Introduction

Glioma is the most common malignant tumor of the central nervous system [1]. It is an aggressive tumor characterized by a high propensity for invasion and poor prognosis despite treatment with the currently recommended treatment comprising of surgical resection followed by adjuvant chemotherapy and radiotherapy [2]. The average survival time for glioma patients is 12–15 months [3]. Gene therapy refers to the introduction of a therapeutic gene or manipulation of a disease-related gene, and is a promising approach to treatment of malignant glioma [4, 5].

Among all types of gene therapy (GT), suicide gene therapy [6] and antiangiogenic gene therapy [7] have proved to be effective against glioma tumor. Cancer is characterized by multiple gene mutations and disrupted molecular mechanisms [8]. Therefore, gene therapy targeting single gene may have limited therapeutic outcomes, whereas combined (double) gene therapy may benefit from synergistic effects [4].

The most extensively studied suicide GT against glioma is herpes simplex virus thymidine kinase (HSV-TK), which catalyzes the phosphorylation of nucleoside analog ganciclovir (GCV). Because of TK catalyzation, GCV can be converted into a toxic metabolite which inhibits DNA replication, cell proliferation and induces cell apoptosis [9, 10]. Endostatin (ES) is an angiogenesis inhibitor which opposes antiangioma activities, with the most
probable mechanism of restricting tumor microvasculature development [11, 12]. The effect of combined TK and ES gene therapy on glioma has not yet been investigated.

In this study, we constructed recombinant plasmids expressing TK, ES alone or in combination, and analyzed their potential effects on cell proliferation, migration, and apoptosis of human umbilical vein endothelial cells (HUVECs) ECV304 and C6 rat glioma cell line. In addition, we determined the potential therapeutic effect of these recombinant plasmids in rats bearing C6 glioma tumor. Our findings may contribute to the development of high efficacy double-gene-targeted therapy against glioma.

Materials and Methods

Reagents
DMEM culture medium was obtained from GIBCO Inc., Grand Island, NY. Fetal bovine serum (FBS) was purchased from HyClone, Logan, UT. Anti-CD34 and antienodostatin antibody were bought from Santa Cruz Biotechnology, Santa Cruz, CA. Annexin V-FITC assay was obtained from Imgenex, San Diego, CA. Ganciclovir (GCV) was obtained from Roche, Basel, Switzerland.

Construction of recombinant plasmids
The internal ribosome entrysite (IRES) vector was purchased from ClonTech, Mountain View, CA and the pCMV-TK encoding the HSV-tk gene was obtained from Dr. Li Chen the Department of Pharmacology, College of Basic Medical Sciences, Jilin University, China. Full-length TK gene was amplified using pCMV-TK as a template. Full-length rat endostatin cDNA was amplified based on sequence provided by Genbank (GenBank accession No.: NM_009928). pTK-IRES was constructed by cloning PCR-amplified TK fragments into the NheI and EcoRI sites of pIRES vector. pIRES-endostatin (ES) recombinant vector was constructed by inserting ES sequence at the restricted SalI and NotI sites of pIRES vector. pTK-IRES-ES recombinant plasmid expressing both TK and ES was constructed by inserting TK and ES sequences at the corresponding sites of pIRES vector as described in Fig.S1. The inserted sequence in all vectors was confirmed by DNA sequencing.

Cell culture and transfection
Human umbilical vein endothelial ECV304 cells and rat C6 glioma cells were provided by Professor Liankun Sun the Department of Pathophysiology, College of Basic Medical Sciences, Jilin University, China. Cells were maintained in DMEM culture medium containing 10% FBS, and were cultured in a 5% CO2 incubator at 37°C. Furthermore, transfection was conducted when cells attained approximately 90% confluence. ECV304 cells were transfected with pIRES, pIRES-ES, or pTK-IRES-ES plasmids, respectively, using Lipofectamine2000 transfection protocol according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). Untransfected cells were used as normal control.

