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• *BRIEF REPORTS* •

Adenovirus-mediated FasL gene transfer into human gastric carcinoma

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Abstract

AIM: To evaluate the possible value of FasL in gastric cancer gene therapy by investigating the effects of FasL expression on human gastric cancer cell line.

METHODS: An adenoviral vector encoding the full-length human FasL cDNA was constructed and used to infect a human gastric cancer (SGC-7901) cell line. FasL expression was confirmed by X-gal staining, flow cytometric analysis and RT-PCR. The effect of FasL on cell proliferation was determined by clonogenic assay, cytotoxicity was detected by MTT assay, and cell viability was measured by trypan blue exclusion. The therapeutic efficiency of Ad-FasL *in vivo* was investigated with a xenograft tumor model in nude mice.

RESULTS: SGC-7901 cells infected with Ad-FasL showed increased expression of FasL, resulting in significantly decreased cell growth and colony-forming activity when compared with control adenovirus-infected cells. The cytotoxicity of anti-Fas antibody (CH-11) in gastric cancer cells was stronger than that of ActD (91±8 *vs* 60±5, $P<0.01$), and the cytotoxicity of Ad-FasL was stronger than that of CH-11 (60±5 *vs* 50±2, $P<0.05$). In addition, G_1-phase arrest (67.75±0.39 *vs* 58.03±2.16, $P<0.05$) and apoptosis were observed in Ad-FasL-infected SGC-7901 cells, and the growth of SGC-7901 xenografts in nude mice was retarded after intra-tumoral injection with Ad-FasL (54% *vs* 0%, $P<0.0001$).

CONCLUSION: Infection of human gastric carcinoma cells with Ad-FasL induces apoptosis, indicating that this target gene might be of potential value in gene therapy for gastric cancer.

Key words: FasL gene; Gene transfer; Apoptosis; Carcinoma; Gastrocellular; Gene therapy

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INTRODUCTION

Gastric cancer is one of the most common digestive tract cancers in China. Although an increasing number of gastric cancer patients have benefited from the development of modern tumor therapies, the prognosis of this disease is still relatively poor. Gastric cancer often resists various treatments, including immunotherapy, wherein deficient tumor-specific T-cell responses result in poor immune response.

In this context, apoptosis mediated by the Fas/FasL system is of great interest to researchers, as cytotoxic T-lymphocytes (CTLs) utilize the perforin/granzyme and Fas/FasL systems to kill cancer cells. Fas is a member of the tumor necrosis factor/nerve growth factor receptor family, whereas the Fas ligand (FasL) is a member of the tumor necrosis factor/nerve growth factor family[1-7]. The FasL is primarily expressed in active CTLs, and induces apoptosis of Fas-expressing tumor cells[2,6]. Since the Fas/FasL apoptosis pathway is a key mechanism for clearing tumor cells, researchers are currently seeking methods for triggering FasL expression in tumor cells via gene therapy, thus 'marking' them for Fas/FasL-mediated apoptosis[5,8]. Accordingly, we used adenoviral gene transfer to trigger high-level FasL expression in SGC-7901 (human gastric cancer) cells to investigate the possible use of FasL in gastric cancer gene therapy.

MATERIALS AND METHODS

Cell line

The human gastric cancer cell line SGC-7901 was obtained from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Medium 199 (Gibco) supplemented with 10% fetal bovine serum.

Recombinant retroviral vector construction

The recombinant FasL retroviral vector was constructed in our laboratory. The FasL gene expression cassette includes the CMV promoter, a full-length FasL cDNA (Jingmei

Company, Shenzheng, China) and the SV40 polyA signal sequences. This cassette was inserted into the E1 region of an adenoviral genome lacking the viral E1 and E3 sequences. Briefly, the FasL cDNA was inserted into the pAdCMV shuttle plasmid (kindly provided by Dr. Daru Lu) and co-transfected with pJM17 (Microbix Biosystems Inc., Canada) into human embryonic 293 cells (provided by Dr. Lu) using the Lipofectin reagent (Gibco). The FasL expression cassette was then transferred into the adenovirus genome by homologous recombination. The control virus Ad-LacZ was constructed in the same manner. Virus proliferation, purification and titering were performed as described by He *et al*[9].

