Histone deacetylase inhibitor trichostatin A enhances the antitumor effect of the oncolytic adenovirus H101 on esophageal squamous cell carcinoma in vitro and in vivo

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Abstract. Replication-selective oncolytic virotherapy provides a novel modality to treat cancer by inducing cell death in tumor cells but not in normal cells. However, the utilization of oncolytic viruses as a stand-alone treatment is problematic due to their poor transduction efficiency in vivo. H101 was the first oncolytic adenovirus (Ads) to be approved by the Chinese FDA, and exhibits modest antitumor effects when applied as a single agent. The multiple histone deacetylase inhibitor trichostatin A (TSA) has been demonstrated to potently enhance the spread and replication of oncolytic Ads in several infection-resistant types of cancer. The present study aimed to investigate the antitumor effects of H101 in combination with TSA on esophageal squamous cell carcinoma (ESCC) in vitro and in vivo, and determine the mechanisms underlying these effects. H101 and TSA in combination increased the survival of mice harboring human ESCC cell line-tumor xenografts, as compared with mice treated with these agents individually. Therefore, TSA may enhance the antitumor effects of H101 in ESCC.

Introduction

Despite advances in the treatment of esophageal squamous cell carcinoma (ESCC), the overall mortality rate for this disease remains high (1). The frequency of late-stage diagnosis, high incidence of postsurgical local-regional recurrence and occurrence of distant metastasis contributes to this high mortality (2,3). At present, the therapeutic strategies for ESCC include surgery, chemotherapy regimens and radiotherapy (4). These treatment methods are unable to eradicate all malignant cells, and are associated with frequent side effects (5-7). Therefore, numerous investigations have focused on developing alternative interventions, including tumor-specific replicating viruses (4).

The well-characterized modified adenovirus (Ads), H101 oncolytic Ads, varies from wild-type Ads in that the E1B 55 kDs gene and the E3 region are deleted (8,9). This approach is able to produce viral agents with the ability to selectively enter and replicate in tumor cells, consequently leading to cancer cell lysis with minimal damage to surrounding normal cells (10). It is hypothesized that the infecting oncolytic virus (OV) may spread through a solid tumor and eliminate it through the release of progeny virions and activation of the antitumoral immune response (11-14). However, clinical trials in patients with head and neck cancer have revealed that the efficacy of this treatment is limited when it is utilized as a single modality, potentially due to inefficient intratumoral viral dispersal and the barriers imposed by the tumor microenvironment (15). Therefore, oncolytic Ads H101 requires combination with another modality to improve its antitumor activity (9,14,16-18).

During transcription, histone acetylation/deacetylation is a major regulator of chromatin structural dynamics (19). Histone deacetylase inhibitors (HDACIs) block the activity of histone deacetylases, leading to the increased acetylation of histones and causing non-histone proteins to form a compact and transcriptionally repressed chromatin structure (20-22). HDACIs have been reported to inhibit the ability of tumor cells to mount a productive antiviral response (23-25). At present, trichostatin A (TSA) is considered the most promising HDCAI...
for tumor treatment, functioning as a potent inhibitor of cyclin D1 with the ability to arrest cell-cycle progression (26).

In the present study, the ability of TSA to augment the oncolytic activity of H101 was evaluated. The results suggested that the HDACI TSA potently and selectively enhanced the replication of H101 virions in ESCC in vitro and in vivo. Furthermore, the mechanism underlying TSA-mediated enhancement of the oncolytic activity of H101 was examined.

Materials and methods

Cell culture. The EC1 human esophageal carcinoma cell line was provided by The Department of Cell Biology, Hong Kong University (Hong Kong, China). This cell line has been demonstrated to be poorly-differentiated squamous cell carcinoma (27). EC1 cells were propagated in monolayer culture in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% inactivated fetal bovine serum (56˚C; 30 min; Hyclone Laboratories, Logan, UT, USA), 1x10⁵ U/l penicillin and 100 µg/l streptomycin in a humidified atmosphere with 5% CO₂ at 37˚C.

Reagents and treatments. TSA was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and dissolved in dimethyl sulfoxide (DMSO) to produce a 5 mM stock solution, which was stored at -20˚C. Control cells were treated with DMSO in parallel during each experiment.

