#ASO-2010-05-0746# (highlighted manuscript)

Induction of the mitochondria apoptosis pathway by phytohemagglutinin erythroagglutinating in human lung cancer cells

Wei-Ting Kuo[a], Yung-Jen Ho[b], Shyh-Ming Kuo[c], Feng-Huei Lin[d], Fuu-Jen Tsai[e], Yueh-Sheng Chen[b,e†], Guo-Chung Dong[d†], Chun-Hsu Yao[b,e*†]

[a]Graduate Institute of Clinical Medical Science, China Medical University, Taichung 40402, Taiwan

[b]Department of Biomedical Imaging and Radiological Science, China Medical University, Taichung 40402, Taiwan

[c]Department of Biomedical Engineering, I-Shou University, Kaohsiung 84001, Taiwan

[d]Division of Medical Engineering Research, National Health Research Institutes, Miaoli 35053, Taiwan

[e]School of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

*** Correspondence and reprint request to:**

Dr. Chun-Hsu Yao, Department of Biomedical Imaging and Radiological Science, China Medical University, Taichung 40402, Taiwan;

Tel: 886-4-22053366 ext. 7806; Fax: 886-4-2205-4179;

Email: chyao@ctust.edu.tw; chyao@mail.cmu.edu.tw

†**These authors contributed equally to this work**

Synopsis

In this paper, PHA-E was used to induce apoptosis of A-549 lung cancer cells, and the possible signal transduction pathway was determined.~~and determine the possible signal transduction pathway.~~ The results show that PHA-E can induce growth inhibition and cytotoxicity of lung cancer cells, which is mediated through an activation of the mitochondria apoptosis pathway.

Abstract

Background

De-regulation of apoptosis will influence the balance of cell proliferation and cell death, resulting in various fatal diseases that can include cancer. In prior research reports related to cancer therapy, phytohemagglutinin, a lectin extracted from red kidney beans, demonstrated the ability to inhibit the growth of human cancer cells. However, one of its isoforms, erythroagglutinating has yet to be evaluated on its anti-cancer effects.

Methods

PHA-E was used to induce apoptosis of A-549 lung cancer cells and the possible signal transduction pathway was elucidated, ~~in order to determine the possible signal transduction pathway,~~ as measured by the MTT assay, G6PD release assay, flow cytometry, and Western blot analysis.

Results

PHA-E treatment caused a dose-dependent increase of cell growth inhibition and cytotoxicity on A-549 cells. In Annexin V/PI and TUNEL/PI assay, we found that the rate of apoptotic cells was raised as the concentration of PHA-E increased. Treatment of A-549 cells with PHA-E resulted in enhancing the release of cytochrome c, which thus activated an increase in caspase 9 and caspase 3, the up-regulation of Bax and Bad, the down-regulation of Bcl-2 and phosphorylated Bad, and finally the inhibition of the epidermal growth factor receptor and its downstream signal pathway PI3K/Akt and MEK/ERK.

Conclusions

Our study has demonstrated that PHA-E can induce growth inhibition and cytotoxicity of lung cancer cells, which is mediated through an activation of the

mitochondria apoptosis pathway. These results suggest that PHA-E can be developed into a new therapeutic treatment that can be applied as an effective anti-lung cancer drug in the near future.

Keywords: Apoptosis; Lung cancer; Phytohemagglutinin Erythroagglutinating; Epidermal growth factor receptor

Introduction

Lung cancer, generally divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), is the leading cause of cancer-related deaths in both men and women worldwide [1, 2]. NSCLC accounts for approximately 85% of lung cancer cases, and is furthermore histologically subdivided into adenocarcinoma (50%), squamous cell carcinoma (30%) and large cell carcinoma (20%) [3]. Therefore, it is critical to study new and effective drug treatments, and develop novel therapeutic strategies for the management of lung cancer. Past studies have revealed that both growth inhibition and apoptosis induction are important determinants in response to anti-cancer therapy [4].

Apoptosis, programmed cell death, is a physiological mechanism that is required for maintaining cell numbers and removing unnecessary cells [5, 6]. The two major pathways that trigger apoptosis are an extrinsic pathway initiated by death receptors and an intrinsic pathway that occurs through the mitochondria. The extrinsic pathway is activated at the cell surface when a particular ligand binds to its respective death receptors. The intrinsic pathway involves the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm by the loss of mitochondrial membrane potential (MMP). Cytochrome c activates caspase 9, which in turn cleaves

downstream caspases such as caspase 3, and finally results in apoptosis [7-11]. Additionally, apoptosis pathway of cells was also controlled by Bcl-2 family proteins [12, 13]. Morphological and biochemical features of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, and the final stage includes the engulfment by macrophages or neighboring cells [5]. De-regulation of apoptosis can affect the balance of cell proliferation and cell death, resulting in various diseases that can include cancer [14, 15]. Thus, drugs devised to re-stimulate apoptosis might be an effective method in fighting against cancer [16, 17].

