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Anti-human hepatocellular carcinoma effects of tumor necrosis factor-related apoptosis-inducing ligand *in vivo* and *in vitro*[1]

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KEY WORDS apoptosis; *bcl*-2 genes; tumor necrosis factor; membrane glycoproteins; hepatocellular carcinoma; molecular cloning; reverse transcriptase polymerase chain reaction

ABSTRACT

AIM: To investigate the effect of over-expression of Bcl-2 protein on Trail protein-induced apoptosis in human hepatoma cells, and the cytotoxicity of Trail protein on human hepatoma cells *in vitro* and *in vivo*. **METHODS**: The Trail gene was cloned and expressed in *E coli*. The cytotoxicity of the recombinant Trail protein was assayed on human hepatoma cells *in vitro* and *in vivo*. The cell viability was assessed by trypan blue exclusion. The stable human hepatoma cells clone in which Bcl-2 protein over-expressed was established by transfecting eukaryotic expression plasmid pcDNA3-Bcl-2 into BEL-7404 human hepatoma cells, and was selected with G418 400 mg·L^{-1}. **RESULTS**: The recombinant Trail protein actively killed human hepatoma cells tested in this study such as BEL-7404, BEL-7402, and SMMC-7721. Over-expression of Bcl-2 protein could inhibit apoptosis induced by Trail in BEL-7404 human hepatoma cells *in vitro*. It was obvious that the purified recombinant Trail protein could inhibit tumor formation of BEL-7404 human hepatoma cells in nude mice. **CONCLUSION**: The recombinant Trail protein could kill human hepatoma cells *in vitro* and *in vivo*. Over-expression of Bcl-2 protein could inhibit Trail-induced apoptosis in BEL-7404 human hepatoma cells. The results suggested that Trail might be a potential agent for the liver cancer therapy.

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INTRODUCTION

Tumor necrosis factor (TNF) is the prototypic member of a family of cytokines that interact with a corresponding set of receptor that form the TNF receptor (TNFR) family. Signals induced by these interactions serve such diverse functions as differentiation, proliferation, activation, or induction of apoptosis[1-3]. Three of these ligands, CD95L, TNF, and LTα, have received particular attention because they can induce apoptosis in transformed cells and activated lymphocytes. These molecules were once believed to have potential utility in cancer therapy. But their toxic effects on normal tissues *in vivo* limited their potential widespread use in the treatment of cancer.

TNF-related apoptosis-inducing ligand (Trail) is a type Ⅱ transmembrane protein that was initially identified based on the homology of its extracellular domain with CD95L (28 % identical), TNF (23 % identical), and LTα (23 % identical)[4]. Trail induces apoptosis in a wide variety of transformed cells *in vitro*. To date four receptors for Trail had been identified, including DR4[5], DR5/TRAIL1-R2/TRICK2[6-8], TRID/DcR1/TRAIL-R3[6,7,9], and TRAIL-R4/ DcR2[10,11] called TRAIL-R1 through R4, respectively. Both Trail-R1 and Trail-R2 contain intracytoplasmic death domains and mediate apoptosis in most sensitive targat cell lines. In contrast, neither Trail-R3 nor Trail-R4 (which contain a truncated cytoplasmic death domain) mediates cellular death.

The previous reports demonstrated that Trail could kill most of the transformed cell lines, but had no or weak effect on normal cells[4,12-14]. These results indicated that Trail might prove to be a powerful cancer therapeutic agent. The previous papers proved that Trail could induce apoptosis in chlangiocarcinoma cells[15] and breast adenocarcinoma cells[16] *in vitro* & *in vivo*. In the present study, Trail gene was cloned and expressed. The cytotoxicity of purified recombinant Trail proteins were assessed on the hepatoma cells including BEL-7404, BEL-7402, SMMC-7721, HepG2, Hep3B, and L02, an

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immortal cell of normal human liver, *in vitro*. The effect of Bcl-2 on Trail-induced apoptosis in BEL-7404 cells was determined. The cytotoxicity of purified recombinant Trail on subcutaneous implanted human hepatocellular carcinoma cells was examined *in vivo*, and the potential for Trail in the treatment of HCC was also characterized.