Adenovirus transduction efficiency

Gastric carcinoma cells were infected with Ad-LacZ at various multiplicities of infection (MOIs). After 48 h, cells were fixed in 40 g/L formaldehyde for 6-8 h and then treated with X-gal solution (1 mg/mL X-gal in a solution of 0.1 mol/L PBS, 1.3 mmol/L $MgCl_2$, 3 mmol/L $K_3Fe(CN)_6$) for 2 h or overnight in a 37 ℃ incubator. The percentage of blue cells was then determined.

FasL gene transfection

Cells were passaged at a density of 10^5/well. For viral infection, cells were incubated with virus suspensions (at various MOIs) and 8 μg/mL polybrene (Sigma) for 2-3 h at 37 ℃, washed twice with fresh medium, and further incubated for 48 h. Then cells were passaged and incubated in medium containing 1 mg/mL G418 (Gibco) for selection of transfectants. The medium was changed every 3-4 d until anti-G418 cells appeared.

Detection of exogenous FasL expression

Cells were harvested, washed with PBS, and treated with FITC-conjugated FasL antibody (Jackson ImmunoResearch Lab) or control serum for 0.5-1 h at 4 ℃. Cells were once again washed with PBS, and FasL protein expression was confirmed by FCM analysis (Becton Dickinson, San Jose, CA). For FasL gene expression, RT-PCR was performed with the kit according to the manufacturer's instructions (Shanghai Shanggong Company, Shanghai, China). The primers were: forward 5'-CTGAATTCTGACTCACCA-GCTGCCATGC-3', reverse 5'-TACTCGAGCTATTAGA-GCTTATATAAGCCG-3'.

Clonogenic assay

SGC-7901 cells were infected with Ad-FasL or Ad-LacZ (100 MOI). After 24 h, the cells were seeded in 6-well plates at 500 cells/well. After being incubated for 2-3 wk, cells were stained with 0.1% crystal violet and counted under a microscope. Colonies of more than 50 cells were counted for all clonogenic assays.

Cell cycle and apoptosis assays

Cells infected with Ad-FasL or Ad-LacZ (more than 10^6 cells) in either suspension or adhesion were harvested and fixed in 70% ethanol for 3 h. Cells were then treated with 50 μg/mL RNase for 1 h at 37 ℃, and stained with 100 μg/mL PI for 20-30 min prior to FCM analysis.

MTT assay

SGC-7901 cells were treated with 100 ng/mL anti-Fas antibody (CH-11; PharMingen), 50 ng/mL actinomycin D (ActD; Sigma), and infected with Ad-FasL (100 MOI) or treated with anti-Fas antibody combined with Ad-FasL infection. Cytotoxicity was determined by MTT assay. About 10^{3-4} cells/well were plated in 96-well plates and incubated overnight in 100 μL of culture medium. After 2-3 d, 20 μL of MTT solution (5 mg/mL) was added to each culture well. After incubating for 4 h at 37 ℃, the MTT was removed and 200 μL of dimethyl sulfoxide (Sigma) was added and mixtures were shaken, the crystals were fully dissolved (about 10 min). The A value of each well was detected using a microculture plate reader (Huandong Cesium Electron Tube Company) with a test wavelength of 490 nm. Cell survival rate (SR) was expressed as the following equation: SR=(A in experimental group/A in control group)×100%. Results were expressed as mean±SD; the Student's t-test was used for statistical analysis.

