Gene therapy for human ovarian cancer cells using efficient expression of Fas gene combined with $\gamma\delta$ T cells

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Abstract. Low tissue specificity and efficiency of exogenous gene expression are the two major obstacles in tumor-targeted gene therapy. The Fas cell surface death receptor (Fas)/Fas ligand pathway is one of the primary pathways responsible for the regulation of cell apoptosis. The aim of the present study was to explore whether the regulation of tumor specific promoters and a two-step transcriptional amplification system (TSTA) assured efficient, targeted expression of their downstream Fas gene in human ovarian cancer cells, and to assess the killing effect of $\gamma\delta$ T cells on these cells with high Fas expression. Three shuttle plasmids containing different control elements of the human telomerase reverse transcriptase (hTERT) promoter and/or TSTA were constructed and packaged into adenovirus 5 (Ad5) vectors for the expression of exogenous Fas gene. The human ovarian cancer cell line SKOV3 and a control human embryonic lung fibroblast cell line were transfected with Ad5-hTERT-Fas or Ad5-hTERT-TSTA-Fas. Fas mRNA and protein expression were examined by reverse transcription-quantitative polymerase chain reaction and western blot analysis. $\gamma\delta$ T lymphocytes were isolated, cultured and mixed at different ratios with SKOV3 cells with Fas expression in order to assess the killing effect of $\gamma\delta$ T cells. hTERT promoter induced the specific expression of FAS gene in SKOV3 cells, and the TSTA strategy increased FAS expression by 14.2-fold.

The killing effect of $\gamma\delta$ T cells increased with the expression level of Fas and the effector-target cell ratio. The killing rate for SKOV3 cells with high FAS expression was 72.5% at an effector-target cell ratio of 40:1. The regulators of hTERT promoter and TSTA assure the efficient and targeted expression of their downstream Fas gene in SKOV3 cells. The killing effect of $\gamma\delta$ T cells for ovarian cancer cells with relatively high Fas expression was improved.

Introduction

Low tissue specificity and efficiency of exogenous gene expression are the two major obstacles in tumor-targeted gene therapy. It is possible to achieve specific and efficient expression of a target gene in tumor cells by the regulation of tumor-specific promoters (TSP) and two-step transcriptional amplification (TSTA) (1,2). Multiple promoters have been used in targeted gene therapy for ovarian cancer, including secretory leukocyte protease inhibitor (SLPI), the ovary-specific promoter OSP1 and the human epithelial tissue-specific promoter, transcription factor HES1. However, these promoters are neither ovarian cancer-specific nor epithelial-specific, and are often active in normal cells (3). The human telomerase reverse transcriptase (hTERT) promoter is only activated in ovarian cancer cells exhibiting high telomerase activity, and therefore is a good candidate TSP for gene therapy to treat ovarian cancer.

The selection of efficient target genes and vectors is crucial for gene therapy. The Fas cell surface death receptor (Fas) gene promotes cell apoptosis through multiple pathways. The Fas/Fas ligand (FasL) pathway is associated with not only the occurrence and development of cancer but also the chemosensitivity of cancer cells to certain chemotherapy reagents (4). Furthermore, since the Fas/FasL pathway is one of the primary pathways responsible for the regulation of cell apoptosis, immune effector $\gamma\delta$ T cells expressing FasL specifically target and kill cells expressing Fas. However, Fas is expressed at low levels or is not expressed at all in certain ovarian cancer cells, leading to reduced Fas-mediated cell apoptosis and drug resistance of these cells. Increasing the intracellular Fas level

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may be an efficient approach for gene therapy to treat ovarian cancer. Nevertheless, the exogenous gene is often expressed at low levels at target tumor sites.

In the present study, recombinant adenoviral vectors Ad5-hTERT-Fas and Ad5-hTERT-TSTA-Fas expressing Fas under the regulation of the hTERT promoter and a TSTA system were constructed in order to study the regulation of the hTERT promoter and TSTA system on Fas expression in the human ovarian cancer cell line SKOV3. The effect of $\gamma\Delta$ T cells on SKOV3 cells with Fas expression was also studied at different effect-target cell ratios.

