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SALF REGULATES MITOCHONDRIA- AND CASPASE-DEPENDENT APOPTOTIC PATHWAYS IN CERVICAL CANCER CELLS

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Key words: AMPs, SALF, cervical cancer, antitumor activity, apoptotic pathway.

ABSTRACT

Antimicrobial peptides (AMPs) are a new source of potential anticancer drugs. One such AMP is the shrimp anti-lipopolysaccharide factor (SALF), an AMP isolated from *Penaeus monodon*; the SALF was previously reported to induce tumor cell death. However, the mechanism by which the SALF regulates these pathways remains unclear. In this study, we identified that the SALF modulates mitochondria- and caspase-dependent apoptotic pathways in human cervical cancer cells. The SALF was found to inhibit the growth of four cervical cancer cell lines: HeLa, HeLa 299, C33A, and Ca ski. In addition, a fluorescence-activated cell sorting (FACS) analysis revealed that the SALF induced death and G2/M phase arrest in HeLa and HeLa 299 cells. The SALF itself was found to be localized to the cytosol and nuclei of HeLa cells by confocal imaging. Staining with acridine orange/ethidium bromide and analysis of DNA fragmentation confirmed that the SALF induced apoptosis in HeLa cells. Moreover, caspases-3 and -9 were found to be involved in SALF-induced apoptosis, through the use of various caspase inhibitors in vitro and immunohistochemistry in vivo. Finally, the SALF was found to induce depolarization of mitochondrial membranes with associated translocation of the AIF to nuclei, and to affect levels of Bcl-2 family proteins in HeLa cells. Taken together, our findings indicate that the SALF induces mitochondria- and caspase-dependent apoptotic pathways in HeLa cells. We suggest that the SALF may be an effective treatment for cervical cancer, either alone or in combination with traditional therapies.

I. INTRODUCTION

Antimicrobial peptides (AMPs) play key roles in innate immunity and display antitumor activities (Zasloff, 2002; Brown and Hancock, 2006) due to their membrane-destabilizing and apoptosis-inducing effects (Mader et al., 2005; Mader et al., 2009). One of the AMPs is a natural shrimp product that offers hopeful new choices for developing more-effective chemotherapeutic drugs for cancers. The shrimp anti-lipopolysaccharide factor (SALF) has anti-inflammatory response functions (Lin et al., 2010a) and anti-cervical cancer properties, and displays minimal toxicity in animals. Previously, we showed that supplementation with the SALF resulted in dose-dependent inhibition of cervical tumor growth (Lin et al., 2010b). However, the exact mechanism of the inhibitory effect on cervical cancer cell growth by the SALF is not well understood. So, understanding the mechanism of the SALF could lead to improved therapeutic benefits and approaches.

Apoptosis plays a vital role in regulating cell growth and leads to morphological and biochemical changes (Ghobrial et al., 2005). In principle, apoptotic mechanisms are often divided into mitochondrial-dependent and -independent pathways. The mitochondrial-independent pathway, also called the extrinsic-mediated pathway, is activated on cell surfaces of ligand-gated death receptors that include Fas and death receptors (Ashkenazi and Dixit, 1998; Schmitz et al., 2000). When ligands trigger extracellular death receptors, such as CD95, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-R1, and TRAIL-R2, the death domains attract TNF receptor 1-associated death domain (TRADD) proteins and Fas-associated death domain (FADD) proteins which bind to cytoplasmic death receptors. It then activates the initiator of caspases-8 and -10, also called the death-inducing signaling complex (DISC), through Bid cleavage and induces irreversible cell death (Kischkel et al., 1995; Scaffidi et al., 1998; Krammer, 1999; Yu et al., 1999; Sprick et al., 2000; Kischkel et al., 2001; Thorburn, 2004).

On the other hand, the mitochondrial-dependent pathway is

Paper submitted 12/10/13; revised 06/16/14; accepted 07/31/14. Author for correspondence: Jen-Leih Wu (e-mail: jlwu@gate.sinica.edu.tw) and Jyh-Yih Chen (e-mail: zoocjy@gate.sinica.edu.tw).
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regulated by caspase activation and mitochondrial pore formation. Caspases play important roles in apoptosis, proliferation, differentiation, and inflammation (Evan and Vousden, 2001; Siegel, 2006). During this process, mitochondrion outer membranes (MOMs) undergo permeabilization which causes loss of the mitochondrial membrane potential (MMP) and releases apoptotic proteins into the cytosol, such as cytochrome (Cyt) c and apoptosis-inducing factor (AIF) (Rathmell and Thompson, 1999; Martinou and Green, 2001; Zamzami and Kroemer, 2001; Savill and Fadok, 2007). MOMs are also regulated by Bcl-2 family proteins (Youle and Strasser, 2008), that include Bcl-2 and Bcl-XL, which have antiapoptotic roles that maintain the integrity of mitochondrial membranes (Vaux et al., 1998). In contrast, Bak and Bax initiate conformational changes in MOMs in response to stimulation of cell apoptosis. This leads to oligomerization of MOMs (Green and Kroemer, 2004). One of the released apoptotic proteins is Cyt c that binds to apoptotic protease-activating factor (Apaf)-1 (Li et al., 1997). Caspase-9 then binds to Apaf-1 to form a high-molecular-weight complex called the apoptosome (Zou et al., 1999). In addition, this interaction activates caspase-3 and triggers its reversible apoptotic program (Mueller et al., 2003).

In the present communication, we conducted an investigation on the mechanism responsible for inhibiting cervical cancer cell proliferation and apoptosis using HeLa, HeLa299, C33A, and Ca ski cell lines as an in vitro cell-culture model. We report that the SALF induced apoptosis in cervical cancer cells and mitochondrion-dependent apoptosis cell death in aggressive cervical cancer cells. We also checked progression of the deregulated cell cycle in cervical cancer cells. Interestingly, the SALF induced apoptotic cell death of cervical cancer cells which was mediated through modulation of the expression levels of pro- and antiapoptotic proteins. Also, the SALF induced MMP loss and activated caspase-3 and -9 pathways in HeLa cells. Thus, our studies provide insights into the mechanism by which the SALF induces apoptosis in HeLa cells.

II. MATERIALS AND METHODS

1. Peptide

The cyclic peptide, the SALF (Ac-ECKFTVKPYLKRFQVYYKGRMWCP-NH2), was synthesized and purified by GL Biochemistry (Shanghai, China) to a purity grade of >95%. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments.

