—Regular Article—

Induction of Caspase-3-Dependent Apoptosis in Human Leukemia HL-60 Cells by δ-Elemene

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δ-Elemene, an antitumor component, is a chemical compound isolated from *Curcuma wenyujin*, a Chinese traditional herb. We examined whether δ-elemene could inhibit cell growth and cell cycle progression and induce apoptosis in human leukemia HL-60 cells. The results demonstrated that δ-elemene induces significant apoptosis of HL-60 cells, as shown by MTT assay, annexin V (AnV) binding of externalized phosphatidylserine (PS), and the mitochondrial probe JC-1 using flow cytometry. HL-60 cells treated with δ-elemene showed high percentages in the early apoptotic and late apoptoctic/necrotic stages, as well as caspase-3 activation of HL-60 cells. By monitoring the changes in cell cycle profiles, we confirmed that δ-elemene could interfere with the cell cycle in the G2/M phase and induce apoptosis in HL-60 cells in a time-dependent manner. Caspase-3 plays a direct role in proteolytic cleavage of the cellular proteins responsible for progression to apoptosis. Therefore we examined apoptosis in HL-60 cells after exposure to δ-elemene and measured caspase-3 activities with or without Z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk, a broad-spectrum caspase inhibitor) pretreatment using flow cytometric analysis. The results showed that δ-elemene could induce caspase-3 activation as detected by the decrease in δ-elemene-induced caspase-3 activities after treatment with z-VAD-fmk. In the present study, δ-elemene activated typical caspase-dependent apoptosis in HL-60 cells, as demonstrated by an inhibitory effect of z-VAD-fmk on this cell death. During δ-elemene-induced apoptosis, cytochrome c and apoptosis-inducing factor were released into the cytosol and BAX was translocated from the cytosol to mitochondria. However, these were not prevented by z-VAD-fmk. In conclusion, our study demonstrated that δ-elemene could induce G2/M cell cycle transition and trigger apoptosis through a caspase-3-dependent pathway.

Key words——apoptosis; HL-60 cell; cell cycle; caspase-3; cyt c; *Curcuma wenyujin*

INTRODUCTION

Therapeutic approaches to human leukemia include irradiation, hyperthermia, and chemotherapy. Unfortunately, an unacceptably high proportion of affected patients still die and many questions remain unanswered about the optimum way to treat the disorder. While chemotherapy is the major treatment to improve prognosis and long-term survival, its value is limited by toxicity or lack of efficacy.

δ-Elemene, a component isolated from the traditional Chinese medicinal herb *Curcuma wenyujin*,[1,2] exists as an essential oil mixture of β-, γ-, and δ-elemene (Fig. 1) and exerts antitumor activity by inducing apoptosis in several types of cell.[3,4] Our previous study showed that δ-elemene does not suppress bone marrow cells and normal liver cell line WRL-68.[5] Thus it is relatively safe as an antitumor component. However, the effect of δ-elemene on HL-60 cells has not yet been investigated and whether it can trigger apoptosis in HL-60 cells was not clear.

Apoptosis is programmed cell death and it is typically accompanied by the activation of a class of death proteases (caspases).[6,7] The activation of the

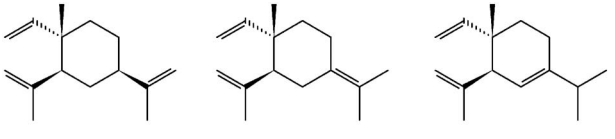

Fig. 1. Chemical Structure of β-Elemene, γ-Elemene, and δ-Elemene (MW: 204.35)

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caspase cascade is involved in chemical- and agent-induced apoptosis.[8] Caspase-3 has been shown to be a key component involved in the underlying mechanisms of apoptosis and relies on the action of the initiator caspases including caspase-8 and caspase-9 for its action.[9] However, there is no available information to address the exact mechanism of action of δ-elemene on cell cycle progression and of caspase-3 on δ-elemene-induced apoptosis in HL-60 cells. Thus, the present study investigated the effects and possible mechanism of action of δ-elemene on the cell cycle progression and regulatory molecules of HL-60 cells.

Apoptosis along the mitochondrial pathway is initiated by the translocation of Bax into mitochondria.[10] This is followed by an increase in mitochondrial membrane permeability ($\Delta\psi$m) and a release of cytochrome c (cyt c) and apoptosis-inducing factor (AIF) from mitochondria into the cytosol. In the cytosol, cyt c activates postmitochondrial caspase cascades, caspase-3 and caspase-9, which leads to apoptotic cell death.[11] AIF is a caspase-independent apoptotic effector that, on translocating from the cytosol to the nucleus, causes chromatin condensation and large-scale DNA fragmentation, leading to apoptotic cell death.[12] Furthermore, it was shown that δ-elemene stimulates the release of cyt c from mitochondria in human colorectal adenocarcinoma DLD-1 cells to induce the intrinsic apoptotic pathway.[5] However, the mechanism underlying δ-elemene-induced intrinsic apoptosis is currently unclear. In this study, we addressed this issue by conducting an analysis of the effect of δ-elemene on mitochondrial permeability transition, BAX activation, and the effects of caspase inhibitors on δ-elemene-induced apoptosis in human leukemic HL-60 cells.

MATERIALS AND METHODS

Cell Culture The human leukemia cell line (HL-60) was purchased from the American Type Culture Collection (ATCC, #CCL-240, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco, Grand Island, NY, USA). Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

δ-Elemene and β-elemene were isolated from the essential oil of *Curcuma wenyujin* using the method reported previously[13] (assay >97%, GC). Cells were treated with either δ-elemene or β-elemene at the concentrations indicated. δ-Elemene was prepared as previously described.[2–4]

Determination of Antiproliferative Activity Two hundred microliters of HL-60 cells at a density of 1×10^5/ml containing various concentrations of δ-elemene and β-elemene were plated in each well of 96-well plates. The cells were cultured for 12 and 24 h at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (50 ml of 2 mg/ml) was added per well and the cultures were continued for an additional 4 h. The medium was aspirated after centrifugation at 1000 rpm for 10 min, the cells were dissolved in 100 ml of DMSO, and the optical density (OD) at 570 nm was determined in each well with a 96-well plate reader. The inhibition rate (IR%) was calculated as follows: Inhibition(%) = [A570(control) − A570(drug)]/A570(control) × 100.

