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Induction of Apoptosis by Aqueous Extract of *Cordyceps militaris* Through Activation of Caspases and Inactivation of Akt in Human Breast Cancer MDA-MB-231 Cells

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Cordyceps militaris **is well known as a traditional medicinal mushroom and has been shown to exhibit immunostimulatory and anticancer activities. In this study, we investigated the apoptosis induced by an aqueous extract of *C. militaris* (AECM) *via* the activation of caspases and altered mitochondrial membrane permeability in human breast cancer MDA-MB-231 cells. Exposure to AECM induced apoptosis, as demonstrated by a quantitative analysis of nuclear morphological change and a flow cytometric analysis. AECM increased hyperpolarization of mitochondrial membrane potential and promoted the activation of caspases. Both the cytotoxic effect and apoptotic characteristics induced by AECM treatment were significantly inhibited by z-DEVD-fmk, a caspase-3 inhibitor, which demonstrates the important role of caspase-3 in the observed cytotoxic effect. AECM-induced apoptosis was associated with the inhibition of Akt activation in a time-dependent manner, and pretreatment with LY294002, a PI3K/Akt inhibitor, significantly increased AECM-induced apoptosis. The results indicated that AECM-induced apoptosis may relate to the activation of caspase-3 and mitochondria dysfunctions that correlate with the inactivation of Akt.**

Keywords: *Cordyceps militaris*, apoptosis, caspase-3, Akt

Mitochondria-mediated apoptosis is regulated by the Bcl-2 family of proteins, which can promote (pro-apoptotic Bax members) or inhibit (anti-apoptotic Bcl-2 members) apoptosis [15]. Bcl-2 preserves the integrity of the outer mitochondrial membrane and thereby prevents the release of pro-apoptotic factors from mitochondria [24, 26]. Although the effects of Bcl-2 on pro-apoptotic factor release are well described, other functions of Bcl-2 in the upstream and downstream regulation of apoptosis remain poorly understood. Bcl-2 seems to inhibit many agent-induced apoptosis through inactivation of caspase-3 [12, 23].

Akt represents a major downstream target of phosphatidylinositol 3-kinase (PI3K) [28], and has been linked, through both indirect and direct mechanisms, to a wide variety of anti-apoptotic functions [3, 17]. Thus, it is tempting to speculate that PIK inhibitors promote the lethal effects of other agents by blocking the activation of Akt and one or more of its downstream targets, thereby lowering the threshold for mitochondrial damage and apoptosis [25, 32].

Many natural dietary extracts in mushrooms have been shown to be potent in the treatment of various cancer cells *in vitro* by induction of cell cycle arrest and apoptosis [7, 8, 10, 30]. Mixed extracts from mushrooms might contain different chemotherapeutic materials with more than one mechanism of action, thus possessing a combination of different chemotherapeutic effects. It is therefore very important to identify combination effects of mixed extracts from mushrooms. *Cordyceps* or Dong-Chong Xia-Cao (winter worm-summer grass) has been used as a tonic for longevity, endurance, and vitality for thousands of years by the Chinese [34]. *Cordyceps* extract has been reported to have immunomodulatory and antitumor effects [13, 14, 19, 31]. Our previous results also demonstrated that *C. militaris*, a species of *Cordyceps*, has potent cytotoxic effects against cancer cells and immunostimulatory effects [11, 22].

In the present study, in order to further evaluate the cytotoxic effects of *C. militaris* in cancer cells, we investigated the effect of an aqueous extract of *C. militaris* (AECM)-induced apoptosis in MDA-MB-231 human breast carcinoma cells. Our data indicated that AECM-induced apoptosis was associated with modulation of pro- and anti-apoptotic members of the Bcl-2 family, loss of mitochondrial membrane permeability, and activation of caspase-3. In addition, AECM decreased the Akt activation,

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and inhibition of the PI3K/Akt pathway enhanced the AECM-induced apoptosis.

Materials and Methods

Reagents

4,6-Diamidino-2-phenylindole (DAPI), propidium iodide (PI), and the specific mitochondrial dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were obtained from Sigma (St. Louis, MO, U.S.A.). Caspase activity assay kits were obtained from R&D Systems (Minneapolis, MN, U.S.A.). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL, U.S.A.). Z-DEVD-fmk (caspase-3 inhibitor) was obtained from Calbiochem (San Diego, CA, U.S.A.). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.) and GIBCO-BRL (Gaithersburg, MD, U.S.A.), respectively. All other chemicals not specifically cited here were purchased from Sigma.

