Coxsackievirus and adenovirus receptor promotes antitumor activity of oncolytic adenovirus H101 in esophageal cancer

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Abstract. Esophageal cancer is an intractable disease due to late diagnosis, high incidence of post-surgical locoregional recurrence and frequent distant metastasis. Oncolytic adenovirus (Ad) vectors are a promising method for cancer treatment. The H101 virus is a recombinant Ad which has replication-selective properties and replicates only in tumor cells. The coxsackievirus and adenovirus receptor (CAR) is considered a surrogate marker that monitors the outcome of Ad-mediated gene therapy. Accumulating evidence indicates that CAR expression levels are lower in various types of tumors such as ovarian, lung, breast and bladder when compared to their normal counterparts. In this study, we reported that trichostatin A (TSA) induced the expression of CAR in esophageal squamous cell carcinoma (ESCC) cell lines through the MAPK/ERK1/2 signaling pathway. The expression levels of CAR were positively related with the antitumor activity of H101. Our results suggest that TSA increases the antitumor activity of the oncolytic adenovirus H101 through the MAPK/ERK pathway.

Introduction

Esophageal cancer patients have a survival rate lower than 40% in the first year due to late diagnosis, high incidence of post-surgical locoregional recurrence and frequent distant metastasis. For patients undergoing potentially curative surgery, 5-year survival rates are lower than 25% (1-3). Therefore, new strategies with better antitumor activity, such as gene therapy are required (4-6).

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Oncolytic viruses (OVs) are members of a class of adenoviruses (Ads) that selectively replicate in tumor cells and lyse infected cells. OVs have been extensively investigated as novel anticancer agents. A variety of strategies have been developed to establish selectivity to transformed cells, including deleting the viral genome or replacing the viral endogenous promoters through cancer-selective promoters (7,8). Collectively, these approaches produce viral agents which have the ability to replicate in target tumor cells and kill these cells by virtue of replicating in them. Viral oncolysis may also be achieved through infecting adjacent tumor cells through the release of progeny virions and the activation of antitumoral immune responses. Theoretically, the infecting OVs may spread through a solid tumor and eliminate the tumor (9). The H101 virus produced by Shanghai Sunway Biotech also contains a deletion in the *E1B 55* gene and E3 region. This recombinant Ad has a replication-selective property and replicates only in tumor cells. Although there are several promising advances involving OVs, their clinical efficacy in human trials has failed to fulfill high expectations, and single-agent efficacy of OVs remains to be improved. The coxsackievirus and adenovirus receptor (CAR) is considered a surrogate marker to monitor the outcome of OVs (10).

CAR, which functions as a primary receptor for both the coxsackie B virus (CVB) and Ads, plays a crucial role in gene transfer efficacy (11). Recent studies have indicated that CAR levels are closely related with Ad attachment, infection or transgene expression (12). Furthermore, CAR is also considered a surrogate marker for monitoring and/or predicting the outcome of Ad-mediated gene therapy. Accumulating evidence indicates that CAR expression levels are lower in several types of tumors, such as ovarian, lung, breast and bladder when compared to their normal counterpart (13-16).

Histone deacetylation is an important component of the epigenetic regulation of gene expression (17). Histone deacetylation also regulates CAR expression. Kitazono *et al* (18) demonstrated that the histone deacetylase inhibitors (HDACi) FR901228 (depsipeptide), trichostatin A (TSA) and valproic acid (VPA) increased the protein levels of CAR in 6 cancer cell lines, they found that the small molecular inhibitors regulate CAR expression through histone deacetylation modification.

In the present study, the protein level of CAR in esophageal squamous cell carcinoma (ESCC) cell lines and its

correlation to the antitumor efficiency of oncolytic Ad H101 were investigated. The effect of TSA on the protein level of CAR and the related signaling pathway were also evaluated.

Materials and methods

Cell culture. Positive control, HeLa cells and ESCC cell lines, EC9706 and EC1, were propagated in a monolayer culture in RPMI-1640 medium supplemented with 10% fetal inactived bovine serum (56˚C, 30 min), $1x10^5$ U/l penicillin and 100 mg/l streptomycin in an incubator with a humidified atmosphere of 5% CO_2 at 37˚C.

