ORIGINAL ARTICLE

Enhanced antitumor effect of combining TRAIL and MnSOD mediated by CEA-controlled oncolytic adenovirus in lung cancer

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Lung cancer, especially adenocarcinoma, is one of the leading causes of death in the world. Carcinoembryonic antigen (CEA), a superb non-small-cell lung cancer marker candidate, showed a beneficial effect in cancer therapy with oncolytic adenovirus in recent studies. Cancer-targeting dual gene-virotherapy delivers two therapeutic genes, linked by a connexon, in the replication-deficient vector instead of one gene so that they can work in common. In this study, we constructed a tumor-specific oncolytic adenovirus, CD55-TRAIL-IETD-MnSOD. The virus has the fusion protein complementary DNAs for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and for manganese superoxide dismutase (MnSOD) complementary DNA linked through a 4-amino acid caspase-8 cleavage site (IETD), and uses a CEA promoter to control virus E1A express. This is the first work to use a CEA promoter-regulated oncolytic adenovirus carrying two therapeutic genes for cancer research. Its targeting and anticancer capacity was evaluated by *in vitro* and *in vivo* experiments. The results indicated that CD55-TRAIL-IETD-MnSOD caused more cell apoptosis than CD55-TRAIL or CD55-MnSOD alone, or their combination *in vitro*, with low cytotoxicity of normal cells. In the A549 tumor xenograft model in nude mice, data showed that CD55-TRAIL-IETD-MnSOD could effectively suppress tumor growth than single gene groups, with no histological damage in liver, spleen or kidney tissues. Thus, the CEA-regulated dual-gene oncolytic virus CD55-TRAIL-IETD-MnSOD may be a novel potential therapy for lung cancer.

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INTRODUCTION

In China, lung cancer incidence and mortality have increased during the past few decades.¹ The morbidity of lung cancer has increased at the rate of 26.9% per year to become the leading cause of cancer-related death in our country. Therefore, the exploration of novel and effective therapy methods for lung cancer is imperative.

Cancer-targeting gene-virotherapy (CTGVT) is a novel strategy of combined gene therapy and virotherapy together that is constructed by inserting an antitumor gene into an oncolytic viral vector (OV).^{2,3} An oncolytic viral vector, such as the commonly used ZD55 in our group, instead of a replication-deficient vector, can target and replicate several hundred-fold in cancer cells, leading to several hundred-fold expression of the inserted gene in cancer cells.⁴ Therefore, the therapeutic effects of CTGVT (OV-gene) have been significantly improved in recent publications.⁵ However, there remain some issues in CTGVT strategy such as the restricted targeting of OV and inefficient antitumor effects of a single antitumor gene. Consequently, the cancer-targeting dual gene-virotherapy (CTGVT-DG) strategy was exploited by us.⁶ Previous studies proved that the combination of ZD55-TRAIL and Ad-K5,⁷ or ZD55-TRAIL and ZD55-MnSOD,⁸ or ZD55-TRAIL and ZD-IL-24,⁸ as well as ZD55-TRAIL-IETD-Smac,⁹ co-expression of the therapeutic genes TRAIL and Smac by a linker had a much stronger antitumor effect *in vitro* and *in vivo*.

However, improving the tumor specificity of OV is another goal, and transcriptional targeting can be a crucial strategy to attain this goal. Carcinoembryonic antigen (CEA), a type of acidoglycoprotein with human embryo antigen specificity, is mainly expressed in fetal gastrointestinal epithelial tissue, pancreas and liver, but only at low levels in normal tissue. Its concentration is increased in colorectal cancer^{10–12} and gastric carcinoma.¹³ Furthermore, some studies have found that the serum CEA also increases in patients with lung cancer,^{14–16} and its level is also an important predictive factor for the postoperative survival rate in patients with non-small-cell lung cancer.^{17,18} These implied that CEA has the potential for targeting and modifying CTGVT in lung cancer.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a typical member of the tumor necrosis factor superfamily, and is currently being investigated as a potential therapeutic agent against many types of tumor cells via its death receptors and subsequent inducement of tumor cell apoptosis.^{19,20} Previous study reported that OV-mediated TRAIL could significantly suppress human hepatocellular carcinoma,²¹ lung adenocarcinoma²² and colorectal cancer.⁸ More than that, its combination with manganese superoxide dismutase (MnSOD) could completely eliminate a colorectal tumor xenograft²³ via enhanced apoptosis. Some evidence also showed that MnSOD has effective antitumor ability in pancreatic,²⁴ colorectal²⁵ and hepatocellular carcinoma.²⁶ Based on the results using the separate OV-mediated delivery of