Antibodies

Antibodies against Bcl-2, Bcl-xL, Bax, poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-8, caspase-9, and Akt were purchased form Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and the antibody against phospho-Akt was purchased from Cell Signaling (Beverly, MA, U.S.A.). The antibody against β-actin was obtained from Sigma. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham.

Cell Culture and Growth Study

Human breast cancer cell lines (MCF-7 and MDA-MB-231) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO_2. *C. militaris* was supplied by Daejeon University Oriental Hospital (Daejeon, Korea) and the aqueous extract of *C. militaris* (AECM) was prepared as previously described [19]. For the growth study, cells were cultured in the absence and presence of variable concentrations of AECM for 24 h. The cells were trypsinized, washed with phosphate-buffered saline (PBS), and the viable cells were scored with a hemocytometer through the exclusion of trypan blue.

Flow Cytometry Analysis for Measurement of the Sub-G1 Phase

The cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol, and stored at 4°C. Prior to analysis, the cells were washed once again with PBS, suspended in a cold PI solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber; Becton Dickinson, San Jose, CA, U.S.A.), and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence. The sub-G1 population was calculated to estimate the apoptotic cell population.

Nuclear Staining with DAPI

After treatment with AECM, the cells were harvested, washed with PBS, and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5 mg/ml DAPI solution for 10 min at room temperature. The cells were washed 2 more times with PBS and analyzed under a fluorescent microscope (Carl Zeiss, Germany).

Detection of Apoptosis by Annexin-V FITC Staining

The cells were washed with PBS and resuspended in annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. Aliquots of cells were incubated with annexin-V fluorescein isothiocyanate (FITC), mixed, and incubated for 15 min at room temperature in the dark. PI at a concentration of 5 μg/ml was added to distinguish the necrotic cells. The apoptotic cells (V^+/PI) were measured within 1 h by a flow cytometer.

Measurement of Mitochondrial Membrane Potential (MMP) Loss

The MMP ($\Delta\Psi_m$) of intact cells was measured by a flow cytometer with the lipophilic cationic probe JC-1. JC-1 is a green dye used to elucidate the membrane potential of energized mitochondria by promoting the formation of red-fluorescent JC-1 aggregates. The ratio of JC-1 depends only on the membrane potential, with a decrease being indicative of membrane depolarization. Briefly, the cells were harvested, loaded with 2 μg/ml of JC-1 at 37°C for 20 min, and then analyzed using a flow cytometer.

Protein Extraction and Western Blot Analysis

Cells were harvested, washed once with ice-cold PBS, and gently lysed for 20 min in ice-cold lysis buffer (20 mM sucrose, 1 mM EDTA, 20 mM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mg/ml pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml aprotinin). Supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Samples were stored at −80°C or immediately used for immunoblotting. Aliquots containing 30 μg of total protein were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes for immunoblot analysis using the indicated primary antibodies. HRP-conjugated secondary antibodies were detected using an enhanced ECL detection system.

Determination of Caspase Activity

Caspase activities were determined by colorimetric assays using caspase-3, -8, and -9 activation kits according to the manufacturer's protocol. The kits utilize synthetic tetrapeptides labeled with *p*-nitroanilide (*p*NA) (DEVD-*p*NA for caspase-3, IETD-*p*NA for caspase-8, and LEHD-*p*NA for caspase-9, respectively). Briefly, the cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37°C. The caspase activity was determined by measuring changes in absorbance at 405 nm using a microplate reader.

Results

AECM Induces Growth Inhibition and Apoptosis in MDA-MB-231 Cells

We have recently shown that AECM was able to inhibit cell growth in human cancer cells [11, 22]. Here, we further assessed the effect of AECM on human breast cancer cell death. First, MCF-7 and MDA-MB-231 cells were treated with the indicated concentration of AECM and subjected