Materials and methods

Materials. The plasmids pBCVP2G5-luc-NSN, carrying galectin 4 (GAL4)VP2 fusion protein and G5E4TATA elements, and pBTdel279, carrying the hTERT core promoter, were provided by Dr. Yue Song, ShengJing Hospital of China Medical University (Shenyang, China). The plasmid vector pMD18-T was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The shuttle plasmid pDC316 and recombinant adenovirus backbone plasmid pBHGloxde-IE13cre were purchased from Applied Genetic Technologies Corporation (Alachua, FL, USA). The AdEasy adenoviral vector systems were purchased from Applied Biological Materials, Inc. (Richmond, BC, Canada). The HEK293 cells used for co-transfection were purchased from Applied Genetic Technologies Corporation. Antibodies used for the detection of $\gamma\Delta$ T cell subsets and anti-human FasL-IgG1 monoclonal antibody (catalog no. MA5-17073) were purchased from eBioscience, Inc. (San Diego, CA, USA). Reagents for western blot analysis were purchased from Applygen Technologies, Inc. (Beijing, China). The antibodies used for western blotting were a mouse anti-human Fas monoclonal antibody (Abcam, Cambridge, UK; catalog no. 15285; 1:500) and a β -actin monoclonal antibody (Abcam; catalog no. ab8226; 1:1,000). The quantitative real time-polymerase chain reaction (PCR) kit was purchased from Takara Biotechnology Co., Ltd.

Cell lines. The human ovarian carcinoma cells (SKOV3) and the control human embryonic lung fibroblast cells (HELFI) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SKOV3 and HELFI cells were cultured in Dulbecco's modified Eagle's medium, Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Construction of recombinant plasmid vectors carrying the hTERT promoter and/or TSTA regulatory elements and the Fas gene. pBCVP2G5-luc-NSN, pBTdel279, pCDNA3-Fas carrying the Fas gene and the shuttle plasmid pDC316 were transfected into JM109 *Escherichia coli* competent cells (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) using the Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following amplification, the bacterial plasmids were extracted and purified using the Wizard Plus SV Minipreps DNA Purification

System (Promega Corporation, Madison, WI, USA). Verification of pBTdel279 was performed using plasmid DNA as a template, and target fragments were sequenced using the primer pair 5'-TTG ATA TCG ACC CCC GGG TCC GCC CGG AGC A-3' and 5'-CTG AAT TCG CTG CCT GAA ACT CGC GCC GCG AG-3' containing EcoR V/EcoR I restriction enzyme sites. Verification of pCDNA3-Fas was performed using plasmid DNA as a template and the T7 promoter sequence 5'-TAA TAC GAC CTA CTA TAG GG-3' as a primer. Starting prior to the insertion site of the Fas gene, the target fragment was sequenced using the terminal ending method. At the same time, given that the insertion of Fas gene has Xba I and Kpn I enzyme sites, a fragment of ~1,000 bp was obtained following Xba I (catalog no. R0145, New England BioLabs, Inc., Ipswich, MA, USA) and Kpn I enzyme (catalog no. R0142; New England BioLabs, Inc.) digestion. Verification of pBCVP2G5-luc-NSN was performed as follows: Analysis of the plasmid profile revealed that it is possible to obtain two fragments of 2,284 and 6,487 bp, respectively, following enzyme digestion using Nhe I (catalog no. R0131S; New England BioLabs, Inc.) and Bsu36I (catalog no. R0524S; New England BioLabs, Inc.). For enzyme digestion, PCR products were digested with XbaI and Kpn I (10 U) or Nhe I and Bsu36I (10 U) for ≥ 3 h in 50 μ l and purified with the Qiagen PCR purification kit according to the manufacturer's protocol. For the ligation reactions, the purified vector and PCR fragments were incubated overnight at 16°C using T4 DNA ligase (Thermo Fisher Scientific, Inc., catalog no. EL0012). The insert: Vector ratio was 3:1. 1 μ l (400 U/ μ l) T4 ligase and 2 μ l 10 ligation buffer were used in a total volume of 20 μ l. pDC316-hTERT and pDC316-G5E4T were constructed using the restriction enzymes in a total volume of 20 μ l, as recommended by the manufacturer (New England BioLabs, Inc.) and ligation reactions with DNA ligase (Thermo Fisher Scientific, Inc., catalog no. EL0012). The shuttle plasmid pDC316-hTERT-Fas, pDC316-hTERT-GAL4VP2 and pDC316-G5E4T-Fas were also constructed. Construction of shuttle plasmids was performed as follows: The plasmid target sequence was used as a template, and primer pairs containing corresponding restriction enzyme sites were used to amplify the target sequence by PCR using Hercules II Fusion DNA Polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA) (Table I). The PCR product of target sequence fragments was electrophoresed and collected using the Takara Agarose Gel DNA Purification kit (Takara Biotechnology Co., Ltd), and then ligated into the connection vector pMD18-T using Solution I of the Takara DNA Ligation kit (Takara Biotechnology Co., Ltd). The connection vector was used to transfect competent JM109 cells (Sigma Aldrich) using the Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and bacterial colonies were selected for clone culture. For verification of the target sequence of the pMD18-T vector, the RV-M sequence of 5'-GAG CGG ATA ACA ATT TCA CAC AGG-3' and the M13-47 sequence of 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3' were used as primers to amplify positive clones by PCR. The obtained connection vector plasmid containing the target sequence was digested using restriction enzymes *EcoRV* (catalog no. R0195T, New England BioLabs, Inc.) and *EcoR I*, (catalog no. B0101S, New England BioLabs, Inc.) corresponding to the enzyme sites in the PCR primers, and

