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Antitumor Effect of Antisense Ornithine Decarboxylase Adenovirus on Human Lung Cancer Cells

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Abstract Ornithine decarboxylase (ODC), the first enzyme of polyamine biosynthesis, was found to increase in cancer cells, especially lung cancer cells. Some chemotherapeutic agents aimed at decreasing ODC gene expression showed inhibitory effects on cancer cells. In this study, we examined the effects of adenoviral transduced antisense ODC on lung cancer cells. An adenovirus carrying antisense ODC (rAd-ODC/Ex3as) was used to infect lung cancer cell line A-549. The 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay was used to analyze the effect on cell growth. Expression of ODC and concentration of polyamines in cells were determined by Western blot analysis and high performance liquid chromatography. Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling was used to analyze cell apoptosis. The expression of ODC in A-549 cells was reduced to 54%, and that of three polyamines was also decreased through the rAd-ODC/Ex3as treatment. Consequently, cell growth was substantially inhibited and terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling showed that rAd-ODC/Ex3as could lead to cell apoptosis, with apoptosis index of 46%. This study suggests that rAd-ODC/Ex3as has an antitumor effect on the human lung cancer cells.

Key words polyamine; putrescine; biosynthesis; antisense technology; gene therapy; A-549 lung cancer cell line

The three polyamines, spermidine, spermine and putrescine, which have a low molecular weight and a simple chemical structure, are positively charged aliphatic amines under physiological conditions. The polyamines interact with various macromolecules, both electrostatically and covalently. Consequently, they have a variety of cellular effects. They are known to be critically involved in cell growth and have been implicated in the process of cell transformation [1,2]. However, the level of polyamines is high in cancer cells and tissues, and rapid tumor growth has been associated with remarkable elevation of polyamine biosynthesis and accumulation [3,4].

Ornithine decarboxylase (ODC) is the first and the rate-controlling enzyme in polyamine biosynthesis, which decarboxylates *L*-ornithine to form diamine putrescine. The complete structure and nucleotide sequence of the ODC gene from mammals are known, with 12 exons and 11 introns [5]. Active mammalian ODC is a homodimer with two-fold symmetry. Subunits have a molecular weight of approximately 51 kDa and the polypeptide chain consists of 461 amino acid residues. ODC becomes activated after treatment with chemical carcinogens and tumor promoters, as well as in cells transformed by various oncogenes, such as *v-src*, *neu* and *ras* [1,6,7]. The level of ODC protein was elevated in various cancers [8–10] and related to recurrence [11]. Some chemotherapeutic agents (e.g., difluoromethylornithine), aimed at inhibiting the activity of ODC, have appeared and taken on inhibitory effects on tumor growth *in vitro* or *in vivo* [12,13], although they show dose-limiting toxicity. Stable transfection of human lung squamous carcinoma cell line LTEP-78 with antisense ODC-expressing plasmid has shown to be related with the reversion of malignant

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phenotypes of human lung squamous carcinoma cells [15]. Taken together, these findings suggest that ODC might provide an important target for the development agents that inhibit carcinogenesis and tumor growth.

Lung cancer is one of the most frequently diagnosed cancers in the world. Metastatic lung cancer is essentially resistant to systemic cytotoxic chemotherapy, and external beam and radioisotope radiotherapy offers only symptom palliation. Clearly the development of novel therapies, such as gene therapy, is a high priority. Some studies have proved that lung carcinomas have greatly elevated polyamine levels [15]. Adenoviral vectors are among the most promising gene transfer vehicles for direct *in vivo* gene therapy for a diverse array of human diseases [16].

In this study, we used a replication-deficient recombinant adenovirus to efficiently deliver a 120 bp antisense ODC (rAd-ODC/Ex3as), which is complementary to the initiation codon, and tested the effects of antisense ODC on lung cancer. The data presented here showed that adenovirus-mediated gene transfer of antisense ODC could significantly inhibit growth of lung cancer cells.

Materials and Methods

Cell culture and reagents

The A-549 lung cancer cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, USA). β-actin antibody and ECL Western blot detection kit were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Monoclonal antibody of ODC was made in our laboratory. Polyamine standards (putrescine, spermidine and spermine) were from Sigma. All other reagents were of reagent grade.