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Table 1. Sequences of primers

Primers	Sequences
CEA	Forward: 5'-CCGCTCGAGCTGGAGAGCATGGGGAGAC-3' Reverse: 5'-TACGTAGTCTCTGCTGTCTGCTCTGT-3'
MnSOD	Forward: 5'-AGTTGGCATTGAGACAGACAAGCACAGCCTCCCGACC-3' Reverse: 5'-CCGCTCGAGTTACTTTTGAAGCCAGT-3'
TRAIL-IETD	Forward: 5'-GGAATTCATGGCTATGATGGAGGTCCA-3' Reverse: 5'-GGTCTGGGGAGGCTGTGCTTGTCTGTCTCAATGCCAACT-3'
Ad 5 virus	Forward: 5'-CGCGGGAAACTGAATAAGAG-3' Reverse: 5'-ACCGCCAACATTACAGAGTCG-3'
CEA real-time PCR	Forward: 5'-CTCTGCTCACAGCCTCACTTC-3' Reverse: 5'-TTCGGATCCACTCTTTCACCTT-3'
GAPDH real-time PCR	Forward: 5'-TTCGGATCCACTCTTTCACCTT-3' Reverse: 5'-TGGAAGATGGTGATGGGAT-3'

Abbreviations: CEA, carcinoembryonic antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

TRAIL or MnSOD, it may be more effective to use one OV to deliver a bicistronic arrangement of both TRAIL and MnSOD. It has also been shown that the isoleucine-aspartate-threonine-glutamate (IETD) sequence encoding one of the caspase-8 cleavage sites forms a good link between two therapeutic genes, without affecting their function, and specifically the antitumor activity of TRAIL in the CTGV-DG strategy.⁹

In this study, we used the CEA promoter to regulate E1A expression of OV (ZD55) and first constructed a cancer-targeting dual gene-virus CD55-TRAIL-IETD-MnSOD carrying two therapeutic genes, TRAIL and MnSOD, linked by the tetrapeptide linker IETD. We further investigated the antitumor effect of the novel construct in lung adenocarcinoma for the first time. The experiments indicated that CD55-TRAIL-IETD-MnSOD could more effectively suppress tumor growth than either CD55-TRAIL or CD55-MnSOD or their combination, both *in vivo* and *in vitro*. The oncolytic virus enhanced tumor cell apoptosis and did not cause toxicity effects on normal cells.

MATERIALS AND METHODS

Cells and culture

Human lung bronchial epithelial cell line BEAS-2B and human embryonic kidney cell HEK293 (containing the E1A region of adenovirus) were obtained from ATCC (American Tissue Culture Collection, Rockville, MD, USA). Human colorectal cancer cell line SW480, normal human liver cell line QSG-7701, human hepatoma cell lines HepG2, Hep3B, Bel-7404 and SMMC-7721, human breast cancer cell line MCF-7, human gastric cancer cell line BGC-823, human cervical carcinoma cell line Hela and human lung carcinoma cell lines A549, NCI-H460, NCI-H1299 and NCI-H1975 were purchased from the Shanghai Cell Collection (Shanghai, China). The HEK293 and BEAS-2B were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL). The cancer cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. All cell lines were incubated at 37 °C in a humidified air atmosphere with 5% CO₂.

Plasmid and adenovirus construction

The adenovirus type 5 vector pXC2, adenovirus packaging vector pBHGE3, shuttle vector pShuttle, pCA13-TRAIL, pCA13-EGFP and pCA13-MnSOD were preserved in our laboratory. The oncolytic adenovirus plasmid pZD55 was constructed previously using site-directed mutation to delete the E1B 55-kDa gene.²³ The novel shuttle vector pSD55 was obtained by inserting the expression cassette of pZD55 into pShuttle. The CEA promoter sequence was amplified by PCR from pGL3-CEAp. The PCR product was digested with *Xho*I and *Sna*BI, and then cloned into pXC2 to get the pXC2-CEA. Next, the CEA-E1A in pXC2-CEA was subcloned into pSD55 to obtain the pAdCEAp-D55 (briefly pCD55). The pCA13-TRAIL-IETD-

MnSOD was assembled and constructed from pCA13-TRAIL and pCA13-MnSOD plasmids with the primer sequences of TRAIL-IETD and IETD-MnSOD by overlap PCR. The PCR products were digested with *Bgl*II and the TRAIL-IETD-MnSOD expression cassette was inserted into the corresponding site of pSD55 to obtain the pSD55-TRAIL/MnSOD. Similarly, pSD55-TRAIL, pSD55-MnSOD and pSD55-EGFP were constructed. All the recombinant plasmids were confirmed by PCR, restriction enzyme digestion and DNA sequencing. All the primer sequences for PCR are shown in Table 1.

