B-Cell Lymphoma-2 Over-Expression Protects δ-Elemene-Induced Apoptosis in Human Lung Carcinoma Mucoepidermoid Cells *via* a Nuclear Factor Kappa B-Related Pathway

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Received March 9, 2011; accepted May 8, 2011; published online May 27, 2011

δ-Elemene, an antitumor component, is a chemical compound isolated from *Curcuma wenyujin*, a Chinese traditional herb. We examined whether δ-elemene could affect apoptosis in human lung carcinoma mucoepidermoid NCI-H292 cells, and test whether and how the over-expression of B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma extra large (Bcl-xL) could off-set the effect of δ-elemene on cell growth. The result demonstrated that δ-elemene significantly induced apoptosis of NCI-H292, as shown by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, DNA fragmentation measurement, Annexin V (AnV) binding of externalized phosphatidylserine and the mitochondrial probe JC-1 using flow cytometry. Treatment of NCI-H292 with δ-elemene increased both p38 mitogen-activated protein kinase (MAPK) and inducible nitric oxide synthese (iNOS) levels, suggesting these two molecules maybe relate to the apoptotic effect of δ-elemene. The cells with Bcl-2 or Bcl-xL over-expression showed an elevation of nuclear factor kappa B (NF-kappa B) activity, accompanying a significant reduction of δ-elemene-induced apoptosis. Furthermore, inhibition of NF-kappa B by IkBαSR, which is a powerful inhibitor of NF-kappa B, restored the ability of δ-elemene to induce apoptosis in the cells transfected with Bcl-2. These data strongly indicated that the apoptotic effect of δ-elemene on NCI-H292 was closely associated with the activity of NF-kappa B, which was up-regulated by Bcl-2 and Bcl-xL. In conclusion, δ-elemene induced apoptosis in NCI-H292 cells. The apoptotic effect of δ-elemene could be significantly offset by over-expression of either Bcl-2 or Bcl-xL. Bcl-2 and Bcl-xL were able to increase the activity of NF-kappa B, which was a known anti-apoptotic molecule in human lung cancer cells.

Key words apoptosis; B-cell lymphoma-2; lung cancer; nuclear factor kappa B; *Curcuma wenyujin*

Elemene is a naturally occurring compound that can be isolated from the traditional Chinese medicinal herb *Curcuma wenyujin*, which is used to treat tumors in Chinese folk medicine.[1,2] Elemene exists as an essential oil mixture of β-, γ- and δ-elemene (Fig. 1). The major antitumor active component, β-elemene, possesses broad-spectrum clinical activity in treatment of various tumors and is known to induce apoptosis.[3] δ-Elemene is another isomeric compound of β-elemene with a different site of double bond. Our previous study was shown that δ-elemene exerts antitumor activity by inducing apoptosis in several type cells,[4—6] and possesses no signs of bone marrow cells and normal liver cell lines WRL-68[7] suppression. However, the effect of δ-elemene on human lung cancer cells has not yet been investigated.

Nuclear factor kappa B (NF-kappa B), a redox-sensitive transcription factor, is regulated by various apoptotic stimuli or inhibitors. A number of reports have shown that NF-kappa B is inhibited by apoptosis-inducing agents in human cancer cells.[8—10] Like B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma extra large (Bcl-xL), NF-kappa B itself may serve as a pro-survival agent in various circumstances.[11] The activation of NF-kappa B is known to induce the expression of Bcl-2 and Bcl-xL.[12—16] Inducible loss of NF-kappa B activity is associated with the down- regulation of anti-apoptotic Bcl-2 family members and the occurence of apoptosis. Furthermore, the promoter regions of both Bcl-2 and Bcl-xL genes contain binding sites for NF-kappa B.[15—17] On the other hand, Bcl-2 can also stimulate or restore NF-kappa B activity.[18,19]

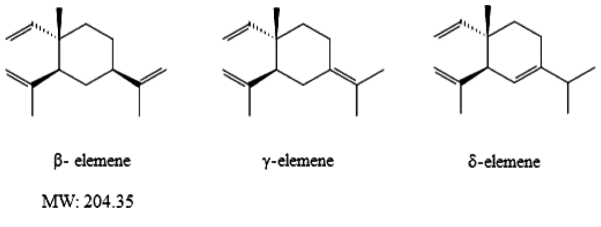

Fig. 1. Chemical Structure of β-Elemene, γ-Elemene, and δ-Elemene

In the present experiment, we examined whether δ-elemene, an effective antitumor component from *Curcuma wenyujin*,[1—3] could affect apoptosis in human lung cancer cells. In order to test whether and how the over-expression of Bcl-2 and Bcl-xL offsets the effect of *Curcuma wenyujin* on cell growth, we transfected human lung cancer cells with either Bcl-2 or Bcl-xL gene.

MATERIALS AND METHODS

Cell Culture Human lung carcinoma mucoepidermoid cell line NCI-H292 was purchased from American Type Culture Collection (ATCC, #CRL1848, MD, U.S.A.), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen), 100 U/ml of peni-

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cillin, and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY, U.S.A.). 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 in Ref. 1) (assay >97%, GC). Cells were treated with either δ-elemene or β-elemene at concentrations indicated. δ-Elemene was prepared as previous description.[2—4)]

Determination of Anti-proliferation Activity A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantify cell death/viability. Cell death was also determined by DNA fragmentation assay kit (Roche Molecular Biochemicals, Mannheim, Germany), which measures apoptotic cell death by detection of bromodeoxy uridine (BrdU)-labled DNA fragments in the cytoplasm of affected cells. Briefly, DNA fragments in the cytoplasm were labeled with BrdU. After the BrdU-labeled DNA fragments were then isolated from the cells using the solutions provided by the kit, the BrdU-labeled DNA fragments were then detected using an enzyme-linked immunosorbent assay (ELISA) method. The entire assay was performed according to the manufacturer's instruction. Appearance of DNA fragments has been considered as a hallmark of apoptosis.

