Original Article Killing effect of TNF-mediated by conditionally replicating adenovirus on esophageal cancer and lung cancer cell lines

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Abstract: Objective: The killing effect of TNF mediated by conditionally replicating adenovirus SG502 on human cancer cell lines was assessed by in vivo and in vitro experiments. Methods: The recombinant adenovirus SG502-TNF was used to infect human lung cancer cell line A549 and human esophageal cancer cell line TE-1. The expression of the exogenous gene and its inhibitory effect on the tumor cell lines were thus detected. Tumor transplantation experiment was performed in mice with the purpose of assessing the inhibitory effect of the adenovirus on tumor cells and tumor formation. The targeting of the adenovirus and the mechanism of tumor inhibition were discussed by in vivo imaging technology, HE staining and TUNEL assay. Results: Recombinant adenovirus SG502-TNF targeted the tumor cells specifically with stable expression of TNF, which produced a killing effect on tumor cells by regulating the apoptotic signaling pathway. Conclusion: Recombinant adenovirus SG502-TNF possessed significant killing effect on TE-1 cells either in vivo or in vitro. This finding demonstrated the potential clinical application of adenovirus SG502.

Keywords: Adenovirus, tumor necrosis factor, cancer cells

Introduction

Tumors are malignancies that pose serious threat to human health. The conventional therapies for tumors such as surgery, chemotherapy and radiotherapy cause great injury to human body, and none of them can ensure radical cure of the tumors [1]. Genetic therapy for tumors is gaining popularity in recent years, and the rationale is to transfer exogenous genes into the target cells of the human body. The success of genetic therapy depends on the use of safe, reliable and convenient gene transfer method [2, 3]. Since tumor occurrence and progression are the results of combined actions of multiple genes, genetic therapy that aims to revive the functions of one or several tumor suppressor genes can hardly work in practice [4]. As more knowledge is gained concerning the structure and functions of viral genes, it is now possible to engineer the viruses genetically. Breakthrough has been made in the application of viruses to tumor treatments, including the oncolytic viruses that target the tumor cells and cause no damage to normal cells.

Oncolytic viruses that have been developed and currently under clinical trials are adenovirus (AV), Newcastle disease virus (NDV), Herpes simplex virus-1 (HSV-1) and Reovirus (RV). Among them, adenovirus is the most thoroughly researched with respect to its gene structure and expression regulation [5-8]. Adenovirus is a non-integration virus with several subtypes. Compared with other viral vectors, adenovirus is safer and has lower pathogenicity and larger capacity to carry exogenous genes [9].

Tumor necrosis factor (TNF) is the most potential bioactive factor ever discovered. Besides directly killing or inhibiting tumor cells, TNF mediates the cytotoxic effect of monocytes and macrophages. However, the defects such as short half-life and severe toxic and side effects restrict its application [10]. We assessed the inhibitory effect of recombinant adenovirus SG502-TNF that was capable of expressing TNF on human lung cancer cells and human esophageal cancer cells in vivo and in vitro. On this basis we evaluated the feasibility of applying this recombinant virus to clinical trials.

 Table 1. Primers sequences of RT-PCR

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Primers	Sequences (5'-3')
hTERT F	GCTGCTCAGGTCTTTCTTTTATG
hTERT R	CAAGTGCTGTCTGATTCCAATG
hTNF F	CACGCTCTTCTGCCTGCTG
hTNF R	GGCTTGTCACTCGGGGTTC
h actin F	TGACGTGGACATCCGCAAAG
h actin R	CTGGAAGGTGGACAGCGAGG

Materials and methods

Cell lines and cell culture

Human lung cancer cell line A549 and human esophageal cancer cell line TE-1 were purchased from Cell Bank of Shanghai Institutes for Biological Sciences. The cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 1% Penicillin/Streptomycin (Gibco) and 10% FBS (Gibco) and placed in humidified incubator at 37°C with 5% CO₂.

Titration of recombinant adenovirus

SG502-TNF containing luciferase reporter gene and conditionally replicating recombinant adenovirus SG502-GFP were prepared by our team. A549 cells and TE-1 cells were inoculated to a 96-well plate at a density of 8-10×10³ cells per well. After culture in a CO₂ incubator for 24 h, the cell convergence reached 60% approximately. The recombinant adenovirus was subjected to ten-fold serial dilution in RPMI 1640 medium, with the addition of 100 µl viral dilution into each well (10⁻¹-10⁻⁷). Eight replicates were set up for each gradient, and blank control groups were established. After culture for 48-96 h in an incubator, the expression of GFP and the cytopathic effect (CPE) were observed under the inverted fluorescence microscope. The 50% tissue culture infectious dose (TCID50) was calculated using Reed-Muench method. Then A549 cells and TE-1 cells were infected by SG502-TNF and SG502-GFP at 10 TCID50/ml.