2. In Vitro Cytotoxicity Assay

Cervical cancer cells were cultured in 96-well plates at 5000 cells per well, and treated with the SALF. PBS treatment served as a control. At the end of treatment, a mixture (20 μl) of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazinemethosulfate (PMS) (Promega, Mannheim, Germany) was added for 2 h at 37°C. The optical density was measured on a microtiter plate reader at 570 nm. Experiments were repeated three times. Results are expressed as a percentage of the inhibition rate of viable cells, and values of the PBS-treated group were deducted from the experimental results.

3. Acridine Orange/Ethidium Bromide (Ao/EtBr) Staining

Typical apoptotic cells were detected and stained with Ao/EtBr. After treatment with the SALF, HeLa cells were washed with PBS. Then these cells were stained with Ao/EtBr that used cold DPBS containing 1 μg/ml EtBr and 1 μg/ml Ao for 15 min at 37°C. Double staining can discriminate live cells from apoptotic and necrotic cells. Cells were washed with PBS and subsequently observed. Photographs were taken with a fluorescence microscope.

4. DNA Fragmentation Assay

HeLa cells were cultured in the absence and presence of the SALF. Cells were collected, and DNA was extracted using a Suicide Track DNA Ladder Isolation Kit (Calbiochem, Darmstadt, Germany). DNA fragments were separated by 2% agarose gel electrophoresis, and stained with EtBr. Then, the DNA fragments were visualized under UV light.

5. Western Blot Analysis

Briefly, 10 μg of proteins was loaded on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and blotted with primary antibodies reactive to the detected proteins of Cyt c, AIF, Bid, Bax, Bad, Bim, Bik, Bak, puma, β-actin, cyclooxygenase (Cox)-IV, and PCNA, followed by the secondary antibodies of goat anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG labeled with alkaline phosphatase (AP) for 0.5 h at room temperature. After three washings with PBS and Tween-20 (PBS/T) for 10 min, membranes were incubated with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) in a toluidinium salt substrate at room temperature.

6. Annexin V and Propidium Iodide (PI) Double Staining by Flow Cytometry

HeLa cells were cultured in the absence and presence of the SALF and different caspase inhibitors. Cells were collected and fixed with ice-cold 70% ethanol at 4°C overnight. Cells were washed with DPBS buffer twice to remove any residual ethanol. We used annexin V to assess phosphatidylserine (PS) exposure, and PI for the cell viability analysis. Cells were analyzed with Gallios software (Beckman Coulter, CA, USA).

7. MMP Detection by Flow Cytometry

Changes in the MMP in HeLa cells after treatment with the SALF were determined by flow cytometry using 1,1′,3,3,3′-hexamethylindodicarbo-cyanine iodide (DilC1). Detection followed the instructions of the manufacturer. The monomeric form emits light at 658 nm after excitation at 633 nm, and

results were analyzed by Gallios software (Beckman Coulter).

8. Cell Cycle Analysis by Flow Cytometry

In order to determine the effect of the SALF on the cell cycle, a fluorescence-activated cell-sorting (FACS) analysis was carried out. For propidium iodide (PI) staining, cells were seeded in 6-well plates at a density of 10^4 cells/ml. After 24 h of attachment, cancer cells were treated with the indicated SALF concentrations for different time intervals. Floating and attached cells were harvested, washed in PBS, fixed in ice-cold ethanol (70% v/v), and stored at -20°C. For analysis, cells were washed in PBS and suspended in PI (25 mg/ml) in PBS with RNase A (200 mg/ml). Cells were analyzed by Gallios software (Beckman Coulter).

9. Confocal Fluorescence Microscopy

To detect AIF release, samples were treated with the SALF and imaged with a confocal microscope. Images of AIF (green) and DAPI (blue) were separately obtained and then merged.

10. Immunohistochemistry

Tumors were removed, and paraffin sections were created using primary antibodies of anti-activated caspases-3 and -9 by immunohistochemistry that was performed at the Taipei Institute of Pathology (http://www.tipn.org.tw/). Photomicrographs were taken under a fluorescence microscope.

11. Statistical Analysis

For the statistical analyses, multiple-group comparisons were evaluated using an analysis of variance (ANOVA) in SPSS software (Chicago, IL, USA). Differences were defined as significant at $p < 0.05$.

III. RESULTS

1. The SALF Exhibited Cytotoxic Properties in Cervical Cancer Cells

To determine the cytotoxicity of the SALF, human cervical cells (C33A, Ca ski, HeLa 299, and HeLa cells) were treated with different dosages of the SALF (0, 25, 50, 100, and 200 μg/ml) for 24 h (Fig. 1(a)). Cell inhibitory percentages were 36.41% ± 4.13%, 52.60% ± 1.86%, 60.26% ± 2.95%, and 68.06% ± 3.71% in C33A, Ca ski, HeLa 299, and HeLa cells treated with 100 μg/ml of the SALF, respectively. Also, we detected cell inhibitory rates of SALF-treated cells. Cells showed decreased viability in a time-dependent manner (Fig. 1(b)). We then wanted to know whether or not the cytotoxicity in these cells was caspase-dependent. We used a general caspase inhibitor (Z-VAD-FMK) to pretreat HeLa cells. The results showed that the cell inhibition rate significantly decreased in SALF-induced HeLa cell death (Fig. 1(c)). Cell inhibitory percentages were 38.08% ± 4.14%, 29.95% ± 4.79%, 28.32% ± 3.03%, and 22.28% ± 2.72% in C33A, Ca ski, HeLa 299, and HeLa cells, respectively. These data

