with BAPTA/AM, an intracellular $Ca^{2+}$ chelator, at 37°C for 30 min reduced the magnitude of the magnolol-induced elevation of cytosolic free $Ca^{2+}$ (Figure 3c). Moreover, pretreatment of Hep G2 cells at 37°C for 30 min with U73122 (a phospholipase C inhibitor), which inhibits the hydrolysis of phosphatidylinositol biphosphate to inositol triphosphate [20], significantly attenuated the magnolol-induced elevation of $[Ca^{2+}]_i$ (Figure 3d).

### Fas was Involved in Magnolol-Induced Caspase-8 Activation

It has been suggested that caspase-8 is associated with apoptosis involving a so-called death receptor [21]. Hep G2 cells express both Fas and Fas ligand [22]. Therefore, it is reasonable to speculate that Fas might be involved in caspase-8 activation in magnolol-treated Hep G2 cells. To address this issue, Hep G2 cells were treated with ZB4, an antibody antagonistic to Fas that binds to the Fas ligand binding site on a Fas receptor and blocks Fas activation. We then examined DNA content frequency histograms, caspase levels, and caspase activity. Figure 4a–c shows representative DNA content frequency histograms from DMSO-, magnolol-, and ZB4 + magnolol–treated Hep G2 cells. In response to 100 μM magnolol treatment, the $G_0/G_1$ subpopulation, an indicator of apoptosis, was increased significantly (Figure 4b and d). We also found that pretreatment with ZB4 at a concentration of 250 ng/mL suppressed the magnolol-induced increase in the $G_0/G_1$ subpopulation (Figure 4c and d). It is clear that ZB4 partially reversed magnolol-induced apoptosis by 15–20%. ZB4 also inhibited magnolol-mediated caspase-8 activation, as evidenced by Western blot analysis and a caspase activity assay (Figure 4e). In contrast, pretreatment with ZB4 had no effect on magnolol-induced caspase-9 activation (Figure 4f).

### Inositol Triphosphate–Mediated Pathway was Involved in Magnolol-Induced Caspase Activation

To study further the effect of elevated $[Ca^{2+}]_i$ on the development of apoptosis, Hep G2 cells were pretreated with 20 μM U73122 (a phospholipase C inhibitor) for 30 min, followed by 100 μM magnolol treatment. Figure 5a–c shows representative DNA content frequency histograms from DMSO-, magnolol-, and U73122 + magnolol–treated Hep G2 cells. Figure 5c and d shows that pretreatment of these cells with U73122 for 30 min resulted in the inhibition of the magnolol-induced increase in the $G_0/G_1$ subpopulation. The graphs presented as Figure 5e and f show that magnolol-induced activation of caspase-8 and caspase-9 was inhibited substantially by pretreatment of Hep G2 cells with U73122. These findings suggested that the inositol triphosphate–mediated signaling pathway might be involved in the magnolol-induced increase of cytosolic free $Ca^{2+}$, which, in turn, led to apoptosis in Hep G2 cells through activation of caspase-8 and caspase-9.

## DISCUSSION

A commonly held view is that uncontrolled cell proliferation in malignant tissues derives from a combination of two circumstances: increased cell multiplication unresponsive to normal control processes and decreased occurrence of the normal process of cell death, that is, apoptosis. Our present state of knowledge, however, offers little insight regarding the intrinsic or extrinsic signals that might govern the opposing processes of cell multiplication and cell death to maintain normal cell populations. While we were engaged in our investigations of these matters, we discovered that the substance magnolol, derived from a herbal medication, has the potent effect of inducing apoptosis in two human cancer cell types, Hep G2 and COLO 205, but not in human nontransformed cells, such as gingival fibroblasts and HUVEC. In that initial part of our study, we found that even though magnolol, at the concentrations of 0–50 μM, strongly inhibited growth and proliferation in these cells, it did not have a perceptible influence on apoptosis. While continuing this investigation, we found that magnolol at the higher concentration of 100 μM used for treatment periods of 36 h or more could indeed produce the characteristic signs of apoptosis.