Quantitation of Cell Apoptosis Apoptotic cells were also evaluated using a Annexin-V fluorescein isothiocyanate (FITC) kit (Alexis, Switzerland) and JC-1 (300 nM, Molecular Probes) assay by flow cytometry (FACScan, Becton Dickinson, CA, U.S.A.). Glutathione (GSH) was added 1 or 3 h prior to the treatment with δ-elemene and β-elemene. Data were analyzed using LYSIS II software. $(AnV^+)PI^-$ cells were considered early apoptotic, and $(AnV^+)PI^+$ cells were considered late apoptotic and necrotic.

DNA Transfection Stable transfection was performed using cationic lipid reagent, LipofectAMINE (Life Technologies, Rockville, MD, U.S.A.). The experiment was carried out according to the manufacturer's instruction. NCI-H292 cells (80—85% confluence) were transfected with 500 ng of plasmid DNA in serum-free conditions. After the cells were incubated for 6 h in serum-free medium containing DNA and LipofectAMINE, an equal volume of growth medium containing 20% serum was then added without removing the transfection mixture. The supernatant was changed with complete medium at 24 h following the start of transfection. At 72 h after transfection, the cells were passaged into the selective medium containing geneticin selective antibiotic, G418. The concentration of G418 for the selection was previously determined by dose–response assay. Positive stably-transfected cells were selected by G418 (0.4 mg/ml) after 2 months.

Western Blot Analysis Cell samples were homogenized with ice-cold PBS and then lysed in a solution containing 8 M urea, 0.1 M $Na_2H_2PO_4$ and 0.01 M Tris–HCl. Supernatants were obtained after centrifugation at 10000×g. Proteins were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were then electrophoretically transferred from the gel onto nitrocellulose membranes and the membranes were blocked for 1 h in phosphate buffered saline (PBS)-Tween buffer containing 5% dry milk powder (fat free) at room temperature. The membranes were then incubated with a primary antibody for 1 h. After washing, the membranes were incubated with a secondary antibody, immunoglobulin G (IgG)-horse-radish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Finally, they were treated with the reagents in the chemiluminescent detection kit (ECL system, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions. Anti-human actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used to detect human actin, which was used as a control for equal loading. Anti-human antibodies: Bcl-2, Bcl-xL, p65, inducible nitric oxide (iNOS) and p38 mitogen-activated protein kinase (p38 MAPK) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The densities of the protein bands corresponding to the size were determined with a GSP-700 scanner with Quantity One image software (Hercules, CA, U.S.A.).

Nuclear Protein Extraction and NF-kappa B Activity Assay Nuclear protein was isolated according the procedure described.[19)] Briefly, NCI-H292 cells were harvested, washed in PBS, and collected by centrifugation. The cell pellet was resuspended in 0.5 ml of 10 mM Tris–HCl (pH 7.5)/5 mM $MgCl_2$/0.05% (v/v) Triton X-100 and lysed with 20 strokes in a homogenizer. The homogenate was centrifuged at 10000×g for 15 min at 4 °C. The pellet was obtained. The nuclei pellet volume was estimated and the pellet was resuspended in an equal volume of 10 mM Tris–HCl (pH 7.4)/5 mM $MgCl_2$, followed by the addition of 1 nuclear pellet volume of 1 M NaCl/10 mM Tris–HCl (pH 7.4)/4 mM $MgCl_2$. The lysing nucleus was left on ice for 30 min and then centrifuged at 10000×g for 15 min at 4 °C. The supernatant (nuclear extract) was removed and 80% glycerol was added so that the final glycerol concentration was 20% (v/v). The concentration of the nuclear protein was determined. NF-kappa B activity was measured by an enzyme immunoassay kit from Oxford Biomedical Research (Oxford, MI, U.S.A.), which employed an oligonucleotide containing the DNA binding NF-kappa B consensus sequence. If there is any NF-kappa B presented in the sample, it will specifically bind to the oligonucleotide coated on the plate and the DNA-bound NF-kappa B can be selectively recognized by the antibody to NF-kappa B subunit, p50 or p105.

Inhibition of NF-kappa B A recombinant replication-deficient adenovirus, Ad5IkB, contains an IkB construct in which serines 32 and 36 are mutated to alanines, driven by the cytomegalovirus promoter-enhancer. This mutant IkB cannot be phosphorylated, and therefore irreversibly binds to NF-kappa B, preventing its activation.[20,21)] Ad5IkB (a generous gift from Dr. Z. M. Liu) was used as previously described.[21)] The adenovirus, Ad5LacZ, which contains the *Escherichia coli* β-galactosidase gene, was used as a control. Both viruses were grown in 293 cells and purified as the previous description.[21)] For adenovirus infection, sub-confluent cells (*ca.* 80%) were infected with virus in serum-free medium at multiplicity of infection (MOI) of 10—500 for 12 h. The free virus was then washed off and fresh media containing serum was added to the cells. The cells were cultured for another 12 h before experiments.

Statistical Analysis All results were obtained in at least three independent experiments. Data were expressed as means±standard error. Representative data were analyzed for statistical significance by software SPSS11.5. Differences among groups were analyzed by One-way analysis of variance (ANOVA), multiple comparisons used LSD and SNK's

test for homogeneity of variance and Dunnett's T3-test for heterogeneity of variance. $p<0.05$ was considered statistically significant.

RESULTS

δ-Elemene Induced Cell Death in Dose- and Time-Dependent Manners δ-Elemene induced NCI-H292 cell death in a dose-dependent manner between the concentrations of 0 and 400 μM (Fig. 2A). At the concentration of 400 μM, over 80% of the cells were dead after 24-h treatment. Compared with 12-h treatment with 200 μM of δ-elemene, the cell death was gradually increased to about 2.4 folds and 2.9 folds after 24-h and 48-h treatments with δ-elemene respectively. Both the dose- and the time-dependent manners were similar to the results obtained when the cells were treated with β-elemene (Fig. 2).

