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Retrovirus-mediated herpes simplex virus thymidine kinase gene therapy approach for hepatocellular carcinoma

GAO DING CHENG, WEI AN*, JIE DAI

Department of Cell Biology, Capital University of Medical Sciences, Beijing 100054, China

ABSTRACT

The therapeutic effect of herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) system on hepatocellular carcinoma was studied in this experiment. The tk-containing retroviral recombinants were used to infect hepatoma cells (BEL-7402) and the cells were treated with ganciclovir (0-1000 μg/ml). The results showed that HSV-tk gene could be efficiently transferred in vitro into hepatoma cells and stably expressed. The growth potential of the tk-containing cells was significantly inhibited by GCV ($P < 0.01$) as compared to the non-tk-containing cells. The antitumor effect of HSV-tk/GCV system was also produced ex vivo in tk-containing tumor of nude mice as characterized by a marked decrease in tumor growth after GCV treatment contrary to a progressive enlargement of non-tk-containing tumors. Although the histological examination demonstrated that the efficiency of the gene transfer was less than 30%, the killing effect of HSV-tk/GCV system on hepatocellular carcinoma was still significantly generated. The proper mechanism of HSV-tk gene therapy on hepatic tumor referred as "bystander effect" in therapeutic approach has not been found in this study and required to be explored further.

Key words: *HSV-tk, retrovirus, gene therapy, hepatocellular carcinoma.*

INTRODUCTION

* Correspondence: AN Wei, Department of Cell Biology, Capital University of Medical Sciences, You An Men, Beijing 100054, China

Tel: 086-010-63291921 Fax:086-010-63051130 e-mail: anwei@cpums.edu.cn

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, causing an estimated 1,250,000 death toll per year worldwide[1]. The poor prognosis encountered in treatment of such carcinoma is mainly caused by late diagnosis and insufficiency of effective strategies, especially for advanced-staged patients. However, recent knowledge of pathogenesis of HCC at molecular level provides an alternative approach when considering gene therapy as treatment for HCC. Among the various gene therapy strategies in cancer, it was reported that thymidine kinase (tk) gene originated from herpes simplex virus, referred as HSV-tk, was often used both in vivo and in vitro[2-4]. The target cancer cells, after transfected by HSV-tk, can be killed with treatment of ganciclovir drug (GCV). Ganciclovir is nucleotide analog which, upon phosphorylated by HSV-tk, could act as inter-metabolic substance and thereafter terminate DNA chain of the replicating cancer cells[5]. Hence, HSV-tk gene could induce ultimately a direct "killing" or "suicide" effect in the transfected cells. The cells engineered to express HSV-tk exhibit the increased sensitivity to GCV as compared with parent cells due to the unavailability of the mammalian cellular tk to catalyze GCV as a substrate. In this approach, a retrovirus-mediated gene transfer system is widely used with treatment of glioma including the clinical trial[6],[7].

We had previously constructed a retroviral vector containing HSV-tk gene driven by 5'-LTR, designated as XM6-tk[8]. Herewith, we report the therapeutic effect of this system on HCC in vitro and ex vivo. The purpose of this study is to explore an alternative but effective method for treatment of hepatic carcinoma.

MATERIALS AND METHODS

Cell lines and culture conditions

The ectropic and amphotropic packaging cell lines, ψ-2 and PA317, human hepatic carcinoma cell line BEL-7402 were maintained in DMEM (Gibco/BRL) supplemented with 10% fetal bovine serum (Hyclone) at 37°C, 5% CO_2 and 100% humidity. The medium was replaced with fresh medium every 2-3 d.

Packaging of retroviral recombinant and transfection of cells

XM6-tk was constructed as described previously[8] and transfected into Y-2 cells using routine calcium-phosphate coprecipitation. The vector-containing supernatant from transfected cells was used to cross-infect PA317 cells. The transduced PA317 cells were grown under G418 (400 mg/ml) selection to obtain the single-cell clusters. As a parallel, PA317-LacZ was also constructed with the same method. The supernatant of the G418-resistance clusters with titers beyond 1×10^6 cfu/ml was used to transfect BEL-7402.

