Biomaterials 32 (2011) 9766–9775

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Suicide gene therapy using reducible poly (oligo-D-arginine) for the treatment of spinal cord tumors

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ARTICLE INFO

Article history:
Received 1 July 2011
Accepted 31 August 2011
Available online 15 September 2011

Keywords:
Suicide gene therapy
Cancer gene therapy
Reducible polymer
Spinal cord tumor

ABSTRACT

Suicide gene therapy based on a combination of herpes simplex virus-thymidine kinase (HSV-tk) and ganciclovir (GCV) has obstacles to achieving a success in clinical use for the treatment of cancer due to inadequate thymidine kinase (TK) expression. The primary concern for improving anticancer efficacy of the suicide gene therapy is to develop an appropriate carrier that highly expresses TK *in vivo*. Despite great advances in the development of non-viral vectors, none has been used in cancer suicide gene therapy, not even in experimental challenge. Reducible poly (oligo-D-arginine) (rPOA), one of the effective non-viral carriers working *in vivo*, was chosen to deliver HSV-tk to spinal cord tumors which are appropriate targets for suicide gene therapy. Since the system exerts toxicity only in dividing cells, cells in the central nervous system, which are non-proliferative, are not sensitive to the toxic metabolites. In the present study, we demonstrated that the locomotor function of the model rat was maintained through the tumor suppression resulting from the tumor-selective suicide activity by co-administration of rPOA/HSV-tk and GCV. Thus, rPOA plays a crucial role in suicide gene therapy for cancer, and an rPOA/HSV-tk and GCV system could help promote *in vivo* trials of suicide gene therapy.

1. Introduction

Spinal cord tumors are caused by abnormal growth of tissue found inside the bony spinal column, which is a primary component of the central nervous system (CNS), and these tumors exert pressure on sensitive tissues and impair function, resulting in pain, sensory changes, and motor problems [1]. The first tests for the diagnosis of spinal cord tumors are neurological examinations and imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and position emission tomography (PET) [2]. Surgery, radiotherapy, and chemotherapy are the three most commonly used treatments. Gene therapy and immunotherapy are the most studied experimental treatments for future clinical trials [3]; among them, considerable attention has been focused on suicide gene therapy [4,5].

Suicide gene therapy based on gene-directed enzyme prodrug therapy (GDEPT), one of the promising alternatives to conventional chemotherapy, requires a non-toxic prodrug and tumor-specific transfection of a gene to produce a foreign enzyme that is necessary for activation of the prodrug to its cytotoxic form [6,7]. A combination of herpes simplex virus-thymidine kinase (HSV-tk) and ganciclovir (GCV) is the most well-known strategy among various suicide gene therapies [8,9]. HSV-tk and GCV are non-toxic to human cells when they exist in cells separately. When GCV is administered to cells that produce HSV-tk, thymidine kinase (TK) metabolizes GCV into a monophosphate form and cellular kinases continue to convert it to triphosphate GCV, which is a highly active form that induces an apoptotic pathway [10]. The active GCV leads to apoptosis through inhibition of DNA replication that occurs only in dividing cells, suggesting that this system selectively exerts toxicity in cancer cells because adult brain cells or neurons are usually non-proliferative [11]. In particular, cells in the CNS are known not to divide [12,13]; thus, brain or spinal cord tumors are appropriate targets for suicide gene therapy. In addition, non-transfected cancer cells adjacent to the HSV-tk expressing cells also die through a bystander effect that allows movement of the

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doi:10.1016/j.biomaterials.2011.08.089

active GCV from the transduced cells to the surrounding cells via gap junctions [14,15]. Once the active GCV moves through the gap junction, the adjacent dividing cells undergo apoptosis regardless of a lack of direct introduction of HSV-tk or GCV, but apoptosis does not occur in non-dividing cells because of the mechanism of action of the metabolites. Although suicide gene therapy using a combination of HSV-tk and GCV is a promising approach for the treatment of spinal cord tumors, there has been few study of this approach due, in part, to the absence of suitable gene carriers other than viral vectors.

