

Therapeutic effect of targeted Fas-expressing adenoviruses method combining $\gamma\delta$ T cells in a mouse model of human ovarian carcinoma

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Abstract. The present study aimed to investigate the therapeutic effect and safety of targeted use of Fas-expressing adenoviruses combined with $\gamma\delta$ T cell-mediated killing to treat human ovarian cancer xenografts in BALB/c mice. Shuttle plasmids containing control elements of human telomerase reverse transcriptase promoter and two-step transcriptional amplification system were constructed and packaged into adenovirus-5 vectors to generate expression of an exogenous Fas gene. A mouse xenograft model of human ovarian carcinoma was constructed. A total of 35 BALB/c mice were randomly divided into five groups, which were injected with PBS, $\gamma\delta$ T cells, Fas-expressing adenoviruses, taxol, or Fas-expressing adenovirus and $\gamma\delta$ T cells. The weight and volume of tumors in mice in each group was monitored. Tissue sections of the various tissues of mice in the Fas-expressing adenovirus and $\gamma\delta$ T cells group was compared with those in the PBS group to evaluate the safety of Fas-expressing adenovirus and $\gamma\delta$ T cells in the treatment of human ovarian cancer xenograft tumors. The results of the present study indicated that mice in all treatment groups were alive at the end of the treatment course. Tumor weight and volume was the highest in the PBS group, followed successively by the adenovirus group, the $\gamma\delta$ T cell group, the adenovirus and $\gamma\delta$ T cell group, and the taxol group. The weight and volume inhibition rate in adenovirus

and $\gamma\delta$ T cell group were significantly higher compared with in the PBS group ($P<0.05$). Pathological observation of tissue samples revealed that none of vital organs in the adenovirus and $\gamma\delta$ T cell group developed any evident morphological changes during treatment, when compared with healthy controls. In conclusion, the combined therapy with Fas-expressing adenoviruses and $\gamma\delta$ T cells is efficient and safe for the treatment of mouse human ovarian carcinoma xenografts.

Introduction

Low tissue specificity and efficiency of exogenous gene expression are two major obstacles to tumor-targeted gene therapy. Previous studies have suggested that the regulation of a tumor-specific promoter (TSP) and two-step transcriptional amplification system (TSTA) is able to markedly improve the specificity and efficiency of expression of a target gene in tumor cells (1,2). Although numerous promoters have been used in targeted gene therapy for ovarian cancer, including secretory leukocyte protease inhibitor, ovarian-specific promoter and human epithelial tissue-specific transcription factor promoter, these promoters are neither ovarian cancer-specific nor epithelium-specific, and may even be active in normal cells (3). By contrast, the human telomerase reverse transcriptase (hTERT) promoter is only activated in ovarian cancer cells with high telomerase activity, and therefore is highly suitable for the gene therapy of ovarian cancer (4,5). However, the activity of tumor-specific promoters is often too weak to mediate the desired gene therapy (6). Recent studies have shown that the recombinant TSTA containing a transcriptional activator (RTA) may effectively enhance the activity of tumor-specific promoters (7,8). RTA is an important transcription factor that controls the switch from the latent to the lytic cycle and regulates immediate-early gene expression (9). The TSTA system, composed of a transcriptional activator GAL4-VP16 (Activator) and an end-biotinylated G5E4T regulatory element (a small promoter that is responsive to GAL4), has markedly enhanced the activities of the corresponding TSP (8,10).

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The selection of an appropriate target gene is crucial for efficient gene therapy. The Fas cell-surface death receptor gene (Fas) regulates cell apoptosis primarily through the Fas/Fas ligand (FasL) signaling pathway, and thus is associated with the occurrence and development of ovarian cancer (11), and the chemosensitivity of cancer cells to certain chemotherapy reagents, including cisplatin, epirubicin and paclitaxel (12,13). Therefore, increasing the expression of Fas may directly activate tumor cell apoptosis. It is known that the immune effector $\gamma\delta$ T cells, with abundant surface FasL, are able to specifically target and kill Fas-expressing cells by activating the Fas/FasL apoptotic pathway (7). However, Fas is expressed at markedly low levels, or not at all, in certain ovarian cancer cells, leading to decreased Fas-mediated cell apoptosis and drug resistance in these cells. Enhancing intracellular Fas levels may be an efficient approach for ovarian cancer gene therapy.

