RESEARCH ARTICLE

Fused Polypeptide with DEF Induces Apoptosis of Lung Adenocarcinoma Cells

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Abstract

   To analyze the effects of a new unknown peptide DEF on the growth of tumor cells, a fused polypeptide TAT-DV1-DEF was designed and synthesized. The lung adenocarcinoma cell line GLC-82 treated with TAT-DV1-DEF was analyzed with a cell counting kit 8, and the location of polypeptides in cells was observed under laser confocal microscopy. The efficiency of polypeptide transfection and changes in nuclear morphology were analyzed by flow cytometry and fluorescence microscopy, respectively. Finally, the mechanism of tumor cell growth inhibition was evaluated by Western blotting. We found that TAT-DV1-DEF could significantly inhibit the growth of the lung adenocarcinoma cell line GLC-82, but not the normal human embryonic kidney cell line HEK-293. Polypeptides were found to be mostly localized in the cytoplasm and some mitochondria. The efficiency of polypeptide transfection in the two cell types was approximately 99%. Apoptotic nuclei were observed under fluorescence microscopy upon treatment with polypeptides and DAPI staining. Western blot analyses indicated that the polypeptide inhibition of tumor cell growth was apoptosis dependent. In the present study, we demonstrated that fused polypeptides could induce apoptosis of the lung adenocarcinoma cell line GLC-82, indicating that the new unknown peptide DEF has antitumor effects.

Keywords: Fused polypeptide - lung adenocarcinoma - apoptosis - antimicrobial peptide

Introduction

   Apoptosis is a very important procedure in eukaryotic development and cell homeostasis. That it is inappropriate to failure of activate the apoptotic program is related to inflammatory and autoimmune diseases, while apoptosis occurring early has been associated with neurodegenerative disease (Kashiwagi et al., 2007). During the past 30 years, there have been many studies on the efficacy of apoptosis as a potential target for cancer therapy and the mechanism by which the failure of tumor cells to undergo apoptosis leads to malignant potential and chemotherapeutic resistance (Huerta et al., 2006).

   Lung cancer has a high morbidity and mortality rate. Worldwide, lung cancer is the primary cause of cancer-related death, accounting for approximately 12.5% of newly diagnosed cancers (World Cancer Research Foundation International) and 1.3 million deaths worldwide each year (Jadus et al., 2012). Unfortunately, there are no effective therapies for lung cancer, and there has been little change in the overall 5-year survival rate since the 1970s (Marshall 2011). Currently available treatments for lung cancer are problematic, and therefore, there is a pressing need to develop appropriate treatments that are effective and have fewer side effects. This need has led to research on lung cancer treatments that target specific cellular signaling pathway proteins associated with tumors (Movassaghian et al., 2013).

   The fused polypeptide strategy is a trial of targeted therapy for malignant tumors. Our previous studies have shown that this strategy is feasible and demonstrated that the fused polypeptide containing the human immunodeficiency virus 1 transactivator of transcription protein (TAT), DV3 and BH3, which could specially inhibit the growth of colon cancer cells by inducing apoptosis in vitro and efficiently inhibited tumor growth in nude mice in vivo. DV3 is come from DV1 which is a synthetic peptide composed of D-configuration amino acids that has a strong binding ability with CXCR4 and shows very significant inhibition of CXCR4 activity. BH3 is the only domain of PUMA, a p53 upregulated modulator of apoptosis (Liu et al., 2009).

   Antimicrobial peptides (AMPs) are a class of polypeptides from natural sources, which exist in widely from microbes to human (Martin et al., 1995). AMPs not only kill quickly the invading microorganisms, some AMPs but also kill some tumor cells (van et al., 2012). DEF is a new unknown peptide which composes of 10

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amino acid residues. It is the product of a new gene that was fished from Scallop with 5'RACE (rapid amplification of cDNA end, RACE) when we study AMPs from bivalve (Liang et al., 2012). There is uncertain that it could inhibit the growth of tumor cells like some AMPs (van et al., 2012). In view of these, we tried to design and synthesize the fused polypeptide with DEF including TAT and DV1, which help DEF enter tumor cell more easily, quickly and specially. It is more accurately to study the antitumor effects of DEF if it could enter cells effectively. The present study has determined that the fused polypeptide could enter the lung adenocarcinoma cell line GLC-82 with high effectively. Most exciting of all, we find that the fused polypeptide could inhibit the growth of GLC-82 via inducing apoptosis and co-locate with some mitochondria, suggesting that DEF has antitumor effects.

