Rejection of adenovirus infection is independent of coxsackie and adenovirus receptor expression in cisplatin-resistant human lung cancer cells

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Abstract. The adenovirus vector-based cancer gene therapy is controversial. Low transduction efficacy is believed to be one of the main barriers for the decreased expression of coxsackie and adenovirus receptor (CAR) on tumor cells. However, the expression of CAR on primary tumor tissue and tumor tissue survived from treatment has still been not extensively studied. The present study analyzed the adenovirus infection rates and CAR expression in human lung adenocarcinoma cell line A549 and its cisplatin-resistant subline A549/DDP. The results showed that although the CAR expression in A549 and A549/DDP was not different, compared with the A549, A549/DDP appeared obviously to reject adenovirus infection. Moreover, we modified CAR expression in the two cell lines with proteasome inhibitor MG-132 and histone deacetylase inhibitor trichostatin A (TSA), and analyzed the adenovirus infection rates after modifying agent treatments. Both TSA and MG-132 pretreatments could increase the CAR expression in the two cell lines, but the drug pretreatments could only make A549 cells more susceptible to adenovirus infectivity.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers in many countries, and treatment outcomes for NSCLC patients are still disappointing. Most patients receiving chemotherapy do not respond, resulting in disease progression. Thus, inherent or acquired drug-resistance leads to treatment failure. In recent years, progress in chemotherapy and molecular target-based therapy have altered the standard therapy for NSCLC (1-3). Gene therapy has become an attractive regimen, in addition to conventional therapy (4). Adenovirus is a useful agent for cancer gene therapy due to its high potential for gene transfer, ease of high titer production and demonstrated safety in clinical trials (5). Thus, recombinant adenovirus vectors are widely used in preclinical and clinical gene therapy, particularly in NSCLC gene therapy (6). Moreover, results have suggested synergism between chemotherapy and adenovirus p53 gene therapy with no increased side effects (7). However, in a multicenter phase II study, intratumoral adenoviral p53 gene therapy appears to provide no additional benefit in patients receiving an effective first line chemotherapy for advanced NSCLC (8).

The combination of adenovirus with its receptor is a key step for virus infection and sequent biological effect. Adenovirus can infect cells since it uses the knob domain of the fiber binding to its cellular receptor, the coxsackie and adenovirus receptor (9). Several lines of evidence showed the relationships between CAR expression and adenovirus infection (10,11), and low levels of CAR in tumors are thought to be one of the reasons for poor adenovirus infection (12-14). It has become evident that CAR expression is often low in various types of tumors, and some biological or chemical agents could result in an increase in CAR expression in several tumor cell lines, making them more susceptible to adenovirus infectivity (15-17).

Adenoviral vector-mediated gene transfer is highly effective, but large differences regarding transduction efficiencies among different cell lines and between *in vitro* and *in vivo* gene transfer have been reported. To evaluate the potential of adenovirus vector-based gene therapy on cisplatin-resistant lung cancer, the present study examined the adenovirus infection rates and CAR expressions in A549 and its cisplatin-resistant subline A549/DDP.

Materials and methods

Cells culture, antibodies and chemicals. The human lung adenocarcinoma cell lines A549 and its cisplatin-resistant

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Abbreviations: CAR, coxsackie and adenovirus receptor; rAd.EGFP, recombinant adenovirus encoding enhanced green fluorescent protein; MOI, multiplicities of infection

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subline A549/DDP, were propagated in monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. The transformed embryonic kidney cell line 293 was grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/l), 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were grown at 37˚C in a 5% CO_2 humidified incubator. Antibodies were commercially available, including mouse monoclonal antibody against CAR (Upstate Biotechnology, Charlottesville, VA, USA), rabbit polyclonal antibody against CAR, mouse monoclonal antibody against p53, mouse monoclonal antibody against p21, mouse monoclonal antibody against α-tubulin and mouse monoclonal antibody conjugated with HRP against actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Histone deacetylase inhibitor trichostatin A (TSA) and proteasome inhibitor MG-132 was purchased from Sigma Chemicals (St. Louis, MO, USA). Each agent was prepared as a stock solution in dimethyl sulphoxide (DMSO) medium such that the final concentration of DMSO exposed to cells was <0.1%. In all experiments, control cells were treated with 0.1% DMSO. Each experiment was repeated at least three times.