Southern and Northern analysis

Genomic DNA was isolated from PA317, PA317tk, BEL-7402 and BEL-7402tk cells, respectively. 20μg of each cell DNA were digested with 20 U BamH I. After electrophoresis, DNA were blotted onto nylon membrane (Bio-Rad). Parallel with DNA extraction, total cellular RNA of the abovementioned cells was extracted with guanidium-phenol-chloroform[9]. 20 μg RNA were denatured with formaldehyde and immobilized on nylon membrane through a slot manfold (Bio-Rad). The filters were hybridized with [α-^{32}P]dCTP-labeled 3.4 kb BamH I fragment of pHSV-106 vector

which encodes full length of HSV-tk. The Southern and Northern blot signals on the filters were visualized by autoradiography on the X-ray films and analyzed with imaging densitometer (GS-700 Bio-Rad).

Cell proliferation assay

A non-radioactive proliferation assay, MTS colorimetric method, was utilized to evaluate cell growth as described previously. The assay is based on the cellular conversion of the tetrazolium salt, MTS [3-(3,5-dimethylthialzol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt], into a formazan product that is soluble in tissue culture medium[10]. Briefly, the transfected and non-transfected cells (as control) were inoculated into 96-well plates. 4 h after attachment, GCV (0, 10, 100, 1000 μg/ml) was added. MTS reagent (Promega) was added into the medium 2 h prior to measurement and the absorption was determined with Microplate Reader (Bio-Rad) at 490 nm.

Ex vivo treatment

Hepatic tumor model was established in 12 male BALB/c nude mice as follows: 1×10^7/ml cultured BEL-7402 cells were mixed ex vivo with the equal density of either PA317 or PA317tk cells, respectively. 200 ml of each cell mixture (2×10^6 cells) were inoculated subcutaneously into defined zones of the mice, i.e. PA317/BEL-7402 in cephalical zone and PA317 tk/BEL-7402 in caudal zone. 10 d after the inoculation, the tumors were visually grown up, and then the mice were divided randomly into two groups (6 animals in each group). The treatment group was initiated by GCV administration twice daily (30 mg/kg) via peritoneal route and the control was given normal saline (NS) twice daily for 14 d. The measurements of tumor growth in all mice were performed twice per week and lasted for 35 d. The tumor sizes were calculated as reported previously[11][11]. All animals were killed on 35 d after inoculation of tumor cells. A part of tumor sample was removed and fixed with glutaraldehyde. Subsequent preparation of electron microscope specimen were consistent with standard methods.

Statistical analysis

Each value was expressed as X $\pm$S. All data were analyzed with F test using the SPSS computer program.

RESULTS

Efficiency of gene transfer and expression of tk gene

To evaluate the efficiency of gene transfer, we constructed retroviral vector containing reporter gene that encodes b-galactosidase (lacZ) and co-transferred it into PA317. The supernatant of the transfected cells (PA317-lacZ), with titer consistent to that of PA317tk (1×10^6 cfu/ml), was used to infect BEL-7402 and followed by X-gal staining (Fig 1). Since the essential titer of retrovirus for efficient delivery of gene to target cells was 5×10^5 cfu/ml[12], this titer of 1×10^6 cfu/ml was theoretically adequate to produce satisfied gene transfers. It was obviously seen in Southern blot that a 3.4 kb band was generated when hybridizing genomic DNA of PA317tk and BEL-7402tk with HSV-tk BamH I fragment (Fig 2a). Northern analysis result indicated that tk gene could be stably expressed in those cells identically (Fig 2b). Meanwhile, We also investigated gene transfer ex vivo by co-

injecting PA317-LacZ and BEL-7402 cells in nude mice. 10 d later, the tumor was removed, sectioned and stained with X-gal. The transfected BEL-7402 cells (blue) and non-transfected BEL-7402 cells were counted respectively under microscope. Because of asymmetrical distribution of tumor cells within solid tumors, the staining consistency might probably vary in some regions. The average gene transfer efficiency obtained in this study ex vivo was calculated as 30 %.