DNA Fragmentation in NCI-H292 Cells Treated with δ-Elemene The cell death induced by either δ-elemene or β-elemene was mainly caused by apoptosis, as the similar result was obtained by DNA fragmentation assay (Fig. 3). The occurrence of DNA fragment is a typical Marker for apoptosis. δ-Elemene is able to significantly induce DNA fragmentation in a dose- and time-dependent manner. There was 4.4 fold increase in 200 μM δ-elemene for 24 h with vector control cells (Fig. 3A); and the amount of DNA fragmentation was elevated after treatment for 12 h (3.9 fold that of the vector control), and it peaked at 48 h (5.1 fold that of the vector control) after treatment with 200 μM δ-elemene (Fig. 3B).

Cell Death Induced by δ-Elemene Is in Manner by Apoptosis Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine (PS) externalization on the outer membrane of apoptotic cells and the mitochondrial transmembrane potential ($\Delta\psi_m$).

Results showed that apoptosis was triggered after 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 23.4% 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 control, more death events were observed in δ-elemene treated cells for 48 h. Such result was similar to that of $\Delta\psi_m$, which started to decrease at 6 h following treatment with δ-elemene and the reduction occurred in a time-dependent manner. Compared with control cells, NCI-H292 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. The trends of increasing inci-

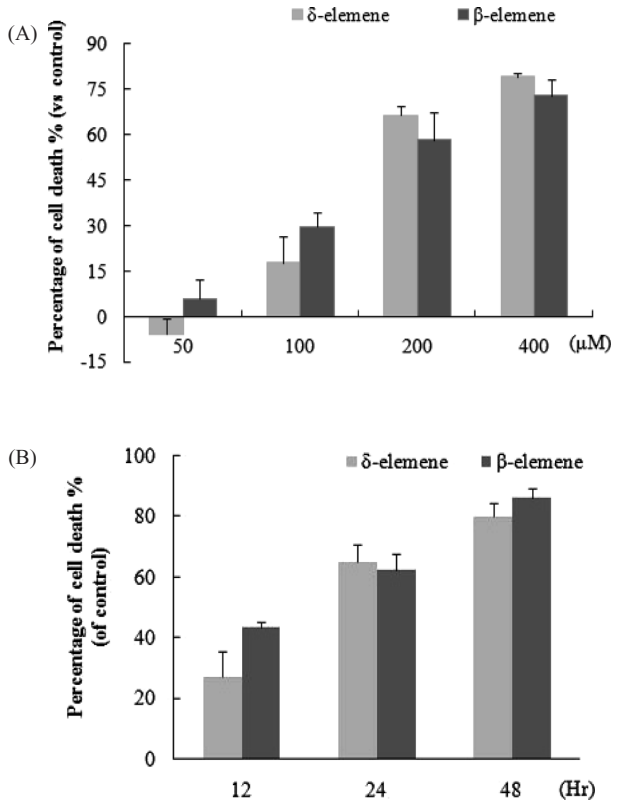

Fig. 2. Cell Death Induced by δ-Elemene and β-Elemene

After NCI-H292 cells were treated by 0—400 μM of δ-elemene and β-elemene for 24 h (A), the cell was harvested and the MTT assay was performed to measure cell death. For the time–course study, the cell was treated with either 200 μM of δ-elemene and β-elemene for 12, 24 and 48 h (B). At the end of the time point, the cell death was measured by MTT assay. Control cells were treated with vehicle only. The result of MTT was expressed as cell viability, which was calculated by the following formula: inhibition (%)=[A_{570} (control)−A_{570} (drug)]/A_{570} (control)×100%. Each experiment was repeated at least 3 times.

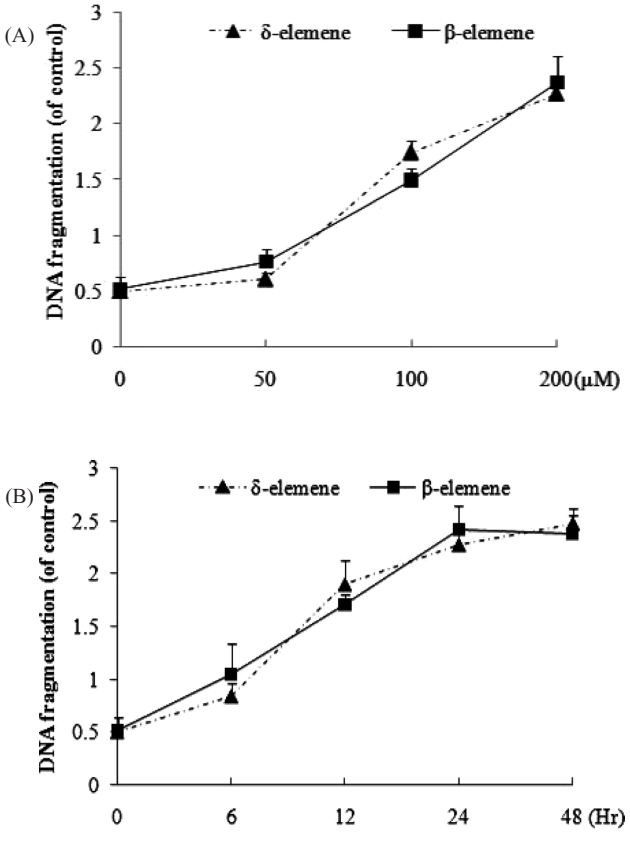

Fig. 3. Effect of δ-Elemene on DNA Fragmentation in NCI-H292 Cells

Cells were treated with various concentrations of δ-elemene and β-elemene for 24 h (A) or with 200 μM of δ-elemene at indicated periods (B). The DNA fragmentation was determined by cellular DNA fragmentation ELISA assay. DNA fragments from damaged cells were released into the culture supernatant and labeled by BrdU. The BrdU-labled DNA was quantified using a monoclonal antibody against BrdU. Each point represents a mean of three independent experiments with triplicate wells. The result of DNA fragmentation detection assay was expressed as Apoptotic Index, which was calculated by the following formula: [1−absorbance (test well-background)/absorbance (control-background)]×100%.

