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Aloe-emodin 誘發氧化性傷害對肺癌細胞凋亡之探討

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1. Aloe-emodin induced apoptosis through generation of reactive oxygen species in human lung carcinoma cells (**已投稿 food and chemical toxicology)**
2. The release of nucleophosmin from nucleus: involvement in Aloe-emodin induced human lung nonsmall carcinoma cell Apoptosis **(已投 International Journal of Cancer 且已接受並在列印中)**

Aloe-emodin induced apoptosis through generation of reactive oxygen species in human lung carcinoma cells

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Abstract

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) is an extremely potent inducer of apoptosis in H460 cells. It has been demonstrated that aloe-emodin-induced cell death was accompanied by degradation of conventional and novel PKC isozymes protein levels in our previous study, but the mechanisms of aloe-emodin-induced cell death have not been well understood. This study found in H460 cells that oxidative stress primarily mediates the strong proapoptotic effect of aloe-emodin. Furthermore, the result suggests that superoxide anions, which are especially produced via NADPH oxidase and xanthine oxidase, may mediate aloe-emodin-induced H460 cell apoptosis. In this study, the apoptosis-inducing effect of aloe-emodin did not significantly affect the protein amount of antioxidant enzymes, such as Cu/Zn SOD and Mn SOD, but could enhance the activity of total SOD and Mn SOD. These results suggest that aloe-emodin induces H460 cell death by its prooxidant, such as inducing ROS formation, and antioxidant, such as activation of SOD enzyme, activity. The effects of aloe-emodin on H460 cell apoptosis were suspected to result from the prooxidant rather than the antioxidant action of aloe-emodin.

1. Introduction

Aloe-emodin, an active component contained in the root and rhizome of *Rheum palmatum L*. (Polygonaceae), was found to have anti-tumor activity (Kupchan and Karim, 1976; Yang et al., 1999; Pecere et al., 2000; Lee, 2001). Our previous study demonstrated that aloe-emodin induced cytotoxicity, which was indicative of a typical apoptosis on lung carcinoma cell lines. Our findings indicate that downregulation of conventional and novel protein kinase C (PKC) isoforms is involved in aloe-emodin-induced apoptosis of lung carcinoma cell lines (Lee, 2001). However, the mechanisms of the aloe-emodin-produced anticancer effects remain unknown.

Flavonoids have been found to possess prooxidant and antioxidant action, which are on intimate terms with their polyphenolic structure. Some evidences suggested that polyphenols-inducing cell toxicity was suspected to result from the prooxidant or the antioxidant action. The more OH substitutions these are on their structures the stronger the prooxidant and antioxidant activities (Lee et al., 2002; Shen et al., 2002). Since the structure of aloe-emodin contains polyphenolic structure, this study would demonstrate the role that prooxidative or antioxidative action of aloe-emodin play in aloe-emodin-induced H460 cell apoptosis. Free radicals are a family of molecules, which modulate several important physiological functions including proliferation and

apoptosis. Previous studies reported that reactive oxygen species (ROS) participated in cancer and apoptosis through inducing DNA damage (Chen et al., 1998; Varbiro et al., 2001; Arai et al., 2003; Wu et al., 2004). Superoxide dismutase (SOD) is a well-known antioxidative enzyme with the activity to convert superoxide to hydrogen peroxide, and at least two types of SOD have been identified, one is mitochondrial Mn SOD, and the other is cytosolic Cu/Zn SOD, which can be inhibited by cyanide. Antioxidant enzymes can antagonize initiation and promotion phases of carcinogenesis and they are reduced in many malignancies. The most commonly decreased enzyme is the mitochondrial Mn SOD. Mn SOD is reduced in a variety of tumor cells and has been proposed to be a new type of tumor suppressor gene (Kiningham and Clair, 1997; Matés and Sánchez-Jiménez, 2000). This study would also demonstrate the role of various reactive oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radical, in aloe-emodin-induced H460 cell apoptosis.

2. Materials and Methods

2.1. Materials

N-acetyl-L-cycteine, allopurinol, aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone), antipain, aprotinin, aspirin, chelerythrine, diphenylene iodonium (DPI), dithiothreitol, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), glutathione, insulin-like growth factor-1, leupeptin, D-mannitol, N-nitro-L-arginine methyl ester (L-NAME), pepstatin, phenylmethylsulfonyl fluoride, tiron and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Company (St. Louis, MO); anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Amersham (Buckinghamshire). Cu/Zn SOD and Mn SOD antibodies were purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA).

2.2. Cell culture

The human lung non-small cell line H460 were grown in monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) containing 5 % fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD) and 2 mM glutamine (Merck, Darmstadt, Germany) at 37℃in a humidified atmosphere comprised of 95 % air and 5 % CO_2. When H460 cells were treated with aloe-emodin, the culture medium containing 1 % fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

2.3. Trypan blue dye exclusion assay

H460 cells were seeded at a density of 5×10^4 cells/well onto 12-well plate (Falcon, Franklin Lakes, NJ) 48 h before drugs treated. Drugs were added to medium, at various indicated times and concentrations. The control cultures were treated with 0.1 % DMSO (dimethylsulfoxide; Merck). After incubation, cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining cell population with Trypan blue (Sigma). One part of 0.2 % Trypan blue dissolved in PBS was added to one part of the cell suspension and the number of unstained (viable) cells was counted.

2.4. Protein preparation

Protein was extracted as previously described (Lee, 2001). Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4℃. Lysates were clarified by centrifugation at 100,000 × g for 30 min at 4℃ and the resulting supernatant was collected, aliquoted (50 μg/tube) and stored at -80℃ until assay. The protein concentrations were estimated with the Bradford method (Bradford, 1976).

2.5. Fluorescence microscopic measurements of reactive oxygen species *(ROS) production*

This study used 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, molecular Probes) to detect intracellular generation of ROS. H460 cells were loaded with 5 μM CM-H_2DCFDA for 30 min in the dark. After loading, cells were washed with warm PBS. During loading, the acetate groups on CM-H_2DCFDA are removed by intracellular esterase, trapping the probe inside the H460 cells. Cells loaded with CM-H_2DCF were treated with aloe-emodin (40 μM) and analyzed by fluorescence microscopy. Production of ROS can be measured by changes in fluorescence due to intracellular production of CM-DCF (5-(and-6)-chloromethyl-2',7'-dichlorofluorescein) caused by oxidation of CM-H_2DCF. CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

2.6. Evaluation of superoxide dismutase (SOD)

Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Sonicate the cell pellets in cold 20 mM HEPES (Sigma) buffer, pH7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose (Sigma). Samples were then centrifuged for 5 min at 1,500 g and 4℃. The supernatant was aspirated and the total SOD and Mn SOD activity was assayed spectrophotometrically at 450 nm with a commercial kit (Cayman chemical). A standard curve of SOD solution (from 0.025 to 0.25 U/ml) was run for quantification. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical.