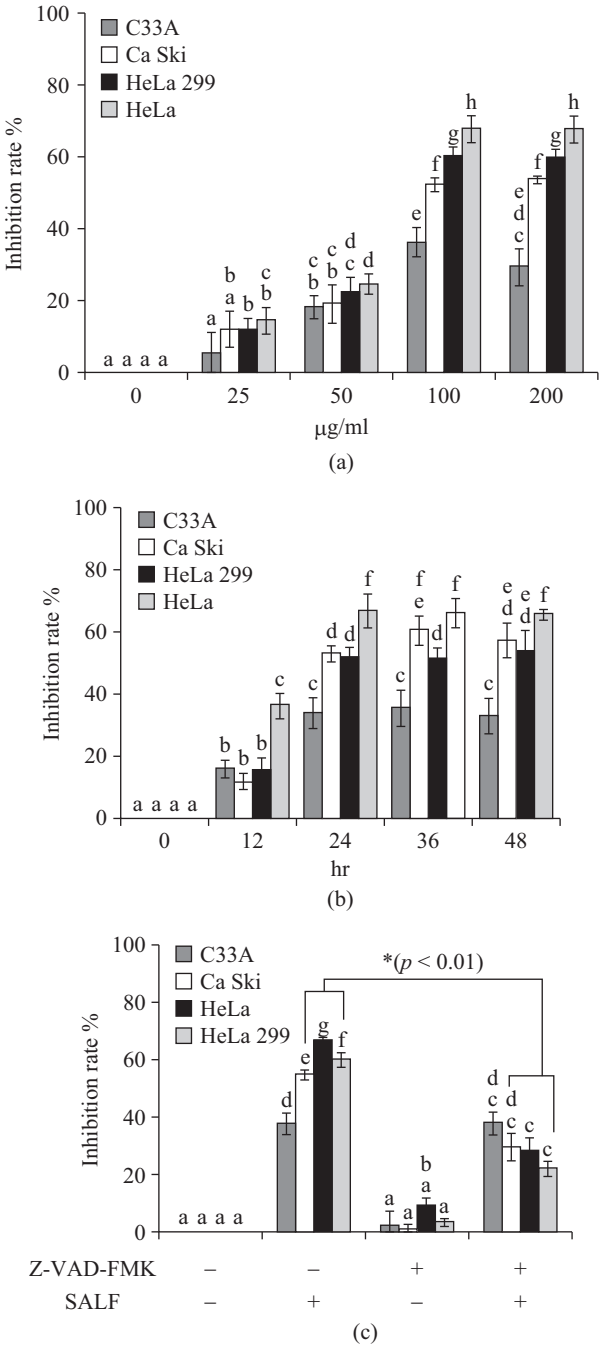

Fig. 1. Effects of the shrimp anti-lipopolysaccharide factor (SALF) on the viability of different cervical cancer cell lines. (a) Cells (5000 cells/well) in a 96-well dish were cultured overnight and then incubated with different indicated concentrations (0, 25, 50, 100, and 200 μg/ml) of the SALF for 24 h. Cell viability was determined by an MTS assay. (b) Cells (5000 cells/well) in a 96-well dish were cultured overnight and then incubated with 100 μg/ml of the SALF for different times (0, 12, 24, 36, and 48 h). Cell viability was determined by an MTS assay. (c) Effects of pretreatment with a general caspase inhibitor (Z-VAD-FMK) on the cytotoxicity of the SALF. Cells (5000 cells/well) in a 96-well dish were grown overnight, pretreated with 50 μM of a general caspase inhibitor for 2 h, and then incubated with 100 μg/ml of the SALF in the presence and absence of Z-VAD-FMK for 24 h. Cell viability was determined as described above.

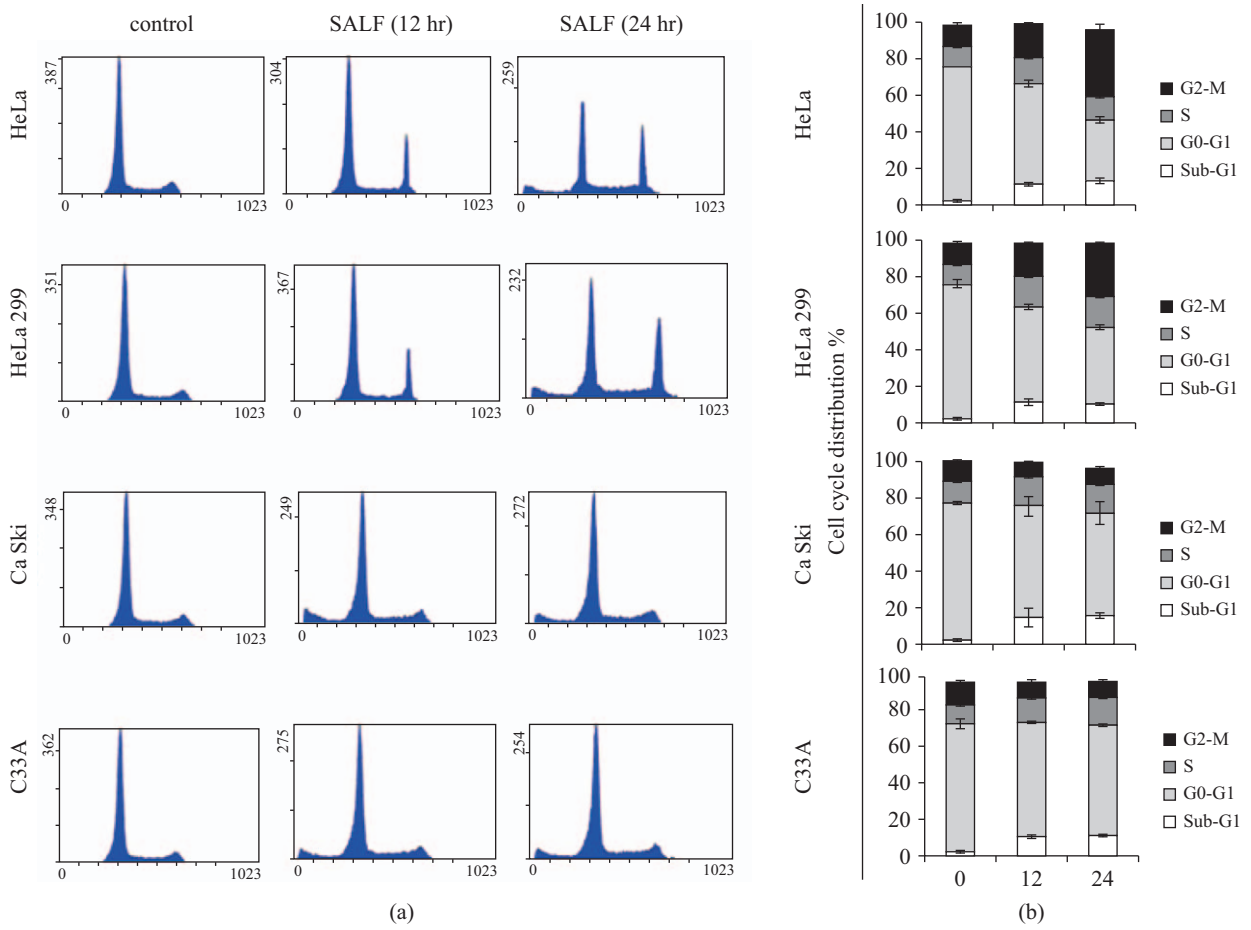

Fig. 2. The shrimp anti-lipopolysaccharide factor (SALF) induces cell cycle arrest in cervical cancer cell lines. (a) Cervical cancer cells were incubated for increasing intervals (12 and 24 h), and DNA distribution was analyzed with propidium iodide staining by flow cytometry. (b) Percentages of cells in the G0/G1, S, and G2/M phases were calculated. The results are the mean ± SD of three experiments.

suggested that the SALF inhibited cervical cancer cell growth and induced caspase-dependent apoptosis in Ca ski, HeLa 299, and HeLa cells.