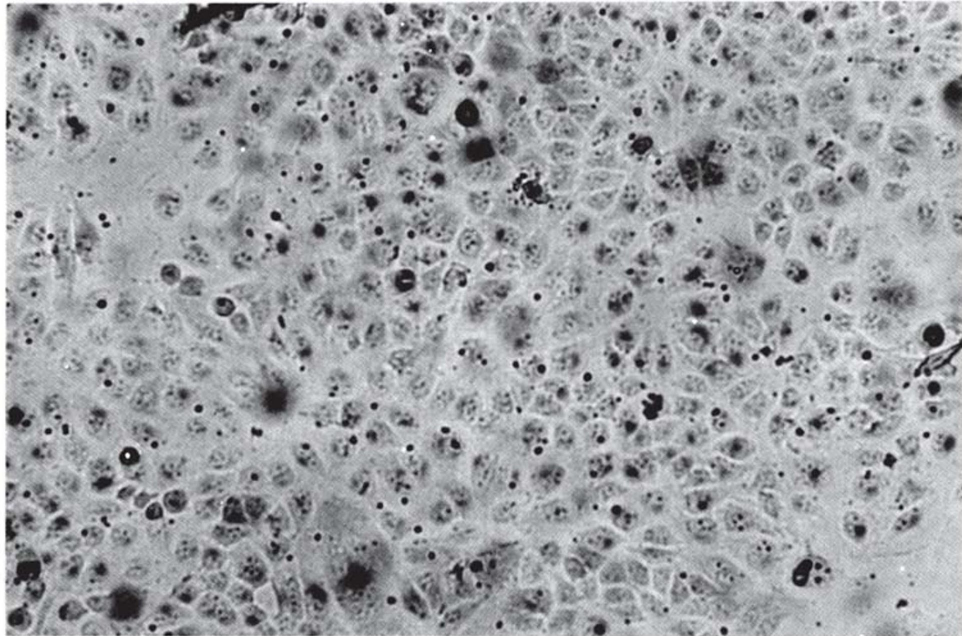


Fig 1.
Hepatic tumor cells BEL-7402 transfected with the supernatant of PA317-LacZ. Almost all the cells turned blue with X-gal staining. It suggested that the gene transfer efficiency of retrovirus was about 100% in vitro.

In vitro effect of gene therapy

The potential utility of HSV-tk gene therapy for the hepatic carcinoma in vitro was determined by the ability of recombinant virus expressing tk to confer sensitivity to GCV in the infected cells and resultant inhibition of cell growth. The results showed that the growth of BEL-7402tk cells was significantly declined after administration of GCV (10, 100, 1000 μg/ml), $P < 0.01$ (Fig 3). On the contrary, even up to 1000 μg/ml of GCV alone could not result in significant killing effect on non-transfected BEL-7402 (Fig 4). It implied that the increased sensitivity to GCV in BEL-7402tk up to 100-fold was due to functional expression of HSV-tk gene and its metabolic influence on DNA of replicating

hepatic carcinoma cells. Meanwhile, it was revealed in Fig 5 that the detachment and ultimate lysis phenomenon of BEL-7402 tk cells were clearly observed with treatment of GCV (100 μg/ml), but these morphological changes were not found in the tk negative cells.

