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Regular paper

pVAX1 plasmid vector-mediated gene transfer of soluble TRAIL suppresses human hepatocellular carcinoma growth in nude mice

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The extracellular domain of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) may function as a soluble cytokine to selectively kill various cancer cells without toxicity to most normal cells. We used a high-biosafety plasmid pVAX1 as a vector and constructed a recombinant plasmid expressing the extracellular domain (95–281 aa) of human TRAIL fused with signal peptides of human IgGγ, designated as pVAX-sT. Transduction of human BEL7402 liver cancer cells with pVAX-sT led to high levels of sTRAIL protein in the cell culture media and induced apoptosis. The therapeutic potential of pVAX-sT was then evaluated in the BEL7402 transplanted naked mouse model. Subsequent intratumoral administration of naked pVAX-sT resulted in the expression of soluble TRAIL in the sera and the tumor site, as well as effective suppression of tumor growth, with no toxicity to liver. In conclusion, the successful inhibition of liver cancer growth and the absence of detectable toxicity suggest that pVAX-sT could be useful in the gene therapy of liver cancer.

Keywords: soluble TRAIL, gene therapy, pVAX1, hepatocellular carcinoma, naked DNA

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo-2L) is a typical member of the tumor necrosis factor family, inducing apoptosis by activating death receptors (Wiley *et al*., 1995; Diana *et al*., 2004). A number of studies have shown that both the membrane-bound and the soluble extracellular domain of TRAIL can induce apoptosis in a wide variety of transformed cell lines and tumor cell lines without affecting most normal cells (Ashkenazi *et al*., 1999; Walczak *et al*., 1999; Kagawa *et al*., 2001) *in vivo*. The selective induction of apoptosis in cancer, but not in normal cells, has prompted investigation into the use of TRAIL in cancer therapy. But to be effective, proapoptotic therapy with TRAIL in tumor-bearing mice requires prolonged administration and high doses of recombinant protein (Ashkenazi *et al*., 1999). In addition, production of the functional polypeptide has proven difficult, perhaps because of its physical properties and because of variations in the purification procedures utilized by different laboratories (Wang *et al*., 2004; Shen *et al*., 2003). Gene therapy can overcome the above weakness and become a potential method for cancer therapy. Although viral vectors have been preferentially applied in sTRAIL-mediated gene therapy, nonviral vectors offer several advantages. Such vectors are simpler, more amenable to large-scale manufacture, and potentially safer (risk of replication eliminated, inflammatory response potentially reduced). So in this study, we used a high-biosafety plasmid vector pVAX1 expressing TRAIL 95–281, to examine the effect of sTRAIL in BEL7402 cells both *in vitro* and *in*

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Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; NS, normal saline (0.9% sodium chloride solution); TRAIL/Apo-2L, tumor necrosis factor-related apoptosis-inducing ligand; sTRAIL, soluble tumor necrosis factor-related apoptosis-inducing ligand; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride; TUNEL, terminal nucleotidyl transferase (TdT)-mediated nick end labeling.

vivo. The results indicate that pVAX-sT may provide an effective form of gene therapy for liver cancer.

MATERIAL AND METHODS

Mice and cell lines. Naked BALB/c mice, 5–6 weeks old, were obtained from Shanghai Slac Laboratory Animal (China). All mice were housed in the Animal Facilities, Institute of Immunology Shandong University under pathogen-free conditions. Mice were culled into experimental groups of equivalent average postprandial glucose level and body mass at the beginning of the study. We give assurance of humane practice in animal maintenance and experimentation; and we subscribe to the concept of using every acceptable method in the performance to minimize the use of animals and to prevent animal distress. This study has been approved by The Animal Ethical Committee of Shandong University.

The hepatocellular carcinoma cell line BEL7402 and COS-7 were cultured at 37°C in RPMI 1640 medium and DMEM (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal calf serum, 50 U/ml penicillin or streptomycin.