Cell viability assay. EC1 cell lines were seeded at a density of 5x10⁵ cells/well in 96-well microtiter plates. The cells were incubated at 37˚C with 5% CO₂ for 24 h and then were treated with TSA at various concentrations (0.1, 0.3 and 0.5 µM; prepared from a stock solution dissolved in DMSO) for 24, 48 and 72 h. Cells treated with identical concentrations of DMSO were used as controls. A total of 4 h prior to absorbance evaluation, 10 µl Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added to each well and incubated at 37˚C for 1 h. Absorbance was determined at a wavelength of 450 nm for each well using an enzyme-labeling instrument (Multiskan GO; Thermo Fisher Scientific, Inc.). All experiments were performed independently in triplicate.

Apoptosis assay. Following incubation with or without TSA at various concentrations (0.1, 0.3 and 0.5 µM) for 48 h, EC1 cells were harvested using 2.5 g/l trypsin and washed twice with PBS. A total of 1x10⁵ cells were stained with fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) using an Annexin V-FITC kit (Beckman Coulter, Inc., Brea, CA, USA), according to the manufacturer's protocol. Subsequently, the apoptosis of 1.5x10⁴ stained cells was quantified using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Each experiment was performed in triplicate. BD CellQuest™ software version 3.0 (BD Biosciences) was used to calculate the proportion of apoptotic cells. Negative staining for Annexin V and PI indicated viable cells; early apoptotic cells were positive for Annexin V and negative for PI, whereas late apoptotic cells were positive for Annexin V and PI.

In vitro H101 oncolytic Ads replication assay. EC1 cells were cultured on 6-well plates (5x10⁵ cells/well) at 37˚C for 24 h prior to infection with H101 Ads at a multiplicity of infection (MOI) of 100, and in the presence or absence of 0.3 µM TSA. The cells and the supernatants were harvested 24, 48 and 72 h following infection, freeze-thawed 3 times and serially diluted. HEK293 cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China) were seeded at a density of 1x10⁵ (100 µl) cells/well with 2% DMEM in 96-well microtiter plates. Each sample (cells and supernatants) that was diluted serially 10 times with 2% DMEM was added to 96-well microtiter plates at 37˚C for 10 days. Each titer was repeated 10 times. The same volume of 2% DMEM was added as a control. Viral titers were calculated by infecting serially diluted virus particles in HEK293 and determined using the limiting dilution method (4) (determination of the 50% infective dose in tissue culture using HEK293 cells).

Co-treatment of EC1 cells with TSA and H101 oncolytic Ads. EC1 cells were seeded at a density of 5x10⁵ cells/well at 37˚C in 96-well microtiter plates. Following culture at 37˚C for 24 h, cells were incubated with H101 Ads at an MOI of 100 in the presence or absence of 0.3 µM TSA for 24, 48 and 72 h. Cell viability was evaluated using CCK-8. A total of 10 µl of the CCK-8 solution was added to each well and incubated at 37˚C for 2 h. Absorbance was determined at a wavelength of 450 nm for each well using an enzyme-labeling instrument (Multiskan G0). All experiments were performed independently in triplicate.

Animal treatments. Nu/nu athymic female mice, 4-6 weeks old and weighing 18-22 g, were obtained from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China). All animals were housed in specific pathogen free laminar airflow boxes at a temperature of 25-26˚C, with a humidity of 50%, and administered sterile food and water ad libitum. The mice were treated in accordance with the Guide for the Care and Use of Laboratory Animals of Henan Province, China, and experimental procedures were approved by the Medical Ethics Committee of Zhengzhou University (Zhengzhou, China). To obtain xenograft tumors, a 4x10⁶ EC1 cell resuspension (200 µl) was injected subcutaneously into the dorsal right flank of the athymic mice. The animals were monitored for tumor growth every other day. Upon reaching the required mean tumor volume of ~100 mm³ (volume = length x width² x 0.5), a total of 24 mice were randomly assigned to the following 4 groups (6 mice per group): The TSA alone group, the H101 alone group, the TSA and H101 combination treatment group, and the control group. The treatment protocol comprised of TSA (0.3 µmol/l, 200 µl TSA) administered as intratumoral injections 3 days prior to H101 injection. The H101 treatment protocol comprised of 100 µl H101 (1x10⁶ plaque-forming units) administered as intratumoral injections on days 2, 7, 11, 15 and 19. The control group received five injections of 100 µl PBS on days 2, 7, 11, 15 and 19. Tumor size was measured every 7 days using Vernier calipers. The mice from each group were sacrificed by cervical dislocation on day 21.

