Tumor necrosis factor-related apoptosis-inducing ligand gene on human colorectal cancer cell line HT29

Xiang-Ming Xu, Chao He, Xiao-Tong Hu, Bing-Liang Fang

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand, is also called Apo-2L, a new member of TNF family. It was first identified through a search of an expressed sequence tag (EST) database using a conserved sequence contained in many TNF family members. TRAIL is a type II transmembrane protein whose extracellular region forms a soluble molecule on cleavage. Both membrane-bound TRAIL and soluble TRAIL rapidly induce apoptosis in a wide variety of tumorogenic cells via interaction with the death receptors DR4 and DR5. However, unlike its relatives, TNF and CD95L, TRAIL appears to induce apoptosis only in tumorogenic or transformed or virus infected cells and not in normal cells. Because of its selective cellular toxicity, TRAIL may act as a safe agent for tumor gene therapy[1,2]. In our experiment, human colorectal cancer cell line HT29 was transfected with binary adenovirus-mediated TRAIL gene. Bax gene was used as positive control, LacZ gene was used as the vector control, and cells treated with PBS only were used as a mock control. To evaluate the therapeutic efficiency of TRAIL on human colorectal cancer cell line HT29, reversemicroscope, MTT method and flow cytometry were used.

MATERIALS AND METHODS

Materials

Adenoviral vectors and cell lines: Ad/GT-TRAIL, Ad/GT-Bax, Ad/GT-LacZ and Ad/PGK-GV16 were presented by Dr. Bingliang Fang (Department of Thoracic and Cardiovascular surgery, The University of Texas M.D .Anderson Cancer center). Human embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA (293 cell) was maintained in our laboratory (Clinical research institute of Sir Run Run Shaw Hospital, Zhejiang University). Human colorectal cancer cell line HT29, a kind gift from Dr. Junhui Cui (Medical School, Zhejiang University).

Methods

Construction, amplification and titration of adenoviruses

Adenoviral vectors Ad/GT-Bax, Ad/GT-LacZ and Ad/PGK-GV16 were constructed as described previously[14,15]. Ad/GT-TRAIL, an adenoviral vector expressing TRAIL, was also constructed. Briefly, a cDNA containing the entire coding sequence of human TRAIL was inserted into an expression cassette driven by a GT promoter to generate shuttle plasmid pAD/GT-TRAIL. This shuttle plasmid was then cotransfected into 293 cells along with a 35-kb Clal fragment from adenovirus type 5. Then, recombinant vector Ad/GT-TRAIL was generated by homologous recombination and plaque-purified. To produce large quantities of the virus, when 293 cells (transformed primary human embryonal kidney cells) are grown to 60-70 % confluency on 75 cm2 bottle, the media were changed using RPMI1640 supplemented with 10 % FBS and antibiotics. Purified virus is added to the bottles at a multiplicity of infection (MOI) of 100:1. Every 15 min gently rotate the bottles to redistribute the liquid over the entire bottle, and rotate the bottles to allow for even distribution of the viral...
suspension. After one hour incubation, add 15 ml fresh media to each bottle. Incubate the bottles in a 37 °C, 5 % CO2 incubator for 48-72 h, harvest the cells when the complete cytopathic effect (CPE) is evident (95-100 % cells rounded with 10-20 % floating). The cells should be lifted off the bottle by gently pipetting medium over them and collect cells into 50 ml centrifuge tubes. Centrifuge in a clinical centrifuge at room temperature for 3-4 min at 1 500 rpm. The supernatant may be placed directly at -70 °C for further infection. The cell pellet may also be placed directly at -70 °C until ready for purification. After three cycles of freeze-thaw the cell pellets, remove the cellular debris by centrifuging in a clinical centrifuge at 2 500 rpm for 5 min. The titer determined by the absorbency of the dissociated virus at A260 nm (one A260 units=1×1012 viral particles/ml) was used in this study. All of the viral preparations were found to be free of the E1+ adenovirus by PCR assay and endotoxin by testing with a Limulus amebocyte lysate endotoxin detection kit.

