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Effect of 5- AZn-2 ′-deoxycytidine on proliferation of human lung adenocarcinoma cell line A549 *in vitro*

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ABSTRACT

Objective: To explore effect of 5-AZn-2 ′-deoxycytidine on proliferation of human lung adenocarcinoma cell line A549 *in vitro*. **Methods:** Superoxide dismutase (SOD) activity was measured by hydroxylamine colorimetric method. Inhibition effect of 5-AZn-2′ deoxycytidylic acid at different concentration and different time on growth of A549 cell was determined by MTT assay. Methylene dioxyamphetamine (MDA) was measured by thiobarbituric acid colorimetric method. Effect of 5-AZn-2′ deoxycytidylic acid on apoptosis of A549 cell was determined by Hoechst 33258 dyeing detection. **Results:** 5-AZn-2′ deoxycytidylic acid had significant inhibition effect on proliferation of A549 cells *in vitro*, and the inhibition was notably dependent on time and dosage during 48-72 h; SOD level was significantly lower than those of control group ($P<0.05$, $P<0.01$), MDA level was significantly higher than those in the control group ($P<0.05$, $P<0.01$). A549 cells began to be in apoptosis after using 5-AZn-2′ deoxycytidylic acid. **Conclusions:** 5-AZn-2′ deoxycytidylic acid has significant inhibition effect on growth of A549 cell, and can lead the change of lipid peroxidation. It indicates that the mechanism has relationship with A549 cell cycle tissue and induction factor of apoptosis.

1. Introduction

Lung cancer is a kind of cancer with the highest mortality rate around the world. According to clinical manifestations, lung cancer can be categorized into non-small cell lung cancer and small cell lung cancer. Greatly different from epithelial cancer (such as adenocarcinoma and squamous cell carcinoma) in biomorphology and development, small cell lung cancer is highly malignant, spreads rapidly in the early period after onset, and is very sensitive to chemotherapy and radiotherapy. Chemotherapy, radiotherapy, surgery and biotherapy are the major treatment modalities of small cell lung cancer. The key step of small cell lung cancer treatment is to know cell changes in cancer tissue. 5-Aza-2′-deoxycitydine (DAC), a DNA methyltransferase inhibitor, has been used to deteriorate hypermethylated gene expression, inhibit the growth of various cells[1-3], accelerate tumor necrosis factor-mediated malignant melanoma cell apoptosis and promote the expression of quiescent gene MAGE-1[4-6]. In addition, DAC can inhibit DNA and protein synthesis in tumor cells by inhibiting intracellular dihydrofolate reductase expression[7,8]. This can stop the growth of cancer cells and even lead to cellular death. This study investigated the effects of DAC on the proliferation, apoptosis and lipid peroxidation of human lung adenocarcinoma epithelial cell line A549 and the underlying mechanisms in order to provide theoretical evidence for clinical use of DAC.

2. Materials and methods

2.1. Materials

2.1.1. Cells and their culture

Human lung adenocarcinoma epithelial cell line A549 (China Center for Type Culture Collection, Zhejiang University, China) was cultured with DMEM (Gibco,

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Carlsbad, CA, USA) containing 10% fetal bovine serum (Sijiqing, Zhejiang Tianhang Biological Technology Co., Ltd., China) in an incubator at 37 ℃, 5% CO_2 with saturated humidity and then digested with 0.25% trypsin for passage.

2.1.2. Drugs and reagents

DAC (Shanghai Hualian Pharmaceutical Co., Ltd., Shanghai, China) was prepared into 1 mol/L solution with physiological saline and diluted into the required concentrations with DMEM when use. MTT solution was purchased from Huamei Bioengineering Co., Ltd., China. Hochest33258 and dimethyl sulfoxide (DMSO) were purchased from Sigma, St. Louis, MO, USA.

2.2. Methods

2.2.1. Cell line and cell culture

Human lung adenocarcinoma epithelial cell line A549 was cultured with RPMI-1640 culture medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37 ℃, 5%CO_2 with saturated humidity. When cells reached 80% confluence, they were digested with 0.25% trypsin for passage.

2.2.2. DAC preparation

100 mmol/L DAC stock solution was prepared with a small amount of DMSO by applying an ultrasonic wave. Prior to experiment, DAC was diluted to the required concentration (DMSO final concentration < 0.1%) with RPMI-1640 culture medium.

2.2.3. MTT assay

Human lung adenocarcinoma epithelial cell line A549 at the log phase of growth were seeded into a 96-well plate, 2×10^3 cells/well. After 12 h of culture, these cells were incubated with DMSO (control group) or different concentrations (0, 0.25, 0.5, 2.5, 12.5 μmol/L) of DAC solution (DAC groups) at 37 ℃ and 5% CO_2 under saturated humidity for 12, 24, 48 and 72 h. Five parallel wells were designated for each group. After removal of supernatant, 20 μL MTT solution (5 g/L) was added to each well and A549 cells were incubated at 37 ℃ for 4 more hours. Supernatant was removed, then 150 μL DMSO was added to each well and A549 cells were oscillated for 10 min. Absorbance at 490 nm was determined through the use of ELISA reader. Each experiment was repeated three times. Cell growth curve was drawn and cell growth inhibitory rate was calculated according to the formula: Cell growth inhibitory rate (%) = (1-absorbance in the DAC group/absorbance rate in the control group) ×100%.