Phytohemagglutinin (PHA) is the lectin derived from red kidney beans (Phaseolus vulgarus), and phytohemagglutinin erythroagglutinating (PHA-E or E_4) belongs to the PHA isoform groups [18, 19]. The content of PHA in red kidney beans is reported to be 0.4-1.2% [20]. PHA consists of four subunits which are grouped into E- and L-subunits. E-subunit (for erythroagglutinating) appears to be involved in erythrocyte agglutination, and the other type L-subunit (for leucoagglutinin) is involved in lymphocyte agglutination. These subunits are synthesized alongside in the endoplasmatic reticulum and then randomly combined to form five isolectins that are classified the structures L_4, L_3E_1, L_2E_2, L_1E_3, and E_4. All of these isolectins are glycoproteins of approximately 125 kDa [21]. PHA is usually used for the activation

of normal T-cells [22], and has been demonstrated to exhibit antifungal, antiviral, and HIV-1 reverse transcriptase inhibitory activities [23, 24]. In prior literature related to cancer therapy, PHA was also reported to inhibit the growth of human cancer cells [25, 26]. Also, the PHA-L_4 has also been reported to exert its therapeutic effects through the ability to exhibit direct antitumor cytotoxic effects, to enhance the antineoplastic effect of radiation and chemotherapy, and to decrease the liability to malignant transformation [27]. However, PHA-E has never been evaluated on its anti-cancer effects against lung cancer cells. In this study, we aimed at evaluating the ability of PHA-E to induce apoptosis and investigating its effects and on the signal transduction in lung cancer cells A-549.

~~Phytohemagglutinin (PHA), the lectin derived from red kidney beans (Phaseolus vulgarus), has been demonstrated to inhibit the growth of human cancer cells [18]. PHA-E (erythroagglutinating) belongs to the PHA isoform groups [19]. This study evaluated the ability of PHA-E to induce apoptosis and its stimulated effect on the signal transduction pathway in lung cancer cells~~.

Materials and methods

Materials

PHA-E was purchased from Sigma (USA). The anti-bodies for α-Tubulin (B-7), p-PI 3-kinase p85α (Tyr 508), p-MEK-1/2 (Ser 218/Ser 222), p-ERK (E-4), Bad (C-7), Bax (P-19), Bcl-2 (C-2) and cytochrome c (7H8) were purchased from Santa Cruz (USA). The anti-bodies for phospho-Akt (Ser473), phospho-Bad (Ser155) and cleaved caspase 9 (Asp353) were purchased from Cell Signaling (USA). The anti-body for caspase 3 was purchased from Millipore (USA). The anti-bodies for EGFR and phospho-EGFR (Y1068 and Y1101) were purchased from Abcam (USA), and EGF itself was purchased from Upstate (USA). The cytotoxicity assay kit, Annexin V conjugates, APO-BrdU™ TUNEL assay kit, propidium iodide solid and DAPI dilactate were all purchased from Invitrogen (USA).

Cell culture

A-549 (human lung adenocarcinoma) cells were obtained from BCRC (Taiwan). Cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum,

2 mM L-glutamine, and 1.5 g/L sodium bicarbonate. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment. Typically, adherent cells were harvested with trypsin and re-suspended in a serum-containing medium before use in the assays as described below.

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) ~~MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)~~ assay was performed to determine the effect of PHA-E on cell growth. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by mitochondrial succinate dehydrogenase of living cells. This reduction occurs only when mitochondrial reductase enzymes are active so that they can be directly related to the number of viable cells. The crystals can be dissolved in DMSO, and the concentration determined by microplate reader at 570 nm. The result is proportional to the cell numbers.

Briefly, A-549 cells were seeded at 10,000 ~~10000~~ cells/well in 24-well plates and incubated for 24 h ~~hr~~. The medium was then replaced with serum-free medium, PHA-E (20-160 µg/ml) and co-cultured for 48 h ~~hr~~. After treatment with PHA-E, the

medium was then replaced with 0.5 mg/ml MTT medium and incubated for 4 h ~~hr~~. The MTT solution was removed from the wells and the formazan crystals were dissolved in DMSO. Finally, the concentration was determined by a microplate reader.