**MTT assay of cell growth**

Cell growth was measured by MTT methods. Cells were seeded onto 96-well plates at a density of 10^4 in 100 µl of medium per well. When HT29 cells were grown to 80 % confluency, PBS, Ad/GT-LacZ, Ad/GT-Bax, Ad/GT-TRAIL, Ad/GT-LacZ+Ad/PGK-GV16 (1:1), Ad/GT-Bax+Ad/PGK-GV16 (1:1), Ad/GT-TRAIL+Ad/PGK-GV16 (1:1) were added respectively. The optimal MOI was determined by infecting HT29 cell with Ad/GT-LacZ+Ad/PGK-GV16 (1:1) and assessing the expression of β-galactosidase via X-gal staining. The MOI that resulted in >85 % of cells being stained blue were used in this experiment. The MOI was 1 000 for HT29 cells. Unless otherwise specified, Bax was used as positive control, LacZ was used as the vector control, and cells treated with PBS only were used as a mock control. At 0, 1, 3 and 5 days, cells were incubated with 0.5 % MTT for 4 hours. The medium was then removed as a mock control. At 0, 1 d, 3 d and 5 d, cells were incubated with 0.5 % MTT for 4 hours. The absorbance of the reaction solution at 490 nm was measured. These data were used to make growth curves. The cell suppression percentage=(1-absorbance of experimental group/absorbance of control group)×100 %.

**Assay of apoptosis (FCM)**

HT29 cells were seeded onto 6-well plates at a density of 4×10^4 in 2.5 ml of medium per well. The cells were given the same treatment as we did in the MTT assay. The MOI was 1 000, reverse microscope was used to watch the morphological changes. Cells were harvested by trypsinization at 5 d after treatment, then FCM analysis for cell surface molecules was performed using Annexin V Kit (Immunotech, Annexin-FITC), according to the following procedure: wash cells twice with cold PBS, resuspend cells in 1×Binding Buffer at a concentration of 1×10^6/ml. Transfer 100 µl to a 5 ml culture tube, add 5 µl Annexin V-FITC and 10 µlPI, gently vortex the tube and incubate for 15 min at room temperature in the dark. Add 400 µl 1×Binding Buffer to each tube, analyze by flow cytometry as soon as possible (within one hour). PI/Annexin V-negative cells were regarded as living cells; PI-positive/Annexin-negative cells were regarded as injured others; PI-negative/Annexin V-positive cells were regarded as early apoptotic cells; PI/Annexin V-positive cells were regarded as late apoptotic or secondary necrotic ones.

**Statistical analysis**

Differences among the treatment groups were assessed by paired t-test using SPSS10.0 statistical software. P<0.05 indicates significant difference.

**RESULTS**

**Titers of recombinant adenovirus**

All adenoviral vectors titer determined by optical absorbency at A260nm were 1×10^{10} viral particle/ml (vp/ml).

**Morphological changes of recombinant adenovirus on HT29 cell**

When HT29 were transfected with Ad/GT-TRAIL, Ad/GT-Bax, obviously morphological changes of HT29 were found. These changes were found to be much more early and obviously when Ad/PGK-GV16 was cultured together. Over 80 % of the cells showed signs of cytopathology, and became rounded and detached. However, when HT29 were transfected with Ad/GT-LacZ, Ad/GT-LacZ+Ad/PGK-GV16, no significant morphological changes were found, the cells remained in monolayers with normal morphology.