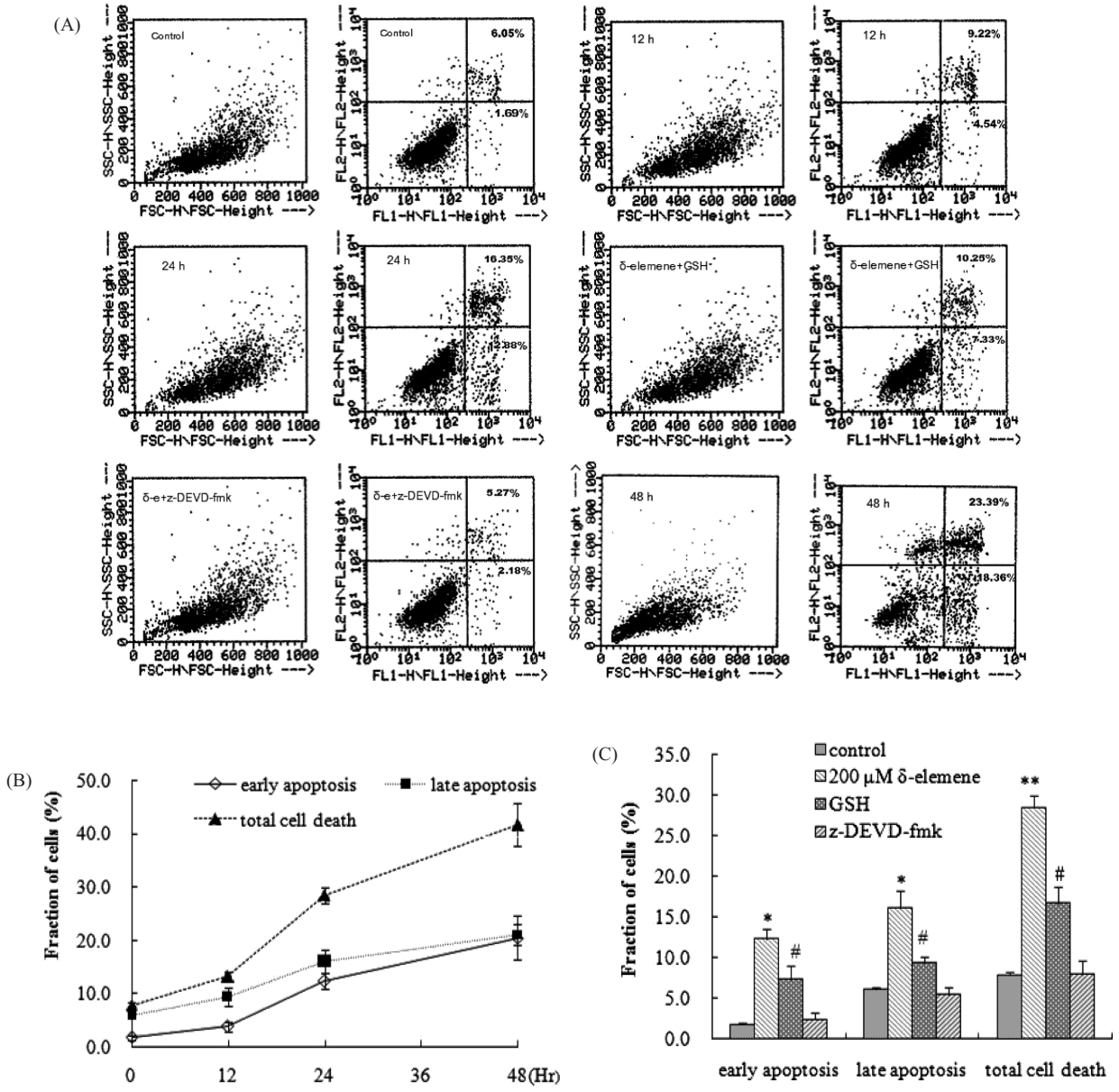

Fig. 4. Apoptosis and Necrosis in NCI-H292 Cells Treated with δ-Elemene

NCI-H292 cells were cultured with 200 μM of δ-elemene for indicated time, and stained with FITC-conjugated Annexin V (AnV) and PI, followed by the 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 total. Early apoptotic populations are found in the lower-right quadrants, while necrotic or late apoptotic cells are localized in the upper-right quadrant. (B) The percentage of early apoptosis, late apoptosis and total cell death in NCI-H292 cells with treatment of 200 μM δ-elemene for indicated periods. (C) The percentage of early apoptosis, late apoptosis and total cell death in NCI-H292 cells with treatment of 200 μM δ-elemene, GSH or z-DEVD-fmk. For the experiment using GSH and z-DEVD-fmk, NCI-H292 cells were pretreated with 1 mM GSH or with 2 μM z-DEVD-fmk 1 or 2 h prior to the treatment by 200 μM of δ-elemene for 24 h. Values represent Mean$\pm$S.D. of 3 independent experiments performed. $*p<0.05$, $**p<0.01$ *vs.* control; $\#p<0.01$ *vs.* 200 μM δ-elemene.

dences of compromised $\Delta\psi_m$ for 6, 12 or 24 h were 1.94, 3.50 and 6.42 fold with respect to the untreated control, respectively (Fig. 5B). Furthermore, the rate of early apoptosis in NCI-H292 cells treated with δ-elemene was inhibited by GSH or z-DEVD-fmk (Fig. 4C) for 24 h.

