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Inhibitory effect of antisense RNA of ornithine decarboxylase gene on human esophageal squamous carcinoma cell line Eca109

Research Article

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Abbreviations: 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, (MTT); cytomegalovirus, (CMV); Difluoromethylornithine, (DFMO); fetal bovine serum, (FBS); Ornithine decarboxylase, (ODC); triphosphate-biotin nick end-labeling, (TUNEL)

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Summary

To investigate the *in vitro* inhibitory effect of rAd-ODC/Ex3as on human esophageal carcinoma cells. The infection rate of rAd-ODC/Ex3as was measured with the aid of GFP expression. Western Blot technique was used to observe the inhibition of ODC expression in infected tumor cells. The malignant phenotype of Eca109 cell line was assessed by growth curve. TUNEL was used to analyze cell apoptosis. Approximate 65% of Eca109 cell line were infected with rAd-ODC/Ex3as when MOI reached 50. The expression of ODC was inhibited in the infected tumor cells. rAd-ODC/Ex3as could inhibit Eca109 cell line growth and invasive ability at 20 of MOI. TUNEL proved that rAd-ODC/Ex3as can lead to cell apoptosis. rAd-ODC/Ex3as could inhibit effectively the expression of ODC gene and the growth of of esophageal squamous carcinoma cell line Eca109 *in vitro*, and induce apoptosis. It may be one of the promising medicines for antisense gene therapy in esophageal cancer.

I. Introduction

The polyamines, spermidine, spermine and the diamine precursor, putrescine, are positively charged aliphatic amines at physiological conditions, have a low-molecular weight and a simple chemical structure. They interact with various macromolecules, both electrostatically and covalently and, as a consequence, 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 (Auvinen et al, 1992; Moshier et al, 1993). On the other hand, the level of polyamine is high in cancer cell and tissues, and rapid tumor growth has been associated with remarkable elevation of polyamine biosynthesis and accumulation (Marton and Pegg, 1995; Pegg et al, 1998).

Ornithine decarboxylase (ODC) is the first and the rate-controlling enzyme in polyamine biosynthesis. It decarboxylates L-ornithine to form diamine putrescine. Complete structure and nucleotide sequence of ODC gene from mammalians is known for human (Moshier et al, 1990), which have 12 exons and 11 introns. Active mammalian ODC is homodimer with 2-fold symmetry. Subunits have molecular weight of about 51kDa and the polypeptide chain consists of 461 amino acids. ODC becomes activated after treatment with chemical carcinogens and tumor promoters, as well as in cells transformed by various oncogens, such as v-src, neu and ras (Pegg et al, 1988; Sistonen et al, 1989; Auvinen et al, 1992). The level of ODC was reportedly elevated in various cancers (Glikman et al, 1987; Upp JR, Jr. et al, 1988; Love et al, 2003) and related to recurrence (Love et al, 2003). Some chemotherapeutic agents, such as Difluoromethylornithine (DFMO), which aimed to inhibit the activity of ODC have appeared and taken on inhibitory effects on tumor growth *in vitro* and *in vivo* (Umemoto, 1989; Zagaja et al, 1998), though showing dose-limiting

toxicity. Stable transfection of human lung squamous carcinoma cell line LTEP-78 with antisense ODC-expressing plasmid DNA has been shown too related with the reversion of malignant phenotypes of human lung squamous carcinoma cells (Guan et al, 1996). Taken together, these findings suggest that ODC may provide an important target for the development agents that inhibit carcinogenesis and tumor growth.

Esophageal cancer is one of the most frequently diagnosed cancers in the world. Metastatic esophageal cancer is essentially resistant to systemic cytotoxic chemotherapy, while 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 had proved that lung cancer had greater elevated polyamine levels (Carlisle et al, 2002). Because Adenoviral vectors are among the most promising gene transfer vehicles for direct, in vivo gene therapy for the treatment of a diverse array of human disease (Meager, 1999). In this study, we used a replication-deficient recombinant adenovirus to efficiently deliver a 120bp antisense ODC which is complementary to initiation codon and tested the effect of antisense ODC on esophageal cancer. The data presented here show that adenovirus-mediated gene transfer of antisense ODC could significantly inhibit growth of esophageal cancer cells.