G6PD release assay

Glucose-6-phosphate dehydrogenase (G6PD) ~~G6PD~~ release assay was performed to determine the effect of PHA-E on cell death. The number of dead cells was assessed by measuring the release of cytosolic enzyme G6PD ~~glucose-6-phosphate dehydrogenase (G6PD)~~ from damaged cells into the surrounding medium, using a cytotoxicity assay kit and following the manufacturer's instructions. The assay quantifies plasma membrane integrity as released G6PD, based on the G6PD-dependent conversion of $NADP^+$ to NADPH and subsequent reduction of resazurin to resorufin, which emits fluorescence at 590 nm after excitation at 530 nm.

Briefly, A-549 cells were seeded at 10,000 ~~10000~~ cells/well in 24-well plates and incubated for 24 h ~~hr~~. The medium was then replaced with a serum-free medium, PHA-E (20-160 μg/ml), which was added and co-cultured for 48 h ~~hr~~. After homogenized, only 50 μl/well of the supernatant medium was transferred to 96-well plates, then 50 μl of a resazurin/reaction mixture was added to each well. After

light-protected incubation for 20 min,~~-~~ at 37°C, measurements were made by using the fluorescence microplate reader.

Annexin V/PI assay

Annexin V assay was used to quantify the number of apoptotic cells by flow cytometry. In apoptotic cells, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V labeled with a fluorophore can identify apoptotic cells by binding to PS exposed on the outer leaflet.

Briefly, cells were harvested after treatment of PHA-E, washed in a cold PBS and re-suspended in 100 µl binding buffer. Samples were incubated with 5 µl Annexin V and 2 µl of the 100 µg/ml PI in a dark environment for 15 min at room temperature. The volume was adjusted to 200 µl with a binding buffer, and fluorescence was then measured with a flow cytometry ~~cytometer~~.

TUNEL/PI assay

Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) ~~TUNEL (terminal deoxynucleotide transferase dUTP nick end labeling)~~ assay was used to

identify cell apoptosis. In apoptotic cells, nuclei that are activated eventually degrade their nuclear DNA into fragments. The TUNEL assay kit can detect the DNA fragmentation of these apoptotic cells.

After treatment of PHA-E, the harvested cells were briefly fixed on ice for 15 min with 1% formaldehyde, then followed with an overnight permeabilization in 70% ethanol at -20°C. The 70% ethanol was removed and the fixed cells were washed.

This process was followed by incubation with a DNA-labeling solution at 37°C for 60 min. An antibody solution was subsequently added and the cells were treated in a dark environment for 30 min at room temperature. Finally, a PI/RNase solution ~~used to detect their specific stage of cell cycle~~ was added to the cells, which were maintained in the dark environment for an additional 30 min at room temperature. Fluorescence was measured with a flow cytometry ~~cytometer~~.

Western blot analysis

After treating the cells with PHA-E, they were then rinsed twice with PBS. The cells were then directly solubilized in a lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) containing a protease inhibitor cocktail for 5 min. on ice. The cells were then scraped and the lysate was

collected in a eppendorf tube. The lysate was cleared by centrifugation at 12,000~~12000~~g for 30 min. at 4°C, and the protein concentration in the supernatant was determined by the Bradford method (Bio-Rad protein assay).

For Western blotting, equal amounts of proteins were resolved over 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ~~SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)~~ and transferred onto a polyvinylidene fluoride (PVDF) ~~PVDF (polyvinylidene fluoride)~~ membrane. The nonspecific sites on blots were blocked by incubating them in a blocking buffer (5% nonfat dry milk/TBS, pH 7.4) for 1 h ~~hr~~ at room temperature. Incubation was performed with an appropriate monoclonal primary antibody in TBS overnight at 4°C, and then incubated with a horseradish peroxidase ~~(HRP)~~ conjugated secondary antibody for 1 h ~~hr~~ at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence system.

Statistical analysis

Numerical data were presented as mean ± standard deviation. Statistical differences among samples were evaluated by one-way analysis of variance (ANOVA) followed by *post hoc* Fisher's LSD multiple comparison test. Statistical significance

was considered at a probability $P < 0.05$.

Results

PHA-E results in growth inhibition and cell death

In this study, we first evaluated the cell viability of E-PHA on A-549 cells by MTT assay. As shown in Figure 1a, PHA-E treatment of A-549 cells resulted in cell growth inhibition by 13, 27, 41 and 49% at the doses of 20–160 µg/ml. To investigate cytotoxicity, we used an assay that measured the leakage of the enzyme G6PD ~~glucose-6-phosphate dehydrogenase (G6PD)~~ from the cytoplasm into the culture medium. PHA-E treatment (20-160 µg/ml; 48 h ~~hr~~) caused a dose-dependent increase of fluorescence in A-549 cells (Fig. 1b). Because the resulting fluorescence signal is proportional to the amount of G6PD release, PHA-E treatment results in cytotoxicity of A-549 cells.