In a preliminary study, the recombinant adenoviral vectors Ad5-hTERT-Fas and Ad5-hTERT-TSTA-Fas that markedly express Fas under the regulation of hTERT promoter and TSTA system, respectively, were successfully constructed in SKOV3 cells transfected with these adenoviruses (7). The marked killing effect of $\gamma\delta$ T cells on SKOV3 cells with high Fas expression was also confirmed (7). The present study further measured the therapeutic effect of Fas-expressing adenoviruses combined with $\gamma\delta$ T cell-mediated killing in a mouse xenograft model of human ovarian cancer. In recent years, with increasingly more clinical studies on tumor gene therapy, the safety of gene therapy has received considerable attention (14-16). In the present study, the safety of the combined therapy of adenoviral Fas expression and $\gamma\delta$ T cells was therefore evaluated in mice with human ovarian cancer xenografts.

Materials and methods

Materials and animals. The plasmid pBCVP2G5-luc-NSN carrying GAL4VP2 and G5E4TATA elements and pBTdel279 carrying the hTERT core promoter were provided by Dr Yue Song, ShengJing Hospital of China Medical University (Shenyang, China). *EcoRV* restriction enzyme (cat. no. R0195L), *EcoRI* restriction enzyme (cat. no. R0101S), *BglII* restriction enzyme (cat. no. R0144S), *SalI* restriction enzyme (cat. no. R3138S), *SacI* restriction enzyme (cat. no. R3156S), *NotI* restriction enzyme (cat. no. R3189S) and *SpeI* restriction enzyme (cat. no. *SpeI*; all from New England Biolabs, Beverly, MA, USA). The plasmid vector pMD18-T was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The shuttle plasmid pDC316 and recombinant adenovirus backbone plasmid pBGloxdelE13cre were purchased from AGTC, Gene Technology Company, Ltd. (Beijing, China). The AdEasy adenoviral vector systems were purchased from Applied Biological Materials, Inc. (Richmond, BC, Canada). For co-transfection, 293 cells were purchased from AGTC Gene Biotech. The human ovarian carcinoma SKOV3 cell line and $\gamma\delta$ T cells were provided by the Gynecological Oncology Laboratory in the Beijing Union Medical College Hospital (Beijing, China). Female BALB/c nude mice were provided by the Experimental Animal Center in the Beijing Union Medical College Hospital.

Cell culture. SKOV3 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine. A total of 293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. All cells were incubated in an atmosphere of 5% CO₂ at 37°C.

Construction of recombinant plasmid vectors carrying hTERT promoter and/or TSTA regulatory element and Fas gene. Plasmid pBCVP2G5-luc-NSN, pBTdel279, pCDNA3-Fas carrying the Fas gene and shuttle plasmid pDC316 were transfected into *Escherichia coli* JM109 competent cells (Promega Corporation, Madison, WI, USA) using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h, according to the manufacturer's protocol. Following amplification, bacterial plasmids were extracted and purified using the Wizard Plus SV Minipreps DNA Purification system (Promega Corporation). To verify transfection of pBTdel279, using plasmid DNA as the template, the target fragment was amplified using Qiagen Multiplex PCR kit (Qiagen China Co., Ltd., Shanghai, China) with the following primer sequences: 5'-TTGATATCG ACCCCCGGGTCCGCCCGGAGCA-3'; and 5'-CTGAAT TCGCTGCCTGAAACTCGCGCCGCGAG-3', containing an *EcoRV/EcoRI* restriction enzyme sites. Verification of pCDNA3-Fas was performed using plasmid DNA as template and the T7 promoter sequence 5'-TAATACGACCTACTATA GGG-3' as a primer, starting prior to the insertion site of the Fas gene, the target fragment was sequenced using the terminal ending method (17). At the same time, given that the inserted Fas gene has *XbaI* and *KpnI* enzyme sites, it was possible to obtain a fragment of ~1,000 bp upon enzyme digestion.