A safe and efficient gene carrier is a prerequisite for successful anticancer therapy based on the HSV-tk/GCV combination; however, viral vectors are still widely used in suicide gene therapy for brain or spinal cord tumors [16,17]. Despite their high transfection efficiency, viral vectors may cause immune reactions or uncontrollable gene expression, through which the transfected gene is continuously expressed in the cells [18–20]; thereby, severe side effects may occur in the CNS. On the other hand, genes transfected by non-viral vectors are known to express the gene for a couple of days in dividing cells; therefore, the expression levels of the genes are expected to be quite low in normal CNS cells that do not divide. Attempts to develop sufficient non-viral carriers have focused on enhancing transfection efficiency *in vivo* because one of the disadvantages of the use of non-viral carriers is poor gene expression *in vivo*. In recent studies, reducible poly(oligo-D-arginine) (rPOA) showed strong gene expression after local administration *in vivo*, whereas branched polyethylenimine (bPEI; 25 kDa), a very effective cationic polymer used in gene delivery, was insufficient in *in vivo* experiments [21,22]. The rPOA composed of a Cys-9-arginine-Cys repeating unit includes an internal disulfide linkage that is reduced in reductive environments of cytoplasm. In particular, cytosolic glutathione (GSH) triggers reduction of the disulfide bond, through which the reducible polymer is converted to its counterpart, which does not condense nucleic acids as well as the oxidized form [23]. It is already known that transgene expression depends on the GSH level in the cytoplasm, and the GSH level is relatively high in cancer cells compared to normal cells [24,25]. In addition, rPOA further proved its ability to express the transgene in brain or spinal cord in a previous study that demonstrated rPOA was capable of expressing a therapeutic gene in brain tissue and treating hypoxia-ischemic brain injury through gene expression [26]. Consequently, as one of the effective non-viral carriers for *in vivo* gene delivery, rPOA is considered a powerful tool for the delivery of HSV-tk to spinal cord tumors that will result in high levels of gene expression and less toxicity to normal CNS cells.

In this study, suicide gene therapy based on a combination of HSV-tk and GCV was chosen for the treatment of spinal cord tumors, and rPOA was used as a non-viral carrier to deliver the HSV-tk. The anticancer effects of the system were confirmed in spinal cord and brain tumor cell lines, and the *in vitro* results were further extended to *in vivo* experiments in an animal model. C6 glioma cells were inoculated in the T5 regions of rats in order to develop a spinal cord tumor model, and tumor growth was confirmed using MRI. Finally, the anticancer effect and mechanism of action of suicide gene therapy based on HSV-tk and GCV were evaluated through observing locomotor function, measuring tumor volume, and performing TUNEL assays in the animal model.

2. Materials and methods

2.1. Materials

HSV-tk (pORF-HSV-tk) was obtained from Invivogen (San Diego, CA) and Cys-(D-R9)-Cys (CRRRRRRRRRC, Mw 1628) was purchased from Peptron (Daejeon, Korea). GCV and PEI (branched form, Mw 25 kDa) were obtained from Sigma–Aldrich Co. (St Louis, MO). Plasmid luciferase (pLuc, pGL3-promoter, 5010 bp) and the luciferase assay kit were from Promega (Madison, WI). N2A (neuroblastoma), C6 (glioma), U87 (glioma), F98 (glioma), and 9L (gliosarcoma) cells were purchased from American Type Culture Collection (Rockville, MD). Cyanine 5.5 (Cy5.5) was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were analytical grade.

2.2. Preparation of rPOA

The rPOA was prepared as previously described [21,22]. In brief, Cys-(D-R9)-Cys was polymerized into rPOA in PBS containing 30% DMSO at a final concentration of 57 mM. HEPES buffer was added to terminate the reaction after 6 days and the resulting peptide was purified and lyophilized.

2.3. HSV-tk condensation

Various amounts of rPOA and PEI were mixed with 1 μg of HSV-tk, and then the samples were incubated for 30 min at room temperature. After the incubation, the samples were electrophoresed on 0.8% (w/v) agarose gel for 30 min at 100 V in 0.5% TBE buffer solution. Ratios were expressed as N/P ratios of the cationic polymers to DNA for all data.