Compounds. TSA was purchased from Sigma Corporation. Cell lines were treated with 0.1, 0.3, 0.5 and 1.0 μmol/l TSA for 24 and 48 h. The protein levels of CAR in EC1 cells were detected with immunocytochemical analysis, flow cytometry and western blot analysis. The positive rate of CAR protein expression evoked by different concentrations of TSA in EC9706 and EC1 cells was analyzed using flow cytometry (FCM).

Immunocytochemical analysis. Cells ($5x10^4$) were seeded on glass coverslips in 24-well plates. After a 24-h-incubation, the cells were treated with different concentrations of drugs for 48 h, then fixed with 4% paraformaldehyde for 30 min. Cells were then treated with 3% H_2O_2 for 30 min to remove the endogenous peroxidase activity. Cells were incubated with rabbit anti-CAR monoclonal antibody (1:200; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. After washing 3 times with PBS, the cells were incubated at room temperature with biotin-labeled secondary antibody for 1 h. The resultant immune activity was developed in 0.5% 3,3'-diaminobenzidine hydrochloride (DAB) for 3 min. Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant antiserum. HeLa cells were used as the positive control (19).

Flow cytometric analysis. Cells were incubated with different concentrations of TSA for 48 h, and were then washed with PBS and harvested with 2.5 g/l trypsin and resuspended in PBS containing 1% BSA. The cells were resuspended in PBS with a concentration of $1x10^6$/ml. The rabbit against human anti-CAR monoclonal antibody was incubated with the cells for 30 min in the dark at 4˚C. After being washed with PBS-BSA, cells were incubated in a 1:100 dilution of FITC-labeled goat anti-rabbit IgG (Sigma) for 1 h at 4˚C. Cells ($1x10^5$/ml) were analyzed immediately using FACS.

Western blotting. Cells were washed twice in cold PBS, collected and then lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and protease inhibitor mixture). After 20 min on ice, the lysates were spun at 14,000 rpm in a microcentrifuge at 4˚C for 10 min. The supernatants were used as whole cell extracts. Cell lysates (50 μg) were separated on a 10% SDS-polyacrylamide gel and transferred on polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with 5% non-fat dried milk and were incubated with the primary antibodies against CAR (1:200), or anti-p44/42 ERK1/2 Ab (1:1,000, Thr202/Tyr204; Cell Signaling Technology, Inc.) at 4˚C overnight. HRP-IgG secondary antibody was added and incubation was carried out for 2 h at room temperature. An ECL chemiluminescent detection kit (ECL; Pierce Biotechnology, Inc.) was used to detect target proteins. The bands were subjected to densitometry for quantitation using Bio-Rad Quantity One software.

Viral replication. Cells were seeded in 6-well plates ($5x10^5$/well) for 24 h before the infection and were then infected with H101 Ads at a multiplicity of infection (MOI) of 1. The cells and the supernatants were collected 48 h after the infection and freeze-thawed thrice, serially diluted and titered by the limiting dilution method [determination of the 50% tissue culture infective dose ($TC\ ID_{50}$) using HEK293 cells].

Cell viability assay. Cells were seeded in 96-well plates ($5x10^3$/well). After a 24-h incubation, cells were infected with H101 at a MOI of 0.01, 0.1, 1, 10 and 100. Cells treated with PBS were used as the negative control. Each data point was obtained in quintuple. After a 72-h infection, the cells were washed with PBS to remove the free virus in the medium. Cell viability was measured using the Cell Counting Kit-8 (CCK-8). Control absorbance was designated as 100%, and cell viability was expressed as a percentage of the control absorbance.

MTT assay. Cells were seeded in 96-well plates at a density of $5x10^3$/well. After incubation for 24 h, the medium was removed and replaced with medium containing various concentrations of TSA. Cells treated with identical concentrations of DMSO (diluent for depsipeptide) were used as the control. After 24 or 48 h of incubation with TSA, cell viability was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The number of cells was determined using an enzyme-linked immunosorbent assay at 490 nm wavelength using an enzyme-labeling instrument. Data was obtained in triplicate.

