TK gene combined with mIL-2 and mGM-CSF genes in treatment of gastric cancer

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INTRODUCTION
Gastric cancer is a common malignancy in China. However, all the efforts of conventional treatments including extended resection, radiation and chemotherapy have a little influence on the improvement of its survival. In searching for a new way to the treatment of such a malignant disease, the gene therapy was introduced and displayed its promising. One of the landmark discoveries is the application of suicide gene to cancer cells. It converted a nontoxic prodrug into a cell-killing compound. The herpes simplex virus type I thymidine kinase (HSV-tk) and the Escherichia coli cytosine deaminase (CD) was popularly used as transfected suicide gene.

The expressed products of these genes are enzymes, which can convert the nontoxic anti-ancer drugs into toxic ones, and disrupt the synthesis of target DNA. The product of TK gene can phosphalate the ganciclovir (GCV), and it was further phospholated by endogenous kinase that leads to the formation of cytotoxic ganciclovir triphosphate. Interestingly, neighbor tumor cells that do not express the suicide gene were also killed in the presence of prodrg. This phenomenon is called the “bystander effect”[1-10].

Cytokines play important roles in the anti-tumor immune responses. IL-2 can activate the NK, LAK cells and CD8+ T lymphocytes. The activated CD8+ T lymphocytes can kill tumor cells directly. GM-CSF can promote the antigen presentation to macrophage and dendritic cells in the anti-tumor immune reaction[6-14].

The aim of this study was to boost the anti-tumor effect to achieve long-term survival and tumor eradication in model by the combination of TK/GCV with IL-2 and GM-CSF.

MATERIALS AND METHODS

Materials
The retroviral vector pLxSN was purchased from the Genetech. The HSV-TK gene was provided by Dr. Bingya Liu. LacZ gene was purchased from Promega. MFC cell line was derived from the 615 murine carcinoma of proximal stomach, and obtained from the Drug Research Institute of Chinese Science Academy. PA317 cell and NIH3T3 cells were cultured in this laboratory. Ganciclovir was purchased from Shanghai Roche Company, DMEM from Gibso, and G 418 from Promega.

Methods
Vectors and cell lines The retroviral vector is pLxSN. TK gene was inserted into the multiple cloning site between EcoRI and BamHI I, which was under the control of long terminal repeat (LTR), and the neomycin resistance gene was driven by an SV40 promoter. The report gene LacZ was inserted as same as TK gene. The murine IL-2 (Mil-2) and murine GM-CSF (mGM-CSF) were cloned from murine spleen tissue, and was confirmed by DNA sequencing. They were inserted into multiple cloning site of the pIRES vector through the EcoRI I and BamHI I, and driven by the cytomegalovirus (CMV) promoter.

MFC cells were maintained in DMEM (Dubecco’s modified essential medium), supplemented with 10% FBS (Hangzhou
Sijiqing Biotech Company), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. PA317 cell was used as the packaging cell, and NIH 3T3 cell was used to assay the virus titre.

**Packaging cells transfection, clone selection and supernatant preparation** The retrovirus plasmids containing TK and LacZ gene were transduced into the PA317 packaging cell line by lipofectamine (Gibco). Clones were isolated by G418 selection. After 48hs of lipofection, the media was replaced by the media contain G418 (600 µg/ml). The media was changed every 3 days. Most cells died after 2 weeks and the transfected cells survived. Culture and generate the selected anti-G418 cells. Collect virus suspension of four generations. To infect the NIH 3T3 cell with the virus suspension in different titres. Calculate the virus titres.

**Infection of MFC gastric carcinoma cell line** Infection was performed in suspension by a 30 minutes incubation of MFC cells with virus dilutions in 1 ml of PBS, supplemented with 4 µg/ml polybrene. To change the medium with DMEM which contained G418 48hs later, and repeated it every 3 days. Cells started to die after one week. The infected cells survived ultimately and formed cell clones.

**Histological analysis and immunohistochemical studies** Samples of tumor and surrounding tissues were fixed with formalin for 24 h, wax embedded. Sections were obtained with a microtome, and stained with haematoxylin-eosin for histological analysis. The frozen samples were incubated for 15 minutes in phosphate buffered saline (PBS), 1 % bovine serum albumin (BSA), and then overnight at 4°C with monoclonal antibodies diluted in PBS/1 % BSA.

**Statistical analyses** The tumor volumes were performed using the variance analysis. $P<0.05$ was considered to be statistically significant.

**RESULTS**

**Plasmid transduction and virus supernatant collection** The TK gene retrovirus vector plasmid was transduced into packaging cells with lipofectamine and maintained for 5-7 days in culture medium containing G418 600 mg/ml, and many cells started to die. After cultured for 2-3 weeks, some adherent cells formed cell clones contrasted with the dead cells.

The supernatants of every clone were collected and filtered after the cell clones were selected and expanded, the number of retroviral particles produced by the different cell clones was measured by NIH 3T3 cells. The maximum titer was 2x10⁵ cfu/ml.

**In vitro cytotoxicity and bystander-effect** After infected by the virus supernatant, many MFC cells began to die. Some adhesive cellsimmerged 3 weeks later, and formed cell clones.