Analysis of Cell Division Cycle Profile Cells were treated with 200 μM of δ-elemene for 0, 3, 6, 12, 24, and 48 h. Cells (1×10^6) were fixed with cold 70% (v/v) ethanol at 4°C overnight. Fixed cells were washed with PBS twice and treated with 0.5 ml of 50 μg/ml propidium iodide (PI) in PBS and 50 μl of RNase A for 30 min at ambient temperature in the dark. Cell cycle analysis was performed using flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA). Cells labeled with PI were detected at the FL2 channel with a doublet discrimination module. Thirty thousand events were acquired. Subsequent analyses were performed using the ModFit LT software (version 3.1 SP1, Verity Software House, Topsham, ME, USA).

Measurement of Phosphatidylserine Externalization Phosphatidylserine (PS) externalization was examined with a two-color analysis of FITC-labeled annexin V binding and PI uptake using flow cytometry.[14] Briefly, HL-60 cells were plated at 5×10^4/ml in RPMI-1640. Flasks were incubated with 200 μM of δ-elemene. Cells were harvested, suspended at 1×10^6/ml, and washed two times with ice-cold PBS after 12, 24, and 48 h. The cells were suspended in 300 μl of dilute binding buffer from an annexin-V FITC kit (Alexis, Lausen, Switzerland), then 5 μl of PI and 5 μl of annexin-V-FITC were added. The tubes were gently mixed and kept in the dark on ice for 10 min before analysis with flow cytometry. Data were analyzed using LYSIS II software. (AnV^+)PI^- cells

were considered early apoptotic, and $(AnV^+)PI^+$ cells were considered late apoptotic and necrotic. HL-60 cells were also pretreated with or without the cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk for 3 h before δ-elemene treatment for 48 h.

Mitochondrial Transmembrane Potential ($\Delta\psi$m) Measurement The mitochondrial membrane potential was analyzed using JC-1, a lipophilic cationic fluorescence dye. JC-1 is capable of selectively entering mitochondria and is a dual emission probe, which reversibly changes color from green (FL-1) to greenish orange (FL-2) as the mitochondrial membrane becomes more polarized.[15] In the treatment of cells with 200 μM of δ-elemene for 6, 12, and 24 h, HL-60 cells were suspended in 200 μl of RPMI medium, after which JC-1 (300 nM, Molecular Probes) was added for 15-min incubation at 37℃ in the dark. The cells were then harvested with trypsin (0.25%) and washed once with PBS. Following suspension in PBS, the cells were subjected to flow cytometry analysis.

Quantitation of Active Caspase-3 Expression HL-60 cells were treated for 0, 3, 6, 12, 24, and 48 h with 200 μM of δ-elemene. One million cells were treated with 500 μl 1×FACS Permeabilizing Solution for 15 min at ambient temperature in the dark. Permeabilized cells were washed with PBS and stained with 20 μl of PE-conjugated polyclonal antibody against the active form of caspase-3 for 30 min at ambient temperature in the dark. Cells were washed again with PBS and then fixed in 400 μl of 1% paraformaldehyde (PFA) in PBS. The fluorescence signal was detected at the FL2 channel with flow cytometry. Ten thousand events were acquired. HL-60 cells were also pretreated with or without the cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h before δ-elemene treatment for 48 h.

Preparation of Proteins in the Mitochondrial and Cytosolic Fractions Proteins were prepared as previously described[16] with minor modifications. The cells were washed twice in ice-cold PBS and resuspended in five volumes of ice-cold extract buffer (Western blotting analysis Hepes-KOH 20 mM, MgCl2 1.5 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, and PMSF 0.1 mM, pH 7.5). The resuspended cells were homogenized with 10 strokes of a Teflon homogenizer. The homogenates were centrifuged twice at 750 g for 10 min at 4℃. The supernatants were centrifuged at 10000 g for 15 min at 4℃ to obtain the mitochondrial pellets. Cytosolic fractions were obtained after further centrifugation at 100000 g for 1 h at 4℃. The protein concentrations of the resulting supernatants and mitochondrial fractions were measured and stored at −70℃.

Western Blotting Analysis Briefly, 10 μg of protein from the mitochondrial or cytosolic fraction and 40 μg of total protein from treated cells were loaded onto 12% sodium dodecyl sulfate (SDS)-polyacylamide gels, and the separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). To detect the levels of Bax, cyt c, and AIF, a mouse-anti-human monoclonal Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse-anti-human monoclonal cyt c antibody (BioVision Research Products, Mountain View, CA, USA) and mouse-anti-human monoclonal AIF antibody (Santa Cruz Biotechnology) were employed with a horseradish peroxidase-conjugated goat-anti-mouse IgG antibody (Santa Cruz Biotechnology). The goat-anti-human actin antibody was used to detect the levels of actin, which was used as a control for equal loading. Detection of the target protein signal was achieved using an ECL system (Amersham Biosciences).

Statistical Analysis All results were obtained in at least three independent experiments. Data are expressed as mean±standard error. Representative data were analyzed for statistical significance with SPSS 11.5 software. Differences among groups were analyzed using one-way ANOVA, multiple comparisons used the LSD and SNK tests for homogeneity of variance and Dunnett's T3-test for heterogeneity of variance. A value of $p<0.05$ was considered statistically significant.

RESULTS

δ-Elemene-induced Cell Death in Dose- and Time-Dependent Manners δ-Elemene induced HL-60 cell death in a dose-dependent manner between the concentrations of 0 and 400 μM (Fig. 2A). At the concentration of 400 μM, more than 80% of the cells were dead after 24-h treatment. Compared with 12-h treatment with 200 μM of δ-elemene, cell death gradually increased to about 1.4-fold and 1.6-fold after 24-h and 48-h treatment with δ-elemene, respectively (Fig. 2B).