Materials and Methods

Fused polypeptide synthesis

The polypeptides of the study were chemically synthesized by the GL Biochem Ltd. (Shanghai, China). The purity of all polypeptides exceeds 95%. The polypeptides sequences are as follows: TAT-DV1-DEF (RRRQR RKKRG GGG LG*ASW HRPDK CCLGY QKRRL P* GGG MRAGY ISVPG), TAT-DV1 (RRRQR RKKRG GGG LG*ASW HRPDK CCLGY QKRRL P*), DEF (MRAGY ISVPG); italic letters stand for D-configuration amino acids. TAT-DV1 and DEF were used as control peptides, and TAT-DV1-DEF was the expected functional molecule. FITC was conjugated to the amino-end of the polypeptide to aid in localization studies. The peptides were dissolved in sterile phosphate buffer saline for use.

Cell culture

The human lung adenocarcinoma cell GLC-82 and human embryonic kidney fibroblasts HEK-293 were obtained from the State Key Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences (Beijing, China). GLC-82 cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (RPMI 1640, Gibco, Invitrogen, Paisley, UK) and HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 incubator (Liang 1985, Movassaghian et al., 2013, Yu et al., 2006).

Estimate the Cytotoxicity of TAT-DV1-DEF, TAT-DV1 and DEF

To investigate the biologically activity of TAT-DV1-DEF, CCK-8 (Cell Counting Kit 8, CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) was used to estimate the cell survival rate of TAT-DV1-DEF treatment. In addition, treatment with the control peptide was also analyzed with a CCK-8. The CCK-8 was used as previously described and following the manufacturer's protocol (Liu et al., 2009). The polypeptides were used at a concentration of 80 µmol/l. After 72 hours of peptide incubation, the absorbance of all wells was measured with

a microplate reader (Bio-Rad 680; Bio-Rad, Hercules, CA) at a measured wavelength of 450 nm and a reference wavelength of 630 nm. The experiment was repeated in triplicate. Survival Rate (%) = ($A_{treated}$ − A_{blank}) × 100/ ($A_{control}$ − A_{blank}).

Distribution of polypeptides in cells

To determine the distribution of the polypeptide in cells, laser scanning confocal microscopy was used to observe the green fluorescence from the FITC conjugated to the polypeptides as previously described (Liu et al., 2009) and following the manufacturer's protocol (Hao et al., 2007). Briefly, the lung adenocarcinoma cell line GLC-82 and the human embryonic kidney cell line HEK-293 were cultured on coverslips in 35 mm plates with the three polypeptides at 40 µmol/l for 1.0 hour, and then, the cells were stained with the mitochondria dye MitoTracker® Red CMXRos (Invitrogen, Carlsbad, CA). After being dyed, the cells were washed three times with fresh, pre-warmed culture medium; then, the medium was discarded and the cells were co-cultured with pre-warmed medium containing 4% methanol at 37°C for 15 minutes. The cells were washed three times with PBS, and the coverslips were placed on slides. All slides were observed using laser scanning confocal microscopy (Leica TCS SP5; Leica Microsystem, Wetzlar, Germany).

Transfection efficiency of the fused polypeptides was confirmed by flow cytometry

To confirm the transfection efficiency of the three fused polypeptides, flow cytometry was used to capture the green fluorescence from the FITC conjugated to the polypeptides as previously described (Liu et al., 2009). Briefly, GLC-82 and HEK-293 were co-cultured respectively with 40 µmol/l three fused polypeptides for 1.0 hour. The cells were then harvested, dispersed and analyzed with a fluorescence-activated cell sorter (BD FACSCanto™II; Becton, Dickinson and Company, Franklin Lakes, NJ USA) using FACSDiva Version 6.1.2 software. The percentages of transfected cells in each population were determined from at least 1×10^4 cells. The control cells were not treated with fused polypeptide.