II. Materials and methods

A. Cell culture and reagents

Esophageal cancer Eca109 cell line was obtained from Chinese Academy of Science. Cells were cultured in DMEM or RPMI 1640 medium supplemented with 10% heat-inactived fetal bovine serum (FBS), 100U/ml penicillin, and 100μg/ml streptomycin. MTT was purchased from Sigma, MO. β-actin antibody and ECL Western blotting detection system were obtained from Santa Cruz, CA. Monoclonal antibody of ODC was made in our lab. Other reagents were all of reagent grade and obtained from Chinese companies.

B. Adenovirus and infection condition

The recombinant adenovirus rAd-ODC/Ex3as, containing the cytomegalovirus (CMV) promoter and GFP gene, was constructed by reversely inserting a 120bp cDNA fragment of ODC into the multiple clone sites (Zhang et al, 2003), rAd-ODC/Ex3as was purified by ultracentrifugation in cesium chloride step gradients (Prevec et al, 1991). The titer of the viral stock, measured in plaque-forming unit (pfu)/ml, was determined to be 8.5×109pfu/ml by a method published previously (Wei et al, 2000),and the frozen stock was confirmed to have retained their titer. The control virus rAd-GFP was same to rAd-ODC/Ex3as but no 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°C for 2 hours. The necessary amount of culture medium with 5% fetal bovine serum was then added and the cells were incubated for the desired times.

C. MTT assay

Firstly MTT assay was employed to assess transduction efficiency of rAd-ODC/Ex3as in Eca109 cell line. Briefly, cells were seeded at density of 5000 cells/well in 96-well plates and grown overnight. On the next day the cells were infected by a wide range of viral titres, from 1 to 100 pfu/cell (MOI, multiplicity of infection). After 48 hours of incubation, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added (50μg/well) for 4 hours. Formazan products were solubilized with DSMO, and the optical density was measured at 570nm. To observe the effect of 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 different infective efficiency, A-549 cells were infected by 50 and 25 MOI respectively. All experiments were performed in sextuple. After 24, 48, 72, 96 and 120 hours, cell viability was measured by absorbance at 570nm as described previously.

D. Western blotting analysis of ODC proteins

Eca109 cell line was infected with rAd-ODC/E3as by 50 MOI in 1640 medium containing 5%FCS for 48 hours. The cells were washed three times with ice-cold PBS and collected with a cell scraper. Total cell lysates were prepared in extraction buffer containing 0.05M Tris (pH8.0), 0.15M NaCl, 0.02% Sodium Azide, 0.1% SDS, 100μg/ml PMFS (phenylmethylsulfonyl fluoride), 1μg/ml aprotinin and 1%NP-40. The extracts were subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1h at room temperature in PBS containing 1% powdered milk. Mouse anti-ODC monoclonal antibody was added at a dilution of 1/500 and incubation was continued overnight at 4°C. The secondary antibody was horseradish peroxidase-conjugated antimouse IgG antibody (Zhongshan Beijing China). Antibody reactive bands were revealed using the ECL Western blotting detection system (Santa Cruz CA). The content of each protein sample was controlled by means of β-actin. For quantitation of bands, we used Nikon digital camera and SmartView analysis software.

E. HPLC analysis of polyamine pools

Eca109 cell line was infected with rAd-ODC/Ex3as at the MOI of 50. After 48 hours, cells were trypsined and washed with PBS twice. Intracellular polyamine were extracted from cell pellets with 10% trichloroacetic acid, dansylated, and measured by reverse phase HPLC as described previously (Fu et al, 1998).

F. TUNEL was used to analyze cell apoptosis

Terminal deoxynu-cleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) was used to detect apoptotic cells. TUNEL was performed with the kit according to the manufacture's instruction.

G. Statistical analysis

Statistical analysis was performed using Statview J 5.0 software (SAS Institute Inc., San Francisco, CA). A significant difference was defined as $p<0.05$.

III. Results

A. Inhibitory effects of rAd-ODC/Ex3as on Eca109 cell line

There was dose-dependent growth inhibition in Eca109 cell line, which reflected the transduction efficiency of the adenovirus to esophageal cancer cell line. We chose 50 MOI of adenovirus to infect Eca109 cell line. Under these conditions, rAd-ODC/Ex3as was more suppressive of growth than the control rAd-GFP virus, while rAd-GFP had no obviously toxic effect on cells. We

examined the *in vitro* growth inhibition of rAd-ODC/Ex3as in Eca109 cell line using cell growth curves as described in "Material and Methods". Antisense ODC had an impact on the growth of esophageal cancer cells. RAd-ODC/Ex3as in both of the cells inhibited their proliferation by ~50% when compared with the control virus and no virus-treated groups (**Figure 1**).