2.7. Western blot analysis

Samples were separated by various appropriate concentrations (12 and 15 %) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA). The SDS-separated proteins were equilibrated in transfer buffer (Tris-HCl 50 mM, pH 9.0-9.4, glycine 40 mM, 0.375 % SDS [Bio-Rad], 20 % methanol [Merck]) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA). The blot was blocked with a solution containing 5 % nonfat dry milk in Tris-buffered saline (Tris-HCl 10 mM, NaCl 150mM [Sigma]) with 0.05 % Tween 20 (TBST; Merck) for 1 h, washed and incubated with antibodies to β-actin (1:5000 [Sigma], the detection of β-actin was used as an internal control in all of the data of Western blotting analysis), Cu/Zn SOD (1:1000; Calbiochem) and Mn SOD (1:1000; Calbiochem). Secondary antibody consisted of a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG (for Cu/Zn SOD and Mn SOD; Jackson ImmunoResearch). The enhanced chemiluminescent (NEN Life Science Products, Boston, MA) detection system was used for immunoblot protein detection.

2.8. Statistical analysis

Results were analyzed for statistical significance by analysis of variance with repeated measures and a Student's *t* test. A *P*-value of less than 0.05 was considered significant for all tests.

3. Results

3.1. Effect of aloe-emodin on intracellular ROS in H460 cells

ROS producing and scavenging activities play import roles in the drug-induced apoptosis. In order to demonstrate the role that ROS play in aloe-emodin-induced apoptosis, production of ROS was examined by using an oxidant-sensitive fluorescent probe, CM-H_2DCFDA. The results showed that treatment with 40 μM aloe-emodin had significantly increased the intensity of the DCF signal as compared with those in the control during treatment with aloe-emodin for 1, 4 and 8 h (Fig. 1). The concentration of 40 μM of aloe-emodin was used to inducing H460 cell apoptosis in our serial studies. The DCF signal also observed in H460 cells treatment with 6 μM chelerythrine (Fig. 1). Chelerythrine was used as a positive control of the generation of ROS. This result indicates that ROS producing is involved in aloe-emodin or chelerythrine-induced cell death in H460 cells.

3.2. The role of superoxide anion in aloe-emodin-induced apoptosis in H460 cells

In order to investigate whether superoxide anion is induced by aloe-emodin, this study used superoxide anion scavenger and inhibitors of xanthine oxidase, cyclooxygenase, nitric oxide synthase and NADPH oxidase, which are generally recognized to be responsible for progressive production of superoxide anion, on aloe-emodin-induced cell death in H460 cells. A scavenger of superoxide, 1 mM tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid) could slightly prevent aloe-emodin-induced cell death in this study (Fig. 2). Both pretreatment with 3 μM diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, or 2 mM allopurinol, a xanthine oxidase inhibitor, show significant protective effect on aloe-emodin-induced cell death (Fig. 2). However, prior treatment of cells with 1 mM aspirin, a cyclooxygenase inhibitor, or 1 mM N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, for 1 h only had a slight recover the decreased viability induced by following treatment with 40 μM aloe-emodin for 24 h (Fig. 2). These results indicate that O_2^- is involved in aloe-emodin-induced cell death in H460. Furthermore, production of superoxide anion induced by aloe-emodin may be mainly released via NADPH oxidase and xanthine oxidase in these cells.

3.3. The role of intracellular hydrogen peroxide in aloe-emodin-induced apoptosis in H460 cells

To demonstrate whether hydrogen peroxide (H_2O_2) is involved in aloe-emodin-induced cell death, H460 cells were pretreated with antioxidants such as N-acetyl-L-cysteine and glutathione before aloe-emodin treatment. Since N-acetyl-L-cysteine stimulates glutathione synthesis and glutathione peroxidase reduces hydrogen peroxide in the presence of glutathione, both N-acetyl-L-cysteine and glutathione reduce intracellular hydrogen peroxide. Treatment of H460 cells with N-acetyl-L-cysteine (NAC; 5 mM), for 1 h did not significantly affect aloe-emodin (40 μM, 24 h)-induced cell death (Fig. 3). However, 40 μM aloe-emodin-induced H460 cell death was slightly prevented by 5 mM glutathione pretreatment (Fig. 3). These results indicate that the production of H_2O_2 is not a major determinant in aloe-emodin-induced cell death in H460. Interesting, both NAC and glutathione significantly inhibited chelerythrine-induced H460 cell death (Fig. 3), suggesting that aloe-emodin and chelerythrine induce H460 cell apoptosis by distinct mechanisms, even though both could induce ROS production.

3.4. The role of hydroxyl radical in aloe-emodin-induced apoptosis in H460 cells

This study investigated whether hydroxyl radical was involved in aloe-emodin-induced cell death in H460 cells. Dimethylsulfoxide (DMSO, 3 %), a hydroxyl radical scavenger, could affect aloe-emodin-induced cell death (Fig. 4), while higher concentrations of DMSO alone caused damage to H460 cells (data not shown). Pretreatment of H460 cells with 1 mM D-mannitol, another hydroxyl radical scavenger, showed no inhibitory effect on aloe-emodin-induced cell death (Fig. 4). These results indicated that hydroxyl radical producing might not involve in aloe-emodin-induced H460 cell death.

3.5. Effects of aloe-emodin on superoxide dismutase (SOD) activity in H460 cells

Results described above suggested that aloe-emodin-induced apoptosis mediated by its prooxidant activity. The antioxidant properties of aloe-emodin, such as the activity of SOD (an antioxidant enzyme), were also detected in aloe-emodin-induced H460 cells apoptosis. This study measured total SOD and Mn SOD activity in H460 cells treated with 40 μM aloe-emodin for various time points. 40 μM aloe-emodin stimulates a biphasic change in total SOD activity. The initial phase of the aloe-emodin response exhibited a significant increase in total SOD activity compared to those of control cells at 8 h of aloe-emodin treatment. The second phase followed by a significant decay after treatment with aloe-emodin for 8 h (Fig. 5A). To further investigate whether the activation of Mn SOD by aloe-emodin could be linked to aloe-emodin-induced H460 cell apoptosis, 1 mM potassium cyanide, which will inhibit Cu/Zn SOD, was added to the assay samples. As shown in Fig. 5A, after 4 h treatment with aloe-emodin also induced a biphasic increased in the activity of Mn SOD. These results suggest that the antioxidant properties of aloe-emodin in cell death are mediated by an increase the activity of antioxidant enzymes. Our study also demonstrated the expression of Cu/Zn SOD and Mn SOD protein levels during aloe-emodin-induced apoptosis by Western blotting techniques. The protein levels of

Mn SOD and Cu/Zn SOD were not changed during treatment with 40 μM aloe-emodin for 24 h (Fig. 5B).