**Cell growth inhibition of recombinant adenovirus on HT29 cell**

Cell viability was measured by MTT assay as showed in Figure 1 and Figure 2. Cultured with Ad/GT-TRAIL, Ad/GT-Bax, Ad/GT-LacZ, the cell growth of HT29 was inhibited by 52.5 %, 30.3 % and 10.5 % respectively (Table 1). When Ad/PGK-GV16 was added, the cell growth inhibition rates were 85.2 %, 61.5 % and 12.1 % respectively (Table 2). There were significant difference between TRAIL, Bax and LacZ, and PBS treatment. Ad/GT-TRAIL and Ad/GT-TRAIL+ Ad/PGK-GV16, Ad/GT-Bax and Ad/GT-Bax+ Ad/PGK-GV16 (P<0.05). There were also shown significant difference between TRAIL and Bax treatment when combined with Ad/PGK-GV16. The result showed TRAIL was more efficient to inhibit cell growth than Bax on HT29.

**Table 1 Cell growth inhibition of recombinant adenovirus on HT29 cell**

| Group               | OD (x±s)  | Cell growth inhibition rates (%) |
|---------------------|-----------|---------------------------------|
| Ad/GT-GT-TRAIL     | 0.57±0.12 | 52.5                            |
| Ad/GT-Bax          | 0.84±0.08 | 30.3                            |
| Ad/GT-LacZ         | 1.07±0.13 | 10.5                            |
| PBS                 | 1.20±0.17 | 0.0                             |

*<0.05, vs LacZ, PBS groups; <0.05, vs Bax group.*

**Table 2 Cell growth inhibition of recombinant adenovirus on HT29 cell when combined with Ad/PGK-GV16**

| Group               | OD (x±s)  | Cell growth inhibition rates (%) |
|---------------------|-----------|---------------------------------|
| Ad/GT-TRAIL+Ad/PGK-GV16 | 0.18±0.03 | 85.2                            |
| Ad/GT-Bax+Ad/PGK-GV16 | 0.46±0.09 | 61.5                            |
| Ad/GT-LacZ+Ad/PGK-GV16 | 1.05±0.07 | 12.1                            |
| PBS                 | 1.20±0.17 | 0.0                             |

*<0.05, vs LacZ, PBS groups; <0.01, vs Bax group.*

**Apoptosis detected by flow cytometry**

Apoptosis was detected by flow cytometry (Figure 3). The percentage of apoptotic cells treated with Ad/GT-TRAIL, Ad/GT-Bax, Ad/GT-LacZ and PBS (120 h after treatment) were 16.5 %, 14.7 %, 7.45 %, and 6.15 % respectively (Table 3). There were significant difference between Ad/GT-TRAIL, Ad/GT-Bax and Ad/GT-LacZ, PBS group (P<0.05). When combined with Ad/PGK-GV16, the percentage of apoptotic cells were significantly increased (P<0.05), 35.9 %, 29.0 % and 11.2 % respectively (Table 4). There were no significant difference between Ad/GT-TRAIL and Ad/GT-Bax treatment,
but there were significant difference between Ad/GT-TRAIL+Ad/PGK-GV16, Ad/GT-Bax+Ad/PGK-GV16 and Ad/GT-LacZ+Ad/PGK-GV16, PBS treatment (P<0.01).

**Table 3** The percentage of apoptotic cells of recombinant adenovirus on HT29 cell after 5 d treatment

| Group | The percentage of apoptotic cells (%) ± s |
|-------|------------------------------------------|
| Ad/ GT-TRAIL | 16.50±1.13^a |
| Ad/ GT-Bax   | 14.70±0.46 |
| Ad/ GT-LacZ  | 7.45±0.75  |
| PBS          | 6.15±0.23  |

^aP<0.05, compared with LacZ, PBS groups.

**Table 4** The percentage of apoptotic cells of recombinant adenovirus on HT29 cell when combined with Ad/PGK-GV16 after 5 d treatment

| Group | The percentage of apoptotic cells (%) ± s |
|-------|------------------------------------------|
| Ad/ GT-TRAIL +Ad/ PGK-GV16 | 35.90±3.32^b |
| Ad/ GT-Bax+Ad/ PGK-GV16    | 29.00±1.66^b |
| Ad/ GT-LacZ+Ad/ PGK-GV16   | 11.23±1.14  |
| PBS                        | 6.15±0.23  |

^bP<0.01, compared with LacZ, PBS groups.