To evaluate apoptosis with flow cytometry

To determine whether TAT-DV1-DEF could induce apoptosis, flow cytometry was used to analyze the sub-G1 cell population as previously described (Liu et al., 2009). Briefly, 1×10^5 cells were cultured in 35 mm plates for 24 hours. The medium was discarded and replaced with fresh culture medium containing 20, 40, or 80 µmol/l TAT-DV1-DEF. After 72 hours of incubation, all cells including floating cells were harvested and fixed with 70% ethanol at 4°C overnight. The cells were cultured in RNase A/PBS (100 µg/ml) at 37°C for 30 minutes after being washed twice with PBS. The fluorescence-activated cell sorting (FACS) (BD FACSCanto™II; Becton, Dickinson and Company, Franklin Lakes, NJ USA) was used to detect the fluorescence from cells which DNA was labeled with propidium iodide (50 µg/ml) and FACSDiva Version 6.1.2 software was used to analyze. The percentage of sub-G1 cells in every test was determined from at least 1×10^4
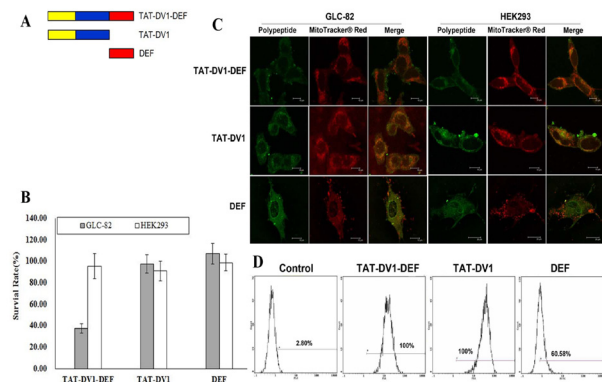
Figure 1. Fusion Peptides Penetrate Cells and Inhibit Cell Growth. A. Schematic structure of peptides used in the study. B. The bar of survival rates of cells treated with the fusion peptides. The two cell lines GLC-82 and HEK-293 were co-cultured with 80 µmol/l polypeptides TAT-DV1-DEF, TAT-DV1 and DEF for 72 hours respectively and the survival rates were compared with analysis of variance. Results indicated that TAT-DV1 and DEF had no effects on these two cell lines ($P > 0.05$). Only TAT-DV1-DEF inhibited the growth of GLC-82 significantly ($P < 0.01$), but not of HEK293 ($P > 0.05$). C. Distribution of polypeptides in cells. The green color shows the fluorescein isothiocyanate-labeled peptides and the red color shows the mitochondria stained with Mito Tracker® Red CMXRos. The yellow color represents the co-localization of peptides and mitochondria. Scale bars stand for 10 µm. D. Histogram of peptides transfection efficiency after co-cultured for 1.0 hour with GLC-82. The GLC-82 was transfected with TAT-DV1-DEF and TAT-DV1 high-efficiently (about 100%), indicating the two polypeptides are able to penetrate the cell membrane of GLC-82 efficaciously. The DEF has about 60% transfection efficiency

cells. The control group was treated with PBS, and the other two groups were treated with 80 µmol/l TAT-DV1 and DEF.

DAPI staining to reveal changes of nucleus

To observe the morphological changes in the cells after treatment with TAT-DV1-DEF, fluorescence microscopy was used to analyze the blue fluorescence from cells stained with DAPI (4, 6-diamidino-2-phenylindole, DAPI) as previously described (Liu et al., 2009). GLC-82 cells were seeded onto coverslips in 35 mm dishes as 5×10^4 cells each dish. After 24 hours, the cells were kept on culturing with fresh culture medium containing 80 µmol/l TAT-DV1-DEF. The cells were washed with PBS and fixed in pre-chilled methanol at 4°C for 10 minutes after 0, 48, and 72 hours respectively. The cells stained with 1 µg/ml DAPI for 15 minutes after washed with PBS, and then followed by PBS washing for 5 minutes. If there were nuclear shrinkage and chromatin condensation or fragmentation, the cells were defined apoptosis morphologically.

Western blot

To further demonstrate that the polypeptide could induce apoptosis of lung adenocarcinoma cells, western blotting was used to analyze the key molecules in the apoptosis pathways as previously described (Liu et al., 2009). Cells were co-cultured with 20, 40, and 80 µmol/l TAT-DV1-DEF for 72 hours and harvested. All cells

were lysed with lysis buffer with a protease inhibitor cocktail (Protease inhibitor cocktail 50×, Applygen Technologic, Peking, China) for 40 minutes. The total protein solutions were supernatant from cell lysates were centrifuged at 12, 000 rpm at 4°C for 20 minutes, and the protein concentrations were determined. Equal amounts of the total protein were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were split two parts. The two parts were incubated respectively with antibody against Caspase-3 (1:500, Cell Signaling Technology, Danvers, MA) and antibody against β-actin (1:2, 000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and then with the corresponding secondary antibody (1:2, 000, Zhongshan Golden bridge Bio-technologic, Peking, China) conjugated to horseradish peroxidase. Super Signal ECL (Applygen Technologic, Peking, China) was used to visualize the antibody binding and recorded with X film.