to MTT assays. As shown in Fig. 1A, treatment with 0.2–1.6 mg/ml of AECM for 24 h resulted in only a slight decrease in cell viability in MCF-7 cells. Notably, MDA-MB-231 cells treated with AECM showed significantly more reduced cell viability than the treatment did in MCF-7 cells. To investigate the viability decrease in MDA-MB-231 cells, we first evaluated the amount of cells with sub-G1 DNA content using flow cytometric analysis. Treatment with AECM (0.8 mg/ml) constantly resulted in significant accumulation of cells with sub-G1 DNA content (Fig. 1C). In addition, treatment with 0.8 mg/ml of AECM showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with AECM (Fig. 1B). Additionally, flow cytometry analysis with annexin V and PI staining was used to determine the magnitude of apoptosis elicited by AECM. As shown in Fig. 1D, the annexin-V positive cells increased in the AECM-treated MDA-MB-231 cells compared with the untreated control cells. These results suggest that AECM significantly induced apoptosis in human breast MDA-MB-231 cells.

AECM Modulates Pro- or Anti-Apoptotic Proteins and Induces Loss of MMP

To determine the role of the Bcl-2 family in AECM-mediated apoptosis, we investigated the effect of the protein levels of anti-apoptotic Bcl-2 and Bcl-xL, and pro-apoptotic Bax by Western blot analysis. As shown in Fig. 2A, treatment with AECM resulted in a decrease in the levels of Bcl-2 and an increase of Bax in MDA-MB-231 cells exposed to 0.2–0.8 mg/ml of AECM for 24 h, in a dose-dependent fashion. However, the levels of Bcl-xL remained unchanged. In addition, we investigated the effect of AECM on the levels of MMP by measuring JC-1 dye retention. Treatment of cells with AECM significantly induced reduction in the MMP levels, and may lead to apoptosis in MDA-MB-231 cells (Fig. 2B). These data demonstrate the importance of a mitochondrial amplification step in AECM-induced apoptosis.

Inhibition of Caspase-3 Activity Restores Cell Apoptosis by AECM

It has been shown in recent studies that caspases are very important regulators of apoptosis induced by apoptosis stimuli [1, 2]. As shown in Fig. 3A, Western blot analysis reveals that treatment with AECM significantly decreased pro-caspase-3, -8, and -9 in a concentration-dependent manner. Next, cell lysates containing equal amounts of total protein from cells treated with AECM were assayed for *in vitro* caspase activity. As shown in Fig. 3B, treatment with AECM for 24 h significantly increased the activities

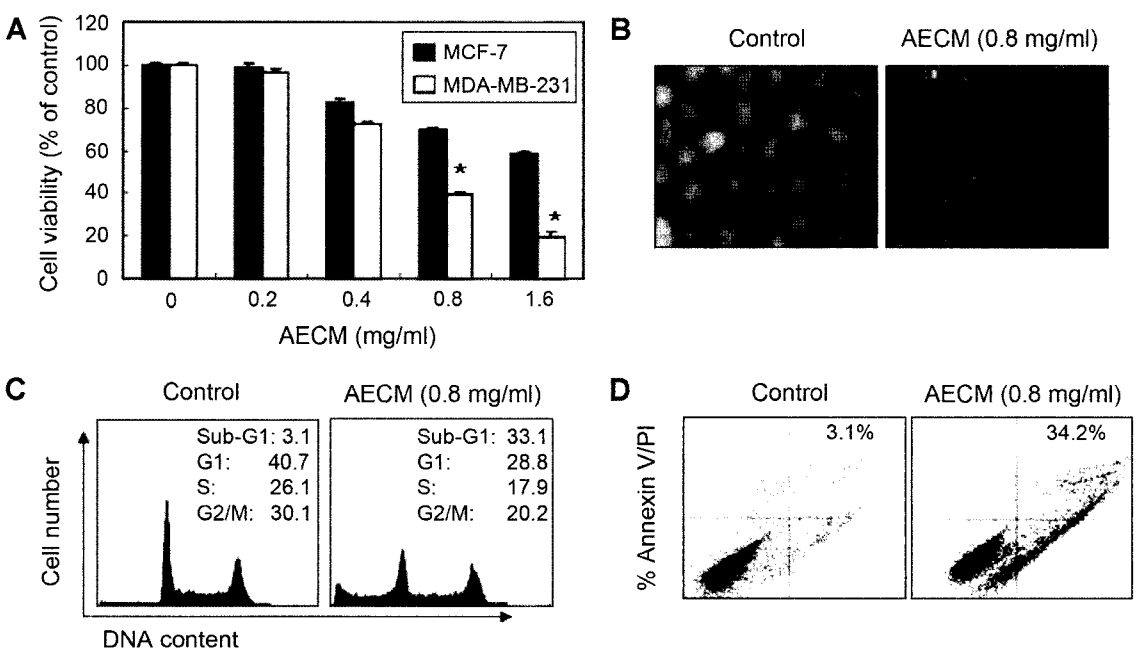

Fig. 1. AECM inhibits proliferation and induces cell death in breast cancer cells. The cells were seeded at 2×10^5 cells/ml and then treated with the indicated concentrations of AECM for 24 h.