Determination of the effects of recombinant plasmids on ECV304 cells
Forty-eight hours after transfection, the mRNA and protein expression levels of ES were evaluated by reverse transcription polymerase chain reaction (RT-PCR) and Western
blotting, respectively. The influence of recombinant plasmid on cell proliferation was examined by MTT assay, and growth inhibition rate was calculated using the following equation:

\[
\text{Growth inhibition rate (\%)} = \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right) \times 100\% .
\]

Cell migration capability was evaluated by wound healing assay and Transwell chamber system. Cell apoptosis was assessed by Annexin V/PI double staining followed with flow cytometric analysis (Becton–Dickinson Biosciences, Drive Franklin Lakes, New Jersey, USA). Data were obtained from three independent experiments.

Figure 2. Endostatin (ES) and Thymidine kinase–Endostatin (TK-ES) inhibited migration of ECV304 cells. (A) The extents of cell migration without (upper panel) and with (lower panel) inhibition evaluated by wound healing assay during 48 h. (B) The extents of cell migration for untransfected cells (upper left), pIRES transfected cells (upper right), pIRES-ES transfected cells (lower left), and pTK–internal ribosome entry site (IRE)-ES transfected cells (lower right) evaluated by Transwell chamber system. (C) Original magnification (×100) of cell migration for untransfected cells (control), pIRES transfected cells (empty vector), pIRES-ES transfected cells (pIRES-ES), and pTK–IRE-ES transfected cells (pTK–IRE-ES). \( N = 3 \). ** indicates \( P < 0.01 \) when compared with normal control group.
Determination of the effects of recombinant plasmids on C6 cells

Forty-eight hours after transfection, the mRNA expression of TK was determined using RT-PCR. In order to establish pTK-ES/ganciclovir (GCV) system, single cell suspension of C6 cell lines was prepared and these cells were seeded onto 96-well plate at a density of $1 \times 10^5$ cells/mL in 100 μL culture medium, and transfection was performed on the next day. On day two after transfection, cells were exposed to different concentrations of GCV (0, 0.1, 1, 10 or 100 μg/mL). The culture medium was changed after every 2 days. Three days after culture, cell morphology was examined under phase-contrast microscope. Cell proliferation was determined by MTT assay. The ultrastructural changes were evaluated by transmission electron microscope. Cell cycle was determined by flow cytometric analysis 48 h after the initial GCV treatment. Three days after GCV exposure, cell apoptosis was evaluated by Hoechst 33258 staining and Annexin V/PI double staining followed with flow cytometric analysis. Data were obtained from three independent experiments.

Establishment of rat C6 glioma and drug administration

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee guidelines of Jilin University (Changchun, People’s Republic of China). C6 glioma was produced in Wistar rats as previously described [13]. Seven days after C6 cell inoculation, rats were examined by magnetic resonance imaging (MRI). After confirmation of the tumor mass, animals were randomly divided into five groups, each group comprising of nine animals. In normal control, empty vector control, pTK-IRES/GCV, pIRES-ES, and pTK-IRES-ES/GCV group, rats were given 15 μL of PBS, pIRES,
pTK-IRES, pIRES-ES, and pTK-IRES-ES by intratumoral injection under stereotaxic apparatus. Drug administration was repeated 7 days after initial injection. In pTK-IRES/ GCV and pTK-IRES-ES/GCV groups, animals received daily intraperitoneal injection of GCV at a dose of 50 mg/kg/d. GCV injection was administered 3 days post plasmid injection, and was continued for 8 days. Experimental procedures for animal studies are described in Fig.S2.

Examination

The activity and behavior of animals in all experimental groups was monitored. MRI examination was carried out on Day 21 after tumor cell inoculation. The largest coronary and horizontal plane of the tumor was selected. The maximum length (L), width (W), height (H) was measured by MRI. The tumor size was calculated using the following equation: Tumor size (mm$^3$) = n × L × W × H / 6.

