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Original Article

Andrographolide inhibits proliferation of human lung cancer cells and the related mechanisms

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Abstract: This study is to determine effect of Andrographolide (AD) on the growth of the non-small cell lung cancer cell line H3255. Expression of vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1), and the activity of protein kinase C (PKC) were also detected. The H3255 cells were treated with 1.0, 2.5, or 5.0 μM AD for 24 h. MTT assay was performed to examine cell viability. Levels of VEGF and TGF-β1 were detected by ELISA. The ATPase activity and PKC activity were tested. AD treatments decreased cell viability via a concentration-dependent manner, leading to decreases in the Na^+-K^+-ATPase activities ($P < 0.05$). AD also increased levels of the DNA fragmentation and releasing of lactate dehydrogenase. AD also reduced VEGF and TGF-β1 levels, and inhibited protein kinase C activities in H3255 cells ($P < 0.05$). AD inhibits proliferation of lung cancer cells via a concentration-dependent manner by a mechanism related to reducing levels of VEGF and TGF-β1. Thus, AD might be a potent anti-lung cancer agent.

Keywords: Andrographolide, lung cancer cells, Na^+-K^+-ATPase activity, protein kinase C, vascular endothelial growth factor, transforming growth factor β1

Introduction

Lung cancer is the leading cause of the cancer-related deaths worldwide. The non-small cell lung cancer (NSCLC) accounts for more than 80% of the total numbers of the lung cancer cases. The clinical symptoms might be not obvious in many patients with an early stage of NSCLC, which may lead to the loss of opportunity of surgery [1].

Na^+-K^+-ATPase, a key enzyme in tricarboxylic acid cycle, is a heterodimer composed of one α-subunit and one β-subunit. The α-subunit is a transmembrane protein, which mediates the exchange of intracellular Na^+ and extracellular K^+ [2, 3] and thus plays a key role in maintaining dynamic balance of the ions on both sides of membrane. Therefore, various factors that influence the activity of Na^+-K^+-ATPase may cause changes of the mitochondrial transmembrane potential and consequent release of apoptosis-related molecules [4, 5]. It has been reported that Na^+-K^+-ATPase has important activities in mediating cell adhesion and signal pathway activation among the incidence of lung cancer. Therefore, there are significant correlations between the Na^+-K^+-ATPase activities and the development of cancer occurrence, which highlights that the Na^+-K^+-ATPase might be an important target of the anti-cancer drugs for NSCLC [6]. In addition, the vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) are also involved in the adhesion of cancer cells to promote tumor metastasis [7, 8].

The novel molecular therapy may target at inhibition of the specific molecular pathway in the process of tumor growth and invasion. So, development of the anti-cancer therapeutic agent with low toxicity is important for NSCLC, since it can be used independently or in combination with other therapeutics to increase the sensitivity of radiotherapy and chemotherapy for NSCLC [9, 10].

Andrographolide (AD), a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*, is known to possess multi-

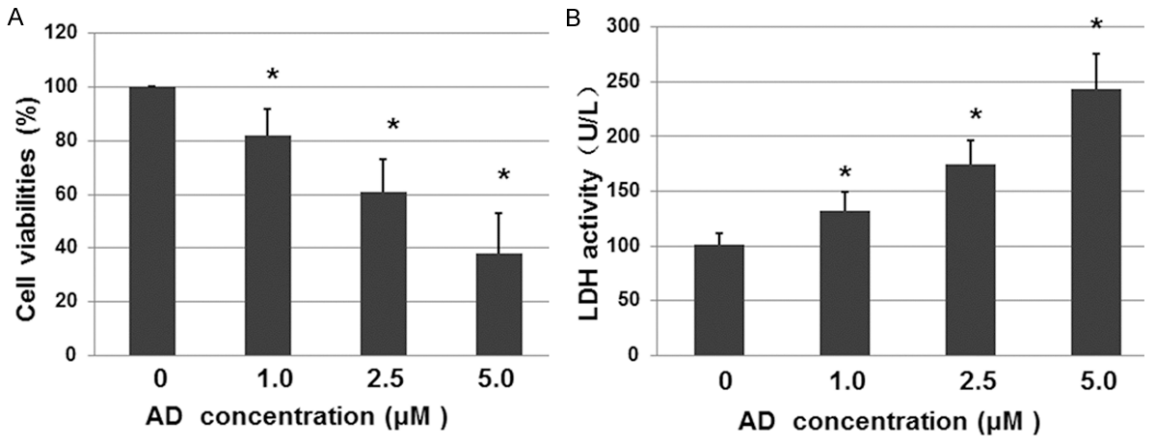

Figure 1. Effect of different concentration of AD on the cell viability and LDH activity of the H3255 cells. The cells treated with RPMI-1640 medium containing a final concentration of less than 0.5% DMSO served as the control. The H3255 cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. A. Effect of different concentration of AD on cell viability. *, $P < 0.05$ vs Control group. B. Effect of different concentration of AD on LDH activity in H3255 cells. *, $P < 0.05$ vs the control group.