Effect of δ-Elemene on HL-60 Cell Cycle Profiles HL-60 cells were exposed to 200 μM of δ-elemene

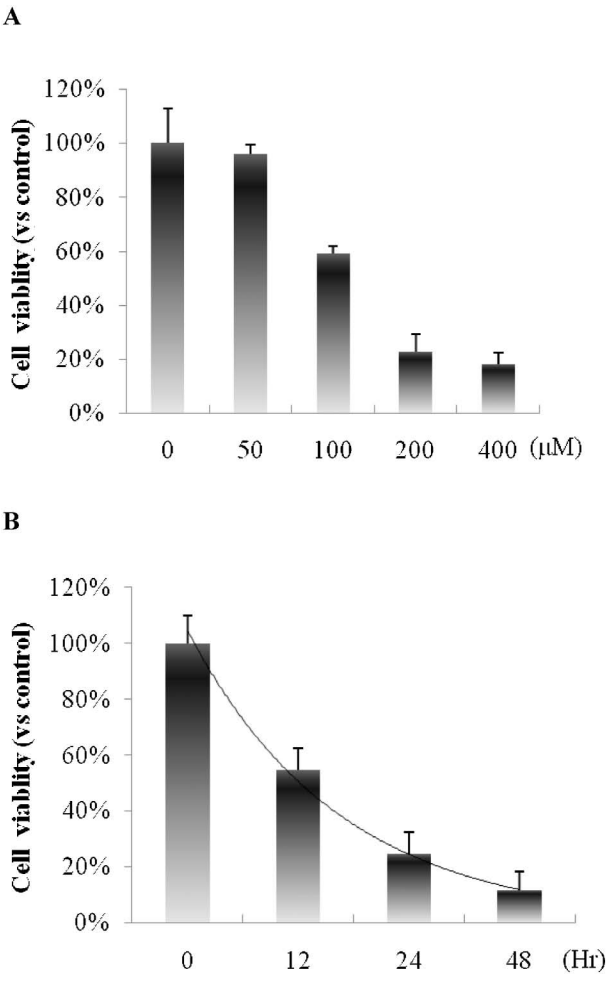

Fig. 2. Cell Death Induced by δ-Elemene

After HL-60 cells were treated with 0–400 μM of δ-elemene for 24 h (A), the cells were harvested and the MTT assay was performed to measure cell death. For the time-course study, the cells were treated with 200 μM of δ-elemene for 12, 24, and 48 h (B). Then cell death was measured in the MTT assay. Control cells were treated with vehicle only. The results of the MTT assay are expressed as cell viability, which was calculated using the following formula: Inhibition (%) = [*A* 570 (control) − *A* 570 (drug)] / *A* 570 (control) × 100%. Each experiment was repeated at least 3 times.

for the indicated times, washed, and harvested. The cells were fixed and stained with PI and RNase A, then analyzed using flow cytometry (FACS). The cell percentage in each phase (G0/G1, S, and G2/M) of the cell cycle was calculated and shown in Fig. 3. Starting from 6 h, the proportion of cells in the G0/G1 phase began to decline ($p<0.05$). The fall in cells in G0/G1 was accompanied by an increase in cells in S ($p<0.05$) and G2/M ($p<0.05$) phases. As time progressed, the decrease in cells in G0/G1 and the increase in cells in G2/M became more prominent. A trend of decreasing S phase cells was noted at 24 h; at or beyond 24 h, diminishing cells in G0/G1 and S phases and accumulating cells in G2/M were observed.

Cell Death Induced by δ-Elemene Apoptosis was triggered after δ-elemene treatment for 12 h (Fig. 4A). The percentage of early apoptosis represented by $(AnV)^+PI^-$ cells was significantly increased by δ-elemene treatment in a time-dependent manner with 36.4% at 48 h (Fig. 4B). Total cell death, which comprised early apoptotic and late apoptotic cells, calculated by the summation of the $(AnV)^+PI^-$ and $(AnV)^+PI^+$ cell populations, was also increased at 24 h. Compared with the untreated controls, more death events were observed in δ-elemene-treated cells after 48 h. (Fig. 4C, $^*p<0.01$, $^{**}p<0.001$ *vs.* control).

Caspase-3 Activation Induced by δ-Elemene in HL-60 Cells δ-Elemene-induced expression of the active form of caspase-3 was determined as other evidence of apoptosis. Caspase-3 was activated when HL-60 cells were incubated with 200 μM of δ-elemene for 6 h (Fig. 5). The minimum up-regulation was found in cells treated for 3 h, which was 1.55-fold increase over the controls ($p<0.001$). Very marked up-regulation of active caspase-3 could be observed in the 48-h groups, where there was a 10.56-fold increase compared with control cells.

z-VAD-fmk Inhibited δ-Elemene-induced Apoptosis and Caspase-3 Activation in HL-60 Cells To examine whether caspase-3 activation is involved in the apoptosis triggered by δ-elemene, some HL-60 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk 3 h before δ-elemene treatment. The HL-60 cells were treated with 200 μM of δ-elemene with or without z-VAD-fmk pretreatment, and then examined at different times to analyze the time-dependent inhibition of δ-elemene-induced caspase-3 activation by z-VAD-fmk. The same conditions and procedures were then used to analyze the time-dependent inhibition of paclitaxel-induced apoptosis by z-VAD-fmk. The results are shown in Fig. 6. After treatment with δ-elemene and z-VAD-fmk, inhibition of δ-elemene-mediated caspase-3 activation (12 h: $p<0.05$; 24 h: $p<0.01$; 48 h: $p<0.01$) was accompanied by the marked attenuation of δ-elemene-induced apoptotic cell death (12 h: $p<0.01$; 24 h: $p<0.01$; 48 h: $p<0.001$). The results also indicate that the activation of caspase-3 contributes to δ-elemene-induced apoptosis.

Mitochondrial Pathway Involvement in Apoptosis Induced by δ-Elemene To examine whether δ-ele-