From the analysis of the plasmid profile, it was known that it is possible to obtain two fragments (2,284 and 6,487 bp) following enzyme digestion using the enzymes *NheI* and *Bsu36I*. PDC316-hTERT and pDC316-G5E4T were constructed using a series of enzyme digestion and ligation reactions. Double digest samples were set up with 6 μ l DNA, 1 μ l 10x Buffer, 0.5 μ l restriction endonuclease, and distilled water to 10 μ l reaction volumes. Following the double digest, the samples were incubated at 37°C for 3 h. Digested products were ligated with T4 DNA ligase (New England BioLabs, Inc., Ipswich, MA, USA) using a 5:1 insert DNA to vector DNA ratio. A total of 10 μ l reaction volumes were set up, including 1 μ l T4 DNA ligase, 1 μ l 10x Ligase Buffer (New England Biolabs) and 20 ng plasmid DNA in distilled water. Tubes were incubated overnight at 4°C, and products were visualized by gel electrophoresis and stored at -20°C. The shuttle plasmid pDC316-hTERT-GAL4VP2 and pDC316-G5E4T-Fas were constructed as previously described (7).

Construction of shuttle plasmids. As presented in Table I, plasmid DNA with target sequences were used as a template and primer pairs containing corresponding restriction enzyme sites to amplify target sequences using the polymerase chain reaction (PCR). The thermocycling conditions were: 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 64°C for 45 sec and 72°C for 60 sec, and finally 72°C for 7 min. The PCR product of the target sequence fragment was electrophoresed and collected using an Agarose Gel DNA Purification

Table I. Construction of shuttle plasmids.

Plasmid	Target sequence	Template plasmid	PCR primers for target sequence, forward and reverse	Restriction enzymes used	Verification method	Vector plasmid	Restriction enzyme processing of vector plasmid	Verification of constructed plasmid
pDC316-hTERT	hTERT promoter	pBTdel279	5'-TTGATATCGACCCCG GGTCCGCCCGGAGCA-3' and 5'-CTGAATTCGCTGC CTGAAACTCGCGCCG CGAG-3'	<i>EcoRV</i> and <i>EcoRI</i>	Sequencing using RV-M/M13-47 primer pair	pDC316	Complement terminals following <i>XbaI</i> digestion and cut MCMV promoter using <i>EcoRI</i> following ethanol precipitation <i>BglII</i> and <i>SacI</i>	Sequencing using PCR primers and enzyme cutting using <i>EcoRV/EcoRI</i>
pDC316-hTERT-GAL4VP2	GAL4VP2+ PA gene	pBCVP2G5-luc-NSN	5'-GTAGATCTGAAGCTA GCCTCCTGAAAGATG-3' and 5'-TAGTCGACTAGT GGGCCCGGATCCAG ACAT-3'	<i>BglII</i> and <i>SacI</i> (<i>NotI-SpeI</i>)	Sequencing using P1 primer 5'-AAGTGCGA CATCATCATC-3'	pDC316-hTERT		Sequencing using P2 primer 5'-TTCTAGCCTT GATCCAC-3' and enzyme cutting using <i>XbaI/EcoRI</i>
pDC316-G5E4T	G5E4T fragment	pBCVP2G5-luc-NSN	5'-TAGATATCAGGTGAC ACTATAGAAATACAAG-3' and 5'-GTGAATTCAACA GTACCCGGAATGC-3'	<i>EcoRV</i> and <i>EcoRI</i>	Sequencing using M13-47 primer	pDC316	Complement terminals following <i>XbaI</i> digestion and cut MCMV promoter using <i>EcoRI</i>	Sequencing using PCR primers
pDC316-G5E4T-Fas	Fas gene	pCDNA3-Fas	5'-TATGAATTCGCCGCC ACCATGCTGGGCATCT GGAC-3' and 5'-GCTGAGCTCTAGAC CAAGCTTGGATTTC-3'	<i>EcoRI</i> and <i>SacI</i>	Sequencing using RV-M/M13-47 primer pair	pDC316-G5E4T	<i>EcoRI</i> and <i>SacI</i>	Sequencing using P3 primer