Statistical analysis. All results are presented as the means ± standard deviation (SD). Statistical analysis was performed by ANOVA using SPSS 11.0 software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CAR expression in ESCC cell lines. CAR expression levels have been found to be lower in several types of tumors. To investigate the expression levels of CAR in ESCC cell lines, we performed immunocytochemistry assay, flow cytometry and western blot analysis. EC9706 and EC1 cell lines were evaluated with HeLa cells used as the positive control. CAR was expressed in the 3 cell lines examined. In the immunocytochemistry assay, brown coloration was not noted in the EC9706 and EC1 cells compared with the positive control cells (Fig. 1A). The positive rate of CAR expression in the 2 ESCC cell lines was lower compared to the HeLa cells as detected with FCM (Fig. 1B). The percentages of CAR expression were 21.00±2.00, 9.67±3.05 and 74.67±9.45% in the EC9706, EC1 and HeLa cells, respectively. Western

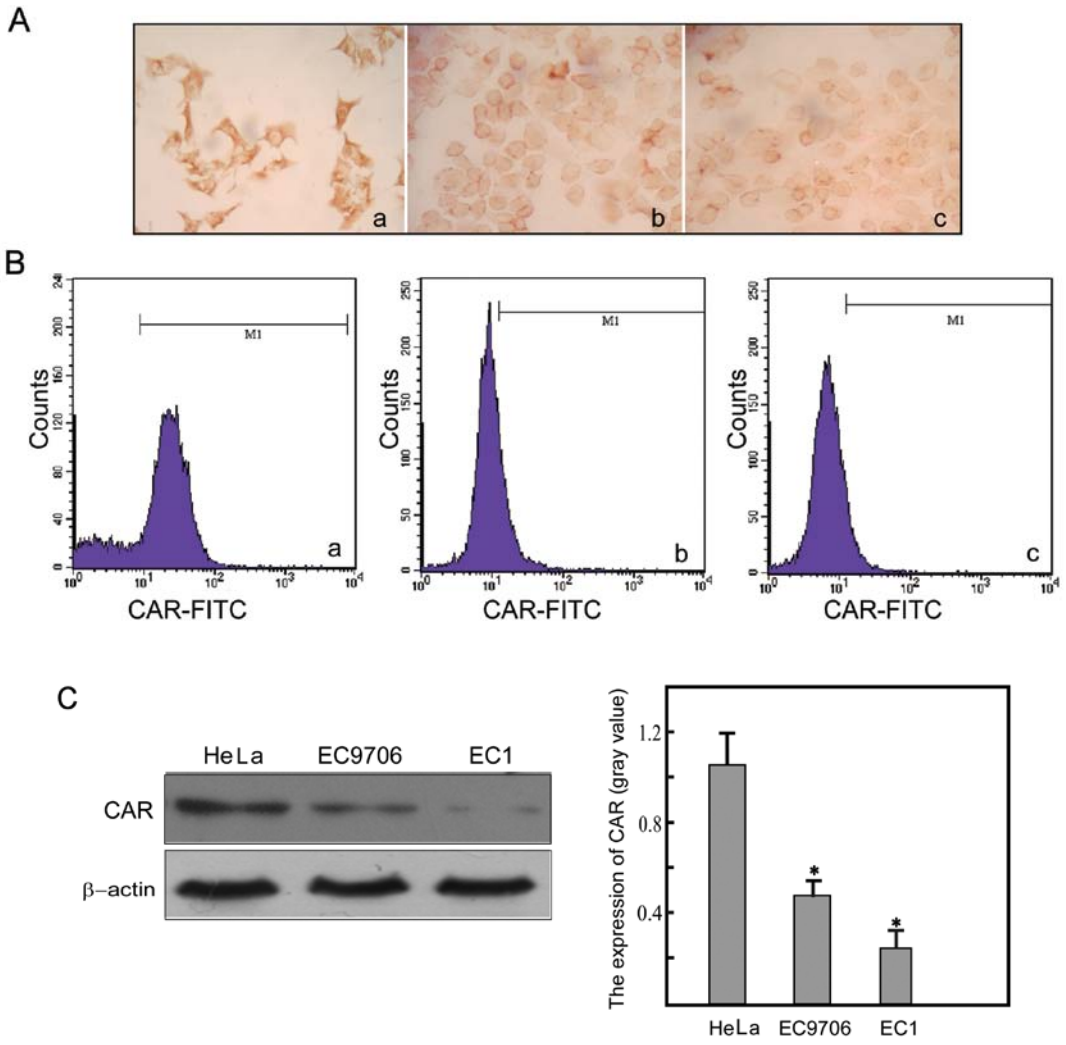

Figure 1. Protein level of CAR in esophageal carcinoma cell lines. Levels of CAR expression in the 3 types of cells were measured by (A) immunocytochemical analysis (magnification, x400), (B) flow cytometric analysis and (C) western blot analysis. a, HeLa; b, EC9706 and c, EC1 cells. This assay was performed in triplicate. Values represent the means ± SD (n=3). *P<0.05 vs. HeLa cells as the positive control.