**DISCUSSION**

Colorectal cancer is one of the malignant tumors that threaten human life severely. The main treatments of colorectal cancer are surgical resection, chemotherapy and radiotherapy. Because of local recurrence and metastasis, the 5-year survival rate after surgical resection is about 50%. Gene therapy is a new method of introducing genetic material into cells developed in modern medicine and molecular biology. It is certain that occurrence of colorectal cancer is the result of interaction between hereditary and environmental factors, research on colorectal gene therapy has been undertaken in recent years, including suicide gene therapy (HSV-TK/GCV system,CD/5-FC system), tumor suppressor gene, immunogene therapy and anti-VEGF gene therapy. The idea of them is to induce apoptosis of tumor cells [6-9].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a new member of the TNF family, showed a promising result for tumor gene therapy because of its selective cytotoxic effect. TRAIL is present on many normal cell surfaces. It appears to induce apoptosis only in tumorigenic or transformed cells or virus infected cells but not in normal cells [10,11]. It has five receptors: DR4[12], DR5[13], DcR1[14], DcR2[15], OPG[16] (Osteoprotegerin). Both member-bound TRAIL and soluble TRAIL induce apoptosis rapidly via interaction with death receptors DR4, DR5. DcR1, with no death domain and DcR2, with a truncated death domain are not capable of inducing apoptosis, however by competing for TRAIL, they are capable of inhibiting TRAIL-induced apoptosis, thereby protecting normal cells from the cytotoxic effect[17,18]. In addition, it is important for tumor gene therapy that TRAIL may elicit bystander effects either through interaction of surface TRAIL molecules with receptors on neighboring cells or through the action of soluble TRAIL from the TRAIL-expressing cells[19].

Recent studies showed that TRAIL induced apoptosis in a wide variety of tumor cell lines, both in *vitro* and in *in vivo*. Because of the TRAIL gene’s high apoptotic activity and its toxic effect on packaging 293 cells, constructing an adenoviral vector that can express TRAIL remain to be a problem. In this study, we constructed adenoviral vectors expressing the human TRAIL gene using a binary vector system that allows expression of a highly apoptotic gene. Briefly, the TRAIL gene’s promoter was replaced by GT, a synthetic promoter consisting of five GAL4-binding sites and a TATA box, which has very low transcriptional activity *in vitro* and *in vivo* when placed in a adenoviral backbone. Moreover, the transgene activity can be substantially induced *in vitro* and *in vivo* by administering this construct along with an adenoviral vector (Ad/PGK-GV16) expressing a GT transactivator, namely, the GAL4-VP16 fusion protein[20]. This means TRAIL protein expression could be induced by coinfecting target cells with Ad/GT-TRAIL and Ad/PGK-GV16. One potential problem that may arise in using this system is that not all the target cells may be transduced by both the transgene-expressing and transactivator-expressing vector. Our early study showed in *vitro* transduction of H1299 cells with Ad/CMV-LacZ or Ad/GT-LacZ+Ad/PGK-GV16(1:1) at the same MOI showed equivalent blue cells by X-gal staining, which suggested that transduction efficiency may not be hampered by using two vectors. The binary adenoviral vector system was effective for expressing high levels of the proapoptotic gene, it was also confirmed in Bax gene study[21]. Here Bax gene was used as positive control. The results showed Ad/GT-TRAIL and Ad/GT-Bax were able to induce apoptosis of HT29 and inhibit its growth. When combined with Ad/PGK-GV16, the effects were enhanced significantly, it was testified again that the binary adenoviral vector system was simple and effective for
expressing high levels of the proapoptotic gene. This method may provide an alternative approach for colorectal cancer gene therapy. Our result also provides experimental evidences for preclinical research on colorectal cancer gene therapy. There are several advantages using adenoviral vector: high transduction efficiency; high expression of report gene; high viral titer; simpler to construct recombinant virus; insert size up to 8kb. Direct intratumoral injection of recombinant adenoviral vector can induce tumor cell apoptosis, suppress tumor progression and even ablate the tumor with TRAIL gene’s antitumor and bystander effect. Recent studies showed that repeated i.v. injection of recombinant and biologically active TRAIL induced tumor cell apoptosis, suppressed tumor progression, and improved survival in mice bearing colorectal cancer model (DLD-1), with no detectable toxicity[26]. Whereas toxic effects of TRAIL on human hepatocytes in vitro were observed, concern must be raised about the potential toxicity of TRAIL, especially when administered systemically. A non-tagged, soluble, native-sequence form of TRAIL(14,281) failed to induce hepatotoxicity in cynomolgus monkeys[24]. A recombinant, soluble version of the ligand fused to a trimerizing leucine zipper (amino acids 95-281) also lacked hepatotoxicity in mice[14]. More recently, a polyhistidine-tagged recombinant soluble form of TRAIL (amino acids114-281) was reported to induce apoptosis in cultured human hepatocytes[29]. Although the recent report of hepatocyte death after treatment with TRAIL in vitro must be taken seriously, it is important that the limitations of the model system used in these experiments are also understood[23]. Fortunately, further research showed that the proapoptotic activity of the TRAIL gene is mainly elicited via membrane-bound TRAIL, and soluble factors contribute little to antitumor and bystander effect. The possible explanation is that the effects of the soluble TRAIL may be dose-dependent, conformation-dependent. It has been reported that only the trimerized recombinant TRAIL is the most effective. With direct intratumoral injection of TRAIL, the release of soluble TRAIL from such TRAIL-expressing tumor cells is not substantial enough to cause liver damage, and toxicity may be reduced by vector-targeting strategies. TRAIL has a strong bystander effect, but our preliminary data showed the bystander effect was mediated by the membrane-bound TRAIL, and it was cell-contact dependent. Therefore, efficient delivery of the TRAIL gene into as many cancer cells as possible is still an important goal in treating cancer patients.