A. Cell viability was determined by MTT assay. Each time point represents the means±SD of three independent experiments. The significance was determined by the Student's *t*-test (*$p<0.05$ *vs.* vehicle control). **B.** After being treated with AECM for 24 h, MDA-MB-231 cells were fixed and then stained with DAPI. The nuclear morphology was photographed under fluorescence using a blue filter. Magnification, ×400. **C.** To quantify the degree of apoptosis induced by AECM, MDA-MB-231 cells were evaluated for sub-G1 DNA content, which represents the fractions undergoing apoptotic DNA degradation, using a flow cytometer. **D.** MDA-MB-231 cells treated with AECM for 24 h were also assessed for apoptosis by staining with annexin V-FITC and PI double staining. PBS (0.1%) used as a vehicle control did not affect cell viability. Each point represents the mean of three independent experiments.

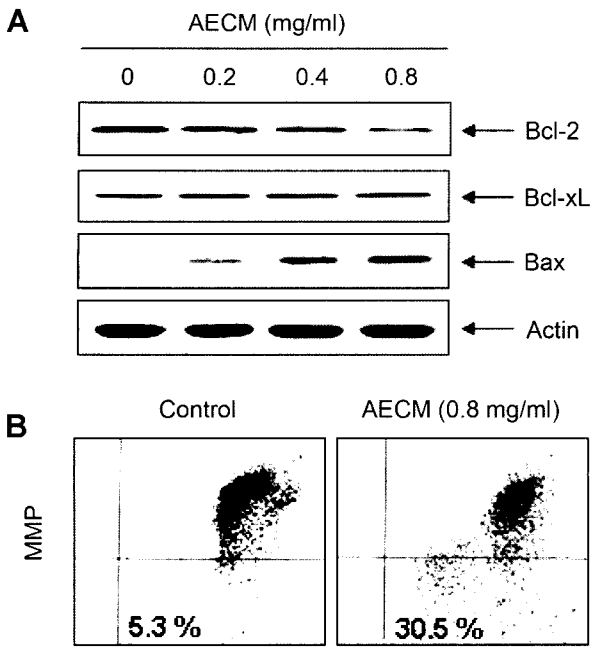

Fig. 2. Induction of Bax, inhibition of Bcl-2, and loss of MMP by treatment with AECM in MDA-MB-231 cells.
A. MDA-MB-231 cells were treated with the indicated concentration of AECM for 24 h. The cells were lysed and the cellular proteins were then separated in SDS-polyacrylamide gels, transferred to nitrocelluloses, and probed with anti-Bcl-2, anti-Bcl-xL, and anti-Bax antibodies. Proteins were then visualized using an ECL detection system. Actin was used as the internal control. A representative study is shown, and two additional experiments yielded similar results. **B.** The cells were treated with 0.8 mg/ml of AECM for 24 h and stained with JC-1 and incubated at 37°C for 20 min. The mean JC-1 fluorescence intensity was detected using a flow cytometer. Data represent the mean of representative experiments performed at least three times.