Table I. Construction of shuttle plasmids.

Plasmid	pDC316-hTERT	pDC316-hTERT-Fas	pDC316-hTERT-GAL4VP2	pDC316-G5E4T	pDC316-G5E4T-Fas
Target Sq	hTERT promoter	Fas gene	GAL4VP2+PA gene	G5E4T fragment	Fas gene
Template plasmid	pBTdel279	pCDNA3-Fas	pBCVP2G5-luc-NSN	pBCVP2G5-luc-NSN	pCDNA3-Fas
PCR primers of target Sq (contain corresponding restriction enzyme sites)	Forward: 5'-TTGAT ATCGACCCCGGG TCCGCCCGGAGCA-3' Reverse: 5'-CTGAATT CGCTGCCTGAACT CGCGCCGCGAG-3' ^a	Forward: 5'-TATGAAT TCGCCGCCACCATGC TGGGCATCTGGAC-3' Reverse: 5'-GCTGAGCT CTAGACCAAGCTTTG GATTTC-3'	Forward: 5'-GTA GATCTGAAAGCTAG CCTCCTGAAAGATG-3' Reverse: 5'-TAGTCGACT AGTGGGCCGCGATC CAGACAT-3'	Forward: 5'-TAGAT ATCAGGTGACACT ATAGAATACAAG-3' Reverse: 5'-GTGAAT TCAACAGTACCGG AATGC-3'	Forward: 5'-TATGA ATTCGCCGCCACC ATGCTGGGCATCTGGAC-3' Reverse: 5'-GCTGAGCTCTA GACCAAGCTTTGGGATTTC-3'
Restriction enzymes to cut target Sq	EcoR V & EcoR I	EcoR I & Sac I	Bgl II & Sal I (Not I-Spe I)	EcoR V & EcoR I	EcoR I & Sac I
Verification of target Sq	Sequencing using RV-M/M13-47 primer pair	Sequencing using RV-M/M13-47 primer pair	Sequencing using P1 primer 5'-AAGTGC GA CATCATCATC-3'	Sequencing using M13-47 primer	Sequencing using RV-M/M13-47 primer pair
Vector plasmid	pDC316	pDC316-hTERT	pDC316-hTERT	pDC316	pDC316-G5E4T
Restriction enzyme processing of vector plasmid	Complement terminals following Xba I digestion and cut MCMV promoter using EcoR I following ethanol precipitation	EcoR I & Sac I	Bgl II & Sac I	Complement terminals following Xba I digestion and cut MCMV promoter using EcoR I	EcoR I & Sac I
Verification of constructed plasmid	Sequencing using PCR primers and enzyme cutting using EcoR V/EcoR I	Sequencing using P3 primer 5'-CAGTCTTCCTC AATTCCA-3'	Sequencing using P2 primer 5'-TTCTAGCCTTGATT CCAC-3' and enzyme cutting using Xba I/EcoR I	Sequencing using PCR primers	Sequencing using P3 primer