MTT assay. Cells were plated into 96-well tissue culture plates overnight. Then, cells were treated with cisplatin, MG-132 or TSA at the indicated concentrations, respectively, for 72 h. Four hours before the end point, a medium containing 0.5 mg/ml MTT was added. Finally DMSO was added to each well and mixed thoroughly to dissolve the crystals of MTT formazan. Results were quantified using a LabSystems Multiskan MS at 540 nm wavelength. Control absorbance was designated as 100%, and cell survival was expressed as a percentage of control absorbance.

FACS assay. The effect of MG-132 and TSA on CAR or GFP expression in cell lines was examined with FACS (Becton-Dickinson, Franklin Lakes, NJ, USA). Cells were incubated with MG-132 or TSA for the indicated hours. Before analysis, cells were washed, trypsinized and resuspended in phosphate-buffered saline (PBS). Cells ($2x10^5$) were incubated with antibody against CAR at a 1:200 dilution for 1 h at 4˚C, followed by FITC-labeled goat anti-mouse IgG for 30 min at 4˚C. After fixation, $3x10^4$ cells were analyzed by FACS. Analysis was performed using LYSYS II software (Becton-Dickinson). To analyze the GFP expression, cells were trypsinized, washed and fixed for analysis.

Transfer assay. Recombinant adenovirus encoding enhanced green fluorescent protein (rAd.EGFP) was purchased from Sino-Gene (Shanghai, China). The cytomegalovirus promoter was used to drive the transcription of EGFP. Titers of recombinant viruses were determined by plaque forming assay in the 293 cells. All vectors were prepared, purified and stored at -80˚C.

The rAd.EGFP transfer assay for the potential of adenovirus transfer detected with a fluorescence microscope and FACS. Cells ($5x10^4$) were plated into 24-well plates overnight at 37˚C. Next day, fresh medium or fresh medium containing MG-132 or TSA cells were replaced. Twenty-four hours later, medium was removed and cells were washed once with PBS. Then, cells were infected with rAd.EGFP at indicated MOI for 2 h. Subsequently, cells were washed and overlaid with growth medium for the indicated time periods.

For fluorescence microscope analysis, a Leica fluorescence stereo microscope equipped with a 50 W mercury lamp was used. Selective excitation of GFP was produced through a D425/60 bandpass filter. Emitted fluorescence was collected through a long pass filter on a Hamamatsu cooled charged coupled device camera. FACS analysis and MTT assay were as mentioned above.

Western blot analysis. Cell lysates were prepared by SDS lysis buffer. Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of protein was separated by electrophoresis on a 10.5% SDS polyacrylamide gel. The proteins were electrotransferred from gel to nitrocellulose membrane. The membrane was blocked with 5% dry milk solution for 30 min, and then incubated with primary antibody for 2 h at room temperature. After washed, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, membrane was detected with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences Europe, Freiburg, Germany) according to the manufacturer's instructions.

RT-PCR analysis. Total RNAs were isolated from the cells using TRIzol procedure (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 4 μg of total RNA with SuperScript RT kit (Invitrogen Life Technologies). The reverse transcription PCR exponential phase was determined from 18 to 36 cycles to allow semi-quantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved an initial denaturation at 94˚C for 5 min followed by cycles predetermined for each type of cDNA: 28 cycles for GAPDH and 30 cycles for CAR. Primer sequences were designed as follows: sense primer, 5'-ATA AAG CCA CCA CCG CCA CC-3' and antisense primer, 5'-TGG TCA CAG CTT TCG CAG CC-3' for human GAPDH (internal control); sense primer, 5'-AGC CTT CAG GTG CGA GAT GTT ACG-3' and antisense primer, 5'-TAC GAC AGC AAA AGA TGA TAA GAC-3' for human CAR. The thermal cycle was defined by denaturation at 94˚C for 5 min, followed by indicated cycles with denaturation at 94˚C for 30 sec, annealing at 60˚C for 60 sec and extension at 72˚C for 45 sec, and then a final elongation at 72˚C for 5 min. The PCR products were analyzed on 1% agarose gels and visualized by ethidium bromide staining.

