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• *GASTRIC CANCER* •

Anti-gastric cancer active immunity induced by FasL/B7-1 gene-modified tumor cells

Shi-Ying Zheng, De-Chun Li, Zhi-De Zhang, Jun Zhao, Jin-Feng Ge

Shi-Ying Zheng, Jun Zhao, Jin-Feng Ge, Department of Cardiothoracic Surgery, The First Affiliated Hospital of Suzhou University, Suzhou 215006, Jiangsu Province, China
De-Chun Li, Zhi-De Zhang, Department of General Surgery, The First Affiliated Hospital of Suzhou University, Suzhou 215006, Jiangsu Province, China
Supported by the Medical Scientific Foundation of Jiangsu Province, No. H200147
Correspondence to: Dr. Shi-Ying Zheng, Department of Cardiothoracic Surgery, The First Affiliated Hospital of Suzhou University, Suzhou 215006, Jiangsu Province, China. syzheng88@ sina.com
Telephone: +86-512-65263570
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Abstract

AIM: To study the activation of cytotoxic T lymphocytes (CTLs) against gastric cancer cells induced by FasL/B7-1 (FB-11) gene-modified tumor cells, and to explore whether co-expression of FasL and B7-1 in SGC-7901 tumor cells could initiate synergistic antitumor effect.

METHODS: FasL and B7-1 genes were transfected into human SGC-7901 gastric cancer cells with adenovirus vectors. The positive clones were selected by G418. FasL and B7-1 genes were detected by flow cytometry and RT-PCR. Abdominal infiltrating lymphocytes and sensitized spleen cells were obtained from mice that were immunized with SGC-7901/FB-11 or wild type SGC-7901 cells intraperitoneally, and cytotoxicity of these CTLs against tumor cells was determined by MTT assay.

RESULTS: Flow cytometry and RT-PCR showed that FasL and B7-1 genes were highly expressed. FasL and B7-1 transfected cancer cells had a high apoptosis index. DNA laddering suggested that FasL and B7-1 genes induced gastric cancer cell apoptosis. $FasL^+/B7\text{-}1^+$SGC-7901 cells (SGC-7901/FB-11) were inoculated subcutaneously in the dorsal skin of C57BL/6 mice and then decreased their tumorigenicity greatly (z = 2.15-46.10, P<0.01). SGC-7901/FB-11 cell-sensitized mice obtained protective immune activity against the rechallenge of wild type SGC-7901 cells (z = 2.06-44.30, P<0.05). The cytotoxicity of CTLs induced by SGC-7901/FB-11 cells against SGC-7901 was significantly higher than that of CTLs activated by wild-type SGC-7901 cells (84.1±2.4% *vs* 30.5±2.3%, P<0.05).

CONCLUSION: FasL and B7-1 genes can effectively promote the activity of CTLs against gastric cancer cells. FasL/B7-1 molecules play an important role in CTL cytotoxicity.

Key words: Gastric cancer; FasL gene; B7-1 gene; Gene therapy; Synergistic effect

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INTRODUCTION

The immune surveillance and killing activity of immunoactive cells on tumor cells play crucial roles in tumor immunity. However, oncologists and immunologists have long been faced with a tough task, i.e., how to successfully induce anti-tumor cytotoxic effects mediated by antigen specific T lymphocytes[1-3].

Research has revealed that one major reason for the low responsiveness of cytotoxic T lymphocytes (CTLs) to cancerous cells lies in the loss of interaction between costimulatory factors CD28 and CD152 and B-7 molecules on tumor cell surface which results from the low expression of the costimulatory factors of B-7 family on the surface of tumor cells[3-6]. This precludes CTLs partially activated after recognition of antigens from being fully activated, making it impossible to generate cytokines with immunological attacking activity against tumor cells and membrane lysis signals (from the Fas-FasL system) as well[4-7].

In the present study, we constructed the recombinant adenovirus vector (AdV) containing human FasL and B7-1 genes (termed FB-11), which was used to transfect human gastric cancer cells. Transfection rates and expression of the two genes were determined, and the capability of inducing tumor-specific CTLs from peripheral blood T lymphocytes (PBT) and tumorigenicity in mice of the FasL/B7-1-modified gastric cancer cells were observed. This provides an experimental base for the combination of tumor immunology and gene therapy in the treatment of cancer.

MATERIALS AND METHODS

Animals, cells, materials

C57BL/6 (H-2b) inbred mice at the age of 6-8 wk were bought from the Shanghai Laboratory Animal Research Center of Chinese Academy of Sciences. SV40 promoter (P_{SV40}) driven human B7-1 cDNA was presented by Professor Daru Lu at the Institute of Molecular Genetics, Fudan University, Shanghai. Rat-anti-mouse monoclonal

antibody (mAb) against B7-1 was purchased from Pharmingen Corporation, San Diego, CA, USA. Alkaline phosphatase-conjugated goat-anti-rat IgG was obtained from Organon Teknika Corporation, Durham, NC, USA. G418 was the product of Gibco. CMV promoter (P_{CMV}) containing human FasL cDNA and its labeled antibody were manufactured by Jinmei Company, Shanghai, China.

Human gastric cancer cell line SGC-7901 was provided by the Institute of Cell Biology of Chinese Academy of Sciences, Shanghai. The cells were grown in RPMI-1640 supplemented with 10% fetal calf serum at 37 ℃ in a 50 mL/L CO_2 atmosphere.

