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Valproic acid enhances anti-tumor effect of mesenchymal stem cell mediated HSV-TK gene therapy in intracranial glioma

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ABSTRACT

Suicide gene therapy of glioma based on herpes simplex virus type I thymidine kinase (HSV-TK) and prodrug ganciclovir (GCV) suffers from the lack of efficacy in clinical trials, which is mostly due to low transduction efficacy and absence of bystander effect in tumor cells. Recently, stem cells as cellular delivery vehicles of prodrug converting gene has emerged as a new treatment strategy for malignant glioma. In this study, we evaluated the anti-glioma effect of suicide gene therapy using human bone marrow mesenchymal stem cells expressing HSV-TK (MSCs-TK) combined with valproic acid (VPA), which can upregulate the gap junction proteins and may enhance the bystander effect of suicide gene therapy. Expression of HSV-TK in MSCs was confirmed by RT-PCR analysis and the sensitivity of MSCs-TK to GCV was assessed. A bystander effect was observed in co-cultures of MSCs-TK and U87 glioma cells by GCV in a dose-dependent manner. VPA induced the expression of the gap junction proteins connexin (Cx) 43 and 26 in glioma cell and thereby enhanced the bystander effect in co-culture experiment. The enhanced bystander effect was inhibited by the gap junction inhibitor 18-β-glycyrrhetinic acid (18-GA). Moreover, the combined treatment with VPA and MSCs-TK synergistically enhanced apoptosis in glioma cells by caspase activation. In vivo efficacy experiments showed that combination treatment of MSCs-TK and VPA significantly inhibited tumor growth and prolonged the survival of glioma-bearing mice compared with single-treatment groups. In addition, TUNEL staining also demonstrated a significant increase in the number of apoptotic cells in the combination treated group compared with single-treatment groups. Taken together, these results provide the rational for designing novel experimental protocols to increase bystander killing effect against intracranial gliomas using MSCs-TK and VPA.

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1. Introduction

Malignant gliomas are the most common and aggressive primary intracranial tumors in adults. While conventional treatments including surgical resection, irradiation and chemotherapy may extend survival by weeks, the prognosis of malignant glioma patients is still poor [1].

A number of experimental treatments have been developed to improve the survival of malignant glioma patients [2–4]. Herpes simplex virus thymidine kinase gene (HSV-TK)/ganciclovir (GCV) gene therapy has been considered as one of the promising therapeutic strategies for malignant gliomas. The therapeutic potential is enhanced by the bystander effect that consists of the transfer of phosphorylated GCV as a cytotoxic metabolite from TK-expressing cells toward neighboring TK negative cells. Transfer of phosphorylated GCV requires cell contact and is mainly mediated through gap junctions. Connexin (Cx) 43 and 26, one member of Cx family, is considered as a gap junctions (GJ), which are formed of connexins [5].

Despite impressive results in experimental studies for malignant glioma, clinical trials using HSV-TK/GCV treatment have failed [6]. One of the main problems is the insufficient distribution of the gene therapy vehicles, mostly viruses, over the whole volume of the tumor. A second problem is that both the conversion of the prodrug into the cytotoxic compound and its delivery to neighboring tumor cells not expressing the suicide gene suffers from low efficiency. To overcome these problems, we used human bone marrow mesenchymal stem cells (BM-MSCs) as HSV-TK gene delivery vehicles with innate glioma targeting migration capabilities and valproic acid (VPA) to enhance gap junction communication in glioma cells.

VPA, an anti-epileptic drug and an anticancer drug, inhibits histone deacetylase (HDAC) and induces tumor cell differentiation,

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apoptosis, and growth arrest [7,8]. VPA has been examined as an HDAC inhibitor (HDACI) in preclinical and clinical trials for solid tumors and leukemias [9,10]. Recently, HDACI compounds like phenyl butyrate have been reported to modulate the gap junction component Cx43 expression and enhance gap junction communications in glioma cells. Furthermore, VPA enhanced tumor cell kill in adenovirus-HSV/TK mediated suicide gene therapy in a HNSCC xenograft mouse model [11].

This study examined for the first time the potential of VPA to increase the therapeutic efficiency of the bystander effect by enhancing gap junction communication in glioma cells, using combined therapy with MSCs-TK and VPA. Although further studies are needed to determine the exact mechanism underlying the expression of gap junction by VPA, these results suggest that the clinical therapeutic efficacy of MSC-mediated HSV-TK gene therapy for malignant glioma can be enhanced by combination with VPA.