Results

Cytotoxicity studies. Cell lines were plated at 7,000 cells/well into 96-well plates. Cisplatin was added the next day, and the plates were assayed after 72 h. The analysis was performed by the MTT assay for the cisplatin-resistant subline cells and its parental cells. The results of MTT assays showed that the IC_{50} (inhibitory concentration 50%) of cisplatin to A549 is 1.47 μg/ml, and the IC_{50} of cisplatin to A549/DDP is 15.11 μg/ml. Based on these results, we recorded that the resistant factor of A549/DDP was 10.28, and the model of cisplatin-resistant subline was established.

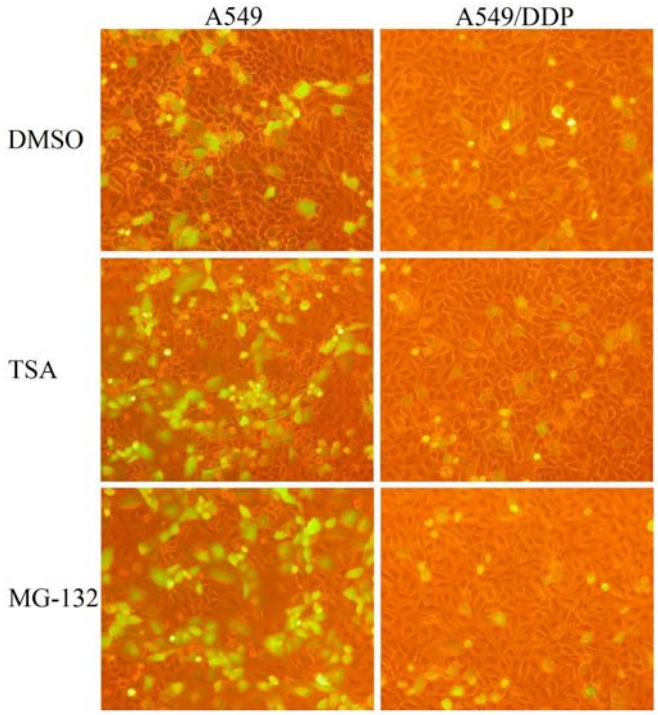

Figure 1. Fluorescence microscope analysis of expression of GFP in adenovirus infected control and TSA or MG-132-treated cells. A549 or A549/DDP cell lines were treated with DMSO, TSA or MG-132 before infection with 2 MOI of rAd.EGFP. The infected cells were grown for 48 h and virus-mediated gene delivery was determined with fluorescence microscope. Original magnification, x200.

CAR expressions and rAd.EGFP infection rates of A549 and A549/DDP. A549 and A549/DDP cells were infected with rAd.EGFP at different MOIs, and GFP expression was analyzed in the two cell lines. Compared with A549, A549/DDP showed resistance to adenovirus infection. For example, with 2 MOI of rAd.EGFP, transfer rate of adenovirus was (29.5±2.3%) in A549 and (6.6±0.9%) in A549/DDP cells (Figs. 1 and 2). The difference was significant in statistical analysis (p=0.004). Nevertheless, FACS analysis of the two cancer cell lines indicated that CAR expression levels of A549 and A549/DDP cells were not different (Fig. 3).