4. Discussion

Reactive oxygen species (ROS) play an important role in the initiation and progression of cancer and its ability to induced apoptosis has also been discussed enthusiastically. Specifically, oxidative damage to DNA is considered an essential step in cancer development or apoptosis (Chen et al., 1998; Varbiro et al., 2001; Arai et al., 2003; Wu et al., 2004). ROS, which include hydroxyl radical, superoxide anion and hydrogen peroxide, are involved in drug-induced cancer cell apoptosis (Shen et al., 2003; Wenzel et al., 2003). Yamamoto et al. (2001) have also demonstrated that ROS, possibly hydrogen peroxiode, play an important role in mediating chelerythrine-induced rapid cardiac myocyte apoptosis. Since many reports have demonstrated to anthraquinones-induced generation of reactive oxygen species, this study explored the relationship between the ROS and apoptosis in H460 cells induced by aloe-emodin (an anthraquinone compound) (Mueller et al., 1998; Chen et al., 2002). This study demonstrated that aloe-emodin induced ROS production during H460 cell apoptosis. The results indicate that DPI (NADPH oxidase inhibitor) and tiron (superoxide anion scavenger) partially inhibited aloe-emodin-induced cell death. By contrast, D-mannitol (a hydroxyl radical scavenger) or NAC (an antioxidant) did not significantly affect aloe-emodin-induced cell death. Thus, superoxide anion rather

than hydroxyl radical or hydrogen peroxide may mediate aloe-emodin-induced apoptosis in H460 cells.

Xanthine oxidase, cyclooxygenase, NOS and NADPH oxidase are generally recognized to be responsible for progressive production of superoxide anion as potential sources of ROS (Matsunaga et al., 2003). In this study, pretreatment with aloe-emodin with DPI, a selective inhibitor of NADPH oxidase, or allopurinol, a xanthine oxidase inhibitor, partially inhibited cell death induced by treatment with aloe-emodin in H460 cells. However, treatment with aspirin or L-NAME had slightly protective effect on aloe-emodin-induced cell death. It indicates that superoxide anion formation induced by aloe-emodin may be mainly released via NADPH oxidase and xanthine oxidase in these cells.

Polyphenolic antioxidants are scavengers of free radicals and modifiers of various enzymatic functions. The structure of aloe-emodin is anthraquinone and contains two phenolic structures. Therefore, this study also demonstrated that reduction of antioxidant enzymes such as superoxide dismutase was observed in h460 cells treated with aloe-emodin. SOD is a well-known antioxidative enzyme with the activity to convert superoxide to hydrogen peroxide, and at least two types of SOD have been identified, one is mitochondrial Mn SOD, and the other is cytosolic Cu/Zn SOD, which can be inhibited by cyanide. Antioxidant enzymes can antagonize initiation and

promotion phases of carcinogenesis and they are reduced in many malignancies. The most commonly decreased enzyme is the mitochondrial Mn SOD. Mn SOD is reduced in a variety of tumor cells and has been proposed to be a new type of tumor suppressor gene (Kiningham and Clair, 1997; Matés and Sánchez-Jiménez, 2000). Results of the present study indicated that total cellular SOD and Mn SOD activity had a significant increase in H460 cells in the presence of aloe-emodin. Although the activity of the radical-scavenging enzyme SOD was enhanced by aloe-emodin, H460 cells still died. Therefore, the effects of aloe-emodin on H460 cell apoptosis were suspected to result from the prooxidant rather than the antioxidant action of aloe-emodin. Based on the above data, the biological effects of aloe-emodin may dependent upon the net balance of prooxidative and antioxidative effect.

Summary, aloe-emodin is an extremely potent inducer of apoptosis in H460 cells. This study found in H460 cells that oxidative stress primarily mediates the strong prooxidative effect of aloe-emodin. Furthermore, the result suggests that superoxide anions, which are especially produced via NADPH oxidase and xanthine oxidase, may mediate aloe-emodin-induced H460 cell apoptosis.

Acknowledgments

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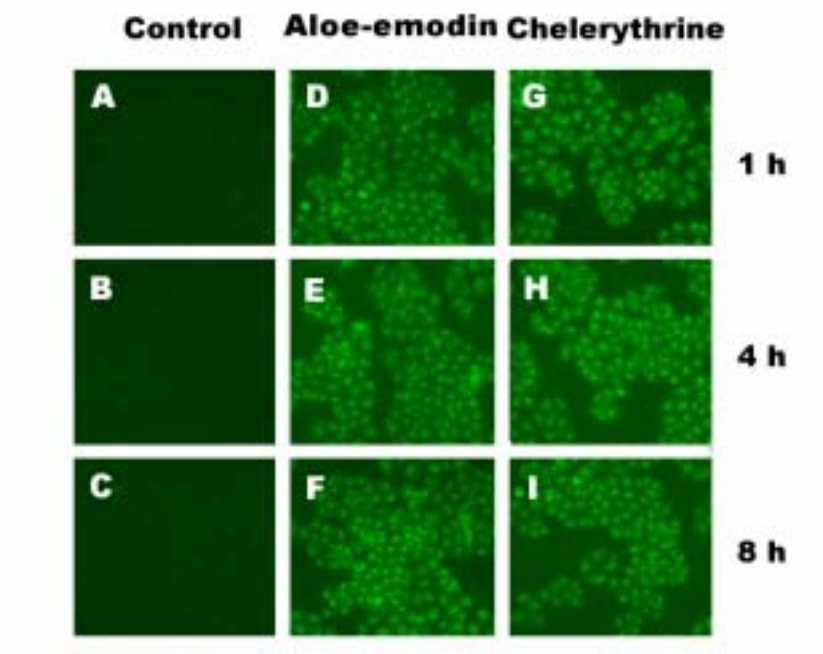

Fig. 1. Effects of aloe-emodin and chelerythrine on ROS production in H460 cells. Cells were cultured for 48 h before drug treatment in 12-well plates. Intracellular levels of ROS were examined by 5-H_2DCFDA (5 μM) for 30 min in the dark, washed and then treated with 0.1 % DMSO (A, B and C), 40 μM aloe-emodin (D, E and F) or 6 μM chelerythrine (G, H and I) with in the presence of 1 % serum at 37℃ for 1, 4 and 8 h and cells were washed and observed by using fluorescence microscopy. Results are representative of three independent experiments. All photographic exposures were for 10 s.