δ-Elemene Upregulated the Expression of p38 MAPK and iNOS but Downregulated the Levels of Bcl-2 and Bcl-xL To understand how δ-elemene regulates cell death, we measured several cell death-related molecules in NCI-H292 cells treated with δ-elemene. By Western blot analysis, we found that the expression of p38 MAPK and iNOS was much higher in the cells treated with δ-elemene than those treated with vehicle, whereas the levels of p65, Bcl-2 and Bcl-xL were lower (Fig. 6). The similar expression patterns of these molecules analyzed were also observed in the cells treated with β-elemene (Fig. 6).

Over-Expression of Bcl-2 and Bcl-xL Protected Cell Death Induced by δ-Elemene To investigate how the over-expression of Bcl-2 and Bcl-xL affects the cell death in NCI-H292 induced by δ-elemene, we transfected NCI-H292 cells with Bcl-2, Bcl-xL, or pcDNA3.1 plasmids and the cells were named as NCI-H292/Bcl-2, NCI-H292/Bcl-xL and NCI-H292/mock cells respectively. Positive clones were selected by G418 and the over-expression of Bcl-2 or Bcl-xL was confirmed using Western blot (Fig. 7).

In order to clarify whether the over-expression of Bcl-2 or

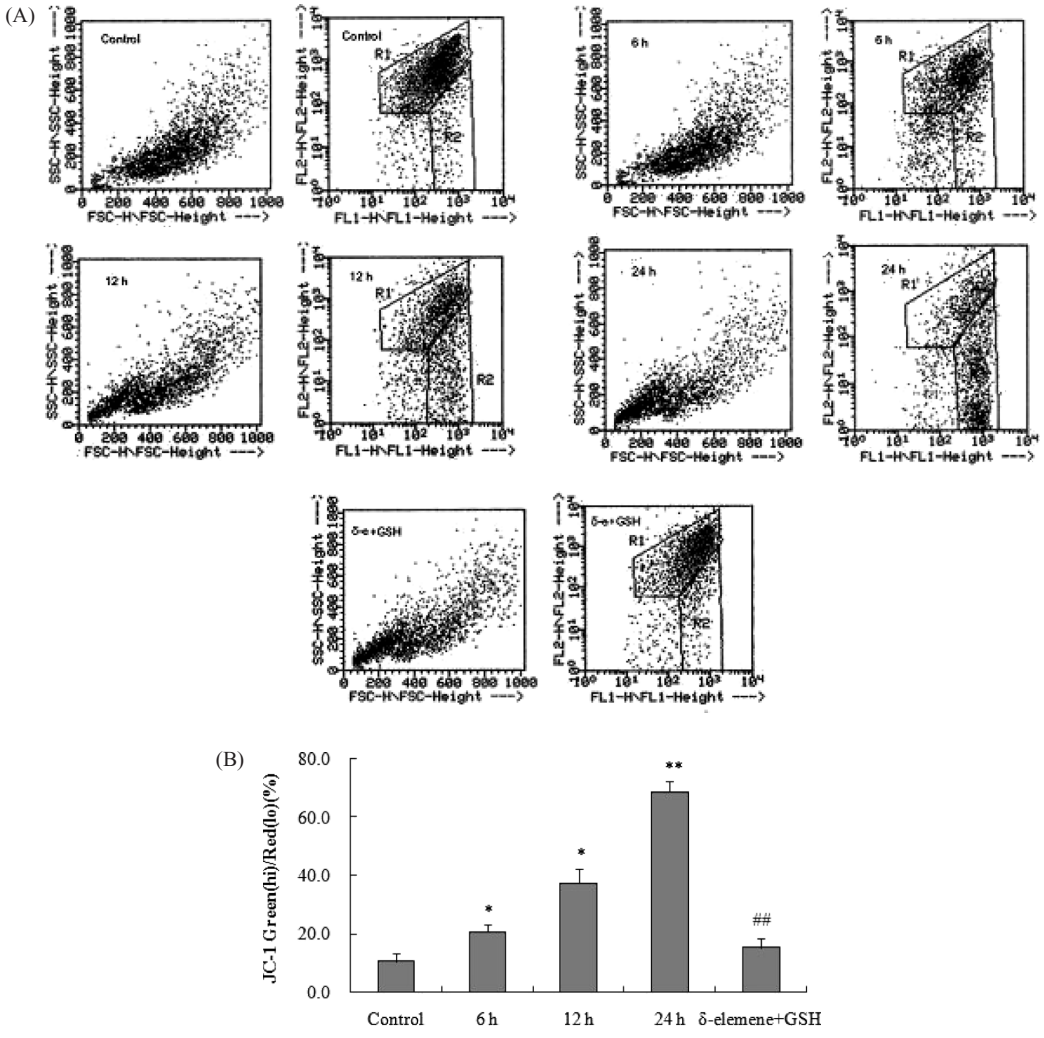

Fig. 5. Reduction in $\Delta\psi_m$ of NCI-H292 Cells Treated with δ-Elemene

NCI-H292 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 °C and analyzed by flow cytometry. In the experiment of the inhibition of GSH on the reduction in $\Delta\psi_m$, the NCI-H292 cells were pretreated with 1 mM GSH 1 h prior to treatment with 200 μM of δ-elemene for 24 h. (A) After δ-elemene treatment, depolarization of $\Delta\psi_m$ became evident as indicated by increased cell polulation in R2. Cells found in region R1 had little scattering properties typical of apoptosis. (B) The mean and SEM of the results were obtained from three independent experiments. Mitochondrial damage was most marked at 24 h post δ-elemene treatment (* $p<0.05$, ** $p<0.01$ *vs.* control; ## $p<0.01$ *vs.* 200 μM of δ-elemene for 24 h).