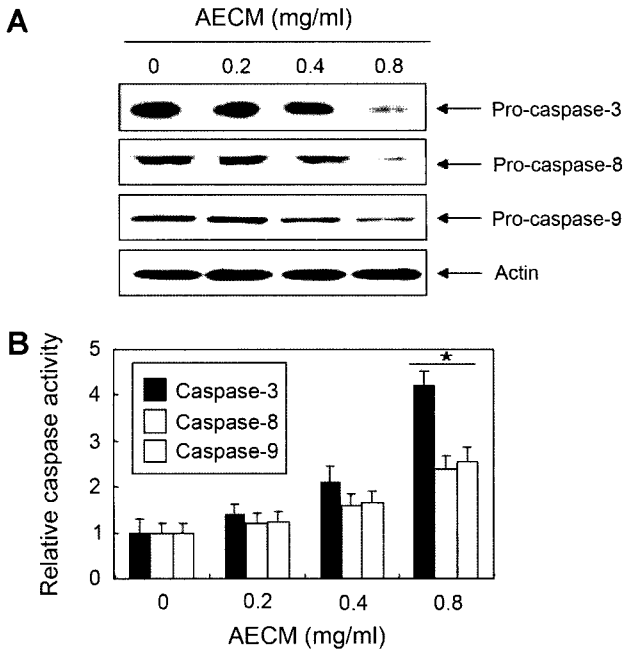

Fig. 3. Activation of caspases by AECM treatment in MDA-MB-231 cells.
A. After being treated with the indicated concentration of AECM for 24 h, the cells were lysed and the cellular proteins were then separated in SDS-polyacrylamide gels, transferred to nitrocelluloses, and probed with anti-caspase-3, -8, and -9 antibodies. Proteins were then visualized using an ECL detection system. Actin was used as the internal control. A representative study is shown, and two additional experiments yielded similar results. **B.** The cell lysates from the cells grown under the same conditions as (**A**) were assayed for *in vitro* caspase-3, -8, and -9 activity using DEVD-*p*NA, IETD-*p*NA, and LEHD-*p*NA, respectively, as substrates. The released fluorescent products were measured using a flow cytometer. Each point represents the mean±SD of three independent experiments. The significance was determined by the Student's *t*-test (*$p<0.05$ *vs.* untreated control).

of caspase-3, -8, and -9 in MDA-MB-231 cells. To further evaluate the significance of caspase activation in AECM treatment, we used a general and potent inhibitor of caspase-3: z-DEVD-fmk. As shown in Fig. 4A, AECM-induced cell death was significantly suppressed by z-DEVD-fmk, indicating that the AECM-induced apoptosis was mediated by caspase-3 activation. In addition, treatment with AECM markedly induced caspase-3 activation and cleavage of caspase-3, whereas z-DEVD-fmk pretreatment significantly inhibited activation or cleavage caspase-3. These results clearly suggest that caspase-3 closely regulates AECM-mediated apoptosis in MDA-MB-231 cells.

Blockage of the Akt Pathway Increases Apoptosis by AECM

In order to investigate the significance of the Akt pathway in response to AECM treatment, we performed time-dependent experiments to determine the expression and phosphorylation levels of Akt. As shown in Fig. 5A, the levels of phosphorylated Akt significantly decreased in response to AECM at 12 h. Total Akt protein levels remained constant throughout the course of the experiment. We next investigated whether inactivation of the Akt pathways is necessary for apoptosis induced by AECM treatment. The PI3K inhibitor LY294002 (an Akt-upstream inhibitor) was used to determine whether the inhibition of Akt activation was responsible for the induction of apoptosis. As shown in Fig. 5B, pretreatment with LY294002 alone did not induce apoptosis; however, treatment with the LY294002 and AECM resulted in a marked increase in apoptosis, as determined by the cells' chromatin condensation. We next analyzed apoptosis by annexin-V and PI staining in order to further elucidate the relationship between the Akt pathway and AECM-induced apoptosis. As shown in Fig. 5C, treatment with LY294002 significantly induced apoptosis in the presence AECM. We also found that co-treatment of LY294002 with AECM resulted in a significant cleavage of PARP or activation of Akt levels compared with other agents used alone. These results indicate that AECM-induced apoptosis may be associated with downregulation of the Akt signaling pathway.