Although our data showed TRAIL and Bax gene can significantly inhibit the growth of HT29, the percentage of apoptotic cell are not very high. It has been shown that different kinds of tumors, even different cell lines of the same tumor have different sensitivity to TRAIL, which had relation to level of FLIP, low expression or mutation of DR4 gene, activation or not of NF-kB, overexpression of Bcl-2, etc. However with the addition of chemotherapeutic agent or immunoregulator[24,25], TRAIL-resistant tumor cells recovered its sensitivity to TRAIL. For example, addition of actinomycin D to TRAIL-resistant melanomas resulted in decreased intracellular concentrations of FLIP, which correlated with their acquisition of TRAIL sensitivity[25]. The same results were found, when treated with Doxorubicin or 5-Fu to induce apoptosis of breast cancer, Doxorubicin or Camptothecin in human hepatocellular carcinoma dramatically augmented TRAIL-induced cytotoxicity[26]. In experiment of the antitumor activity of recombinant TRAIL on mice bearing human colon carcinoma, GlimiaB[29] found that these tumors displayed a differential sensitivity to TRAIL in vivo that paralleled their susceptibility to TRAIL-induced apoptosis in vitro. It demonstrated that TRAIL alone was a potent antitumor agent in vivo, and its activity could be significantly enhanced in combination with the chemotherapeutic agent CPT-11. However, one of these reports also mentioned that normal cells could be sensitized to TRAIL-induced apoptosis[30]. This suggests that such a combination treatment may also increase toxicity. Nevertheless such an increase in toxicity in response to this combination therapy may be avoided if TRAIL expression can be limited locally to tumors. With the combination of TRAIL and chemotherapy, the dose of chemotherapeutic agent could be reduced, and thus decreased the side effects of chemotherapy. In order to explore a more effective approach to deliver TRAIL gene for colorectal treatment, or to evaluate the effect of TRAIL gene on liver metastasis (through liver metastasis models of human colorectal carcinoma established in nude mice)[31], further research on it is necessary.

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Edited by Xu JY