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World J Gastroenterol 2004;10(5):654-659
World Journal of Gastroenterology
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• LIVER CANCER •

Anti-liver cancer activity of TNF-related apoptosis-inducing ligand gene and its bystander effects

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Received: 2003-03-20 **Accepted:** 2003-06-02

Abstract

AIM: To observe the anti-liver cancer activity of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene and its bystander effects on hepatocellular carcinoma (HCC) cell line SMMC7721.

METHODS: Full-length cDNA of human TRAIL was transferred into SMMC7721 cells with a binary adenoviral vector system. Polymerase-chain reaction following reverse transcription (RT-PCR) was used to determine the expression of TRAIL gene. Effects of the transfected gene on proliferation of SMMC7721 cells were measured by MTT assay. Its influence on apoptosis was demonstrated by fluorescence-activated cell sorting (FACS). The bystander effect was observed by co-culturing the SMMC7721 cells with and without the transfected TRAIL gene at different ratios, and the culture medium supernatant from the transfected cells was also examined for its influence on SMMC7721 cells.

RESULTS: The growth-inhibition rate and apoptotic cell fraction in the cells transfected with the TRAIL gene, Bax gene or only LacZ gene were 91.2%, 48.0%, 28.8% and 29.1%, 12.5%, 6.6%, respectively. The growth-inhibition rate of transfection with these three sequences in normal human fibroblasts was 6.1%, 45.5% and 7.6%, respectively, indicating a discriminative inhibition of TRAIL transfection on the cancer cells. In the co-culturing test, addition of the transfected TRAIL to SMMC7721 cells in proportions of 5%, 25%, 50%, 75% and 100%, resulted in a growth-inhibition of 15.9%, 67%, 80.2%, 86.4% and 87.7%, respectively. We failed to observe a significant growth-inhibition effect of the culture medium supernatant on SMMC7721 cells.

CONCLUSION: TRAIL gene transferred by a binary adenoviral vector system can inhibit proliferation of SMMC7721 cells and induce their apoptosis. A bystander effect was observed, which seemed not to be mediated by soluble factors.

He C, Lao WF, Hu XT, Xu XM, Xu J, Fang BL. Anti-liver cancer activity of TNF-related apoptosis-inducing ligand gene and its bystander effects. *World J Gastroenterol* 2004; 10(5): 654-659
http://www.wjgnet.com/1007-9327/10/654.asp

INTRODUCTION

TRAIL, first identified by searching an expressed sequence tag (EST) database with a conserved sequence contained in many tumor necrosis factor (TNF) family members, appears to induce apoptotic cell death only in tumorigenic or transformed cells and not in most of normal cells[1,2]. TRAIL has five receptors, including two death receptors DR4 and DR5, two decoy receptors DcR1 and DcR2, one soluble receptor osteoprotegerin. TRAIL is expressed constitutively in many normal tissues, which suggests that normal cells contain mechanisms that protect them from apoptosis induced by TRAIL. One explanation reported was that the decoy receptors DcR1 and DcR2 and another receptor osteoprotegerin could compete with DR4 and DR5 for TRAIL binding[3,4]. Furthermore, TRAIL has a synergistic effect with chemotherapeutic drugs to kill tumor cells and cause substantial tumor regression[5-7]. Much evidence have shown that repeated intravenous injection of a recombinant, biologically active TRAIL protein could induce tumor cell apoptosis, suppress tumor progression, and improve the survival of animals bearing solid tumors without any detectable toxicity in nonhuman primates[5,8]. Therefore, it appears that TRAIL may act as a potent anticancer agent. Furthermore, TRAIL can elicit apoptotic bystander effects on malignant cells. However, few researches of anti-liver cancer activity and bystander effects of TRAIL have been carried out up to date. Here, we first transferred human TRAIL gene to liver cancer cell line SMMC7721 with a binary adenoviral vector system, and assessed the anti-liver cancer activity of TRAIL gene and explored its bystander effects. In addition, we also assessed the toxicity of TRAIL to normal human fibroblasts (NHFB).

