Peptide Micelle-Mediated Delivery of Tissue-Specific Suicide Gene and Combined Therapy with Avastin in a Glioblastoma Model

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ABSTRACT: Bevacizumab (Avastin) is an angiogenesis inhibitor used as a treatment for various cancers. In this study, the combination therapy of Avastin and glioblastoma-specific thymidine kinase gene [pEpo–NI2–SV–herpes simplex virus thymidine kinase(HSVtk)] was evaluated in a glioblastoma animal model. The R7L10 peptide was used as a gene carrier of pEpo–NI2–SV–HSVtk. Gel retardation assays confirmed that R7L10 formed stable complexes with pEpo–NI2–SV–HSVtk. R7L10 protected DNA from nuclease digestion. R7L10 had lower transfection efficiency than polyethylenimine (PEI; 25 kDa). However, the *in vitro* and *in vivo* toxicity assays showed that R7L10 had lower cytotoxicity than PEI, suggesting that R7L10 is safer than PEI. For the combination therapy, Avastin was injected intravenously and the pEpo–NI2–SV–HSVtk/R7L10 complexes were injected intratumorally in the glioblastoma animal model. Tumor growth was most effectively inhibited by the combination therapy of Avastin and the gene. The immunostaining results confirmed that the HSVtk genes were expressed in the groups with the pEpo–NI2–SV–HSVtk/R7L10 complex. The terminal deoxynucleotidyl transferase dUTP nick end labeling assay showed a higher level of apoptotic cells in the combination group than the pEpo–NI2–SV–HSVtk/R7L10 complex or Avastin group. In conclusion, the combination of Avastin and the glioblastoma-specific HSVtk gene has a higher antitumor effect than single therapy of Avastin or HSVtk after intratumoral administration in glioblastoma animal model. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:1461–1469, 2015

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INTRODUCTION

Glioblastoma multiforme (glioblastoma) is a highly aggressive primary brain tumor.[1] Surgery, chemotherapy, and radiotherapy are conventional treatments for glioblastoma. In spite of advances in the conventional therapies, the recurrence rate of glioblastoma is very high and the expected lifespan of patients is very short. This suggests that an improved therapeutic method with higher and longer therapeutic effect should be developed. Gene therapy has been developed as an alternative option for glioblastoma therapy.[1–4] Suicide genes such as the herpes simplex virus thymidine kinase (HSVtk) gene have been delivered using delivery vectors such as adenoviral vectors.[5,6] In addition to viral vectors, nonviral vectors have also been evaluated for the delivery of the HSVtk gene in glioblastoma animal models.[7,8] Nonviral vectors include cationic polymers, liposomes, and peptides.[9] Nonviral carriers are nonimmunogenic, less-toxic, easy-to-produce, easily modified, and repeatedly injectable. Because of these advantages, many nonviral vectors have been developed and evaluated in various disease models. Typical nonviral vectors are polyethylenimine (PEI; 25 kDa) and Lipofectamine. Although these vectors can deliver DNA into the cells *in vitro*, they have high toxicity, limiting their *in vivo* application.[10,11] To avoid high toxicity of the carriers, short amphiphilic peptide micelles were developed for gene delivery.[12–15] The R7L10 amphiphilic peptide forms micelles in aqueous solution. R7L10 is composed of a 7-arginine stretch and a 10-leucine stretch. The R7L10 peptide micelles have hydrophobic cores and positively charged surfaces. Thus, a hydrophobic drug can be loaded into the cores of the R7L10 peptide micelles. At the same time, DNA can be complexed with the positive surface of the micelles, forming the DNA/R7L10 complex. These peptide micelles are useful for delivery of hydrophobic drugs and therapeutic genes. One *in vitro* study showed that R7L10 delivered the luciferase gene into C6 glioblastoma cells more efficiently than poly-L-lysine.[15]

Gene therapy with the HSVtk gene has been under clinical trials, but researches have supported the idea that the combination of chemotherapy and gene therapy may improve the efficiency of glioblastoma therapy. In this study, we evaluated the combination therapy of Avastin and the glioma and hypoxia dual-specific HSVtk gene. Bevacizumab (Avastin) is an angiogenesis inhibitor that targets vascular endothelial growth factor-A.[16] Avastin has been used for various types of cancers, including colorectal, lung, and breast cancers. Recently, Avastin was approved for use in glioblastoma therapy in the United States.[17] The glioma and hypoxia dual-specific HSVtk, pEpo–NI2–SV–HSVtk, was previously developed by combining the erythropoietin (Epo) enhancer and the nestin intron 2 (NI2).[8] The Epo enhancer increased the transcription level in hypoxic tissue. Previously, the Epo enhancer was used to increase gene expression in ischemic myocardium and injured spinal cord.[18–20] Solid tumors such as glioblastoma have a hypoxic region in their core, because of lack of blood supply. The Epo enhancer responds to the low-oxygen concentration in the

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core of the tumors and enhances gene expression.[8] NI2 was reported to increase gene expression in neural stem cells or glioblastoma.[21–23] Combination of the Epo enhancer with NI2 increased gene expression in hypoxic glioblastoma cells, not in neural stem cells, as neural stem cells are not under hypoxic condition. Therefore, the dual-specific expression vector may be useful for the treatment of glioblastoma with specific enhancement of gene expression in hypoxic glioblastoma tissue. Our previous report showed that pEpo–NI2–SV–HSVtk induced gene expression specifically in hypoxic glioblastoma cells and enhanced the therapeutic efficacy of glioblastoma gene therapy.[8]

The current study has two purposes. First, the therapeutic effect of the pEpo–NI2–SV–HSVtk/R7L10 complex was evaluated *in vitro* and *in vivo*. The physical characteristics and transfection efficiency of the complex was evaluated through various assays. In addition, the toxicity and therapeutic efficacy of the pEpo–NI2–SV–HSVtk/R7L10 complex were measured *in vitro* and *in vivo*. Second, the combination therapy of Avastin and pEpo–NI2–SV–HSVtk/R7L10 complex was evaluated in the glioblastoma animal model. Avastin was administrated systemically and the pEpo–NI2–SV–HSVtk/R7L10 complex was administrated locally into the tumor. The tumor size measurement, immunohistochemistry (IHC), and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed to evaluate the therapeutic effect of the combination therapy.