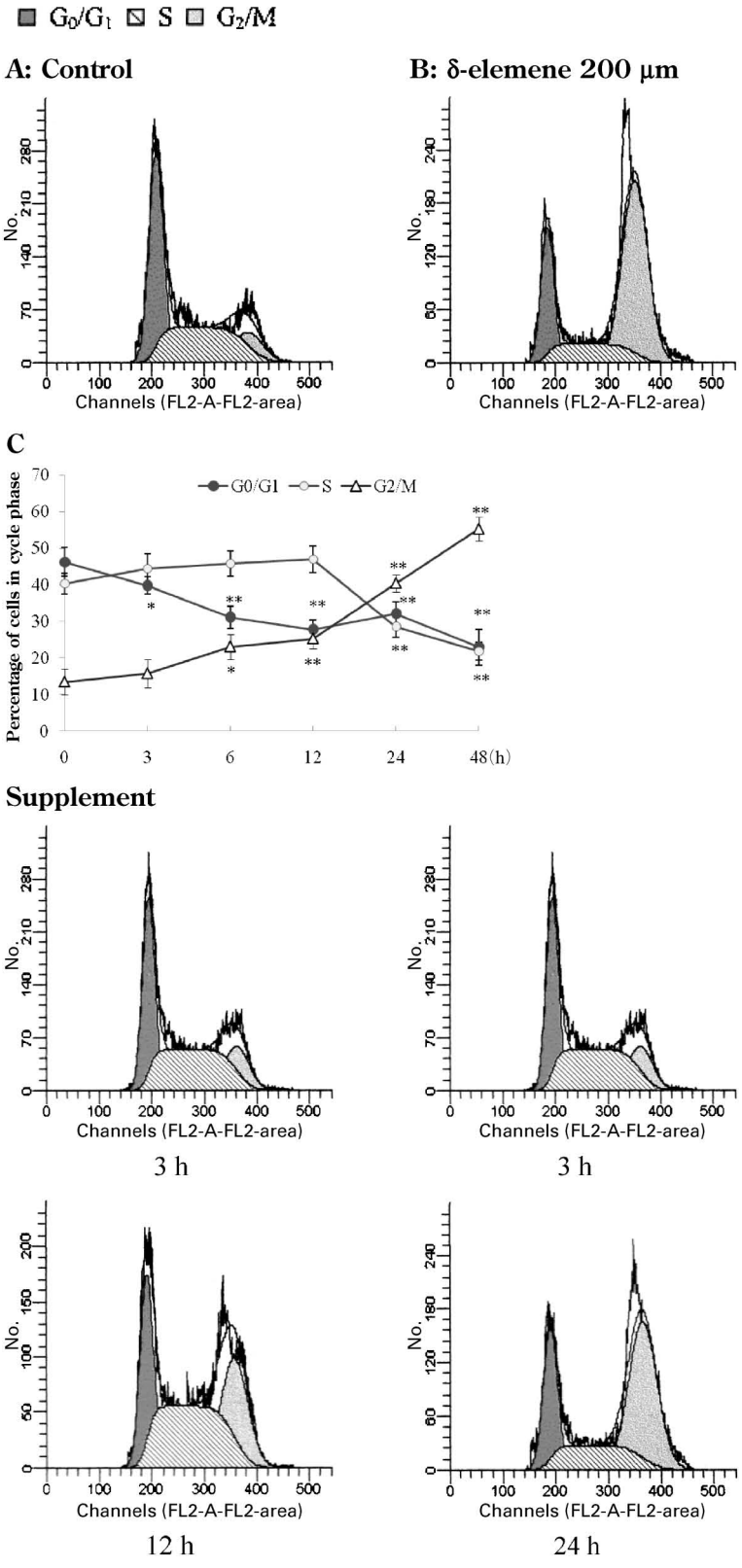

Fig. 3. δ-Elemene Gradually Interfered with HL-60 Cells in G_2/M, with a Concomitant Decrease in G0/G1 and S Populations

HL-60 cells were treated with 200 μM of δ-elemene for the indicated periods. (A) Histograms demonstrate cell number *versus* DNA content of viable cells in different groups as indicated. (B) The proportion of cells in each of the three phases of the cell cycle was determined using ModFit LT. Values represent mean$\pm$S.D. of 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control group.

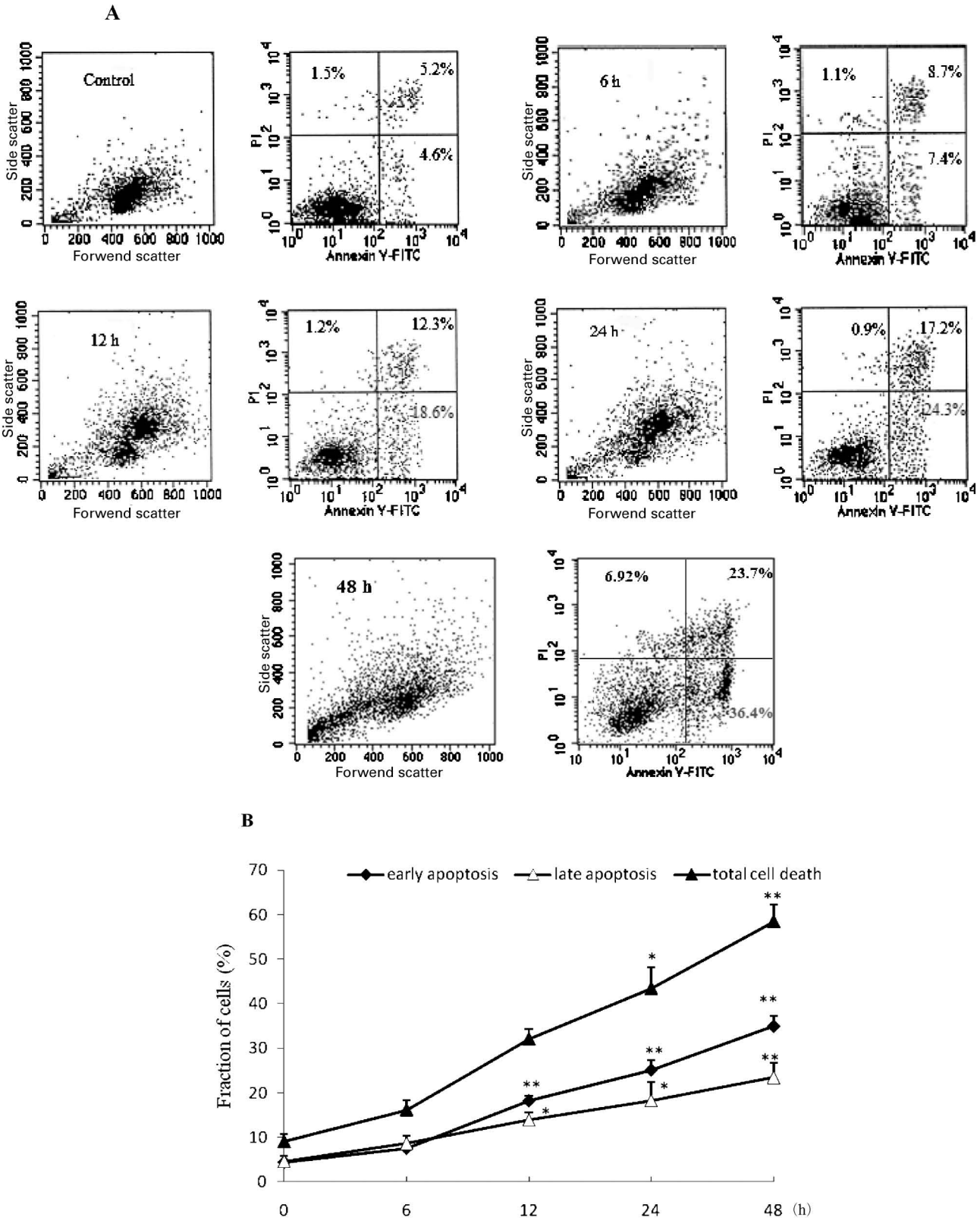

Fig. 4. Apoptosis and Necrosis in HL-60 Cells Treated with δ-Elemene

Cells were cultured with 200 μM of δ-elemene for the indicated times, and stained with FITC-conjugated annexin V (AnV) and PI, followed by flow cytometric analysis. (A) The light scattering and staining profiles of a representative experiment as shown in panels I and II. The numerals in the quadrants indicate the percentages of cells in that particular quadrant with respect to the total. Early apoptotic populations are found in the lower-right quadrants, while necrotic or late apoptotic cells are localized in the upper-right quadrants. (B) The percentage of early apoptosis, late apoptosis, and total cell death in HL-60 cells with treatment with 200 μM of δ-elemene for the indicated periods. Values represent mean $\pm$ S.D. of 3 independent experiments.