2. The SALF Arrests Cell-Cycle Progression

In order to examine the cell-cycle distribution in SALF-treated cervical cancer cells, we used flow cytometry to analyze and calculate the percentages of cells in the G0/G1, S, and G2/M phases. Typical time-dependent G2/M arrest in HeLa and HeLa 299 cells was observed with 100 μg/ml SALF treatment (Fig. 2(a)). G0/G1 phase DNA contents decreased from 72.83% ± 0.06% to 33.27% ± 1.48%, whereas the G2/M DNA contents increased from 11.80% ± 1.73% to 35.80% ± 3.33% in HeLa cells. G0/G1 phase DNA contents decreased from 74.07% ± 2.15% to 42.27% ± 0.93%, whereas G2/M DNA increased from 11.37% ± 1.46% to 28.80% ± 0.26% in HeLa 299 cells (Fig. 2(b)).

3. Characterization of SALF Localization and the Study of Its Effects on Apoptosis in HeLa Cells

To further characterize localization of the SALF in HeLa cells, we used the SALF labeled with FITC to study peptide localization by immunocytochemical staining. We observed that the SALF was localized in the cytosol of HeLa cells after 24 h of treatment. Accumulation of the SALF increased with time, especially in areas surrounding nuclei at 72 h (Fig. 3(a)). These results suggest that the SALF functions like a lytic peptide which breaks down plasma membranes. In order to investigate the type of cell death induced by the SALF, cells were stained with Ao/EtBr. With Ao/EtBr, one can identify viable, apoptotic, and necrotic cells based on the color and appearance. Viable cells are green with undamaged nuclei. Bright-orange chromatin indicates non-viable cells. The appearance of cell shrinkage and DNA with condensation indicates apoptosis. Samples treated with 100 μg/ml of the SALF for 24, 48, and 72 h were stained with Ao/EtBr, and we observed that cell shrinkage and DNA with condensation appeared in SALF-treated HeLa cells at 24 h (Fig. 3(b)). To further characterize the end stage of apoptosis induced by the SALF, we examined whether treatment with the SALF induced a typical ladder pattern of inter-nucleosomal DNA fragmentation in HeLa cells. As shown in Fig. 3(c), DNA

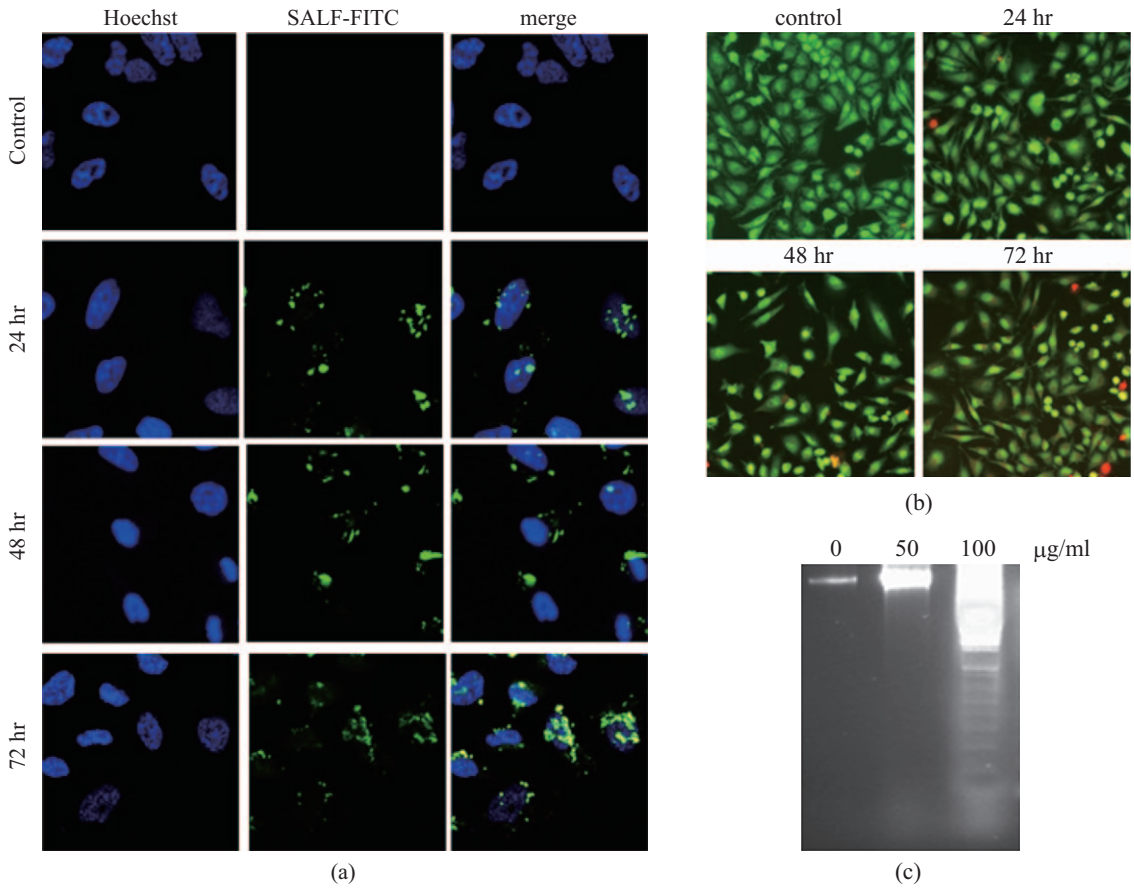

Fig. 3. The shrimp anti-lipopolysaccharide factor (SALF) induced apoptosis in HeLa cells. (a) We studied the distribution of the SALF coupled with FITC after HeLa cells were treated with the SALF for 24, 48, and 72 h. The green signal indicates FITC-labeled SALF, while the blue signal indicates nuclei. (b) Identification of apoptotic HeLa cells using acridine orange-ethidium bromide (Ao/EtBr) staining and the effect induced by the SALF at 24, 48, and 72 h. (c) Cells were treated with the SALF (0, 50, and 100 μg/ml), and genomic DNA was extracted and resolved on 2% agarose gels. Apoptotic DNA fragmentation was visualized by EtBr staining.

fragmentation was observed after treating HeLa cells with the SALF, and it was found to occur in a dose-dependent manner. These data suggest that the SALF induces apoptosis in HeLa cells.