Adenovirus and infection condition

The recombinant adenovirus rAd-ODC/Ex3as, containing the cytomegalovirus promoter and green fluorescent protein (GFP) gene, was constructed by reversely inserting a 120 bp cDNA fragment of the ODC gene into the multiple clone sites [17]. rAd-ODC/Ex3as was purified by ultracentrifugation in cesium chloride step gradients [18]. The titer of the viral stock, measured in plaque-forming units (pfu) per ml, was determined to be 8.5×10^9 pfu/ml by a method published previously [20], and the frozen stock was confirmed to have retained its titer. The control virus rAd-GFP was constructed in the same way as rAd-ODC/Ex3as but without the targeted gene inserted in the polylinker. Viral stocks were suitably diluted in serum-free medium to obtain the desired pfu, added to cell monolayers of lung cancer cells and incubated at 37 ºC for 2 h. The necessary amount of culture medium with 5% FBS was then added and the culture was incubated for the desired time.

MTT assay

MTT assay was used to assess the transduction efficiency of rAd-ODC/Ex3as in A-549 cells. Briefly, cells were seeded at a density of 5000 cells/well in 96-well plates and grown overnight. The next day, these cells were infected by virus solutions at different multiplicity of infection (MOI), from 1 to 100 pfu/cell. After 48 h of incubation, MTT was added (50 μg/well) and incubated for 4 h. Formazan products were dissolved in dimethylformamide, and the absorbance was measured at 570 nm.

To observe the effect of the adenovirus on cell proliferation, MTT assay was also used to draw cell growth curves. Cells were inoculated at a density of 4000 cells per well, under which control cells remained subconfluent and in exponential phase growth for the duration of the assay. Due to a different infective efficiency, A-549 cells were infected at an MOI of 50. All experiments were carried out six times. After 24, 48, 72, 96 and 120 h, absorbance at 570 nm was measured respectively.

Invasion assay

Invasion was assessed by the ability of A-549 cells infected with rAd-ODC/Ex3as to traverse a matrigel-coated membrane as follows. Transwell inserts (8 μm in pore size; Corning Costar, Cambridge, USA) for six-well culture plates were coated with matrigel at a final concentration of 0.7 mg/ml (Becton Dickinson, Bedford, USA). Cells were trypsinized, and 1.5 ml of cell suspension (5×10^5 cells/ml) was added in triplicate wells. The lower chamber was filled with RPMI 1640 medium containing 10% FBS. These chambers were incubated for 6 h at 37 ºC with 5% CO_2 to allow cells to migrate through the matrigel-coated membranes. The non-migrated cells were scraped from the upper surface of the membrane with cotton swabs. Cells on the bottom side of the membrane were fixed and stained with hematoxylin-eosin. Five representative fields in each well were counted under a microscope (magnification,

40×) to determine the number of migrated cells.

Western blot analysis of ODC proteins

A-549 cells were infected with rAd-ODC/Ex3as at an MOI of 50 in RPMI 1640 medium containing 5% FBS for 48 h. The cells were washed three times using ice-cold phosphate-buffered saline (PBS) and collected with a cell scraper. Total cell lysates were prepared in extraction buffer containing 0.05 M Tris (pH 8.0), 0.15 M NaCl, 0.02% sodium azide, 0.1% sodium dodecylsulfate, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1% NP-40. The extracts were subjected to 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in PBS containing 1% nonfat milk powder. Mouse anti-ODC monoclonal antibody was added at a dilution of 1:500 and incubation continued overnight at 4 ºC. The secondary antibody was horseradish peroxidase-conjugated antimouse immunoglobulin G antibody (Zhongshan, Beijing, China). Antibody reactive bands were revealed using the ECL Western blot detection system (Santa Cruz). For quantitation of bands, a digital camera (D50; Nikon, Tokyo, Japan) and SmartView analysis software were used. β-actin was used as the internal control.

High performance liquid chromatography (HPLC) analysis of polyamine pools

A-549 cells were infected with rAd-ODC/Ex3as at an MOI of 50. After 48 h, cells were trypsinized and washed twice with PBS. Intracellular polyamines were extracted from cell pellets with 10% trichloroacetic acid, dansylated and measured by HPLC using muBondapak C18 column (Santa Cruz) as described previously [21].