2.2.4. Cell morphology and cell apoptosis rate

Cell apoptosis in each group was detected using Hoechst 33258 staining. A549 cells (2×10^4/well) were seeded in each well in which a piece of sterile coverslip was pre-placed and five parallel wells were used for each group. When adhering to culture plate, A549 cells were treated with different concentrations of DAC (0, 0.25, 0.5, 2.5, 12.5 μmol/L) or DMSO for 24, 48, 72 and 96 h. Culture medium was removed, then A549 cells were collected, washed and evenly smeared. After natural drying, the cells were treated with 0.05 mg/L Hoechst33258 and 0.1 mg/L calcium- and magnesium-free thiomersal for 40 min. After mounting, the cells were observed under the fluorescence microscope, photographed and counted.

2.2.5. Detection of superoxide dismutase (SOD) and malondialdehyde (MDA) activities in A549 cells

In strict accordance with the instructions of reagent kits provided by Nanjing Juli Biomedical Engineering Institute (Nanjing, China), SOD activity was measured by hydroxylamine chromatometry and MDA activity by thiobarbituric acid-reactive-substances assay.

2.3. Statistical analysis

All data were expressed as mean±SD and statistically processed with SPSS16.0 software (SPSS, Chicago, IL, USA). t test was used. A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects of DAC on A549 cell proliferation

DAC at a concentration of 0.5, 2.5 and 12.5 μmol/L significantly inhibited the growth of A549 cells in a time- and dose-dependent manner ($P<0.01$; Figure 1).

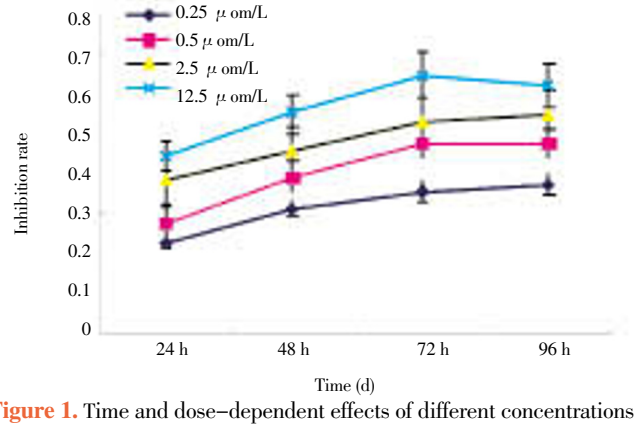

Figure 1. Time and dose-dependent effects of different concentrations of DAC on A549 cell proliferation.

3.2. Effects of DAC on SOD and MDA activities in A549 cells

The SOD activity in the DAC (0.5, 2.5 and 12.5 μmol/L) groups was significantly, dose-dependently lower compared with the control group ($P<0.05$, $P<0.01$). The MDA activity in

the DAC (0.5, 2.5 and 12.5 μmol/L) groups was significantly, dose-dependently higher compared with the control group ($P<0.05$, $P<0.01$) (Table 1).

Table 1

Effects of DAC on SOD and MDA activities in the A549 cells (Mean±SD, $n=10$).

Group	SOD (U/mg)	MDA (nmol/mg)
Control	72.23±2.40	24.23±3.20
DAC 0.25 μmol/L	69.26±2.70	32.26±3.20
DAC 0.5 μmol/L	56.42±4.40a	37.43±5.20a
DAC 2.5 μmol/L	52.64±2.50a	45.52±4.30a
DAC 12.5 μmol/L	49.45±3.20b	57.58±3.40b

a: $P<0.05$, b: $P<0.01$, *vs.* control group.

3.3. Effects of DAC on A549 cell apoptosis rate and morphology

Five visual fields were randomly selected under high-fold magnification for counting total cells and apoptotic cells. Cell apoptosis rate was calculated according to the formula: cell apoptosis rate = number of apoptotic cells/total cell number×100%. Cell apoptosis rate reached its peak in the DAC (0.5, 2.5, 12.5 μmol/L) groups at 48 and 72 h, which was significantly higher compared to the control group ($P<0.05$, $P<0.01$) (Table 2). Hoechst33258 fluorescent staining showed that after 72 h of DAC treatment, cell fluorescent staining was light and even and no apoptotic cells were observed in the control group (Figure 2A); cell apoptosis signs including karyopyknosis, chromatic agglutination, vacuole and apoptotic body were observed in the DAC (0.5, 2.5 and 12.5 μmol/L) groups (Figures 2B-D), indicating that DAC induced apoptosis of A549 cells.