Generation, identification, purification and titration of adenovirus

The oncolytic adenoviruses CD55-TRAIL, CD55-MnSOD, CD55-TRAIL/MnSOD and CD55-EGFP were generated by homologous recombination in *Escherichia coli* strain, BJ-5183. Briefly, the shuttle plasmid pSD55-TRAIL, pSD55-MnSOD, pSD55-EGFP or pSD55-TRAIL/MnSOD and adenovirus packaging backbone plasmid pAdeasy-1 were co-transfected into BJ-5183, and the recombinant adenovirus genomes pSD55-TRAIL, pSD55-MnSOD, pSD55-EGFP or pSD55-TRAIL/MnSOD were obtained. After being digested with *Pac*I, each of them was transfected into HEK293 cells using Effectene transfection reagent (Qiagen, Germantown, MD, USA). After homologous recombination in HEK293 cells, the CEA-regulated E1B 55-kDa gene deletion oncolytic adenoviruses CD55-TRAIL, CD55-MnSOD, CD55-EGFP and CD55-TRAIL/MnSOD carrying the TRAIL, MnSOD, EGFP and TRAIL/MnSOD genes, respectively, were generated. Each recombinant adenovirus was amplified by infecting HEK293 cells and purified by cesium chloride gradient ultracentrifugation. The virus titers were determined by the 50% tissue culture infectious dose (TCID₅₀) method in HEK293 cells and calculated as plaque-forming units (PFUs) per ml.

Assay of virus infection with reporter gene

Various cells were seeded in 24-well plates at a density of 5×10^4 cells per well and infected with CD55-EGFP at the indicated 0.1, 1 and 10 multiplicity of infection (MOI). After 48 h, enhanced green fluorescent protein (EGFP) expression was detected using an Olympus fluorescence microscope with an Olympus digital camera apparatus DP70 (Tokyo, Japan).

Quantitative real-time PCR

Total RNA was isolated from prepared lung cancer cells or normal cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For analysis of CEA expression, complementary DNA was synthesized by qPCR RT Kit (Toyobo, Osaka, Japan) as described by the manufacturer. Quantitative real-time PCR was performed using SYBR Green kit (Qiagen). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used for normalization. The primer sequences of CEA and GAPDH are shown in Table 1. Real-time PCR was run on the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Each reaction was run in triplicate and repeated three times. We used the $\Delta\Delta C_t$ method with relative quantification of gene expression to determine CEA mRNA expression levels.

Apoptotic cell staining and cell viability assay

Cells were plated on 6-well plates and treated with various recombinant adenoviruses at 10 MOI. After 48 h, cells were incubated with Hoechst 33342 (Beyotime, Nantong, China) for 10 min, washed twice with phosphate-buffered saline (PBS) and observed under a fluorescence microscope. Cell viability was determined by methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich, St Louis, MO, USA) assay according to the manufacturer's instructions (Biosharp, Hefei, Anhui, China) at the indicated MOI and time.

Western blot analysis

Cells were incubated with CD55-EGFP, CD55-MnSOD, CD55-TRAIL or CD55-TRAIL-IETD-MnSOD at 10 MOI. After 48 h, cell lysates were harvested and the protein concentration was determined by Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, an equal amount of total protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes after being boiled for 5 min. The membranes were blocked with 5% nonfat dry milk and incubated with corresponding primary antibodies overnight. After incubation with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence, each expressed protein was detected. Actin was used to control for equal gel loading. Caspase-8, caspase-3, caspase-9, poly-ADP-ribose polymerase, TRAIL, BAX and Bid antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). E1A, Actin and Smac antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Flow cytometric analysis

Cells were trypsinized and harvested after different treatment. Aliquots of cells were resuspended in 500 μ l of binding buffer and stained with Annexin V FITC/PI (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. A fluorescence-activated cell sorting (BD Biosciences) assay was performed immediately after staining.

Animal experiment

Animal experiments were performed according to the guide for regulations and standards of Experimental Animal of the US Department of Agriculture and the National Institutes of Health. Female BALB/c nude mice ~4 weeks old were purchased from the Shanghai Experimental Animal Center (Shanghai, China). A549 cells were injected subcutaneously into the right flank of nude mice to establish the tumor xenograft model.