MATERIALS AND METHODS

Cell lines and culture conditions

Human embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA (293 cell) and human liver cancer cell line SMMC7721 were obtained from the Key Laboratory of Infective Diseases under Ministry of Public Health (Zhejiang University), normal human fibroblasts (NHFB) from normal human bone marrow were cultured; Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (NHFB with 20% fetal calf serum) in a 5% CO_2 atmosphere at 37 ℃.

Adenoviral vectors

Adenoviral vectors Ad/GT-Bax, Ad/GT-LacZ and Ad/PGK-GV16 were constructed as described previously[9,10]. Ad/GT-TRAIL, an adenoviral vector expressing TRAIL, was also constructed as described previously[9]. Amplification, titration, and quality analysis of all of the vectors were performed as described previously[9,10]. The titer determined by the absorbency of dissociated viruses at $A_{260\ nm}$ (one $A_{260\ nm}$ unit=10^{12} viral particles/ml) was used in the study, whereas the titers determined by plaque assay were used as additive information.

Transgene expression of TRAIL

As determined in preliminary experiments, cells were

coinfected with Ad/GT-TRAIL or Ad/GT-LacZ and Ad/ PGK-GV16 at a ratio of 1:1. The optimal MOI was determined by infecting each cell line with Ad/GT-LacZ +Ad/PGK-GV16 and the expression of β-galactosidase was assessed via X-gal staining. The MOI that resulted in >80% of blue stained cells were used in this experiment. These MOI were 1000 particles for SMMC7721 and NHFB. Unless otherwise specified, Ad/GT-LacZ and Ad/PGK-GV16 were used as the vector control for Ad/GT-TRAIL and Ad/PGK-GV16. Ad/GT-Bax and Ad/PGK-GV16 were used as the positive control. Cells treated with PBS only were used as a blank control.

1×10^6 SMMC7721 cells were plated on 6-well plates and infected with Ad/GT-TRAIL+ Ad/PGK-GV16 or Ad/GT-LacZ + Ad/PGK-GV16. Forty-eight hours after infection, the cells were harvested and washed in PBS. RNA was extracted from the cells using Trizol reagent (Life Technology Inc.) and reversely transcribed to cDNA. The PCR conditions for cDNA amplification were 35 cycles of at 95 ℃ for 45 s, at 58 ℃ for 45 s, at 72 ℃ for 45 s, forward primer: 5' -AGA CCT GCG TGC TGA TCG TG-3' , and reverse primer: 5' -TTA TTT TGC GGC CCA GAG CC-3' . The PCR products were separated in a 10g/L agarose gel and visualized by ethidium bromide staining.

Cell viability

Cell viability was assessed using MTT assay (Amresco) according to the manufacturer' s protocol. The 5×10^3 SMMC7721 and NHFB cells were inoculated in to 96-well plates, with 3 parallel teams. Twenty-four hours after inoculation, the cells were infected with Ad/GT-TRAIL+ Ad/PGK- GV16, Ad/GT-Bax+ Ad/PGK- GV16, Ad/GT-LacZ + Ad/PGK-GV16 at MOI of 1000, and treated with PBS. At the 1st, 3rd and 5th day after infection, the cells were incubated with 5mL/L MTT for 4 h. Then the medium was removed and 150 μl of sterilized DMSO solution was added, followed by incubation at 37 ℃ for 4 h. The absorbance of the reaction solution at 490 nm was measured. These data were used to make growth curves. The cell growth inhibition rate was (1-absorbance of experimental group/absorbance of control group)×100%.

Apoptosis

Cell apoptosis was assessed by observing morphology and using the Annexin Vkit (Immunotech, Annexin-FITC) according to the manufacturer' s protocol. The 5×10^4 SMMC7721 cells were inoculated in to 6-well plates. Twenty-four hours after inoculation, the cells were infected with adenoviruses at MOI of 1000. Then the cell morphology was observed with a reversed microscope every day. On the 4th day, the cells were harvested by trypsinization, washed in PBS and labeled with ANNEXIN V and propidium iodide (PI) according to the manufacturer' s protocol. Finally, they were subjected to flow cytometry to determine the extent of cell death.