2. Materials and methods

2.1. Stem cell culture and reagents

Human MSCs derived from the bone marrow were purchased from Lonza (Walkersville, Maryland, USA). The MSCs were subcultured at a concentration of 5×10^4 cells/cm^2 in MSC growth medium (Lonza) and used for experiments during passages 5–8. MSC growth medium was supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and supplement mix (Lonza). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. GCV was acquired from InvivoGen (San Diego, CA, USA). Gap junction inhibitor 18 beta-glycyrrhetinic acid (18-GA) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Adenoviral vectors and infection

The recombinant adenoviral vector encoding the gene for green fluorescent protein (Ad-GFP) and HSV-TK (Ad-HSV-TK) was constructed and produced using the Ad-Easy vector system, following the manufacturer's instructions (Quantum Biotechnologies, Carlsbad, CA, USA). MSCs were infected with 50 multiplicity of infection of Ad-GFP or Ad-HSV-TK.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared from MSCs using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using 2 μg total RNA and oligo (dT) primer and Superscript II polymerase for reverse transcription PCR (Invitrogen). PCR amplifications consisted of a total of 20 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min with a first denaturation at 94 °C for 7 min, and final extension at 72 °C for 7 min. Target primers sequences used were as follows: HSV-TK forward: 5′-CGCGAACATCTACACCACAC-3′, HSV-TK reverse: 5′-GTTATACAGGTCGCCGTTGG-3′ and GAPDH forward: 5′-TCCATGACAACT TT GGTATCG-3′, GAPDH reverse: 5′-TGTAGCCAAATT CGTTGTCA-3′.

2.4. Assessment of cell viability by MTT assay

To test the sensitivity to GCV, wild-type MSCs, MSCs-GFP or MSCs-TK were cultured in the MSC growth medium containing various concentrations of GCV (0.1–1000 μg/ml) and were incubated for 3 days. The viability of cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) assay. Briefly, MTT tetrazolium salt added to the cells and incubated for 30 min at 37 °C. The formazan dye formed by viable cells was solubilized in isopropanol. Aliquots of the solutions were transferred to 96-well microplates. The absorbance at 570 nm was measured with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For co-culture experiments, MSCs-GFP and MSCs-TK (1×10^4 cells) were plated in the Transwell inserts (0.4 μm pores ; Costar, NY, USA) containing various concentrations of GCV (0.1–1000 μg/ml) and then U87 cells (1×10^4 cells) were grown in the lower well of the Transwell plates. VPA and 18-GA were added to the lower wells. After 3 days, the viability of U87 cells in the lower well was also analyzed by MTT assay.

2.5. Western blotting

Connexin (Cx) 43 antibody (Cell Signaling Technology, Danvers, MA, USA), β-actin antibody (Sigma–Aldrich), and Cx 26 antibody (Abcam, Cambridge, MA, USA) were used for the analyses. Cells were rinsed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM dithiothreitol, and 50 μg/ml phenylmethanesulfonylfluoride). Insoluble material was removed by centrifugation at 12,000 rpm for 5 min at 4 °C. The proteins in the supernatant were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the resolved proteins were transferred to a nitrocellulose blot membrane. Each blot was blocked using TBS-0.05% Tween 20 containing 5% skim milk, incubated with the appropriate antibodies, and incubated with the secondary antibodies conjugated to horseradish peroxidase (HRP). The bands were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6. Animals and intracranial glioma model

Nude mice (6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) were used in accordance with institutional guidelines under the approved protocols. For the intracranial implantation of human glioma cells in the brain of mice, animals were stereotactically inoculated with 1×10^5 U87 cells (in 3 μl phosphate buffered saline, PBS) into the right frontal lobe (2 mm lateral and 1 mm anterior to bregma, at 2.5 mm depth from the skull base) using a Hamilton syringe (Hamilton Company, Reno, NV, USA) and a microinfusion pump (Harvard Apparatus, Holliston, MA, USA).

2.7. Animal tumor size and survival evaluation

Tumor size was determined as described previously [12]. Briefly, brains from mice given therapeutic treatment at day 35 after tumor inoculation were serially sectioned (18 μm-thick, obtained every 200 μm into the tumor) and then stained with hematoxylin and eosin (H&E). The section with the maximum tumor area was calculated via a computer using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). For survival experiments, intracranial glioma-bearing mice were randomly divided into four groups after tumor implantation and treated with intratumoral injections of saline (PBS), intraperitoneal injections of VPA (300 mg/kg, i.p), MSCs infected with Ad-HSV-TK (MSCs-TK) and combination therapy (MSCs-TK and VPA). GCV (50 mg/kg) was injected 1 day after MSCs transplantation and continued everyday for 7 days.

2.8. Histological evaluation of apoptosis by TUNEL assay

Mouse brains were perfused with PBS followed by 4% paraformaldehyde and postfixed overnight. Fixed brains were embedded, snap frozen in liquid nitrogen, and stored at −70 °C until use.

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