When the tumors grew to 80–120 mm³ in size, mice were assigned to six groups ($n = 8$) at random. Different adenoviruses (5×10^8 PFUs) or PBS was injected once every other day for a total of four times in an intratumoral manner. From the end of the injection, tumor size was measured by the Vernier caliper every 5 days. The tumor volume (mm³) was calculated as (length $\times$ width²)/2. On day 7 after virus injection, tumors were harvested for histopathological and transmission electron microscopy, immunohistochemistry (IHC) and terminal deoxynucleotidyltransferase-mediated dUTP biotin nick end labeling (TUNEL) assays were performed.

Histopathology, IHC and TUNEL assay

Tumor tissue, liver, kidney and spleen were harvested and fixed in 4% paraformaldehyde, dehydrated with gradient ethanol and embedded in paraffin wax, and then cut in 5 μ m sections, dewaxed and rehydrated according to a standard protocol. For histopathology analysis, the sections were stained with hematoxylin and eosin. For IHC analysis, the tumor sections were incubated with rabbit monoclonal anti-TRAIL antibodies at 1:200 dilutions, washed with PBS and incubated with the avidin-biotin-peroxidase complex reagent (Vector Laboratories, Burlingame, CA, USA) and detected with diaminobenzidine tetrahydrochloride solution containing 0.006% hydrogen peroxide. Hematoxylin was used as a counterstain. For the TUNEL assay, an *in situ* apoptosis detection kit (Sino-American Biotechnology Co., Luoyang, China) was used to stain in apoptotic cell tumor tissue sections according to the manufacturer's instructions. All sections were counterstained with hematoxylin.

Transmission electron microscopy analysis

Tumor tissue was cut in 1 mm³ pieces, fixed with 2.5% glutaraldehyde overnight and washed with PBS three times 5 min each time. Then, 1% osmium tetroxide was used to fix the tumor again. After being rinsed in 0.1 M phosphate buffer, all of the samples were dehydrated in a graded series of ethanol and propylene oxide washes, embedded in pure acetone with embedding buffer in a ratio of 1:2 at room temperature for 3 to 4 h and cured at different gradients of temperature and time. The sections were double-stained with uranyl acetate and lead citrate, examined and photographed with a Jeol JEM-1230 transmission electron microscope (JEOL Ltd, Akishima, Japan).

Statistical analysis

All experimental data were presented as mean $\pm$ s.d. Statistical analysis was performed using a one-way analysis of variance and compared at a given

Figure 1. Schematic diagram of recombinant oncolytic adenovirus CD55 transgene. The transgenes included EGFP, TRAIL, MnSOD and TRAIL/MnSOD. The wild-type adenovirus 5 was used as the backbone. EGFP, enhanced green fluorescent protein; ITR, inverted terminal repeat; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

condition by Student's *t*-test. Results were considered to be statistically significant when $*P < 0.05$, to be very significant when $**P < 0.01$ and to be highly significant when $***P < 0.001$.

RESULTS

Construction and characterization of oncolytic adenovirus

Based on the study of CTGVT-DG, we constructed a novel oncolytic adenovirus CD55-TRAIL-IETD-MnSOD. The novelty displays three characteristics, including the CEA promoter regulating E1A gene, E1B 55-kDa gene deletion and delivery of the bicistronic genes TRAIL and MnSOD. The control single gene oncolytic viruses CD55-TRAIL, CD55-MnSOD and CD55-EGFP were also constructed for comparison (Figure 1).

To verify the CEA characteristic, we used real-time quantitative PCR to detect the relative expression in a variety of cancer cell lines. The results showed that the expression of CEA mRNA was enhanced in SW480, Hep3B and HepG2 cell lines, especially in the A549 cell line (Figure 2a). Similarly, more EGFP expression mediated by the CEA-regulated oncolytic adenovirus CD55-EGFP was also observed in A549 cells than in Hep3B or SW480 cells in a dose-dependent manner, but minimally in the normal cell QSG-7701 (Figure 2b). In addition, infected A549 cells with CD55-EGFP, CD55-MnSOD, CD55-TRAIL, CD55-MnSOD plus CD55-TRAIL and CD55-TRAIL-IETD-MnSOD at the indicated MOI displayed a significant production of adenovirus protein E1A, and the transgenes TRAIL and MnSOD protein (Figure 2c).