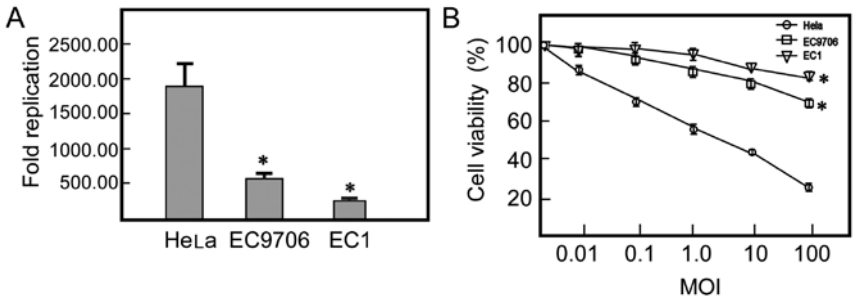

Figure 2. Comparison of the oncolytic and cytolytic effects of H101 in the 3 types of cells. (A) Replication of H101 in the 3 cell lines. Cells were infected with H101 Ads at a MOI of 1. The cells and the supernatants were collected 48 h after infection and titered by the limiting dilution method. Data indicate the reproductive multiple of H101 in the 3 cell lines. (B) Cell viability assay. Cells were treated with H101 at a MOI of 0.1, 1, 10 and 100 pfu/cell for 72 h. Cell viability was analyzed using the CCK-8 assay kit. This assay was performed in triplicate. Values represent the means ± SD (n=3). *P<0.05 vs. HeLa cells as the positive control.

blot analysis also demonstrated that the CAR protein level decreased in the ESCC cell lines, compared to the positive control (Fig. 1C).

Replication of H101 and cytotoxicity of H101 in ESCC cell lines. Decreased expression of the CAR protein on the surface of cancer cells, which is required for efficient virus entry into target cells, is a potential explanation for the limited activity. We examined the replication of H101 in the 2 ESCC cell lines and the HeLa cells. In the CAR-deficient ESCC cell lines, H101 displayed poor replication ability. After 48 h of infection, titers increased 538.33- and 240.55-fold in the EC9706