Human embryonic kidney cell line 293 containing E1 region of human adenovirus type 5 (Ad5) was bought from GeneTherapy Unit of Baxter Healthcare Company, USA, and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37 ℃ in a 50 mL/L CO_2 atmosphere.

Construction of recombinant adenovirus

Recombinant adenovirus (rAd) carrying green fluorescence protein (GFP) gene (Ad-GFP) was purchased from GeneTherapy Unit of Baxter Healthcare Company, USA. E1/E3-deleted replication defective Ad5 vectors containing PCMV driven human FasL cDNA and PSV40 driven human B7-1 cDNA were constructed by Jinmei Corporation and Professor Daru Lu at the Institute of Molecular Genetics of Fudan University, Shanghai. This vector was termed FB-11. Amplification and purification of rAd and measurements of concentration, titer and infection rate were performed as previously described[8].

Transfection of gastric cancer cells with adenoviral vector

SGC-7901 cells were seeded in six-well plates at a density of 5×10^5 cells/well. Culture media were aspirated after 12 h and Ad-GFP in 0.8 mL of culture medium was added to each well at various MOIs (0, 12.5, 25, 50, and 100, respectively). After soaking for 1-2 h, the viral solution was aspirated and 2 mL of culture medium was added to each well. After 12 h, the numbers of GFP^+-cancer cells was counted under fluorescence microscope. Each counting was repeated thrice and transfection rates were calculated.

Measurement of expression of B7-1 gene by flow cytometry

SGC-7901 gastric cancer cells were grown in 60-mm dishes at a density of 1×10^6 cells/dish. After 24 h, the cells were infected with FP-11 at a MOI of 50 for 48 h, trypsinized and washed twice with Hank's solution, and resuspended in 100-200 μL Hank's solution. This was followed by the addition of 20 μL of AV-FITC labeled mouse-anti-human B7-1 mAb solution, incubated at 4 ℃ for 30 min, washed twice in Hank's solution, and resuspended in 1 mL of PI containing Hank's solution. The expression of B7-1 gene delivered by FB-11 on the surface of the two cancer cell lines was determined by AV/PI bivariate flow cytometry.

Measurement of expression of FasL gene by RT-PCR

Total RNA of FB-11-transfected SGC-7901 cells (termed SGC-7901/FB-11, respectively) was extracted by TRIzol RNA isolation method (Life Technologies, Gaithersburg, MD, USA). The RNA was then reversely transcribed into cDNA with Moloney mouse leukemia virus (MoMLV) reverse transcriptase (GIBCO-BRL) and Oligo-dT (GIBCO-BRL). The human FasL cDNA was amplified with primers as follows: sense primer (483-503): 5' CTGGGGATGTT-TCAGCTCTTC 3'; antisense primer (713-693): 5' CTTC-ACTCCAGA AAGCAGGAC 3'.

cDNA amplification was conducted in a 50-μL reaction volume comprising four sorts of dNTP (200 μmol/L each), 2.5 mmol/L $MgCl^2$, 2.5 U Taq polymerase and sense and antisense primers (0.4 μmol/L each). Amplification was performed for 35 cycles, each consisted of 30 s at 94 ℃, 1 min at 55 ℃, and 1.5 min at 72 ℃. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide (EB) staining, revealing a 231 bp band corresponding to human FasL gene fragment. Furthermore, PCR products were analyzed by restriction enzyme mapping.

Inhibitory effects of FB-11 on SGC-7901 cell growth

SGC-7901 cancer cells were plated in 24-well plates at a density of 1×10^6 cells/well, and infected with FB-11 or Ad-GFP at a MOI of 50 after 24 h. Subsequent growth of the two cell lines was monitored daily. The cells were trypsinized, stained with trypan blue, and counted by a hemacytometer, and the growth curve was plotted.

Measurement of apoptosis in FB-11-transfected gastric cancer cells by Hoechst33342 staining

Suspended FB-11-transfected SGC-7901 gastric cancer cells were passed through filters and adjusted to a concentration of 5×10^5 cells/mL. Then the cells were fixed in 1% formaldehyde in 1× phosphate buffered solution (PBS) for 30 min at 4 ℃. After washing twice in 1× PBS, 0.5 μg/mL Hoechst33342 was added and incubated at 37 ℃ for 2 h. After being washed in 1× PBS, the number of apoptotic cells was counted under fluorescence microscope. In detail, a total of 200 cells were observed and only cells with typical characteristics of apoptosis were counted. Apoptosis index (AI) was calculated according to the following formula:

$$AI(\%)=\frac{\text{Number of apoptotic cells}}{\textit{Total}\text{ number of observed cells}}\times100\%$$

Analysis of DNA fragments

SGC-7901 cells infected with FB-11 or Ad-GCP at a MOI of 100 were collected (above 10^6 cells). NP40 lysis buffer (1% NP40, 20 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 7.4) was added and stood still for 10 s. Supernatant was collected after centrifugation, SDS was added to a concentration of 1% and RNase to a concentration of 50 μg/mL. After incubation at 56 ℃ for 1-2 h, protease K was added to a concentration of 2.5 μg/mL and incubated at 37 ℃ for 2 h or overnight. DNA was precipitated by ethanol, dissolved in 10-20 μL of TE buffer, and subsequently analyzed by 1% agarose gel electrophoresis.