MATERIALS AND METHODS

Cell culture and reagents Human hepatocellular carcinoma cell line BEL-7404, BEL-7402, SMMC-7721, L02, HepG2, and Hep3B (Shanghai Cell Bank, Chinese Academy of Sciences) were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10 % newborn bovine serum (Evergreen, Hangzhou, China), penicillin 100 kU/L and streptomycin 100 mg/L. Cell viability was assessed by trypan blue exclusion. Anti-Bcl-2 antibody was purchased from Santa Cruz Biotechnology Inc. Diazobenzidine (DAB) and G418 were obtained from SIGMA.

Construction of expression plasmid Trail coding 114-281 amino acid was amplified by RT-PCR. In brief, RNA 2 μg total extracted from Hela cells was reversely transcripted using 200 ng 6-mer random primers in a volume of 50 μL reaction for 2 h at 37 ℃ in water bath. Then 1 μL of reverse transcription product was added into 50 μL reactive mixture containing 10 pmol forward primer, 10 pmol reverse primer, 1 × reaction buffer, and 2 unit Taq polymerase . The reaction runs for 32 cycles, each consisted of 94 ℃ denaturing for 30 s, 55 ℃ annealing for 30 s, 72 ℃ extension for 1 min with final extension at 72 ℃ for 10 min. The primer of trail cDNA fragment was: sense 5′-GGAATTCCAGTGAGAGAAAGAGGTCC-3′, antisense 5′-CGGGATCCCGTTTCCAGGTCAGTTAGCC-3′. PCR products of 521 bp was digested by *EcoR* Ⅰ and *BamH* Ⅰ, and inserted into *EcoR* Ⅰ and *BamH* Ⅰ site of plasmid pBluescript SK^+ followed by dideoxy sequencing. To obtain Trail expression plasmid, RT-PCR products were subcloned into prokaryotic expression vector pT7-6His (QIAGEN) in frame with upstream of a sequence encoding a His6 Tag.

Expression and purification of Trail protein Prokaryotic expressing plasmid pT7-6His-Trail was transformed into *E coli* BL21 (DE3), and induced to express 6His-Trail fusion protein at 37 ℃ under isopropyl-1-thio-β-*D*-galactoside (IPTG) 0.1 mmol/L induction for 4 h. The cell culture was harvested and washed with sonication buffer for 3 times. Then the cells were resuspended in sonication buffer and incubated on ice for 30 mins after adding lysozyme 1 g/L and phenylmethylsulfonyl fluoride (PMSF). The cells were sonicated on ice and centrifuged at 12 000 × *g* for 30 min at 4 ℃. The supernatant was collected and mixed with pre-equilibrated Ni^+-NTA resin in sonication buffer for binding at 4 ℃ for 1 h. After washing the mixture for 3 times, the protein was eluted with elution buffer (imidazole 0.3 mol/L in sonication buffer). The eluate was collected, and imidazole was removed by dialysis in PBS. The precipitate was also collected and washed with washing buffer for three times. The purity of purified recombinant protein was inspected by PAGE electrophoresis and the concentration of purified recombinant protein was determined by Bradford method. Finally, the purified recombinant protein was stored at −20 ℃ in 100 μL fractions.

Analysis of apoptosis The induction of apoptosis of BEL-7404 hepatoma cells treated with purified recombinant fusion protein in different doses and duration was assessed by examination of cellular morphological changes using light microscopy and nuclear division stained with PI under fluoroscope. In brief, the harvested cells were washed three times with D-Hanks' solution and fixed in sodium citrate buffer at 4 ℃ for 30 min. Staining solution 500 μL (propidium iodide 50 μg/mL, RNase 10 mg/L) was added to 1×10^6 cells and incubated at 4 ℃ for 15 min in dark before the microscope examination.