MATERIALS AND METHODS

Synthesis of R7L10 and Preparation of the Plasmid DNA

R7L10 was chemically synthesized and purified by C18 reverse-phase chromatography by Peptron (Daejeon, Korea). R7L10 was amidated at the carboxyl terminus to form the amide group. Amidation of the carboxyl terminus increased the hydrophobicity of the leucine stretch. This peptide was dissolved in deionized water at 5 mg/mL and stored at −80°C.

The plasmid DNA, pEpo–NI2–SV–HSVtk, was previously constructed.[8] pEpo–NI2–SV–HSVtk and pβ-Luc were propagated in JM109 *Escherichia coli (E. coli)* and purified by the QIAGEN Plasmid Maxiprep Kit (QIAGEN, Valencia, California).

Gel Retardation Assay

A fixed amount of DNA (0.5 μg) was mixed with increasing amounts of R7L10 or PEI in deionized water. After 30 min of incubation at room temperature, the complexes were analyzed in a 1% (w/v) agarose gel with ethidium bromide.

Heparin Competition Assay

A fixed amount of DNA (0.5 μg) was mixed with gene carrier at 1:5 (DNA–R7L10) and 1:1 (DNA–PEI) weight ratios in deionized water, based on the *in vitro* transfection assay (R7L10) and the previous reports (PEI).[24,25] After 30 min of incubation, increasing amounts of heparin were added to the DNA/carrier complexes. The complexes were analyzed by a 1% (w/v) agarose gel with ethidium bromide.

DNase I Protection Assay

The DNA/carrier complexes were prepared at their optimal ratios. DNase? (5 units; Promega, Madison, Wisconsin) was added to the naked DNA (5 μg) or DNA/carrier complexes. The reaction mixtures were incubated for the indicated time period at 37°C. Fifty microliter of the reaction mixtures was taken at 0, 45, and 90 min after the incubation with DNase I, mixed with 50 μL of 2× stop solution (200 mM EDTA, 20 mM NaCl, 1% SDS, 0.5 mg/mL heparin), and incubated at 60°C overnight. Then, the samples were analyzed in 1% agarose gel and the position of DNA in the gel was visualized on a UV illuminator.

Cell Culture and Preparation of the DNA/Carrier Complexes

The C6 rat glioblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Seoul, Korea) containing 1% (v/v) penicillin and 10% (v/v) fetal bovine serum at 37°C in a 5% CO_2 incubator. For *in vitro* transfection assay, we optimized the ratio between DNA and R7L10 by preparing DNA/R7L10 complexes at various weight ratios. For comparison of the transfection efficiencies with other carriers, the DNA/R7L10 complexes were prepared at a 1:5 weight ratio (DNA–carrier). The DNA/PEI complexes were prepared at a 1:1 weight ratio, based on previous reports. The DNA/Lipofectamine complexes were prepared at a 1:5 (w/v) ratio, as recommended in the manufacturer's manual. All the complexes were prepared in DMEM at a concentration of 0.5 mg DNA/mL for *in vitro* transfection. The complexes were incubated for 30 min at room temperature for complex formation.

Luciferase Assay

The C6 cells were seeded at a density of 7.5×10^3 cells/well in 24-well plates and incubated 24 h at 37°C in a 5% CO_2 incubator. After incubation, the DNA/carrier complexes were added to the cells, and the cells were incubated with the transfection mixtures for 4 h. The amount of DNA was fixed at 0.5 μg/well. After transfection, the transfection mixtures were replaced with fresh media. After an additional 44 h, the cells were washed with phosphate-buffered saline (PBS) and lysed with reporter lysis buffer (Promega). The cell extracts were harvested and transferred to microcentrifuge tubes. The extracts were centrifuged at 12,000 g for 5 min to remove cell debris. Luciferase activities of the samples were measured using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Protein concentrations in the extracts were measured with a BCA assay kit (Pierce, Iselin, New Jersey). The values for the luciferase activity were reported as relative light units (RLU)/mg protein.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The C6 cells were seeded at a density of 7.5×10^3 cells/well in 24-well plates and incubated 24 h at 37°C in a 5% CO_2 incubator. After the incubation, the cells were transfected with the DNA/carrier complexes. The amount of DNA was fixed at 0.5 μg. Then, the transfection mixtures were replaced with fresh media. Ganciclovir (GCV; 50 μg/mL) was added to the cells and the cells were incubated for an additional 44 h at 37°C. After 44 h, 40 μL of MTT {[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]} solution (5 mg/mL) was added to each well. Next, the cells were incubated for an additional 4 h. The MTT-containing medium was removed and 750 μL of dimethyl sulfoxide was added to dissolve the formazan crystals formed by the live cells. The absorbance was measured at 570 nm using a microplate reader for cell viability. Cell viability

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