Prodrug-activating Gene Therapy with Rabbit Cytochrome P450 4B1/4-Ipomeanol or 2-Aminoanthracene System in Glioma Cells

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Abstract

Objective We determined the cytotoxic properties of cytochrome P450 4B1 (CYP4B1) activated 4-ipomeanol (4-ipo) and 2-aminoanthracene (2-AA) in rat glioma to verify the CYP4B1/4-ipo or 2-AA system for prodrug-activating gene therapy.

Methods The cyp4B1 cDNA was cloned into pcDNA3.1/Hygro from rabbit lung total RNA (pcDNA-cyp4B1). Lentiviral vector encoding firefly luciferase (fLuc) was infected into C6 (rat glioma), and the fLuc-expressing cell was selected (C6-L). After transfection with pcDNA-cyp4B1 vector into C6-L, the single clone expressing cyp4B1 gene was selected (C6-CL). Prodrug for various concentrations of 4-ipo or 2-AA was treated for 72 h and 96 h. The cell survival rate of C6-CL was determined using MTT assay and trypan-blue dye exclusion methods.

Results By RT-PCR analysis, fLuc and CYP4B1 expression was detected in C6-CL, but not in C6. MTT assay and trypan-blue dye exclusion showed that IC₅₀ of C6-CL was 0.3 mM and <0.01 mM after 4-ipo or 2-AA treatment at 96 h or 72 h exposure, respectively. Cell survivals of C6-

CL were more rapidly reduced after treatment with 4-ipo or 2-AA than those of C6-L cells. The cell survival rate with MTT and trypan-blue dye exclusion assay was well correlated with fLuc activity in C6-CL cells.

Conclusion CYP4B1-based prodrug-activating gene therapy may have the potential to treat glioma and the cytotoxic effects of CYP4B1 enzyme activated 4-ipo or 2-AA in C6, and could be clearly determined by bioluminescent activity in C6-CL.

Keywords Cytochrome P450 4B1 · Firefly luciferase · Glioma · Prodrug-activating gene therapy

Introduction

The conventional cancer therapy is chemotherapy, surgical resection and/or radiotherapy. Chemotherapy using cytotoxic drugs has some problems because of the lack of tumor selectivity resulting in toxicity to normal tissues. To enhance the tumor selectivity of cytotoxic drugs, prodrug-activating gene therapy is designed based on the transduction of tumor cells with suicide genes encoding for prodrug-activating enzymes that render target cells susceptible to prodrug treatment [1, 2]. The first comprises foreign enzymes of non-mammalian origin, with or without human homologues. Examples are viral thymidine kinase [3], bacterial cytosine deaminase (CD) [4], and purine nucleoside phosphorylase (PNP) [5]. The second category consists of enzymes of human origin that are absent or are expressed only at low concentrations in tumor cells, such as cytochrome P450 (CYP) and deoxycytidine kinase (dCK) [6]. The most commonly used enzyme gene is the herpes simplex virus type 1 thymidine kinase (HSV1-tk). HSV1-tk converts the nucleoside analog ganciclovir (GCV) into a

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toxic metabolite and allows selective elimination of HSV1-tk transduced cells both in vitro and in vivo. The CYP enzymes are a family of heme-containing proteins that are known to modify a wide variety of compounds [7]. Families 1–4 are found mainly in those tissues exposed to xenobiotic chemicals, liver, lung, and skin. Studies on species including mouse, rat, rabbit, hamster, and human show certain commonalities of CYP isoforms of the lung [8]. All species have isoforms of the subfamilies (1A, 2B, and 4B). In contrast, little is known about the rabbit cytochrome P450 4B1 gene (CYP4B1) as a prodrug-activating gene therapy system. This enzyme activates the prodrug 4-ipomeanol (4-ipo), a naturally occurring substituted furane, and the toxic metabolite is suggested to be a furane epoxide (Fig. 1) [9]. It can also convert aromatic amines, such as 2-aminoanthracene (2-AA), probably to highly reactive unsaturated dialdehyde intermediates [7]. For both activated drugs, DNA alkylation seems to be the main mechanism of cellular toxicity. The CYP4B1/4-ipo or CYP4B1/2-AA systems have already been studied as a gene therapy approach for tumors. Rainov et al. demonstrated that the CYP4B1/4-ipo or CYP4B1/2-AA suicidal gene therapy systems can generate a strong cytotoxicity for brain tumor cells in vitro and in vivo [10].