Statistical analysis

The data are presented as $\overline{\chi} \pm SD$. The independent-samples t-test was used to evaluated means compare between two groups. One-way analysis of variance was used for the mean comparisons among three or more groups. A *P*-value of < 0.05 was considered statistically significant.

Results

TAT-DV1-DEF could inhibit the growth of lung adenocarcinoma cancer cells but not HEK-293 cells

The polypeptides TAT-DV1-DEF, TAT-DV1 and DEF were synthesized chemically (Figure 1A). And their suppressive effects on cancer cells were confirm. The results displayed that TAT-DV1 and DEF did not inhibit the growth of two cell lines GLC-82 and HEK-293, while TAT-DV1-DEF significantly inhibited the growth of human lung adenocarcinoma cell line GLC-82, the survival rate of the TAT-DV1-DEF treated lung adenocarcinoma cells was $37.64 \pm 4.21\%$ ($P < 0.01$, Figure 1B). TAT-DV1-DEF did not inhibit the growth of the human embryo kidney cell line HEK-293 cell line and the survival rates of these two cell lines in the TAT-DV1 and DEF treated groups ranged from 90 to 113% ($P > 0.05$, Figure 1B).

TAT-DV1-DEF mostly localizes in the cytoplasm and co-localizes with some mitochondria

This fused polypeptide was predicted to localize mostly in the cytoplasm with TAT-DV1 based on our previous study (Liu et al., 2009). Hence, laser scanning confocal microscopy was used to analyze the green fluorescence FITC from the amino-end of TAT-DV1-DEF and the red fluorescence from the mitochondria specific tracking dye MitoTracker® Red CMXRos as a reference dye. The results indicated that the fused polypeptide TAT-DV1-DEF localized mainly in the cytoplasm in the two cell types and co-localized with some mitochondria after these cells were co-cultured with the polypeptide for 1.0 hour (Figure 1C).
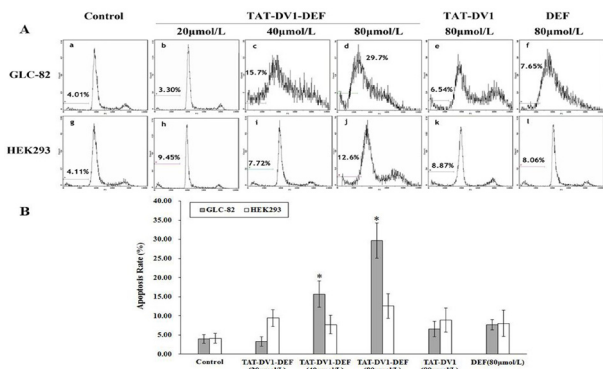
Figure 2. Apoptosis Rate Was Obtained by flow Cytometry Analysis. A. The histogram of apoptosis in different conditions. a~f stand for GLC-82 cell line, and g~l for HEK-293 cell line. B. Bars of apoptosis rate. *stands for $P < 0.05$ vs control group, there were no difference among the other groups
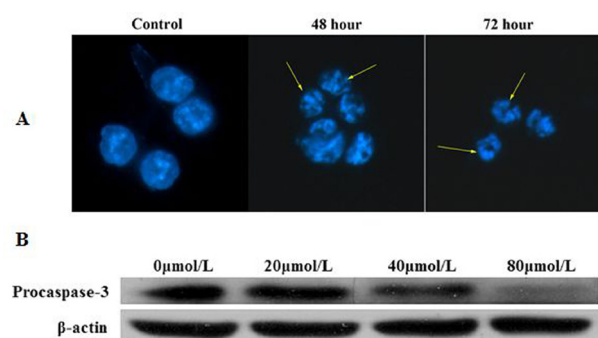
Figure 3. Apoptosis Assay after Peptide Treatment. A. DAPI staining after peptide treatment. Apoptotic changes of GLC-82 nuclear under fluorescence microscope after treatment with 80 µmol/l TAT-DV1-DEF and DAPI staining. After 48 hours of treatment, nuclei showed characteristic crimpled nuclear membrane, condensed chromatin. After 72 hours of treatment, the nuclei exhibited remarkable membrane break, chromatin condensation and fragmentation. All of these signs are representative of apoptosis. B. Change of expression of Caspase-3 was analyzed by western blot. Detection of key molecules in apoptosis activation after GLC-82 treated with TAT-DV1-DEF for 72 hours. The procaspase-3 displayed a decrease by treatment. Actin was detected as loading control