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

Adenovirus	Viral titer, VP/ml	Target gene identification by polymerase chain reaction	TCID ₅₀ , IU/ml
Ad5-htert-GAL4VP2	1.4x10 ¹¹	Correct	5.0x10 ⁹
Ad5-G5E4T-Fas	2.7x10 ¹¹	Correct	5.6x10 ⁹

TCID₅₀, half-maximal tissue culture infective dose.

kit (Takara Biotechnology Co., Ltd.), and then ligated into a connection vector pMD18-T using Solution I of a DNA Ligation kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. The connection vector was used to transfect competent JM109 cells and select bacterial colony for clone culture. For verification of the target sequence in the pMD18-T vector, the RV-M sequence (5'-GAGCGGATAACAATTTACACAGG-3') and M13-47 sequence (5'-CGC CAGGGTTTTCCCAGTCACGAC-3') were used as primers to amplify positive clones using 2X *Taq* PCR Master Mix (Qiagen) and the fragment was sequenced. The PCR reaction protocol was programmed as: Initial pre-denaturation step at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 sec, an annealing step at 59°C for 40 sec, an extension at 72°C for 45 sec and a final extension at 72°C for 10 min. Next, the obtained connection vector plasmid containing the target sequence was constructed using a series of enzyme digestion and ligation reactions as described above. The target sequence was electrophoresed and collected. At the same time, the vector plasmid was digested using the corresponding restriction enzyme, and then electrophoresed and collected. The target sequence was ligated into the vector plasmid and the constructed plasmid was transfected into competent JM109 cells using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h, according to the manufacturer's protocol. The plasmid-positive clone culture was collected and verified by fragment PCR sequencing or enzyme digestion.

Adenoviral packaging and purification. As described previously (7), the packaging and purification of adenovirus vectors Ad5-hTERT-GAL4VP2 and Ad5-G5E4T-Fas (Benyuan Zhengyang Gene Technology Co., Ltd., Beijing, China) was performed using the AdEasy adenoviral vector systems (cat. no. 240009; Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, 293 cells which obtained 60-70% confluency in fresh DMEM containing 10% fetal bovine serum (FBS; Gibco) were co-transfected with recombinant shuttle plasmid (pDC316-hTERT-Fas, pDC316-hTERT-GAL4VP2 or pDC316-G5E4T-Fas) and adenoviral backbone plasmid pBHGloxdelE13cre, using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The plaques were scraped off at 7-10 days following transfection, collected by centrifugation (30,000 x g at 4°C for 16 h) and resuspended in PBS. The suspension was frozen in liquid nitrogen and thawed at 37°C three times to release the virus. The supernatant containing recombinant adenoviruses was collected by centrifugation (12,000 x g at 4°C for 15 min), purified by chromatography, and stored at -20°C in PBS (cat.

no. P5368-10PAK; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The target gene in the recombinant adenoviruses was confirmed using 2X *Taq* PCR Master Mix (Qiagen) (Table II). The PCR reaction protocol was programmed as described above. The viral titer and half-maximal tissue culture infective dose of each viral stock was measured (Table II).

Construction of mouse xenograft model of human ovarian cancer. SKOV3 cells in the exponential growth phase were collected and diluted into a 2.5x10⁷/ml cell suspension. A total of 38, 4-week-old female BALB/c mice weighing 14-16 g were purchased from the Laboratory Animal Centre of Guangxi Medical University. All mice were housed in a specific pathogen-free clean room in a temperature-controlled (24-26°C) room at 60±5% humidity under a 12-h light/dark cycle. Mice were provided with distilled water *ad libitum* and fed with OVA-free food. Mice were given a subcutaneous injection of 0.2 ml SKOV3 cell suspension at the back of the neck. The tumor xenografts were measured every 4 days until their diameters reached 5-6 mm. The Institutional Review Board of Liuzhou Maternal and Child Health Hospital approved the present study.

Treatment. A total of 35 mice were randomly selected from the 38 mice with xenograft tumors, and randomly divided into five groups (n=7), which were injected with PBS, $\gamma\delta$ T cells, Fas-expressing adenoviruses (TSTA group), taxol, and Fas-expressing adenovirus with $\gamma\delta$ T cells (TSTA+ $\gamma\delta$ T group) (Table III). The total weight and tumor size of each mouse were measured, and tumor volume was calculated prior to treatment.