Tumor-selective cytopathic effect of CD55-TRAIL-IETD-MnSOD

To investigate the tumor-specific cytopathic effects, as well as to confirm the toxicity results, various human lung carcinoma cell lines and human lung normal cell line BEAS-2B were transduced with different OVs CD55-MnSOD, CD55-TRAIL and CD55-TRAIL-IETD-MnSOD at an MOI of 10, and combined treatment with CD55-MnSOD and CD55-TRAIL each at an MOI of 5. The results by MTT assay showed ~40–70% cell death in cancer cell lines 96 h after infection with CD55-TRAIL-IETD-MnSOD, whereas BEAS-2B cells showed little cell death after infection with any of the viruses (Figure 3a). Among all lung cancer cells, CD55-TRAIL-IETD-MnSOD exhibited the most cytotoxic effect in A549 cells. Furthermore, cytopathic effect was examined in A549 cells by staining with crystal violet. CD55-TRAIL-IETD-MnSOD induced the most obvious cytopathic effect in tumor cells compared with the CD55-TRAIL or CD55-MnSOD or their combination (Figure 3b, left). Moreover, there was no distinct change in BEAS-2B cells after being transduced with different viruses (Figure 3b, right), suggesting that the CEA-regulated dual-gene-targeting oncolytic virus may not be toxic to normal cells.

Selective induction of cell apoptosis by CD55-TRAIL-IETD-MnSOD

To further investigate whether the mechanism of the novel construct OA is mediated by apoptotic induction, we first performed the Hoechst assay to detect morphologic change of apoptosis on A549 cells. The result indicated that treatment with different viruses, especially the treatment with CD55-TRAIL-IETD-MnSOD, resulted in apoptosis, characterized by chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies (Figure 4a). Inspiringly, there was little apoptosis

Figure 2. Characterization of oncolytic adenovirus CD55-TRAIL-IETD-MnSOD. **(a)** Real-time quantitative PCR (qPCR) to detect the relative background expression of carcinoembryonic antigen (CEA) in a variety of cancer cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. **(b)** Four high-expression CEA cancer cell lines, detected by qPCR, and the normal cell QSG-7701 were infected with different viruses at the indicated multiplicity of infection (MOI; 0.1, 1 and 10). After 72 h, EGFP expression was observed by a fluorescence microscope. **(c)** Western blot was used to detect the E1A, TRAIL and MnSOD expression. A549 cells were infected with various viruses at 10 MOI, with untreated cells as controls. After 48 h, cell lysates were prepared for analysis. Actin was used as protein loading control. EGFP, enhanced green fluorescent protein; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Figure 3. Tumor-selective cytopathic effect of CD55-TRAIL-IETD-MnSOD. **(a)** Four human lung carcinoma cell lines (A549, NCI-H460, NCI-H1299 and NCI-H1975) and human lung bronchial epithelial cell line BEAS-2B were infected with CD55-EGFP, CD55-MnSOD, CD55-TRAIL and CD55-TRAIL-IETD-MnSOD at the multiplicity of infection (MOI) of 10, or with CD55-TRAIL combined with CD55-MnSOD at an MOI of 5. After 24, 48, 72 or 96 h of infection, cell viability was measured by methyl thiazolyl tetrazolium (MTT) assay. There was no significant difference between each group in BEAS-2B cell lines. Data are presented as mean $\pm$ s.d. ($n=3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). **(b)** The tumor cells A549 and the normal cells BEAS-2B were infected with different viruses at the indicated MOIs; in combination therapy, the MOIs of CD55-TRAIL and CD55-MnSOD were halved. After 4 days, cells were stained with crystal violet, and results are shown with the photograph. EGFP, enhanced green fluorescent protein; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

in normal cells BEAS-2B. Similarly, apoptosis was also detected by flow cytometry assay in A549 cells (Figure 4b). Cells were infected with CD55-MnSOD, CD55-TRAIL, combined CD55-MnSOD with CD55-TRAIL (at a ratio of 1:1), and CD55-TRAIL-IETD-MnSOD at 10 MOI for 48 h. Apoptosis in the treatment group with CD55-TRAIL plus CD55-MnSOD or with CD55-TRAIL-IETD-MnSOD was much greater than other groups. Consistent with the above, western

blotting analysis showed a gradient decrease of procaspase-8, procaspase-9 and procaspase-3 in total protein when treated by CD55-EGFP, CD55-MnSOD, CD55-TRAIL, combination of CD55-MnSOD and CD55-TRAIL as well as CD55-TRAIL-IETD-MnSOD, respectively (Figure 5c). Furthermore, we observed an increased release of SOD-2 and Smac from mitochondria into the cytosol in cells treated with TRAIL/MnSOD compared with cells treated with