Cell proliferation assay

SGC-7901 cells (2.5-3.0×10^3 cells/well) were plated in 24-well plates (3 wells for each test) and infected with various MOIs of Ad-LacZ or Ad-FasL for 24 h. Cells were harvested every 2 d, and the living cell rate (LR) was measured by trypan blue exclusion assay. LR was expressed as the following equation:

LR=number of living cells/(number of living cells+number of dead cells)×100%. Results were expressed as mean±SD; ANOVA was used for statistical analysis with SPSS 10.0 software. $P<0.05$ was considered statistically significant.

Animal model

BALB/c nude mice (The Shanghai Institute of Cancer Research) were subcutaneously injected with SGC-7901 cells at 1-5×10^7 cells/mouse. When tumors grew to 0.5 cm in diameter, the mice were randomly divided into treated and control groups (n = 5). Tumor volumes were calculated by [(1/2)×(longest diameter)×(shortest diameter)] as described previously. Tumors were measured every 5 d for 6 wk. Growth curves were drawn and the percentage of tumor inhibition was calculated (treated group/control group×100%).

RESULTS

FasL gene transfection

Anti-G418 colonies were obtained after screening for 2 wk. The selected clone containing the FasL gene was named as SGC-7901-FasL, and the control clone containing the blank vector was designated as SGC-7901-vect. There was no morphologic difference between cultures of these two cells.

Determination of FasL protein expression

FCM analysis revealed that SGC-7901-FasL cells expressed FasL on their surface, whereas SGC-7901-vect did not (Figure 1).

Determination of FasL mRNA expression

Total RNA was extracted from the test and control cells,

and FasL-specific primers were used for amplification by RT-PCR. The expected 231-bp fragment was amplified from SGC-7901-FasL cells, but not from SCG-7901-vect cells (Figure 2).

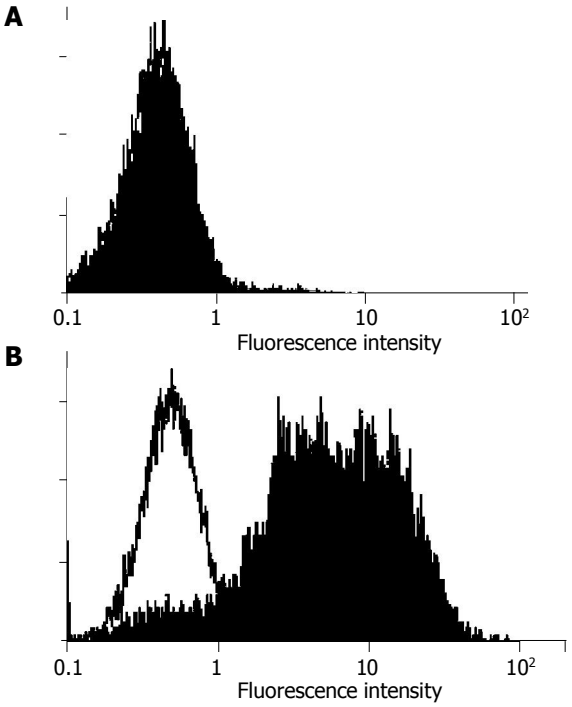

Figure 1 FasL expression on the surface of adenovirus infected SGC-7901 cells. **A**: Ad-LacZ (titer 4.0×10^5 CFU/mL); **B**: Ad-FasL (titer 2.8×10^5 CFU/mL).

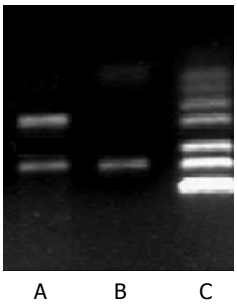

Figure 2 FasL expression as examined by RT-PCR. A: SGC-7901-Fas-L (the FasL amplicon has a size of 231 bp); B: SGC-7901-vect (negative control); C: DNA marker.