Comparison of therapeutic effects. The therapeutic effects of different treatments were compared according to the following. i) Morphological observation of the surface morphology and texture of the xenograft, and activity and other conditions of mice. ii) Tumor growth curve: the major axis (a) and minor axis (b) of the xenograft tumors were measured with a Vernier caliper every 4 days. The mean tumor volume in each group was calculated as $V = a \times b^2/2$, and the tumor growth curve was created. iii) Volume and weight inhibitory rates: All mice were sacrificed at 24 days after the start of treatment. The size and weight of the xenograft tumors were measured, and volume and weight inhibitory rates were calculated using the following formulae: Weight inhibition rate = (mean tumor weight in PBS group - mean tumor weight in the treatment group)/mean tumor weight in PBS group x100, and volume inhibition rate = (mean tumor volume in PBS group - mean tumor volume in the treatment group)/mean tumor volume in the PBS group x100.

Table III. Summary of treatment groups of mice with ovarian xenograft tumors.

Group	Treatment
PBS	Multi-point intratumoral injection of PBS twice a week, 200 μ l/injection four times (days 1, 4, 7 and 11)
Taxol	Multi-point intratumoral injection of taxol twice a week, 200 mg/kg/injection four times (days 1, 4, 7 and 11)
TSTA	Multi-point intratumoral injection of Ad5-hTERT-GAL4VP2 and Ad5-G5E4T-Fas once a week, TCID ₅₀ 1x10 ⁹ IU/injection twice (days 1 and 7)
$\gamma\delta$ T	Multi-point intratumoral injection of $\gamma\delta$ T cells once a week, 2x10 ⁷ cells/injection twice (days 1 and 7)
TSTA+ $\gamma\delta$ T	Multi-point intratumoral injection of Ad5-hTERT-GAL4VP2 and Ad5-G5E4T-Fas once a week, TCID ₅₀ 1x10 ⁹ IU/injection twice (days 1 and 7) and multi-point intratumoral injection of $\gamma\delta$ T cells once a week on the following day of adenoviral injection, 2x10 ⁷ cells/injection twice (days 2 and 8)

TCID₅₀, half-maximal tissue culture infective dose; TSTA, two-step transcriptional amplification system.

Safety evaluation of TSTA+ $\gamma\delta$ T cells. The safety of the combined treatment of Fas-expressing adenovirus+ $\gamma\delta$ T cells was evaluated by pathological observation. Briefly, tissue samples of xenograft, liver, kidney, spleen, intestines, heart and ovary of mice in the TSTA+ $\gamma\delta$ T cell group were collected, fixed with 10% neutral buffered formalin overnight at 4°C, embedded in paraffin and cut into 4 mm sections. The sections in the TSTA+ $\gamma\delta$ T cell group were compared with those in the PBS control group to evaluate the safety of using Fas-expressing adenovirus with $\gamma\delta$ T cells in the treatment of human ovarian cancer xenograft.

Statistical analysis. All statistical analyses were performed using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA). The difference in volume and weight inhibitory rates among the treatment groups was compared by univariate analysis of variance. The difference between a treatment group and the PBS group was further compared using the Fisher's least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Construction of mouse xenograft model of human ovarian cancer. At 2 weeks after the injection of SKOV3 cells, subcutaneous xenograft tumors with a diameter of 4-6 mm developed. The oval hard xenograft tumors had a uniform nodule size and smooth surface and exhibited slow expansive growth.

General conditions of mice. During the course of treatment, there was no significant difference in the general conditions of mice, including eating and mental status. No significant loss of appetite or weight was observed in any mouse. No mice succumbed to disease throughout the 24-day observation period. At 24 days, mice were sacrificed and autopsy analysis identified no ascites or pleural/abdominal metastasis of the tumor. A large necrotic area was detected in the center of the xenograft tumors in the TSTA+ $\gamma\delta$ T group, but no morphological changes in the tumor were observed in any other group.