4. Apoptosis of SALF-Induced HeLa Cells Requires Caspase Activities

To further confirm the involvement of caspases in SALF-induced apoptosis, we used various caspase inhibitors that completely blocked activation of their corresponding caspases. Those inhibitors were BAPTA (a calcium chelator), NAC (a reactive oxygen species inhibitor), Z-VAD-FMK (a broad caspase inhibitor), Z-DEVD-FMK (a specific caspase-3 inhibitor), Z-IETD-FMK (a specific caspase-8 inhibitor), and Z-LEHD-FMK (a specific caspase-9 inhibitor). We found that Z-VAD-FMK, Z-LEHD-FMK, and Z-DEVD-FMK significantly suppressed SALF-induced apoptosis. Survival percentages were 41.02% ± 3.26%, 87.99% ± 5.08%, 73.01% ± 9.37%, and 70.57% ± 5.31% in groups of only SALF-treated HeLa cells, Z-VAD-FMK pretreatment, Z-LEHD-FMK pretreatment, and Z-DEVD-FMK pretreatment, respectively. However, the survival percentage was 38.65% ± 3.95% in the group with Z-IETD-FMK pretreatment (Fig. 4(a)). Similarly, pretreatment with Z-VAD-FMK, Z-LEHD-FMK, and Z-DEVD-FMK attenuated SALF-induced apoptotic phenomena (Fig. 4(b)). Apoptotic percentages were 78.57% ± 5.35%, 69.03% ± 14.22%, 29.43% ± 20.41%, and 28.43% ± 7.92% in the group of only SALF-treated HeLa cells, Z-IETD-FMK pretreatment, Z-DEVD-FMK pretreatment, and Z-LEHD-FMK pretreatment, respectively (Fig. 4(c)). Those results indicate that the SALF activated caspases-3 and -9, but not caspase-8.

5. The SALF Induced Mitochondrial Membrane Depolarization Associated with AIF Translocation to Nuclei and Modulated Expressions of Bcl-2 Family Proteins in HeLa Cells

Activation of caspases-3 and -9 by the SALF suggested that the mitochondrial apoptotic pathway is involved in SALF-induced apoptosis in HeLa cells. Mitochondria play a key role in apoptosis induction. Therefore, we investigated the effects of the SALF on the MMP using DilC1. DilC1 is a mitochondrion-specific voltage-dependent dye. It can enter