Tumor growth inhibition rate was calculated using the following equation: tumor growth inhibition rate = (Control tumor size – Sample tumor size) / Control tumor size × 100%. Five rats were randomly selected from each group for analyzing survival time. The survival curve was drawn and animal survival was compared between different groups. Four animals in each group were used for histological examination. Briefly, on Day 22 after tumor cell inoculation, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. The rat brain was removed, fixed in 10% formaldehyde, and stained with hematoxylin–eosin (HE). Samples were immunostained with anti-CD34 antibody using a SP kit as per the manufacturer’s instructions (Maixin Biotech, Fuzhou, China), and the microvascular density (MVD) was measured according to a previously published method [14]. In areas with most intense CD34-positive neovascularization, micrographs were captured under ×200 magnification. Any endothelial cell or its cluster was considered as a single countable microvessel. The absolute number of the quantified microvessels per area was considered as the MVD value.

Statistical analysis

Data were analyzed by SPSS 14.0 software (IBM SPSS New York, U.S.), and are expressed as mean ± standard deviation (SD). Statistical significance of intergroup differences was determined using analysis of variance (ANOVA) and $\chi^2$ test. Animal survival was determined using Kaplan–Meier analysis and compare by means of the LogRank test. $P < 0.05$ or $P < 0.01$ was considered as significantly different.

Results

ES and TK-ES inhibits the proliferation of ECV304 cells

In order to determine the effects of pIRES-ES and pTK-IRES-ES plasmids on ECV304 cells, these were transfected with pIRES empty vector, pIRES-ES or pTK-IRES-ES plasmids. It was observed that mRNA and protein expression levels of ES were greatly upregulated in cells expressing TK and TK-ES, whereas untransfected normal control or cells carrying empty vector did not express TK. With cells incubated with different concentrations of Ganciclovir(GCV), cell proliferation was examined by MTT assay after 3 days of GCV treatment. The survival rates in different groups showed that there was no significant cell growth inhibition for untransfected cells or cells carrying empty vector exposed to GCV, and there was a significant reduction in cell survival in cells those expressing TK or TK-ES when administered increased doses of GCV. The survival rate of cells expressing TK or TK-ES was only 11.2% and 8.3%, respectively, after exposure to GCV at a dose of 100 μg/mL. $N = 3$. RT-PCR, reverse transcription polymerase chain reaction.
38.1%, respectively, at 72 h after transfection. No significant difference was observed with respect to the rate of cell proliferation between normal control and empty vector groups ($P > 0.05$).

**ES and TK-ES inhibits the migration of ECV304 cells**

Cell migration capacity was determined by wound healing assay and Transwell chamber system. After scratching, the healing of the gap was completed within 48 h by cell migration toward the center of the gap (Fig. 2A). However, cells expressing ES or TK-ES exhibited a decreased rate of “healing” of the gap, suggesting ES or TK-ES may inhibit cell migration in ECV304 cells. This finding was confirmed by Transwell chamber system. The number of migrated cells was remarkably reduced in cells transfected with pIRES-ES or pTK-ES as compared to that in the normal control or empty vector groups ($P < 0.01$).

**ES and TK-ES increases apoptosis of ECV304 cells**

Using Annexin V/PI double staining followed by flow cytometric analysis, we found that transfection of pIRES-ES or pTK-RES-ES significantly enhanced the percentage of apoptotic cells when compared with control (Fig. 3). It should be noted that no difference in the number of apoptotic cells between empty vector and normal control groups ($P > 0.05$). The percentages of apoptotic rate were 12.4%, 31.4%, and 35.9% for empty vector, pIRES-ES, pTK-RES-ES experimental groups, respectively.

**TK/GCV and TK-ES/GCV inhibits the proliferation of C6 cells**

To understand the effects of pTK-RES and pTK-RES-ES plasmids on rat glioma cells, C6 cells were transfected
with pIRES empty vector, pTK-IRE or pTK-IRE-ES plasmids. The mRNA expression of TK was greatly upregulated in cells expressing TK and TK-ES, whereas untransfected normal control or cells carrying empty vector did not express TK (Fig. 4A). Besides, when untransfected cells or cells carrying empty vector were exposed to GCV, no significant cell growth inhibition was observed (Fig. 4B). In contrast, there was reduced cell survival in those expressing TK or TK-ES when administered increased doses of GCV. The survival rate of cells expressing TK or TK-ES was only 11.2% and 8.3%, respectively, after exposure to GCV at a dose of 100 μg/mL.