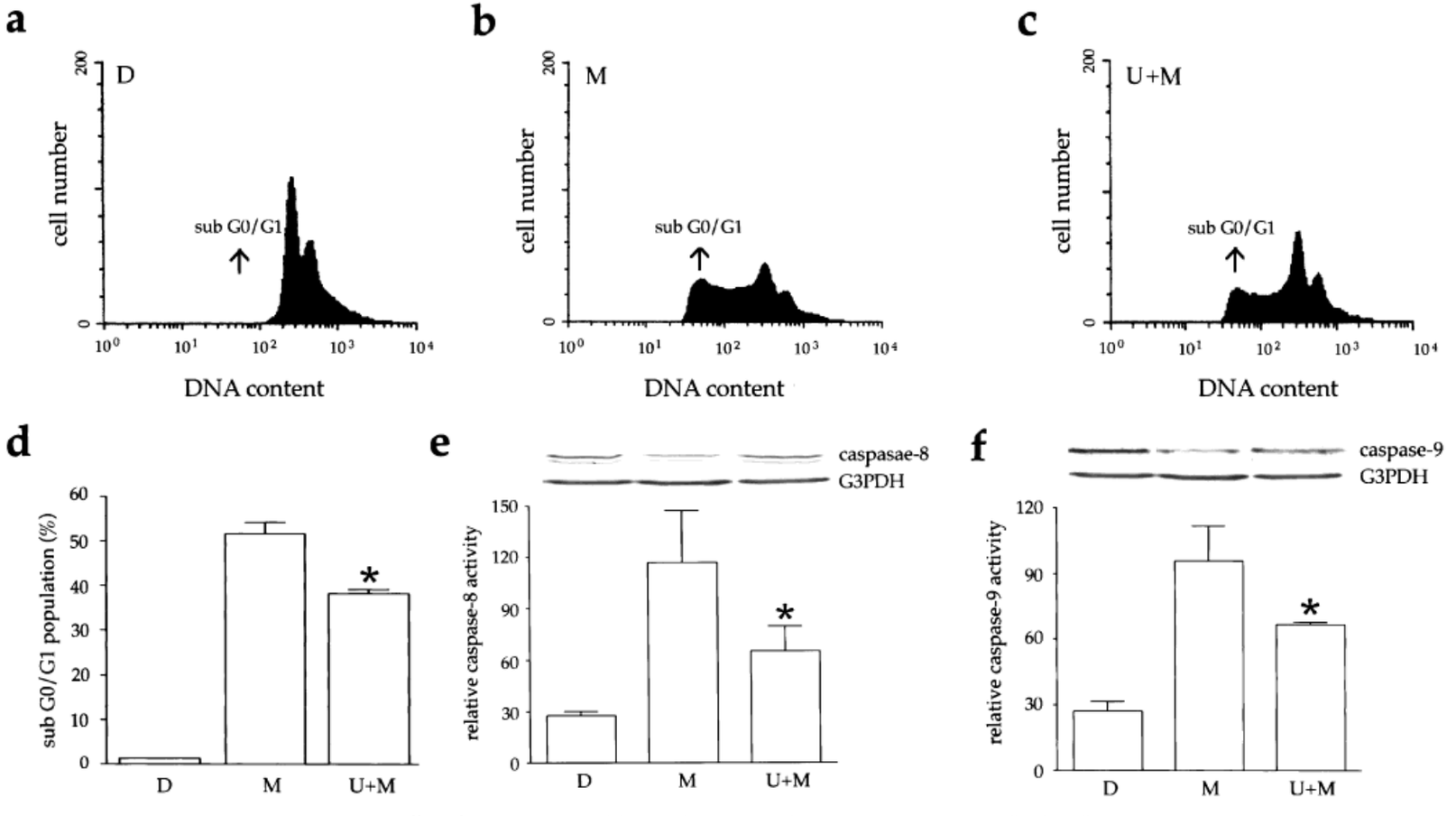


Figure 5. Effect of U73122 on magnolol-induced apoptosis and caspase activity in Hep G2 cells. The cells were grown in MEM supplemented with 1% FCS. Shown are representative DNA content frequency histograms of Hep G2 cells treated with 0.1% DMSO (a), 100 μM magnolol (b), or 20 μM U73122 + 100 μM mangolol (c). (d) The average $G_0/G_1$ subpopulation of 0.1% DMSO-treated versus magnolol-treated versus magnolol + U73122–treated Hep G2 cells. Pretreatment of Hep G2 with 20 μM U73122 partially reversed magnolol-induced activation of caspase-8 (e) and caspase-9 (f). The activation of caspase-8 and caspase-9 was evidenced by the degradation of pro-enzymes of caspase-8 and caspase-9, as detected by Western blot analysis; increased caspase activity was examined by a caspase activity assay. Values are means ± S.E. (n = 3). Comparisons were subjected to Student's *t* test. Significance was accepted at $P < 0.05$. Asterisk, magnolol–treated group different from U73122 + magnolol–treated group. D, 0.1% DMSO; M, 100 μM magnolol in 0.1% DMSO; U, U73122.

These findings show for the first time that magnolol can induce apoptosis in cultured cells.

Apoptosis is a cell-suicide mechanism that requires specialized cellular machinery. A central component of this machinery is a proteolytic system involving caspases, a highly conserved family of cysteine proteinases with specific substrates [17]. How caspases