Transient transfection (using electroporation method) The apparatus used in the experiment was Gene Pulser™ (BIORAD). Jurket cells in exponential stage were pooled, rinsed twice with D-Hanks' saline solution and suspended in D-Hanks' solution at $0.5 \times 10^{10} - 1.0 \times 10^{10}$ L^{-1}. The *Bcl*-2 gene expressing plasmid pCDNA-3-*Bcl*-2 was added to final concentration of 30 mg/L and incubated on ice for 10 min. Then cells were electroporated one time at 500 V, 25 μF, and 100 Ω, and followed by incubation on ice for another 10 min. Appropriate amount of cells were inoculated into RMPI-1640 medium and developed at 37 ℃ in 5 % CO_2. Meantime, the plasmid pcDNA-3 as a control was also transfected into BEL-7404 cells.

Western blot analysis The Bcl-2 protein expression was determined by Western blot analysis. Preparation of protein extraction and Western blot were based on Oltvai method[17]. In brief, cells were washed twice with D-Hanks' solution and lysed in following

buffer with fresh protease inhibitors [KCl 142.5 mmol/L, $MgCl_2$ 5 mmol/L, HEPES 10 mmol/L (pH 7.2), egtazic acid 1 mmol/L, 0.2 % NP-40, PMSF 0.2 mmol/L] for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 12000 × *g* for 10 min. Protein concentration was determined by Bradford's method. For Western blot, 10 μg of total protein was subjected to 12 % SDS-PAGE and electrotransfered at 4 ℃ for 2 h onto Nitrocellulose membrane. After blocking at 37 ℃ for 2 h with TBST (Tris-buffered saline containing 0.5 % Tween-20) containing 5 % nonfat milk, membrane was incubated with anti-Bcl-2 antibody (1 : 100, Sino-America Biotech) at 37 ℃ for 2 h. Membrane was subsequently incubated with HRP conjuncated second antibody (1 : 2000, Vector) at room temperature for 2 h. Finally, membrane was developed with diaminobenzidine (Amersico) and H_2O_2. Membrane was washed with TBST for 3 times between each step.

Treatment of BEL-7404-implanted mice with recombinant fusion Trail protein Male BALB/c-nude mice 7-week-old (Shanghai Institute of Materia Medica, Certificate № 122) were implanted subcutaneously 5×10^6 BEL-7404 cells. Recombinant fusion Trail protein in 200 μL TBS (6 mg/kg) or 200 μL TBS as control were injected intravenously into nude mice respectively everyday for 10 d from the third day after implantation of BEL-7404 cells. After 5 weeks, all the mice were killed and the solid tumors were measured in length and width. The size of the solid tumors were calculated with the formula: $V = (L \times W^2) \times 0.5$ (V = volume; L = length; W = width).

Statistics All data were expressed as $x \pm s$. P value was evaluated by t test.

RESULTS

Construction of prokaryotic expression plasmid To obtain the cDNA coding 114-281 AA of Trail protein, a pair of primer shown in materials and methods was designed according to the reported Trail sequence. Total RNA 2 μg extracted from HeLa cell line was reversely transcripted. The product was used as the templet to run PCR. A predicted 521 bp band was obtained (Fig 1) and subcloned into plasmid pBluescipt SK$^+$ for sequencing.

The sequencing result was identical to the reported sequence of Trail. Then the fragment was recovered

Fig 1. Cloning of Trail by RT-PCR. M: DNA marker; 1: 521bp band (RT-PCR product); 2: pBluescript SK$^+$-Trail cut by *EcoR* I and *BamH* I; 3: negative control RT-PCR with total RNA as template.

from pBluescript SK$^+$ Trail with *EcoR* I and *BamH* I digestion and inserted into the expressing vector pT7-6His.

Expression and purification of Trail The recombinant plasmid pT7-6His-Trail was transformed into *E coli* BL21 (DE3). LB 1000 mL culture was prepared to induce expression of Trail protein under IPTG 0.1 mmol/L for 4 h. The cells were harvested, washed three times, and sonicated in sonication buffer. The supernatant was loaded onto the pre-equilibrated Ni^{++}-NTA resin column. As shown in the Fig 2, the cells expressed a predicted protein (M_r = 21000). The expressed Trail protein existed not only in soluble form (Fig 2, lane 3) but also in inclusion body form (Fig 2, lane 4). The purity of purified recombinant protein Trail (Fig 2, lane 1 or lane 2) is over 95 %.