2.4. Size and zeta potential measurements

Five micrograms of HSV-tk were mixed with rPOA at N/P ratios of 7.0, 14.0, and 21.0 in 1 ml HEPES buffer. Dynamic light scattering was performed to measure the mean diameters and surface zeta potentials of the polyplexes using Zetasizer-Nano ZS (Malvern Instruments, Worcestershire, UK). The mean diameters and zeta potentials of the rPOA/HSV-tk polyplexes were measured three times in triplicate.

2.5. Stability of HSV-tk in serum

In order to see whether rPOA protects HSV-tk in serum, rPOA/HSV-tk and PEI/HSV-tk polyplexes were prepared at N/P ratios of 7.0 and 14.0. After 30 min of incubation, mouse serum was added to the polyplexes at a final concentration of 50% (v/v) and incubated for an additional 2 h at 37 °C and shaking at 150 rpm. After the reaction, heparin was added to the polyplexes in the presence of 10 mM EDTA. After 1 h, the mixtures were electrophoresed on 0.8% (w/v) agarose gel for 30 min at 100 V in 0.5% TBE buffer solution.

2.6. In vitro transfection efficiency and cytotoxicity

DMEM was chosen for C6 and N2A; RPMI 1640 was for U87; MEM was for F98 and 9L. The culture media contained 10% FBS and 1% penicillin-streptomycin, and cells were incubated at 37 °C under 5% CO2. C6, N2A, U87, 9L, and F98 cells were seeded on 24-well plates at a density of 4.0×10^4 cells/well. After 24 h of incubation, the culture media were replaced with 50 μl of plain media containing polyplexes prepared by mixing 1 μg pLuc and rPOA or PEI at an N/P ratio of 14.0. After one day, cells were washed with PBS and lysed with 150 μl reporter lysis buffer for 20 min. The cells were harvested and centrifuged for 3 min at 13,000 rpm. Luciferase RLU of the cells was measured using a 96-well plate luminometer, and the results were expressed as RLU/mg of cell protein determined by the DC protein assay kit (Bio-Rad, Hercules, CA) with a BSA standard. Cytotoxicities of rPOA/HSV-tk or PEI/HSV-tk polyplexes were examined using CCK-8 (Dojindo Laboratory, Tokyo, Japan) according to the protocol described above. In addition, cytotoxicity of GCV alone was investigated by adding various concentrations of GCV to the cells 4 h after cell seeding. After an additional 20 or 44 h of incubation, cell viability was determined using CCK-8 according to manufacturer's instructions.

2.7. In vitro anticancer efficacy of the GCV and rPOA/HSV-tk polyplex combination system

When cell confluence reached 60%, the culture media were replaced with 1 ml of plain media containing rPOA/HSV-tk or PEI/HSV-tk polyplexes. The polyplexes were prepared with 1 μg HSV-tk and rPOA at an N/P ratio of 14.0 or PEI at an N/P ratio of 7.0. After 24 h, N2A, C6, U87, 9L and F98 cells were seeded on 24-well plates at a density of 4.0×10^4 cells/well. After 4 h of incubation, the culture media were replaced with 100 μl of plain media containing various amounts of GCV. After 48 h incubation, cell viability was determined using CCK-8.

2.8. Spinal cord tumor model and in vivo anticancer study

The Animal Care and Use Committee of the Medical Research Institute of Yonsei University College of Medicine approved all of the protocols. All experiments were performed according to international guidelines on the ethical use of animals, and the number of animals used was minimized. The spinal cord tumor model was induced in adult male Sprague–Dawley rats (250–300 g; Orient Bio, Kyungki-do, Korea). After anesthesia (Zoletil, 50 mg/ml), laminectomy was performed at the T5 level and C6 glioma cells (1.0×10^5 cells in 5 μl PBS) were injected into the T5 position using a Hamilton syringe (Hamilton, Bonaduz, Switzerland). Five days after