contribute to this process is not understood fully. It has been suggested that the terminal stages of apoptosis occur through the activation of caspases and that different initiator caspases mediate distinct sets of signals. By Western blot analysis, we showed that 100 μM magnolol induced activation of caspase-8 and caspase-9 in Hep $G_2$ cells, as evidenced by decreases in stainable procaspase-8 and procaspase-9, and increases in caspase-8 and caspase-9 enzyme activities (Figure 4e and f). The active forms of caspase-8 and caspase-9 can cleave and activate downstream caspases, such as caspase-3 (Figure 2a), which eventually leads to apoptosis [23].

Caspase-9 is involved in death induced by cytotoxic agents [24,25]. Zou et al. [18] postulated that Cyto *c* may activate caspases by binding to Apaf-1, which interacts with and activates caspase-9. Cyto *c* release sometimes can contribute to Fas-mediated apoptosis by amplifying the effects of caspase-8 to activate downstream caspases [26]. The active caspases, on the other hand, can promote Cyto *c* release and thereby amplify the signal for apoptosis [27]. In response to 100 μM magnolol, cytosolic Cyto *c* levels began to increase in about 12 h (Figure 2b), and significant activation of caspase-8 and caspase-9 occurred about 24 h later (Figure 2a). This finding suggested that the magnolol-mediated increase of cytosolic Cyto *c* could bring about activation of caspase-8 and caspase-9.

