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GENE DELIVERY

Hypoxia/hepatoma dual specific suicide gene expression plasmid delivery using bio-reducible polymer for hepatocellular carcinoma therapy

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ABSTRACT

Gene therapy is suggested as a promising alternative strategy of hepatocellular carcinoma (HCC, also called hepatoma) therapy. To achieve a successful and safe gene therapy, tight regulation of gene expression is required to minimize side-effects in normal tissues. In this study, we developed a novel hypoxia and hepatoma dual specific gene expression vector. The constructed vectors were transfected into various cell lines using bio-reducible polymer, PAM-ABP. First, pAFPS-Luc or pAFPL-Luc vector was constructed with the alpha-fectoprotein (AFP) promoter and enhancer for hepatoma tissue specific gene expression. Then, pEpo-AFPL-Luc was constructed by insertion of the erythropoietin (Epo) enhancer for hypoxic cancer specific gene expression. *In vitro* transfection assay showed that pEpo-AFPL-Luc transfected hepatoma cell increased gene expression under hypoxic condition. To confirm the therapeutic effect of dual specific vector, herpes simplex virus thymidine kinase (HSV-TK) gene was introduced for cancer cell killing. The pEpo-AFPL-TK was transfected into hepatoma cell lines in the presence of ganciclovir (GCV) pro-drug. Caspase-3/7, MTT and TUNEL assays elucidated that pEpo-AFPL-TK transfected cells showed significant increasing of death rate in hypoxic hepatoma cells compared to controls. Therefore, the hypoxia/hepatoma dual specific gene expression vector with the Epo enhancer and AFP promoter may be useful for hepatoma specific gene therapy.

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1. Introduction

Hepatoma is the most common type of a primary cancer of the liver which is mainly caused by viral hepatitis infection or liver cirrhosis. Currently, liver transplantation and conventional treatments such as surgical resection, chemotherapy and radiotherapy are counted as the best treatment options of hepatoma. However, the current therapies can be applied only to early stages of cancer cells, but not to chronic hepatitis or liver cirrhosis [1,2]. Moreover, recurrence of tumor after the surgical treatment also brings down survival rates of the patients [3,4]. For these reasons, hepatoma remains one of the most difficult tumors to cure. In order to overcome the limitation of current therapy methods, gene therapy has been suggested as a potential novel approach for hepatoma.

Suicide gene therapy is based on the introduction of a gene into tumor cells with pro-drug. Recently, herpes simplex virus thymidine kinase (HSV-TK) gene and cytosine deaminase (CD) gene are widely used for suicide genes. In HSV-TK and ganciclovir (GCV) pro-drug (TK/GCV) system, the TK gene is delivered and expressed in tumor cells, converting to the administrated GCV pro-drug into toxic phosphorylated form. The GCV inhibits DNA synthesis, resulting in killing of dividing cancer cells. In addition, the cancer cells are coupled by gap junctions, which enhance the cancer cell killing by the transfer of toxic GCV to neighboring cancer cells that do not express TK. This phenomenon was known as the "bystander effect" [5]. Therefore, the TK/GCV gene therapy is one of the powerful strategies for cancer gene therapy.

Although the prospective results were observed in many researches, further strategies are required to overcome the problems of current gene therapy such as toxicity and non-specific death of normal tissues [6]. Most of all, controllable therapeutic gene is an important factor to avoid side-effects in normal tissues [7]. For this purpose, several tissue-specific promoters have been developed and alpha-fectoprotein (AFP) gene promoter is being used for hepatoma targeting gene expression [8,9]. AFP is a main plasma protein of mammalian fetal serum, and produced during fetal development and immediately decreased after birth. Although, normal AFP gene is silent in adult liver, most of hepatoma cells re-express it and produce abundant amount of AFP. Because of this feature, increasing the levels of AFP in blood is widely used as an important marker for hepatoma diagnosis [10].

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Fibrogenesis of hepatoma chronic injury causes damaged blood system, which leads to the low blood supply to the liver and consequently leads to hypoxia. The hypoxic regions of hepatoma also induce high proliferation of tumor cells by expression of vascular endothelial growth factor (VEGF). This dual-aspect of hypoxia in hepatoma suggests that hypoxia regions are the critical targeting feature for hepatoma gene therapy [11]. Therefore, we hypothesize that the combination of regulatory elements for hepatoma tissues and cancer hypoxia may be useful for maximum gene expression with high specificity to hepatoma.

Additionally, delivery carrier is one part of non-viral gene therapy system along with therapeutic gene expression vector. Currently, more than 70% of approved clinical trials have used viral vectors for gene therapy [12]. Viral vectors have high gene delivery efficiency, but they have problems such as immunogenicity and safety. For these reasons, development of non-viral vectors was promoted as an alternative gene delivery carrier. Previously, an arginine-grafted poly(cystaminebisacrylamide-diaminohexane) (ABP) polymer was developed by our group. Because of the disulfide bonds, ABP can be biodegraded in the intracellular cytoplasm. The ABP showed higher transfection efficiency in comparison with polyethylenimine (25 kDa, PEI25K) with insignificant cytotoxicity [13]. Recently, to improve the ABP efficiency, new modified dendrimer type poly(amido amine) (PAMAM) conjugated ABP named "PAM-ABP" was developed [14].

In this study, a novel hypoxia/hepatoma dual specific gene expression vectors, pEpo-AFPL-Luc and pEpo-AFPL-TK, were constructed and delivered by bio-reducible non-toxic polymer, PAM-ABP. The transfection and gene expression efficiency of PAM-ABP/pEpo-AFPL-Luc system was evaluated by luciferase assay. In addition, therapeutic effect of dual specific vector was verified using TK gene for hepatoma gene therapy.