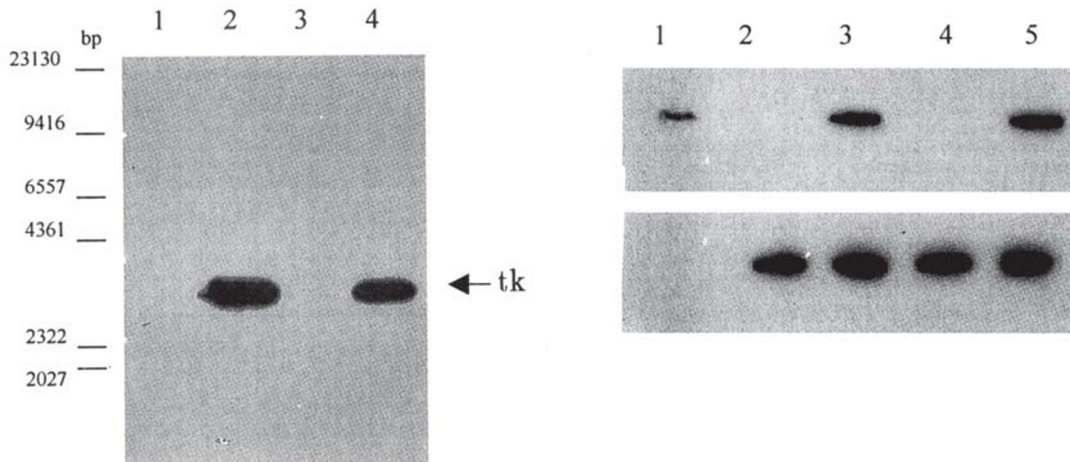
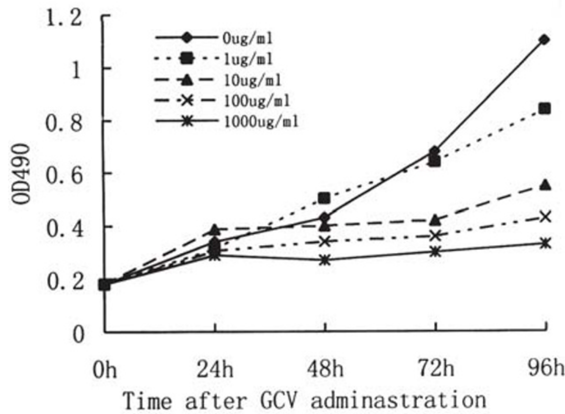


Fig 2a. (left)
Southern blot analysis of HSV- tk integration into the genomic DNA of host cells. The DNA were extracted from the cells, digested with Bam HI and eletrophoresed in 1.0 % agarose gel. After blotting onto the membrane, the genomic DNA was hybridized with tk DNA and probe subjected to autoradiography. The lane 1 to 4 represents DNA of wild-type PA317, PA317tk, BEL-7402 and BEL-7402tk, respectively.
Fig 2b. (right)
RNA slot blot of the HSV-tk gene in the transduced BEL-7402 cells. Slot 1 represents pHSV-106 plasmids DNA; Slot 2 to 5, represents total RNA of wild-type PA317, PA317tk, BEL-7402 and BEL-7402tk, respectively. 40 mg of RNA were hybridized with HSV- tk probe (upper) and S26 control probe (bottom).

Ex vivo effects of gene therapy

Subcutaneous tumors were established in vivo in nude mice as described in ``Materials and Methods". The tumors grew up to ca. $1\times 1cm^2$ at the 10th day after the cell inoculation (Figures not shown). The cytohistological examination showed that the solid tumors were mainly composed of two kinds of cells, PA317 and BEL-7402.

In the N.S. treatment group, the hepatic tumors originated from the tk-transfected as well as non-tk-transfected BEL-7402 grew progressively in consistent speed. This indicated that DNA manipulation on host genome caused by tk gene transfer did not impact proliferative characterization of tumor cells. However, as seen in the GCV treated group, tumors in caudual zone of mice where BEL-7402tk was injected were regressed (Fig 6). Fig 7 demonstrated the results obtained from treatment of mice carrying BEL-7402 hepatic carcinoma or control followed by either GCV (30 mg/kg) or NS. Tumor suppression effect