^aPrimer pair used for sequencing verification of plasmid pBTdel279. hTERT, human telomerase reverse transcriptase; GAL4, galectin 4; Fas, Fas cell surface death receptor; PCR, polymerase chain reaction; Sq, sequence.

Table II. Summary of target gene identification, viral titer and TCID₅₀ of 3 recombinant adenoviruses.

Adenovirus	Viral titer (VP/ml)	Target gene identification by PCR	TCID ₅₀ (IU/ml)
Ad5-htert-GAL4VP2	1.4x10 ¹¹	Correct	5.0x10 ⁹
Ad5-htert-Fas	3.8x10 ¹¹	Correct	3.9x10 ⁹
Ad5-G5E4T-Fas	2.7x10 ¹¹	Correct	5.6x10 ⁹

TCID₅₀, tissue culture infective dose; PCR, polymerase chain reaction; hTERT, human telomerase reverse transcriptase; GAL4, galactin 4; Fas, Fas cell surface death receptor.

the target sequence was electrophoresed and collected. At the same time, the vector plasmid was digested using corresponding restriction enzymes (*EcoRV*, catalog no. R0195T and *EcoR I*, catalog no. B0101S, New England BioLabs, Inc.), and then electrophoresed and collected. The target sequence was ligated into the vector plasmid using a DNA ligase enzyme (Sigma-Aldrich) and the constructed plasmid was transfected using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The plasmid was collected following positive clone culture. For verification of the constructed plasmid, fragment PCR sequencing using Herculese II Fusion DNA Polymerase (Agilent Technologies) or enzyme digestion was used (using *EcoRV*, catalog no. R0195T and *EcoR I*, catalog no. B0101S, New England BioLabs, Inc.). The reaction conditions of PCR using a DNA polymerase were as follows: 1 cycle at 95°C for 5 mins; 5 cycles at 95°C for 30 sec, 58°C for 45 sec and 72°C for 3 mins; 35 cycles of 95°C 30 sec, 95°C for 45 sec, 72°C for 3 mins and a final extension at 72°C for 10 mins. The reaction conditions of enzyme digestion and ligation reactions were the same as above.

Adenoviral packaging and purification. The packaging and purification of the adenovirus vectors Ad5-hTERT-GAL4VP2, Ad5-hTERT-Fas and Ad5-G5E4T-Fas was performed using the AdEasy adenoviral vector system, according to the manufacturer's protocol. Briefly, HEK293 cells at 60-70% confluence in fresh Dulbecco's modified Eagle's medium (Sigma Aldrich) containing 10% fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Inc.) were co-transfected with recombinant shuttle plasmids (pDC316-hTERT-Fas, pDC316-hTERT-GAL4VP2 or pDC316-G5E4T-Fas) or negative control plasmid (pDC316 empty vector) (Vigene Biosciences Inc., Rockville, MD, USA) and adenovirus backbone plasmid pBGloxdelE13cre using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The plaques were scraped off 7-10 days following transfection, collected by centrifugation (5 mins at 7,000 x g at 4°C), and resuspended in PBS. The cell suspension was frozen in liquid nitrogen and thawed at 37°C 3 times. The supernatant containing recombinant adenoviruses was collected by