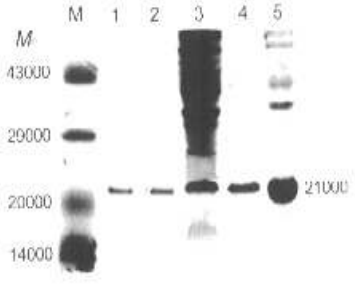

Fig 2. SDS-PAGE of recombinant Trail protein. M: Protein standard marker; 1, 2: Purified Trail; 3: Supernatant; 4: Inclusion body; 5: Cell total protein.

Activity assay of recombinant fusion protein Trail on hepatoma cells To investigate the biological activity of soluble purified recombinant protein Trail,

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human hepatocellular carcinoma BEL-7404 cell was selected as a target for responsiveness assay. The result showed that recombinant protein Trail could cause a remarked reduction in cell number, morphological change, and chromatin condensation, which were characteristic of apoptosis, in BEL-7404 cells (Fig 3A, B, C). We then screened several hepatoma cell lines using Trail protein 0.1 mg/L. The percentage of apoptosis was measured by trypan blue exclusion method. As shown in Fig 4, all the cells were treated with purified recombinant Trail protein 0.1 mg/L for 6 h. The rate of apoptosis was 82 %, 78.4 %, 87.5 %, 54.8 %, 47.3 %, 43.7 %, and 6.2 % in BEL-7404 cells, BEL-7402 cells, SMMC-7721 cells, HepG2 cells, Hep3B cells, L02 cells, and ES cells repectively. These data indicated that the hepatoma carcinoma cells such as BEL-7404 cells, BEL-7402 cells, SMMC-7721 cells were sensitive to recombinant fusion protein Trail, while the cytotoxicity of recombinant fusion protein Trail on normal cells, for example, ES cells was weaker.

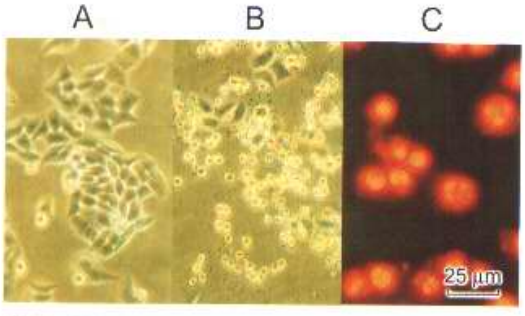

Fig 3. Morphological change of cells and the percentage of apoptosis induced by Trail protein 100 mg/L for 6 h on BEL-7404 cell line. A: BEL-7404 cells (× 100); B: Trail-treated BEL-7404 cell (the cell detached from dish and became shrunken, × 100). C: the nucleus was divided into several small nucleuses stained with PI (× 400).

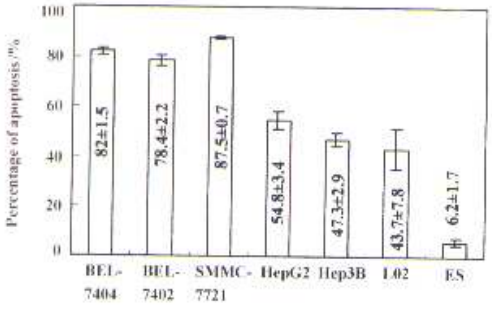

Fig 4. Cytotoxicity of Trail on several cell lines. All the cells were treated with purified recombinant protein Trail 0.1 mg/L for 6 h. $n = 6$. $\bar{x} \pm s$.