The aim of the study was to analyze and measure the cytotoxic effect of CYP4B1/4-ipo or CYP4B1/2-AA by bioluminescent activity in order to assess the therapeutic efficacy and usefulness of these systems for cancer therapy strategies.

Materials and methods

Cell line

Rat glioma cell line C6 was purchased from the American Type Culture Collection (ATCC). The cells were cultured in

Fig. 1 4-Ipomeanol and its relevant metabolite activated by CYP4B1. 4-Ipo is a furan derivative produced by the common sweet potato in the presence of typical mold, *Fusarium solani*. CYP4B1 activates 4-ipo, and the toxic metabolite is suggested to be a furane epoxide [9]

Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (WelGENE, Korea), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and antibiotics (100 units/ml penicillin G and 10 µg/ml streptomycin) (Invitrogen) at 37°C in a humidified 5% CO₂ atmosphere.

Cloning of rabbit CYP4B1 cDNA and construction of CYP4B1 expression vector

Total RNA was extracted from New Zealand white rabbit lung with total RNA Isolation using TRI reagent (MRC, USA). By using the CapFishing™ Full-length cDNA Premix Kit (Seegene, USA), the full length cDNAs was synthesized from the total RNA of rabbit lung. Synthetic oligonucleotide primers (5' primer, ATGCTCGGCTTCCTCTCCCGCCTG and 3' primer, CTACTTCTCAGCCTTGGGGCCCAGAGG) were designed based on the reported rabbit CYP4B1 cDNA sequence (GenBank accession no. AF332576). The coding region of rabbit CYP4B1 cDNA was amplified from the full-length cDNAs of rabbit lung using the CapFishing™ cDNA Isolation Kit (Seegene). The resulting PCR product was gel purified and cloned into *E. coli* cloning vector pMD18-T. The recombinant plasmid was transformed into *E. coli* DH5α, and the transformants were confirmed by DNA sequencing. To construct the CYP4B1-expressing vector in animal cells, pcDNA3.1/Hygro (Invitrogen) vector was chosen and digested with BamHI and XbaI. CYP4B1 gene was PCR amplified using primer set (Cyp4B1-S: ATAGGATCCATGCTCGGCTTCCTCTCC, Cyp4B1-A: ATATCTAGACTACTTCTCAGCCTTGGGG) and digested with BamHI and XbaI. The purified CYP4B1 product was ligated into pcDNA3.1/Hygro, and the expression construct was named pcDNA-cyp4B1.

Establishment of C6-fLuc (C6-L) using the lentiviral vector

One day prior to infection with lentiviral vector, C6 cells were seeded in six-well plates at a density of 5×10^5 cells/well. After overnight culture, the cells were pre-incubated with serum-free medium for 4 h. The cells were infected by exposing the cell monolayer to the lentivirus for 6 h in the presence of 8 µg/ml of Polybrene (Sigma, St Louis, MO) with gentle shaking every 30 min. After 6 h incubation, lentivirus-infected cells were added with complete media and grown overnight. After 24 h incubation in the complete media, the cells were plated to 1 cell/well and kept in a humidified incubator for 7 days. Then, C6-L was selected with high fLuc activity for following experiment (C6-L).

Establishment of C6-fLuc-CYP4B1 (C6-CL) using the plasmid transfection

pcDNA-cyp4B1 vector expressing CYP4B1 under CMV promoter was transfected into C6-L cells. C6-L cells were seeded in six-well plate at the concentration of 3×10^5 cells/well 1 day prior to plasmid transfection. After overnight culture, 4 μ g of DNA and 10 μ l of lipofectamine 2000 (Invitrogen) gently mix and exposed to monolayer cells according to manufacturer's recommendation. The CYP4B1-expressing cells were selected by treatment of 500 μ g/ml of Hygromycin (Sigma) (C6-CL).