Detection of cell proliferation by MTT assay

The tumor cells infected by recombinant adenovirus for 24 h were digested and inoculated to a 96-well plate at the density of 1×10^4 cells per well. Cell culture continued for 24 h with 5 replicates for each group. Then 20 µl 5 mg/ml MTT solution (Sigma, USA) was added into each well, and the cell culture continued for another 4 h. The culture medium was discarded, 150 µl DMSO was added into each well, and the cells were oscillated on a shaker at room temperature at low speed for 10 min. The absorbance was detected using a microplate reader at the wavelength of 490 nm, with the setting of blank wells by adding DMSO.

HE staining

At 7 days after infection, the cells were digested and adjusted to a density of 2×10^4 cells/ml. The coverslips were prepared and fixed in 4% paraformaldehyde for 20 min. After HE staining and air drying, the coverslip was sealed with neutral balsam. For in vitro experiment, the tissues were fixed in 4% paraformaldehyde, subjected to serial alcohol dehydration, transparentized with dimethylbenzene, embedded in paraffin and sectioned. HE staining was performed, followed by serial alcohol dehydration, transparentization in dimethylbenzene and sealing with neutral balsam.

Detection of mRNA expression of TERT and TNF

At 24 h after infection, the A549 and TE-1 cells were digested and centrifuged. Total RNA was performed using TRIzol reagent (Qiagen, USA). For tissues infected in vivo, 1 ml TRIzol was added for 50-100 mg of tissues to prepare homogenate, which was then centrifuged at 4°C at 12000 g for 10 min with insoluble discarded. Total RNA was extracted, and the extracted RNA was reverse transcribed into cDNA. The mRNA expressions of telomerase revere transcriptase (TERT) and tumor necrosis factor (TNF) were detected by using SYBR green I PCR (Eppendorf). The sequences of primers are shown in Table 1, with PCR conditions set as follows: 94°C 4 min, 94°C 20 sec, 60°C 30 sec, 72°C 30 sec, 35 cycles, 3 replicates for each sample.

TERT and detection of protein expression of TNF

At 24 h after infection, A549 cells and TE-1 cells were digested and centrifuged. The cells were washed with PBS once and added with RIPA lysis buffer. Then the cells were vortex oscillated, resuspended and placed in ice bath



Figure 1. Fluorescence detection after infections with recombinant adenovirus in A549 and TE-1 cells.



Figure 2. Observation of CPE after infections with recombinant adenovirus in A549 and TE-1 cells.

for 5 min. The tissues infected in vivo were cut into 1 mm² pieces and homogenized by adding RIPA lysis buffer. The lysed cells or tissues were centrifuged at 4°C at 12000 g for 5 min, with supernatant collected for SDS-PAGE and Western Blot detection. The gels were scanned and processed with Labworks4.6 software (UVP) to calculate the gray value of the target bands.

Tumor transplantation experiment

BALB/C nude mice aged 4-6 weeks were purchased from Experimental Animal Center of Third Military Medical University, regardless of gender. They were randomly divided into three groups, blank group, control group and experimental group, with 4 mice in each group. TE-1 cells were digested, washed with PBS buffer twice and adjusted to a density 1×107 cells/ ml. Subcutaneous inoculation was performed to the axilla with the amount of 1×106 cells. For other groups, PBS, SG502-GFP and SG502-shRNA/ TNF were injected into the tail vein, respectively. The short and long diameters of the tumors were measured every 3 days after inoculation, and the tumor growth curve was plotted. When the signs auguring death occurred (e.g., arched back and restricted mobility), the mice were sacrificed by cervical dislocation to harvest the tumors.

binant adenovirus							
Cells	Group	OD1	0D2	0D3	OD4	0D5	Average
TE-1	Mock	0.4802	0.4998	0.5194	0.503	0.5182	0.50412
	GFP	0.4812	0.476	0.4667	0.4752	0.4831	0.47644
	TNF	0.4233	0.4371	0.4252	0.4495	0.4597	0.43896
A549	Mock	0.5343	0.5127	0.5306	0.5125	0.5247	0.52296
	GFP	0.4936	0.4822	0.4805	0.4898	0.499	0.48902
	TNF	0.4198	0.4182	0.4233	0.4146	0.4271	0.4206

Table 2. Results of MTT assay in two cells after infection with recom-



with HRP-conjugated streptavidin working solution at 37°C for 30 min. DAB substrate was added for color development in the dark for 10 min. The tissues were counterstained with hematoxylin for 1 min and washed with water to restore the blue color before serial alcohol dehydration and transparentization in dimethylbenzene. The slides were sealed with neutral balsam, and one drop of PBS or glycerol was added. The cell apoptosis was observed under the optical microscope.