MFC cells expressing TK gene were assayed for sensitivity to GCV. From the second day of culture with the medium containing GCV, the TK gene transfected cells began to die, and almost all the cells died at the seventh day. The untransfected cells in control group had no marked death.

The TK gene transfected MFC cells expressing marked bystander effect. A few transfected cells can cause many co-cultured cells to death combined with GCV (50 u/ml). Twenty percent of the TK gene transfected cells could kill 70-80 % of total cells (Figure 1).
In vivo experiment

In vivo analyses of TK/GCV and cytokines were performed in 615 mice implanted with the mouse carcinoma MFC cell line in proximal stomach. The retrovirus supernatant was injected into the tumors, and the cytokine genes were injected into the tumor surrounding tissues as indicated in the “materials and methods”. There has no significant inhibition of tumor growth in control group although treated with peritoneum injection of GCV. The group of TK gene without use of GCV also had no inhibition effect on tumor growth. In the TK/GCV group, tumor growth was significantly suppressed (P<0.01). In the animal groups treated with both TK/GCV and mIL-2 or mGM-CSF, there was a further significant reduction of the residual tumor size as compared to the group treated with TK/GCV (P<0.05).

There was further more decrease of tumor size in the group of TK/GCV combined with both cytokines. The tumors diminished in 7 mice of this group (Figure 2).

Histological and immunohistochemical analyses

There were great many tumor cells with mitoses in the sections of control group. The TK/GCV group showed lots of necrotic cells, and some of them accompanied by bleeding. But active tumor cells could also be seen in this group. There was massive infiltration of inflammation cells surrounding the necrotic area of the tumor treated with TK + cytokine, but not in those areas of animals treated with TK alone. Tumor cells diminished in most animals treated with TK/GCV + mIL-2 + mGM-CSF. There were a few residuum tumor tissues in part of these animals, but few mitoses phase can be seen, with great many of inflammatory cells.

Immunohistochemical analyses revealed that the infiltrates were mainly CD8+ lymphocytes in the tumor boundary area of animals treated with TK+mIL-2 or TK+ mIL-2 + mGM-CSF. The number of CD8+ lymphocytes was approximately equal in the TK + mGM-CSF and TK+mIL-2 + mGM-CSF groups. But few mitoses phase can be seen, with great many of inflammatory cells.

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In vivo expression of transfected genes

By RT-PCR analyses, TK gene and cytokine genes all can be expressed in vivo by virus transfection or liposome transduction.

DISCUSSION

Transfer suicide genes into tumors has emerged as an attractive gene therapy for the selective elimination of cancer cells. The suicide genes encode non- mammalian enzymes that can convert nontoxic prodrugs into cellular toxic metabolites. The most widely used suicide gene is HSV-tk, which confers prodrug GCV into phosphorylated GCV. The GCV monophosphate is further phosphorylated by cellular kinase, forming GCV triphosphate, which inhibits cellular DNA synthesis and lead to cell death. The “bystander effect” caused by TK gene can strongly enhance its killing capacity. Many researchers believe that necrosis of tumor cells is the mechanism of tumor killing effect caused by the metabolites of prodrugs, but the activated CTL can kill tumor cells as well. There also have many people think that apoptosis take an important role in the procession. In our studies, necrosis was shown in the prodrug used tumor tissues, some of them with bleeding. This might be the vascular endothelials transfected by suicide genes. In vitro experiment showed that 20% gene transfected cells rendered 80% of total cells to death. The mechanism of bystander effect has unclear. It has been hypothesized that the following factors may be concerned with the mechanism. (1) Gap junction: the toxic product of suicide gene was transferred from transfected cells into the surrounding untransfected ones. Studies demonstrated that the bystander effect of TK gene was via the gap junction. The converted phosphorylated GCV can get into the contact cells by gap junction, which needs the direct cell contact. (2) Apoptosis: the apoptotic acetes that released by the transfected cells engulfed by the surrounding cells. (3) Immune mechanism: tumors cells killed by TK/GCV can release tumor antigens. The tumor cell derived antigens were taken up by APCs (antigen presenting cells), and then presented to the CD8+ T lymphocytes. It in turn activated tumor-specific CD8+ cytolytic T cells. The immunohistochemistry shows tremendous aggregation of CD8+ and CD2+ lymphocytes surrounding the tumor tissue.

Chen et al reported that cytokine gene IL-2 acted synergistically with the suicide gene to induce a systemic antitumor immunity. The immunity resulted in regression of local tumor and protection against distant site challenge of parental tumor cells. The antitumor immunity was attributed to IL-2 mediated activation and proliferation of CD8+ CTLs.

TK/GCV gene therapy led to death of the tumor cells. The tumor antigens were then available to the immune system, and might activate an anti-tumor immune response. The local expression of mGM-CSF enhanced the inflammatory response and antigen presentation. Expressed mIL-2 activated and enhanced the proliferation of T lymphocytes. Combination of mIL-2 with mGM-CSF can synergistically stimulate the anti-tumor immune response.

The experimental results confirmed that TK/GCV gene therapy could kill tumor cells markedly. If combined with mIL-2 and mGM-CSF genes, they could boost the anti-tumor reaction, and produce powerful anti-tumor effects.

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