Over-expression of Bcl-2 inhibited Trail-induced apoptosis in BEL-7404 cells Many genes such as the members of *Bcl-2* family and TNF family regulate apoptosis. In mammalian cells *Bcl-2* was the first anti-apoptosis gene that could inhibit apoptosis induced by many signals, for example ultra-violet radiation, anti-tumor drugs, and deprivation of growth factor. In order to investigate the effect of Bcl-2 protein over-expression on Trail-induced apoptosis in hepatocellular carcinoma cells, the expression of Bcl-2 protein was screened in several hepatocellular carcinoma cells with Western blot. As shown in Fig 5, all the inspected hepatocellular carcinoma cells did not express Bcl-2 protein. Then we transfected eukaryotic recombinant plasmid pCDNA-3-Bcl-2 and pcDNA-3 as a control into BEL-7404 cells by electroporation method. Stable clone overexpressing Bcl-2 protein was obtained by selection under 400 mg/L G418 (Fig 5, Lane 7). Purified recombinant Trail protein 0.1 mg/L was used to act on BEL-7404-Bcl-2 cells, parent BEL-7404 cells, and the BEL-7404 cells transfected with control plasmid pcDNA-3 for 6 h. As shown in Fig 6, 82 % parent BEL-7404 cells underwent apoptosis, while only 7.5 % apoptosis occurred in BEL-7404-Bcl-2 cells. The results indicated that Bcl-2 protein could counteract the cytotoxicity of Trail in hepatocellular carcinoma BEL-7404 cells.

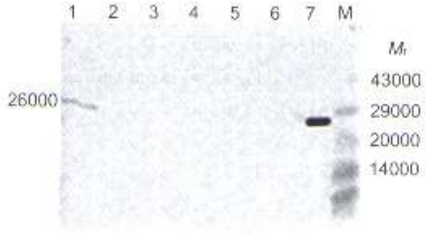

Fig 5. Expression of Bcl-2 in several hepatoma cell lines. 1: L02; 2: HepG2; 3: Hep3B; 4: BEL-7402; 5: BEL-7721; 6: BEL-7404; 7: BEL-7404 transfected with eukaryotic recombinant plasmid pcDNA-3-Bcl-2; M: marker.

To determine whether the tumoricidal activity of Trail observed *in vitro* could be demonstrated *in vivo*, BALB/c-nude mice were injected subcutaneously with 5×10^6 BEL-7404 cells. After 3 d, experimental mice were injected intravenously with 6 mg/kg recombinant

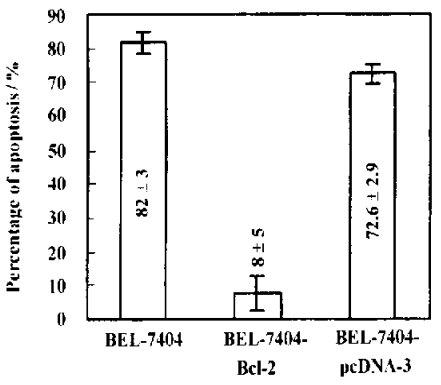

Fig 6. Percentage of apoptosis induced by Trail 0.1 mg/L in BEL-7404 cells and BEL-7404-Bcl-2 cells for 6 h. $n=6$. $\bar{x} \pm s$.

Trail protein in 200 μL TBS once a day for ten days, at the same time the control mice were injected only with 200 μL TBS. Five weeks later only one experimental nude mouse had a smaller solid tumor in contrast to control mice in which five in six had bigger solid tumors (Tab 1). All solid tumors were measured in length and width and the size of the solid tumor were calculated with the formula mentioned in methods and materials. The ability of recombinant fusion protein Trail inhibiting the growth of subcutaneously implanted hepatoma cell was very obvious.

Tab 1. Tumoricidal effects of Trail on human hepatocellular carcinoma cell BEL-7404 in nude mice. $n=6$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control group.

Implanted cells	Treatment of mice	Time/week	Tumor incidence	Mean tumor size/mm^3
BEL-7404	TBS	5	6/6	126 ± 32
BEL-7404	Trail in TBS	5	1/6	6 ± 8^c

DISCUSSION

TNF was initially found to cause hemorrhagic necrosis *in vivo* in some mouse tumors and kill cancer cells *in vitro*, indicating its potential for cancer therapy[13]. However, it became apparent that TNF rarely induced direct cytolysis in tumor cells *in vivo*. Instead, injection of TNF into animal activates the immune system and kills the animal within a few minutes to hours, causing ischemic and hemorrhagic lesion in various tissues[3]. CD95 could induce apoptosis and was also proposed as an anti-cancer drug[16]. However, a similar agonistic CD95 was shown to kill mice by causing massive hemorrhagic necrosis in the liver[18].