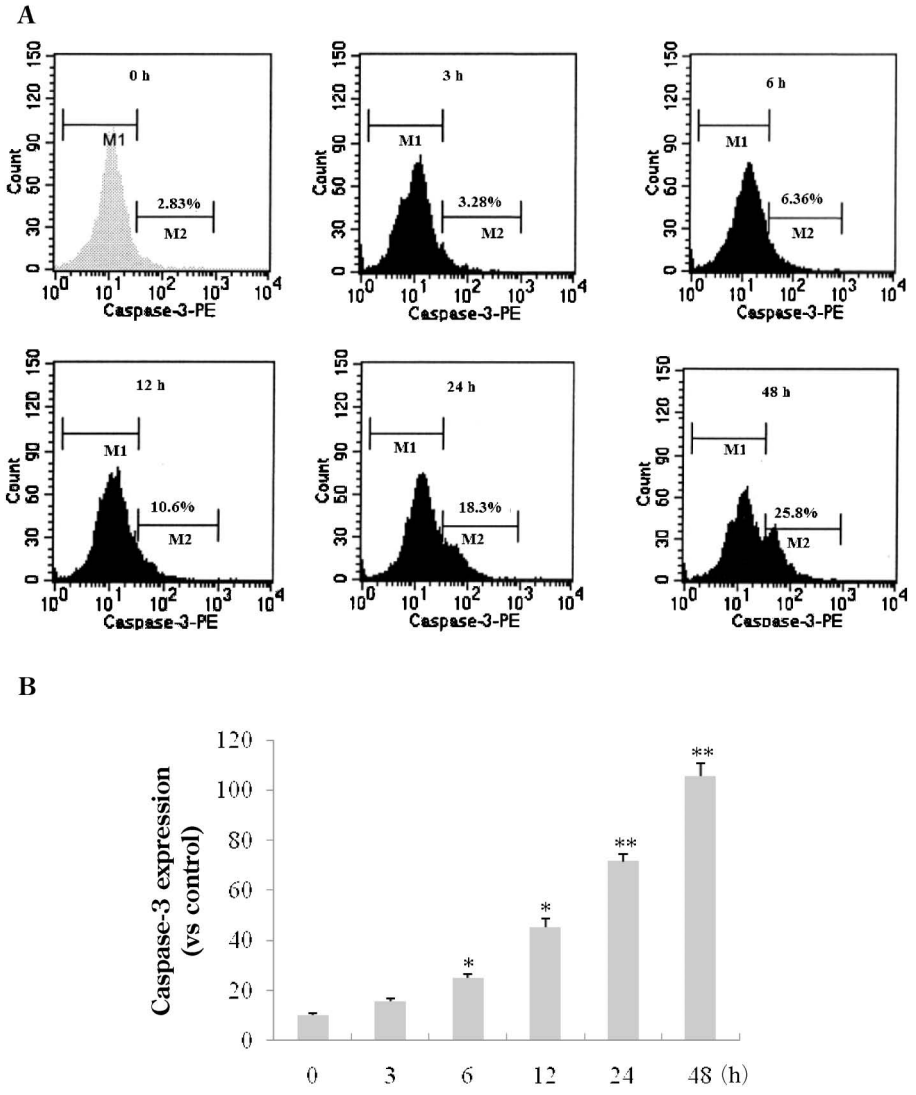

Fig. 5. Activation of Caspase-3 with δ-Elemene in HL-60 Cells

Cells were cultured with 200 μM of δ-elemene for 0, 3, 6, 12, 24, or 48 h and assayed for active caspase-3 expression by flow cytometry. (A) Representative histograms from 3 independent experiments. The percentages signify the expression of active caspase-3. (B) Marked activation of caspase-3 was found in δ-elemene treatment groups after 6 h. Values represent mean $\pm$ S.D. of 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with control cultures.

mene could induce a change in $\Delta\psi$m, we measured $\Delta\psi$m in HL-60 cells treated with this compound. Following treatment with δ-elemene, a reduction in $\Delta\psi$m of the cells was observed (Fig. 7A). The $\Delta\psi$m started to decrease at 6 h following treatment with δ-elemene, and the reduction occurred in a time-dependent manner. Compared with control cells, HL-60 cells with 200 μM δ-elemene treatment for 6 h exhibited green JC-1 fluorescence, which is consistent with a loss of mitochondrial membrane polarization. As shown in Fig. 7B, the trends of increasing incidences of compromised $\Delta\psi$m for 6, 12, or 24 h were 1.34$\pm$0.27-, 2.00$\pm$0.50-, and 4.64$\pm$1.51-fold with respect to the untreated control cells, respectively (Fig. 7B, $^{*}p<0.05$, $^{**}p<0.01$, respectively, *vs.* untreated controls).