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

The ODC proteins produced from Eca109 cell line after infection with rAd-ODC/Ex3as were examined by Western immunoblot analysis. The ODC expression in the cells infected with rAd-ODC/E3as substantially more reduced than in the cells infected with rAd-GFP or no virus-treated cells. The results analyzed by SmartView software showed that ODC expression in Eca109 cell line infected with rAd-ODC/Ex3as accounted for 40% of that in cells treated with rAd-GFP (**Figure 2**). HPLC also exhibited a decrease of concentrations of the three polyamines: putrescine (put), spermidine (spd),spermine (spm), especially of putrescine (**Table 1**).

C. TUNEL assay for apoptosis

To examine the mechanism by which rAd-ODC/Ex3as may retard esophageal cancer cell growth *in vitro*, we used TUNEL to detect the effect of the rAd-ODC/Ex3as on apoptotic cells at 48 (**Figure 3**) and 72 hours after infection. As shown in **Table 2**, the rate of apoptosis in cells infected by rAd-ODC/Ex3as was significantly high in comparison to infected by rAd-GFP or no virus-treated cells ($p<0.05$).

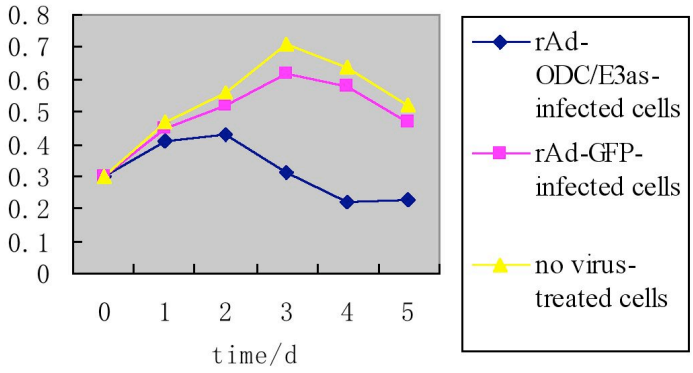

Figure 1. The effect of rAd-ODC/Ex3as on growth of Eca109 cell line. Growth curves of cells was drawn after Eca109 cell line were infected with either rAd-ODC/Ex3as or rAd-GFP at MOI of 50, and absorbance was measured everyday in a period of 5 days.RAd-ODC/Ex3as in both of the cells inhibited their proliferation by ~50% when compared with the control virus and no virus-treated groups.

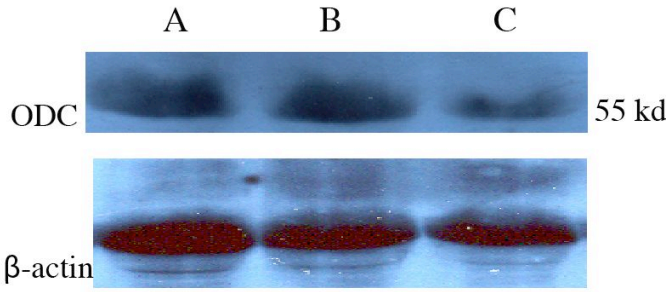

Figure 2. Western blotting analysis for ODC expression in Eca109 cell line after transduction of rAd-ODC/E3as or rAd-GFP. Eca109 cell lines were infected with adenoviruses at MOI of 50 . After 48 hours, 2×10^6 cell were collected in 300μl extraction buffer. 40μl protein extraction was added to SDS-PAGE. **A**. rAd-ODC/Ex3as-infected Eca109 cell lines. **B**. No virus-treated Eca109 cell lines. **C**. rAd-GFP-infected Eca109 cell lines

Table 1. Polyamine Pools of Eca109 cell lines

Cell line and Treatment	Polyamine polls (pmol/10^6cell)		
	Put	Spd	Spm
Eca109 cell line	590	1560	1489
+rAd-GFP	525	1463	1672
+rAd-ODC/E3as	254	1189	1321