Cell apoptosis analyzed by terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL)

TUNEL assay kit was supplied by Santai Biological Company (Beijing, China) and used to detect apoptotic cells, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out using Statview J 5.0 software (SAS Institute, San Francisco, USA). A significant difference was defined as $P<0.05$.

Results

Dose-dependent growth inhibition of A-549 cells by rAd-ODC/Ex3as

There was dose-dependent growth inhibition in A-549 cells (**Fig. 1**). Due to differential sensitivity, we chose an MOI of 50 for the adenovirus to infect A-549 cells. Under these conditions, the growth of A-549 cells is fast, but rAd-ODC/Ex3as was more suppressive on cell growth (with a suppressive rate of 60%) than the control rAd-GFP virus, which had no obvious toxic effect on cell growth.

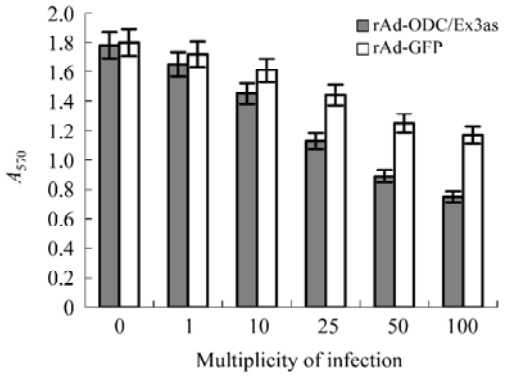

Fig. 1 Effects on A-549 cells with different infective efficiencies of adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as) and control virus rAd-GFP

Due to differential sensitivity, we chose a multiplicity of infection (MOI) of 50 for the adenovirus to infect A-549 cells. The result shows dose-dependent growth inhibition of A-549 cell growth.

Time dependence of the inhibitory effects of rAd-ODC/Ex3as on lung cancer cells

We examined the *in vitro* growth inhibition of rAd-ODC/Ex3as in A-549 cells using cell growth curve as described in "Material and Methods". Antisense ODC had an impact on the growth of lung cancer cells. rAd-ODC/Ex3as inhibited their proliferation by approximately 50% compared with the control virus or no virus-treated group (**Fig. 2**).

Effect of rAd-ODC/Ex3as on invasion ability of A-549 cells

We used a matrigel invasion model to study the effect of the rAd-ODC/Ex3as on invasion. A-549 cells infected with rAd-ODC/Ex3as (18.20±8.62) invaded the matrigel-coated membrane to a much lesser extent than rAd-GFP-infected cells (36.40±8.76) or no virus-treated cells (39.80±

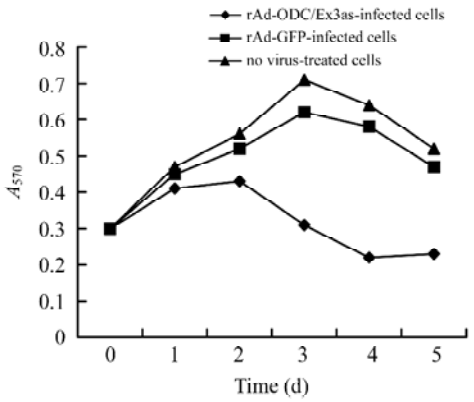

Fig. 2 Effects of adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as) on growth of A-549 lung cancer cells

Growth curves of cells were drawn after A-549 cells were infected with either rAd-ODC/Ex3as or control virus rAd-GFP at a multiplicity of infection of 50. Absorbance was measured every day for a period of 5 d. rAd-ODC/Ex3as inhibited A-549 cell proliferation by approximately 50%, compared with the control virus- or no virus-treated group.

6.88) (**Fig. 3**).

TUNEL assay for apoptosis

To examine the mechanism by which rAd-ODC/Ex3as might retard lung cancer cell growth *in vitro*, we used TUNEL to detect the effect of rAd-ODC/Ex3as on cell apoptosis at 48 h (**Fig. 4**) and 72 h after infection. As shown in **Table 1**, the rate of apoptosis in cells infected by rAd-ODC/Ex3as was significantly higher than in cells infected by rAd-GFP or no virus-treated cells (P<0.05).