Reverse transcription-PCR analysis for gene expression

Total RNA was prepared from cells using total RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's protocol. Purified RNA was used for a template of the one-step RT-PCR kit (Qiagen, USA) with CYP4B1 primer (Cyp4B1-S: CTTCCATTACGACGTGCTGA, Cyp4B1-AS: TCATGCACATGGTCAGGTAG) or fLuc primer (fLuc-S: CGCCTTGATTGACAAAGGATGG, fLuc-AS: GGCCTTTATGAGGATCTCTCT). The samples were then subjected to 15 min of denaturation at 94°C, 35 amplification cycles (30 s at 94°C, 30 s at 50°C, and 30 s at 72°C), and an additional 10 min at 72°C. β -Actin was amplified as a control using the same samples and same reaction condition with β -actin primers (actin-S: GTGGGGCGCCCCAGGCACCAGGGC, actin-AS: CTCCTTAATGTACGCACGATTTC). The amplified products were analyzed by 2% EtBr-stained agarose gel electrophoresis.

Prodrug-induced cytotoxicity

MTT assay

C6-CL cells were seeded into 96-well plates in triplicate at a density of 5×10^3 cells/well. After 24 h, cell culture media containing various concentrations of 4-ipo (Futurchem, Korea) or 2-AA (Sigma) ranging from 0 to 1 mM or 0 to 0.1 mM were added and incubated for 96 h or 72 h. The number of surviving cells was determined by MTT assay kit (Sigma). Briefly, for MTT 10 μ l of reagent was added to each well and incubated for 2 h at 37°C. When purple precipitate was clearly visible under the microscope, detergent reagent was added to all wells, and the plate was placed in the dark at room temperature for 2 h. The color development was measured and analyzed by ELISA reader (Bio-Rad) with a dual filter (595 nm and 655 nm).

Trypan-blue dye exclusion

C6-CL cells were seeded into six-well plates in triplicate at a density of 1×10^5 cells/well. After 24 h, cell culture media with various concentrations of 4-ipo or 2-AA ranging from 0 to 1 mM or 0 to 0.1 mM were added and incubated for 96 h or 72 h. The number of surviving cells was determined by cell count. Trypan-blue solution (Sigma) was mixed with cells, adding 20 μ l of trypan-blue solution to 20 μ l of cell suspension. The suspension was loaded into a C-Chip (Digital Bio., Korea) and scored with a microscope. Cells that stained blue were scored as nonviable.

Luciferase activity assay

C6-CL cells seeded into 24-well plates in duplicate at a concentration of 5×10^4 cells/well. After 24 h incubation, cell culture media containing various concentrations of 4-ipo ranging from 0 to 1 mM were added and incubated for 96 h. The cells were treated with 200 μ l of lysis buffer. A luciferase assay kit (Luciferase Assay Kit, Applied Biosystems) was used for the evaluation of luciferase activities by using a luminometer (Spectramax-L; Molecular Devices, Sunnyvale, CA).

Statistical analysis

Data are presented as means $\pm$ standard deviation. Statistical analyses were performed using an unpaired Student's t test.

Results

Generation of rat glioma cell expressing fLuc and CYP4B1 gene

To monitor the therapeutic intervention of CYP4B1/4-ipo or CYP4B1/2-AA with fLuc reporter gene activity, C6-CL clone expressing the CYP4B1 and fLuc gene was generated. C6 cells were infected with lentiviral vector expressing fLuc gene, and one clone among 80 clones was selected with the highest fLuc gene expression. C6-L was transfected with pcDNA-cyp4B1 expressing CYP4B1 under CMV promoter, and CYP4B1-expressing cell was selected by treatment with Hygromycin for 3 weeks (C6-CL).

Expression of CYP4B1 and fLuc gene in C6-CL

The gene expression of the fLuc and CYP4B1 in C6-CL cells was analyzed with RT-PCR. CYP4B1- and fLuc-specific primers were designed to produce PCR products with 650 and 399 bp, respectively (Fig. 2). The CYP4B1

Fig. 2 Identification of CYP4B1 and fLuc gene expression in C6-CL. Confirmation of CYP4B1 and fLuc gene expression in C6-CL cells by RT-PCR analysis. RT-PCR products of CYP4B1 and fLuc were 650 bp and 399 bp, respectively. The murine beta-actin product was 540 bp amplified PCR product as internal control. CYP4B1 and fLuc gene was expressed in C6-CL cells, but not in C6 by RT-PCR

and fLuc specific DNA band was detected in C6-CL, but not in C6.