Bystander effects

Bystander effects of the TRAIL gene were assayed by MTT as follows: 5×10^4 SMMC7721 cells were washed in PBS and plated on 80-mm dishes, cultured with fresh RPMI 1640. Twenty-four hours later, the cells were infected with adenoviruses at MOI of 1000. Another 24 h after infection, the cells were harvested as the transferred SMMC7721 cells (SMMC7721/TRAIL cells). SMMC7721/TRAIL and SMMC7721 cells were suspended in 2×10^4/ml of RPMI 1640 medium. SMMC7721/TRAIL and SMMC7721 cells were mixed with different ratios, SMMC7721/TRAIL cells accounted for 0, 5%, 25%, 50%, 75% and 100%, respectively. Mixed cells (1×10^4) were inoculated in to 96-well plates with 3 parallel teams each ratio. Four days later the cell viability was determined by MTT assay.

Mechanism of bystander effects

In this study we tried to discover the effect of soluble factors on bystander effects of TRAIL. The 1×10^6 SMMC7721 cells were washed in PBS and plated on 80-mm dishes, cultured with fresh RPMI 1640. Twenty-four hours later, the cells were infected with Ad/GT-TRAIL+ Ad/PGK- GV16, and then cultured for another 24 h. Finally, the dishes were sent to be centrifuged and the medium was collected. This medium was filtrated with a 0.22 μm filter membrane. Other dishes of non-infected SMMC7721 cells were cultured with this filtrated medium. We made the RPMI 1640 medium as blank control and Ad/GT-TRAIL+ Ad/PGK- GV16 as positive control. Four days later, the cell viability was assessed with MTT assay.

Statistical analysis

Statistical analysis was performed with SPSS 10.0, the cells viability and the cell apoptosis ratio were determined by paired *t*-test. Statistical significance was set when $P<0.05$.

RESULTS

Virus titers

The titers of viruses determined by A_{260} were 1×10^{10}particles/mL.

Transgene expression of TRAIL

The expression of TRAIL gene with this system was confirmed *in vitro* in human liver cancer cell line SMMC7721 by RT-PCR. Treatment of cells with Ad/GT- TRAIL+ Ad/PGK-GV16 resulted in a strong TRAIL-specific band, whereas infection with control vectors resulted in undetectable expression (Figure 1). It indicated that TRAIL gene was transferred into the SMMC7721 cells and the binary adenoviral vectors were effective.

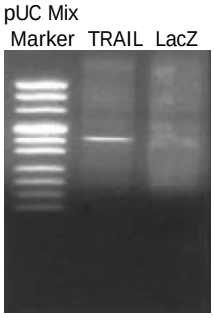

Figure 1 Expression of TRAIL tested with RT-PCR assay.

Cell viability

In cultured SMMC7721 cells, the cell viability (MTT) study showed a significant difference in cell killing effects between lines responsive to treatment with TRAIL-expression vectors versus control vectors, blank controls, even positive controls (Table 1, Figure 2). In cultured NHFB cells, the cell viability study showed a significant difference in cell killing only between TRAIL-expression vectors and Bax-expression vectors (Table 2, Figure 3). These results demonstrated that treatment with TRAIL gene could effectively elicit cell killing in cultured human liver cancer cells but not in normal human fibroblasts. TRAIL gene was more effective than Bax gene in killing cultured human liver cancer cells. Bax gene was obviously toxic to normal human fibroblasts.

Table 1 Cell growth-inhibition of SMMC7721 cells

Groups	OD(mean±SD)	Cell growth-inhibition rate (%)
PBS	1.25±0.20	0
TRAIL	0.11±0.02[ac]	91.2
Bax	0.65±0.13[a]	48.0
LacZ	0.89±0.04	28.8

[a]$P<0.05$ *vs* PBS, [c]$P<0.05$ *vs* Bax.

Table 2 Cell growth-inhibition of NHFB cells

Groups	OD(mean±SD)	Cell growth-inhibition rate (%)
PBS	0.66±0.02	0
TRAIL	0.62±0.02[c]	6.1
Bax	0.36±0.02[a]	45.5
LacZ	0.61±0.03	7.6

[a]$P<0.05$ *vs* PBS, [c]$P<0.05$ *vs* Bax.