Effect of rAd-ODC/Ex3as on expression of ODC and polyamine pools in cell lysate

The ODC proteins produced in the A-549 cells infecting with rAd-ODC/Ex3as were examined by Western blot. The ODC expression in the cells infected with rAd-ODC/Ex3as was substantially reduced compared with that of the cells infected with rAd-GFP or no virus-treated cells (**Fig. 5**). The results analyzed by SmartView software

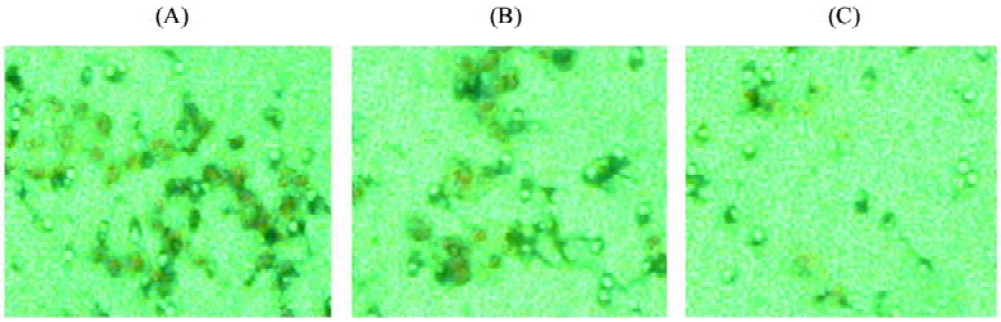

Fig. 3 Effect of adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as) on invasion ability of A-549 lung cancer cells

(A) No virus-infected A-549 cells invaded the matrigel-coated membrane to a high extent. (B) Control virus rAd-GFP-infected A-549 cells invaded the matrigel-coated membrane to a lesser extent, compared with (A). (C) A-549 cells infected with rAd-ODC/Ex3as invaded the matrigel-coated membrane to a much lesser extent, compared with (A) or (B).

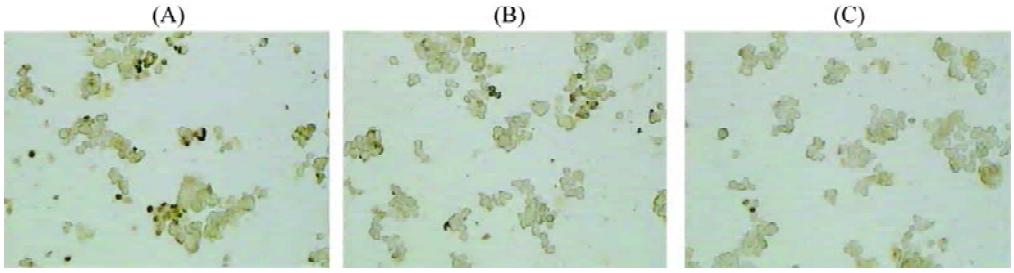

Fig. 4 Effects of adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as) on the apoptosis of A-549 lung cancer cells 48 h after infection

Cells were observed under a microscope (magnification, 100×). Apoptotic cells were colored brown. (A) A-549 cells infected with rAd-ODC/Ex3as. (B) A-549 cells infected with control virus rAd-GFP. (C) No virus-treated A-549 cells.

Table 1 Apoptosis rate of A-549 lung cancer cells at 48 h and 72 h after infection with control virus (rAd-GFP) or adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as)

Group	Apoptosis rate (%)		*P*
	48 h	72 h	
No virus	5.10±0.72	7.60±0.65	
rAd-GFP	8.70±0.94	11.30±0.70	
rAd-ODC/Ex3as	23.10±1.51	56.40±2.26	<0.05

Date were represented as mean±SD.

Table 2 Polyamine pools of A-549 lung cancer cells after infection with control virus (rAd-GFP) or adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as)

Group	Polyamine pool (pmol/10^6 cells)			*P*
	Putrescine	Spermidine	Spermine	
A-549	540±12	1390±16	1425±21	
rAd-GFP	485±9	1275±14	1470±18	
rAd-ODC/Ex3as	235±5	1160±10	1215±13	<0.05

Date were represented as mean±SD.