Therapeutic efficacy of 4-ipo or 2-AA treatment in C6-CL cells

The survival rates of C6-CL and C6-L were determined by MTT assay (Fig. 3a) and trypan-blue dye exclusion assay (Fig. 3b). As shown in Fig. 3, the survival rates of

C6-CL cell was more rapidly decreased with increasing 4-ipo or 2-AA dose than that of C6-L. Dose/response evaluation for therapeutic efficacy of 2-AA or 4-ipo treatment for 72 h or 96 h in C6-L and C6-CL cells suggested that 2-AA may induce a stronger cytotoxicity than 4-ipo in C6-L as well as C6-CL. C6-CL cells were more sensitive to 4-ipo or 2-AA treatment than those of C6-L ($p < 0.005$ except for 2 mM 4-ipo treatment). MTT assay and trypan-blue dye exclusion showed that a 50% lethal dose (IC_{50}) of C6-CL was 0.3 mM and < 0.01 mM after 4-ipo or 2-AA treatment at 96 h or 72 h exposure, respectively.

Correlation of fLuc activity and prodrug sensitivity in C6-L and C6-CL

To investigate the possibility of determining the cell survival of C6-CL with fLuc activity, fLuc activity of C6-CL after treatment with 4-ipo was measured. A dose-dependent reduction in luciferase activity in C6-CL cells with 4-ipo were observed as compared with those of C6-L ($p < 0.005$, Fig. 4). Cell survival rates with MTT assay and trypan-blue dye exclusion were found to be well correlated with fLuc activity in C6-CL cells ($R^2 = 0.96$ for fLuc/dye exclusion, $R^2 = 0.91$ for fLuc/MTT) as shown in Fig. 5.

Fig. 3 2-AA or 4-ipo induced cytotoxicity in C6-CL. Dose-dependent cytotoxicity of 2-AA or 4-ipo was determined by MTT assay (a) and trypan-blue dye exclusion assay (b) in C6-CL cells. (a) Dose-dependent cell survival rate of C6-L (♦) and C6-CL (■) cells for different doses of 2-AA (left panel) or 4-ipo (right panel) by MTT assay. Cell survival rate was measured at 72 h after 2-AA treatment or 96 h after 4-ipo treatment for C6-CL cells and C6-L cells. (b) Dose-

dependent cell survival rate of C6-L (♦) and C6-CL (■) cells for different doses of 2-AA (left panel) or 4-ipo (right panel) by trypan-blue dye exclusion assay. C6-CL exhibited a higher sensitivity in 2-AA or 4-ipo treatment than those of C6-L. Survival rate (%) was calculated with relative produced formazan absorbance for MTT assay or viable cell number for dye exclusion assay of prodrug-treated C6-CL compared to that of prodrug non-treated C6-CL.

Fig. 4 fLuc activity in C6-L and C6-CL after treatment with 4-ipo. fLuc activity in C6-CL cells was dose-dependently reduced with increasing 4-ipo dose, but not in C6-L cells. The fLuc activity ratio (%) was calculated with relative fLuc activity of 4-ipo-treated C6-L or C6-CL cells compared to those of 4-ipo non-treated C6-L or C6-CL cells, respectively

Discussion

Rabbit CYP4B1 was considered as an ideal candidate for gene therapy because it bio-activates 4-ipo or 2-AA to cytotoxicity, and the equivalent human enzyme may have no activity against these compounds [10, 11]. Advantages of the CYP4B1/4-ipo or CYP4B1/2-AA system observed in the glioma model include the low concentrations of prodrug needed to efficiently kill tumor cells expressing CYP4B1, the relatively low toxicity of the prodrug 4-ipo in humans [12, 13], and the good penetration of the small lipophilic 4-ipo molecule through the blood-brain barrier [10]. Rainov et al. reported that the rabbit CYP4B1 gene transduced glioma cell was shown to exhibit high sensitivity to 2-AA and also reported a remarkable bystander effect in which nontransduced cells in close proximity to transduced cells are killed, enhancing the antitumor effects [10]. This system was applied to the treatment of hepatocellular carcinoma [14]. To radiation-induced cytotoxicity of CYP4B1/4-ipo, a CYP4B1 expression system under control of radiation-inducible EGR1 promoter was applied, and the relative survival fraction of EGR1-CYP4B1 transfected cells exhibited a decrease with increasing radiation dosage, whereas no effect was observed in control cells [15]. To monitor therapeutic transgene expression, fusion genes of