**TK-ES/GCV induces S phase arrest and apoptosis in C6 cells**

Flow cytometric analysis revealed that transfection of pTK-IRE-ES plasmids led to S phase cell arrest when incubated with GCV for 48 h. The percentage of cells in S phase was greatly increased in cells expressing TK-ES when compared with empty vector control (Empty vector, 20.23%; pTK-IRE-ES, 39.24%) (Fig. 5A, B). However, the percentage of cells at G0/G1 or G2/M phases was reduced in cells expressing TK-ES. Moreover, administration of GCV induced morphological changes in cells transfected with pTK-IRE-ES plasmids, observed as cell shrinkage and poor adherence. Electron microscopy revealed that normal control cells treated with GCV exhibited abundant microvilli, regular nucleus shape, clear nucleolus, and enriched intracellular organelles in cytosol (Fig. 5C). While cellular changes such as absence of microvilli, irregular nucleus, nuclear condensation, nuclear chromatin concentration and edge accumulation, mitochondrial cavitation, rough endoplasmic reticulum swelling as well as lysosomal accumulation were observed in cells expressing TK-ES after GCV incubation. Hoechst 33258 staining further confirmed that cell apoptosis was indeed induced by TK-ES/GCV (Fig. 5C). Besides, compared to normal control, TK-ES/GCV resulted in a dramatical increase in percentage of early apoptotic cells (Empty vector, 3.8%; pTK-IRE-ES, 40.1%) as determined by Annexin V/PI double staining followed by flow cytometric analysis (Fig. 5D). These findings suggest that TK-ES/GCV induced apoptosis in C6 cells.

**Therapeutic effects of recombinant plasmids in C6 glioma rats**

After 6 days of C6 glioma cell inoculation, rats showed decreased water and food intake, showed poor response, signs of mental fatigue, and occasional seizure attack, with reduced strength in left limb muscle, and unsteady walking. Recombinant plasmid therapy (pIRES-ES, pTK – internal ribosome entry site(IRES)-ES transfected cells (pTK-IRE-ES) and pTK-IRE-ES transfected cells (pTK-IRE-ES)). (A) Tumor volumes measured by magnetic resonance imaging (MRI) at 7th and 22nd Days of post-C6 glioma cell inoculation. Recombinant plasmid therapy significantly inhibited tumor growth in C6 glioma rats. N = 5 for each experimental group. * indicates P < 0.05, ** indicates P < 0.01 compared with model control; # indicates P < 0.05 compared with either pIRES-ES or pTK-IRES. (B) Cumulative animal survival from Kaplan–Meier analysis was presented, and pTK-IRE-ES/Ganciclovir(GCV) therapy appeared to be most efficient in inhibiting tumor cell growth.
morphological alteration and necrosis in tumor cells, as compared to that in the other groups (Fig. 7A). In addition, pIRES-ES and pTK-IRES-ES/GCV therapy significantly reduced the MVD value of tumor tissues as compared to that of empty vector groups ($P < 0.01$) (Fig. 7B).

**Discussion**

The tumorigenesis and progression of malignant glioma involves complex mechanisms involving multiple genes. Combined gene therapy is a promising therapeutic approach to treatment of glioma [15]. IRES-based vector, which allows engineering of double- or triple-gene constructs from a single mRNA, have been widely investigated in preclinical and clinical studies [16]. In this study, we, for the first time, designed a double-gene dicistronic expression constructs by inserting a suicide gene (TK) and an antiangiogenic gene (ES) at the appropriate sites of pIRES vector. In a eukaryotic system, TK and ES get transcribed from a single promoter in the same cell, which avoids the interferences of gene transcription from different promoters. Besides IRES-based expression vector has many advantages over fused protein expression vectors, thus ensuring the sustenance of biological activities of TK and ES. Both RT-PCR and Western blotting revealed expression of TK and ES in both ECV304 and rat C6 glioma cells.