Construction and detection of the recombinant plasmid pVAX-sT. The sTRAIL sequence encoding TRAIL amino acids from 95 to 281 was amplified from pEGFP-TRAIL provided by Prof. B. Fang (Huang *et al*., 2003). Primers for sTRAIL fragment amplification were designed according to the sequence in GeneBank. In order to get a secret protein, the signal sequence of human IgG γ chain was added to the 5′ end of the forward primer, and sequences recognized by *Kpn*I and *Eco*RI were respectively added to the forward and reverse primer: forward(P1) -5′ CGGGGTACCATGGAAGCCCCAGCTCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAACCTCTGAGGAAACCATT 3′ reverse (P2) -5′ GCCGGAATTCAGCCAACTAAAAAGGCCC 3′. (The oligonucleotide primers were synthesized by Shanghai BioAsia Bio-technology Co. Ltd, China). The PCR fragment was cloned into pVAX1 (Invitrogen, Carlsbad, CA, USA) and named pVAX-sT. The construct was verified by sequencing.

Expression of pVAX-sT *in vitro*. The expression of pVAX-sT *in vitro* was checked by RT-PCR, Western blotting and ELISA. The COS-7 cells were transfected with 1 μg of pVAX-sT or pVAX1, respectively, by lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) and incubated for 24 h before the culture media and cells were collected separately. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol and revers transcribed to cDNA for RT-PCR. The cells were lysed in 200 μl of lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 62.5 mM sucrose, 5 mM EDTA, 1 mM PMSF). Equal amounts of protein were separated by SDS/PAGE, transferred to nitrocellulose membrane (Novex, San Diego, CA, USA). The membrane was incubated with an anti-hTRAIL mAb (BD Biosciences CA, USA). For detection of secreted TRAIL, the medium of the TRAIL-expressing cell cultures was assayd by ELISA (Diaclone, France).

The apoptotic effect of pVAX-sT on BEL7402 cell line. The BEL7402 cells were transfected with pVAX1 or pVAX-sT by lipofectamine. Twenty-four hours post transfection, apoptosis was assessed by observing cell morphology and by using the Annexin V kit (Immunotech, Marseille, France) according to the manufacturer's protocol.

Establishment of tumor xenografts and assessment of growth. BEL7402 cells 1×10^7, were administered in the s.c. tissue of the left axillary of 6-week-old male nude mice. The length (a) and width (b) of tumor were measured by caliper regularly. The tumor volumes were then calculated using the formula: volume=$a\times b^2\times 0.52$. When tumor size reached approx. 50 mm^3 (about 15 days post-inoculation), animals were randomized into three groups and plasmids were administered by intratumoral injection. Groups 1 and 2 received 20 μg naked pVAX-sT or pVAX1, respectively, twice a week, four times altogether, and group 3 mice received NS as control. The experiment was done thrice with 8 animals in each group.

Expression of sTRAIL *in vivo*. Gene-treated tumor sites were harvested 3 days after the final treatment. RT-PCR was used to detecte the sTRAIL mRNA. Tissue from the tumor sites (50 mg) was homogenized in 0.5 ml of general extraction PBS containing 0.1% Triton X-100 and 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN, USA), and sonicated. Supernatant was removed after centrifugation and frozen at –20°C until ELISA assay. In order to assay the association of anti-tumor effects and the expression level of sTRAIL, peripheral blood was prepared from epicanthic and the serum was used to detect the expression of sTRAIL with ELISA.

Detection of the apoptosis of BEL7402 cells *in vivo*. The propidium iodide (PI) and terminal nucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) assays were carried out as previously described (Widlak *et al*., 2003) to detect the apoptosis of BEL7402 cell *in vivo*.

Toxicity of pVAX-sT *in vivo*. The toxicity of pVAX-sT was examined in non-tumor-bearing mice following the intramuscle injection of 20 μg naked pVAX-sT. The general status of the naked mice was observed. The serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were meas-

ured 25 days after transduction and the histopathology of the heart, liver, spleen, lung, and kidney was studied.

Statistical analysis. Results were expressed as mean values ± standard deviation (S.D.), and Student's *t*-test was used for evaluating statistical significance. A value of $P<0.05$ was taken as statistically significant.