PHA-E induces apoptosis

We next confirmed whether PHA-E induced growth inhibition and cytotoxicity of A-549 cells is mediated through the induction of apoptosis, and ~~. We~~ quantified cell apoptosis by flow cytometry. The percentage of apoptotic, dead and live cells were

analyzed by Annexin V/PI assay. Early apoptotic cells were the Annexin V-positive but PI-negative cells, while late apoptosis were the Annexin V-positive and PI-positive cells. The apoptotic cells were the sum of early apoptosis and late apoptosis. Dead cells were the PI-positive but Annexin V-negative cells, live cells were the PI- negative and Annexin V-negative cells (Fig. 2a). As shown in Table 1, after treatment with PHA-E at 20, 40, 80 and 160 μg/mL for 48 h ~~hr,~~, the apoptotic cells were 7.4±2.7%, 18.2±2.5%, 22.6±4.4% and 35.6±1.3%~~5.9%, 15.8%, 20.2% and 36.5%~~, respectively, which were higher than the control at 2.6±0.3%~~2.9%~~. Dead cells were a dose-dependent increase and live cells were a reduction. In addition, the same trend of apoptosis rate was determined by TUNEL/PI assay. PHA-E treatment of A-549 cells resulted in 6.4%, 7.1%, 8.9% and 14.6% of apoptotic cells at the doses ~~a dose of 20, 40, 80 and 160 μg/mL~~, respectively (Fig. 2b).

PHA-E increases the expression level of cytochrome c, cleaved caspase 9 and cleaved caspase 3

The mitochondria play an important role in apoptosis by releasing apoptogenic factors such as cytochrome c. The release of cytochrome c can activate caspase 9 and in turn trigger caspase 3. Next, we assessed the effect of PHA-E on cytochrome c,

cleaved caspase 9 and cleaved caspase 3. As shown in Figure 3, cytochrome c was increased after treatment with PHA-E by Western ~~western~~ blot analysis. The expression level of cleaved caspase 9 and cleaved caspase 3 were also enhanced when the PHA-E dose was raised.

PHA-E results in up-regulation of Bad and Bax and down-regulation of phospho-Bad and Bcl2

Because Bcl-2 family members are the major regulators of apoptosis, we next determined the effect of PHA-E on the protein levels of Bad, phospho-Bad, Bcl-2 and Bax in A-549 cells. The Western blot analysis exhibited an increase in the expression of Bad and Bax concomitant with a decrease in the expression of phospho-Bad and Bcl-2 in a dose-dependent fashion (Fig. 4), further confirming the induction of the apoptotic process.

PHA-E inhibits the expression level of EGFR and phosphorylation of EGFR, PI3K, Akt, MEK and ERK

EGFR is expressed at high levels in a wide range of tumor types and in most lung

cancers, and is associated with lower rates of survival. We assessed the effect of PHA-E on EGFR and its specific phosphorylation sites Y1068 and Y1101. Treatment of A-549 cells with PHA-E resulted in a dose-dependent decrease in the specific phosphorylation sites Y1068 and Y1101, and the expression level of EGFR also reduced (Fig. 5a). Downstream signaling pathways of EGFR include the PI3K/AKT and MEK/ERK pathways ~~pathway~~. We next determined the effect of PHA-E treatment to A-549 cells on the expression of phosphorylation of PI3K, Akt, MEK and ERK, and we found that these molecules investigated were all inhibited ~~We found that PHA-E inhibited phosphorylation of PI3K, Akt, MEK and ERK~~ (Fig. 5b).

Discussion

~~As a result of its poor prognosis, lung cancer is the leading cause of cancer-related deaths in the world. Therefore, the development of novel anti-cancer drugs that can provide effective drug treatments is necessary to enhance the quality of life. It is important to develop treatments that can effectively trigger cell death in tumor cells during cancer therapy. Previous studies have demonstrated that effective treatment concerning anti-cancer drugs is largely dependent on their ability to inhibit growth and induce apoptosis in cancer cells [20].~~

~~PHA has been reported to possess anti-cancer effects on several human cancer cells [18], but one of its isoforms, PHA-E PHA- E, had not been thoroughly explored until this study.~~

As a result of its poor prognosis, lung cancer is the leading cause of cancer-related deaths in the world. Therefore, an early diagnosis and aggressive therapy are important to maintaining a patient's quality of life. Once diagnosed, a patient's anti-lung cancer therapy can usually consist of surgery, radiotherapy and chemotherapy. As scientific research develops, a significant progress in the development of targeted therapy drugs in conjunction with radiochemotherapy can have a better outcome on anti-cancer treatment [28]. It is important to channel this research towards the development of treatments that can effectively trigger cell death in tumor cells during cancer therapy. The reason being is that previous studies have

successfully demonstrated that effective anti-cancer drugs are largely dependent on their ability to inhibit growth and induce apoptosis in cancer cells [29].