Tumor growth curve. The tumor growth curves for all treatment groups are presented in Fig. 1. The tumors grew slowly in the first 12 days in all groups. The tumor in the PBS, TSTA

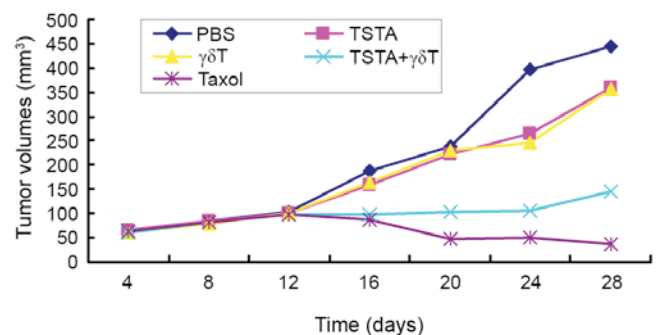


Figure 1. Growth curve of the xenograft tumors in different treatment groups.

and $\gamma\delta$ T groups began to grow rapidly after 12 days, whereas the growth of tumors in the TSTA+ $\gamma\delta$ T group remained slow and the tumor volume in the taxol group was decreased.

Combination of TSTA and $\gamma\delta$ T inhibits tumor weight. To investigate the effects of TSTA and $\gamma\delta$ T on the tumor growth, the tumor weight was observed and the weight-inhibiting rate was analyzed. The results revealed that the tumor weight in the TSTA+ $\gamma\delta$ T and taxol groups were significantly increased compared with the TSTA or $\gamma\delta$ T single-treatment groups ($P < 0.05$; Table IV). Furthermore, the tumor weight inhibitory rates of TSTA+ $\gamma\delta$ T (50.9%) and taxol group (79.0%) were obviously increased compared with those of the single-treatment groups (Table IV).

Combination of TSTA and $\gamma\delta$ T inhibits tumor volume. The effects of TSTA and $\gamma\delta$ T on the tumor volume were also observed in the present study. The results indicated that the tumor volume in the TSTA+ $\gamma\delta$ T and taxol groups were significantly increased compared with the TSTA or $\gamma\delta$ T single-treatment groups ($P < 0.05$; Table V). Furthermore, the tumor volume inhibitory rates of TSTA+ $\gamma\delta$ T (60.3%) and taxol (88.6%) groups were markedly increased compared with those of the single-treatment groups (Table V).

Safety evaluation of Fas-expressing adenovirus and $\gamma\delta$ T cells. Tissue sections of the xenograft, liver, kidney, spleen, intestines, heart and ovary of mice in the TSTA+ $\gamma\delta$ T group were compared with those in the PBS group to evaluate the safety of

Table IV. Comparison of weight inhibitory rates in different treatment groups.

Group	Mean tumor weight, g	Weight inhibitory rate, %
PBS	0.573 \pm 0.015	-
TSTA	0.507 \pm 0.012	10.5
$\gamma\delta$ T	0.497 \pm 0.013	14.0
TSTA+ $\gamma\delta$ T	0.276 \pm 0.016 ^{a,b}	50.9
Taxol	0.120 \pm 0.011 ^{a,b}	79.0

^aP<0.05 vs. TSTA single-treatment group. ^bP<0.05 vs. $\gamma\delta$ T single-treatment group. TSTA, transfected with Fas via the two-step transcriptional amplification system.

Table V. Comparison of volume inhibitory rates in different treatment groups.

Group	Mean tumor volume, mm ³	Volume inhibitory rate, %
PBS	402.29 \pm 9.83	-
TSTA	361.43 \pm 6.87	10.2
$\gamma\delta$ T	360.00 \pm 8.32	10.5
TSTA+ $\gamma\delta$ T	159.71 \pm 11.93 ^{a,b}	60.3
Taxol	45.86 \pm 4.87 ^{a,b}	88.6

^aP<0.05 vs. TSTA single-treatment groups; ^bP<0.05 vs. $\gamma\delta$ T single-treatment groups. TSTA, transfected with Fas using the two-step transcriptional amplification system.

the combined treatment for human ovarian cancer xenograft. None of the vital organs in TSTA+ $\gamma\delta$ T group developed any evident morphological changes during the treatment when compared with the PBS controls. A large necrotic area was detected in the center of the xenograft tumors in the TSTA+ $\gamma\delta$ T group, but was not observed in the PBS group.