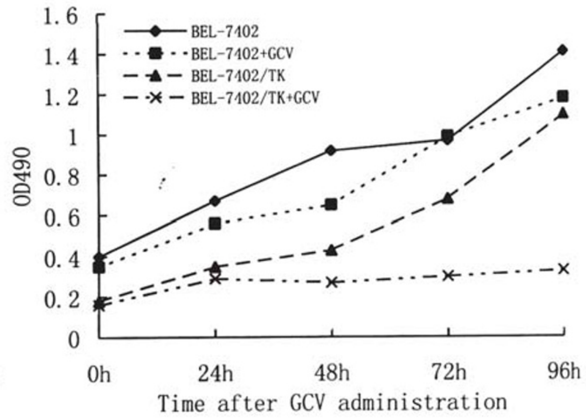


Fig 3.
The effect of GCV with various concentrations on the BEL-7402tk cells. The figure indicated that the GCV at 10 μg/ml significantly inhibited the growth of the BEL-7402tk cells ($P < 0.01$). As compared with wild-type BEL-7402 cells indicated in Fig 4, it suggested that the sensitivity of BEL-7402tk cells to GCV was increased up to 100-fold.

Fig 4.
The efficiency of HSV- tk/GCV system to hepatocellular carcinoma in vitro. After treatment initiated with GCV, only the tk-transduced cells showed growth inhibition. It was obviously proved that tk gene transfer into BEL-7402 did not influence the proliferation feature of hepatic tumor cells.

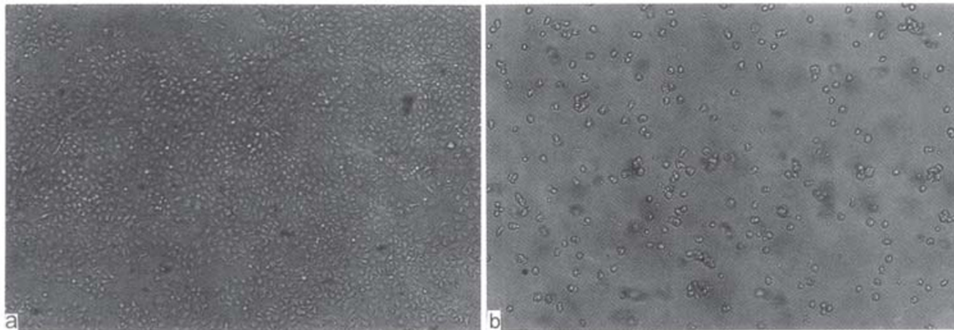


Fig 5.
Wild-type and tk-transduced BEL-7402 cells after treated with GCV (100 μg/ml). (a) BEL-7402 cells; (b) BEL-7402tk cells. The figure demonstrates profound phenomena of death, such as detachment and lysis.

occurred exclusively in BEL-7402tk-induced tumor accompanied with GCV treatment. The growth of other tumors was not markedly inhibited. The ex vivo transfection of β-galactosidase gene as stained by X-gal allowed us to evaluate definitively the efficiency of

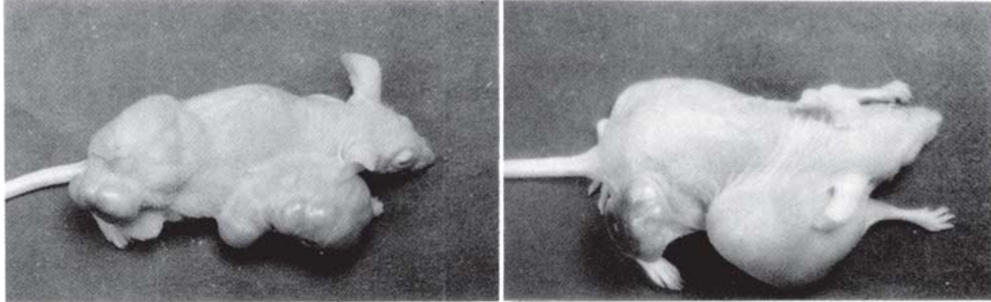


Fig 6.
The effect of HSV-tk/GCV system on the tumors growth. The nude mice were administrated intraperitoneally with either normal saline (left) or GCV (right) ten days after inoculation of tumor cells described in "Materials and Methods". It was seen in the right figure that tumor in caudal region (tk positive) was smaller than that in the cephalical region, where the non-tk transfected PA317 cells were inoculated.