centrifugation at 3,000 x g for 10 mins at 4°C. Adenovirus contained in the supernatant was filtered through 0.45 μ m filter and pooled with the buffer (40 mM Tris, 2 mM MgCl₂, 5% glycerol (v/v), pH 8.0) to obtain a homogeneous adenovirus stock for purification experiments. Chromatography was performed on an AKTA Basic low-pressure liquid chromatography system. The column was equilibrated with 2 column volumes (CV) loading buffer (40 mM Tris, 2 mM MgCl₂, 0.3 M NaCl, 5% glycerol (v/v), pH 8.0) at a flow rate of 0.5 ml/min. Injection of 100 μ l of sample was followed by a linear gradient of 0.3 M to 1 M NaCl for 10 CV. The column was cleaned with 2 CV of 0.5 M NaOH and then the recombinant adenoviruses were stored at -20°C in buffer (20 mM Tris-HCl, pH 7.8, 75 mM NaCl, 2 mM MgCl₂, 5% Trehalose, 0.0025% Tween-80 in 1 l ddH₂O). The presence of the target gene in the recombinant adenoviruses was confirmed by PCR with Herculese II Fusion DNA Polymerase (Agilent Technologies), the viral titer and TCID₅₀ of each viral stock was measured (Table II).

Transfection of adenovirus. The human ovarian carcinoma cell line SKOV3 and the control human embryonic lung fibroblast cell line HELF were transfected with recombinant adenoviruses using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, SKOV3 and HELF cells at the exponential phase were digested with trypsin (100 μ g/ml; Sigma Aldrich), collected by centrifugation (5 min at 1,000 x g on 4°C), inoculated at a density of 8.0x10⁵ cells/bottle in 25 cm² flasks and incubated overnight at 37°C in an incubator with 5% CO₂. Cells were infected with 1 ml adenoviral solution at a multiplicity of infection of 100 pfu/cell. Three experimental groups were prepared including a blank control group cultivated in medium without viruses, a hTERT group transfected with Ad5-hTERT-Fas, and a TSTA group co-transfected with Ad5-hTERT-GAL4VP16 and Ad5-G5E4T-Fas.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted by the conventional TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA extract was quantified using a spectrophotometer at 260 and 280 nm. A ratio of OD₂₆₀ to OD₂₈₀ between 1.8 and 2.0 indicated clean RNA isolates. RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., catalog no. K1622) following the manufacturer's protocol. The cDNA was then used as the template for PCR using a SYBR-Green PCR Master mix kit (Takara Biotechnology Co., Ltd., catalog no. RR820) and a Bio-Rad IQTM5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Primer sequences were as follows: Forward, 5'-TGC CCA AGT GAC TGA CAT CAA-3' and reverse, 5'-GCC ATG TCC TTC ATC ACA CAA-3' for Fas; forward, 5'-AAC CCT AAG GCC AAC CGT GAA-3' and reverse, 5'-TGT CAC GCA CGA TTT CCC TCT-3' for β -actin. Relative target gene expression was quantified according to the 2^{- $\Delta\Delta$ C_q} method and normalized to β -actin as an endogenous control (5). Data are expressed as the mean $\pm$ standard deviation of three independent experiments.

Figure 1. Western blot analysis of Fas cell surface death receptor expression in SKOV3 cells in different transfection groups: 1, blank control group; 2, human telomerase reverse transcriptase group and 3, two-step transcriptional amplification system group.

Western blot analysis. The expression of Fas protein was determined by western blot analysis. Briefly, cells at the exponential phase were collected and lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma-Aldrich) for 10 min at 4°C. Total protein was collected by centrifugation (12,000 × g for 15 mins at 4°C), and quantified using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal amounts of total protein (50 μg) were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes at 200 mA for 6 h. The membrane was blocked in TBS buffer containing 5% skim milk and 0.1% Tween-20 at room temperature for 2 h, and incubated with the appropriate primary antibody overnight at 4°C. The membrane was washed and incubated with peroxidase-labeled goat anti-mouse secondary antibodies (1:10,000, catalog no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. The membranes were washed 3 times with TBST for 5 min. The results were analyzed using an Enhanced Chemiluminescence substrate kit and the Enhanced Chemiluminescence Detection system (GE Healthcare, Chicago, IL, USA). The antibodies used were mouse anti-human Fas monoclonal antibody (Abcam; catalog no. 15285; 1:500) and β-actin monoclonal antibody (Abcam; catalog no. ab8226; 1:1,000).