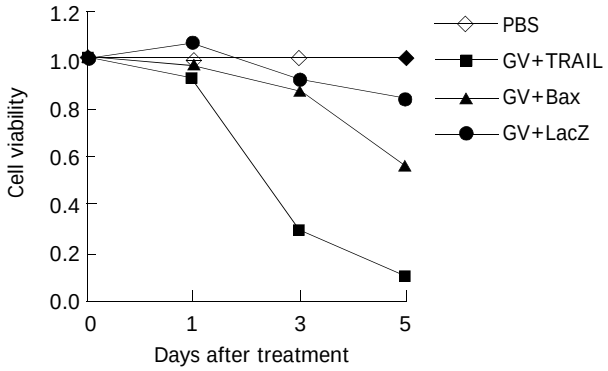

Figure 2 Viability of SMMC7721 cells.

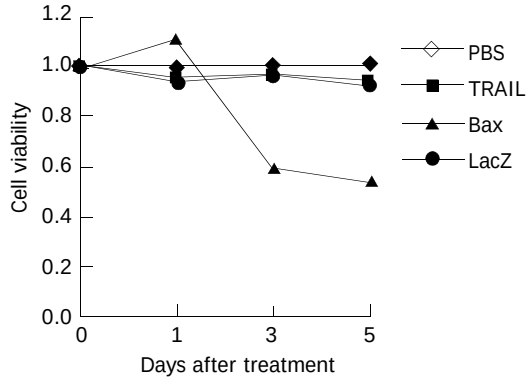

Figure 3 Viability of NHFB cells.

Apoptosis

Morphologic changes of SMMC7721 cells showed cell apoptosis in cells treated with TRAIL gene versus PBS (Figure 4). NHFB cells treated with TRAIL gene and PBS showed no obvious difference in morphology (Figure 5). Assessed by FCM, the apoptosis of SMMC7721 cells infected with TRAIL gene ,Bax gene, LacZ gene and treated with PBS was 29.07%, 12.53%, 6.58%, and 2.94%, respectively. There were significant differences between SMMC7721 cells treated with TRAIL gene and Bax gene, LacZ gene and PBS (Table 3, Figure 6). It indicated that TRAIL gene had a great ability to induce apoptosis of human liver cancer cells. It was also shown that TRAIL gene was more effective than Bax gene in inducing apoptosis of human liver cancer cells.

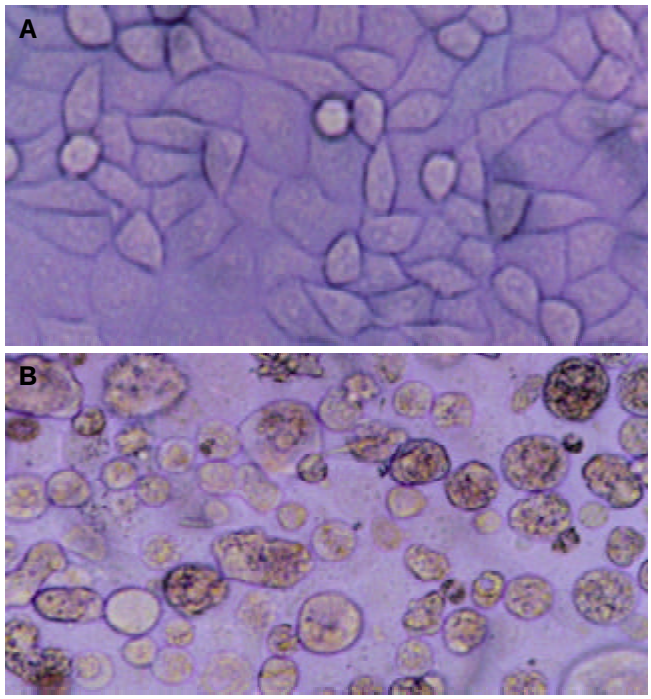

Figure 4 SMMC7721 cells. A: uninfected with Ad/GT-TRAIL+Ad/PGK-GV16, B: infected with Ad/GT-TRAIL+Ad/PGK-GV16.