2. Materials and methods

2.1. Materials

Polyethylenimine (branched, 25 kDa, PEI), heparin and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), phosphate-buffered saline(PBS), lipofectamine 2000, gel extraction kit, total RNA isolation kit, high capacity cDNA reverse transcription kit with RNA inhibitor, DNA purification kit and *Pfx* DNA Polymerase were purchased from Invitrogen (Carlsbad, CA). BCA assay kit was purchased from pierce (Rockford, IL). Luciferase assay kit, 5× reporter lysis buffer, agarose, restriction enzymes, Caspase-Glo® 3/7 assay kit and DeadEnd™ Fluorometric TUNEL System were purchased from Promega (Madison, WI). Huh7 and HepG2 human hepatoma, 293 human embryonic kidney and A549 human lung cancer cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). PAM-ABP was previously synthesized [14].

2.2. Plasmid construction

pSV-Luc was purchased from Promega (Madison, WI). Cloning of the promoters was performed by PCR. An expression vector containing the human alpha-fectoprotein promoter and enhancer, pDRIVE5s-AFP-hAFP, was purchased from InvivoGen (San Diego, CA) and was used as a PCR template. The fragment coding that includes 0.3 kbp AFP promoter (AFPS) and 0.7 kbp AFP enhancer regulatory region with promoter (AFPL) were amplified by PCR using *Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA). The sequences of the primers were as follows: AFPL promoter; forward 5′-CCGCTCGAGATGATTCCCAAATGTCTATCTCTA-3′, backward 5′-CCCAAGCTTGGTTGCTAGTTATTTTGTTATTG-3′ and AFPS promoter; forward 5′-CCGCTCGAGGTTTGAGGAGAATATTTGTTAT-3′, backward 5′-CCCAAGCTTGGTTGCTAGTTATTTTGTTATTG-3′. For cloning convenience, the XhoI and Hind III sites were introduced to forward and backward primers as underlined. Purified promoters were inserted at the XhoI and Hind III site of pSV-Luc, which produced pAFPL-Luc or pAFPS-Luc, respectively. pEpo-SV-Luc was constructed previously [15]. The Epo enhancer was amplified by PCR. For cloning convenience, the NheI site was introduced to the primers. The sequences of the primers were as follows: forward (or backward) 5′-CTAGCTAGCGCGGAGTTAG GGGCGGGATGG-3′. The amplified Epo enhancer was inserted into the upstream of the AFPL promoter, produced pEpo-AFPL-Luc. pSV-TK was constructed previously [16]. pEpo-AFPL-TK was constructed by the insertion of the TK gene at the place of the luciferase gene of the pAFPL-Luc. The resulting vectors, pAFPL-Luc, pAFPS-Luc, pEpo-AFPL-Luc and pEpo-AFPL-TK were confirmed by restriction enzyme study and direct sequencing.

2.3. Complexes preparation

PAM-ABP and plasmid DNA (pDNA) complexes were prepared at various weight ratios by mixing fixed amount of pDNAs (1 μg/well) in PBS. PEI25K/pDNA or lipofectamine/pDNA complexes were prepared at a 1:1 or 2.5:1 weight ratio in PBS, respectively. The volume of mixture was fixed at 50 μl/well. The mixtures were incubated for 30 min at room temperature for complex formation. After incubation, the complexes were treated into each well.

2.4. Measurement of zeta-potential and complex size

PAM-ABP (1 μg/μl) and pAFPL-Luc (1 μg/μl) complexes were prepared at 1:1, 3, 5 and 7 weight ratios. PAM-ABP and a fixed amount of pAFPL-Luc (2 μg) were mixed in 100 μl PBS. After the 30 min incubation at room temperature, the complexes were dissolved in 700 μl H_2O and then particle sizes and zeta potentials were determined by the Zetasizer Nano ZS system (Malvern Instruments, UK). The size and zeta-potential were presented as the average values from 5 runs.

2.5. Gel retardation assay

PAM-ABP/pAFPL-Luc complexes were prepared at various weight ratios by mixing fixed amount of pAFPL-Luc (0.5 μg) with increasing amounts of PAM-ABP (0.5, 1, 1.5, 2, 3, 4, 5, and 6) in PBS. After 30 min incubation, the samples were electrophoresed through a 1% agarose gel containing SYBR gel staining solution for 40 min.

2.6. Serum stability assay

PAM-ABP/pAFPL-Luc or PEI25K/pAFPL-Luc complexes were prepared at a 5:1 or 1:1 weight ratio in PBS and incubated at room temperature for 30 min. After incubation, fetal bovine serum was added to the complexes at a 50% final concentration. Complexes were incubated in 37 °C shaking incubator for 0, 30, 60 and 90 min. To dissociate the pDNA from complexes, same volume of 600× heparin solution was added into the complex mixture for 300× final concentration in the presence of 0.01 mol/l EDTA. After 60 min of incubation with heparin, the complexes were analyzed on a 1% agarose gel electrophoresis [17].

2.7. Cell culture and transfection

Huh7 and HepG2 human hepatocellular liver carcinoma cells, A549 human lung cancer cell line and 293T human embryonic kidney cells were maintained in DMEM supplemented with 10% FBS. The cells were cultured at 37 °C in a 5% CO_2 incubator. For the transfection assays, the cells were seeded at a density of 1.0×10^5 cells/well in 12-well plates (Greiner Bio-one, NC). After 24 h incubation, the medium was replaced with fresh DMEM and then, the carrier/pDNA complexes were added to each well. After 4 h incubation, fresh DMEM containing 10% FBS and with or without GCV (100 μg/ml) was replaced. The cells were incubated for an additional 48 h under normoxia (20% oxygen) or hypoxia (1% oxygen) condition.

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