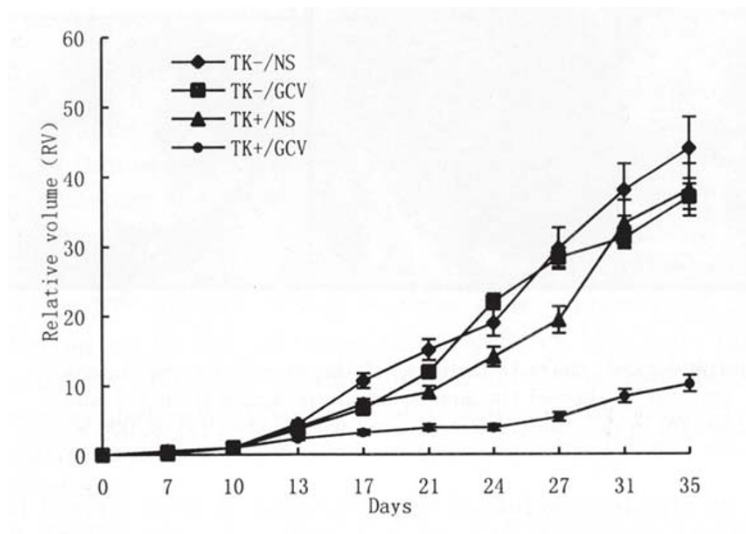


Fig 7.
The efficacy of HSV-tk/GCV system on hepatocellular carcinoma in vivo. The tumor growth was measured twice a week. The growth curves were drawn according to the relative tumor volume. Both the non-tk-containing and tk-containing tumors without GCV treatment revealed consistent growth capacities. Whereas, the tumors with tk were remarkably inhibited after initiation of GCV treatment.

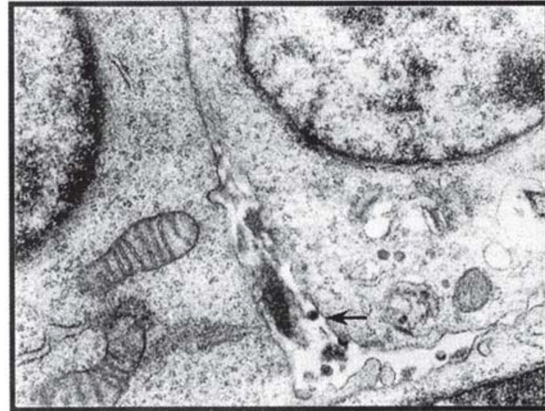


Fig 8.
The retroviral particles surrounded the BEL-7402 cells (marked by arrows). The tumors derived from PA317tk and BEL-7402 cells were sectioned and observed under electron microscope 8,000 × .Figure shows clearly that retrovirus particles were secreted from PA317tk cells into the matrix of tumors to infect its surrounding BEL-7402 cells.

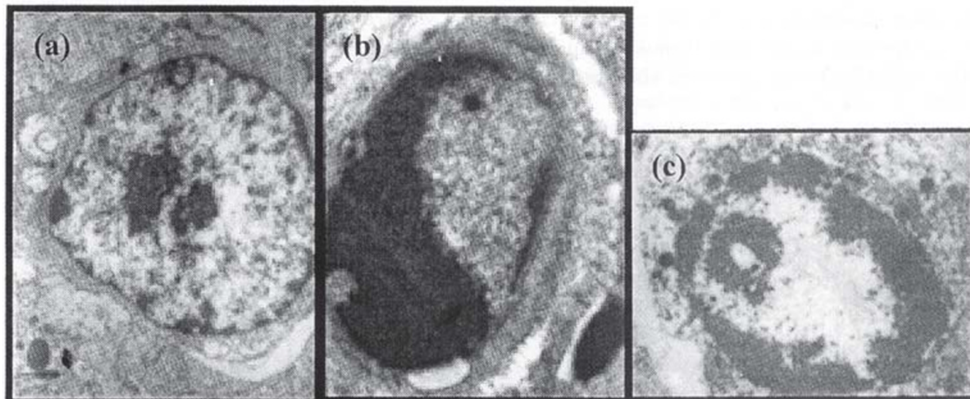


Fig 9.
The morphological characterizations of hepatocellular carcinoma treated with GCV showed the heterochromotin aggregation (b) and karyoschisis (c) in tk^+ tumors as compared with control (a), 8, 000 ×.

in situ gene transfer and relative expression property. The accumulation of β-galactosidase in tumor cells as represented by ca. 30% of X-gal staining was the clear evidence for high-performance of gene transfer in this study.