Bcl-xL in NCI-H292 cells blocks the chemical induction of apoptosis, NCI-H292/Bcl-2, NCI-H292/Bcl-xL and NCI-H292/mock cells were treated with a known cell death inducer, β-elemene (Fig. 8). Following treatment, cell death was measured by MTT assay. The result showed that NCI-H292/Bcl-2 and NCI-H292/Bcl-xL cells had a significantly higher percentage of living cells compared to NCI-H292/mock cells, indicating that the NCI-H292/Bcl-2 and NCI-H292/Bcl-xL transfectants were functional. These cells were then treated with 200 μM of δ-elemene (Fig. 8). While NCI-H292/mock cells displayed marked death in response to δ-elemene treatment, NCI-H292/Bcl-2 and NCI-H292/Bcl-xL cells showed approximately 93% and 85% protection from δ-elemene-induced death respectively.

Over-Expression of Bcl-2 and Bcl-xL Increased NF-kappa B Activity NF-kappa B activity is known to be closely associated with Bcl-2 and Bcl-xL expression.[10—14,16,17] In order to determine how the over-expression of Bcl-2 and Bcl-xL affects the NF-kappa B activity in NCI-H292 cells treated either δ-elemene or β-elemene, the cells transfected with either Bcl-2 or Bcl-xL were incubated with 200 μM of δ-elemene or β-elemene or vehicles. The nuclear protein was isolated after incubation. The cells transfected with an empty pcDNA3.1 vector and the cells without any transfection were used as controls. NF-kappa B activity was significantly higher in the cells with Bcl-2 or Bcl-xL (Fig. 9). It appeared that Bcl-2 was more effective in raising NF-kappa B activity than Bcl-xL.

Inhibition of NF-kappa B Offsetted the Protective Effect Offered by Bcl-2 and Bcl-xL Since the over-expression of Bcl-2 and Bcl-xL increased the activity of NF-kappa B and protected the cells from apoptosis induced by δ-elemene and β-elemene, it would be interesting to know whether the inhibition of NF-kappa B activity could diminish the protective effect offered by Bcl-2 and Bcl-xL. A super-repressor of NF-kappa B activity (Ad5IkB), which is a mutated non-degradable IkBα resistant to phosphorylation and degradation, was used to inhibit the activity of NF-kappa B. NCI-H292/Bcl-2 cells regained their sensitivity to apoptosis induced by δ-elemene after being treated by Ad5IkB (Fig. 10).

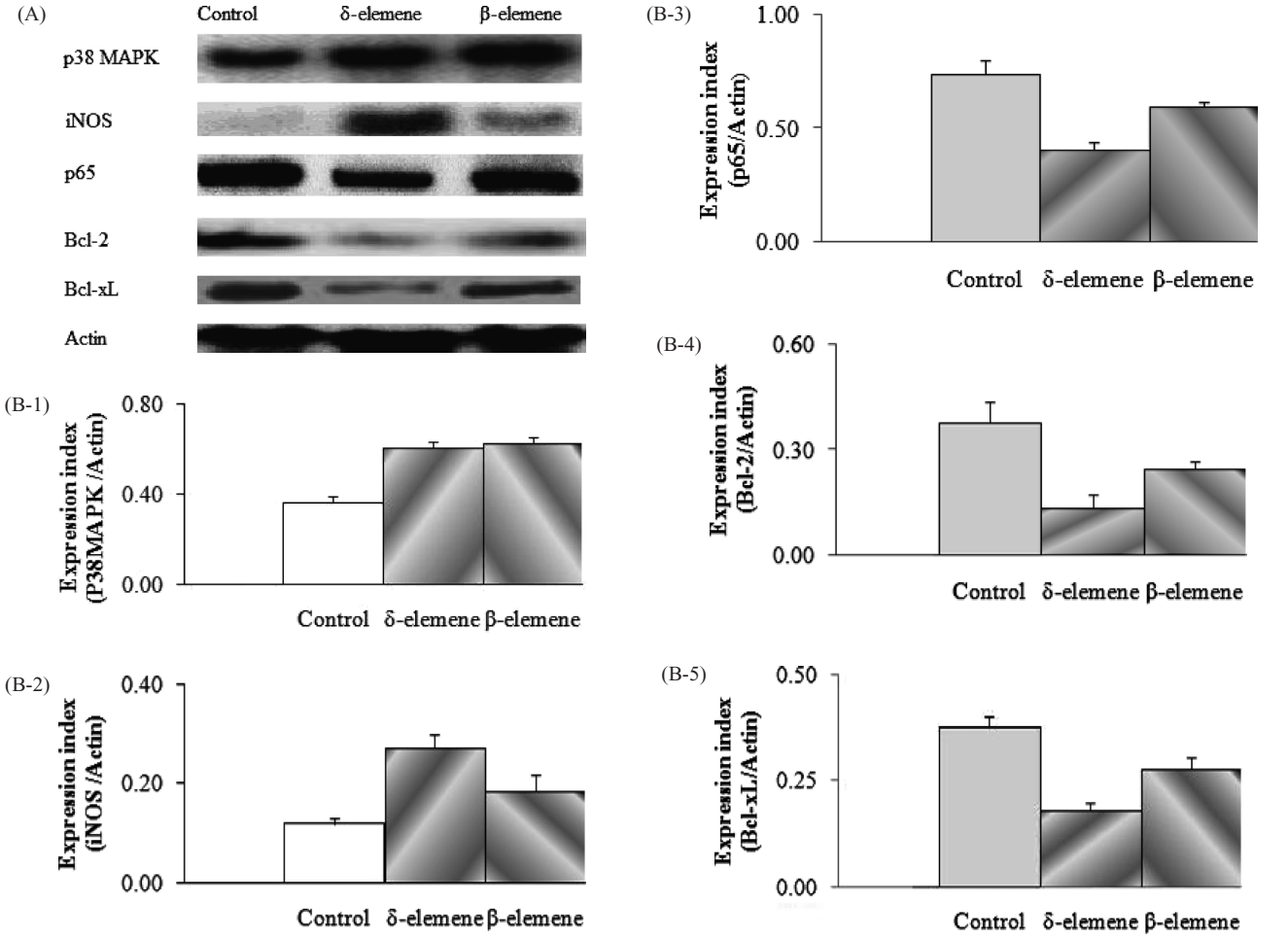

Fig. 6. Effect of δ-Elemene and β-Elemene on the Protein Levels of Bcl-2 and Bcl-xL