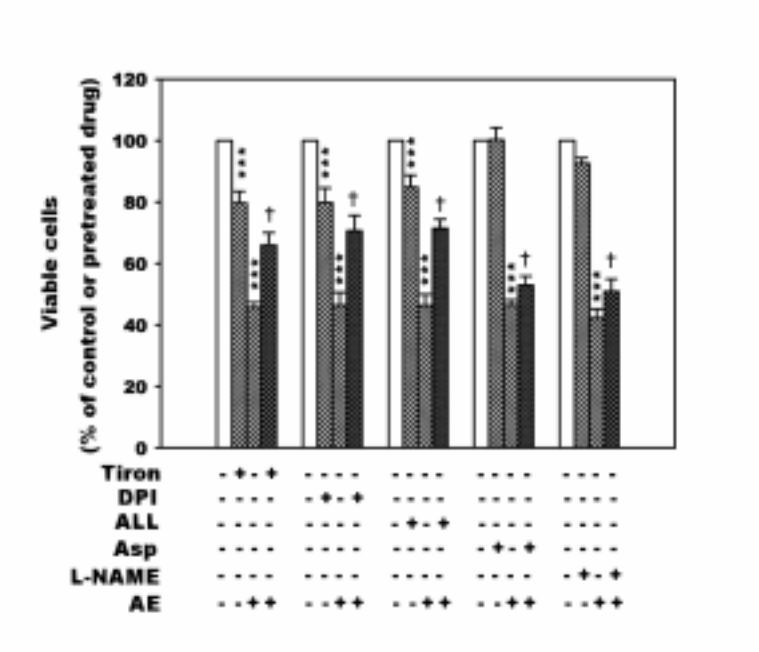

Fig. 2. The role of superoxide anion in aloe-emodin-induced apoptosis in H460 cells. H460 cells were cultured 48 h before drug treatment in 12-well plates. Cells were pretreated with tiron (1 mM), DPI (3 μM), allopurinol (ALL, 2 mM), aspirin (Asp, 1 mM) and L-NAME (1 mM) for 1 h and then 40 μM aloe-emodin (AE) for 24 h, and cells were washed and counted for Trypan blue exclusion with a hemocytometer. The fraction of viable cells was calculated by defining the number of viable cells of aloe-emodin-untreated (0.1 % DMSO or pretreated drug alone) as 100 %. The results are the means ± S.D. of three independent experiments. *** $P < 0.001$ compared to the control values. † $P < 0.001$ compared to the values for aloe-emodin-treated cells.

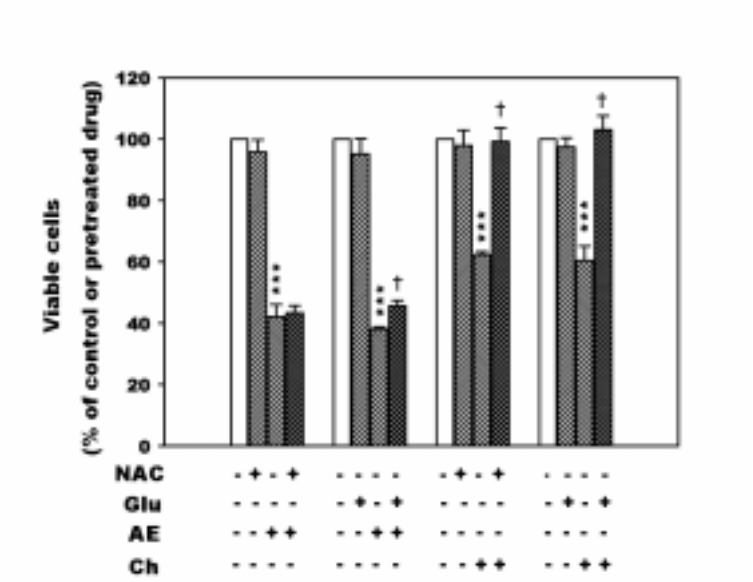

Fig. 3. The role of intracellular hydrogen peroxide in aloe-emodin-induced apoptosis in H460 cells. H460 cells were cultured 48 h before drug treatment in 12-well plates. Cells were pretreated with NAC (5 mM) and glutathione (Glu, 5 mM) for 1 h and then 40 μM aloe-emodin or 6 μM chelerythrine (Ch) for 24 h, and cells were washed and counted for Trypan blue exclusion with a hemocytometer. The fraction of viable cells was calculated by defining the number of viable cells of aloe-emodin- or chelerythrine-untreated (0.1 % DMSO or pretreated drug alone) as 100 %. The results are the means ± S.D. of three independent experiments. *** $P < 0.001$ compared to the control values. † $P < 0.001$ compared to the values for aloe-emodin-treated cells.

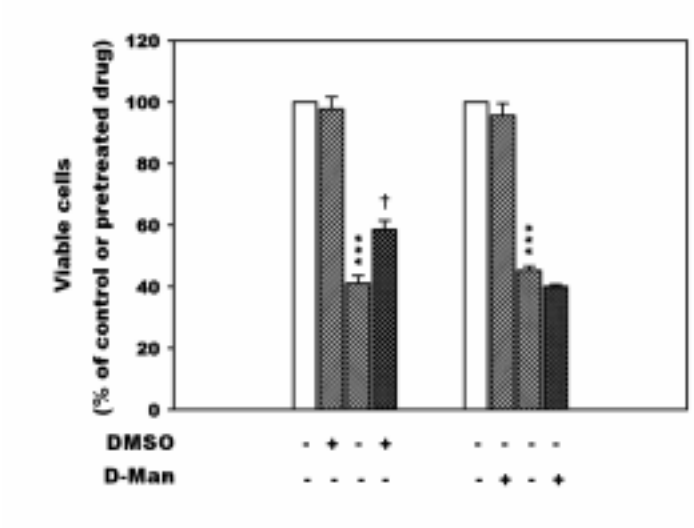

Fig. 4. The role of hydroxyl radical in aloe-emodin-induced apoptosis in H460 cells. H460 cells were cultured 48 h before drug treatment in 12-well plates. Cells were pretreated with DMSO (3 %) and D-mannitol (D-Man, 1 mM) for 1 h and then 40 μM aloe-emodin for 24 h, and cells were washed and counted for Trypan blue exclusion with a hemocytometer. The fraction of viable cells was calculated by defining the number of viable cells of aloe-emodin-untreated (0.1 % DMSO or pretreated drug alone) as 100 %. The results are the means ± S.D. of three independent experiments. *** $P < 0.001$ compared to the control values. † $P < 0.001$ compared to the values for aloe-emodin-treated cells.