Trail, a new member of TNF family, is a newly found cytokine that could induce apoptosis in many tumor cell lines, but not in normal cell lines. As for its specific killing activity on tumor cell line, Trail has been regarded as a potential cancer therapeutic agent. Compared with TNF or CD95, a cytokine that could be cytotoxic to normal tissues *in vivo*, Trail showed lower or no cytotoxicity to normal tissues *in vivo*[16]. These data indicated that Trail might be a potential cancer therapeutic agent.

In order to determine the tumoricidal activity of recombinant protein Trail on liver cancer, the cytotoxicity of recombinant protein Trail was examined on many hepatocellular carcinoma cells. The results showed that all the tested hepatocellular carcinoma cells were sensitive to the recombinant protein Trail *in vitro*. Compared with previous report[19] that HCC cells showed, to a certain extent, resistance to Trail suggested that different source of HCC cells might show different sensitivity to Trail. Otherwise, we found that recombinant protein Trail was toxic to immortal human liver cell L02 though its killing activities on L02 was weaker than that on human hepatocellular carcinoma cells. A similar result was described by Jo[20]. *In vivo*, Trail could inhibit formation of solid tumor of subcutaneously implanted human hepatocellular carcinoma BEL-7404 cells in BALB/c-nude mice. The question remained about the possibility of usage of Trail on treatment of human cancer therapeutics. It is suggested that a mode for test the cytotoxicity of Trail on normal hepatocellular cell *in vivo* must be set up.

Recent paper reported that various cell lines over-expressing the antiapoptotic proteins Bcl-2 or Bcl-XL were not or only marginally protected against Trail induced apoptosis[21]. But there were other reports indicated that the over-expression of Bcl-2 showed inhibitory effect on Trail-induced apoptosis[13]. Our data showed that Trail induced apoptosis in hepatocellular carcinoma cells BEL-7404 could be almost completely inhibited by Bcl-2 protein over-expression. These different results indicated the existence of different signal pathway that transducted death signal from Trail.

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肿瘤坏死因子相关凋亡诱导配体的体内体外抗人肝细胞癌的作用[1]

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关键词 细胞凋亡；*bcl*-2 基因；肿瘤坏死因子；膜糖蛋白类；肝细胞癌；分子克隆；逆转录聚合酶链反应

目的：研究 Bcl-2 蛋白的过表达对 Trail 诱导肝癌细胞凋亡的影响，以及 Trail 蛋白体内和体外对人肝癌细胞的杀伤作用. **方法**：克隆 Trail 基因并在大肠杆菌中表达 Trail 重组蛋白. 检测 Trail 重组蛋白在体内和体外对肿瘤的杀伤作用. 用台盼蓝拒染法检测细胞的凋亡率. 将真核细胞表达质粒 pcDNA3-Bcl-2 转染到人肝癌细胞 BEL-7404 细胞中，并通过 G418 400 mg/L 筛选得到 Bcl-2 蛋白稳定表达的细胞株. **结果**：重组蛋白 Trail 能够有效地杀死人肝癌细胞(包括 BEL-7404，SMMC-7721，和 BEL-7402 等细胞). 在体外，过量表达的 Bcl-2 蛋白能抑制 Trail 重组蛋白对人肝癌细胞 BEL-7404 的杀伤作用. 重组蛋白 Trail 能够有效地抑制人肝癌细胞 BEL-7404 在裸鼠中形成实体瘤. **结论**：在体内和体外，Trail 重组蛋白能够杀死肝癌细胞. Bcl-2 蛋白过表达可以抑制 Trail 重组蛋白对肝癌细胞 BEL-7404 的杀伤作用. Trail 蛋白是一种潜在的肝癌治疗剂.

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