Tumorigenicity test

Seven C57BL/6 mice were randomly divided into test (n = 4) and control (n = 3) groups. The test group received a

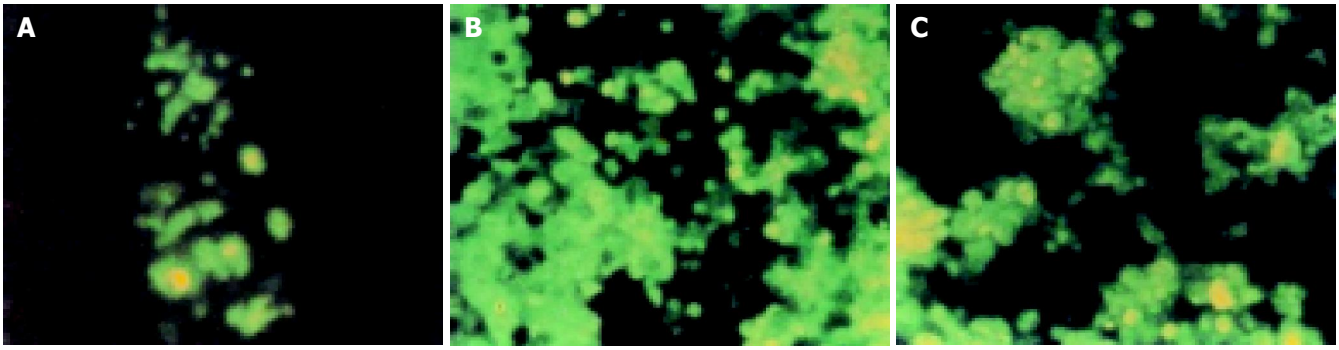

Figure 1 Transfection rates of rAd in gastric cancer cells. **A**: MOI = 20; **B**: MOI = 50; **C**: MOI = 100.

subcutaneous inoculation with 5×10^6 SGC-7901/FB-11 cells in dorsal skin and the control group received an inoculation with the same number of SGC-7901 cells at the same site. The growth of subcutaneous tumors was monitored by measuring long and short diameters with a slide gauge. The size of tumors was expressed in long diameter×short diameter. Tumor sizes of the test and control groups were compared.

Immunological protection test

Four mice sensitized with SGC-7901/FB-11 cells were inoculated subcutaneously in the dorsal skin with 2.5×10^6 wild-type SGC-7901 cells. Meanwhile, four normal mice were inoculated with the same number of SGC-7901 cells at the same site as normal controls. Sizes of the subcutaneous tumors were monitored and compared between the two groups.

In vitro cytotoxic test

Mice were sensitized peritoneously with 5×10^6 SGC-7901 and SGC-7901/FB-11 cells and killed 7 d later. The abdominal cavity was then lavaged with 5 mL Hank's solution, which was subsequently aspirated and depleted of red blood cells. The remaining macrophages and tumor cells were removed by attachment to the wall so that abdominal infiltrating lymphocytes were obtained. In addition, erythrocyte-free single cell suspension of mouse splenocytes was prepared. Cytotoxic activity of activated CTLs was determined by MTT assay.

Statistical analysis

Statistical analysis was performed with SPSS v10.0 for Windows (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was considered statistically significant.

RESULTS

Preparation of high titer recombinant adenovirus (rAd)

Highly concentrated rAd was collected by $CsCl_2$ density gradient super-speed centrifugation. The concentration of rAd calculated according to 1 A_{260} = 1.1×10^{12} particles, was greater than 10^{12} particles/mL with a high purity ($A_{260}/A_{280}>1.3$). The concentrations of FB-11 and Ad-GFP measured by plaque assay were 3×10^{10} and 4×10^{11} pfu/mL, respectively.

Transfection rates of rAd in gastric cancer cells

Gastric cancer cell lines were susceptible to the adenoviral vectors FB-11 and Ad-GFP. The greater the amount of viral particles, the higher were the transfection rates. The transfection rate reached up to above 90% at a MOI of 50 (Figure 1B). But when the amount of adenoviral particles was further increased, the cell growth was suppressed with elevated dead cells (Figure 1C).

B7-1 gene expression

Results from flow cytometry indicated that SGC-7901 gastric cancer cells did not express B7-1 gene (Figure 2). There were 37.5% of cells expressing B7-1 on cell surface on the first day and 80% of cells expressing high level B7-1, 2 d after they were transfected with FB-11 at a MOI of 50.

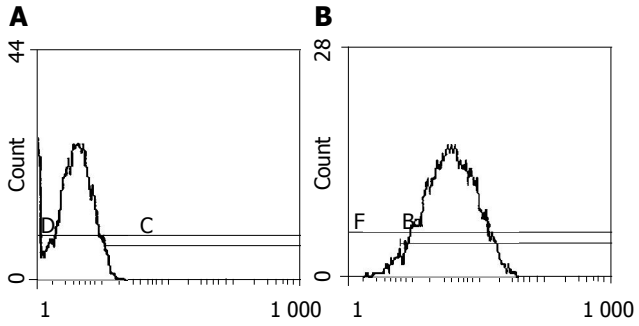

Figure 2 Flow cytometric analysis of B7-1 gene expression. **A**: SGC-7901; **B**: SGC-7901/FB-11.

FasL gene expression

Total RNA of FB-11-modified SGC-7901 cells was extracted and the extracellular segment of FasL gene was amplified by RT-PCR. The length of PCR products was as expected (Figure 3).

Inhibitory and cytotoxic effects of FB-11 on gastric cancer cells

As compared to non-infected cells, the growth of FB-11-transfected SGC-7901 gastric cancer cells was significantly inhibited (Figure 4) with SGC-7901/FB-11 displaying more significant inhibition.