PHA has been reported to possess anti-cancer effects on several different forms of human cancer cells [18]. One of its isoforms, PHE-L, has been used to recognize breast carcinoma metastasis by binding β1,6-branched oligosaccharides [30]. Another isoform, PHA-E, has been proved to bind to the bisecting GlcNAc in pediatric brain tumors which could be a diagnostic tumor marker [31], but it had not been thoroughly explored on lung cancer treatment prior to this study. Our project examined the effects of PHA-E on lung cancer cell A-549. According to our research, the data presented here suggests that PHA-E resulted in a dose-dependent increase in growth inhibition and cell death of A-549 cells (Fig. 1). Apoptosis is associated with cell number reduction and cytotoxicity enhancement, therefore our main objective was to determine whether apoptosis was involved in PHA-E-induced cell death. We used two assays to quantify cell apoptosis by flow cytometry. The apoptotic cells were the sum of early apoptosis (Annexin V-positive and PI-negative) and late apoptosis (Annexin V-positive and PI-positive). Our results suggest that as the concentration of PHA-E ~~was~~ increased, the proportion ~~rate~~ of apoptotic cells was also raised (Fig. 2a, Table 1). In addition, dead cells (PI-positive and Annexin V-negative) and live cells (PI-negative ~~PI- negative~~ and Annexin V-negative) displayed a dose-dependent

increase and decrease according to the results of the G6PD release assay and MTT assay. Another evaluation method, TUNEL/PI assay, determined the same trend of apoptosis induced. Our data suggests that PHA-E induced growth inhibition and cytotoxicity of A-549 cells is mediated through an induction of apoptosis.

The mitochondrial apoptotic pathway is one of the major pathways to initiate apoptosis, which releases pro-apoptotic proteins such as cytochrome c from the mitochondria to cytosol. Cytochrome c then combines with an apoptotic protease-activating factor 1 (Apaf-1) and induces the formation of a complex known as apoptosome. Apoptosome possesses a caspase recruitment domain (CARD), which allows it to bind and process the crucial initiator, caspase 9. Activated caspase 9 then cleaves and activates the downstream effector ~~effectors~~ of apoptotic cell death known as caspase 3 ~~[21]~~[32]. Our objective was to identify whether PHA-E induced apoptosis through an activation of the mitochondrial apoptotic pathway, therefore our study assessed the expression levels of cytochrome c, cleaved caspase 9 and cleaved caspase 3 (Fig. 3). Our data suggests that treatment with PHA-E could promote the release of cytochrome c, which as a result can activate caspase 9 and caspase 3, inducing apoptosis through the mitochondrial apoptotic pathway. Cytochrome c can be released from a remarkable event called mitochondrial outer membrane permeabilization (MOMP), which is regulated by proteins of the Bcl-2 family ~~[22]~~

[33].

Bcl-2 family members are grouped into the following three classes: one class that inhibits apoptosis such as Bcl-2, a second class that promotes apoptosis proteins such as Bax, and a third divergent class of BH3-only proteins such as Bad ~~[23]~~[34]. Although Bcl-2 can prevent cytochrome c release and apoptosis, Bax is shown to trigger cytochrome c release in cells ~~[24]~~[35]. Bad is activated by a loss of phosphorylation ~~[25]~~[36], and it has a conserved BH3 domain that can bind and regulate the anti-apoptotic protein Bcl-2 to promote apoptosis ~~[26]~~[37]. Recent evidence indicates that Bad can affect Bax by directly binding and inhibiting Bcl-2 ~~[27]~~[38]. The present study suggests that treatment with PHA-E caused de-phosphorylation and activation of Bad, which resulted in the activation of Bax and an inactivation Bcl-2, triggering apoptosis (Fig. 4). Activated PI3K/Akt and MEK/ERK pathways have been reported to promote survival signaling by phosphorylating Bad. These phosphorylated residues result in the binding of Bad to 14-3-3 proteins, which subsequently inhibit Bad to bind with Bcl-2 ~~[28, 29]~~[39, 40]. However, PI3K/Akt and MEK/ERK pathways can be activated by the epidermal growth factor receptor (EGFR) ~~[30]~~[41].

