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The use of suicide gene systems in vascular cells *in vitro*

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ABSTRACT

To investigate the efficiency of suicide gene systems on vascular cells, HSV-*tk*/GCV and EC-CD/5-FC systems were established on vascular endothelial cells in vitro by retroviral transduction. Both modified cell lines were highly sensitive to prodrugs, the IC_{50} for GCV was less than 0.4 μM, and IC_{50} for 5-FC was less than 75 μM, while the parental endothelial cells were insensitive even at the highest concentrations of prodrugs in this experiment. Mixed cellular assay showed that significant bystander effect was exhibited in modified endothelial cells. When only 10% or 30% of the mixed cells were tk positive and exposed to 20 μM GCV for 6 days, more than 60% or 90% of the whole population was killed. Similar result was also found in CD positive cells. These results indicated that both HSV-*tk*/GCV and EC-CD/5-FC systems could efficiently suppress endothelial cell growth in vitro.

Key words: *Endothelial cell, suicide gene, HSV-tk/GCV system, EC-CD/5-FC system, bystander effect.*

INTRODUCTION

Recently, more and more evidences have shown that malignant solid tumor growth and metastasis are dependent on the formation of new blood vessels. These blood

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Abbreviations: ganciclovir (GCV); 5-fluorocytosine (5-FC); thymidine kinase (*tk*); cytosine deaminase (CD)

vessels also provide an entry site into the circulation for the neoplastic cells that detach from the tumor mass. Most solid tumors may neither grow beyond a diameter of 3-4mm nor form metastasis if they are deprived of angiogenesis[1]. Thus, it seems reasonable that complete inhibition of angiogenesis might suppress the tumor growth and metastasis. Inhibition of tumor-induced angiogenesis was first proposed as an anticancer approach by Folkman in 1971[2]. In recent publications two strategies for inhibition of angiogenesis have been put forward, anti-angiogenesis and vascular targeting[3]. The key issue of the latter approach is direct destructing the vessels and infarcting areas of the tumor.

In this report, we investigated the possibilities of using suicide gene systems, both HSV-*tk*/GCV and EC-CD/5-FC, as a vascular targeting approach to suppress angiogenesis *in vitro*. It seems that few studies have been done to systematically investigate the efficiency of these suicide genes on endothelial cells.

MATERIALS AND METHODS

Cell culture

Murine pulmonary vascular endothelial cell 1Gll (gift from Dr. Dong Qiang-gang)[4] were cultured in the growth medium DMEM supplemented with 20% FCS, 2m*M* L-glutamine, 1% non-essential amino acids, lm*M* sodium pyruvate, 25m*M* HEPES, freshly added heparin and ECGS (From bovine neural tissue, Sigma) at the final concentration of 100 μg/ml.

Retroviral infection

1G11 cells were infected by incubating with recombinant retrovirus LtkSN or LCDSN (1×10^4 cfu/ml) in the presence of 8 μg/ml polybrene (Sigma) for 24 h. Then the cells were cultured in the medium containing 800 μg/ml G418 for 2 weeks. The representative G418 resistant clones 1G11/R-*tk* and 1G11/R-CD were selected for the further experiments.

Cytotoxic assay in vitro

The cytotoxicity of prodrugs was measured by MTT assay. Target cells were plated into 96-well microplates at 2,000 cells per well. Next day, cells were cultured in 200 μl/well fresh medium containing various concentrations of prodrug. Six days later, medium was replaced with 180 μl fresh medium containing 0.25 mg/ml MTT (Sigma). After 4 h of incubation at 37 ℃, 100 μl solubilizing reagent (20% SDS and 50% DMF in water) was added, then after another 4 h, the A_{595} of each well was measured with a microplate reader with reference filter of 655nm. All tests were performed in six samples, and the percentage of survival was estimated as: % Survival = A / B $\times$100% (A: mean value of A_{595} from cells incubated with prodrug; B: mean value of A595 from cells incubated with medium only.)

Bystander killing effect

Suicide gene positive and negative 1G11 cells were cocultured at 2,000 cells per well in 96-well microplates at ratios of 10:90, 30:70, 50:50, 70:30, 90:10. After 12 h incubation, 20μ*M* GCV and 1,500μ*M* 5-FC were added, respectively. Six days later, the prodrug-mediated growth inhibition was measured.