RESULTS

Successful construction of recombinant plasmid pVAX-sT and expression of sTRAIL *in vitro*

RT-PCR showed specific expression of sTRAIL mRNA in COS-7-pVAX-sT cells but not in COS-7-pVAX1 or non-transfected COS-7 cells (Fig. 1A). Transfection of cells with pVAX-sT resulted in a strong sTRAIL-specific band in Western-blotting (Fig. 1B). ELISA proved the expression of sTRAIL protein in the supernatant of COS-7-pVAX-sT cells reaching (166.7±26.1) pg/ml, significantly higher than in non-transfected COS-7 cells (16.33±5) pg/ml or COS-7-pVAX1 cells (25.5±2.5) pg/ml ($P<0.05$) (Fig. 1C).

Induction of BEL7402 cells apoptosis by pVAX-sT *in vitro*

The BEL7402 cells were transfected with pVAX1 or pVAX-sT by lipofectamine^{TM}2000. A change in the cell morphology was observed and the apoptosis induced by pVAX-sT was evaluated by PI/Annexin V FACS analysis 24 h post-transfection. As shown in Fig. 2A, the morphology was not significantly changed in pVAX1-BEL7402 or non-transfected BEL7402 cells. However, apoptotic morphology was observed in pVAX-sT-BEL7402 cells. The transfected cells were reacted with FITC conjugated annexin V to label apoptotic cells. Of the non-transfected BEL7402 and pVAX1-BEL7402 cells, only 0.89±0.16% and 2.67±0.45% (R2 quadrant, Annexin V^+/PI^-) of the cells were apoptotic, respectively, while 13.47±0.94% pVAX-sT-BEL7402 cells were apoptotic. The difference between BEL7402-pVAX-sT and BEL7402-pVAX1 cells was statistically significant ($P<0.01$) (Fig. 2B, C).

The expression of pVAX-sT *in vivo*

To further test the antitumor effect of pVAX-sT *in vivo*, human hepatocarcinoma xenografts were established in naked mice by inoculating BEL7402 cells subcutaneously into the left axillary of mice as described in ''Materials and Methods.'' Gene-treated tumor sites were harvested 3 days after the final treatment. RT-PCR clearly showed the specific expression of sTRAIL mRNA in the gene therapy group while there was no expression in the control groups (Fig. 3A). To compare the efficiency of gene transfection, expression of sTRAIL transgenic protein in the treated tumor site was evaluated by ELISA. The sTRAIL protein concentrations in resected samples treated with pVAX-sT, pVAX1 or

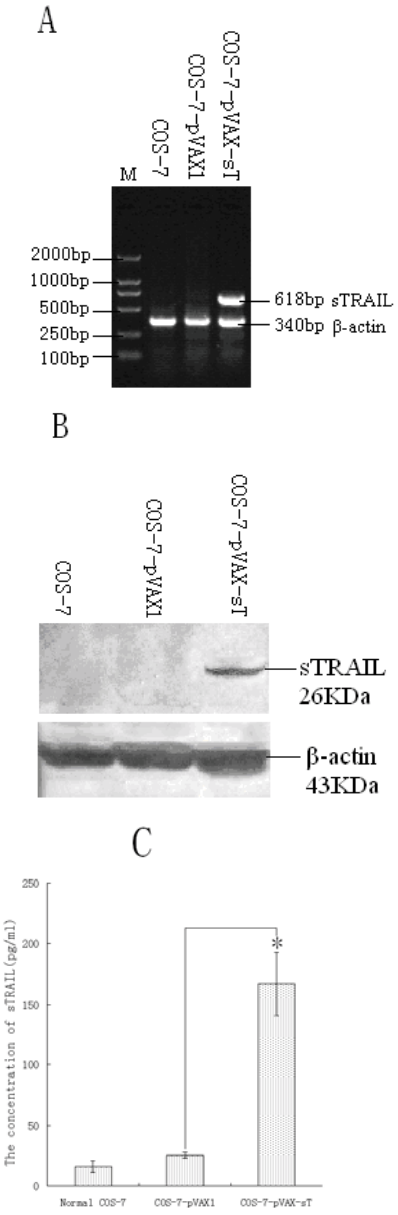
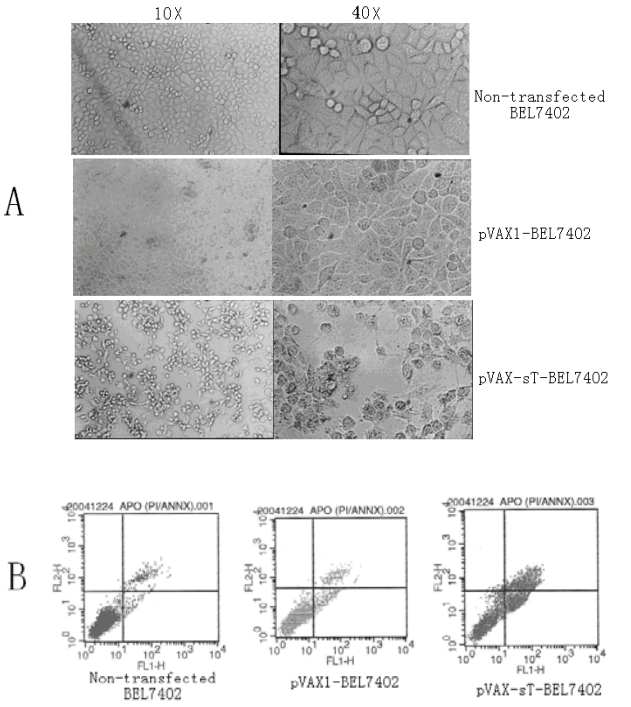