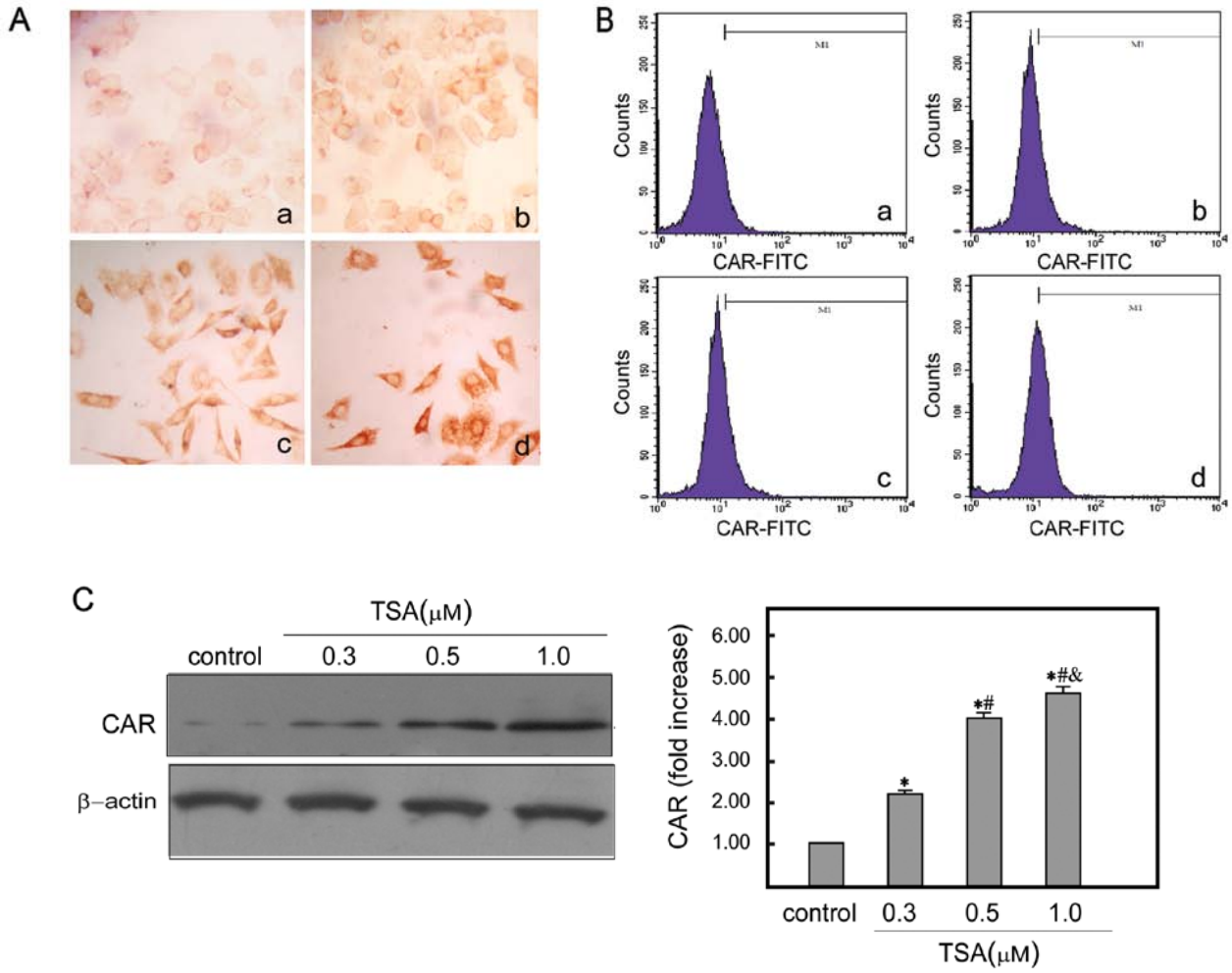

Figure 3. CAR expression in EC1 cells induced by TSA. EC1 cells were treated with the indicated doses of TSA for 48 h. The levels of CAR were visualized by (A) immunocytochemical analysis (magnification, x400), (B) flow cytometry and (C) western blot analysis. a, control group; b, TSA 0.3 μM group; c, TSA 0.5 μM group; d, TSA 1.0 μM group. Data are shown as the means ± SD of values from triplicate experiments. *Significant (P<0.05) change in expression compared to DMSO treatment; #significant (P<0.05) change in expression compared to the 0.3 μM TSA group; &significant (P<0.05) change in expression compared to the 0.5 μM TSA group.

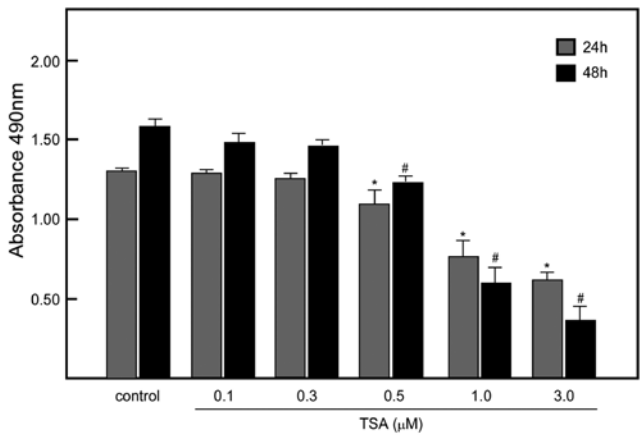

Figure 4. EC1 cell viability following treatment with different doses of TSA for 24 and 48 h. Cell viability was determined by MTT method. This assay was performed in triplicate. Data are shown as the means ± SD of values from triplicate experiments. *Significant (P<0.05) change in EC1 cell viability following treatment with TSA for 24 h compared to DMSO treatment. #Significant (P<0.05) change in EC1 cell viability following treatment with TSA for 48 h compared to DMSO treatment. The data indicate that the cell viability of EC1 cells treated with 0.3 μmol/l TSA for 48 h was 96% more than the control.