Isolation and phenotypic identification of human peripheral blood lymphocytes γδ T. The present study was approved by the Liuzhou Maternal and Child Health Hospital Institutional Review Board (Liuzhou, China) and written, informed consent was provided by all participants. Fresh peripheral blood (30 ml) was collected from healthy women and mixed with lymphocyte separation medium (Sigma-Aldrich). Cells were collected by centrifugation (1,500 × g for 20 mins at 4°C), washed and inoculated into 24-well plates. An appropriate amount of IL-2 was added to each well for a final concentration of 200 U/ml. The medium was replaced with fresh medium every 3–4 days until the mononuclear cells reached the exponential phase, at 10–12 days. γδ T cells with the desired phenotype were collected and diluted for a density of 1 × 10⁷ cells/ml with RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) for subsequent experiments. The phenotype of isolated cells was analyzed by flow cytometry. For flow cytometry, monocyte markers were stained with anti-human FasL-IgG1 monoclonal antibody (dilution, 1:150; eBioscience, Thermo Fisher Scientific, Inc., catalog no. MA5-17073) for

30 min at 37°C, and blood was lysed with 0.87% ammonium chloride (Beckman Coulter, Inc., Brea, CA, USA) for 12 min at room temperature, washed twice with PBS, and fixed with a 100% methanol-based reagent for 10 min at 4°C. The cells were suspended in a tube with a binding buffer (300 ml) and 5 ml Annexin V-fluorescein isothiocyanate (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were added for cell labeling at room temperature in the dark for 15 min. The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo version 9.1 software (TreeStar, Inc., Ashland, Oregon).

Killing activity of γδ T cells against SKOV3 cells. The γδ T cell-mediated killing activity against SKOV3 cells with different Fas expression was assessed. Briefly, SKOV3 cells from the blank control, hTERT and TSTA groups were used as target cells. These cells were inoculated into 96-well plates at a density of 1 × 10⁵ cells/well. γδ T cells were added at an effector-target cell ratio of 5:1, 10:1, 20:1 and 40:1, respectively. The plates were incubated at 37°C for 6 h in an incubator, and Cell Counting Kit-8 reagent (10 μl, Beyotime Institute of Biotechnology, Haimen, China) was added to each well. The plates were incubated for an additional 2 h, and optical density was measured at 450 nm. The killing rate was calculated using the following formula: [OD450 of target cell control - (OD450 of experimental well - OD450 of effector cell control)] / (target cell control × 100%).

Killing activity of γδ T cells blocked with FasL-IgG1 monoclonal antibody. γδ T cells (1 ml; 1 × 10⁷ cells/ml) were incubated with 100 μl anti-human FasL-IgG1 monoclonal antibody (10 μg, dilution: 1:100) at 37°C for 2 h. γδ T cells were collected as effector cells, and their killing activity against SKOV3 cells was determined as described above.

Results

RT-qPCR analysis of Fas mRNA expression. The expression of Fas mRNA in SKOV3 and HELF cells in different transfection groups was compared by RT-qPCR. Fas mRNA expression in SKOV3 cells in TSTA group co-transfected with Ad5-hTERT-GAL4VP16 and Ad5-G5E4T-Fas was 14.2 times higher than in the blank control group (Table III). Fas mRNA expression in HELF cells in the TSTA group was 3.7 times higher compared with the blank control group (Table III).

Western blot analysis of Fas protein expression. The expression of Fas protein in SKOV3 cells in different transfection groups was compared by western blot analysis. The expression of Fas protein was highest in the TSTA group, followed by the hTERT group, with the lowest Fas expression detected in the blank control group (Fig. 1).

γδ T cell-mediated killing against SKOV3 cells. The killing effect of γδ T cells against SKOV3 cells transfected with adenoviruses expressing Fas was evaluated at different effector to target cell ratios. The killing rate was higher with increased Fas expression in SKOV3 cells (Table IV). The killing activity was also stronger at a higher effector to target cell ratio. The highest killing rate (72.5%) was observed in

Table III. Comparison of Fas mRNA expression in SKOV3 and HELF cells in different transfection groups.