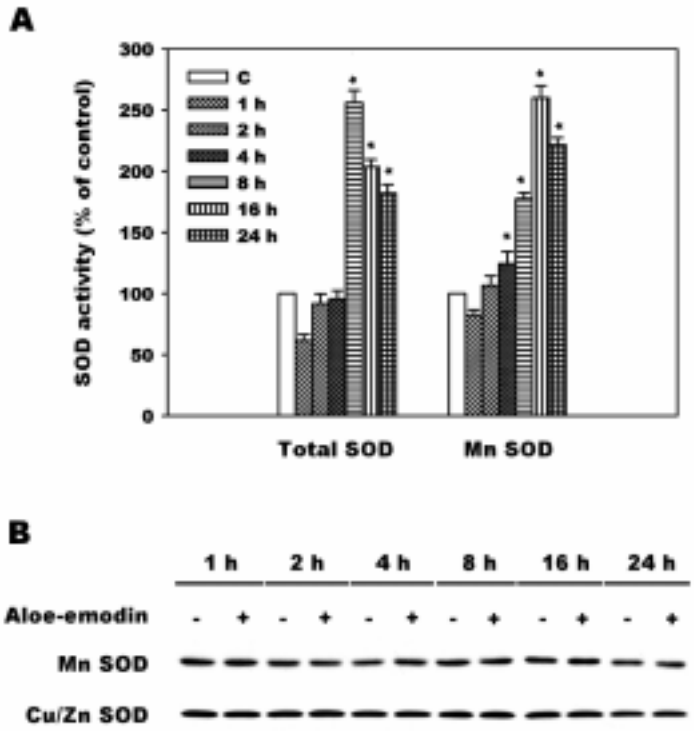

Fig. 5. Effects of aloe-emodin on SOD activity in H460 cells. **A:** Cells were cultured for 48 h before drug treatment in 10 cm dish. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1 % serum for 1, 2, 4, 8, 16 and 24 h. Cell lysates were analyzed by commercial kit. The total SOD activity was assayed by monitoring the absorbance at 450 nm using a plate reader. The addition of 2 mM potassium cyanide to the assay will inhibit Cu/Zu SOD, resulting in the detection of only Mn SOD activity. Data are the mean percent control ± S.D. for 3 separate experiments. * $P < 0.001$ compared to the control values. **B:** The effects of aloe-emodin on the protein levels of Cu/Zn SOD and Mn SOD were detected by Western blot analysis in H460 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence

of 1 % serum for 1, 2, 4, 8, 16 and 24 h. Cell lysates were analyzed by 12 % (Mn SOD) and 15 % (Cu/Zn SOD) SDS-PAGE, and then probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

THE RELEASE OF NUCLEOPHOSMIN FROM NUCLEUS: INVOLVEMENT IN ALOE-EMODIN INDUCED HUMAN LUNG NONSMALL CARCINOMA CELL APOPTOSIS

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d) Key words: Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone); 2-D electrophoresis; human lung nonsmall cell carcinoma cell line H460; nucleophosmin; gene expression; proteomics

e) Abbreviations: Aloe-emodin, 1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; H460 cell, human lung non-small carcinoma cell line; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween; Tris, tris (hydroxymethyl) aminomethane

ABSTRACT

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) is one of the active constituents from the root and rhizome of *Rheum palmatum*. Our previous study has demonstrated that aloe-emodin induced a significant change in the expression of lung cancer cell apoptosis-related proteins compared to those of control cells. However, the molecular mechanisms underlying the biological effects of aloe-emodin remain still unknown. Based on above reasons, we were interested in the change of aloe-emodin-induced total protein expression by proteomics technique during aloe-emodin-induced lung cancer cell apoptosis. The present study applied 2-D electrophoresis to analyze the proteins involved in aloe-emodin-induced apoptosis in H460 cells. We found that the release of nucleophosmin from the nucleus to cytosol and the degradation of nucleophosmin were associated with aloe-emodin-induced H460 cell apoptosis. This study also demonstrated that the gene expression of nucleophosmin remained unchanged after treatment with aloe-emodin. The aloe-emodin-caused increase in the amount of proform and fragment of nucleophosmin in cytoplasm may be one of the important events for aloe-emodin-induced H460 cell apoptosis.

INTRODUCTION

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) is one of the active constituents from the root and rhizome of *Rheum palmatum*. Our previous study demonstrated that 24 h of continuous exposure to 40 μM of aloe-emodin induced a typical apoptosis on lung carcinoma cell line H460. Aloe-emodin-induced apoptosis was characterized by nuclear morphological changes and DNA fragmentation.[1] Many proteins, such as PKC, bcl-2, caspase and MAP kinase family members, have been demonstrated to be involved in aloe-emodin-induced apoptosis.[2] However, the molecular mechanisms underlying the biological effects of aloe-emodin remain still unknown.

Nucleophosmin is a nucleolar phosphoprotein that accumulates in the nucleoplasm of cells. It is significantly more abundant in tumor and proliferating cells than in normal resting cells.[3,4] The putative function of nucleophosmin is ribosome assembly and transport.[5,6] Recently, interest in nucleophosmin has been growing due to its role in DNA repair and in cancer. Nucleophosmin is also a mobile nucleolar protein that shuttles between nucleoli and cytoplasm or shifts from the nucleoli to the nucleoplasm when cells are exposed to certain anticancer drugs.[6,7] Evidence recently has demonstrated that nucleophosmin is down-regulated during drug-induced apoptosis of cancer cells.[8,9] Hsu and Yang (2003) indicated that there was decrease in the level of

cellular nucleophosmin protein and appearance of its degraded product (25 kDa) during the TPA-induced differentiation in human myelogenous leukemia K562 cells.[10] Therefore, nucleophosmin is one of the key elements in the regulation of nucleolar function for cellular differentiation and apoptosis in cancer cells.

Proteomics is now generally accepted as a method to analyze total protein expression and elucidate cellular processes at the molecular level.[11,12] Therefore, proteome analysis allowed the identification of marker proteins that are involved in the induction of apoptotic cell death in cancer cells. Since identification of proteins differentially expressed in apoptotic cells could lead to new insights into pathway or help to investigate the key determinants for aloe-emodin-induced apoptosis, this study applied 2-D gel eletrophoresis to analyze the proteins involved in aloe-emodin-induced apoptosis at protein level in H460 cells. We demonstrated that the release of nucleophosmin from the nucleus to cytosol and the degradation of nucleophosmin were involved in aloe-emodin-induced apoptosis of H460 cells.