NCI-H292 cells were treated with 200 μM of δ-elemene and β-elemene for 24 h and then cell lysates were obtained for Western blot analysis of p38 MAPK (38 kDa), iNOS (130 kDa), p65 (65 kDa); Bcl-2 (28 kDa) and Bcl-xL (32 kDa). Actin protein (43 kDa), which is constitutively expressed, was used as a control. The densities of the protein bands corresponding to the size were determined and the relative amount of the target protein was shown as Expression Index, which was calculated by the formula: the density of the control (actin) band/ the density of the target band.

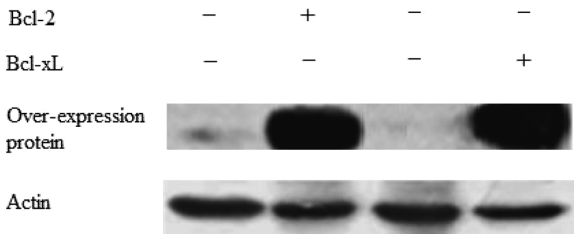

Fig. 7. Over-Expression of Bcl-2 and Bcl-xL in NCI-H292

After stable transfection, NCI-H292/Bcl-2 and NCI-H292/Bcl-xL cells were established. Cell lysates were isolated from the cells and Bcl-2 (28 kDa) and Bcl-xL (32 kDa) levels were determined by Western blot analysis. The cells transfected with an empty vector and with Bcl-2 or Bcl-xL genes.

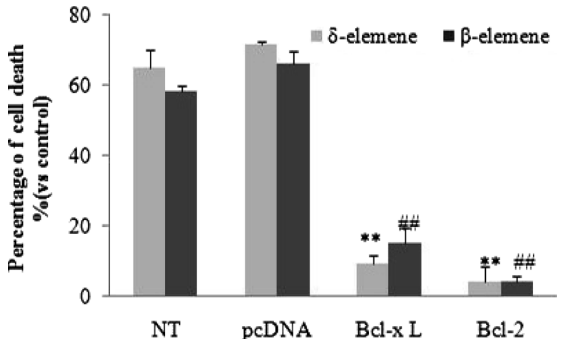

Fig. 8. Protection of Cell Death Induced by δ-Elemene and β-Elemene with Bcl-2 or Bcl-xL

NCI-H292 cells with extraneous genes (Bcl-2 or Bcl-xL) or without were incubated with 200 μM of δ-elemene and β-elemene or vehicles for 24 h. The cell inhibition effect was measured by the MTT assay. The result of MTT was expressed as inhibitory rate (see Fig. 1 for details). NT: No transfection. $^{**}p<0.01$, $^{\#\#}p<0.01$ means δ-elemene or β-elemene compared with the controls.

However, a significant portion of NCI-H292/Bcl-xL cells was not protected after treated by Ad5IkB (Fig. 10), suggesting that Bcl-xL could still function, to a certain degree, to protect the cells from death when the activity of NF-kappa B was inhibited. It is noticed that, cell death induced by δ-elemene or β-elemene in both NCI-H292/Bcl-2 and NCI-H292/Bcl-xL cells were prevented when the cells were treated by Ad5IkB (Fig. 10A), indicating that both Bcl-2 and Bcl-xL lost their anti-apoptotic effects when the activity of NF-kappa B was suppressed. In Fig. 10B, Ad5LacZ, as a control in this experiment, has no effect on the protection of δ-elemene-induced cell death by Bcl-2 or Bcl-xL over-expression.

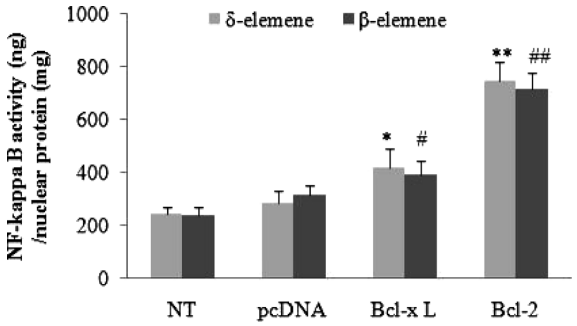

Fig. 9. NF-kappa B Activity in the Cells with Bcl-2 and Bcl-xL Over-Expression

NCI-H292 cell culture was the same as that described in Fig. 4 and cells were with 200 μM of δ-elemene and β-elemene (A) or vehicles for 24 h. After culture, the nuclear protein was isolated for the determination of NF-kappa B activity, which was measured by a commercially-available ELISA kit. Each point represents a mean of three independent experiments with triplicate wells. NT: No transfection. $^{**}p<0.01$, $^{*}p<0.05$ or $^{\#\#}p<0.01$, $^{\#}p<0.05$ means δ-elemene or β-elemene compared with the controls.