Translocation of Bax to mitochondria can alter the outer mitochondrial membrane permeability. It allows the release of pro-apoptotic proteins such as cyt c and AIF into the cytosol. To test whether the apoptosis-inducing effect of δ-elemene was related to the translocation of Bax into mitochondria, we determined the level of Bax in the mitochondrial fraction of HL-60 cells treated with δ-elemene with Western blotting. As shown in Fig. 8, the level of Bax in mitochondria of HL-60 cells treated with δ-elemene increased in a time-dependent manner, implying that the translocation of Bax into mitochondria was in-

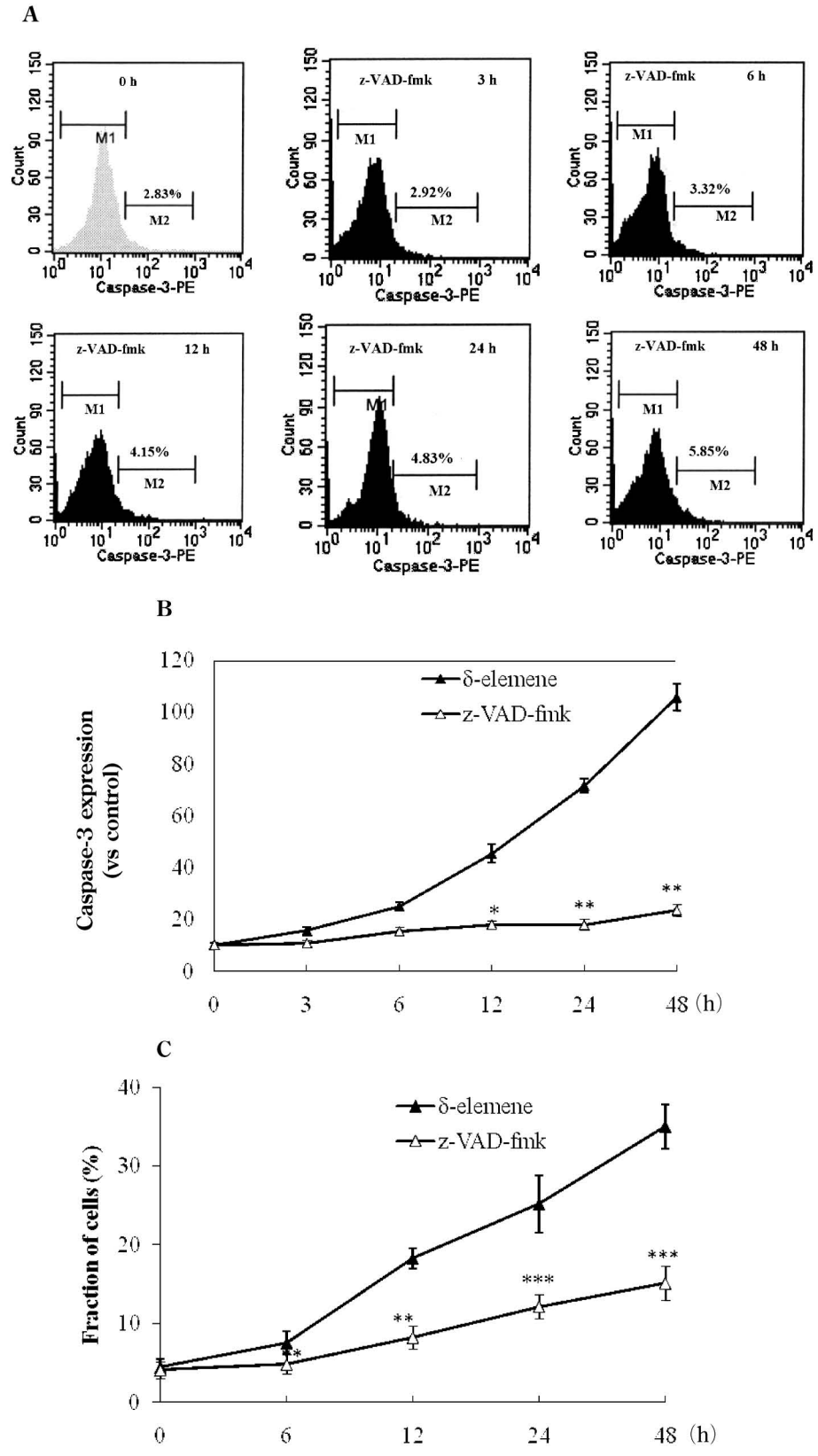

Fig. 6. z-VAD-fmk Inhibited δ-Elemene-induced Apoptosis and Caspase-3 Activation in HL-60 Cells

(A) Time-dependent inhibition of δ-elemene-induced caspase-3 activation by z-VAD-fmk. (B) Time-dependent inhibition of δ-elemene-induced apoptosis by z-VAD-fmk. The results are expressed as mean±S.D. of 3 determinations. $^{**}p<0.01$, $^{***}p<0.001$ compared with 200 μM of δ-elemene.