Immunohistochemical analysis. Tissue sections preserved in 2.5% glutaraldehyde-polyoxymethylene solution at room temperature for 24 h, were dehydrated and embedded in
paraffin following routine methods, and sectioned to 4-µm thick. The sections were deparaffinized in xylene followed by treatment with a graded series of ethanol and distilled water, and thorough rinsing with PBS. Following microwave treatment in citrate buffer (pH 6.0), the container was placed in boiled water for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at room temperature for 10 min. The tissue samples were incubated with a rabbit anti-cocxackie and adenovirus receptor (CAR) monoclonal antibody (dilution, 1:200; catalog no. sc-50462; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. Following washing three times with PBS, the tissue samples were incubated for 30 min with the goat anti-rabbit IgG-horseradish peroxidase secondary antibody (dilution, 1:2,000; catalog no. sc-2004; Santa Cruz Biotechnology, Inc.). Antibody binding was subsequently detected using 0.5% 3,3′-diaminobenzidine hydrochloride (DAB; Sigma-Aldrich; Merck Millipore) at room temperature without light for 3 min. The sections were then washed three times with PBS, counterstained with hematoxalin for 15 sec and dehydrated at room temperature. The images were analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis. Tumor tissues from xenografts of the aforementioned mice were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 100 µg/ml phenylmethysulfonyl fluoride) for tissue homogenization. After 20 min on ice, the lysates were centrifuged at 20,430 x g at 4°C for 10 min. The supernatants were used as whole cell extracts. Cell lysates (50 µg) were separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with 5% non-fat dried milk dissolved in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then incubated with a rabbit CAR monoclonal antibody (dilution, 1:200; Santa Cruz Biotechnology, Inc.) at 4°C overnight. After washing three times with 0.1% TBS-T, the tissue samples were incubated with the aforementioned horseradish peroxidase-conjugated IgG secondary antibody for 2 h at room temperature. A Pierce™ enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) was used to detect the target proteins. The bands were subjected to densitometry for quantitation using the Bio-Rad Quantity One™ software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Quantitative data were expressed as the mean ± standard deviation. One-way analysis of variance was used to compare significant differences amongst the groups. Two-tailed Student's t-tests were used for comparisons between two groups. Data analyses were performed using SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of TSA on the growth of EC1 cells. TSA, an established class I and II HDACI, has been reported to exert numerous antitumor effects by inhibiting cell proliferation and inducing cell apoptosis (24). The present study aimed to examine the ability of TSA to promote the antitumor effects of oncolytic H101, but not to affect cell viability. Therefore, EC1 cells were treated with various concentrations of TSA, and EC1 cell viability and apoptosis were evaluated. As presented in Fig. 1A, the viability of EC1 cells was not significantly inhibited by TSA at doses of 0.1 and 0.3 µM after 72 h of treatment (P=0.542 for 0.1 µM vs. control; P=0.218 for 0.3 µM vs. control). However, the viability of EC1 cells was significantly inhibited at doses >0.5 µM (P<0.001; Fig. 1A). In addition, the proportion of EC1 cells in early apoptosis was not markedly increased at TSA doses ≤0.3 µM (P=0.077 for 0.1 µM vs. control; P=0.350 for 0.3 µM vs. control; Fig. 1B). These results indicated that doses of ≤0.3 µM TSA did not significantly alter EC1 cell viability.