In vivo imaging

Intraperitoneal injection of 7% chloral hydrate (0.5 ml/100 g) and D-luciferin (150 mg/kg, XEnogen) was performed. The mice were anesthetized and scanned by Image Station In-Vivo FX (Kodak) to determine the fluorescence level.

Statistical analysis

Replicates were set up for each experiment. Statistical analysis was performed using SPSS 10.0 software. The means of the two samples were compared with t-test.

Figure 3. HE staining of two cells after infection with recombinant adenovirus.

Results

Apoptosis detection by TUNEL assay

After sectioning and transparentization in dimethylbenzene, the activity of endogeneous peroxidase was destroyed by adding $3\% H_2O_2$. The permeability was increased with Tris-EDTA containing 20 µg/ml proteinase K. Then TdT enzyme and biotin-conjugated dUTP were added along with TUNEL reagent to initiate reaction for 1 h at 37°C in the dark wet box. After the reaction was terminated, $3\% H_2O_2$ was added to destroy the activity of endogenous peroxides. The cells were further incubated

Infection with and titration of recombinant adenovirus

A549 cells and TE-1 cells were infected by SG502-TNF and SG502-GFP, respectively, and distinct fluorescence was observed after infection with SG502-GFP but not with SG502-TNF (**Figure 1**). Both cancer cell lines showed obvious CEP after infections with either recombinant adenovirus (**Figure 2**). According to the calculations with Reed-Muench method, the titer of the two viruses in two cells did not differ significantly. The average measured titer in the



Figure 4. Dissolution curves of products of fluorescence quantitative PCR.

two cells was defined as the actual titer. At 48 h after infection, the titer was $3.8{\times}10^4$ and $3.8{\times}10^5$ TCID50/ml, respectively; at 96 h, the

titer was 2.6×10^5 and 2.6×10^6 TCID50/ml, respectively; the titers at 96 h were taken as the final result.

Cells	Group	Actin	TERT	⊿Ct (TERT)	TNF	⊿Ct (TNF)
TE-1	Mock	20.133	29.698	9.565	24.738	4.606
	GFP	21.406	29.238	7.832	25.656	4.251
	TNF	22.187	27.144	4.957	20.080	-2.108
A549	Mock	19.707	27.401	7.694	23.580	3.874
	GFP	20.152	26.524	6.373	23.519	3.367
	TNF	22.450	26.281	3.831	18.999	-3.451

Table 3. Relative quantification of mRNA expression of TERT and TNFafter infections with recombinant adenovirus



Figure 5. Detection of protein expressions of TERT and TNF after infections with recombinant adenovirus in two cells. Note: 1, 2 and 3 indicate mock, GFP and TNF treatment groups in TE-1 cells, respectively; A, B and C indicate mock, GFP and TNF treatment groups in A549 cell, respectively.



Before treatment

After treatment

Figure 6. In vivo imaging of mice inoculated with TE-1 cells and recombinant adenovirus.Note: From left to right, they are mock group, GFP group and TNF group.

In vitro inhibition of cell proliferation by infection with SG502-TNF

The results of MTT assay are shown in **Table 2**. It can be seen that the cell proliferation ability did not change significantly after infection with SG502-GFP as compared with the normal A549 and TE-1 cells (P>0.05). The proliferation was reduced greatly after infection with SG502-TNF (P<0.05). At 7 days after infection with the dose of 10 TCID50/ml, HE staining of the coverslips indicated that both two cells were lightly stained with cytoplasmic shrinkage as compared with the normal cells. This indicated cell apoptosis (**Figure 3**).

Changes of TERT and TNF expressions after in vitro infection with SG502-TNF

Dissolution curves of the products of fluorescence quantitative PCR indicated that TERT, TNF and actin gene primers amplified the target genes specifically (Figure 4). As shown by the standard curves, the amplification efficiency of all three PCR systems was above 95%. Relative quantification indicated that with normal compared tumor cells, the infection with SG502-GFP did not lead to significant changes of mRNA expressions of TERT and TNF (P>0.05); however, after infection with SG502- TNF, both two cells had a marked increase of mRNA expression of TERT and TNF (P<0.05), as shown in Table 3. Western Blot and grayscale analysis indicated that SG502-GFP infection did not bring about changes of protein expression of TERT and TNF in two cells as compared with normal cells (P>0.05). After infection with SG502-TNF, the expression of TERT protein

decreased dramatically, while the expression of TNF protein increased (P<0.05) (**Figure 5**).

Influence of in vivo SG502-TNF infection on tumor transplantation in mice

TE-1 cells were inoculated to mice along with SG502-GFP and SG502-TNF so as to observe the inhibition of recombinant adenovirus on

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Figure 7. Comparison of tumor size after inoculation with TE-1 cells and recombinant adenovirus.