ple pharmacological activities to treat a variety of infectious diseases [11]. Recent, it has been found that AD has the potential effect of anti-tumor activity, in addition to the antiviral activity [12]. However, the effects of AD on NSCLC and the related mechanisms are still unclear.

In this report, we determine the effect of AD on non-small cell lung cancer cells, and study its possible effects on expression of tumor growth associated biomarkers such as VEGF, TGF-β1, and the activity of protein kinase C (PKC).

Materials and methods

Reagents and cell culture

AD (Andro) was purchased from Sigma-Aldrich Co Ltd. (St. Louis, MO, USA), which was dissolved in dimethylsulfoxide (DMSO) as a stock solution at the concentration of 5 g/L (15 mM). The medium, culture plate, and antibiotics used in the cell culture of the present study were purchased from Invitrogen Co Ltd (Carlsbad, CA, USA). Human lung cancer cell line H3255 was purchased from ATCC. Cell medium was changed every 3 days and the cell density was adjusted to 10^8/L before the experiment.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

H3255 cells (10^8/L) were inoculated in 96-well plates (180 μl/well and six wells per group). After the cell processing, 20 μl of MTT was added to the wells, and incubation continued for an additional 4 h at 37°C. The supernatant was removed, and the cells were oscillated with DMSO (200 μl) photophobically for 15 min. Absorbance was measured at 570 nm using an automatic microplate reader. The cell viability was calculated as follows: OD values of each group/OD value of negative control group × 100%.

Lactate dehydrogenase (LDH) test

LDH activity was determined by colorimetric methods using the semi-automatic biochemical analyzer according to the kit provided by Sigma Corporation.

DNA fragment analysis

DNA fragments from dead cells were analyzed using ELISA. The cells were treated with different concentrations of AD and then harvested with lysis buffer for 30 min. A volume of 20 μl of the supernatant was transferred into each well of a streptavidin-coated 96-well plate. The absorption was measured on an automatic ELISA reader at 405 nm. DNA fragmentation was measured by Enrichment Factor, which calculated as $OD_{sample}/OD_{control}$.

Measurement of Na^+-K^+-ATPase activity

The activity of ATPase was determined by measurement of the amount of phosphate ion (Pi) released from the enzyme-catalyzed reaction.