Figure 1. *In vitro* expression of pVAX-sT in COS-7 cells. COS-7 cells were transfected with pVAX-sT or pVAX1 and incubated for 24 h before the culture media and cells were collected separately. The expression of pVAX-sT *in vitro* was proved by RT-PCR, Western-blotting and ELISA. A. RT-PCR shows specific expression of sTRAIL mRNA in COS-7-pVAX-sT cells but not in COS-7-pVAX1 and non-transfected COS-7 cells. B. Western blot transfection of cells with pVAX-sT results in a strong sTRAIL-specific band (26 kDa). C. ELISA pVAX-sT-transfected COS-7 cells secrete TRAIL into the medium and secreted TRAIL was quantified by ELISA.



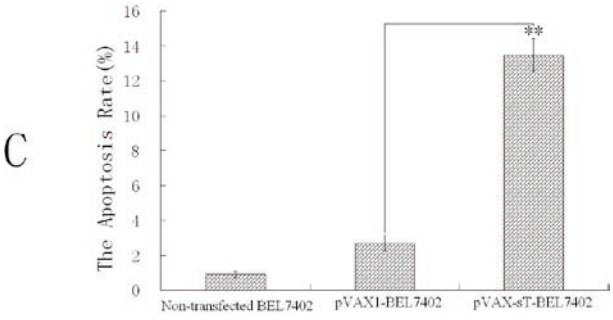


Figure 2. Proapoptoctic effects of pVAX-sT on human hepatoma cell line BEL7402.
The BEL7402 cells were transfected with pVAX1 or pVAX-sT by lipofectamine^{TM}2000. Apoptosis was assessed 24 h post-transfection by observing morphology and using the Annexin V kit. A. Cell morphology is not significantly changed in pVAX1-BEL7402 or non-transfected BEL7402 cells. Apoptotic morphology is observed in pVAX-sT-BEL7402 cells. The apoptosis analysis by annexin V fluorescence. B. shows results of FACS analysis while (C) gives the percentage of apoptotic cells caculated for data in (B).