In literature, dysregulation of human epidermal growth factor receptor (EGFR) pathways by over-expression or constitutive activation can promote tumor processes

including angiogenesis and metastasis and is associated with poor prognosis in many human malignancies [42]. Clinically, EGFR and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation analysis is the most reliable predictor of the lung carcinoma response to EGFR-targeted therapies [43]. In lung cancer, some papers have shown that EGFR was commonly overexpressed, especially in the non-small cell lung cancers [44, 45] but the detail mechanism is not well known. In addition, the activation of its downstream-activated proteins, such as p-Akt and p-STAT3, were correlated with well-differentiated tumor. In this study, we investigated the effects of PHA-E on activated-EGFR and overexpression of p-Akt in lung cancer cells. As we expected, PHA-E could effectively reverse the activation of p-Akt, which is consistent with the previous findings. ~~EGFR is a member of ErbB family of tyrosine kinase receptors (RTK) [31], and it is a transmembrance glycoprotein comprised of extracellular ligand-binding domain, transmembrane domain, intracellular protein tyrosine kinase domain and C-terminal domain. Activation of EGFR occurs when a ligand such as EGF binds to the extracellular domain and induces dimerization of EGFR, leading directly to an activation of tyrosine kinase domain, ensuing autophosphorylation of C-terminal tyrosine residues, and activation of the downstream signaling pathway [32, 33]. Disruption of EGFR pathway results in an activation of the pro-apoptotic protein, Bad [34]. PHA-E has~~

~~been reported to possess inhibition of EGFR auto-phosphorylation [35].~~ Our study suggests that treatment with PHA-E not only caused inhibition of EGFR, but also resulted in blocking the PI3K/Akt and MEK/ERK signaling pathways that promoted the activation of Bad, which can induce A-549 cell apoptosis (Fig. 6).

Conclusions

In this paper, we have found that PHA-E effectively inhibited the growth of A-549 cells and induced their apoptosis. As for apoptosis induction, PHA-E not only caused inhibition of EGFR, but resulted in blocking the PI3K/Akt and MEK/ERK signaling pathways that promoted activation of Bad, which can enhance the release of cytochrome c and activate caspase-9 and caspase-3. However, we also found that PHA-E suppressed tumor growth but the detail mechanisms need further investigation. The multiple approached evidences suggested that PHA-E could be developed into an effective anti-lung cancer drug in the near future, while the animal feeding model, xenograph assays and drug toxicity evaluation are needed before its clinical trials.

~~This study has demonstrated that PHA-E can induce growth inhibition and cytotoxicity of lung cancer cells, which is mediated through the activation of the mitochondria apoptosis pathway. We conducted a thorough MTT assay, G6PD release assay, flow cytometry, and Western blot analysis in order to determine which specific signal transduction pathway was responsible for cell growth inhibition and cytotoxicity on A-549 cells. Our aforementioned research suggests that PHA-E can directly inhibit lung cancer cell growth by triggering the apoptosis process through the mitochondria apoptosis pathway. This evidence suggests that PHA-E could be~~

~~developed into an effective anti-lung cancer drug in the near future.~~

Acknowledgments

The authors would like to thank the *China Medical University*, for financially supporting this research under Contract No. CMU97-230.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-49.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
3. Brambilla E, Travis WD, Colby TV, Corrin B, Shimosato Y. The new World Health Organization classification of lung tumours. Eur Respir J 2001;18:1059-68.
4. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003;4:721-9.
5. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.
6. Lockshin RA, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol 2001;2:545-50.
7. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-12.
8. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269-90.
9. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. Annu Rev Biochem 2000;69:217-45.

10. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000;10:369-77.

11. Ozören N, El-Deiry WS. Cell surface Death Receptor signaling in normal and cancer cells. Semin Cancer Biol 2003;13:135-47.

12. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007;26:1324-37.

13. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47-59.

14. Zornig M, Hueber A, Baum W, Evan G. Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 2001;1551:F1-37.

15. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205-19.

16. Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 2005;5:876-85.

17. Khan N, Afaq F, Mukhtar H. Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis 2007;28:233-9.

18. De Mejía EG, Prisecaru VI. Lectins as bioactive plant proteins: a potential in cancer treatment. Crit Rev Food Sci Nutr 2005;45:425-45.

19. Leavitt RD, Feldsted RL, Bachur NR. Biological and biochemical properties of

Phaseolus vulgaris isolectins. J Biol Chem 1977;252:2961-6.

20. Rüdiger H, Gabius HJ. Plant lectins: occurrence, biochemistry, functions and applications. Glycoconj J 2001;18:589-613.