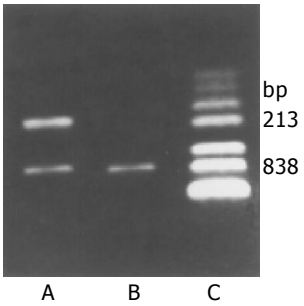

Figure 3 RT-PCR analysis of FasL expression in SGC-7901/FB-11 and SGC-7901. A: SGC-7901/FB-11; B: SGC-7901; C: DNA Marks.

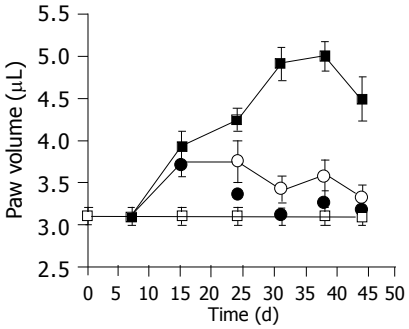

Figure 4 Proliferation inhibition of gastric cancer cell lines. ■: SGC-7901; ○: SGC-7901L/FasL; ●: SGC-7901/FB-11; □: PBS.

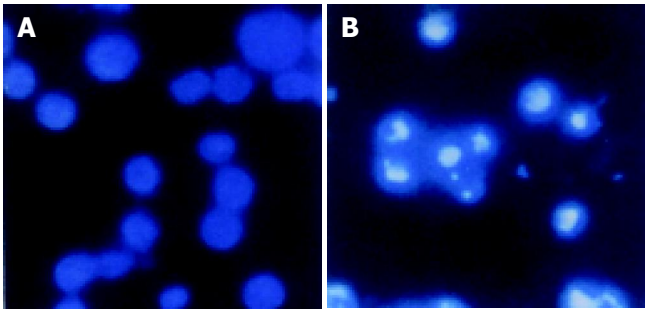

Figure 5 Apoptosis of SGC-7901 cells stained with Hoechst 33342 induced by FB-11 (×400). **A**: SGC-7901/GFP cells; **B**: SGC-7901/FB-11 cells.

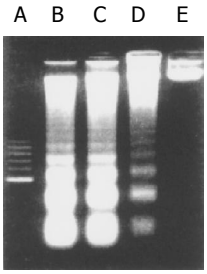

Figure 6 DNA fragmentation of SGC-7901 cells induced by FB-11. A: DNA Marks; B: SGC-7901/FB-11; C: MGC-803/FB-11; D: BGC-823/FB-11; E: SGC-7901.

Apoptosis induced by FB-11 delivery

FB-11- or Ad-GFP-transfected SGC-7901 cells stained with Hoechst33342 were observed under fluorescence microscope. FB-11-transfected cancer cells had a high apoptosis index, but no apoptosis was seen in Ad-GFP-transfected cells (Figure 5). DNA laddering (Figure 6) suggested that FasL and B7-1 genes induced gastric cancer cell apoptosis.

Tumorigenicity of FB-11- transfected gastric cancer cells in nude mice

As seen in Figure 7, SGC-7901 gastric cancer cell lines acquired lower tumorigenicity in C57BL/6 mice after transfected with FB-11. On day 13 after the transfection, a significant difference in size and weight of the transplanted tumor was noticed between FB-11-modified cancer cells and non-transfected cancer cells.

FB-11-mediated protective immunity

SGC-7901/FB-11-sensitized mice were re-inoculated with wild-type cell lines. No tumor growth was seen during the period from day 1 to 32 after the inoculation. But in the control group, measurable tumors occurred 7 d after the inoculation with a slight decrease in size of the tumor in the 2nd wk. Significant differences were revealed between the two groups (Figure 8).

In vitro cytotoxic test

Peritoneal inoculation with either FB-11-transfected SGC-7901 cells or their wild-type counterparts could bring about abdominal lymphocyte infiltration, but the number of lymphocytes and remaining tumor cells in abdominal cavity varied. In the cytotoxic test, SGC-7901/FB-11-activated abdominal infiltrating lymphocytes and splenocytes showed greater cytotoxic activity on SGC-7901 cancer cells than SGC-7901-activated lymphocytes and splenocytes (Figure 9). Moreover, infiltrating lymphocytes induced by SGC-7901/FB-11 had greater killing rates on SGC-7901/FB-11 cancer cells than on the wild-type cell lines (Figure 10). These suggested that transfection with recombinant adenoviral vector FB-11 could enhance the immunogenicity of human gastric cancer cell line SGC-7901.

DISCUSSION

$CD4^+$ and $CD8^+$ T lymphocytes activated by antigens or mitogens from freshly isolated T lymphocytes highly express FasL molecule. FasL and perfurin/granzyme account for the two mechanisms of cytotoxic effects of cytotoxic T lymphocytes (CTLs)[9-12]. $CD8^+$ CTLs, which take on the responsibility of resisting virus and micro-organism invasion and antineoplasm, recognize and kill MHC-I carrying target cells. $CD4^+$ CTLs, which have the activities of immune regulation such as transplantational immunity, eliminate MHC-II carrying cells. However, when the cytotoxic activity of $CD8^+$ CTLs is impaired especially on the occasion that MHC-I molecules are not expressed or are slightly expressed in tumor cells, $CD4^+$ CTLs act as major cytotoxic effectors. The cytotoxic activity of non-specific mitogen activated $CD4^+$ T lymphocytes and mouse splenocytes within 24 h of the activation results mainly from upregulation of FasL expression which binds to Fas receptor, initiating programmed cell death.