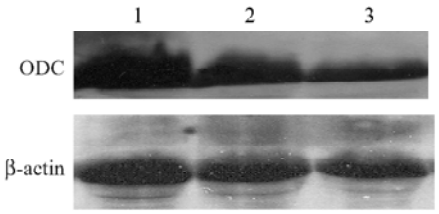

Fig. 5 Western blot analysis for ornithine decarboxylase (ODC) gene expression in A-549 lung cancer cells after transduction of adenovirus carrying antisense ornithine decarboxylase (rAd-ODC/Ex3as) or control virus rAd-GFP

A-549 cells were infected with adenoviruses at a multiplicity of infection of 50. After 48 h, 2×10^6 cells were collected in 300 μl extraction buffer. Forty microliters of protein extraction was added to sodium dodecylsulfate-polyacrylamide gel electrophoresis. 1, no virus-treated A-549 cells; 2, A-549 cells infected with rAd-GFP; 3, A-549 cells infected with rAd-ODC/Ex3as. β-actin was used as the internal control.

showed that ODC gene expression in A-549 cells infected with rAd-ODC/Ex3as accounted for 40% of that in cells treated with rAd-GFP.

Effect of rAd-ODC/Ex3as on polyamine pools in cell lysate

Three polyamines (putrescine, spermidine and spermine) were analyzed by HPLC. It was shown that the concentrations of the three polyamines, especially of putrescine, were signficantly decreased (**Table 2**).

Discussion

Polyamines are aliphatic cations with multiple functions and are essential for life. In normal cells, ployamine levels are intricately controlled by biosynthetic and catabolic enzymes. Multiple abnormalities in the control of polyamine synthesis, metabolism, uptake and function might be responsible for increased levels of polyamines in cancer cells compared to that of normal cells, especially in lung cancer cells [15]. Targeting specific molecules in cells by antisense inhibition was shown to have potential effectiveness in decreasing the protein expression. ODC is one of the most important enzymes in polyamine biosynthesis. The overexpression of ODC in NIH3T3 cells caused transformation of these cells to a malignant phenotype, in essence qualifying ODC as an oncogene [21]. Inhibition of ODC by difluoromethylornithine could compromise cell growth and transformation [23]. *In vitro* studies by Schipper *et al.* using conformationally restricted polyamine analogues showed that these compounds inhibited cell growth, probably by inducing antizyme-mediated degradation of ODC [23]. In addition, Alm *et al.* [24] showed that the ODC gene was a well-defined target gene for *c-myc* and other oncogenes. Therefore, we targeted ODC gene using an antisense gene delivery strategy with a replication-deficient recombinant adenovirus vector. In the present study, we demonstrated that rAd-ODC/Ex3as could inhibit lung cancer growth and lead to the apoptosis of A-549 cells.

MTT assay showed antisense ODC had an impact on the growth of lung cancer cells. rAd-ODC/Ex3as inhibited their proliferation by approximately 60% when compared with the control virus and no virus-treated groups. Western blot analysis showed the expression of the ODC gene was substantially more reduced in the cells infected with rAd-ODC/Ex3as than that in the cells infected with rAd-GFP or no virus-treated cells. A substantial decrease in ODC gene expression resulted in the reduction of polyamine biosynthesis. In addition, the reduction of polyamines might contribute to the marked suppression of cancer cell growth and tumor formation. Recent studies also showed that inhibiting mRNA expression of ODC gene can effectively inhibit the growth of some cancer cells, such as breast,

prostate, colorectal, pancreatic cancer and bladder carcinoma cells [25–28]. These findings suggested that polyamine metabolism and ODC could be potential therapeutic targets in the treatment of some cancers.

To examine the mechanism of antisense ODC inhibiting the growth of lung cancer cells, we demonstrated by TUNEL assay that rAd-ODC/Ex3as infection could contribute significantly to cell apoptosis in comparison to rAd-GFP infected or no virus-treated cells. Some recent studies have demonstrated the inhibition of ODC could lead to induction of apoptosis of some cancer cells [29–31]. Our study indicated the induction of apoptosis was the mechanism of antisense ODC inhibiting the growth of lung cancer cells.

In general, our data suggested that adenoviral vector mediated antisense ODC can lead to induction of apoptosis and inhibition of growth of lung cancer cells *in vitro*. rAd-ODC/Ex3as could be a potential agent against lung cancer. However, further in-depth *in vivo* studies are required.

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