21. Zhang JS, Shi J, Ilic S, Xue SJ, Kakuda Y. Biological Properties and Characterization of Lectin from Red Kidney Bean (Phaseolus Vulgaris). FOOD REV INT 2009;25:12-27.

22. O'Flynn K, Krensky AM, Beverley PC, Burakoff SJ, Linch DC. Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor. Nature 1985;313:686–7.

23. Yashwantrai NV, Flossie WS. The biochemistry of AIDS. Annu Rev Biochem 1991;60:577-630.

24. Ye XY, Ng TB, Tsang WK, Wang J. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (Phaseolus vulgaris) seeds. J Protein Chem 2001;20:367-75.

25. Kiss R, Camby I, Duckworth C, et. al. In vitro influence of Phaseolus vulgaris, Griffonia simplicifolia, concanavalin A, wheat germ, and peanut agglutinins on HCT-15, LoVo, and SW837 human colorectal cancer cell growth. Gut 1997;40:253-61.

26. De Mejía EG, Prisecaru VI. Lectins as bioactive plant proteins: a potential in

cancer treatment. Crit Rev Food Sci Nutr 2005;45:425-45.

27. Wimer BM. Therapeutic activities of PHA-L4, the mitogenic isolectin of phytohemagglutinin. Mol Biother 1990;2:74-90.

28. Nyati MK, Morgan MA, Feng FY, Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer 2006;6:876-85.

29. Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev 1992;11:121-39.

30. Handerson T, Camp R, Harigopal M, Rimm D, Pawelek J. Beta1,6-branched oligosaccharides are increased in lymph node metastases and predict poor outcome in breast carcinoma. Clin Cancer Res 2005;11:2969-73.

31. Rebbaa A, Chou PM, Vucic I, Mirkin BL, Tomita T, Bremer EG. Expression of bisecting GlcNAc in pediatric brain tumors and its association with tumor cell response to vinblastine. Clin Cancer Res 1999;5:3661-8.

32. Von Ahsen O, Waterhouse NJ, Kuwana T, Newmeyer DD, Green DR. The 'harmless' release of cytochrome c. Cell Death Differ 2000;7:1192-9.

33. Sharpe JC, Arnoult D, Youle RJ. Control of mitochondrial permeability by Bcl-2 family members. Biochim Biophys Acta 2004;1644:107-13.

34. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647-56.

35. Fletcher JI, Meusburger S, Hawkins CJ, et. al.~~Fletcher JI, Meusburger S, Hawkins CJ, Riglar DT, Lee EF, Fairlie WD, Huang DC, Adams JM~~. Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. Proc Natl Acad Sci U S A 2008;105:18081-7.

36. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 1996;87:619-28.

37. Chen L, Willis SN, Wei A, et. al.~~Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC.~~ Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 2005;17:393-403.

38. Willis SN, Fletcher JI, Kaufmann T, et. al.~~Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC.~~ Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 2007;315:856-9.

39. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231-41.

40. Datta SR, Ranger AM, Lin MZ, et. al.~~Datta SR, Ranger AM, Lin MZ, Sturgill JF,~~

~~Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME.~~ Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev Cell 2002;3:631-43.

41. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. Cell Mol Life Sci 2008;65:1566-84.
42. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res 2006;12:5268–72.
43. Lacroix L, Besse B, Bidart JM, Bosq J. KRAS status versus EGFR status in lung cancer therapy. Bull Cancer 2009;96:S75-83.
44. Mukohara T, Kudoh S, Yamauchi S, et. al. Expression of epidermal growth factor receptor (EGFR) and downstream-activated peptides in surgically excised non-small-cell lung cancer (NSCLC). Lung Cancer 2003;41:123-30.
45. Tanno S, Ohsaki Y, Nakanishi K, Toyoshima E, Kikuchi K. Small cell lung cancer cells express EGFR and tyrosine phosphorylation of EGFR is inhibited by gefitinib ("Iressa", ZD1839). Oncol Rep 2004;12:1053-7.

~~1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-49.~~

~~2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA~~

Cancer J Clin 2005;55:74-108.

3. Brambilla E, Travis WD, Colby TV, Corrin B, Shimosato Y. The new World Health Organization classification of lung tumours. Eur Respir J 2001;18:1059-68.

4. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003;4:721-9.

5. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.

6. Lockshin RA, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol 2001;2:545-50.

7. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-12.

8. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269-90.

9. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. Annu Rev Biochem 2000;69:217-45.

10. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000;10:369-77.

11. Ozören N, El-Deiry WS. Cell surface Death Receptor signaling in normal and

cancer cells. Semin Cancer Biol 2003;13:135-47.

12. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007;26:1324-37.

13. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47-59.