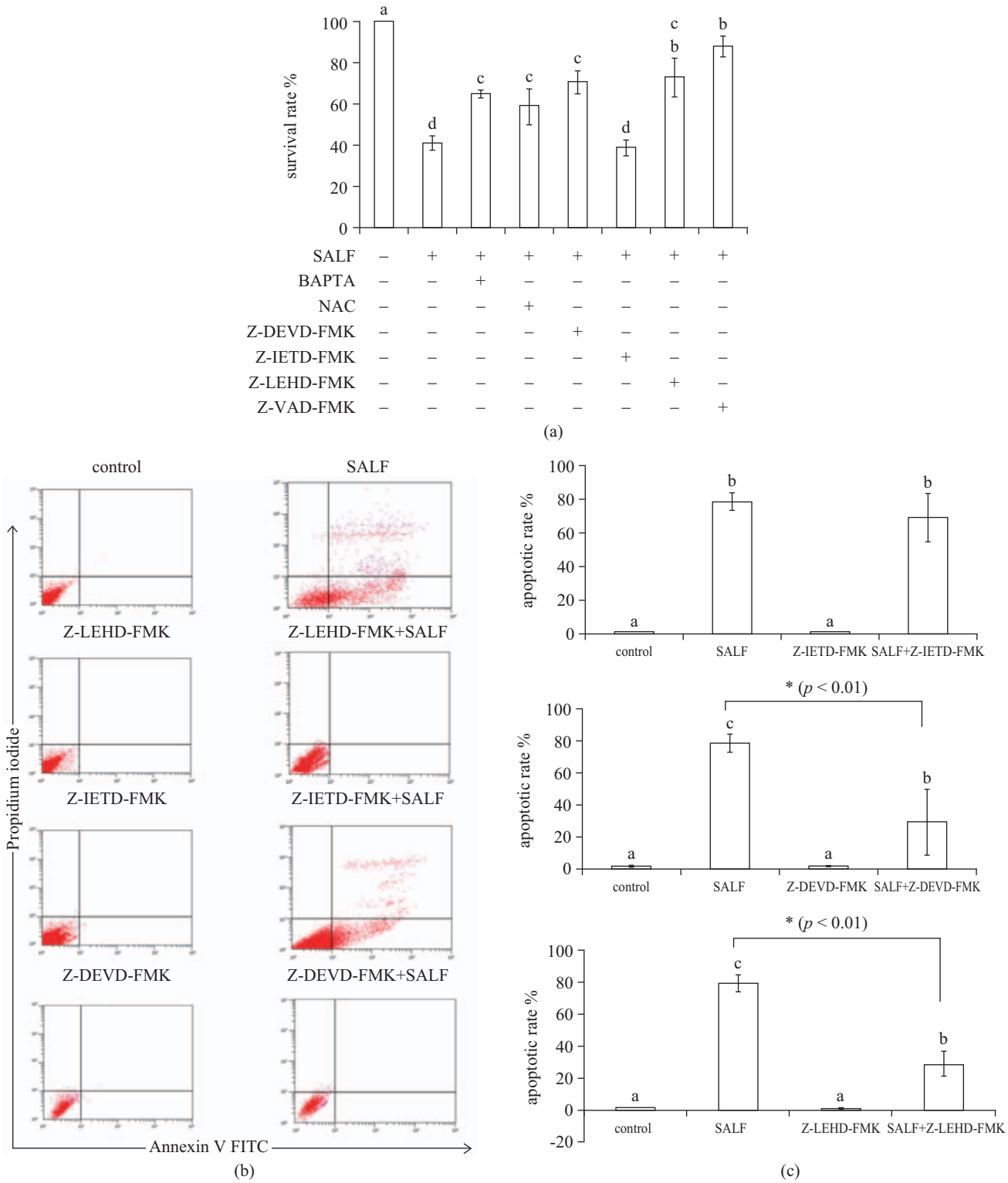

Fig. 4. The shrimp anti-lipopolysaccharide factor (SALF) induced apoptosis in HeLa cells through a caspase-dependent pathway. (a) Cells were pretreated with BAPTA (25 μM), NAC (100 μM), Z-DEVD-FMK (100 μM), Z-IETD-FMK (100 μM), Z-LEHD-FMK (100 μM), or Z-VAD-FMK (50 μM) for 2 h, and then treated with or without the SALF (100 μg/ml) for 24 h. Cell viability was determined by an MTS assay. (b) and (c) Cells were pretreated with Z-DEVD-FMK (100 μM), Z-IETD-FMK (100 μM), or Z-LEHD-FMK (100 μM) for 2 h, and then treated with or without the SALF (100 μg/ml) for 24 h. Cells were harvested and co-stained with FITC-conjugated Annexin V and propidium iodide, and the externalization of phosphatidylserine (PS) was detected using flow cytometry. Data are the mean ± SD from three separate experiments. Differences were defined as significant at $p < 0.05$. Different letters indicate a significant difference between two groups, while the same letter indicates no difference between two groups.

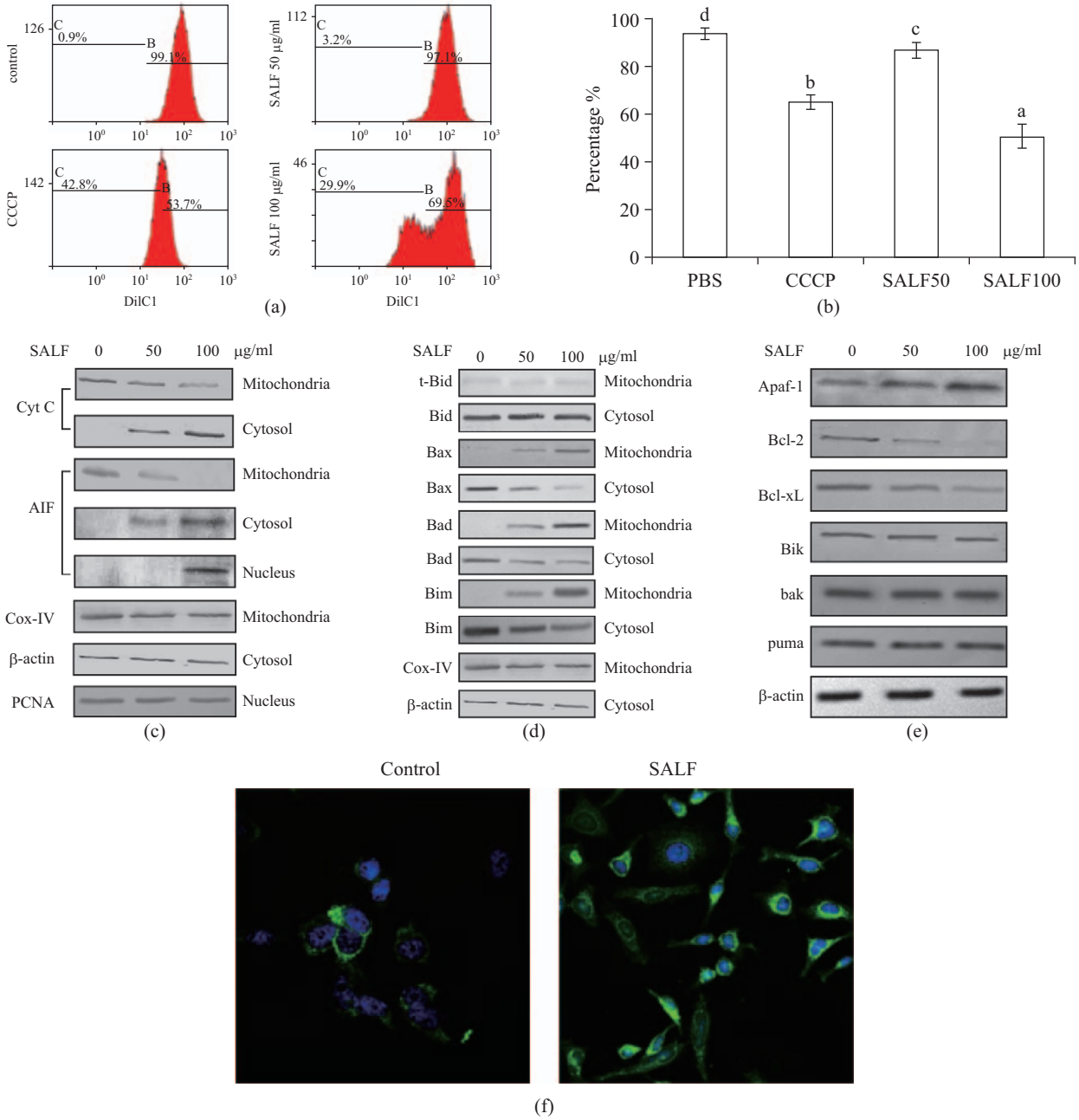

Fig. 5. The shrimp anti-lipopolysaccharide factor (SALF) induced apoptosis in HeLa cells through disruption of a mitochondrion-dependent pathway. (a) HeLa cells were treated with different dosages of the SALF (50 and 100 μg/ml) for 24 h and stained with DilC1, and the mitochondrial transmembrane potential loss was analyzed by flow cytometry, as described in "Methods". CCCP was used as a positive control. (b) The percentage of mitochondrial transmembrane potential loss was calculated. Data are the mean ± SD value from three separate experiments. Differences were defined as significant at $p < 0.05$. Different letters indicate a significant difference between two groups, while the same letter indicates no difference between two groups. (c) HeLa cells were with the SALF (0, 50, and 100 μg/ml) for 24 h. Cellular fractions (cytosolic, mitochondrial, and nuclear) were then isolated using mitochondrial isolation and an NE-PER nuclear and cytoplasmic extraction kit. Samples were boiled in SDS sample buffer. Total protein at 10 μg was loaded into each well of a 12% SDS-polyacrylamide gel for separation by electrophoresis, and transferred to PVDF membranes. Immunoblots were probed with rabbit anti-human apoptosis-inducing factor (AIF) (1:1000), rabbit anti-Cox IV (1:1000), mouse anti-cytochrome C (1:1000), or rabbit β-actin (1:104), and then goat anti-mouse or rabbit alkaline phosphatase (1:104). (d) Cells were treated with different concentrations of the SALF for 24 h and then harvested. Total cell lysates, and mitochondrial and cytosolic fractions were prepared. Equal amounts of protein were separated by 12% SDS-PAGE, transferred to PVDF membranes, and blotted with antibodies specific for Bid, Bax, bad, and bim (at 1:1000). β-Actin and Cox-IV were used as internal controls. (e) Cells were treated with the SALF and harvested as described above. Equal amounts of protein were separated and blotted with rabbit anti-Apaf-1 (1:1000), rabbit anti-bcl-2 (1:1000), rabbit anti-bcl-xl (1:1000), rabbit anti-bik (1:1000), rabbit anti-bak (1:1000), rabbit anti-puma (1:1000), and rabbit β-actin (1:104), and then goat anti- rabbit alkaline phosphatase (1:104). (f) Immunofluorescence confocal microscopy was carried out using anti-AIF to observe translocation of the AIF in HeLa cells. Nuclei were stained with DAPI.