Figure 4. Selectively induced cell apoptosis and mechanism of action of CD55-TRAIL-IETD-MnSOD. **(a)** Cancer cells A549 (the upper two rows) and normal cells BEAS-2B (the lower two rows) were infected with (A) phosphate-buffered saline (PBS); (B) CD55-EGFP; (C) CD55-MnSOD; (D) CD55-TRAIL; (E) combination of CD55-TRAIL and CD55-MnSOD (at a ratio of 1:1); and (F) CD55-TRAIL-IETD-MnSOD at the multiplicity of infection (MOI) of 10 for 48 h; apoptosis detection was by Hoechst 33342 staining. Chromatin condensation, nuclear shrinkage or nuclei fragmentation (arrows shown) were observed under the inverted fluorescence microscope (original magnification: $\times 200$). **(b)** Flow cytometric analysis detected the percentage of apoptotic cells: treated A549 cells with different viruses at 10 MOI for 48 h; untreated group as control. Data are presented as mean $\pm$ s.d. ($n = 3$, $*P < 0.05$). EGFP, enhanced green fluorescent protein; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Figure 5. Western blot investigated the apoptosis-related protein expression. A549 cells were treated as previously described (see Figure 4 legend). The whole cytosol extracts **(a)**, mitochondrial extracts **(b)** or cell extracts **(c)** were prepared and examined by western blot analysis. Actin or COX IV was used as protein loading control.

TRAIL or MnSOD alone or their combination (Figure 5a). The decrease of Bax or Bid in the cytosol (Figure 5a) and an obvious increase of Bax translocation from cytosol to mitochondria in the same group was also detected (Figure 5b). These findings suggest that the apoptosis resulting from TRAIL/MnSOD may depend on coordination of the caspase amplification loop and the mitochondrial pathway

Antitumoral efficacy of CD55-TRAIL-IETD-MnSOD *in vivo*

The data from the *in vitro* experiment indicated that CD55-TRAIL-IETD-MnSOD could cause lung carcinoma cell apoptosis and killing. The antitumor efficacy of CD55-TRAIL-IETD-MnSOD was also investigated in the A549 xenograft model in nude mice. As the tumor growth volumes show (Figure 6), the CD55-TRAIL-IETD-MnSOD treatment group had a significantly stronger antitumor effect

Figure 6. Antitumoral efficacy of CD55-TRAIL-IETD-MnSOD *in vivo*. A549 cells (5×10^6) were injected subcutaneously into female BALB/c nude mice to establish the tumor xenograft model. When the tumors grew to 80–120 mm³ in size, mice were assigned to six groups ($n=8$) at random. Different adenoviruses (5×10^8 plaque-forming units (PFUs)) or phosphate-buffered saline (PBS) were injected once every other day for a total of four times. Tumor size was measured by the Vernier caliper every 5 days. The tumor volume (mm³) = $1/2$ (length $\times$ width²). Data are presented as mean $\pm$ s.d. ($n=8$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

compared with CD55-TRAIL ($P < 0.05$) or CD55- MnSOD ($P < 0.01$), and the antitumor effect of CD55-TRAIL treatment group was similar to the combination group, CD55-TRAIL and CD55- MnSOD.

Furthermore, to detect the cell death and TRAIL expression in tumor tissues, we performed TUNEL, hematoxylin and eosin staining and IHC assays on day 7 after viral injection. TUNEL staining indicated that the CD55-TRAIL-IETD-MnSOD treatment group induced much more apoptosis than the others, and the combined group of CD55-TRAIL with CD55-MnSOD was observed to induce greater apoptosis than CD55-TRAIL or CD55-MnSOD alone (Figure 7a). Treatment with CD55-EGFP just showed a little apoptosis and no changes in the PBS group. Hematoxylin and eosin staining demonstrated that CD55-TRAIL-IETD-MnSOD caused more severe cytopathic effects than the other viruses (Figure 7a). Meanwhile, hematoxylin and eosin staining for some tissues, liver, kidney and spleen showed no obvious toxicity, implying an undetectable level of cell damage and apoptosis (Figure 7b). In addition, IHC staining demonstrated an enhanced expression of TRAIL in tumor tissues after treatment by both CD55-TRAIL-IETD-MnSOD and by CD55- MnSOD (Figure 7a). Thus, MnSOD could promote the expression of TRAIL protein *in vivo*.