Inhibition of SGC-7901 cell growth by exogenous FasL

Two days after infection, SGC-7901-FasL cells were smaller and became more round in shape. Over the next 4 d, the plasma membranes of these cells blebbed, the cytoplasm and nuclei condensed, and the cells ultimately lysed into membrane-bound apoptotic bodies. Cytotoxicity was determined by MTT 4 d after infection. Ad-FasL cultures showed 84% fewer viable cells than Ad-LacZ infected cultures, suggesting that Ad-FasL was cytotoxic to gastric cancer cells. Clonogenic assay showed that SGC-7901 cells infected with Ad-FasL (100 MOI) did not form colonies, whereas Ad-LacZ cultures formed numerous colonies, demonstrating that expression of FasL significantly decreased colony formation.

Cell cycle and apoptosis in Ad-FasL-infected gastric cancer cells

As shown in Table 1, Ad-FasL-infected cultures showed fewer cells in S or G_2M phase, and more cells in G_1 phase, indicating that FasL could inhibit amplification of gastric tumor cells. A sub-G_1 peak (apoptosis peak; Table 1) appeared 4 d after infection of Ad-FasL, indicating that the FasL gene not only induced G_1 phase arrest, but also induced apoptosis of SGC-7901 cells.

Table 1 Influence of FasL expression on cell cycle (FCM analysis) (mean±SD)

Cell lines	G_1/G_0 (%)	S (%)	G_2M (%)
SGC-7901	58.03±2.16	29.72±1.36	9.16±0.92
SGC-7901-vect	61.12±2.24	30.95±1.22	11.02±0.18
SGC-7901-FasL	67.75±0.39[a]	26.56±0.59[c]	5.69±1.12[e]

[a]$P<0.05$, [c]$P<0.05$, [e]$P<0.05$, SGC-7901-FasL *vs* SGC-7901 or SGC-7901-vect.

Cytotoxicity of FasL to SGC-7901 cells

To further evaluate the possibility of gastric cancer gene therapy with Ad-FasL, SGC-7901 cells were infected with Ad-FasL or treated with anti-Fas antibody, and the resulting cytotoxicity was compared with that of ActD, a RNA synthesis inhibitor known as cytotoxin. The cytotoxicity of the anti-Fas antibody (CH-11) to gastric cancer cells was stronger than that of ActD, and the cytotoxicity of Ad-FasL was stronger than that of CH-11 (Table 2).

Table 2 Relative *in vitro* cytotoxicities of various treatments (mean±SD)

Cell line	Treatment group			
	ActD	Ad-LacZ	CH-11	Ad-FasL
SGC-7901	91±8	68±2	60±5[a]	50±2[b]

[a]$P<0.05$ *vs* CH-11 or (compared with CH-11 or anti-Fas antibody+ActD), [b]$P<0.01$ *vs* ActD (compared with ActD).

Inhibition of Ad-FasL on tumor growth in animal model

Nude mice were subcutaneously injected with SGC-7901 cells, then intratumoral injections of Ad-FasL were administered. The growth of tumor infected with Ad-FasL was inhibited by 54%, suggesting that Ad-FasL was a viable gene therapy candidate (Figure 3).

DISCUSSION

Although a growing number of tumor patients have benefited from modern oncotherapeutic methods, there is still a need to improve therapies for malignant tumors. Gene therapy is expected to join surgical, radiological and chemotherapeutic strategies in future methods of integrated oncotherapy. Pre-clinical studies have confirmed that adenovirus-mediated high level expression of carcinoma-inhibiting genes (such as p53) can inhibit tumor growth, induce apoptosis and increase