RESULTS

Identification of the integration and expression of suicide genes

(Data not shown)

Prodrug-mediated growth inhibition in vitro

The wild type 1G11 cells were used as control in the cytotoxicity assay. As shown in Fig la, the parental 1G11 cells were resistant to GCV even at the highest concentration (200 μM) in the experiment. In sharp contrast, almost all the genetically modified cells (1G11/R-*tk*) were killed when incubated with only 20μM GCV for 6 days. The IC_{50} for GCV was only 0.4 μM. Similar results were found in 1G11/R-CD cells after 5-FC treatment. Transduction of CD gene made endothelial cells become highly sensitive to 5-FC with the IC_{50} of 75 μM, while the control 1G11 cells were insensitive even they were exposed to > 10,000 μM 5-FC for 6 days (Fig lb). These data demonstrated that suicide gene systems, both HSV-*tk*/GCV and EC-CD/5-FC had significant killing effect on endothelial cells.

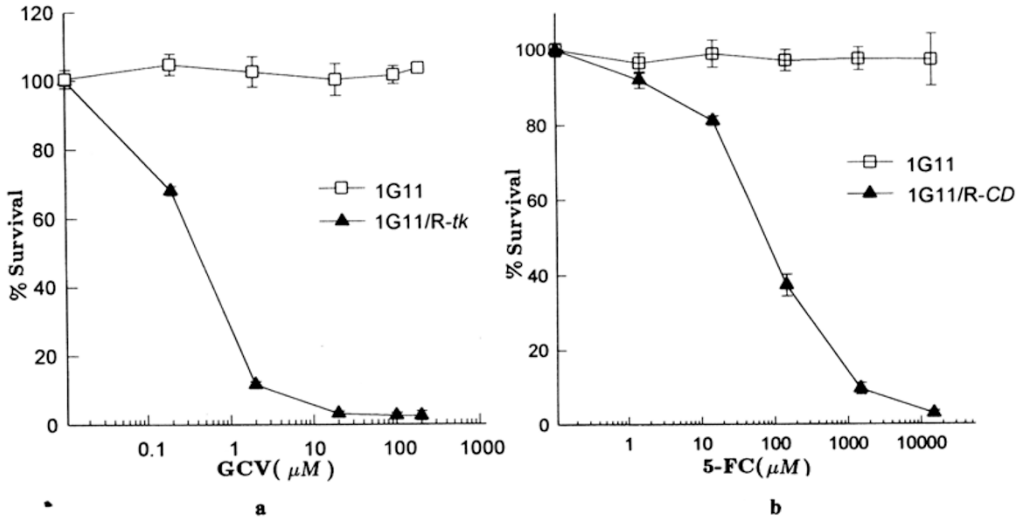

Fig 1. Prodrug-mediated growth inhibition *in vitro*. The cytotoxicity was determined by MTT assay. Each point represents the average ± SD (bars) of five individual determinations. Logarithmic dose-response curves showed that all suicide gene modified 1G11 cells were highly sensitive to prodrugs while wild type 1G11 cells were insensitive. (a) GCV mediated growth inhibition. (b) 5-FC mediated growth inhibition.

Analysis of the bystander killing effect in vitro

The experiments were performed in which suicide gene positive and negative cells were mixed at varying ratios. The significant bystander effect was observed in both HSV-*tk*/GCV and EC-CD/5-FC systems. Fig 2a has shown that as few as 10% or 30% of the mixed cells were HSV-*tk* positive, more than 60% or 90% of the whole population was eradicated after they were exposed to 20 μM GCV for 6 days. The Similar bystander effect was also found in EC-CD/5-FC system, though it was not

as significant as that in HSV-*tk*/GCV system. When CD positive and negative cells were mixed at ratio of 10:90 and cocultured in 1,500 μM 5-FC, more than 50% of the population were killed in vitro (Fig 2b). Taken together, these results indicated that bystander killing effect might be used to kill the endothelial cells which were not transfected by suicide gene.