NS were (192.45±10.34) pg/ml, (25.36±7.41) pg/ml and (30.27±5.36) pg/ml, respectively ($P<0.01$). The sTRAIL concentration in peripheral blood was (97.28±8.72) pg/ml in pVAX-sT-injected mice, significantly higher than in the pVAX1 and NS control groups (20.76±6.53 pg/ml, 15.35±4.26 pg/ml) ($P<0.05$) (Fig. 3B).

pVAX-sT inhibits tumor growth and induces apoptosis *in vivo*

The changes of the tumor volume indicated that naked pVAX-sT could significantly inhibit the growth of hepatoma BEL7402 cells *in vivo* when administered intratumorly. At day 17 the tumor volume was only (0.56±0.07) cm^3, significantly less than in the NS-treated (1.51±0.27) cm^3 or pVAX1-treated group (1.12±0.27) cm3 ($P<0.01$). The inhibition rate was about 63% at day 17 (Fig. 4A). Tumor tissues were stained with H&E (×40). The NS- and pVAX1-injected tissue showed well-preserved proliferated tumor cells. But pVAX-sT-injected tissue showed markedly reduced tumor cells in the center and only small numbers of the cells were present at the periphery (Fig. 4B). The TUNEL assay was done to prove that the anti-tumor action of pVAX-sT was due to their proapoptotic effect. The injec-

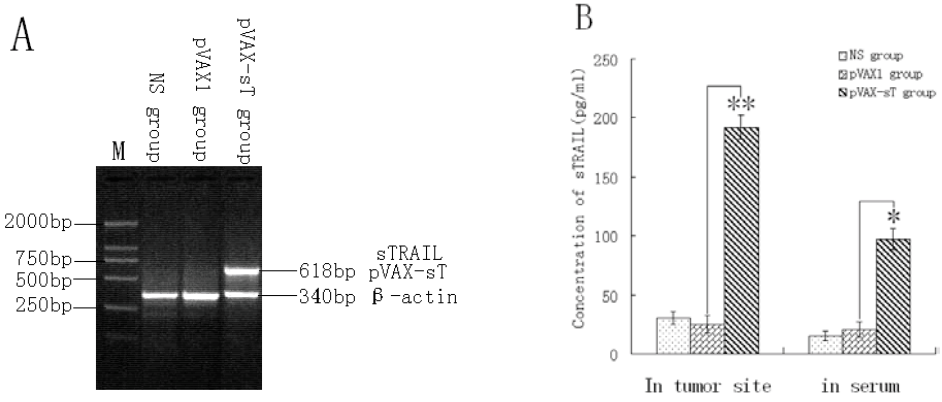


Figure 3. Expression of sTRAIL *in vivo*.
Female nude mice were injected subcutaneously with 1×10^7 BEL7402 cells. Animals were then given an injection of pVAX1 or pVAX-sT at the site of tumor implantation when tumor size reached 50 mm^3 twice a week for two weeks. Tumor sites were harvested 3 days after the final treatment. A. Total RNA of the tumor sites was extracted. RT-PCR clearly showed the specific expression of sTRAIL mRNA in the gene therapy group while there was no expression in the control groups. B. The expression of sTRAIL transgenic protein in the treated tumor site and in serum was evaluated by ELISA. The results show that the concentration of sTRAIL in pVAX-sT-group was significantly higher than in the control groups, both in tumor site and in serum (*$P<0.05$, **$P<0.01$).