The specific cells in the tumor microenvironment, such as endothelial cells, play a pivotal role in providing oxygen and nutrients for the expansion of tumor mass [17, 18]. The crosstalk between glioma cells and host endothelial cells drives the progression of tumor [19, 20]. Considering the close association between tumor cell and endothelial cells, it is proposed that deregulation of angiogenesis may benefit therapeutic outcomes of anticancer therapy when used in combination with surgery, chemotherapy as well as radiotherapy [21]. ES is a potent angiogenesis inhibitor with low-toxicity and a promising potential for clinical use [22, 23]. ES has been shown to suppress the growth of glioma in animal models possibly by suppressing the development of tumor microvasculature [11, 12]. In accordance with these findings, we detected that ES reduced

![Figure 7. Histological analysis and microvessel density (MVD) values of rat tumor tissue by study group. (A) Histological examination of normal rat brain tissues, (B) tumors in model animals, (C) tumors in animals received pTK-IRES-ES treatment (D) MVD values of tumor tissues. **$P < 0.01$ compared with control group. $N = 4$ for each group. MVD, microvessel density. IRES, internal ribosome entry site.](image-url)
proliferation, inhibited migration and induced apoptosis of ECV304 cells. In addition, administration of plasmid expressing ES gene also restricted tumor growth, reduced MDV, and prolonged animal survival in C6 glioma bearing rats. The molecular mechanism by which ES inhibits endothelial cell migration and induces cell apoptosis remains unclear. ES may contribute to reduced migration by modulating several factors, such as by inhibiting expression of integrin, matrixmetalloproteases (MMPs), and cytoskeletal protein tropomyosin [24]. Besides, ES may trigger endothelial cell apoptosis through Shb adaptor protein-dependent tyrosine kinase signal transduction pathway [25]. However, the precise mechanism still needs to be investigated.

Despite the promising therapeutic value of antiangiogenic therapy in glioma patients, increased tumor metastasis is thought to result from an enhanced adaptive resistance [26]. Therefore, combined use of antiangiogenic gene therapy and gene therapy directly targeting glioma cells may improve the therapeutic outcomes. In this study, HSV-TK, the suicide gene most extensively tested in humans [27], was cloned into pIRES vector together with ES gene. To confer cytotoxicity, cells were transfected with plasmids encoding TK (TK alone or TK-ES), followed by GCV incubation. The efficacy of TK/GCV system depends largely on the proportion of proliferating cells, in which TK/GCV inhibits DNA replication and cell proliferation [28]. In our study, the percentage of TK-expressing cells at S phase was greatly increased after GCV exposure, whereas the proportion of cells at G2/M phase was significantly reduced. Moreover, cell apoptosis was dramatically enhanced in TK-expressing cells following GCV treatment. Hence, the antitumor activity of TK/GCV system might be related to its potency in causing S phase arrest and inducing cell apoptosis.

In our study, the in vivo results in rats with C6 glioma further confirmed the efficacy of combined gene therapy. We found that combined therapy greatly inhibited tumor growth, reduced MVD value in tumor tissues, improved disease symptoms and prolonged animal survival time as compared to single gene therapy. It is possible that TK-mediated tumor cell inhibition may benefit ES-modulated tumor microvasculature restriction, and thus lead to synergistic antitumor effects in C6 glioma rats. Our findings are consistent with the results of dual gene therapy for treatment of bladder cancer both in vitro and in vivo studies [29–31]. The combined therapy yielded a significantly increased antitumor activity against bladder cancer when compared with single gene therapy [31].

Our results demonstrate the superior antitumor activity of combined TK and ES gene therapy, mediated, possibly, by inhibition of both angiogenesis and tumor growth. Combined gene therapy could prove to be a valuable treatment modality for malignant glioma.

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Conflicts of interest
None declared.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Plasmid constructs.

Figure S2. Experimental procedures for animal studies. (A) Experimental procedure for animals in pTK-IREs-ES and pTK-IREs groups. (B) Experimental procedures for animals in control (PBS), vector control (pIRES), and pIRES-ES groups.