Figure 8. Tumor growth curves after inoculation with TE-1 cells and recombinant adenovirus.

Table 4. Relative quantification of mRNA ex-
pression of TNF in mice after inoculation with
TE-1 cells and recombinant adenovirus

Group	Actin	TNF	⊿Ct	2 ^(-⊿Ct)
Mock	19.495	21.217	1.722	0.303
GFP	20.537	21.612	1.075	0.475
TNF	20.369	20.417	0.047	0.968

tumor growth. Tumor transplantation experiment indicated that all three treatments resulted in solid tumors. According to in vivo imaging, SG502-GFP and SG502-TNF viruses were concentrated in tumors, which suggested the high targeting ability of SG502 to TE-1 cells (**Figure 6**). GFP group and control group did not differ significantly in tumor size, while the tumor size decreased obviously in TNF group, indicating specific inhibitory effect of SG502-TNF on tumors (**Figure 7**). As found by tumor growth curves, the three groups were ranked as follows in the decreasing order of tumor formation speed: control group \geq GFP group>TNF group

(Figure 8). It was confirmed by the above results that SG502-TNF targeted TE-1 cells with high specificity in vivo, inhibiting tumor growth.

Fluorescence quantitative PCR, Western Blot and grayscale analysis of the tumors indicated that the mRNA and protein expression of TNF in TNF group were higher as compared with the other two groups (**Table 4**; **Figure 9**). HE staining revealed that some cells in TNF group were

deeply stained in the nuclei with cytoplasmic shrinkage and chromatin aggregation (**Figure 10**). This observation indicated cell apoptosis. TUNEL assay found that the number of positively stained brown cells in TNF group was much higher than that of the other two groups (**Figure 11**). It was inferred based on the above results that SG502-TNF infection inhibited TE-1 cell growth and tumor formation probably by promoting cell apoptosis.

Discussion

Viral therapy for tumors is based on high targeting ability, infection efficiency and regulatory capacity of viruses. Genetic engineering of wild-type adenovirus is now possible, typically involving the replacement of natural promoters with tumor-specific promoters or modification of viral structural proteins. These manipulations can regulate the targeting of viruses in hosts, thus weakening the replication of viruses in normal cells while enhancing the replication in tumor cells. So far oncolytic viruses with



Figure 9. Detection of TNF protein expression in mice after inoculation with TE-1 cells and recombinant adenovirus. Note: A1, A2, B1, B2, C1 and C2 indicate the two samples of mock group, GFP group and TNF group, respectively.



Figure 10. HE staining of tumors in mice after inoculation with TE-1 cells and recombinant adenovirus.



Figure 11. TUNEL assay of tumors in mice after inoculation with TE-1 cells and recombinant adenovirus.

transcriptional targeting have been established [11-13].

Adenovirus SG502 experimented in this study was an oncolytic virus with high specificity, efficiency and safety. In vivo imaging of the infected mice showed that the viruses were concentrated in tumors. Thus adenovirus SG502 had high targeting ability to TE-1 cells. In vivo and in vitro experiments with control recombinant adenovirus SG502-GFP indicated that SG502 alone had limited inhibitory effect and pro-apoptotic effect on TE-1 cells and A549 cells. Exogenous gene carried in SG502-TNF is controlled by hTERT promoter, and the recombinant adenovirus SG502-TNF directly acts on tumor cells and mediates the intracellular expression of TNF. In this way the defects of short half-life and severe side effects with TNF are avoided. In vivo inoculation and tumor transplantation experiment in mice indicated that SG502-TNF effectively inhibited the proliferation of TE-1 cells and tumor growth. It was confirmed by HE staining and TUNEL assay using tumor tissue sections that enhanced TNF expression promoted the apoptosis of TE-1 cells. These results were evidences of high targeting specificity of adenovirus SG502 to tumor cells. TNF expressed by the recombinant adenovirus would kill the tumor cells through its regulatory effect on apoptotic signaling pathway.

Adenoviruses as vectors for gene transfer have several shortcomings. Firstly, the infection efficiency is inadequate to kill all tumor cells. The replication-defective adenoviruses can hard-

ly penetrate and diffuse in solid tumors; secondly, not enough animal models are established for experiments; thirdly, exogenous hTERT promoter is less potent than natural promoter. More importantly, such promoters are specific to only one or several tumors, while the tumor occurrence and progression are highly complex [14-17]. Most studies on oncolytic viruses have demonstrated their explicit tumor killing effect with less toxic and side effects [18]. In the future, the therapy utilizing oncolytic viruses for tumors will evolve as new advances are made in technologies of molecular biology of cancers and molecular immunology. It is reasonable to anticipate that the viral therapy will become the fourth major treatment for tumors after surgery, radiotherapy and chemotherapy.

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Disclosure of conflict of interest

None.

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