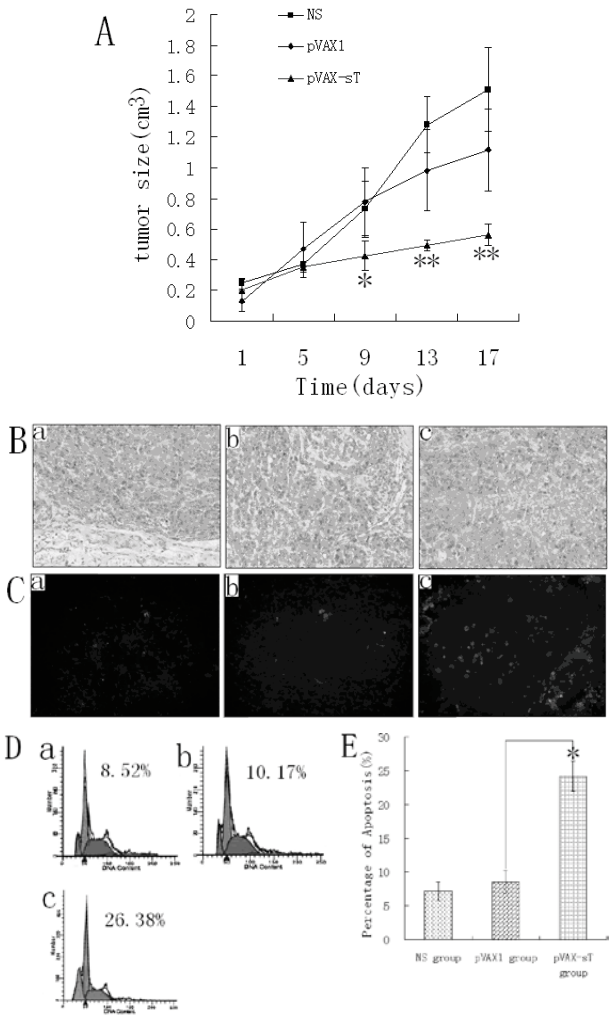


Figure 4. pVAX-sT inhibits tumor growth and induces apoptosis in BEL7402 tumor-bearing nude mice.
Fourteen days after tumor implantation, pVAX-sT was administered by intratumoral injection twice a week for two weeks. The tumor regions were harvested 3 days after the final treatment and analyzed. A. Tumor volume was monitored twice a week. Animals treated with pVAX-sT have significantly smaller tumors than pVAX1-treated or untreated animals. B. Tumor tissues were stained with H&E (×40). The NS (a) and pVAX1-injected tissue (b) showed well preserved and proliferated tumor cells. pVAX-sT-injected tissue (c) showed markedly reduced tumor cells in the center and only small numbers of the cells at periphery. C. TUNEL assay demonstrates that pVAX-sT-injected cells (c) show specific positive stain for apoptotic cells and that NS- (a) and pVAX1-injected cells (b) are not stained for apoptotic cells (×40). D. Representative record of flow cytometric analysis of apoptotic cells by PI. a, NS-injected tumor tissue; b, pVAX1-injected tumor tissue; c, pVAX-sT-injected tumor tissue; pVAX-sT injected tumor tissue induced a distinct sub-G1 peak, which represents the population of apoptotic cells. E. Percentage of apoptotic cells calculated from data in (D). (*$P<0.05$, **$P<0.01$).

tions of pVAX-sT caused profound cell death in the tumor mass *via* apoptosis, whereas no apoptosis was found in tumor tissue receiving pVAX1 or NS injection (Fig. 4C). The PI flow cytometric analysis was done to further determine the apoptotic rate after injection. As shown in Fig. 4D, pVAX-sT expression induced a distinct sub-G1 peak, which represents the population of apoptotic cells. The percentage of apoptotic cells injected with pVAX-sT (24.14±2.24%) was much higher ($P<0.05$) than in the control groups injected with pVAX1 (8.5±1.67%) or NS (7.12±1.4%) (Fig. 4E). These results documented that the pVAX-sT injection induced apoptosis in BEL7402 cells, which coincided with the antitumor effect *in vitro*.

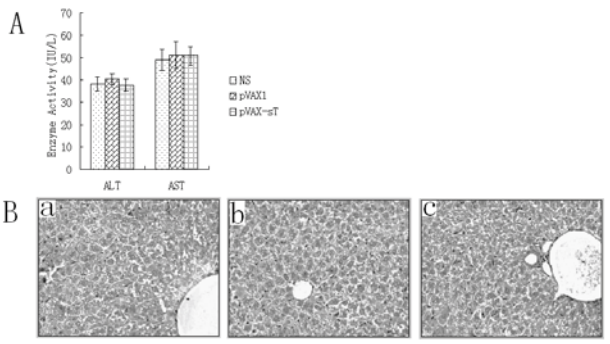


Figure 5. No toxicity of pVAX-sT *in vivo*.
The toxicity of pVAX-sT treatment was examined in non-tumor-bearing mice following the intratumoral injection of 20 μg pVAX-sT naked plasmid. A. Serum levels of ALT and AST in pVAX-sT-treated group were within the reference range 25 days after injection. B. H&E staining of liver tissues. Liver was excised from mice treated with pVAX-sT (c), pVAX1 (b) or NS (a). The results showed there were no obvious lesions in liver.