Furthermore, transmission electron microscopy was also used to detect the representative morphological changes in tumor masses (Figure 8). All the treatment groups resulted in loss of tumor cell architecture except PBS, such as the appearance of nucleus deformation, nuclear collapse and chromatin agglutinated in lumps at the edge of nuclear envelope. However, the CD55-TRAIL-IETD-MnSOD group induced significant late apoptosis: vesicles from cytomembrane budding, increased blebbing of cytoplasm. All the results *in vivo* demonstrated that CD55-TRAIL-IETD-MnSOD could markedly suppress tumor growth and promote tumor cell apoptosis but induce no damage within normal tissues.

DISCUSSION

In this study, the dual-regulated oncolytic adenovirus vector CD55 that can replicate and induce tumor-specific cytotoxicity in CEA-positive lung cancer cell lines with p53 dysfunction was designed based on CTGVT strategy. It has been suggested that the

antitumor effect of TRAIL is limited by overexpression of inhibitors of apoptosis, tumor resistance to TRAIL, side effects and so on.²⁷ Our previous study showed that the combination of MnSOD and TRAIL gene virotherapy could completely eliminate colorectal tumor xenografts, with their synergic effect mediated by their two separate oncolytic adenoviruses.²³ Thus, in this report, we constructed a novel CEA-regulated dual-targeting oncolytic adenovirus harboring both the TRAIL and MnSOD genes (CD55-TRAIL-IETD-MnSOD). In this construct, the four-peptide sequence encoding a caspase-8 cleavage site (IETD) was applied to mediate the double gene of TRAIL and MnSOD expression. We showed that this leads to equivalent amounts of TRAIL and MnSOD because caspase-8 induced by TRAIL can cleave the TRAIL-IETD-MnSOD fusion protein.^{8,9,28} What is more, our group found that the cleaved TRAIL-IETD did not affect the function of TRAIL,⁹ suggesting the potent applied value of OV-mediated TRAIL-IETD gene, where the gene has a synergetic function with TRAIL.

Based on CTGVT-DG, our previous numerous studies found that the combined use of ZD55-TRAIL and ZD55 gene (K5, Smac, MnSOD, interleukin-24 and so on) have achieved complete suppression of xenograft tumors in colorectal cancer and hepatoma.^{8,9,23,29–33} Furthermore, our current data indicated that the treatment with CD55-TRAIL-IETD-MnSOD exerted much better antitumor effect than that of CD55-TRAIL alone, CD55-MnSOD alone or CD55-TRAIL plus CD55-MnSOD with halved dose, either as detected by MTT or crystal violet staining *in vitro* (Figure 3) or in mice *in vivo* (Figure 6). Our results further proved that CD55-TRAIL-IETD-MnSOD induced stronger lung cancer-specific cell apoptosis and caspase expression than CD55-TRAIL, CD55-MnSOD alone or their combination, as shown in Figure 5. Moreover, we found that injection of CD55-TRAIL-IETD-MnSOD did not cause obvious normal cell death *in vitro*, or hepatotoxicity, renal toxicity and spleen toxicity *in vivo* (Figure 7b), as detected by MTT methods and hematoxylin and eosin staining, respectively. The above data showed that our novel construct is an excellent candidate anticancer agent for lung cancer trials.

The use of the CEA promoter in our OVs is also novel. Currently, the core challenges in CTGVT strategy are the control of vector safety and choice of effective anticancer genes. Although the

Figure 7. CD55-TRAIL-IETD-MnSOD induced cell death *in vivo*. Histological analysis of tumor sections or tissues from various treatments of A549 tumor xenografts. Samples were harvested at 7 days after infection. **(a)** The upper row shows TUNEL staining for detecting the apoptosis of tumor section; the middle row shows hematoxylin and eosin (HE) staining for cell necrotic areas in tumors; the lower row shows IHC staining for TRAIL expression of tumors tissues after transduction with different oncoviruses. **(b)** HE staining to detect toxicity of liver, kidney or spleen (original magnification: $\times 200$). IHC, immunohistochemistry; MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP biotin nick end labeling.