MATERIALS AND METHODS

Materials

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone), antipain, aprotinin, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), leupeptin, pepstatin, phenylmethylsulfonyl fluoride and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Company (St. Louis, MO); anti-mouse IgG peroxidase-conjugated secondary antibody were purchased from Amersham (Buckinghamshire). Nucleophosmin antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA); Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA).

Cell culture

H460 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD) containing 5% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD) and 2 mM glutamine (Merck, Darmstadt, Germany) at 37℃ in a humidified atmosphere comprised of 95% air and 5% CO_2. When H460 cells were treated with 40 μM aloe-emodin (Sigma, St. Louis, MO), the culture medium containing 1% fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

Protein preparation

Protein was extracted as previously described.[1] Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (Tris-HCl 50 mM, pH 7.5, NaCl 150 mM, EGTA 1 mM, DTT 1 mM, PMSF 1 mM, sodium orthovanadate 1 mM, sodium fluoride 1 mM, aprotinin 5 μg/ml, leupeptin 5 μg/ml, antipain 5 μg/ml, 1% Nonidet P-40, 0.25% sodium deoxycholate [Sigma]) for 30 min at 4°C. Lysates were clarified by centrifugation at 100,000 × g for 30 min at 4°C and the resulting supernatant was collected, aliquoted (50 μg/tube) and stored at -80°C until assay. The protein concentrations were estimated with the Bradford method.[13]

Western blot analysis

Samples were separated by 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA). The SDS-separated proteins were equilibrated in transfer buffer (Tris-HCl 50 mM, pH 9.0-9.4, glycine 40 mM, 0.375% SDS [Bio-Rad], 20% methanol [Merck]) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA). The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (Tris-HCl 10 mM, NaCl 150mM [Sigma]) with 0.05% Tween 20 (TBST; Merck) for 1 h, washed and incubated with antibodies to β-actin (1:5000 [Sigma], the detection of β-actin was used as an internal control in all of the data of Western blotting analysis) and nucleophosmin (1:250; Innovative Sciences). Secondary antibody consisted of a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham, Buckinghamshire). The enhanced chemiluminescent (NEN Life Science Products, Boston, MA) detection system was used for immunoblot protein detection.

Two-dimensinal gel electrophoresis

The proteins (250 μg) were dissolved in a rehydration buffer (urea 9.8M, CHAPS 0.5%, DTT 10 mM, Bio-lytes 0.2% and a trace of bromophenol blue) to a final volume of 300 μl. The samples were added to the 17 cm IPG strips (pH3-10, linear, Readystrip; Bio-Rad), which were rehydrated for 12 h. After rehydration, the strips were focused for 60000 Vh, starting at 250 V and gradually raising the voltage to 10000 V. Once the IEF was completed, the strips were equilibrated in 6 M Urea containing 2% SDS, 0.375 M Tris (pH 8.8), 20% Glycerol and 130 mM DTT. The 2-D electrophoresis was performed using 12% SDS-PAGE. Gels were stained with standard silver staining protocol.

Database searches

Mass analysis was carried out according to the TurboSequest software (ThermoFinnigan, USA) using the ThermoFinnigan LCQ DECA XP mass spectrometer. Comparisons of the sequences of the spot 1 and nucleophosmin with nucleotide and protein sequences in the GenBank database were done using the NCBI BLAST program.

Immunolocalization of nucleophosmin

H460 cells were seeded at a density of 5×10^4 cells per well onto 12-well plate 48 h before drugs treated. After treatment, cells were fixed with 3.7% formaldehyde (Merck) for 15 min and permeabilized with 0.1% Triton X-100 (Merck). Cells were then incubated 1 h at 37℃ with nucleophosmin antibody dilute 1:50 in TBST solution. The cells were washed with three times with TBST and incubated for 30 min at 37℃ with fluorescein-conjugated anti-mouse IgG antibody diluted 1:50 in TBST. The cells were then washed with TBST and examined by fluorescence microscopy (Olympus IX 70).

Extraction of total RNA

Total RNAs were isolated from control or aloe-emodin-treated H460 cells with RNeasy Mini kit (QIAGEN, USA) according to the manufacturer's descriptions. RNA concentration was quantified using a spectrophotometer at a wavelength of 260 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA was prepared by reverse transcription of 1.5 μg of total RNA. Nucleophosmin and β-actin transcripts were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) with RNA PCR kit (Invitrogen life technologies, USA). The primers of nucleophosmin are 5'-ATGAATGACGAAGGCAGTCC-3' and 5'-GGCAATAGAACCTGGACAACA-3', which amplify a 746 bp product, and β-actin (GenBank accession no. G15871) are 5'-ACAAAACCTAACTTGCGCAG-3' and 5'-TCCTGTAACAACGCATCTCA-3'. The amplification was performed with one denaturing cycle at 95℃ for 5 min, then 30 cycles at 95℃ for 1 min, at 55℃ for 1 min, at 72℃ for 1 min, and one final extension at 72℃ for 10 min. RT-PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

Identification of differentially expressed proteins by 2-D gel

The major purpose of this study was to investigate that how many proteins decreased, disappeared or newly developed after treatment with 40 μM aloe-emodin for 24 h. The protein samples were run on parallel 2-D gels and visualized using silver staining. Many spots were found to vary in intensity between images of control and aloe-emodin-treated cells loaded gels except the pH range of 4.75-6.5 and molecular weight range of 29-95 kDa (Fig. 1). In aloe-emodin-treated cells, the protein expression is significantly decreased compared to the control cells. In the area of insert (pH range of 4.2-5.9 and molecular weight below 19 kDa), three proteins were expressed in aloe-emodin-treated cells whereas diminished or no expression was detected in control cells (Fig. 1). One of these altered proteins was characterized by mass spectrometry. Using the amino acid sequences of spot 1 (Table I) as the query in a basic local alignment search tool (BLAST) of the EST database at the NCBI, the spot 1 was identified as nucleophosmin (pI/Mw: 4.64 / 32575.02, Genbank accession A32915).