Discussion

The Fas gene is one of the most important regulatory genes for apoptosis, and the abnormalities in the Fas/FasL signaling pathway is associated with the occurrence and development of tumor and the sensitivity of tumor cells to certain chemotherapeutic reagents (18-20). However, the level of Fas gene expression is low in or even absent from certain ovarian cancer cells, which decreases the Fas-mediated apoptosis and chemosensitivity of these cells (1,12). Introducing exogenous Fas gene expression into tumor cells has been suggested as an effective method to improve the targeted gene therapy of ovarian cancer (7). However, the low expression of the exogenous Fas gene in tumor cells has been a major obstacle in tumor gene therapy. The tumor-specific hTERT promoter has been successfully used in the gene therapy of ovarian cancer (21,22). Nevertheless, the activity of the hTERT promoter in ovarian tumor cells is often too low for effective targeted

gene therapy (5). The TSTA system, including the transcriptional activator GAL4-VP16 and G5E4T regulatory element (a small promoter that is responsive to GAL4), has markedly enhanced the activity of the corresponding TSP (23,24). Consistent with a previous study, preliminary results demonstrated that the Fas gene was markedly expressed in SKOV3 cells following co-transfection with Ad5-hTERT-GAL4VP2 and Ad5-G5E4T-Fas, whereas Fas expression in the control lung fibroblast cell line HELF was not altered following transfection, indicating the efficient targeted expression of Fas in human ovarian cancer cells using the TSTA system (7).

As a subgroup of T cells, $\gamma\delta$ T cells are able to directly bind to antigens, including polypeptides and lipids, owing to the rich surface expression of FasL, and thus can effectively target Fas-expressing tumor cells, initiating the Fas/FasL apoptotic pathway (25-29). Preliminary results confirmed the strong killing activity of $\gamma\delta$ T cells against adenovirus-mediated Fas-expressing SKOV3 cells (7). The killing activity of $\gamma\delta$ T cells blocked with anti-human FasL-IgG1 monoclonal antibody against SKOV3 cells was markedly decreased. In the present study, the therapeutic effect of targeted Fas-expressing adenoviruses combined with $\gamma\delta$ T cells was evaluated in a mouse xenograft model of human ovarian cancer. Since nude mice are T cell-immunodeficient animals and have no immune effector cells against tumor cells overexpressing Fas, no inhibitory effect was observed in the PBS group. By contrast, the weight and volume inhibition rates (50.9 and 60.3%, respectively) were significantly increased compared with in the PBS group. Furthermore, a large necrotic area was detected in the center of the xenograft tumors in TSTA+ $\gamma\delta$ T group, but this was not observed in the PBS group, indicating that the treatment activated the Fas/FasL apoptotic pathway and directly induced the necrotic lysis of tumor cells.

In recent years, the safety of tumor gene therapy has become a major focus of research (30). There are three main issues concerning the clinical application of replication-defective adenoviral vectors in tumor gene therapy: The construction of recombinant adenovirus with replication ability, the activation of the immune response against adenovirus, and the cytotoxic effect of the adenovirus (31). In the present study, the reporter gene of the injected adenovirus was identified in other regions of the body, despite the local injection of the virus, which might be due to the non-targeted infection of host cells by adenovirus. The safety of the combined treatment of Fas-expressing adenovirus with $\gamma\delta$ T cells was further assessed by the pathological examination of the xenograft, liver, kidney, spleen, intestine, heart and ovary tissue of mice in the TSTA+ $\gamma\delta$ T and PBS groups. None of the vital organs in the TSTA+ $\gamma\delta$ T group developed any evident morphological changes during the treatment compared with the PBS controls, suggesting that treatment with Fas-expressing adenovirus and $\gamma\delta$ T cells was safe in mice. However, further studies are required to validate the results of the present study, which include the measurement of serum biochemical indicators in treated mice.

To conclude, the combination of Fas-expressing adenoviruses and $\gamma\delta$ T cell therapy is efficient and safe for the treatment of mouse human ovarian carcinoma xenografts, which may provide a novel strategy for tumor gene therapy. The therapeutic effect may be improved further when target genes that are more specific for ovarian cancer are identified.

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