and EC1 cells, respectively. By contrast, the replication of H101 increased 1,825.00-fold when compared to the initial titer in the CAR-positive cells. To evaluate the correlation between the expression levels of CAR in the human ESCC cells and the oncolytic activity of H101, we treated ESCC and HeLa cells with H101 at various levels of MOI ranging from 0.01 to 100 pfu/cell. The cell viability rate of the EC9706 and EC1 cells was respectively 69.47 and 84.35% at a MOI of 100. Whereas, HeLa cells treated with H101 dramatically reduced cell viability compared to the ESCC cells at the same MOIs. These results indicated that a low CAR expression in the 2 ESCC cell lines decreased the antitumor activity of oncolytic Ad H101 (Fig. 2).

Effect of TSA on CAR expression in ESCC. TSA is an HDAC inhibitor which blocks the activity of histone deacetylases (HDACs), leading to increased acetylation of histones and other proteins (20). The positive rate of CAR expression in the EC1 cells was lower compared to the rate in the EC9706 cells thus affecting the antitumor activity of H101. Therefore, we modulated CAR expression in the EC1 cells to enhance the efficacy of H101. To investigate whether TSA regulates

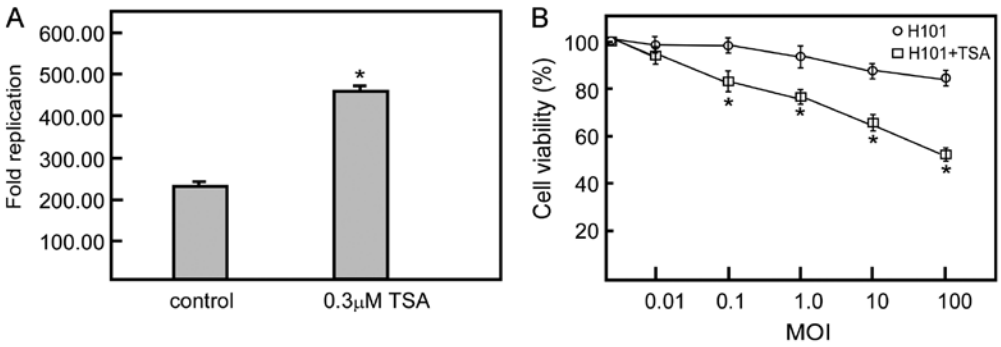

Figure 5. Oncolytic and cytolytic effects of H101 on EC1 cells induced by TSA. (A) Replication of H101 induced by TSA. EC1 cells were treated with or without 0.3 μM of TSA for 48 h. Then cells were infected with H101 Ads at a MOI of 1. The cells and the supernatants were collected 48 h after the infection and titered by the limiting dilution method. (B) Cell viability induced by TSA. EC1 cells were treated with or without 0.3 μM of TSA for 48 h. Cells were then treated with H101 at a MOI of 0.01, 0.1, 1, 10 and 100 pfu/cell for 72 h. Cell viability was analyzed using the CCK-8 assay kit. This assay was performed in triplicate. Values represent the means ± standard deviations (n=3). *P<0.05 vs. the group without TSA treatment.