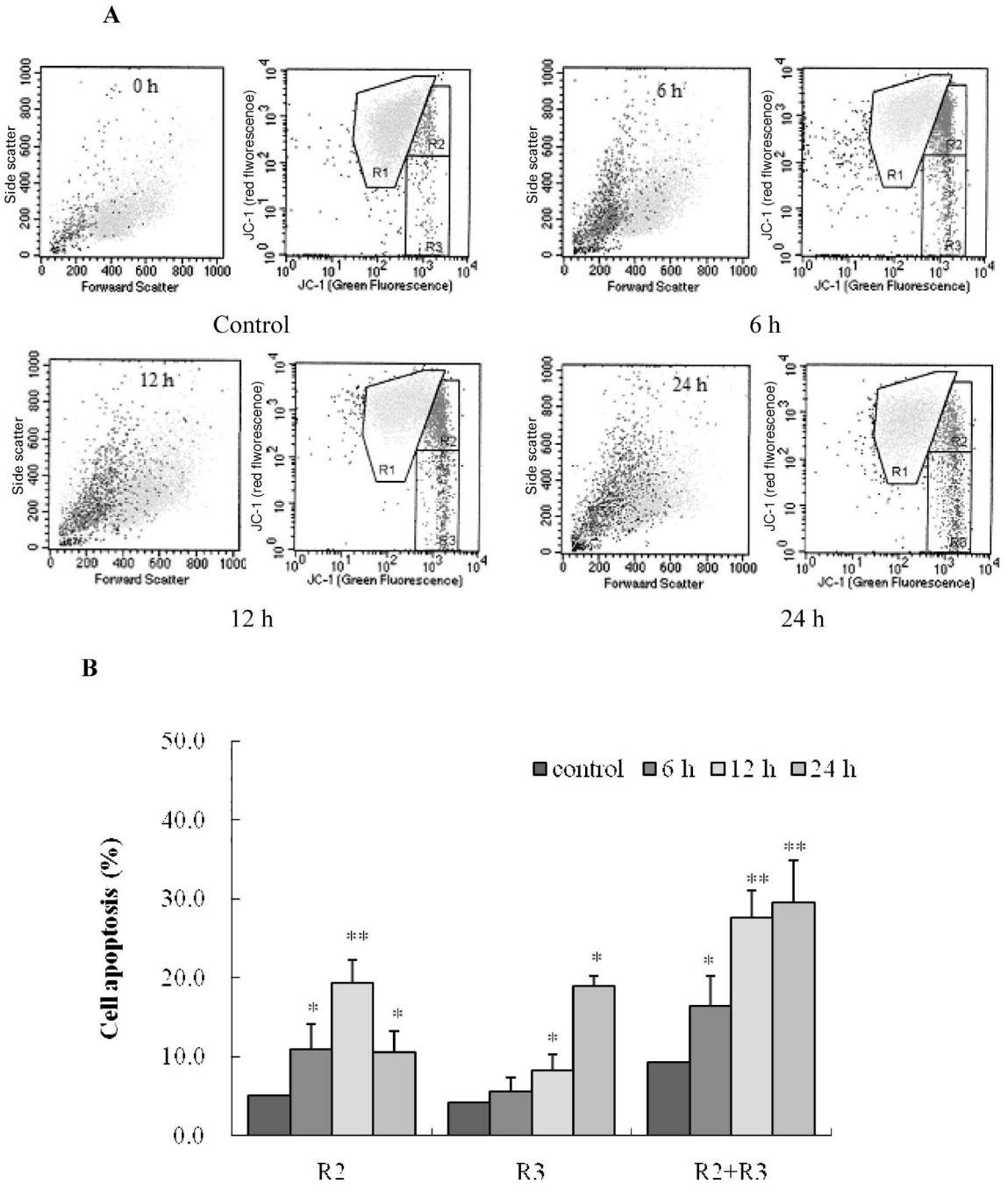

Fig. 7. Reduction in $\Delta\psi$m of HL-60 Cells Treated with δ-Elemene

Cells were treated with 200 μM of δ-elemene for 6, 12, and 24 h. After treatment, cells were stained with JC-1 for 15 min at 37℃ and analyzed using flow cytometry. (A) After δ-elemene treatment, depolarization of $\Delta\psi$m became evident as indicated by the increased cell polulation in R2. Cells found in region R1 had little scattering properties typical of apoptosis. (B) The mean and S.D. of the results were obtained from three independent experiments. Mitochondrial damage was most marked at 24 h after δ-elemene treatment. Values represent mean±S.D. of 3 independent experiments. $^*p<0.05$ compared with control cultures.

volved in cell death induced by δ-elemene. In the case of pretreatment with z-VAD-fmk, the mitochondrial translocation of Bax was not changed (Fig. 8C), indicating that Bax translocation is upstream or independent of caspase activation.

HL-60 cells were treated with 200 μM of δ-elemene for various determined periods of time (Fig. 8). Analysis of cyto c and AIF in the cytosol by Western blotting showed that the δ-elemene levels in these cells had increased in a time-dependent manner (Fig. 8). This implies that after δ-elemene treatment, the release of cyto c and AIF from mitochondria into the cytosol was involved in cell death induced by δ-elemene.

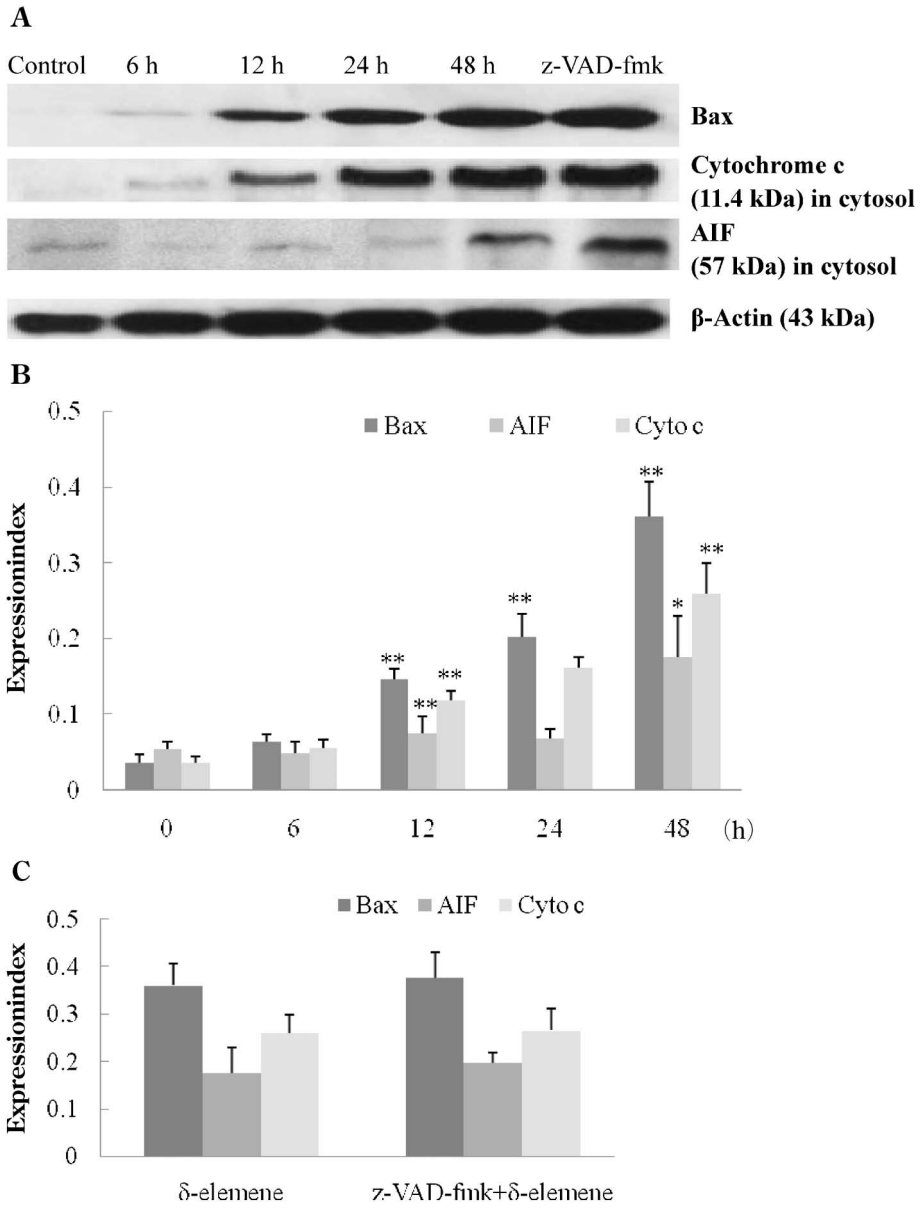

Fig. 8. Effect of δ-Elemene on Bax, Cyt c, and AIF Release from Mitochondria in HL-60 Cells

Translocation of Bax into mitochondria and the release of cyt c and AIF from mitochondria into the cytosol in HL-60 cells treated with δ-elemene. HL-60 cells were treated with 200 μM of δ-elemene for the indicated periods up to 24 h. After treatment, mitochondrial and cytosolic fractions were extracted. (A) Levels of Bax in the mitochondrial fraction and levels of cyt c and AIF in the cytosolic fractions were analyzed with Western blot analysis. (B) Expression of proteins pretreated with 200 μM of δ-elemene for the indicated times. (C) Expression of proteins pretreated with z-VAD-fmk 3 h prior to treatment with 200 μM of δ-elemene for 48 h. Values represent mean$\pm$S.D. of 3 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with control cultures.