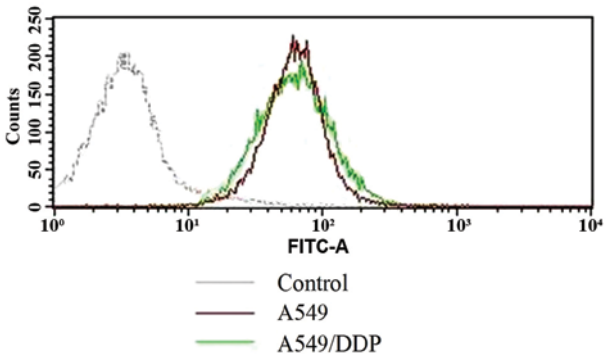

Figure 3. FACS analysis of the CAR expression in A549 and A549/DDP cells.

Induction of CAR expression with MG-132 and TSA. FACS analysis showed that both A549 and A549/DDP cells were CAR-positive cell lines. Thus, we further studied whether the CAR expression could be affected by drugs, such as MG-132 or TSA. Cytotoxicity studies were performed to determine the minimally cytotoxic concentration of MG-132 or TSA for the two cell lines. For A549 cells, the MG-132 concentration, showing no or minimal cytotoxicity that was selected for these studies were 0.7 μmol/l, and the TSA concentration was 30 ng/ml. Whereas, the concentrations of MG-132 for A549/DDP cells was 0.5 μmol/l, and the TSA concentration was also

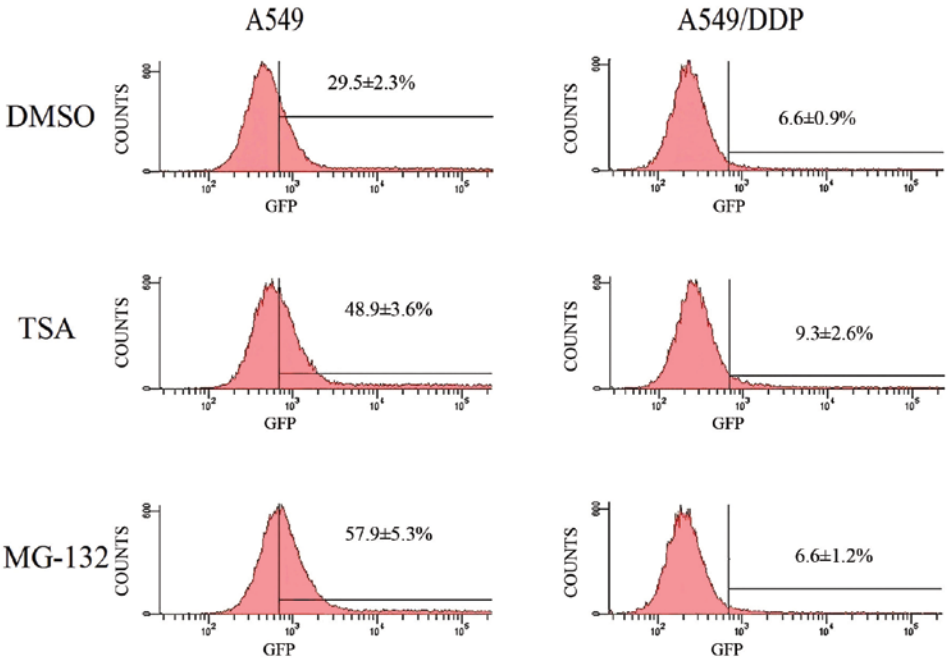

Figure 2. FACS analysis of expression of GFP in adenovirus infected control and TSA or MG-132-treated cells. A549 or A549/DDP cell lines were treated with DMSO, TSA or MG-132 before infection with 2 MOI of rAd.EGFP. The infected cells were grown for 48 h and virus-mediated gene delivery was determined with FACS. Error bars indicate 1 SD.