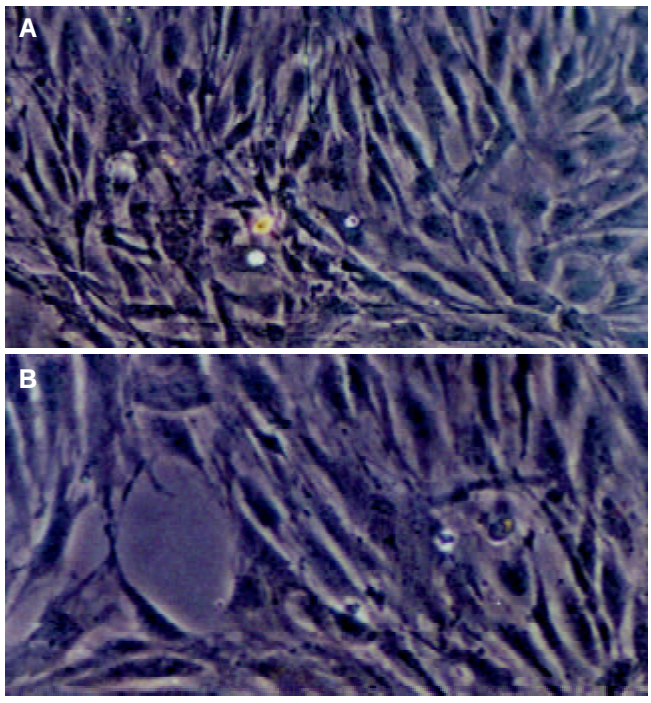

Figure 5 NHFB cells. A: uninfected with Ad/GT-TRAIL+Ad/PGK-GV16, B: infected with Ad/GT-TRAIL+Ad/PGK-GV16.

Bystander effects

The results of MTT assay showed that when transduced SMMC7721/TRAIL cells accounted for 5%, 25%, 50%, 75% and 100% of all cells, 4 days later 15.9%, 67.0%, 80.2%, 86.4%, 87.7% of all cells were killed (Table 4). It showed that partial untransfected cells were killed by bystander effects of TRAIL gene.

Moreover, we found that bystander effects could not be transferred by the medium leached transduced cell components. The viability of SMMC7721 cells cultured with the medium leached transduced cell components was 96%, similar to that

cultured with fresh RPMI 1640 (which was set to be 100%). There were no significant differences between them (P>0.05, Table 5).

Table 3 Percentage of apoptotic SMMC7721 cells

Groups	Percentage of apoptotic cells (%, mean±SD)
TRAIL	29.07±4.96[ace]
Bax	12.53±1.23[ae]
LacZ	6.58±0.49[ac]
PBS	2.94±0.63[ce]

[a]P<0.05 *vs* PBS, [c]P<0.05 *vs* Bax, [e]P<0.05 *vs* LacZ.

Table 4 Bystander effects of TRAIL on SMMC7721 cells

Percentage of SMMC7721 / TRAIL(%)	OD (mean±SD)	Cell growth-inhibition rate (%)
0	0.693±0.028	0
5	0.583±0.036	15.9
25	0.228±0.014	67.0
50	0.137±0.019	80.2
75	0.094±0.009	86.4
100	0.084±0.010	87.7

Table 5 Percentage of apoptotic SMMC7721 cells

Groups	OD (mean±SD)	Cell growth-inhibition rate (%)
PBS	0.846±0.016	0
Cultured medium	0.794±0.027	4.0
TRAIL	0.108±0.010[a]	87.5

[a]P<0.05 *vs* PBS.

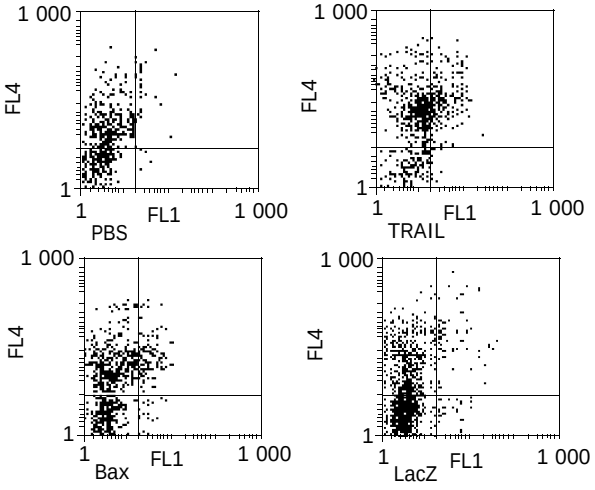

Figure 6 SMMC7721 cell apoptosis.