mitochondria of healthy cells but leaches into the cytosol of cells with MMP dissipation, resulting in a decreased fluorescence intensity. CCCP was used to induce mitochondrial membrane depolarization. As shown in Fig. 5(a), mitochondrial membrane depolarization was visible at 24 h after treatment with 100 μg/ml of the SALF. Percentages of the mitochondrial transmembrane potential were 94.01% ± 2.13%, 65.10% ± 2.81%, 87.02% ± 3.09%, and 50.63% ± 5.05% in the control, CCCP, the SALF at 50 μg/ml, and the SALF at 100 (μg/ml) groups, respectively (Fig. 5(b)). These data suggest that SALF-induced caspase-9 and -3 activation occurs after loss of the MMP in HeLa cells. In order to further analyze whether the SALF affected the release of Cyt c or AIF during apoptosis, we evaluated protein expressions of Cyt c and AIF by Western blotting. We found that the level of cytosolic Cyt c was dose-dependently elevated in SALF-treated HeLa cells, but the level of mitochondrial Cyt c was degraded. Also, AIF was dose-dependently transferred from mitochondria to the cytosol and even into nuclei of HeLa cells exposed to the SALF (Fig. 5(c)). Similarly, immunofluorescence confocal microscopy was used to determine the release of AIF by HeLa cells (Fig. 5(f)). Bcl-2 family proteins maintain a balance between pro- and antiapoptotic members. They are known to control apoptosis that occurs via the mitochondrial pathway. We also examined the effects of the SALF on levels of Bcl-2 family proteins in HeLa cells. Our results showed that the SALF decreased cytosolic levels, but increased mitochondrial levels of the proapoptotic bax, bad, and bim (Fig. 5(d)). However, we found no changes in bid, bik, bak, or puma protein levels. On the other hand, levels of Bcl-2 and bcl-xl were reduced and Apaf-1 increased after SALF treatment (Fig. 5(e)). These findings suggest that the SALF modulates protein levels of Apaf-1, bax, bad, bim, Bcl-2, and bcl-xl in MMP loss and the release of Cyt c and AIF from mitochondria.

6. Detection of Apoptotic Cells in Nude Mice Using Activated Caspase-3 and Caspase-9 Immunohistochemistry

To verify the effect of activated caspases-3 and -9 on SALF-treated HeLa cells in a mouse model, we administered the SALF and determined whether it dose-dependently increased cell apoptosis (Figs. 6(a) and (b)).

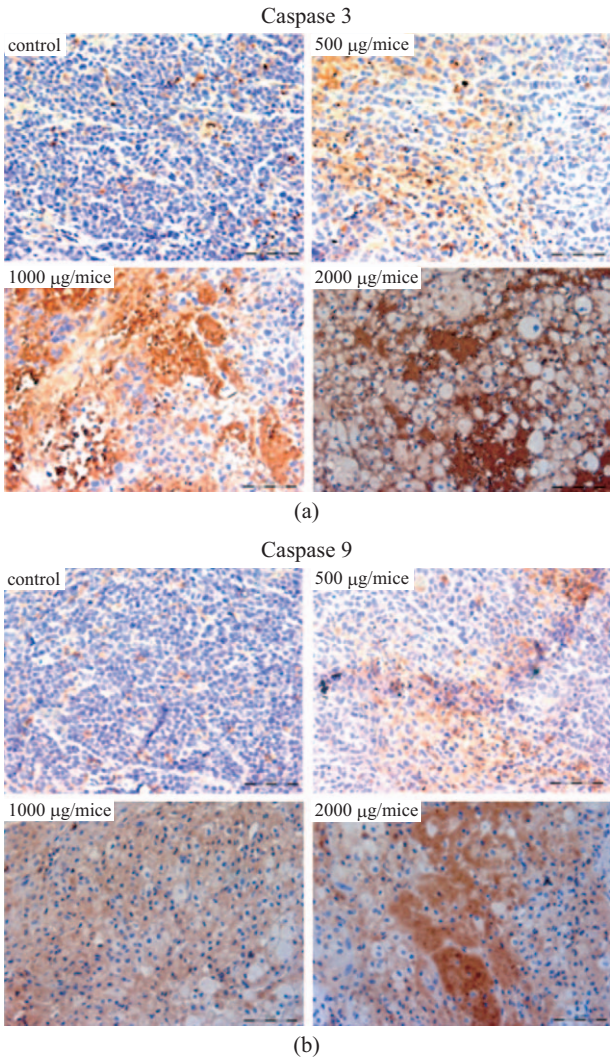

Fig. 6. The shrimp anti-lipopolysaccharide factor (SALF) activated caspase-3 and caspase-9 immunohistochemistry tumor growth in vivo. Paraffin sections were immunostained with antibodies that specifically detected activated caspases-3 and -9.

IV. DISCUSSION

Important resources of anticancer agents are many marine-derived substances which were shown to have various bioactivities. For example, pardaxin from the Red Sea Moses sole was reported to inhibit growth of HeLa and HT1080 cells (Hsu et al., 2011; Huang et al., 2011c). Also, epinecidin-1 and TH1-5 respectively from Epinephelus coioides and Oreochromis mossambicus have antitumor activity (Lin et al., 2009; Chang et al., 2011). Moreover, many marine-derived substances have antibacterial and immunomodulatory activities (Huang et al., 2011a; Huang et al., 2011b; Pan et al., 2009; Pan et al., 2010; Rajanbabu and Chen, 2011; Rajanbabu et al., 2010).