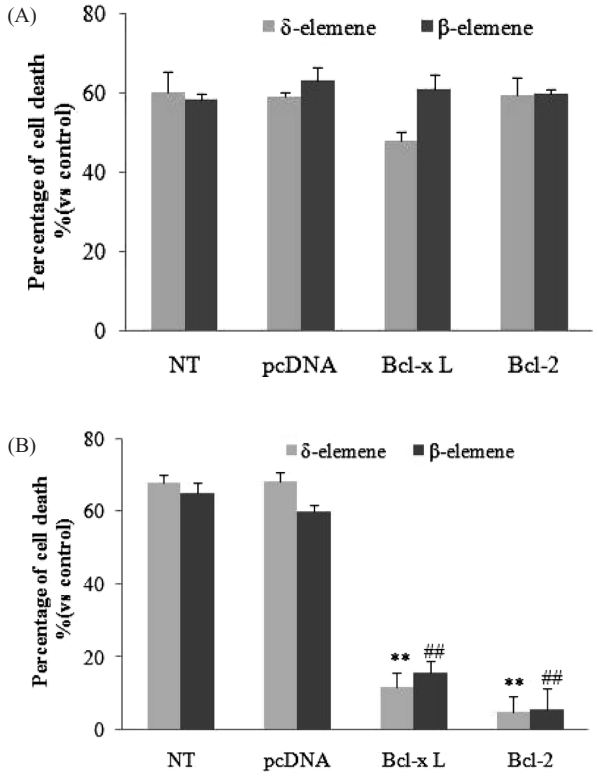

Fig. 10. Attenuation of Bcl-2 and Bcl-xL Protection by the Inhibition of NF-kappa B Activity

The cells were infected with either Ad5IkB (A) or Ad5LacZ (B), as described in Methods. After the infection, the cells were stimulated with 200 μM of δ-elemene and β-elemene or vehicle alone for 24 h. The cell death was measured by the MTT assay. The result of MTT was expressed as Inhibitory rate (see Fig. 1 for details). NT: No transfection. $^{**}p<0.01$,or $^{\#\#}p<0.01$ means δ-elemene or β-elemene compared with the controls.

DISCUSSION

Although δ-elemene, a chemical component isolated from Wenyujin, has been known to inhibit the growth of several types of tumor cells,[4—7] the mechanism is not yet clear and its effect on human lung cancer cells has not yet been studied before. In the present study, we demonstrated that δ-elemene was able to induce cell death *via* an apoptotic pathway and in a similar manner to β-elemene. β-Elemene, as an antitumor drug, is widely applied to clinic in China.

δ-Elemene seems to be able to achieve the same efficiency of β-elemene, as both drugs at these concentrations induced a similar number of cell death and also they were able to generate very similar time–course curves. Therefore, by comparing δ-elemene with a well-known cell growth inhibitor,[22] such as β-elemene, it will help us to evaluate whether δ-elemene can be considered to be an anti-cancer agent.

The occurrence of DNA fragment is a typical Marker for apoptosis. The Annexin-V FITC binding assay was used to distinguish apoptotic cells from necrotic cells.[23] And mitochondria dysfunction has been recognized as a key step in apoptosis.[24] All of these results show that the apoptosis of NCI-H292 cells with δ-elemene is in a time-dependent manner.

In the present study, we found that a number of molecules were altered at protein levels in human lung cancer cells treated by δ-elemene. Both p38 MAPK and iNOS were increased by either δ-elemene or β-elemene treatment.

NF-kappa B has recently emerged as a major player in a variety of human cancers mainly because of its ability to protect transformed cells from apoptosis. We found that accompanying the decreased NF-kappa B activity by δ-elemene was the reduction of two anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL. The finding is not surprising, as both the promoter regions of Bcl-2 and Bcl-xL genes contain binding sites for NF-kappa B and thus their expression is controlled by the activity of NF-kappa B.[14—16] It has been reported that NF-kappa B activity is negatively associated with p38 MAPK. In human melanoma cells, p38 MAPK suppresses NF-kappa B activity, which sensitizes melanoma cells to UV-, ribotoxic and radiomimetic chemicals-induced apoptosis.[25] Although δ-elemene also reduces NF-kappa B activity and elevates the level of p38 in human lung cancer cells, the pathway linked these two molecules needs further study.

The decreased NF-kappa B activity, the reduction of Bcl-2 and Bcl-xL levels in the lung cancer cells treated with δ-elemene were in agreement with a significantly increase in the number of cell death and the amount of DNA fragmentation, a classic indicator of apoptosis. In order to further support our findings, we transfected the cells with Bcl-2 or Bcl-xL genes and tested how these transfectants behaved in response to δ-elemene stimulation. After transfection with either Bcl-2 or Bcl-xL, the cells showed a significant resistance to the δ-elemene treatment and the percentage of cell death was markedly different from those without Bcl-2 or Bcl-xL transfection. And also the activity of NF-kappa B was significantly higher in the cells with Bcl-2 or Bcl-xL genes. The current result may support the notion proposed by the previous reports that Bcl-2 over-expression preserves or restores NF-kappa B and thus inhibits apoptosis.[17,18]

Furthermore, using a super-repressor of NF-kappa B, the sensitivity of the cells with Bcl-2 transfection to δ-elemene treatment is superior to that of with Bcl-xL. Therefore, it appears that the protective mechanism of Bcl-2, to some extent, is much more NF-kappa B- dependent than Bcl-xL, in lung cancer cells in response to δ-elemene stimulation.

Traditional Chinese herb medicine is usually tranquil, both δ-elemene and β-elemene can offer anti-cancer effects prob-

ably *via* a similar mechanism. Nevertheless, further studies need to evaluate δ-elemene as an anti-cancer agent and also its side effects.

CONCLUSION

δ-Elemene induced apoptosis in NCI-H292 cells and this apoptotic effect was associated with the high level of p38 and iNOS expression. The apoptotic effect of δ-elemene could be significantly offset by over-expression of either Bcl-2 or Bcl-xL. Bcl-2, and to the less extent, Bcl-xL, were able to increase the activity of NF-kappa B, which was a known anti-apoptotic molecule in human lung cancer cells. Inhibition of NF-kappa B can restore the ability of δ-elemene to induce apoptosis. Therefore, apoptotic effect of δ-elemene on lung cancer cells is closely related to the activity of NF-kappa B.

Acknowledgments The authors thank Dr. Z. M. Liu for the Ad5IkB and Ad5LacZ adenoviruses and Ms. S. Mo for her technical assistance.

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