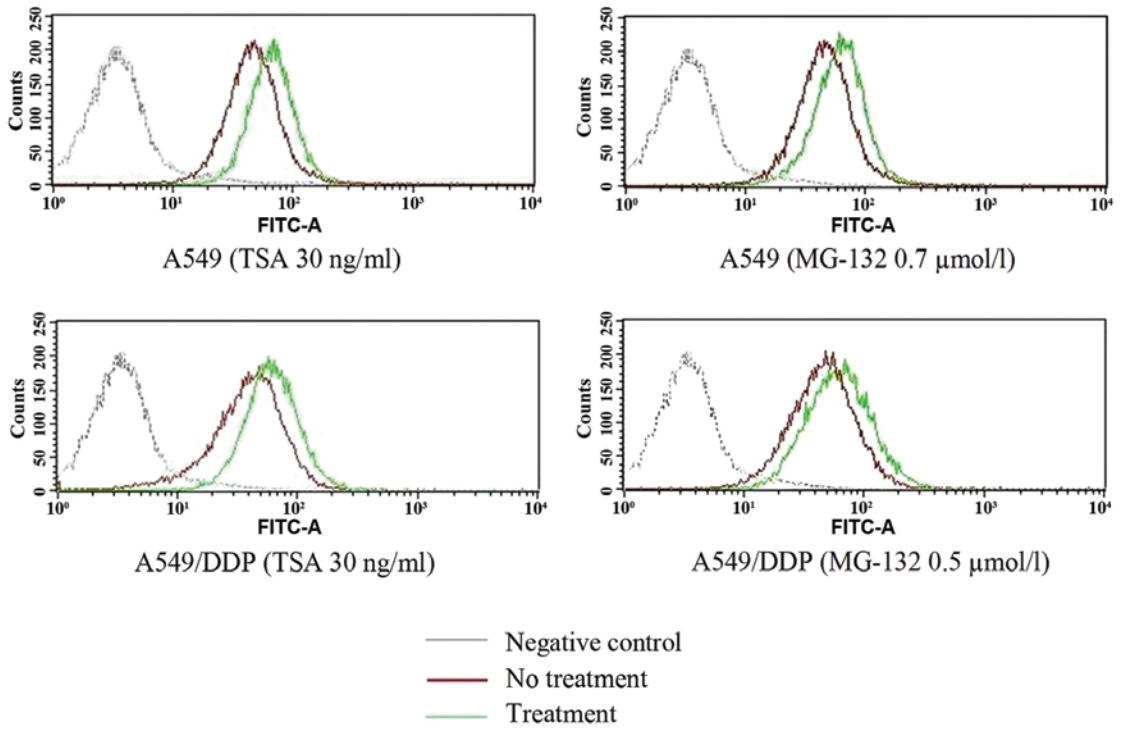

Figure 4. FACS analysis of the CAR expression induced by MG-132 or TSA in A549 and A549/DDP cells.

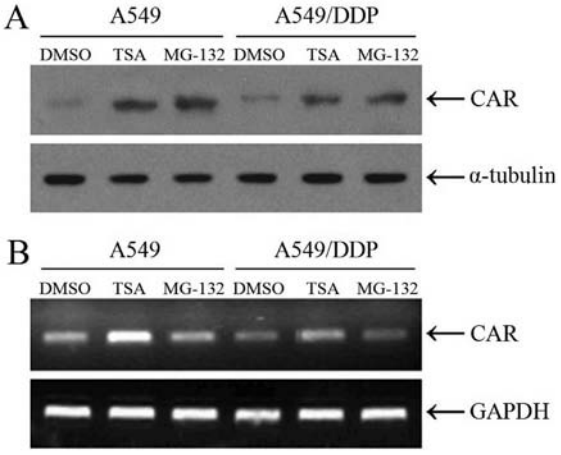

Figure 5. MG-132 and TSA modify CAR expression with different mechanism. (A) Western blotting analysis of CAR protein expression in A549 and A549/DDP cells. (B) RT-PCR of CAR mRNA expression in A549 and A549/DDP cells.