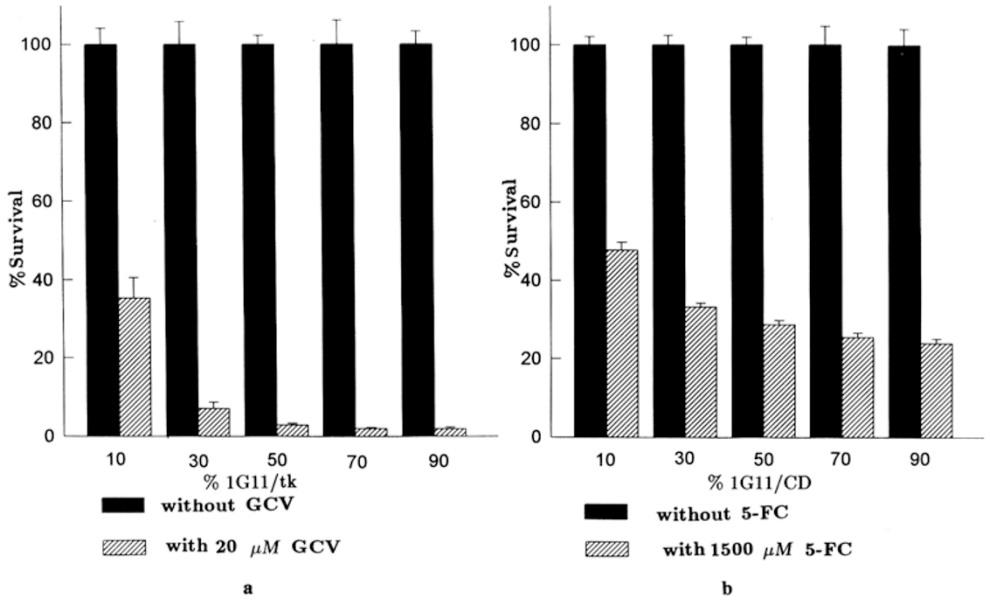

Fig 2. Analysis of the bystander killing effect *in vitro*. Suicide gene positive and negative 1G11 cells were mixed at varying ratios and prodrug-mediated growth inhibition was measured by MTT assay. Data represent: mean ± SD (bars) of five samples. Significant bystander killing effect exhibited in both HSV-*tk* and EC-CD modified 1G11 cells. (a) Mixed 1G11/R-*tk* and 1G11 cells were exposed to 20 μM GCV for 6 days. (b) Mixed 1G11/R-CD and 1G11 cells were exposed to 1,500 μM 5-FC for 6 days.

DISCUSSION

The importance of tumor angiogenesis in the process of solid tumor growth and metastasis has been widely accepted. Vascular endothelial cells provide the supply of nutrients for the growth of the primary tumor mass and the route of intravasation. Though not all angiogenic tumors produce metastasis, the inhibition of angiogenesis prevents the growth of tumor cells at both the primary and secondary sites and thus can prevent the emergence of metastasis[1]. In addition, destroying the blood vessels within tumor might result in the acute ischemic necrosis of the whole nourished area. Also, vascular endothelial cells are similar in different tumors, thus, make it possible to develop a general method for treating various types of cancer.

In this study, we demonstrated the efficiency of using suicide gene systems, HSV-*tk*/GCV or EC-CD/5-FC, on vascular cells *in vitro* by retrovirus transduction. All modified cells were highly sensitive to the prodrugs while the parental endothelial cells were insensitive even at the highest concentrations in the experiment. Significant bystander effect was also observed in the coculture assays, especially rather strong killing effect between *tk* positive and negative endothelial cells.

Many studies have suggested that metabolic cooperation, involving the transfer of low molecular weight molecules between neighboring cells via gap junctions, could account for the bystander effect[5]. Though it has been reported that tumor-derived endothelial cells illustrated tight gap junctions similar to those seen in endothelial cells from normal tissues shown in electron microscopy[6], an analysis of patterns of junctional communication in skin showed that the endothelial cells of small blood vessels are coupled to surrounding stromal cells[7], and the endothelial cells of the vasculature of tumors are similarly coupled to the surrounding cells[8], which should allow toxic products generated by the suicide gene modified tumor cells to damage the blood supply[9]. Conversely, modified endothelial cells can also damage the surrounding tumor cells through its bystander killing effect and accelerate the tumor necrosis[10].

Endothelial cells in tumor blood vessels divide about 500 folds more rapidly than those in normal tissues which mostly represent a highly differentiated cell type[11, 12]. In theory, complete inhibition of angiogenesis should be well tolerated by most adult tissues. Due to the hyperproliferation status of endothelial cells in tumor blood vessels, retrovirus might be an ideal gene transduction vehicle since it could only infect the cells that were actively synthesizing DNA. Also, if cell-specific promoters or regulatory elements were used, the security of this approach might be increased. We have cloned the promoter region of the Fltl gene which has been shown to be almost exclusively expressed in endothelial cells[13] to obtain an endothelial cell-specific regulation element. Our further study will focus on this strategy.

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