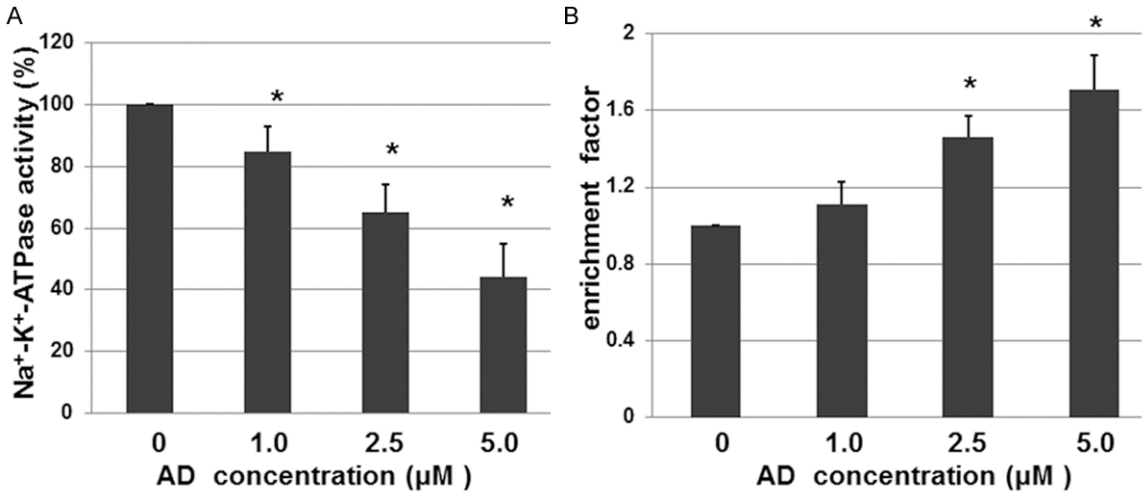

Figure 2. Effect of different concentration of AD on the Na^+-K^+-ATPase activity and DNA fragmentation in the H3255 cells. The cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. The cells treated with medium containing 0.5% DMSO only served as a control. A. Effects of different concentration of AD on Na^+-K^+-ATPase activity in H3255 cells. *, $P < 0.05$ vs Control group. B. Effects of different concentration of AD upon DNA fragmentation (enrichment factor). *, $P < 0.05$ vs the control group.

Each reaction included 100 mM NaCl, 20 mM KCl, 2 mM ATP, 30 mM Tris-HCl buffer (pH 7.4) and freshly isolated mitochondrial extracts from the cells. After ATP was added, the mix was incubated at 30°C for 15 min, then terminated by adding 15% trichloroacetic acid. The released Pi was measured by the absorbance at 640 nm. Each activity unit of ATPase was defined as 1 μM Pi produced from the catalysis of ATP by 1 mg of protein in 1 hour. The ratio of ATPase activity was calculated.

Analysis of VEGF and TGF-β1 levels

The H3255 cells were treated with different concentrations of AD and washed with PBS once. The cell culture was added with TNF-α at a final concentration of 10 μg/L and incubated for 18 h. The concentrations (pg/mg) of VEGF and TGF-β1 in the supernatant of the cell culture were determined with ELISA kit (R&D, USA).

Measurement of PKC activity

The activity of PKC isozymes can be measured intermediately using a pseudosubstrate that is phosphorylated by PKC [13]. The cells after treatment were washed with PBS twice at 4°C and centrifuged at 1000 rpm. After centrifugation, 1 ml of buffer containing 50 mM Tris-HCl, 50 mM β-mercaptoethanol, 10 mM benzamidine, 5 mM EDTA, 1 mM PMSF (pH 7.5) was added to resuspend the cells. PKC activity was detected with the kit (EMD Biosciences, San Diego, USA) (λ = 492 nm).

Statistical analyses

The values obtained from all the groups were given as mean ± standard deviation (mean ± SD). The data were analyzed with the statistical analysis software SPSS 10.0. ANOVA was used for inter-group comparison. The $P < 0.05$ was considered to be statistically significant.

Results

AD decreases the viability of H3255 cells and increases the levels of LDH released by the H3255 cells

To detect the possible effect of AD on viability of H3255 cells, the cells were treated with AD at various concentrations. Control group was difined as the cells treated with RPMI-1640 medium containing a final concentration of less than 0.5% DMSO. The H3255 cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. As shown in **Figure 1A**, AD (1 μM, 2.5 μM or 5.0 μM) inhibited the activity of H3255 cells in a dose-dependent manner. The activity of the H3255 cells treated with 5.0 μM AD decreased to 38% when compared with the control group (**Figure 1A**).

In addition, AD enhanced LDH release from the H3255 cells (**Figure 1B**). When treated with 5.0

Table 1. Effect of different concentrations of AD on VEGF and TGF-β1 expressions (mean ± SD, n = 6) in H3255 cells

	VEGF (pg/mg)	TGF-β1 (pg/mg)
Control (0.1 % DMSO)	172.85 ± 12.75	229.93 ± 9.75
1.0 μM AD	150.32 ± 11.72*	208.23 ± 5.71*
2.5 μM AD	116.36 ± 8.44*	127.52 ± 7.15*
5.0 μM AD	85.62 ± 5.74*	101.87 ± 6.52*

Note: *, P < 0.05 vs the control group.

μM AD, the concentration of released LDH was more than double (243.15 ± 31.77 U/L) than that of control group.

AD decreases the activity of Na^+-K^+-ATPase and increases the DNA fragmentation in the treated cells

As shown in **Figure 2A**, Different concentrations of AD inhibited the Na^+-K^+-ATPase activity in the H3255 cells by a dosage-dependent manner. When treated with 1.0 μM AD, the enzyme activity was decreased to 85% in comparison with the control group. This activity level was decreased to 65%, 44% when treated with 2.5 μM and 5.0 μM AD, respectively (**Figure 2A**).