DISCUSSION

Liver cancer is one of the most malignant cancers with poor prognosis, and about two thirds of patients have been in China[11]. Though many therapeutic methods have been available to its treatment, no one showed a more notable effect than operation. However, the patients who could be radically operated were less than 15%[11]. It is now considered that apoptosis plays an important role in tumorigenesis, and at the same time inducing apoptosis of malignant cells has been prevailing in tumor therapy. There have been clinical reports of primary hepatocellular carcinoma treated with wild-type p53 gene[12].

TRAIL has become an attractive molecule for the treatment of cancer because it could kill tumor cells[5,8]. Studies of recombinant TRAIL protein have revealed that the extracellular portion of TRAIL molecule was sufficient for its antitumor activity but its homotrimerization was necessary for TRAIL protein to retain this activity[5,8], suggesting that the conformational structure of TRAIL is crucial for interaction with its receptors. In this study, we studied whether TRAIL gene could be directly transferred into tumor cells, and whether the expression of biologically active molecules could effectively kill human liver cancer cell lines rather than normal human cells *in vitro*, and whether the bystander effect exists during the process of killing.

It has been reported that a variety of malignant tumors, such as breast carcinoma[13], thyroid carcinoma[14], melanoma[15], glioma[16], multiple myeloma[17,18], colon carcinoma[19] and pancreatic carcinoma[20,21] were all sensitive to TRAIL, but few reports related to liver cancers were available. In this study, binary adenoviral vectors were used to introduce the therapeutic genes into liver cancer cell line SMMC7721 and normal human fibroblasts (NHBF) to evaluate their anti-tumor activity, toxicity and bystander effects.

It was difficult to design an adenoviral vector to transexpress proapoptotic genes because of their high proapoptotic activity and toxicity on packaging 293 cells. Arai *et al*[22] reported that they constructed an adenovirus vector containing Fas-L gene, but it required packaging 293 cells bearing resistance to Fas-L or was decorated with Caspase depressor. Okuyama *et al*[23] designed another system to achieve the transexpression of proapoptotic genes but it was too complicated. We constructed a binary adenovirus vector system, which showed that TRAIL gene had a strong proapoptotic activity on packaging 293 cells. On the other hand this system could augment transgene expressions via a GAL4 gene regulatory system. In brief, we constructed two mated vectors, Ad/PGK-GV16 and Ad/GT-TRAIL, the promoter GT was a synthetic promoter consisting of five GAL4-binding sites and a TATA box, which had a very low transcriptional activity *in vitro* and *in vivo* when it was placed in an adenoviral backbone. But the transgene activity could be substantially induced *in vitro* and *in vivo* by administering this construct in combination with an adenoviral vector (Ad/PGK- GV16) expressing a GT transactivator, the GAL4-VP16 fusion protein[24,25]. A weak promoter PGK would drive expression of the GAL4/VP16 fusion protein (GV16), which in turn would transactivate a minimal synthetic promoter, GAL4/TATA (GT), upstream of a transgene. It has been reported that this system used in CEA-positive cells treated with Ad/CEA-GV16+Ad/GT-LacZ versus Ad/CEA-LacZ had a 20- to 100- fold increase in transgene expression[24]. We determined the ratio of two vectors as 1:1 via X-gal staining with LacZ gene expression. The binary adenoviral vector system was effective for expressing high-level products of the proapoptotic gene. This has been confirmed in Bax gene study[5,26], therefore, Bax gene was used as a positive control in our study. We detected the transgene expression of TRAIL gene by RT-PCR and the result showed quite positive. Another result was that proapoptotic genes TRAIL and Bax had a high toxicity to liver cancer cells while LacZ did not by using this system.