The effect of aloe-emodin on the expression of nucleophosmin in H460 cells

Based on the difference between the theoretical (32.575 kDa), which was found in GenBank database, and apparent (about 18 kDa), which was determined by 2-D gel, molecular weight, we proposed a hypothesis that a degradation of nucleophosmin might have occurred during aloe-emodin-induced apoptosis in H460. To obtain further support for the induction of fragmentation of nucleophosmin by aloe-emodin in H460 cells, monoclonal anti-nucleophosmin, which reacts with the c-terminus of nucleophosmin, was used to detect the proform of nucleophosmin (32.575 kDa) in this study. In two-dimensional gel, the protein at 18 kDa was not found in aloe-emodin-treated cells by Western blot analysis (Fig. 2). However, comparing the control and aloe-emodin-treated cells, we found nucleophosmin to be markedly increased at 32 kDa in aloe-emodin-treated samples (Fig. 2B). The differential expression of nucleophosmin was also analyzed by Western blot analysis of one-dimensional gel. Aloe-emodin-treated samples had a significant increase in the expression of nucleophosmin (32 kDa) (Fig. 3). To elucidate whether the increased nucleophosmin protein level is required during drug-induced apoptosis in lung carcinoma H460 cells, this study used various apoptosis-inducing compounds, such as baicalein, emodin and luteolin. After treatment with baicalein (50 μM, 24 h), emodin (50 μM, 16 h) or luteolin (30 μM, 24 h), the protein levels of nucleophosmin (32 kDa)

were significantly increased by Western blotting analysis (Fig. 3).

Intracellular localization of nucleophosmin

To analyze the intracellular localization of nucleophosmin, immunostaining with an antibody against nucleophosmin was performed. Immunostaining studies verified the altered expression of nucleophosmin in aloe-emodin-treated cells compared to control (Fig. 4). Fluorescence in the control cells and in most aloe-emodin-treated cells exhibited diffuse staining throughout nucleus (arrow), while in a portion of aloe-emodin-treated cells the fluorescence staining was low in nucleus and most intense in the cytoplasm (arrow).

Analysis of nucleophosmin mRNA by RT-PCR

According to the results of proteome or Western blotting, aloe-emodin induced an increase in the nucleophosmin protein level. To elucidate whether the expression of nucleophosmin mRNA is involved in aloe-emodin-induced apoptosis, RT-PCR techniques were used in this study. After H460 cells were treated with 40 μM aloe-emodin for the indicated time intervals, there were no changes in the expression of nucleophosmin mRNA (Fig. 5). This result indicated that aloe-emodin had no significant effect on the mRNA level of nucleophosmin in H460 cells.

DISCUSSION

Many proteins, such as protein kinase C, mitogen-activated protein kinase, bcl-2 and caspase family members, have been demonstrated to be involved in aloe-emodin-induced apoptosis of H460 cells in our previous studies.[1,2] However, the molecular mechanisms underlying the biological effects of aloe-emodin remain unknown. Therefore, detailed knowledge of the molecular changes within apoptotic cells may provide a better understanding of apoptosis and may help to investigate the key determinants. A large-scale screening of proteins, proteome, has become advantageous in analyzing the changes in total proteins of H460 cells during aloe-emodin-induced apoptosis. The present study has found the changes of many protein spots in 2-D gel during aloe-emodin-induced H460 cell apoptosis.

Comparing the proteome of control and aloe-emodin-treated cells, we found a protein spot (pI about 4.7 and m.w. about 18 kDa) markedly increasing in aloe-emodin-treated samples. Analysis with mass spectrometry and the EST database at the NCBI revealed that this protein was nucleophosmin (pI/Mw: 4.64 / 32575.02, Genbank accession A32915). Therefore, we considered that the cleavage of nucleophosmin should be produced during aloe-emodin-induced apoptosis in H460 cells. In the present study, the proform (pI about 5 and m.w. about 35 kDa) of nucleophosmin was detected with the nucleophosmin antibody. Aloe-emodin induced

a significant increase in the protein level of the proform of nucleophosmin in 2-D gel by Western blotting analysis. In this study, aloe-emodin-treated H460 cells also revealed increases in the protein level of nucleophosmin (35 kDa) in 1-D gel. Nucleophosmin is a major nucleolar phosphoprotein that is 20 times more abundant in cancer cells than in normal cells.[14] The abundance of nucleophosmin is directly proportional to cell proliferation.[15] Downregulation of nucleophosmin is associated with anticancer drug-induced apoptosis in various human cancer cells.[8,9] Therefore, our result is not consistent with previous studies in which nucleophosmin frequently overexpressed in a variety of human malignancies and downregulated in drug-induced apoptosis cells. Since aloe-emodin induced the change in the expression of nucleophosmin, we focus the attention on the expression of nucleophosmin in aloe-emodin-induced H460 cell apoptosis. Our study also found that aloe-emodin had no significant effect on the mRNA level of nucleophosmin in H460 cells. Therefore, a direct relationship between nucleophosmin-translocation and apoptosis was determined by immunostaining in this study. We found nucleophosmin (32 kDa) to be markedly translocated from nucleus to the cytoplasm, but not from nucleoli to the nucleoplasm during aloe-emodin-induced H460 cell apoptosis. This result suggested that a certain degree of nucleophosmin-translocation from nucleoli to the cytoplasm should be necessary for the increasing in the proform of nucleophosmin during

aloe-emodin-induced H460 cell apoptosis.

In addition, nucleophosmin is a mobile nucleolar protein that shuttles between nucleoli and nucleoplasm or nucleoli and cytoplasm.[6] Based on the shuttle action, nucleophosmin may recruit protein factors into nucleoli for various functions.[16,17] In the past, many investigators found that nucleophosmin shifts from nucleoli to the nucleoplasm when cells are exposed to certain anticancer drugs.[18,19] Previously, many reports suggested that the nucleolus is one of the target areas of anticancer drugs, such as Adriamycin, actinomycin D, mitoxanthrone and camptothecin. These anticancer drugs localize in nucleoli or have effects on nucleoli, including alter nucleolar structure and inhibit its function.[20,21] Based on the above observations, we proposed the idea that nucleophosmin shifts from nucleoli to the cytoplasm when cells are exposed to aloe-emodin. Firstly, aloe-emodin might induce the destruction of nucleoli structure and function during H460 cell apoptosis. Secondly, the depletion of nucleophosmin from nucleoli or inhibition of the shuttling of nucleophosmin from cytoplasm to nucleus may be responsible for the increase in nucleophosmin during aloe-emodin-induced H460 cell apoptosis. However, the cause effect relationship between nucleophosmin-translocation and apoptosis remains to be investigated.

It is worthy of note that the fragment of nucleophosmin (pI about 4.7 and m.w. about 18 kDa) was not observed in 2-D gel by Western blotting analysis. It seemed to

indicate that the commercial antibody of nucleophosmin, which reacted with the C-terminal of nucleophosmin, could not recognize the fragment at N-terminal of nucleophosmin in this study. The fragment, a predicted m.w. of about 18 kDa, at N-terminal of nucleophosmin was observed in 2-D gel during aloe-emodin-induced H460 cell apoptosis. This result is consistent with other observation that a degraded product of nucleophosmin was detected during the TPA-induced differentiation of K562 cells.[10] To further investigate whether there was a relationship between the increased nucleophosmin protein level and H460 cell apoptosis, this study examined the effect of emodin, luteolin or baicalein, which have been demonstrated to be apoptosis-inducing agents in a series of our studies, on the expression of nucleophosmin. The amount of nucleophosmin induced by emodin, luteolin or baicalein was more intense than that found in untreated cells of H460 cells. This result indicated that the increase, not downregulation, in the protein level of nucleophosmin is involved in drug-induced H460 cell apoptosis.