14. Zornig M, Hueber A, Baum W, Evan G. Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 2001;1551:F1-37.

15. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205-19.

16. Fesik SW. Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 2005;5:876-85.

17. Khan N, Afaq F, Mukhtar H. Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis 2007;28:233-9.

18. De Mejía EG, Prisecaru VI. Lectins as bioactive plant proteins: a potential in cancer treatment. Crit Rev Food Sci Nutr 2005;45:425-45.

19. Leavitt RD, Feldsted RL, Bachur NR. Biological and biochemical properties of Phaseolus vulgaris isolectins. J Biol Chem 1977;252:2961-66.

20. Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev 1992;11:121-39.

21. Von Ahsen O, Waterhouse NJ, Kuwana T, Newmeyer DD, Green DR. The 'harmless' release of cytochrome c. Cell Death Differ 2000;7:1192-9.

22. Sharpe JC, Arnoult D, Youle RJ. Control of mitochondrial permeability by Bcl-2 family members. Biochim Biophys Acta 2004;1644:107-13.

23. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647-56.

24. Fletcher JI, Meusburger S, Hawkins CJ, Riglar DT, Lee EF, Fairlie WD, Huang DC, Adams JM. Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. Proc Natl Acad Sci U S A 2008;105:18081-7.

25. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 1996;87:619-28.

26. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 2005;17:393-403.

27. Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC. Apoptosis initiated when BH3 ligands engage multiple Bcl-2

homologs, not Bax or Bak. Science 2007;315:856-9.

28. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231-41.

29. Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev Cell 2002;3:631-43.

30. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. Cell Mol Life Sci 2008;65:1566-84.

31. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol 2006;7:505-16.

32. Yarden Y. The EGFR family and its ligands in human cancer signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001;37:S3-8.

33. Nyati MK, Morgan MA, Feng FY, Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer 2006;6:876-85.

34. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 2005;8:287-97.

35. Rebbaa A, Yamamoto H, Moskal JR, Bremer EG. Binding of erythroagglutinating phytohemagglutinin lectin from Phaseolus vulgaris to the epidermal growth factor receptor inhibits receptor function in the human glioma cell line, U373 MG. J Neurochem 1996;67:2265-72.

Figure legends

Figure 1: The effects of PHA-E on cell viability and cytotoxicity. A-549 cells were treated with PHA-E (20-160 μg/ml) for 48 h ~~hr~~. (a) The cell viability was determined by MTT assay. (b) Cell cytotoxicity was determined by G6PD release assay. The data shown are mean ± SD for n = 6. *p < 0.05; **p < 0.01 vs. control.

Figure 2: The effects of PHA-E on cell apoptosis by flow cytometry. A-549 cells were treated with PHA-E (20-160 μg/ml) for 48 h ~~hr~~. (a) The percentage of apoptotic cells was detected using Annexin V/PI assay. Q2 quadrant represented late apoptosis, while Q4 quadrant represented an early phase of apoptosis. (b) Apoptosis rate (P2) was considered using TUNEL/PI assay.

Figure 3: The effects of PHA-E on cytochrome c, cleaved caspase 9 and cleaved caspase 3 expression. A-549 cells were treated with PHA-E (20-160 μg/ml) for 6 h ~~hr~~. Equal protein loading was confirmed by probing for α-tubulin.

Figure 4: The effects of PHA-E on the expression of Bcl-2 family members. A-549 cells were treated with PHA-E (20-160 μg/ml) for 6 h ~~hr~~. Expressions of Bad, phospho-Bad, Bcl-2 and Bax. Equal protein loading was confirmed by probing for α-tubulin.

Figure 5: The effect of PHA-E on EGFR and phosphorylation of EGFR, PI3K, Akt, MEK and ERK. The A-549 cells were serum-starved for 24 h ~~hr~~, co-cultured with PHA-E for 3 h ~~hr~~, then treated with 50 ng/ml EGF for 3 h ~~hr~~. (a) The effects of PHA-E on EGFR and its specific phosphorylation sites Y1068 and Y1101~~on~~. (b) The effects of PHA-E on the phosphorylation of PI3K, Akt, MEK and ERK. Equal protein loading was confirmed by probing for α-tubulin.

Figure 6: This model exhibits the PHA-E-induced apoptotic pathway in human lung cancer cells A-549. Treatment with PHA-E causes inhibition of EGFR, and results in the blockage of PI3K/Akt and MEK/ERK pathways. Dephosphorylation of Bad promotes the inhibition of Bcl-2 and the activation of Bax. This process releases cytochrome c, which activates caspase 9 and caspase 3, leading to apoptosis.

Table 1: The percentage of cell populations based on Figure 2a (n=3).