In the case of pretreatment with z-VAD-fmk, the mitochondrial translocation of Bax, the release of cyt c and AIF were not changed (Fig. 8C), indicating that Bax translocation or cyt c release is upstream or independent of caspase activation.

DISCUSSION

Some leukemia cells easily undergo apoptosis, which is mediated by caspase activation, fueling the notion that apoptosis is the goal of chemotherapy.[17,18] Cell death has two characteristics, apoptosis and necrosis. The major difference between the two is the active participation of the cells in the process.[19]

To our knowledge, the antileukemic effects of δ-elemene on the HL-60 cell line had not been documented. Our data suggested that many HL-60 cells treated with δ-elemene were in the early apoptotic and late apoptoctic/necrotic stages, along with caspase-3 activation in the cells. While the externalization of PS in HL-60 cells did not appear until after 12 h, marked

annexin V binding was seen at the end of 24-h incubation. The active form of caspase-3 was significantly up-regulated in the cells at 12 h.

It was reported that δ-elemene could increase the permeability transition pore opening, which is associated with mitochondrial membrane depolarization.[3] Our results showed that δ-elemene-induced mitochondrial damage was found in HL-60 cells. Those cells with perturbed mitochondria were apoptotic or necrotic, as depicted by their distinctive light-scattering properties. Mitochondrial perturbation has been suggested to be an important step in many types of drug-mediated apoptosis.[20,21] We postulate that the mitochondrial integrity compromised by δ-elemene might be one of the initiation steps in the induction of apoptosis in HL-60 cells.

By monitoring the changes in cell cycle profiles, we confirmed that δ-elemene promoted the accumulation of cells in the G2/M phase of the cell division cycle, and at the same time depleted the populations in G0/G1 and S phase. It is suggested that δ-elemene slowed down cell cycle progression by delaying mitosis (M phase). A closer examination of the cell cycle profiles obtained in δ-elemene-treated HL-60 cells revealed that δ-elemene caused a slight increase in S phase cells at 3 h, followed by significant increases in cells in both S and G2/M phases at 12 h. The data indicated that δ-elemene could interfere with the cell cycle at the G2/M phase and induce apoptosis in HL-60 cells in a time-dependent manner.

In some cell lines, caspase-3 plays a direct role in proteolytic cleavage of cellular proteins responsible for progression to apoptosis.[22,23] In an attempt to identify the pathway of apoptosis in HL-60 cells in response to δ-elemene, caspase-3 activation was investigated. The results in Figs. 6 and 7 show that δ-elemene could induce caspase-3 activation as detected by the decrease in the δ-elemene-induced caspase-3 activities after treatment with z-VAD-fmk. Other investigators demonstrated that the ratio between pro- and antiapoptotic proteins determines in part the susceptibility of cells to a death signal.[24,25] The activation of caspase-3 induced poly (ADP-ribose) polymerase cleavage, chromosomal DNA breaks, and finally the occurrence of apoptosis.[26] The broad-spectrum caspase inhibitor z-VAD-fmk had no effect on the δ-elemene-induced increase in cyt c, but inhibited the ensuing caspase-3 cleavage and activity[3] and blocked the apoptosis in this study, which suggests that caspase-3 has a critical role in the apoptosis induced by δ-elemene. As shown in this study, δ-elemene exerts apoptotic effects by acting on caspase-3. δ-Elemene initiates activation of caspases and in turn the caspase activation leads to apoptotic cell death. The mechanism δ-elemene uses to activate caspases on HL-60 cells remains unknown. While we only demonstrated that δ-elemene triggered apoptosis through a caspase-3 pathway, our results should help in further understanding the δ-elemene-induced apoptosis in HL-60 cells, which is important since δ-elemene has a potential role in therapies of human leukemias in the future. Interestingly, these results provide a correlation between caspase-3 activity and δ-elemene-induced apoptosis and deserved to be pursued further.

The signal transduction pathway leads to apoptosis, a highly organized physiological mechanism for destroying injured and abnormal cells[27,28] that may play an important role in some cellular processes of the cell cycle. Furthermore, mitochondrial cyt c releases an important control point in caspase activation and apoptosis.[29] It is suggested that susceptibility to the apoptosis-inducing effects of chemotherapeutic drugs may depend on an intrinsic ability of tumor cells to respond by apoptosis.[30,31]

Mitochondria respond to multiple death stimuli including those in which proapoptotic Bcl-2 family proteins such as Bax and Bak induce mitochondrial membrane permeabilization to cause the release of apoptotic molecules into the cytosol.[32,33] Our study showed that δ-elemene elevated the level of Bax in mitochondria, which was followed by a release of cyt c and AIF from mitochondria into the cytosol by a reduction in the mitochondrial membrane potential. The findings presented here indicate that δ-elemene sequentially induces Bax activation, cyt c release, caspase activation, and apoptosis. z-VAD-fmk could protect HL-60 cells from apoptosis without blocking cyt c release and Bax translocation, indicating that δ-elemene may initiate an intrinsic apoptotic pathway.

Through these experiments, we showed that 1) *in vitro* δ-elemene treatment interfered with the cell cycle at the G2/M phase in HL-60 cells; 2) time- and dose-dependent apoptosis of HL-60 cells was induced by δ-elemene; 3) in HL-60 cells, z-VAD-fmk inhibited the δ-elemene-induced apoptosis and caspase-3 activation; and 4) δ-elemene may stimulate the activation of Bax to induce cyt c release *via* the mitochon-

drial pathway.

In conclusion, we have demonstrated that δ-elemene induces apoptosis in HL-60 cells by activating caspase-3, interfering with the cell cycle at the G2/M phase. Future studies should focus on cell signaling and the biological significance of δ-elemene-induced apoptosis, which may lead to exploring the mechanisms of chemotherapeutic potency of δ-elemene in human leukemias.

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