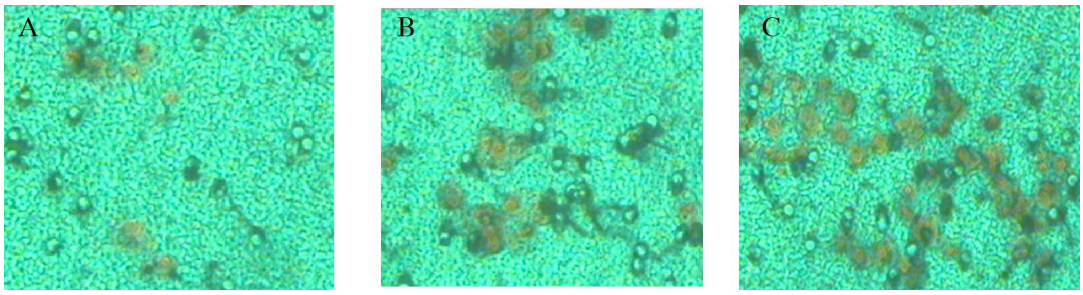

Figure 3. The effect of rAd-ODC/Ex3as on the apoptosis of Eca109 cell lines 48 hours after infection. Cells were observed under 100× microscope. Those brown cells were apoptotic cells. **A**: Eca109 cell lines infected with rAd-ODC/Ex3as, **B**: Eca109 cell lines infected with rAd-GFP, **C**: Eca109 cell lines

Table 2. The rate of apoptosis at 48 and 72 hours after infecting rAd-ODC/Ex3as or rAd-GFP (%) (x±s)

Cell line and Treatment	48h	72h	*p*
Eca109 cell line	5.8±0.63	8.2±0.43	-
+rAd-GFP	9.2±0.87	12.3±0.54	-
+rAd-ODC/E3as	25.6±1.82	64.2±2.42	<0.05

IV. Discussion

Polyamines are aliphatic cations with multiple functions and are essential for life. In normal cells, ploymine 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 as compared to that of normal cells, especially in lung cancer cells (Carlisle et al, 2002). At the same time, targeting specific molecules in cells by antisense inhibition was shown to have potential effectiveness in decreasing the protein expression. ODC is the most important enzyme in polyamine biosynthesis. More recently, the overexpression of ODC in NIH3T3 cells caused transformation of these cells to a malignant phenotype, in essence qualifying ODC as an oncogene (Auvinen et al, 1997). Inhibition of ODC by DFMO could compromise cell growth and transformation (Metcalf et al, 1978). Schipper's recent *in vitro* studies using conformationally restricted polyamine analogues showed that these compounds inhibited cell growth, probably by inducing antizyme-mediated degradation of ODC (Schipper et al, 2000). In addition, Alm and colleageus showed in 2000 that ODC was a well-defined target gene for c-myc and other oncogenes. Therefore, we targeted the ODC by using an antisense gene delivery strategy with a replication-deficient recombinant Ad vector. In the present study, we demonstrated that rAd-ODC/Ex3as could inhibit esophageal cancer growth and lead to the apoptosis of Eca109 cell lines.

MTT assay showed antisense ODC had an impact on the growth of esophageal cancer cells. RAd-ODC/Ex3as in both of the cells inhibited their proliferation by ~60% when compared with the control virus and no virus-treated groups. At the same time, Western blotting showed the ODC expression in the cells infected with rAd-ODC/E3as substantially more reduced than in the cells infected with rAd-GFP or no virus-treated cells. On the other hand a substantial decrease in ODC expression resulted in the reduction of polyamine biosynthesis. In addition, the reduction of polyamines may contribute to the marked suppression of cancer cell growth and tumor formation. Resent studies also showed inhibiting mRNA expression of ODC can effectively inhibits the growth of some cancer cells, such as breast, prostate, colorectal, pancreatic cancer and bladder carcinoma cell (Weeks et al, 2000; Love et al, 2003; Subhi et al, 2004; Wolter et al, 2004). These findings suggest that polyamine metabolism and ODC could be potential therapeutic targets in the treatment of some cancer.

To examine the mechanism of antisense ODC inhibiting the growth of esophageal cancer cells, we demonstrated rAd-ODC/Ex3as infection can contribute significantly to cell apoptosis in comparison to rAd-GFP infected or no virus-treated cells by TUNEL. In the last years, some studies had demonstrated the inhibition of ODC could lead to induction of apoptosis of some cancer cells (Feith et al, 2005; Seiler and Raul, 2005; Stanic et al, 2006). So, our previous study indicated the induction of apoptosis was the mechanism of antisense ODC inhibiting the growth of esophageal cancer cells.

In general, Our data suggest that adenoviral vector mediated antisense ODC can lead to induction of apoptosis and inhibition of growth of esophageal cancer cells *in vitro*. The rAd-ODC/Ex3as could be a potential agent against esophageal cancer, however, further in-depth *in vivo* studies must be warranted.

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