The polypeptides with TAT entered cells effectively

A fluorescence-activated cell sorter was used to monitor the efficiency of TAT-DV1-DEF entry. The three polypeptides were sorted by FITC. The polypeptides with TAT (TAT-DV1-DEF and TAT-DV1) could enter GLC-82 cells with an efficiency of almost 100%, while the peptides DEF could only enter GLC-82 cells with an efficiency of 60.58% for without TAT (Figure 1D).

TAT-DV1-DEF could induce apoptosis of GLC-82 cells

We do not know whether it could induce apoptosis although TAT-DV1-DEF remarkably inhibited the growth of GLC-82 cancer cells. The results from flow cytometry indicated that the fused polypeptide could induce apoptosis of lung adenocarcinoma cells in a dose-dependent manner ($P < 0.05$). However, the apoptosis rates of HEK-293 cells were not significantly different ($P > 0.05$) between the groups treated with the fused polypeptide and the control PBS treatment group. The two control peptides TAT-DV1 and DEF were not significantly different ($P > 0.05$) with

respect to their effects on GLC-82 and HEK-293 cells. These results suggested that only TAT-DV1-DEF had the ability and specificity to induce apoptosis in lung adenocarcinoma cell (Figure 2).

TAT-DV1-DEF induced lung adenocarcinoma cells to produce typical apoptotic nuclear changes

DAPI staining might show nuclear changes of lung adenocarcinoma cells after co-culture with 80 µmol/l TAT-DV1-DEF for 0, 48 and 72 hours respectively. The results were determined from fluorescence microscopy photographs. Nuclear membrane shrinkage, chromatin condensation and marginalization were observed in the 48 hour-treatment group, which indicated apoptosis. In the 72 hour-treatment group the nuclear membrane was completely broken and the chromatin was highly condensed and fragmented, indicating significant apoptotic morphological characteristics (Figure 3A). However, the control group (0 hour group) cells had intact nuclear membranes and clear chromatin, which are indicative of normal nuclear morphology. These morphological observations demonstrated that, similar to what was observed by flow cytometry, the fused polypeptide TAT-DV1-DEF induces apoptosis of the lung cancer cell line GLC-82.

Western blotting confirmed apoptosis

To further demonstrate that the fused polypeptide induced cancer cell apoptosis, western blotting was used to analyze the apoptosis executor caspase-3. There are generally two apoptosis pathways that involve Caspase-9 and Caspase-8. However, the two pathways converge on the same molecular Caspase-3. A change in Caspase-3 (procaspase-3 had also change) in the treated group indicates that the treated cancer cells have undergone apoptosis. The results of western blotting indicated that the procaspase-3 levels decreased as the polypeptide dose increased. This decrease in procaspase-3 levels may have resulted from its activation, indicating that the fused polypeptide did induce cancer cell apoptosis (Figure 3B). We did not detect significant activation of apoptosis pathways by TAT-DV1 or DEF treatment in cancer or normal cells (data not shown).

Discussion

Failure to regulation of apoptosis is one of the main causes of drug resistant cancers. Biotherapy is becoming a new cancer therapy option for its targeted after Anderson et al. first succeeded in curing a girl with severe combined immunodeficiency by using the adenine nucleotide deaminase gene in 1990 (Anderson et al., 1990).

Peptide drugs are promising anticancer agents that have many advantages such as low toxicity, low tissue accumulation, high biological activity and the specific binding of the corresponding molecule.