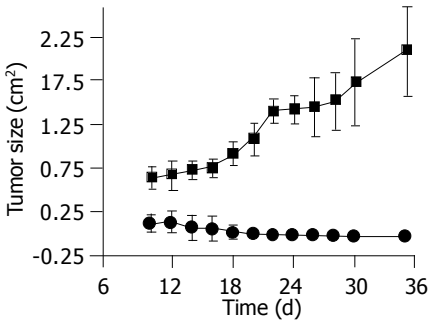

Figure 7 Tumorigenicity of FB-11-transfected gastric cancer cells and tumor size in preventive and control groups. ●: FB-11 preventive group; ■: control group.

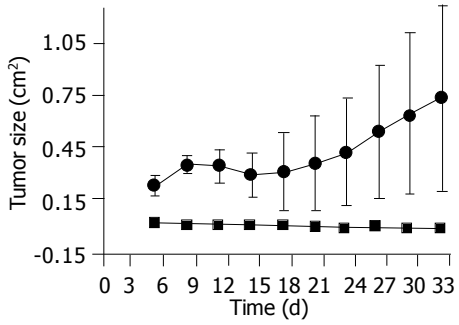

Figure 8 FB-11-mediated protective immunity. ■: SGC-7901/FB-11-sensitized mice group; ●: SGC-7901 mice group (not sensitized with SGC-7901/FB-11).

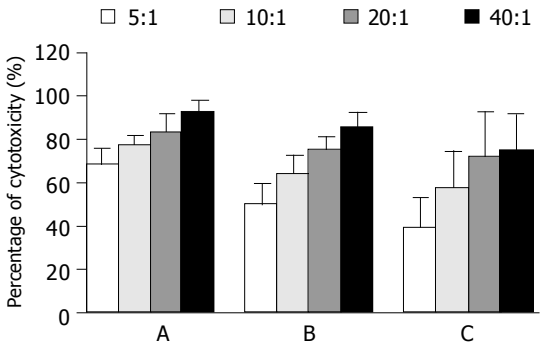

Figure 9 Cytotoxicity of abdominal lymphocyte infiltration against tumor cells induced by FB-11. A: cytotoxicity of SGC-7901/FB-11-induced abdominal lymphocyte infiltration against SGC-7901/FB-11 cells (n = 4); B: cytotoxicity of SGC-7901/FB-11-induced abdominal lymphocyte infiltration against SGC-7901 cells (n = 4); C: cytotoxicity of SGC-7901-induced abdominal lymphocyte infiltration against SGC-7901 cells (n = 4).

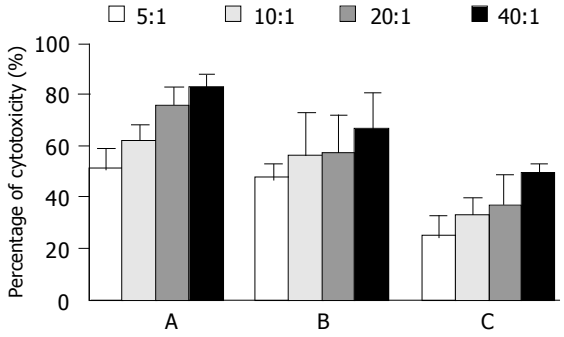

Figure 10 Cytotoxicity of spleen cells against tumor cells induced by FB-11. A: cytotoxicity of SGC-7901/FB-11-induced spleen cells against SGC-7901/FB-11 cells (n = 4); B: Cytotoxicity of SGC-7901/FB-11-induced spleen cells against SGC-7901 cells (n = 4); C: Cytotoxicity of SGC-7901-induced spleen cells against SGC-7901 cells (n = 4).

CTLs are major effector cells in immune system. T cell cytotoxicity is mediated by two independent cytolytic mechanisms[13-15], namely release of perfurin acting on target cells and membrane binding or release of FasL acting on Fas-expressing cells. Degranulization of CTLs and perfurin-induced cytolysis are two separate steps of the specific cytotoxic process, which requires the recognition of T cell receptor (TCR) and MHC molecules on the surface of target cells and involves perfurin, granzyme, Ca^{2+} and ICE family, *etc*. However, the Fas-FasL system mediates a non-specific, non-MHC restricted cytotoxicity. In the absence of Ca^{2+} or perfurin and granzyme, the cytotoxicity of cultured CTLs is completely dependent on the Fas-FasL system. It is now believed that Fas-dependent cytotoxicity of CTLs comprises two steps. The first step is that CTLs recognize Fas carrying cells and are activated to express FasL, the second step is that CTL-expressed FasL binds to target cell-carried Fas, leading to target cell apoptosis. Since FasL-induced apoptosis does not rely on MHC and Ca^{2+}, the cytotoxic activity of $FasL^+$-CTLs is not only broad-spectrum but also Fas^+-cell targeted. Since tumor cells always express Fas, FasL and CTLs are two important weapons against tumors. These are the rationale for the co-delivery of FasL and B7-1 genes in the present study.

FasL is a 40-ku type II trans-membrane protein which is a member of the TNF family. It is used to be believed that only activated T cells express FasL, but later studies demonstrated that this cytokine expresses on several other species of cells including neutrophils, neurons, thyrocytes, retinal stromal cells, salivary gland cells and mouse testicular podocytes[16-21].