As shown in **Figure 2B**, higher concentration of AD increased DNA fragmentation in H3255 cells more profoundly. Treatment with 1.0 μM AD did not significantly produce more DNA fragments than that in the control group. However, treatment with 2.5 μM and 5.0 μM AD induced DNA fragmentation significantly, while the values of enrichment factor were 1.46 and 1.71, respectively (P < 0.05 as compared with the control group).

AD decreases levels of VEGF and TGF-β1

To detect the possible effects of AD on expression levels of VEGF and TGF-β1 in H3255 cells, the cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. The expression levels of VEGF and TGF-β1 in the cells were determined by ELISA. As shown in **Table 1**, 1.0 μM, 2.5 μM or 5.0 μM AD decreased the levels of VEGF and TGF-β1 in a dosage-dependent manner. Treatment with 5.0 μM AD decreased the levels of VEGF and TGF-β1 to 50.4% and 55.7%, respectively, of their levels in the control group (0.1% DMSO only).

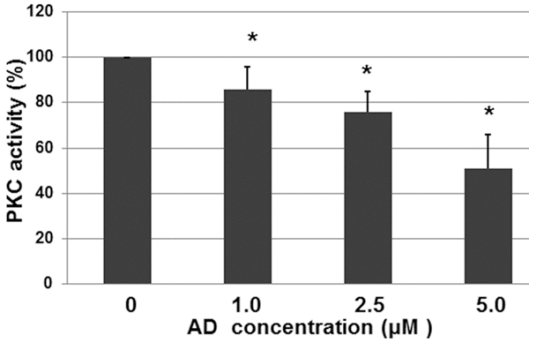

Figure 3. Effect of different concentration of AD on PKC activity in H3255 cells. *, P < 0.05 vs the control group. The cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. The cells treated with medium containing 0.5% DMSO only served as a control.

AD decreases the PKC activities of H3255 cells

To detect the possible effect of AD on PKC activity of H3255 cells, the cells were treated with 0, 1.0, 2.5, or 5.0 μM AD for 24 h. As shown in **Figure 3**, treatments with AD between 1.0 μM and 5.0 μM were demonstrated to inhibit PCK activity in H3255 cells. As shown in **Figure 3**, the treatment with 1.0 μM AD decreased PKC activity slightly, but the treatment with 2.5 μM AD decreased PKC activity to 76%. The treatment with 5.0 μM AD decreased PKC activity even to 51% of that in the control group (P < 0.05).

Discussion

One main aim of tumor treatment is to induce apoptosis of cancer cells, in which DNA fragmentation is a major characteristic of apoptosis [14, 15]. It was demonstrated in this study that AD inhibits proliferation of H3255 cells, possibly by increasing DNA fragmentation, decreasing activity of Na^+-K^+-ATPase and reducing synthesis of VEGF and TGF-β1. In this study, AD showed effects in reducing cell viability and increasing DNA fragmentation. One possible explanation is that AD enters into cells and induces cell apoptosis [16, 17].

In this study, the activity of Na^+-K^+-ATPase in lung cancer cells was decreased by AD, indicating malfunctioning of the α-subunit and/or impairment of mitochondrial membrane, and also suggesting that the mitochondrial dysfunction resulted from AD might induce apoptosis of lung cancer cells [18]. VEGF and TGF-β1 are

both angiogenic factors [19]. TGF-β1 can promote metastasis of Non-Small Cell Lung Cancer (NSCLC) [20]. It is therefore inferred that low expression of VEGF and TGF-β1 may indicate the inhibitory effect of AD on angiogenesis and metastasis of lung cancer cells.

The activation of PCK is related to the cellular signal transduction pathways in tumor cells, which promote proliferation and invasion of tumors [21]. Overexpression and increased activity of PCK can enhance NSCLC metastasis. This study has demonstrated that treatment with AD effectively inhibits PCK activation in lung cancer cells, indicating that AD may potentially down-regulate the activity of the tumor-related pathways and thus retard the progression of lung cancer.

In summary, the present study has demonstrated that treatment with AD can promote apoptosis of lung cancer cells by increasing DNA fragmentation, decreasing Na^+-K^+-ATPase activity, reducing levels of TGF-β1 and VEGF expression, and down-regulating the PCK activity. It is suggested that AD might be a candidate of anticancer drug.

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Disclosure of conflict of interest

None.

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