Interestingly, it was reported that one of the marine-derived substances is an AMP, the SALF, that has various bioactivities, such as antibacterial, anti-parasitic, and anti-inflammatory responses and antitumor activities (Lin et al., 2010a; Lin et al., 2010b; Pan et al., 2007; Pan et al., 2009; Pan et al., 2010). However, the molecular mechanism involved in SALF-induced apoptosis is poorly understood. It has been reported that positively charged AMPs bind to cell plasma membranes containing negatively charged phospholipids such as PG or PS due to strong electrostatic interactions (Melo et al., 2009). Base on this theory, AMPs can disrupt membrane through different mechanisms such as barrel stave, carpet, and toroidal pore. In the barrel stave mechanism, AMPs aggregate in the membrane and form transmembrane pores. In the carpet mechanism, AMPs align parallel to bilayer and produce a

detergent-like effect so that AMPs can disrupt the membrane. The toroidal pore mechanism is similar with barrel stave mechanism.

In this study, the mechanism of cell death induced by the SALF in cervical cancer cells was characterized to confirm its potential use as an anticancer treatment option. We demonstrated in this study that the SALF is a cell cycle modulator which arrests the cell cycle at the G2/M phase (Fig. 2). We also evaluated synthetic SALF compounds as potential therapeutics for cervical cancer (Figs. 1(a) and (b)). Apoptosis was observed at 24 h after SALF treatment (Fig. 3(b)). In addition, Z-VAD-FMK, Z-DEVD-FMK, and Z-LEHD-FMK potently prevented SALF-induced apoptosis. These results indicated that caspase family members are significantly activated following SALF treatment. Furthermore, we also proved that the SALF could increase expressions of activated caspases-3 and -9 by immunohistochemical tumor growth in vivo (Fig. 6). These data indicated that the SALF induced apoptosis in cervical cancer cells through activating a caspase-3 and -9-dependent pathway, resulting in loss of the MMP, and Cyt c and AIF release (Figs. 5-7). In addition, it has been reported that AMP can activate apoptosis inducing factor and induce DNA damage in cancer cells (Ren et al., 2012). Also, we first found that SALF (shrimp AMP) can suppress cervical cancer via apoptosis inducing factor and DNA damages. In Fig. 3(c), we examined dose-dependently DNA fragmentation after treating HeLa cells with the SALF. Also, AIF was transferred from mitochondria to the cytosol in a dose-dependent manner and even into nuclei of HeLa cells exposed to the SALF in Fig. 5(c). AIF is normally in mitochondria of healthy cells. However, it can be released into the cytosol and nuclei, resulting in DNA fragmentation during apoptosis (Susin et al., 1999). Cyt c is also an apoptogenic factor that resides in mitochondrial intermembrane spaces. It acts by forming an apoptosome with Apaf-1 and activated caspase-9 (Li et al., 1997; Zou et al., 1997; Riedl and Salvesen, 2007; Lu et al., 2012). Besides, ROS levels can increase during early apoptotic pathway. ROS also triggers cytochrome c (Cyt c) and AIF releasing from mitochondria (Cregan et al., 2004). Our data showed that the levels of cytosolic Cyt c and AIF were elevated in SALF-treated HeLa cells, but the levels of mitochondrial Cyt c and AIF were degraded (Fig. 5(c)). In addition, the survival percentage of NAC (a reactive oxygen species inhibitor)-preteated group was increased, compared with SALF-treated group (Fig. 4(a)). Therefore, we inferred that ROS might relate with SALF-treated HeLa cells that went through early apoptotic pathway. The X-linked inhibitor of apoptosis (XIPA) directly inhibits caspases-3 and -9 and mediates their ubiquitination and degradation (Deveraux et al., 1997; Huang et al., 2001; Eckelman et al., 2006). Our investigations showed that the SALF also modulated pro- and antiapoptotic proteins that control apoptosis. The balance between them is critical for cell survival and death. The SALF promoted apoptotic protein expression (Bax, Bad, and Bim) from the cytosol to mitochondria and suppressed antiapoptotic protein expressions (Bcl-2 and Bcl-XL) (Figs. 5(d) and (e)). The results suggested that changes in the ratio of pro- and antiapoptotic proteins might contribute to the proapoptotic activity of the SALF. Taken together, these results suggest that SALF treatment generates signature morphologic characteristics of apoptosis in mitochondria in a caspase-3 and -9-dependent manner (Fig. 7).

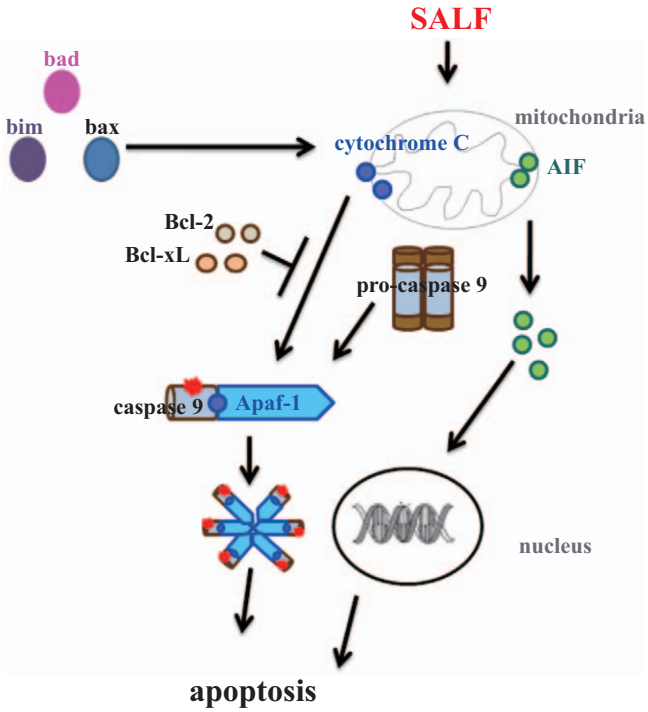

Fig. 7. Schematic representation of the signaling cascade of shrimp anti-lipopolysaccharide factor (SALF)-induced HeLa cell apoptosis.

V. CONCLUSION

In conclusion, the SALF is a peptide that effectively inhibits cellular growth of cervical cancer cells. In the present study, we showed that the SALF mediated both caspase- and mitochondria- dependent pathways during SALF-induced apoptosis in human cervical cancer cells. Our data suggest that the SALF not only induced cervical cancer cell death and cell cycle arrest in G2/M phase in vitro, but also increased activated caspase-3 and -9 in vivo. To sum up, our study provides evidence from molecular studies which shows that the SALF induced the death of human cervical cancer cells. It can improve therapeutic benefits and provide alternative approaches for treating cervical cancer.

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