Histological findings

Histological examination of the residual tumors in mice with tumor regression (GCV treatment) revealed focal necrosis and hemorrhage. However, the progressive tumors in non-GCV treatment developed a typical and massive proliferation. Similarly, non-tk-containing tumors did not appear growth arrest even after GCV administration. It

could be found in electron microscope that the retroviral particles were secreted into intercellular space, where some of them seemed to attach to the membrane of hepatoma cells (Fig 8). Tumors treated with GCV presented morphological changes mainly in nuclei, including heterochromotin aggregation and karyoschisis (Fig 9).

DISCUSSION

The incidence of hepatic cancer rose gradually in recent years in China. Currently surgical operation is considered exclusively as sole option for the treatment of HCC, although less than 20% of patients are considered as candidates for resection[13]. HCC is clearly a disease for which alternative therapies must be developed.

The HSV-tk/GCV gene therapy system might be suitable for the treatment of HCC, which is composed of rapidly dividing cells invading a nonproliferating tissue. One of the most important characteristics of retrovirus is their preferential infection of the dividing cells, hence, the tumor has been primarily considered as a target of retroviral-mediated gene therapy. Similar vectors carrying HSV-tk have been described in recent report for treatment of melanoma[14], lymphoma[15] and glioma cells[5].

Among all the prerequisites dealing with this gene therapy strategy, whether a suicide tk gene could be efficiently introduced into the host cancer cells seems to be of the most important one. According to previous report, the inferior titers obtained in packaging the recombinant retroviral vector were often encountered, and this could result dramatically in failure of therapeutic effect. For this reason, we used cross-infect techniques between $\varphi 2$ and PA317 and obtained a high titer viral stock (1×10^6 cfu/ml). Using reporter gene expression of β-galactosidase, we found that an up to 30% of target tumor cells ex vivo were infected by such a titer retroviral recombinant. In addition, the functional mRNA expression of tk gene was revealed obviously in Northern blot. These results suggested strongly that our retroviral recombinant construct and gene delivery system are optimized for following experiment.

Although at first sight 30% of gene transfer indicated that not all the tumor cells were transfected, the profound antitumor efficacy was indeed produced in this study. Therefore, it seemed that the tumor regression effect was not associated proportionally with efficient gene transfer in all tumor models. As described in many reports of gene therapy for tumors, even so limited number as to 10% of target cancer cells infected, satisfactory antitumor effects were still able to produce[16]. Freeman, et al described the "bystander effect" as the tumor regression was produced when a fraction of tumor mass was genetically modified. Precise mechanism of "bystander effect", though so-far quite unclear, is a possible result from diffusion of toxic GCV metabolites, produced by neighboring nontumor tissue. In this study, we are not attempting to explore in-depth the mechanism of killing effect of HSV-tk/GCV, however, 30% of gene transfer efficacy resulted in the significant tumor suppression in tk-transfected site, implying a possibility of bystander effect, for which we will further follow up the research.

It was reported that HSV-tk/GCV system could induce apoptosis in glioma cells, and apoptosis had been considered a major contribution of GCV killing[17]. However, the typical apoptosis phenomenon had been reported neither in the XC hepatoma model nor in ψ-2 packaging cells in vitro[18]. In our study, the BEL-7402tk cells treated with GCV appeared heterochromotin aggregation and karyoschisis, but characteristics of apoptosis were not found, such as apoptosis particle in electron microscope and expression of apopotosis genes (unpublished observation). This suggested that the killing mechanism of HSV-tk/GCV system vary in different tumor models.

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