Fas and FasL bind in a trimer style, leading to apoptosis of Fas-expressing cells. Interaction between Fas and FasL is currently believed to take place in the following fashions[16,22-24]. Target cell apoptosis induced by T cells is the most common situation, i.e., $FasL^+$-T cells (mostly $CD8^+$) lead Fas-expressing target cells to apoptosis. Induction of apoptosis between T cells (activated T cells express both FasL and Fas so that apoptosis is induced at one or both sides of interacted T lymphocytes) can result in diminution of immune responses. The target cells express both Fas and FasL, leading to apoptotic death at one or both sides of interacted target cells. Fas-FasL-induced apoptosis between different types of cells is called trans apoptosis, while apoptosis-induced between cells of one type is called cis apoptosis. Based on the above-mentioned theory, we transduced FasL and B7-1 genes into human gastric carcinoma cells by adenoviral vector and hoped to achieve cis apoptosis between tumor cells and trans apoptosis in tumor cells induced by T cells whose activation was expanded through B7-1 gene delivery.

Antitumor immunity in human body is mainly T cell-mediated cellular immune response. T cells require three signals to become activated. The first signal is through T cell receptor (TCR) to which specific Ag-MHC complex binds. The binding activates TCR-CD3 complex and then CD3 molecule transducts the binding signal into CTLs. The second signal is co-stimulating signal[25-27]. CTLs are activated by the intercellular contact mediated by CTL surface adhesive molecules. One example is the interaction between CTL surface molecule CD28 and molecule B7-1 on the surface of antigen-presenting cells (APC) and tumor cells, which is thought to have an important role in CTL activation. The third signal is stimulation of cytokines like IL-2, *etc*. One recent great progress in the field of antitumor therapy is the expansion of tumor-specific CTLs by non-specific methods[21]. In preclinical studies, the vitality and specificity of CTL cultures can be maintained by intermittent stimulation by tumor cells or tumor antigens, thus acquiring large quantities of tumor-specific CTLs for anticancer therapy. However, this method cannot be used to expand human tumor-specific CTLs. One reason is the risk of using human cancerous cells as stimuli, and another is that the presence of CTL-recognized human tumor antigens has been confirmed, which is hard to purify such antigens to expand CTLs. Therefore, expansion of tumor-specific CTLs by transfection of genes of non-specific mAbs (anti-CD3 and anti-CD28), cytokines (IL-2) and B7-1 has great prospects in current immunotherapy and gene therapy for the treatment of cancer. But the feasibility of this method in clinical practice lies on the availability of large amounts of expanded tumor-specific CTLs with high antitumor cytotoxicity of clinical standard. In this study, transfection of B7-1 gene promoted expansion and activation of CTLs and transfection of FasL gene gave rise to cis apoptosis in tumor cells. An important innovation of this study is that co-delivery of the two genes results in arrest of tumor cell growth, which has not been reported at home and abroad.

During the effect phase of co-stimulatory molecules, interactions between CD28 and CD86 and between CD152 and CD80 generate cytolytic signals, leading to tumor cell death. Membrane lysis caused by Fas-FasL interaction is one of the effects. Tumor cells do express Fas which is capable of transducting cell apoptotic signals. These are the two prerequisites to the application of anti-Fas antibody and FasL transfection in the treatment of cancer. As yet, FasL gene has not been used in the treatment of gastric cancer. To study the role of the Fas-FasL system in gastric cancer cell apoptosis, Liu measured the expression of Fas and FasL in gastric cancer cells and found that some cells have low expression of Fas but high expression of FasL. This is the same as previously reported that the expression of Fas in some cancer cell lines is downregulated or lost[28]. Many human tumor cell lines have no or low expression of Fas, and this probably results in the incapability of T cells inducing apoptosis of tumor cells, which is a probable reason for the escape of tumor cells from immune surveillance and tumor formation and progression. However, other studies[29,30] have demonstrated that the expression of FasL on tumor cells and islet cells has immune protective action, but FasL-expressing cells were destroyed because of the infiltration of large amounts of granulocytes. Whereas tumor cells have low or no expression of Fas but high expression of FasL on some occasions. It is necessary to explore the mechanisms underlying regulation of Fas/FasL on the surface of tumor cells.

Some cytokines including IFN-γ, TNF-α, and IL-2 upregulate Fas expression, and transfection of FasL in Fas-tumor cells can induce inflammatory reactions[18-20]. In addition, B7-1 overexpression-induced interaction between co-stimulatory molecules increases the expression of cytokines during the effect phase. These suggest that FasL gene transfection can elevate Fas expression in tumor cells (bystander effect indicates that mixed culture of FasL gene-modified cancer cells and parent cells increases the expression of Fas in parent cell lines), and greatly enhances the immunogenicity of parent tumor cell lines. This strengthens the cytolytic effects of CTLs on tumor cells (trans apoptosis).