In summary, we applied 2-D electrophoresis to analyze the proteins involved in aloe-emodin response at protein level in H460 cells. The release of nucleophosmin from the nucleus to cytosol and the degradation of nucleophosmin were observed during aloe-emodin-induced H460 cell apoptosis. This study also demonstrated that the gene expression of nucleophosmin remained unchanged after treatment with

aloe-emodin. The aloe-emodin-caused increase in the amount of proform and fragment of nucleophosmin may be involved in aloe-emodin-induced H460 cell apoptosis.

ACKNOWLEDGEMENTS

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TABLE I-MS/MS ANALYSIS OF TRYPTIC FRAGMENTS FROM SPOT 1 IN ALOE-EMODIN-TREATED SAMPLES

z	MH+	Xcorr	Reference	( )Sequence
3	2929.4	5.35	gi|107221	(-)TVSLGAGAKDELHIVEAEAMNYEGSPIK
2	2145.5	2.49	gi|107221	(-)DELHIVEAEAMNYEGSPIK
2	1569.5	3.43	gi|107221	(-)VDNDENEHQLSLR
2	2928.7	3.18	gi|107221	(-)TVSLGAGAKDELHIVEAEAMNYEGSPIK
1	745.0	1.2	gi|107221	(-)VTLATLK

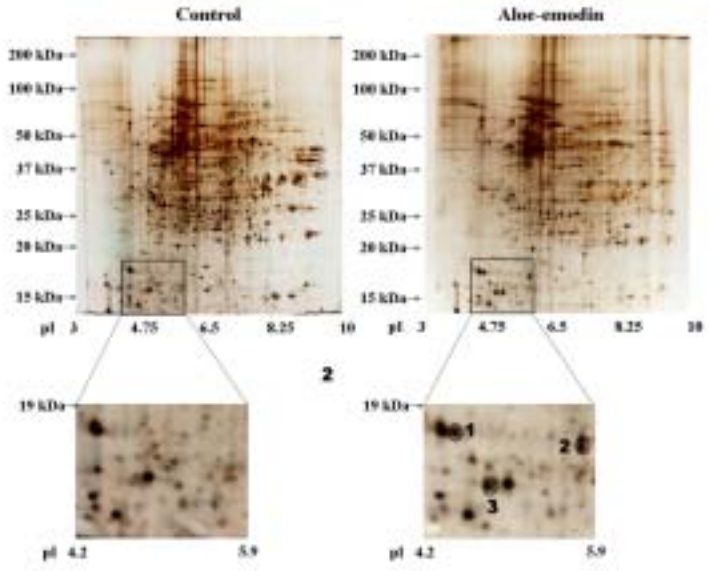

FIGURE 1 – Two-dimensional electrophoresis maps of control or aloe-emodin-treated H460 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1% serum for 24 h. Proteins were separated on a pH 3-10 IPG-strip (17 cm) in the first dimension and on a 12% SDS-polyacrylamide gel in the second dimension. Staining of the protein spots was accomplished by silver nitrate. Magnified views of gels from the control cells or from cells treated with aloe-emodin are the section of pH range of 4.2-5.9 and molecular weight below 19 kDa.

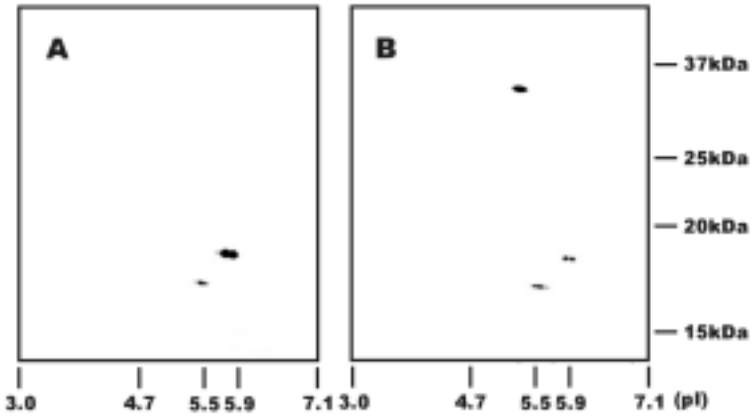

FIGURE 2 –The effect of aloe-emodin on the expression of nucleophosmin of H460 cells. Aloe-emodin-induced the expression of nucleophosmin was detected by Western blotting of 2-D gel. H460 cells were incubated without (A) or with (B) 40 μM aloe-emodin in the presence of 1% serum for 24 h. Proteins were separated on a pH 3-10 IPG-strip (17 cm) in the first dimension and on a 12% SDS-polyacrylamide gel in the second dimension. The 2-D-separated proteins were electrotransferred to Immobilon-P Transfer Membranes and then probed with nucleophosmin antibody as described in Material and Methods. Results are representative of 3 independent experiments.

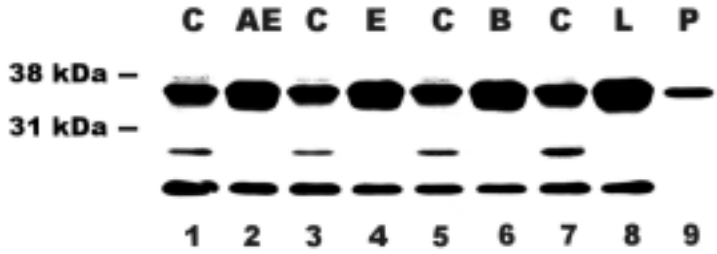

FIGURE 3 –The effect of aloe-emodin, baicalein, emodin or luteolin on the expression of nucleophosmin of H460 cells. Drug-induced the expression of nucleophosmin was detected by Western blotting of 1-D gel. Cell lysates were analyzed by 12% SDS-PAGE and then probed with nucleophosmin antibody as described in Material and Methods. Lane 1, 3, 5 and 7, control cells (DMSO); lane 2, aloe-emodin (AE)-treated cells (40 μM, 24 h); lane 4, emodin (E)-treated cells (50 μM, 16 h); lane 6, baicalein (B)-treated cells (50 μM, 24 h); lane 8, luteolin (L)-treated cells (30 μM, 24 h); lane 9, positive control (HeLa cells). Results are representative of 3 independent experiments.