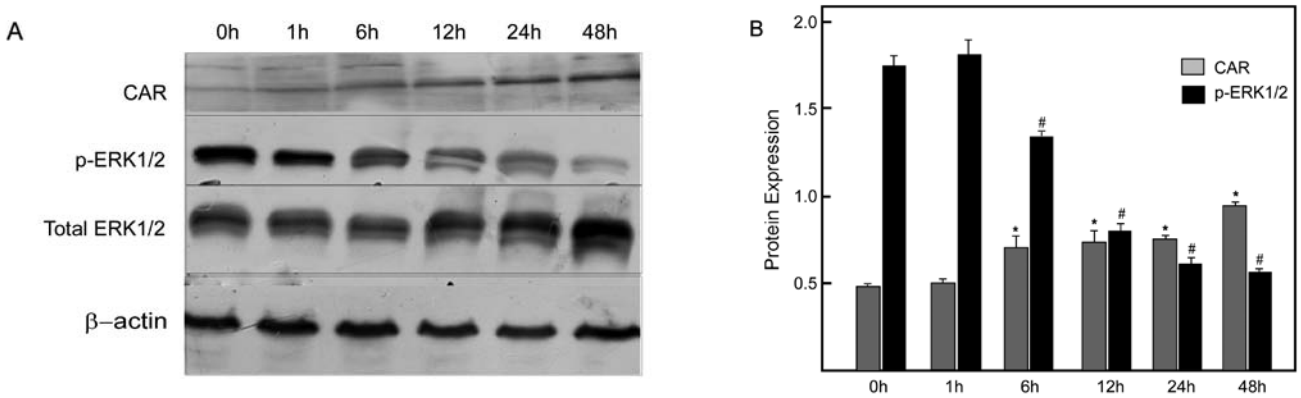

Figure 6. CAR and p-ERK1/2 expression in EC1 cells induced by TSA. (A) EC1 cells were exposed to 1.0 μmol/l TSA for 0, 1, 6, 12, 24 and 48 h and the expression of CAR and the phosphorylation of ERK1/2 were investigated by western blotting. (B) Western blotting results demonstrated that the expression of CAR protein increased and the level of phosphorylation of ERK1/2 decreased in a time-dependent manner. *Significant (P<0.05) change in CAR expression compared to DMSO treatment. #Significant (P<0.05) change in p-ERK1/2 expression compared to DMSO treatment.

CAR expression, we first used FCM to detect the expression of CAR in the EC1 cells induced by TSA. After different doses of TSA treatment, the percentages of CAR-positive cells were 20.93, 27.77 and 40.63% in the 0.3, 0.5 and 1.0 μmol/l treatment groups. Immunocytochemical, flow cytometric and western blot analyses demonstrated that the protein level of CAR increased in a dose-dependent manner in the EC1 cells after TSA treatment (Fig. 3).

TSA at a low concentration did not affect tumor cell viability. TSA has been observed to have antitumor activity as well as alter the growth of tumors. Our purpose was to use TSA to modulate the expression of CAR, but not affect cell viability. We treated EC1 cells with various concentrations of TSA and assessed the cell viability. The data indicated that 96% of cells survived after 0.3 μmol/l TSA treatment for 48 h. Therefore, we conducted the subsequent experiments using 0.3 μmol/l of TSA (Fig. 4).

Replication of H101 after TSA treatment and cytotoxicity of H101 in ESCC cell lines. We next examined the effect of TSA on OV H101 replication. The titer increased 527.46-fold in the EC1 cells treated with 0.3 μmol/l of TSA; the replication of H101 increased in the EC1 cells treated with TSA compared to the untreated control cells. We then examined whether TSA had an effect on the antitumor ability of H101 in EC1 cells. The cell viability of the EC1 cells treated with different doses of H101 was assessed using MTT assay 72 h after treatment with 0.3 μmol/l TSA. Based on a dose-time response curve (Fig. 5), the cell viability was 52.29% in the 0.3 μmol/l TSA and H101 infection (MOI 100) group. However, the cell viability in the H101 infection (MOI 100) group was 84.35%. Furthermore, EC1 cells treated with H101 at the other MOI levels combined with TSA treatment also reduced cell viability (Fig. 5).