In this study, FB-11-transfected human gastric cancer cells had lower tumorigenicity and newly-formed tumors regressed in a short time. The control group had persistently growing tumors, suggesting that FB-11 containing gastric cancer cells acquires cis apoptosis as well as the ability to induce strong immune responses. The mechanism for this reaction is that with the co-stimulatory effects of B7-1, CTL clones specific for the major epitopes on gastric cancer cells are greatly amplified and B7-1 co-stimulatory signaling uncovers the sub-dominant epitopes on the surface of gastric cancer cells, causing the sub-dominant epitope-specific CTL precursors activation and proliferation of and induction of immune reactions[31,32]. However, these sub-dominant epitopes are not able to induce immune reactions in usual cases. Primary tumors in C57BL/6 mice inoculated with FB-11-transfected human gastric cancer cells can regress, and reimplantation of wild-type gastric cancer cell lines in mice sensitized with FB-11-transfected gastric cancer cells cannot result in new tumor formation. The reason for this is that tumor-specific CTL clones are activated by the sub-dominant epitopes on gastric cancer cell surface and the co-stimulatory molecule B7-1. Hereby immune responses occur immediately upon the inoculation with wild-type cancer cells with the same antigens. The activation of T cells requires the co-stimulatory signal of B7-1. But in the post-activation effect phase, most researchers believe that the participation of B7-1 is not essential, which is in contradiction with other studies[33,34,]. In the present study, results of the protective immune test showed that mice sensitized with FB-11-transfected gastric carcinoma cells rejected new implant of wild-type cancer cells, and activated CTLs had efficient cytotoxic activity on FB-11$^+$-cancer cells, suggesting that the cytotoxicity of activated CTLs can be induced without B7-1 co-stimulatory signaling. Results from cytotoxic assay revealed that CTLs activated by FB-11$^+$ gastric cancer cells had greater killing activity on FB-11$^+$ gastric cancer cells than on wild-type cell lines, suggesting that both FasL and B7-1 molecules play crucial roles in effect phase. The probable mechanisms are as follows: The overexpression of FasL recruits great many inflammatory cells, leading to local immune responses. Released inflammatory cytokines

can in turn enhance activation and proliferation of CTLs, binding of B7-1 to CD28 facilitates adhesiveness between lymphocytes and tumor cells, promotes the binding of polypeptide-MHC complex to TCR, in other words, B7-1 can lower the threshold value of T cell responses. Therefore, we propose that the molecule B7-1 is not essential in T cell effect phase, but can boost the cytotoxic activity of T cells.

In conclusion, tumor cells express Fas and FasL. Overexpression of FasL/B7-1 genes in tumor cells do not cause 'immune counteraction' against CTLs, but can elevate Fas expression in tumor cells through inflammatory reactions, which facilitates the performance of CTL cytotoxicity on tumor cells. Overexpression of FasL can increase the immunogenicity of gastric cancer cell lines in the presence of overexpressed B7-1 gene, which promotes the development of antitumor immunity in mice. Overexpression of both tumor immunity-associated gene B7-1 and apoptosis-inducing gene FasL in gastric cancer cell line can generate synergistic anti-tumor activity.

REFERENCES

1 **Grosenbach DW,** Schlom J, Gritz L,Gomez Yafal A, Hodge JW. A recombinant vector expressing transgenes for four T-cell costimulatory molecules (OX40L, B7-1, ICAM-1, LFA-3) induces sustained CD4+and CD8+ T-cell activation, protection from apoptosis, and enhanced cytokine production. *Cell Immunol* 2003; **222**: 45–57

2 **Khatlani TS,** Ma Z, Okuda M, Onishi T. Molecular cloning and sequencing of canine T-cell costimulatory molecule (CD28). *Vet Immunol Immunopathol* 2001; **78**: 341-348

3 **Shankar P,** Schlom J, Hodge JW. Enhanced activation of rhesus T cells by vectors encoding a triad of costimulatory molecules (B7-1, ICAM-1, LFA-3). *Vaccine* 2001; **20**: 744–755

4 **Denfeld RW,** Dietrich A, Wuttig C, Tanczos E, Weiss JM, Vanscheidt W, Schopf E, Simon JC. *In situ* expression of B7 and CD28 receptor families in human malignant melanoma: relevance for T-cell-mediated anti-tumor immunity. *Int J Cancer* 1995; **62**: 259-265

5 **Nagai A,** Nakagawa E, Hatori K, Choi HB, McLarnon JG, Lee MA, Kim SU. Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis* 2001; **8**: 1057–1068

6 **Sharma K,** Wang RX, Zhang LY, Yin DL, Luo XY, Solomon JC, Jiang RF, Markos K, Davidson W, Scott DW, Shi YF. Death the Fas way: regulation and pathophysiology of CD95 and its ligand. *Pharmacol Ther* 2000; **88**: 333-347

7 **Rad AN,** Schlom J, Hodge JW. Vector-driven hyperexpression of a triad of costimulatory molecules confers enhanced T-cell stimulatory capacity to DC precursors. *Crit Rev Oncol Hematol* 2001; **39**: 43–57

8 **Shering AF,** Bain D, Stewart K, Epstein AL, Castro MG, Wilkinson GW, Lowenstein PR. Cell type-specific expression in brain cell cultures from a short human cytomegalovirus major immediate early promoter depends on whether it is inserted into herpesvirus or adenovirus vectors. *J Gen Virol* 1997; **78**(Pt 2): 445-459

9 **Buggins AG,** Lea N, Gaken J, Darling D, Farzaneh F, Mufti GJ, Hirst WJ. Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood* 1999; **94**: 3479-3490

10 **Qiu ZH,** Wu CT, Lao MF, Pan LZ, Li YM. Growth suppression and immunogenicity enhancement of Hep-2 or primary laryngeal cancer cells by adenovirus-mediated co-transfer of human wild-type p53, granulocyte macrophage colony-stimulating factor and B7-1 genes. *Cancer Lett* 2002; **182**: 147–154

11 **McCarthy DO,** Meisner LF, Bourdeau-Heller J, Roberts TR, Wu SQ, Warner TF, Albertini MR. Localization of transfected B7-1 (CD80) DNA in human melanoma cells after particle-mediated gene transfer. *Cancer Genet Cytogenet* 2003; **144**: 106–111