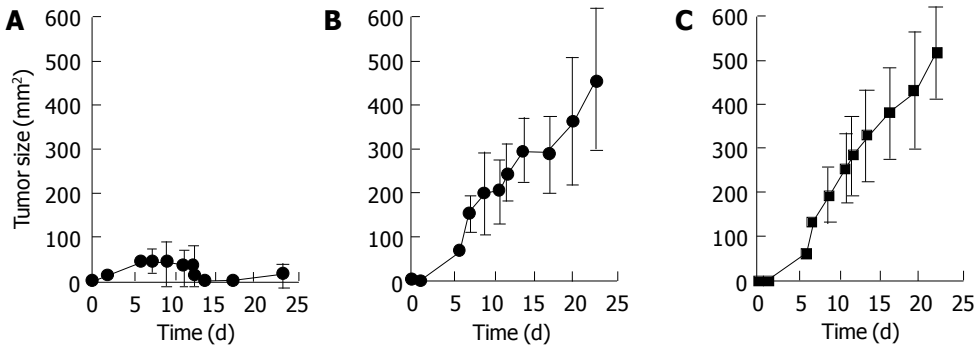

Figure 3 Treatment of SGC-7901 tumor-bearing mice with SGC-7901+FasL cells. **A**: Mice were injected with SGC-7901 cells (5×10^5), followed by treatment with FasL (n = 8); **B**: mice were injected with SGC-7901 cells (5×10^5); **C**: mice were injected with SGC-7901 cells (5×10^5), followed by treatment with PBS (n = 8). Tumor incidence is presented at each time point in Figure. P<0.0001.

tumor tissue sensitivity to radio- and chemotherapy[10-14]. Recent clinical studies in China and abroad have indicated that adenoviral gene therapy is safe and applicable[12-15]. Here, we used this vector to express FasL in cultured human gastric cancer cells. The FasL gene expression cassette includes the CMV promoter, a full-length FasL cDNA, and SV40 polyA signal sequences. A transduction efficiency test using a similarly constructed Ad-LacZ vector illustrated that the adenoviral construct possessed high transduction efficiency. FCM and RT-PCR were used to detect high-level FasL expression in target cells, confirming that the adenovirus vector effectively transfers the FasL gene into tumor cells.

Previous studies[16,17] indicated that binding between FasL and Fas induces receptor trimerization, and apoptosis of Fas-expressing cells. Current theory holds that the signaling responsible for this apoptosis occurs in one of the following three ways: between T cells and target cells, among target cells; or between T cells[18-23]. Fas expression is markedly higher in gastric cancer cells than in normal gastric mucosal cells, implying that Fas participates in the genesis of gastric carcinoma. Fas activation can induce gastric carcinoma cell apoptosis, indicating that the Fas/FasL system might be a good target for gene therapy. In this study, we attempted to induce direct apoptosis of target cells (cis-type apoptosis) by transfecting a highly efficient Ad-FasL expression vector into gastric carcinoma cells (SGC-7901). Expression of FasL inhibited the apoptosis of SGC-7901 cells up to 84%, and significantly inhibited the ability of SGC-7901 cells to form colonies. These results have not been reported in China.

FasL is thought to engage with Fas by inducing receptor trimerization, which then transfers signals to the Fas intracellular death domains (DD). Then, the Fas-associated death domain dimerizes with the DD to transfer an apoptotic signal to Caspase-8, instigating a caspase cascade leading to cell apoptosis[24-26]. In our study, FCM analysis showed that SGC-7901 cells infected with Ad-FasL quickly arrested in the G_1 phase, which was subsequently followed by tumor cell apoptosis. Taken together, these *in vitro* results suggest that FasL gene transfer is capable of inducing gastric tumor cell apoptosis and that it may be a viable candidate for tumor gene therapy.

In our study, an *in vitro* cytotoxicity assay showed that Ad-FasL could significantly inhibit the growth of gastric cancer cells. The inhibition was much stronger than the cytotoxicity conferred by CH-11 treatment, indicating that the Fas/FasL system plays an important role in gastric cancer cell apoptosis[27-29]. In contrast, a SGC-7901 tumor model in nude mice showed only 54% inhibition of tumor growth in response to Ad-FasL infection. This difference between the *in vivo* and *in vitro* response rates might be caused by poor distribution of the recombinant adenovirus in the solid tumor, resulting in lack of target gene transfer to all the tumor cells. Thus, future Fas/FasL gene therapy experiments *in vivo* should focus on stabilizing vectors, increasing transfection efficiency, repeating administration and combining interventional therapy and gene therapy, thereby improving the therapeutic efficacy.

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