30 ng/ml. When A549 cells were incubated with 0.7 μmol/l MG-132 or 30 ng/ml TSA for 48 h, average CAR density was apparently increased, and the same as A549/DDP cells incubated with MG-132 or TSA (Fig. 4).

We further confirmed the CAR expression levels in A549 and A549/DDP cells with western blotting and RT-PCR analysis. The results showed that TSA rather than MG-132 enhanced the CAR mRNA transcription, which indicated that MG-132 modified the CAR expression with post-transcriptional mechanism and TSA with transcriptional mechanism, consist with the fact that MG-132 is a proteasome inhibitor and TSA a histone deacetylase inhibitor (Fig. 5).

Adenovirus infection affected by MG-132 and TSA. To evaluate the effect of MG-132 or TSA on the adenovirus infectious efficiency, GFP density was compared. Treated with MG-132 or TSA at the indicated concentration for 48 h, A549 or A549/DDP cells were infected with rAd.EGFP at multiplicities of infection (MOI) of 0, 0.5, 1, 2, 4 or 8, and incubated in normal medium for another 24 h when analyses was performed. At the indicated time, adenovirus infected cells were photographed under a fluorescent microscope. Then, the infected cells were trypsinized, and further analyzed for GFP expression by flow cytometry.

After MG-132 or TSA treatment, a marked increase in GFP expression of A549 cells occurred with every multiplicity of rAd.EGFP, but the GFP expression was not different in A549/DDP cells after drugs treatments (Fig. 1). For example, with 2 MOI of rAd.EGFP, transfer rate of adenovirus in A549 cells was (29.5±2.3%) in control group (57.9±5.3%) in MG-132 treated group and (48.9±3.6%) in TSA-treated group. In A549/DDP cells, transfer rate of adenovirus was (6.6±0.9%) in control group, (6.6±1.2%) in MG-132-treated group and (9.3±2.6%) in TSA-treated group (Fig. 2).

Discussion

Previously, various studies have suggested that adenoviral vector-mediated gene therapy is more effective in the drug resistant breast or bladder cancer cell lines compared to the parental cell line, which may partially be explained by the efficiency of adenoviral gene transfer (18,19). In the present study, we employed a human lung cancer cell line and its drug resistant sublines to study adenovirus infection sensibility of the two cell lines. The results shown that rAd.EGFP induced higher GFP gene expression in the parental cell line compared

to the drug-resistant cell line, indicating greater efficiency of adenovirus-mediated gene transfer in the parental cell lines compared to the drug-resistant line.

Although patients with advanced non-small cell lung cancer (NSCLC) can be effectively treated initially with combination chemotherapy, many patients receiving chemotherapy do not respond, resulting in disease progression. Furthermore, second line chemotherapy provides little benefit to patients who have relapsed after an initial response. Thus, inherent or acquired drug-resistance leads to treatment failure. Much is known concerning the molecular mechanisms by which tumor cells acquire drug-resistance, but the treatment of drug-resistant tumors remains a significant problem. Gene therapy has become an attractive regimen, in addition to conventional therapy, and recombinant adenovirus vectors are widely used in preclinical and clinical gene therapy (4). The p53 gene is abnormal in 40-74% of NSCLC samples tested (20). Thus, therapeutic approaches involving gene therapy targeting the p53 gene have been explored in preclinical models and clinical trials.