Increased H101 replication and cell cytotoxicity is mediated by TSA and H101 in combination. To examine whether TSA is able to impact H101 replication, end point dilution titrations were performed on HEK293 cells. Following treatment with 0.3 µM TSA for 24, 48 and 72 h, viral titers increased 55.82-fold, 238.84-fold and 527.46-fold in EC1 cells treated with TSA, compared with untreated control cells (P=0.002 for TSA 24 h vs. control; P<0.01 for 0.3 µM vs. control). However, the viability of EC1 cells was significantly inhibited at doses >0.5 µM (P<0.001; Fig. 2A). H101 replication was significantly increased in EC1 cells treated with TSA, compared with the untreated control cells (P=0.002 for TSA 24 h vs. control; P<0.001 for TSA 48 h vs. control; P<0.001 for TSA 72 h vs. control). Subsequently, the antitumor effects of TSA on H101 in EC1 cells were examined. A CCK-8 assay was used to measure the EC1 cell survival rate at 12, 24, 48, 72 and 96 h following treatment. As compared with the H101 monotherapy group, the cell survival rate in the TSA and H101 combination group exhibited a significant decrease at 24, 48 and 72 h (P<0.001; Fig. 2B).

Effect of TSA and H101 combination treatment on the EC1 xenograft model. TSA and H101 in combination enhanced...
tumor cell cytotoxicity in vitro. Therefore, in order to examine whether this treatment is able to inhibit EC1 tumor growth in vivo, tumor-bearing mice were divided into various treatment groups as described in material and methods. As compared with the PBS control group, mice treated with TSA alone did not exhibit tumor regression and there was no significant difference in the tumor volume at the end of treatment (P=0.148). In the TSA and H101 combination treatment group, a significant decrease in tumor volume (286.53±28.99 mm³) was observed, as compared with the untreated controls (1459.79±76.81 mm³) (P<0.001). Furthermore, a significant decrease in the tumor volume was detected in the TSA and H101 combination group, as compared with the two treatments administered individually (both P<0.001). The results indicate that TSA and H101 in combination produce an enhanced antitumor effect, compared with the two treatments administered individually (Fig. 3A). In addition, marked variations were identified in the degree of inflammation and necrosis observed in the tumor specimens in the TSA and H101 combination group, compared with the groups in which TSA and H101 were administered individually (both P<0.001; Fig. 3A and B).

TSA alone, or combined with H101, upregulates the expression of CAR in an EC1 cell- xenograft model. To investigate whether the enhanced antitumor effects of TSA and H101 combined in vitro were achieved via the upregulation of CAR, the expression levels of CAR in the xenograft tumor tissues were detected using immunohistochemistry. An increase in the expression levels of CAR in xenograft tumors was observed in the TSA group and in the TSA and H101 combination group, as compared with the control group and the H101 group (Fig. 4A). Western blot analysis also demonstrated that the CAR protein levels were increased in the TSA group and the TSA in combination with H101 group (both P<0.001; Fig. 4B and C). These results indicated that TSA intratumoral injections may result in increased levels of CAR expression in xenograft tumors in mice.

Discussion

The tumor suppressor gene tumor protein p53 is considered an attractive target for cancer gene therapy (28-30). The human p53 gene is known as the ‘guardian of the genome’ for its roles in regulating the cell cycle, apoptosis and cellular senescence, as well as inducing a variety of activities to maintain genomic stability (31,32). Mutant p53 has been demonstrated to be overexpressed in the tumor tissues of patients with ESCC and its expression levels are correlated with tumor progression (33). Therefore, Ads-mediated p53 cancer gene therapy constitutes a promising treatment approach for patients with ESCC (34). H101 is a recombinant human type 5 Ads with a total deletion of the E1B 55 K gene, which is able to proliferate effectively in p53 mutant cells, but not in p53 wild-type cells (35). However, H101 has limited potential for the eradication of tumors when used as a monotherapy due to its low infection efficiency (10). Therefore, a high degree of viral transduction within the tumor is key to the success of gene therapy approaches. H101 is often used in combination with traditional modalities, including chemotherapy (36). In the present study, the antitumor efficacy of H101 in combination with the HDACI TSA was evaluated.

The H101 OV enters malignant cells through a receptor-mediated endocytosis mechanism (37). CAR is necessary for adenoviral entry into the cell; however, this receptor is frequently downregulated in malignant cells, rendering them less vulnerable to viral attack (38). It has been reported that HDACIs are able to enhance transgene expression, making them suitable for use in conjunction with adenoviral vector-based therapies due to their ability to increase CAR expression levels (39).