Although direct evidence of the involvement of bcl-2 and cytosolic-free $Ca^{2+}$in magnolol-mediated Cyto *c* release is absent, the data of the present study suggested that downregulation of bcl-2 and elevation of cytosolic free $Ca^{2+}$ occurred during magnolol-mediated release of Cyto *c*. It has been suggested that bcl-2 family proteins are involved in the regulation of apoptosis through control of the release of Cyto *c* from mitochondria [19]. Bcl-2 prevents apoptosis by blocking the release of Cyto *c* from mitochondria [28]. Bax, on the other hand, directly induces Cyto *c* release from mitochondria and thereby triggers caspase-9 activation [29]. Treatment of isolated rat liver mitochondria with calcium chloride can trigger the release of Cyto *c* in vitro [30]. The results of the present study showed that elevation of the cytosolic free $Ca^{2+}$ concentration and decrease of the bcl-2 protein level induced by 100 μM magnolol treatment in Hep G2 cells seemed to be responsible for stimulating the release of Cyto *c*. The magnolol-mediated increase in cytosolic free $Ca^{2+}$ was released from the intracellular sources of calcium through inositol triphosphate–mediated pathways but not from $Ca^{2+}$ influx across the plasma membrane. This result differs from that for magnolol-treated rat neutrophils, in which magnolol was found to stimulate $Ca^{2+}$ release from internal stores and $Ca^{2+}$ influx from extracellular sources across the plasma membrane [3]. The discrepancy between these two studies might be due to the differential effects of magnolol on different cell types, such as liver cells versus neutrophils or cancer cells versus nontransformed cells.

The presence of the death receptor Fas and its ligand in Hep G2 cells is in accord with the hypothesis that caspase-8 is involved in magnolol-induced apoptosis in Hep G2 cells [22]. Administration of the anti-Fas antibody (ZB4) prevented caspase-8 activation (Figure 4e) and reduced magnolol-induced apoptosis by 15–20% (Figure 4d). Magnolol-mediated caspase-9 activation, however, was not affected by ZB4 treatment (Figure 4f). These results lend support to the idea that a Fas-mediated pathway is involved in magnolol-induced caspase-8 activation and consequent apoptosis in Hep G2 cells. It is noteworthy that surface expression of Fas

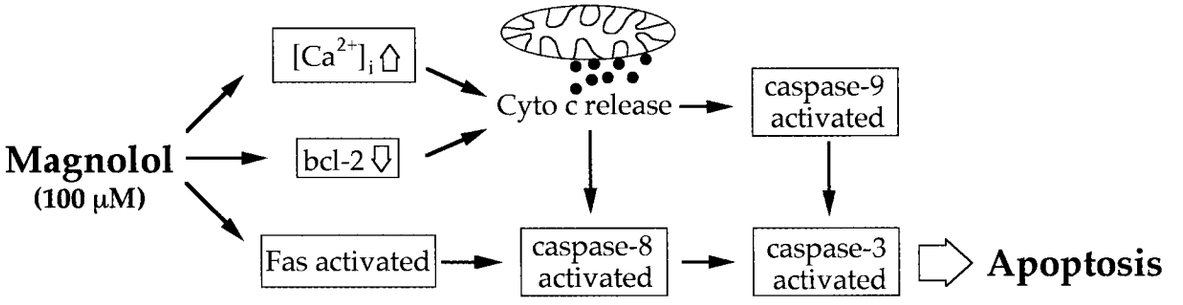


Figure 6. Model for magnolol-induced anticancer activity. In response to 100 μM magnolol administration, Fas was activated and Cyto *c* (solid circles) was translocated from the mitochondria to the cytoplasm through elevation of the cytosolic free $Ca^{2+}$ concentration and bcl-2 downregulation. Caspase-8 was activated by both Fas activation and Cyto *c* release from mitochondria, whereas caspase-9 was activated by Cyto *c* release. The active forms of caspase-8 and caspase-9 can cleave and activate downstream caspases, such as caspase-3, which, in turn, induce apoptosis.

was heterogeneous in malignant cell lines [31]. Therefore, it seems that activation of the Fas-mediated pathway does not always participate in magnolol-induced apoptosis. Whether magnolol activated Fas directly or promoted the action of a Fas ligand, which, in turn, activated Fas, remains to be determined.

Based on the results of the present study, we propose a model of the molecular mechanisms of magnolol-induced apoptosis in malignant cell lines, as shown in Figure 6. Although animal studies of magnolol-mediated anti-tumor action are ongoing, the findings from our previous study of magnolol's anti-cancer effect and the present in vitro studies strongly support the potential applications of magnolol in the treatment of human cancer.

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