The recombinant adenovirus encoding human p53 tumor suppressor gene (rAd.p53) phase I single agent trials in NSCLC demonstrated effective p53 gene transfer, minimal toxicity and transient injected lesion tumor regression (21). Preclinical studies had previously demonstrated synergistic antitumor effect between adenovirus gene therapy and radiotherapy or chemotherapy, which may lead to enhanced antitumoral activity without increased toxicity (22,23). Combination of p53 gene therapy with radiotherapy has been evaluated in NSCLC patients who were not eligible for chemoradiotherapy or surgery (24). Radiation toxicity was not enhanced by adenoviral vector. Intratumoral injection of rAd.p53 in combination with radiation therapy is well tolerated and demonstrates evidence of tumor regression at the primary injected tumor. Since chemotherapy could not be administered to these high risk patients, systemic control of disease was poor, with over 50% of patients experiencing metastatic progression within 1 year. The activity and toxicity of rAd.p53 combined with platinum-based chemotherapy were also evaluated in advanced NSCLC. There was no evidence of increased chemotherapy-related toxicity by adenoviral vector, and some evidence of clinical activity could be observed (7). However, in a multicenter phase II study, intratumoral adenoviral p53 gene therapy appears to provide no additional benefit in patients receiving an effective first line chemotherapy for advanced NSCLC (8). One possible explanation for this observation may be the relatively high efficacy of the first line chemotherapy, and gene therapy should be used in a coordinated fashion in the proper clinical context.

Adenovirus can infect cells since it uses the knob domain of the fiber to bind to its cellular receptor, the coxsackie and adenovirus receptor (CAR), and the efficiency of adenoviral gene transfer is critical for adenoviral vector-based gene therapy (10). The efficiency of adenoviral gene transfer to several tumors, including lung cancer, correlates with the expression of CAR, and low levels of CAR in tumors are thought to be one of the reasons for poor adenovirus infection (12-14). We analyzed CAR expressions in the two cell lines, but no significant differences of CAR expression were observed between the A549/DDP and its parental cell line, suggesting that the differences in gene transfer efficiency between the drug resistant cell lines and the parental cell line may be independent of CAR expression. Despite the known importance of CAR for successful transduction of cells, other unknown mechanism of cell virus interaction may also be important. Variable CAR expressions in tumors may interfere with the interpretation of results of clinical trials, but it is too early to determine whether expressions of CAR in tumor cells are appropriate additional criteria for the enrollment of patients in adenovirus gene therapy trials (25).

It is demonstrated that CAR expression could be induced with biological or chemical agents, which would lead to increased adenovirus-mediated transgene expression (15). Histone acetylation inhibitors could restore the gene expression of the tumor-associated genes that have been transcriptionally silenced by promoter associated histone deacetylation, and use of certain histone deacetylase inhibitors, such as FR901228 (26-28), trichostatin A (29,30), CHAP31, FK228 (31,32), SAHA, MS275 and LBH589 (33-35), resulted in an increase in CAR expression in several tumor cell lines, making them more susceptible to adenovirus infectivity. Other agents, such as chemotherapeutics (15), cytokines (36) and inhibitors of the Raf/MEK/ERK pathway (37) were also reported to have the ability to induce CAR expression in some tumor cell lines. Based on the above experiments, we further modified the CAR expression in the two cell lines with proteasome inhibitors MG-132 or histone deacetylase inhibitors TSA, and the results indicated that the CAR expression in both A549 and A549/DDP could be upregulated. In the parental cell lines, upregulated CAR expression with MG-132 or TSA brought about higher GFP gene expression after rAd.EGFP infection, but the upregulated CAR expression in the drug-resistant cell lines had no help in GFP gene expression, which also certifies that other unknown mechanism of cell virus interaction beside CAR expression may also be important for the gene transfer efficiency.

Though the CAR expression in A549/DDP cells is no less than its parental cells, the cisplatin-resistant subline shows obvious rejection of the adenovirus infection, which implies that the rejection of adenovirus infection is independent of CAR expression in A549/DDP cells. The mechanism of high efficiency of adenoviral gene transfer in the parental cells is unclear, thus needing further investigation. Inherent or acquired drug-resistance is one of the reasons for antitumor chemotherapy treatment failure. Considering that patients enrolled in adenoviral cancer gene therapy trials usually have received chemotherapy or radiotherapy, the tumor cells in those people usually have multiple drug-resistance (MDR) to a certain degree. After MDR has taken place, the impact of MDR on gene therapy is unknown, and requires investigation.

Acknowledgements

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