Although TSA was one of the first HDACIs to be identified, its suboptimal in vivo stability limits its use as a widely administered cancer treatment (40). Furthermore, TSA is more effective at promoting vaccinia virus spread in vitro, compared with TSA derivatives, by increasing the expression levels of CAR in malignant cells, and leading to more efficient cell killing compared with other HDACIs, including vorinistat (40). In the present study, TSA was selected to enhance the antitumor efficacy of H101 with the purpose of evaluating the ability of TSA to enhance H101 viral oncolysis without altering cell viability. Following the treatment of EC1 cells with various concentrations of TSA and the subsequent evaluation of cell viability, the data indicated that a dose of 0.3 µM TSA delivered to EC1 cells was well-tolerated and did not induce apoptosis. However, the treatment of EC1 cells with
Figure 3. Antitumor activity of TSA and H101 co-treatment in an EC1 mouse xenograft model. (A) Tumor volumes from EC1 xenograft tumors in nude mice. (B) EC1 xenograft tumors upon the study termination and the average volume of subcutaneous tumors following treatment with TSA, H101, TSA plus H101 or PBS. All values represent the mean ± SD for six animals per group. *P<0.05. (C) Immunohistological evaluation of histological changes of EC1-derived tumor tissues. Magnification, x100. TSA, trichostatin A; SD, standard deviation.

Figure 4. Expression of CAR in EC1 mouse xenograft tissue upon various forms of treatment. (A) Immunohistochemical evaluation of CAR expression in EC1 xenograft tissue treated with control (PBS), TSA, H101 and TSA+H101 (magnification, x200). (B) Expression levels of CAR in EC1 xenograft tissue as investigated by western blot analysis. Each blot is representative of three independent experiments. (C) Gray values of CAR expression are presented as the mean ± standard deviation. *P<0.05, compared with the with control group. CAR, coxsackie and adenovirus receptor; TSA, trichostatin A; SD, standard deviation.
0.3 µM TSA increased H101 replication and cell cytotoxicity. These results indicate that TSA is able to enhance the antitumor efficacy of H101 in vitro.

Previous studies have indicated that a number of factors may lead to a discrepancy between the efficacy data obtained from cell culture in vitro, and the in vivo data (15,41). In cell culture monolayer, all infectable cells are easily accessed by viruses. By contrast, aspects of the tumor architecture, including fibrotic septa and necrotic areas in tumor tissue, prevent the virus from spreading in vivo (9). This leads to an inconsistency between the efficacy obtained from cell culture experiments in vitro and in clinical trials. Therefore, their efficacy of viral oncology need to be improved (42-45). The present study examined whether the enhanced in vitro tumor cytotoxicity, mediated by TSA and H101 in combination, was also exhibited during EC1 cell tumor growth in vivo. In comparison with the TSA group and the H101 group, a significant decrease in tumor volume in the TSA and H101 combination group was observed. This result indicates that the use of TSA and H101 in combination produced an enhanced antitumor effect in vivo.

Subsequently, the mechanisms underlying the ability of TSA to enhance the antitumor effects of H101 were investigated. Lower expression levels of CAR protein have been reported in ESCC cells (46). In addition, Wei Lu et al proved that an intratumoral injection of chemotherapy in combination with H101 exhibits better antitumor activity to refractory malignant tumors than H101 alone (46). It has become apparent that a major determinant of Ads-mediated gene transfer efficacy is the expression of its primary receptor, CAR, on target cells (47). In order to infect tumor cells efficiently, H101 requires CAR for attachment and αv integrin for internalization (48). In the present study, the expression levels of CAR in the mouse xenograft tumor tissue were increased in the TSA group, and in the TSA and H101 combination group. These results suggest that TSA intratumoral injections may enhance the H101 antitumor effect by increasing CAR expression levels in vivo.

In conclusion, the HDACI TSA is able to enhance the antitumor effect of the OV H101 on ESCC cells in vitro and in vivo. HDACIs combined with OVs may therefore be able to overcome the obstacle of the low infection efficiency of H101 when used as monotherapy.

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