enhanced green fluorescent protein (EGFP) as a reporter gene with HSV-tk and CYP4B1 as therapeutic gene were developed. They also reported that the fluorescence intensity correlated well with the corresponding prodrug sensitivity induced therapeutic gene expression [16].

The objective of the work presented here was to investigate CYP4B1 as a prodrug-activating enzyme in glioma and fLuc gene as a reporter gene to facilitate and provide experimental work in the field of prodrug-activating gene therapy. We simultaneously expressed fLuc and CYP4B1 gene in C6 glioma, and 2-AA or 4-ipo sensitivity by CYP4B1 expression correlates well with fLuc activity in C6-CL (Fig. 5). Thus, determination of the bioluminescent signal may indicate the corresponding CYP4B1 enzyme activity. This observation may help to easily identify gliomas that are resistant to prodrug-activating gene therapy. We demonstrated that the CYP4B1/4-ipo prodrug-activating system effectively induces cell death of glioma cells at low prodrug concentrations (Fig. 3). To evaluate the potential of CYP4B1/4-ipo for prodrug-activating gene therapy of glioma, its therapeutic efficiency was monitored in vitro by bioluminescence when the fLuc gene was used as a bioluminescence reporter gene (Fig. 4).

Cell survival assays are important tools in oncological research and clinical practice to assess the tumor cell sensitivity to anticancer drug. Many assays have been developed to determine cell survival. The most simple test is the dye exclusion assay developed by Weisenthal et al. [17]. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes including trypan blue and eosin, whereas dead cells do not. After trypan blue dye treatment, the viable cell has a clear cytoplasm, but a nonviable cell has a blue cytoplasm in microscopic view. However, this method is still very labor-intensive and subject to observer error. These problems can be overcome by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay on the reduction of MTT to a formazan by viable, but not by nonviable cells [18]. These cell survival assays sometimes yielded different results in the same drug treatment because many different conditions

Fig. 5 Correlation of fLuc activity with cell survival rate in C6-CL cells determined by MTT (a) and dye-exclusion assay (b). Cell survival rate by MTT assay and dye-exclusion assay was compared with fLuc activity after treatment with 4-ipo. Cell survival rate by MTT or dye-exclusion assay was found to be well correlated with fLuc activity in C6-CL cells

have an influence on viability assay. Changes in metabolic activity can give rise to large changes in the reduced MTT amount that is produced by activity of some dehydrogenase enzymes in active mitochondria, while the number of viable cells is constant. We measured the cell survival in CYP4B1 transduced cells with 4-ipo or 4-AA with dye exclusion assay and MTT assay. As shown in Fig. 3, two types of cell survival tests were well correlated, also to bioluminescent assay (Fig. 5). A linear correlation of fLuc activity to cell survival rate was achieved, suggesting that light emission can be used for monitoring the cytotoxic effect of CYP4B1/2-AA or CYP4B1/4-ipo treatment.

In conclusion, we constructed CYP4B1 expressing vector (pcDNA-cyp4B1), C6-CL rat glioma cell expressing simultaneously firefly luciferase and CYP4B1 gene, and observed the expression of CYP4B1 and fLuc by RT-PCR. We also measured the cytotoxic effect of CYP4B1/4-ipo or CYP4B1/2-AA by bioluminescent activity in order to assess the therapeutic efficacy and usefulness of these systems for cancer therapy strategies. CYP4B1-based prodrug gene therapy using 2-AA or 4-ipo may have the potential to be used in glioma management.

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Conflicts of interest statement All authors have no conflicts of interest.

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