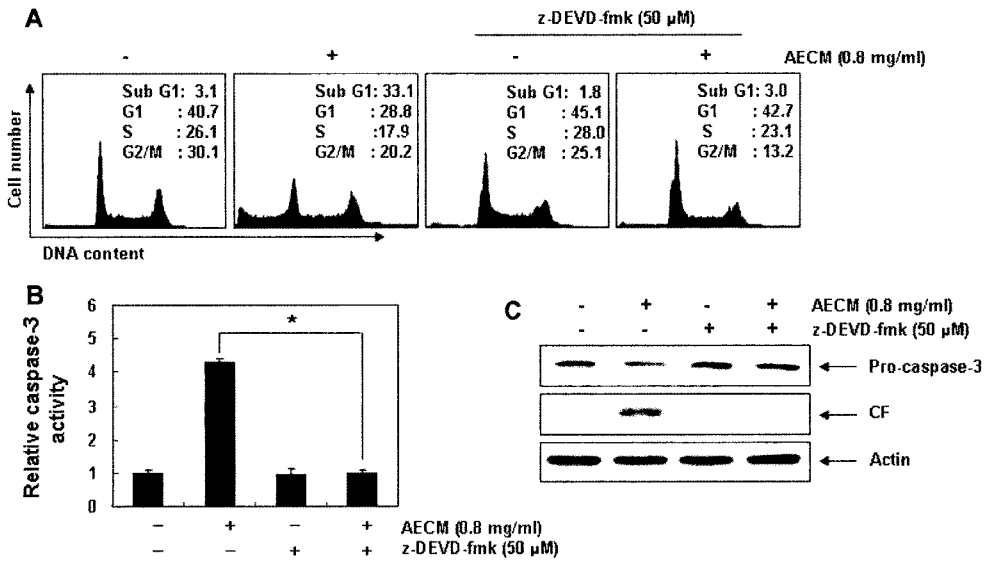

Fig. 4. Inhibition of AECM-induced apoptosis by caspase-3 inhibitor in MDA-MB-231 cells.
Sub-G1 DNA content (**A**), caspase-3 activity (**B**), and Western blotting (**C**) analyses were determined after 24 h in the presence of the caspase-3 inhibitor z-DEVD-fmk (50 μM) for 1 h before AECM (0.8 mg/ml) treatment. Actin was used as an internal control. The results are from one representative experiment of three performed and show similar patterns. Data are expressed as mean±SD of three independent experiments. The significance was determined by the Student's *t*-test (*$p<0.05$ *vs.* untreated control).