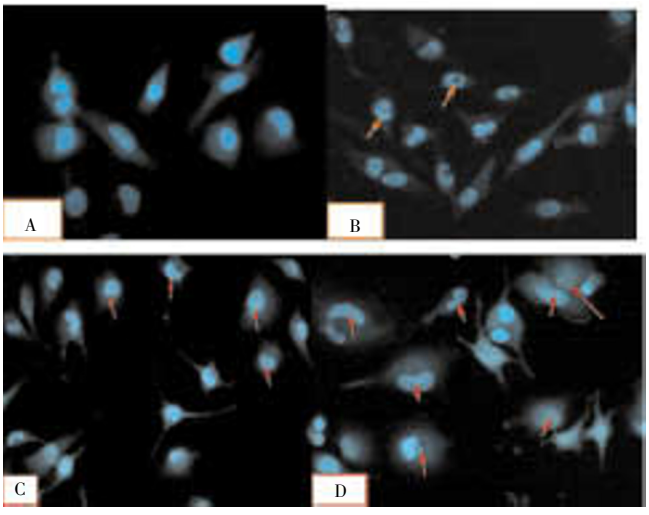

Figure 2. Effects of DAC on A549 cell apoptosis rate and morphology. A: Cell morphology of A549 cells in the control group (×200); B: Morphology of apoptotic A549 cells induced by 0.5 μmol/L DAC for 72 h (×200); C: Morphology of apoptotic A549 cells induced by 2.5 μmol/L DAC for 72 h (×200); D: Morphology of apoptotic A549 cells induced by 12.5 μmol/L DAC for 72 h (×200).

Table 2

Effects of DAC on A549 cell apoptosis rate (n = 5, %)

Group	Cell apoptosis rate (%)		
	24 h	48 h	72 h
Control	2.23±0.52	2.60±0.26	2.50±0.45
DAC 0.25 μmol/L	6.30±1.64	6.92±3.23	22.36±3.44a
DAC 0.5 μmol/L	11.50±2.32a	24.35±6.26b	29.36±5.34b
DAC 2.5 μmol/L	13.50±3.84a	29.87±5.84b	36.54±6.34b
DAC 12.5 μmol/L	16.20±2.53a	34.83±7.44b	42.52±8.26b

a: $P<0.05$, b: $P<0.01$, *vs.* control group.

4. Discussion

The development and apoptosis of tumor cells result from coaction of many proteins and proteases in the human body. Cell apoptosis is the process of programmed cell death and controlled by genes[9,10]. This process can help protect the environment in the human body because only programmed cell growing and apoptosis can balance and stabilize the environment in the human body. This principle is used by gene therapy, a treatment method of tumor, which uses drugs (such as 5-fluorouracil, cisplatin, mitomycin, DAC and Chinese herbal medicine) that can lead to programmed death of tumor cells. Abnormal cell apoptosis can give rise to tumor formation, abnormal immunological function and changes in cell biological characteristics[11-14]. Results from this study showed that DAC inhibited the proliferation of A549 cells as confirmed by MTT assay; that is to say, DAC promoted the death of tumor cells and inhibited the proliferation of tumor cells.

The effects of DAC on A549 cells are greatly different at different doses and for different periods of time, so much care needs to be taken over the dose and periods of time[15,16]. In this study, we also observed the morphology of DAC-treated A549 cells and found that the cancer cells turned from spreading status before DAC treatment to round appearance with apoptotic characteristics including cell shrinkage, nuclear chromatin concentration, nuclear fragmentation, budding and apoptotic body formation after DAC treatment. Cell apoptosis rate reached its peak after DAC (0.5, 2.5 and 12.5 μmol/L) treatment for 48 and 72 h, respectively.

Under normal circumstance, the generation and removal of reactive oxygen species are dynamically balanced. When the organisms are stimulated by some abnormal physiochemical factors, a large amount of reactive oxygen species will be produced, which destroy the balance of oxidation-reduction, and excessive reactive oxygen species will cause lipid peroxidation[17-19]. MDA is the end-product of lipid peroxidation. Increased MDA activity indicates the unbalance between DAC oxidation and anti-oxidation[20,21]. Results from this study showed that MDA activity increased dose-dependently in DAC-treated A549 cells. SOD activity

decreased in DAC-treated A549 cells possibly because DAC inactivated SOD by binding SOD dynamic sites, especially sulfhydryl-containing protein groups. This inevitably decreases the ability of organism to remove free radicals and increases intracellular oxidation, thereby causing oxidative damage[22-25]. Results from this study suggest that DAC anti-cancer drugs are effective in the treatment of lung cancer by causing lung cancer cell apoptosis and changes in lipid peroxidation, providing experimental evidence for their clinical application. The underlying mechanism needs further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

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