12 **Zheng J,** Si L, Song J, Sun X, Yu J, Wang Y. Enhanced immune response to DNA-based HPV16L1 vaccination by costimulatory molecule B7-2. *Antiviral Res* 2003; **59**: 61–65

13 **Seko Y,** Takahashi N, Tada Y, Yagita H, Okumura K, Nagai R. Restricted usage of T-cell receptor Vgamma-Vdelta genes and expression of costimulatory molecules in Takayasu's arteritis. *Int J Cardiol* 2000; **75 Suppl 1**: S77-S83; discussion S85-S87

14 **Fox SA,** Loh S, Thean AL, Garlepp MJ. Identification of differentially expressed genes in murine mesothelioma cell lines of differing tumorigenicity using suppression subtractive hybridization. *Biochim Biophys Acta* 2004; **1688**: 237-244

15 **Fujii H,** Inobe M, Kimura F, Murata J, Murakami M, Onishi Y, Azuma I, Uede T, Saiki I. Vaccination of tumor cells transfected with the B7-1(CD80) gene induces the anti-metastatic effect and tumor immunity in mice. *Int J Cancer* 1996; **66**: 219-224

16 **Nagata S,** Golstein P. The Fas death factor. *Science* 1995; **267**: 1449-1456

17 **Webb SD,** Sherratt JA, Fish RG. Cells behaving badly: a theoretical model for the Fas/FasL system in tumour immunology. *Math Biosci* 2002; **179**: 113-129

18 **Houston A,** O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol* 2004; **4**: 321-326

19 **Lee SH,** Bar-Haim E, Goldberger O, Reich-Zeliger S, Vadai E, Tzehoval E, Eisenbach L. Expression of FasL by tumor cells does not abrogate anti-tumor CTL function. *Immunol Lett* 2004; **91**: 119–126

20 **Toillon RA,** Descamps S, Adriaenssens E, Ricort JM, Bernard D, Boilly B, Le Bourhis X. Normal breast epithelial cells induce apoptosis of breast cancer cells via Fas signaling. *Exp Cell Res* 2002; **275**: 31–43

21 **Arai H,** Gordon D, Nabel EG, Nabel GJ. Gene transfer of Fas ligand induces tumor regression *in vivo*. *Proc Natl Acad Sci USA* 1997; **94**: 13862-13867

22 **Suda T,** Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993; **75**: 1169-1178

23 **Kawasaki M,** Kuwano K, Nakanishi Y, Hagimoto N, Takayama K, Pei XH, Maeyama T, Yoshimi M, Hara N. Analysis of Fas and Fas ligand expression and function in lung cancer cell lines. *Eur J Cancer* 2000; **36**: 656-663

24 **Nafe C,** Cao YJ, Quinones A, Dobberstein KU, Kramm CM, Rainov NG. Expression of mutant non-cleavable Fas ligand on retrovirus packaging cells causes apoptosis of immunocompetent cells and improves prodrug activation gene therapy in a malignant glioma model. *Life Sci* 2003; **73**: 1847-1860

25 **Lang S,** Atarashi Y, Nishioka Y, Stanson J, Meidenbauer N, Whiteside TL. B7.1 on human carcinomas: costimulation of T cells and enhanced tumor-induced T-cell death. *Cell Immunol* 2000; **201**: 132-143

26 **Tamura H,** Dong H, Zhu G, Sica GL, Flies DB, Tamada K, Chen L. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. *Blood* 2001; **97**: 1809-1816

27 **Arlen PM,** Gulley JL, Palena C, Marshall J, Schlom J, Tsang KY. A novel ELISPOT assay to enhance detection of antigen-specific T cells employing antigen presenting cells expressing vector-driven human B7-1. *J Immunol Methods* 2003; **279**: 183–192

28 **Tan LJ,** Jing N, Zhang N, Zhang XR, Qiu DH. Fas/FasL expression of esophageal squamous cell cancinoma, dysplasia tissues and normal mucosa. *Shijie Huaren Xiaohua Zazhi* 2001;

9: 15-19

29 **Reichmann E.** The biological role of the Fas/FasL system during tumor formation and progression. *Semin Cancer Biol* 2002; **12**: 309-315

30 **Shinoura N,** Muramatsu Y, Yoshida Y, Asai A, Kirino T, Hamada H. Adenovirus-mediated transfer of caspase-3 with Fas ligand induces drastic apoptosis in U-373MG glioma cells. *Exp Cell Res* 2000; **256**: 423-433

31 **Ganss R,** Hanahan D. Tumor microenvironment can restrict the effectiveness of activated antitumor lymphocytes. *Cancer Res* 1998; **58**: 4673-4681

32 **McCarthy DO,** Meisner LF, Bourdeau-Heller J, Roberts TR, Wu SQ, Warner TF, Albertini MR. Localization of transfected B7-1 (CD80) DNA in human melanoma cells after particle mediated gene transfer. *Cancer Genet Cytogenet* 2003; **144**: 106-111

33 **Salford LG,** Siesjo P, Skagerberg G, Persson BRR, Larsson EM, Lindvall M, Visse E, Widegren B. Search for effective therapy against glioblastoma multiforme-clinical immunization with autologous glioma cells transduced with the human interferon-γ gene. *International Congress Series* 2002; **1247**: 211-220

34 **Tao G,** Hu J, Zou H, Lin Q, Liu F, Wu Y, Sun Q. Therapeutic antitumor response to cervical cancer in mice immunized with U14 vaccines transfected with costimulatory B7 gene. *Chin Med J (Engl)* 2001; **114**: 623-627

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