TRAIL demonstrated its antitumor activity in a variety of tumors[1,2,5], although different tumors might vary in their sensitivity. This phenomenon was observed in this study as before[27]. SMMC7721 cells showed 29% of apoptosis. In comparison with other cell lines we tested before, HT29 had 24.6% of apoptosis[28], DLD-1, H460 and A549 had 77%, 26% and 43.5% of apoptosis[27], respectively. The mechanism underlying the differential sensitivity of malignant cells to

TRAIL treatment, as well as the differential killing of normal cells versus malignant cells, remain to be delineated. It could be partially explained by the presence of multiple receptors for TRAIL that functioned as either death-inducing or decoy receptors[4,29,30]. Some groups have proposed that expression of decoy receptors confer resistance to normal tissues[29,30], others have suggested that the level of intracellular Caspase/ apoptosis inhibitors including FLIP and Bcl-X_L might result in resistance[15,31,32]. Oppositely, some other studies considered that the levels of DR4, DR5, and DcR1 could not explain this phenomenon[7,15,27,31]. Accordingly, we hold that the presence of decoy receptors is not the exclusive reason for different sensitivity of different cells. Recently, it was reported that a combined treatment with certain chemotherapy reagents could sensitize resistance to TRAIL-induced apoptosis[5,6,33]. But one of these reports also mentioned that normal cells could be sensitized to TRAIL-inducing apoptosis[7], suggesting that such a combination treatment may also increase toxicity.

Our study revealed that treatment with TRAIL gene was nontoxic to normal human fibroblasts cultured *in vitro*. This result was consistent with previous reports[5,8,27]. Recently it was reported that human hepatocytes were very sensitive to apoptosis induced by recombinant TRAIL protein[34]. But the toxicity could arise from histamine or/and leucin taken in combination with recombinant TRAIL protein[35,36]. Another report showed that Z-LEHD- FMK, one of the Caspase inhibitors, could protect hepatocytes from apoptosis induced by TRAIL, but TRAIL gene could induce apoptosis of malignant cells[37]. Now, we can transfer transgenes directly into target cells by target techniques, such as tumor specific promoter hTERT[38], to reduce the toxicity to normal cells.

One of the bottlenecks of gene therapy for malignant tumors is the low transgene efficiency. It is difficult to transfer target genes into each tumor cell. Investigators have attempted to circumvent this limitation by exploiting what was called the bystander effects based on the transfer of vector transgene products from infected to uninfected cells. Though the existence of bystander effects could not increase the transgene efficiency, it could enhance the cell killing capability. Theoretically, treatment with TRAIL may elicit bystander effects either through interaction of cell surface TRAIL molecules with receptors on the neighboring cells or through the action of soluble TRAIL from TRAIL-expression cells. It has been reported that TRAIL gene could exert proapoptotic bystander effects on cancer cells[27,39] as we demonstrated.

TRAIL is a type II membranous protein, it is speculated that membrane-bound TRAIL can be cleaved and then turned into a soluble form. Both membrane-bound TRAIL and soluble TRAIL could rapidly induce apoptosis in a wide variety of tumor cell lines via interaction with the death receptors DR4 and DR5[40,41]. Nevertheless, in our study, the cell killing effects of TRAIL gene were not transferable with the medium of TRAIL-expressing cell cultures. This result suggested that the proapoptotic activity of TRAIL gene was mainly elicited via membrane-bound TRAIL. The soluble factors contributed little to antitumor activity and to the bystander effects of TRAIL gene. One explanation is that the effects of soluble TRAIL may be dose-dependent. In previous reports, the cell killing activity of recombinant soluble TRAIL was demonstrated, but the effect was elicited by high doses of TRAIL. They could not be achieved by spontaneous cleavage of TRAIL from cultured cells.

In conclusion, we found that TRAIL gene was more effective than Bax gene in killing liver cancer cells with bystander effects TRAIL had no toxicity to normal cells. These results suggest that TRAIL gene is more effective as a therapeutic gene of malignant tumors than Bax gene.

More researches should be carried out to reveal the anti-liver cancer activity and bystander effects of TRAIL *in vivo*. It is possible that TRAIL gene will be used in clinical practices as new promoters and neoteric transgene vector systems are developed.

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Edited by Wang XL **Proofread by** Zhu LH