Transfection group	Fas mRNA copies	Fold of increase
SKOV3		
Blank control	927.1	1.0
hTERT	2389.1	2.6
TSTA	13174.4	14.2
HELF		
Blank control	105.6	1.0
hTERT	154.7	1.5
TSTA	387.6	3.7

Fas, Fas cell surface death receptor; hTERT, human telomerase reverse transcriptase; TSTA, two-step transcriptional amplification system.

Table IV. $\gamma\delta$ T cell-mediated killing effect against SKOV3 cells at different effector to target cell ratios.

Parameter	Effector to target cell ratio			
	5:1	10:1	20:1	40:1
OD450 (following calibration)				
SKOV3	1.858	1.858	1.858	1.858
$\gamma\delta$ T	0.158	0.302	0.608	1.197
SKOV3+ $\gamma\delta$ T	1.914	2.206	2.161	2.483
hTERT	1.691	1.691	1.691	1.691
hTERT+ $\gamma\delta$ T	1.670	1.609	1.711	1.934
TSTA	1.672	1.672	1.672	1.672
TSTA+ $\gamma\delta$ T	1.616	1.494	1.569	1.657
Killing rate (%)				
SKOV3	-	-	-	-
$\gamma\delta$ T	-	-	-	-
SKOV3+ $\gamma\delta$ T	5.5	7.2	16.4	30.8
hTERT	-	-	-	-
hTERT+ $\gamma\delta$ T	10.6	22.9	34.8	56.4
TSTA	-	-	-	-
TSTA+ $\gamma\delta$ T	12.8	28.7	42.5	72.5

OD, optical density; hTERT, human telomerase reverse transcriptase; TSTA, two-step transcriptional amplification system.

the TSTA group at an effector to target cell ratio of 40:1 (Table IV).

Killing effects of FasL-IgG1-blocked $\gamma\delta$ T cells against SKOV3 cells. $\gamma\delta$ T cells were blocked with anti-human FasL-IgG1 monoclonal antibody, and the killing effects against SKOV3 cells from different transfection groups was compared. The killing activity of FasL-IgG1-blocked $\gamma\delta$ T cells was markedly reduced (Table V). The killing rate of FasL-IgG1-blocked

Table V. Killing effects of FasL-IgG1-blocked $\gamma\delta$ T cells against SKOV3 cells at different effector to target cell ratios.

Parameter	Effector to target cell ratio			
	5:1	10:1	20:1	40:1
OD450 (following				
SKOV3	1.843	1.843	1.843	1.843
$\gamma\delta$ T	0.148	0.303	0.598	1.156
SKOV3+ $\gamma\delta$ T	1.956	2.089	2.283	2.724
hTERT	1.674	1.674	1.674	1.674
hTERT+ $\gamma\delta$ T	1.727	1.779	2.018	2.388
TSTA	1.668	1.668	1.668	1.668
TSTA+ $\gamma\delta$ T	1.703	1.749	1.974	2.330
Killing rate (%)				
SKOV3	-	-	-	-
$\gamma\delta$ T	-	-	-	-
SKOV3+ $\gamma\delta$ T	1.9	3.1	8.6	14.9
hTERT	-	-	-	-
hTERT+ $\gamma\delta$ T	5.7	11.8	15.2	26.4
TSTA	-	-	-	-
TSTA+ $\gamma\delta$ T	6.8	13.3	17.5	29.6

FasL, Fas cell surface death receptor ligand; IgG, immunoglobulin G; OD, optical density; hTERT, human telomerase reverse transcriptase; TSTA, two-step transcriptional amplification system.

$\gamma\delta$ T cells was only 29.6% at an effector to target cell ratio of 40:1 (Table V).