Our previous study demonstrated that a fused polypeptide could conserve the characteristics of each component peptide. TAT has peptide transduction domains that can not only transfer peptides, nucleic acids, and even metal nanoparticles into a variety of mammalian

cells with high efficiency (approximately 100%) in a very short period of time (the protein transduction can occur within the cell at 10 minutes) but can also do so without any detected toxicity (Nagahara et al., 1998; Snyder et al., 2004). CXC chemokine receptor 4 (CXCR4) and its ligand stromal-derived factor-1 (SDF-1, or CXCL12) are thought play a vital role in the regulation of metastasis of many solid tumors such as lung cancer, breast cancer, prostate cancer and kidney cancer (Balkwill, 2004). CXCR4 is overexpressed in at least 23 types of cancer, including breast cancer, small cell lung cancer, prostate cancer, colon cancer, pancreatic cancer, kidney cancer and non-Hodgkin's lymphoma (Balkwill 2004; Snyder et al., 2005). Because DV1 has very significant inhibition of CXCR4 activity, it is an ideal CXCR4 antagonist because it is composed of D-configuration amino acids, which are less susceptible to degradation by the proteasome in the body, and is thus more promising in clinical applications.

To determine the antitumor effects of DEF, the present study utilized a fused polypeptide including TAT, DV1 and DEF. TAT ensure that the polypeptide entered cells more easily and effectively, and DV1 could enable targeting to the many tumors that overexpress CXCR4. If DEF has antitumor effects the fused polypeptide must be have the same effects. Excitingly, this fused polypeptide retained the TAT characteristic of entering cells efficiently. The results from flow cytometry demonstrated that the two polypeptides containing TAT could enter cells with approximately 100% efficiency after co-culture for 1 hour (Figure 1D). This fused polypeptide could specifically inhibit the growth of the lung adenocarcinoma cell line GLC-82 but not the non-tumor cell HEK-293, which indicates that the inhibition of the fused polypeptide is specific (Figure 1B). In addition, the fused polypeptide could induce apoptosis of lung adenocarcinoma cells as a dose-dependent manner (Figure 2). This finding indicated DEF could inhibit the growth of lung adenocarcinoma cell line GLC-82 via inducing the cells apoptosis (Figure 3). All of that demonstrated that the new peptide has antitumor effects like some AMPs, meantime also implied the ideal of fused peptides as an antitumor treatment is feasible and promising.

Encouragingly, AMPs have become a research topic of interest and their antitumor mechanisms were diverse. Wu et al. studied antitumor activity of pardaxin, a fish antimicrobial peptide and found that pardaxin could inhibit the colony formation of murine fibrosarcoma cell line MN-11. The further study of mechanism of inhibition found that the treated MN-11 cells with pardaxin showed apoptotic bodies containing nuclear fragments, and the chromatin had become condensed or marginalized. Meantime these cells showed that mitochondria appeared hollow, nuclei were condensed, and cell membranes were disrupted. Thus, apoptosis and lytic characters co-exist and are the potential mechanisms of pardaxin-induced cell death (Wu et al., 2012). Likewise, Ceron et al. (2010) found that antimicrobial peptide Cecropin A has antitumor effect. The results displayed that the human promyelocytic leukemia cell HL-60 treated with cecropin A, the cells presented classical characters of apoptosis. But cecropin A-induced apoptosis was independent of Caspase

family members, because the activity of Caspase-8 and -9 were irrelevant. It was related to an increase of reactive oxygen species (ROS) generation. These data indicate that cecropin A is able to induce apoptosis in HL-60 cells through a signaling mechanism mediated by ROS, but independently of caspase activation. Other studies revealed that antitumor effects of AMPs were via cytoplasmic membrane permeabilization and DNA binding (Tian et al., 2013), a necrotic cell death pathway (Lin et al., 2012), disruption of cancer cell membranes (Hsu et al., 2011) et al. Our study has determined that the fused polypeptide with DEF depressed the GLC-82 cell growth via inducing cells apoptosis which was related to Caspase-3 (Figure 3). Meantime, the results from the confocal microscope (Figure 1C) indicated that apoptosis of GLC-82 cell maybe related to mitochondria pathway.

In summary, we succeeded in studying a new unknown peptide DEF has antitumor activity with the strategy of fused polypeptide. It was initially identified that the growth inhibition of lung adenocarcinoma cell line GLC-82 was by inducing tumor cell apoptosis. These results are promising and exciting which will motivate us to continue to study other effects of DEF, such as in anti-microbial, anti-inflammatory.

Acknowledgements

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