No toxicity of pVAX-sT *in vivo*

The toxicity of pVAX-sT treatment was examined in non-tumor-bearing mice following the intratumoral injection of 20 μg of naked pVAX-sT. The serum levels of alanine transaminase and aspartate transaminase were within the reference range 25 days after transduction (Fig. 5A) and histopathologic studies showed no obvious lesions in the heart, spleen, lung, kidney, or liver (Fig. 5B). In addition, body mass, gross appearance, and behavior provided no signs of systemic toxicity.

DISCUSSION

Hepatocellular carcinoma (HCC) is a common primary liver cancer with poor prognosis. Current treatment of small, apparently solitary, nodules of HCC is based on partial hepatectomy or liver transplantation, but there is no efficient treatment for HCC patients with multiple tumor nodules. TRAIL has a cancer-specific killer activity without damaging normal cells and tissues *in vivo*. Basing on pre-

vious studies on TRAIL inducing tumor apoptosis (Rezacova *et al*., 2005), this study further explored gene therapy of hepatoma using recombinant plasmid pVAX-sT. The key point of gene therapy is to establish an effective gene delivering system. Vectors which are used to deliver genes nowadays mainly include viral and non-viral vectors. Because of the limitations of viral vectors such as potent danger, immune response and the limitations of the gene length (Bramson *et al*., 1997; Lehrman *et al*., 2000), more and more researchers pay attention to non-viral vectors. In this paper, a high safety vector pVAX1 authorized by FDA in clinical trial was selected to construct a recombinant vector (Wigler *et al*., 1977). pVAX1 has eukaryotic DNA sequences limited to those required for expression in order to minimize the possibility of chromosomal integration, so it offers high bio-safety. pVAX-sT containing the IgG γ sequence can express soluble sTRAIL and induce BEL7402 cells apoptosis *in vitro* and *in vivo*.

Non-viral vectors can be transferred by physical and chemical methods. Naked DNA injection is another simple and safe gene transfer method. We and others have shown that naked plasmid DNA has low toxicity and high effective antitumor action (Imboden *et al*., 2003; Kim *et al*., 2003; Ma *et al*., 2004). As a consequence, high levels of sTRAIL protein were readily detectable both at the site of vector administration and in the serum of these animals.

The major concern with the application of TRAIL in the treatment of tumors *in vivo* is its controversial role in hepatic cell death (Jo *et al*., 2000; Ichikawa *et al*., 2001; Shi *et al*., 2003). Interestingly, TRAIL studies showing hepatotoxicity are all either with the full-length membrane-bound form of the protein (Ichikawa *et al*., 2001) or, if soluble, in combination with exogenous sequence tags (Jo *et al*., 2000). A histidine-tagged TRAIL has been shown to have an altered protein conformation, reduced stability, decreased solubility, and hepatotoxicity (Lawrence *et al*., 2001). However, the same protein without the histidine tag was able to trimerize adequately, giving it biological activity and neoplastic cell toxicity, with little or no evidence of toxicity to primary human hepatocytes *in vitro* (Lawrence *et al*., 2001). More research work (Sean *et al*., 2001) about liver toxicity of TRAIL indicated that it had no hepatotoxicity upon continuous injection of 120 $mg \cdot kg^{-1}$ for 5 days. In this paper, a high safety vector pVAX1 authorized by FDA for clinical trials, was selected to construct pVAX-sT, and there were no exogenous sequence tags on the expressed recombinant TRAIL. The serum levels of alanine transaminase and aspartate transaminase and the histopathologic studies showed pVAX-sT have no hepatotoxicity, so pVAX-sT is a promising anti-cancer drug.

In summary, the present studies provide the first report of pVAX1-mediated delivery and expression of sTRAIL 95–281, demonstrating that intratumoral injection of naked pVAX-sT results in the presence of sTRAIL in sera and tumor site and leads to a statistically significant reduction in the rate of tumor growth. The pVAX-sT administration did not cause any detectable toxicity to the liver of the injected mice, suggesting that pVAX1-mediated sTRAIL gene therapy may provide a feasible and effective form of treatment for liver cancer.

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