Discussion

At present, low expression of exogenous genes at target sites is one of the major issues in tumor gene therapy (6,7). TSPs have been frequently used in the expression vector system in order for efficient targeted expression of exogenous genes in cancer cells (8,9). To date, several promoters that initiate the targeted expression of exogenous genes have been identified, including SLPI, OSP1, HES1 and hTERT (10,11). Nevertheless, most of these promoters are neither ovarian cancer-specific nor epithelial-specific, including SLPI, OPSI and hESEI. Theoretically, among all TSPs, hTERT promoter is more likely to be efficient in gene therapy for ovarian cancer because telomerase has relatively high activity in ovarian cancer cells, but low activity in normal cells including ovarian epithelial cells. Furthermore, hTERT promoter is only activated in telomerase-positive tumor cells, but is not active in normal telomerase-negative cells (12,13). Nevertheless, the activity of tissue-specific promoters, including the hTERT promoter, is often too low for effective targeted gene therapy. Several strategies have been used to enhance the activity of these promoters including point mutations in the promotor region, deletion mutations removing the parts with transcriptional inefficiency, chimeric promoters formed by the fusion of efficient parts from different promoters, regulation at the post-transcriptional level, and

the use of recombinant transcriptional activator (RTA). In a previous study, RTAs have been used to improve the activity of TSPs (14). The TSTA system contains a transcriptional activator GAL4-VP16 and a G5E4T regulatory element, a small promoter that is responsive to GAL4, and has enhanced the activities of the corresponding TSP (15,16). It has been demonstrated that the expression of firefly luciferase gene (f1) and mutant HSV1 thymidine kinase gene in prostate cancer cells with a TSTA system was increased by 50 and 12 times, respectively, compared with control cells without the system (17). However, the efficiency of the TSTA system is associated with different TSPs and target genes.

In the present study, recombinant adenoviruses carrying an hTERT promoter that regulated the expression of Fas and a TSTA system were successfully constructed in order to achieve high expression levels of the target gene in cancer cells, but low or no expression in noncancerous cells. Although Fas mRNA and protein were expressed in SKOV3 and normal control HELF cells transfected with the constructed adenoviruses, the level of Fas expression in SKOV3 cells was higher than in HELF cells, suggesting that the hTERT promoter initiated ovarian cancer-specific Fas expression and the TSTA system further enhanced the gene expression by improving the activity of the promoter. In a previous study, the TSTA system containing the modified chimeric transcriptional activator GAL4VP2 and the target f1 gene was constructed in a single or in two different adenoviral vectors (16). It was revealed that the f1 expression in cells transfected with two adenoviral vectors was higher compared with single adenoviral group (18-20). In the present study, a similar approach of co-transfection of two different adenoviral vectors was used. However, the expression of Fas in the co-transfection group was only increased by 7 fold compared with the hTERT single adenoviral group, which may be associated with low activity of the hTERT promoter or the low transfection efficiency of adenovirus in the target SKOV3 cells.

Cytotoxic T lymphocytes (CTLs) and natural killer cells serve an important function in the immune system. The anti-tumor effects of CTL cells are achieved by direct lysis or apoptotic induction of target cells. Depending on the T cell receptor (TCR), T lymphocytes can be divided into TCR $_{\alpha\beta}$ and TCR $_{\gamma\delta}$ cells. $\gamma\delta$ T cells directly bind to antigens, including polypeptides and lipids, due to their rich surface FasL, and thus effectively target Fas-expressing tumor cells, which initiates the Fas/FasL apoptotic pathway (21-23). In the present study, the killing activity of effector $\gamma\delta$ T cells against SKOV3 cells was increased with higher effector to target cell ratios. The killing effect was also stronger in SKOV3 cells with higher expression of Fas, which suggested that enhancing the killing effect of immune cells by the upregulation of Fas expression in tumor cells may be a feasible approach for efficient cancer gene therapy. Furthermore, the killing activity of $\gamma\delta$ T cells blocked with anti-human FasL-IgG1 monoclonal antibody against SKOV3 cells was reduced. Nevertheless, these $\gamma\delta$ T cells still exhibited a certain degree of killing activity, as indicated by the clear oncolysis that was observed (data not shown). These results suggested that $\gamma\delta$ T cells killed ovarian cancer cells by direct lysis and apoptotic induction.

In conclusion, the regulators of hTERT promoter and TSTA assured efficient and targeted expression of their downstream Fas gene in SKOV3 cells. The killing effect of $\gamma\delta$ T cells against ovarian cancer cells with high Fas expression was improved. The present study thus sheds light on a potential effective targeted gene therapy for ovarian cancer.

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