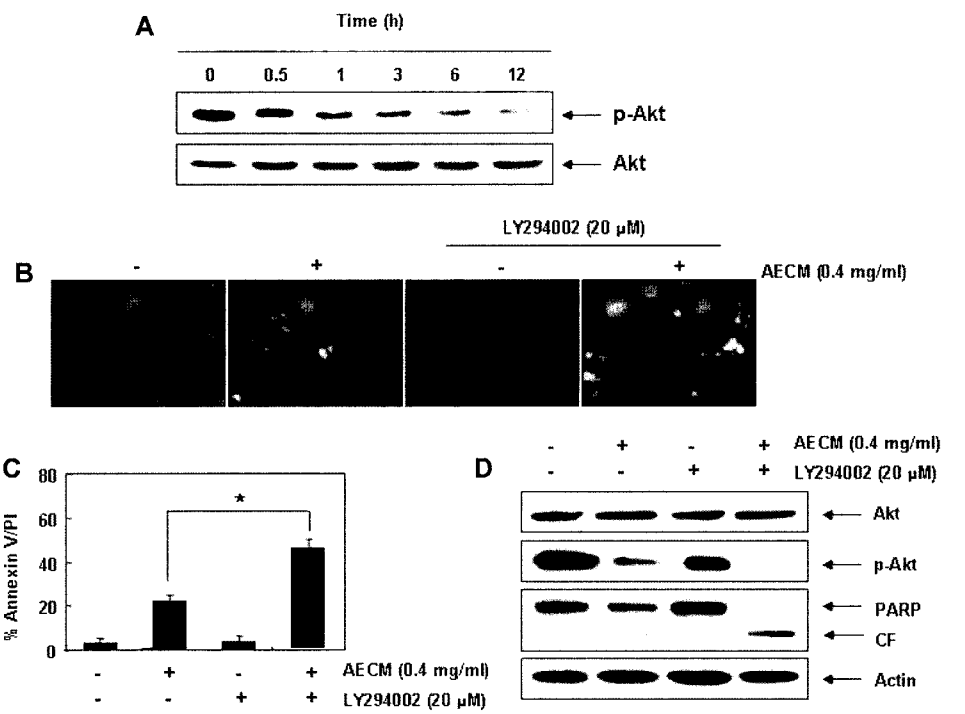

Fig. 5. Inhibition of the PI3K/Akt pathway significantly increased AECM-induced apoptosis in MDA-MB-231 cells.
A. The cells were treated with 0.8 mg/ml of AECM for the indicated times. Equal amounts of cell lysate (30 μg) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the anti-p-Akt and anti-Akt antibodies. The proteins were visualized using an ECL detection system. **B**. The cells were stimulated with 0.4 mg/ml of AECM for 24 h after pretreatment with 20 μM of LY290042 for 1 h. The cells were harvested, fixed, and stained with DAPI solution. Stained nuclei were then observed under a fluorescent microscope using a blue filter. Magnification, ×400. **C.** In a parallel experiment, the cells were also assessed for apoptosis by staining with annexin V-FITC and PI double staining. Each point represents the mean±SD of three independent experiments. The significance was determined by Student's *t*-test (*$p<0.05$ *vs.* untreated control). **D.** Equal amounts of proteins (30 μg) extracted from cells grown under the same conditions as (**B**) were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-Akt, anti-p-Akt and anti-PARP antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control.

Discussion

In this study, we report that AECM treatment significantly induces mitochondrial damage and apoptosis, modulates Bcl-2/Bax proteins, and leads to mitochondrial dysfunction, caspase activation, and PARP cleavage in MDA-MB-231 human breast carcinoma cells. Furthermore, a caspase-3 inhibitor significantly attenuates AECM-induced apoptosis. In addition, we have found that AECM decreased Akt activation and significantly enhanced apoptosis after pretreatment with LY294002, a specific PI3K inhibitor.

Recently, mitochondria have been proposed as a novel prospective target for chemotherapy-induced apoptosis [27]. Since the discovery of Bcl-2, several theories have been proposed to unravel the anti-apoptotic properties of this protein [6]. The anti-apoptotic function of Bcl-2 may be explained by its ability to control several key steps of death signaling. Bcl-2 can form ion channels in biological membranes, and its ion channel activity may control apoptosis by influencing the permeability in the intracellular membranes [21]. Our data demonstrated that AECM treatment significantly inhibits cell growth and induces apoptosis in MDA-MB-231 cells. In order to test the mechanism of AECM-induced apoptosis, we determined the effects of AECM on levels of the Bcl-2 family. Recent studies demonstrated that the Bcl-2 family significantly regulates apoptosis, either as an activator (Bax) or as an inhibitor (Bcl-2) [20, 29, 33]. These results have also suggested that the Bax/Bcl-2 ratio is recognized as a key factor in regulating the apoptotic process. Our data demonstrated that AECM-induced apoptosis is related to increased levels of the Bax protein and the downregulation of Bcl-2, indicating that AECM may increase the Bax/Bcl-2 ratio and induce mitochondrial dysfunction, leading to apoptosis of MDA-MB-231 cells.

The caspase family, aspartate-specific cysteine proteases, also plays a critical role in regulating apoptosis, and the key components of the biochemical pathways of caspase activation have been recently elucidated [18]. Caspase signaling is initiated and propagated by proteolytic autocatalysis, and by the cleavage of downstream caspases and substrates [4]. Specifically, capase-3 is one of the key executioners of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as PARP. PARP is important for cell viability, but the cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [15]. In the present study, we have demonstrated that AECM induces apoptosis through the activation of caspase-3, -8, and -9. In particular, treatment of AECM in the presence of z-DEVD-fmk, a specific inhibitor of caspase-3, inhibited cleavage of caspase-3 and cell death. These data suggest that AECM-induced apoptosis was caused by caspase-3-dependent cell death.

A previous study indicates that the PI3K/Akt signal pathway plays critical roles in regulating cell survival and death in many physiological and pathological settings. The PI3K/Akt pathway is often associated with cell survival through the activation of anti-apoptotic downstream effectors [9, 16]. In order to investigate whether the PI3K/Akt pathway was involved in the AECM-induced apoptosis of MDA-MB-231 cells, we determined the effects of AECM on the activity of Akt. Our results demonstrated that AECM markedly downregulates the PI3K/Akt signaling pathway, and that the inhibition of the PI3K/Akt pathway by pretreatment with LY294002 significantly increases AECM-induced apoptosis. These data strongly suggest that AECM-induced apoptosis is associated with Akt pathways.

In summary, we have demonstrated that AECM significantly induces apoptosis *via* regulation of the Bcl-2 family proteins, loss of MMP, and activation of caspases. Moreover, the inactivation of the PI3K/Akt pathway may perform important roles in AECM-induced apoptosis in MDA-MB-231 cells. These results are expected to further contribute to the understanding of the anticancer activity of *C. militaris*.

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