CTGVT strategy has achieved excellent anticancer effects in the past decades by using an oncolytic adenovirus as the vector that could proliferate and replicate in tumor cells and cause the release of the progeny virus to infect, disrupt and kill more tumor cells,^{31–33} it also lacks the tumor specificity for some cancer types. For example, there is a lack of marker with high specificity for human lung cancer. Recent literature reported that CEA is positively expressed at high frequencies in lung cancer tissues, but not in normal lung tissues.^{14,34} Moreover, postoperative serum CEA level is a more significant prognostic factor than post/preoperative level in non-small-cell cancer patients.³⁵ The results suggested that CEA gene expression is closely related to lung cancer and provides a relatively broad spectrum of molecular targets for lung cancer gene therapy. Our data also showed that there is higher CEA mRNA expression in lung cancer cell lines than in normal cells (Figure 2). The CEA promoter was cloned to regulate the luciferase reporter gene in lung cancer cell lines, and promoter activity was detected by dual-luciferase reporter assay kit (data not shown). Therefore, we constructed a novel oncolytic adenovirus vector CD55 using the CEA promoter to regulate the adenovirus E1a gene. CD55-mediated EGFP fluorescent reporter gene expression in cancer cell lines was shown to gradually increase in a dose-dependent manner. However, in normal cell lines, low EGFP expression was observed, indicating that the novel

OV vector brought tumor-specific proliferating and replicating properties. Thus, specific OV proliferation and targeting expression of anticancer genes in lung cancer cells can be achieved using the CEA promoter to regulate the adenovirus vector carrying the TRAIL-IETD-MnSOD gene with low side effects in normal cells.

In the past few years, an increased interest has been focused on oncolytic virotherapy that made great progresses in the treatment of some malignant diseases.³⁶ Besides the oncolytic adenovirus, the OVs have now expanded to include herpes simplex virus, vaccinia virus, coxsackie virus, respiratory and enteric orphan virus (reovirus), measles virus and so on. In particular, the first oncolytic virus agent (talimogene laherparepvec (T-VEC)) was authorized by the FDA (US Food and Drug Administration) and EFSA (European Food Safety Authority) because T-VEC (genetically modified herpes simplex virus expressing granulocyte-macrophage colony-stimulating factor) improves durable response rate in patients with advanced melanoma phase III trial.³⁷ In addition, many trials of OVs have focused on clinical end points, such as safety, response rate and overall survival, indicating a good prospect.³⁶ Another significant enthusiasm for cancer therapy is precision medicine, cell and immunotherapy. Cancer immunotherapy, virotherapy and gene therapy were added as a fourth weapon after surgery, radiation and chemotherapy, with the intention of also decreasing risks of high mortality and/or causing adverse side

Figure 8. Morphological observation of A549 xenograft tumor tissue by transmission electron microscopy (TEM). Some obvious apoptotic phenomena, such as nucleus deformation, nuclear collapse and chromatin agglutinated in lumps at the edge of nuclear envelope, were observed in the treatment group but not in the control phosphate-buffered saline (PBS)-treated group. However, the CD55-TRAIL-IETD-MnSOD group appeared to show significant late-stage apoptosis, as vesicles from cytomembrane budding and increased blebbing of cytoplasm. MnSOD, manganese superoxide dismutase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

effects. Successful clinical trials of CAR-T,³⁸ anti-CTLA-4 and anti-PD-1,³⁹ and anti-PD-L1⁴⁰ therapy demonstrate such promising results in cancer patients that cancer immunotherapy was selected as the Breakthrough of the Year for 2013 and 2014. Of course, our OV_s also may exert efficient cancer immunotherapy effect by carrying immune-associated genes, such as granulocyte-macrophage colony-stimulating factor.

Although our novel CEA-regulated dual-targeting OV_s have obtained strong antitumor effect in lung cancer cells and in animal models, there are still challenges requiring additional development. The cytotoxicity is still low in lung cancer cells after treatment by CD55-TRAIL-IETD-MnSOD, and it was also not able to completely eradicate tumor xenografts despite CD55-mediated TRAIL and MnSOD efficiently suppressing tumor growth in mice. These disadvantages might be mainly caused by host immune responses to viruses⁴¹ and the drug resistance to oncolytic viruses.⁴² Thus, it is urgent to solve these issues and achieve the significant therapeutic efficacy through combining conventional chemotherapy or radiotherapy as well as immunotherapy in future cancer therapy.⁴³

In conclusion, our successfully engineered OV CD55, with the CEA promoter regulating the targeting oncolytic adenovirus vector, has strong elements regarding both the safety and effectiveness of our CTGVT strategy. This double-targeted CD55 can replicate specifically in CEA-positive lung cancer cells and can efficiently mediate dual anticancer gene TRAIL-IETD-MnSOD expression to maximize the oncolytic effect and suppression of tumor growth and minimize the side effects on normal cells and tissues. Thus, it has broad applications and can become an effective anticancer drug candidate for lung cancer therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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