Inhibition of ERK1/2 signaling increased the expression of CAR. Disruption of MAPK pathway signaling by pharmacological inhibition of MEK has been found to upregulate CAR protein level, resulting in enhanced Ad entry (21). To determine the role of MAPK/ERK1/2 signaling in CAR expression induced by TSA, we examined the correlation between the phosphorylation activity of ERK1/2 and the expression of CAR. Western blotting results demonstrated that the expression of the CAR protein increased and the level of phosphorylation of ERK1/2 decreased in a time-dependent

manner. There was a significant negative correlation between the activity of p-ERK1/2 and the expression of CAR (Fig. 6)

Discussion

OVs are designed or selected to grow preferentially in tumor cells, and are a promising novel therapeutic platform for cancer. H101, a recombinant human 5 Ad, lacks the *E1B 55* gene and also contains a deletion in the E3 region. Before modification, the E1B region of the wild-type Ad 5 expresses early gene products that bind to and inhibit the function of p53, a tumor suppressor. The deletion produces p53-selective replication of OVs which infect tumor cells and induce massive accumulation of normal p53. As a result, the Ad causes direct cytotoxicity only to tumor cells during replication (22). Clinical trials using attenuated strains of these viruses have demonstrated limited toxicity. However, the use of Ad vectors in gene therapy faces a critical prerequisite that single-agent efficacy requires improvement. The relatively low efficiency of recombinant gene transfer to tumor cells contributes partially to the low therapy efficiency. CAR is a surface protein which is involved in Ad binding to the cell surface. Accumulating evidence indicates that CAR expression levels in target cells are a major determinant of gene transfer efficacy with Ads (23-25).

CAR is ubiquitously expressed in most benign epithelial tissues. However, CAR expression is lower in several types of advanced clinical tumors. The level of CAR in ESCC cell lines has not been reported. By using flow cytometric analysis, we discovered that, compared to HeLa cells, EC1 cells had lower surface CAR protein content (Fig. 1). The low CAR protein content resulted in poor adenoviral transduction efficiency. Decreased CAR expression observed in the ESCC cells was parallel with a decreased replication and oncolytic activity of H101 (Fig. 2). Therefore, decreased CAR expression in EC1 cells may also impose an obstacle for Ad-based gene therapy. Thus, restoring the surface expression of CAR will benefit the antitumor effects of OVs.

Histone acetylation plays an important role in epigenetic gene regulation. In general, histone acetylation may cause chromatin relaxation and transcription activation whereas histone deacetylation causes chromatin structure condensation and repression of transcription. Histone deacetylase inhibitors (HDACi) block the activity of histone deacetylases (HDACs) and affect the transcription of specific genes (26,27). CAR transcriptional regulation is modulated mainly through histone acetylation. TSA is one of the most promising HDACi that may increase the levels of CAR in various types of tumor cells such as breast, lung and ovarian (28). The present study demonstrated that TSA regulates the protein content of CAR in a dose-dependent manner. We discovered that 0.3 μmol/l of TSA increases the protein levels of CAR in EC1 cells but does not affect cell viability. The reproductive multiple of H101 was higher in the 0.3 μmol/l TSA and the H101 group compared to the H101 group alone. The cytotoxic activity of H101 increased in the EC1 cells induced by 0.3 μmol/l of TSA compared with the control group. This result may indicate that TSA increases the antitumor activity of oncolytic Ad H101 through the modulation of CAR in EC1 cells.

It was reported that HDACi may induce CAR on the tumor cell surface via accumulation of hyperacetylated nucleosome core histones resulting in transcriptional activation of genes (29). However, the signaling pathway involved in HDACi-induced expression of CAR in tumor cells needs further investigation. The MAPK/ERK1/2 signaling pathway plays a key role in mediating signals from membrane receptors to the nucleus, and is involved in multiple physiological processes, including cell growth, cell differentiation, cell proliferation and apoptosis (30,31). Once activated, ERK1/2 translocates onto the nucleus to phosphorylate related transcription factors and regulates their activity. Disrupting MAPK pathway signaling by pharmacological inhibition of MEK upregulates CAR expression (32,33). In this study, we observed that there were negative correlations between the phosphorylation of ERK1/2 and the protein level of CAR induced by TSA. This observation is consistent with the report that constitutively active MEK may direct the activation of HDAC4. TSA may decrease the activity of HDAC4 and in turn upregulate the acetylation level of histone H4 through inhibiting the activation of ERK1/2.

In conclusion, human ESCC cell lines have low CAR protein levels. TSA may increase CAR protein levels in human ESCC cells, which may benefit the antitumor activity of oncolytic Ad H101 in ESCC cells. The signaling transduction involved in TSA-induced CAR occurs possibly through interrupting the MAPK/ERK pathway.

Acknowledgements

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