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Effects of CD/5-FC Suicide Gene Therapy System on Human Malignant Glioma Cells *in Vitro*

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Abstract The purpose of this paper is to investigate the antitumor effects of cytosine deaminase/5-fluorocytosine (CD/5-FC) suicide gene therapy system on human malignant glioma cells *in vitro*. The pCMVCD plasmid was constructed through the CD gene insertion in the multicloning site of eukaryotic expression vector pcDNA3.0, and confirmed by restriction endonuclease digestion/gene sequencing. The construct was subsequently transfected into the U251 human malignant glioma cells by using LipofectAMINE2000-mediated method. Resistant clones (named U251/CD cells) were isolated by screening with G418 presence. U251/CD cells were incubated with 5-FC in different concentrations to determine viability ratios (or cytotoxicity assay), measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The concentrations of 5-fluorouracil (5-FU) in the media were measured by high-performance liquid chromatography (HPLC) detector. Our results suggested that the untreated U251 cells were insensitive to 5-FC, with the IC50 about 6500 μmol/L. After transfection, the IC50 was dramatically reduced to about 10 μmol/L. Therefore, gene transfection made G418-resistant clones (U251/CD cells) be highly sensitive to 5-FC. HPLC analysis showed that 5-FU was detected in U251/CD cell medium. Study on U251 cells genetically modified by CD gene in vitro will play an essential role in glioma gene therapy *in vivo*. In conclusion, our results indicated that the CD/5-FC system was feasible to treat glioma.

Key words cytosine deaminase; 5-fluorocytosine; gene therapy; malignant glioma

Introducing a suicide gene into malignant tumor cells proposes an attractive approach for human gene therapy[1]. Suicide genes typically encode for nonmammalian enzymes that convert nontoxic prodrugs into highly toxic metabolites. Therefore, systemic application of the nontoxic prodrug results in the production of the active drug at the tumor site. Escherichia coli cytosine deaminase (CD), which is expressed in yeasts and bacteria but not in mammalian cells, converts the antifungal agent 5-fluorocytosine (5-FC) into the highly toxic 5-fluorouracil (5-FU)--a very common antitumor drug[2]. Consequently, mammalian cells transfected with CD gene induce growth inhibition and cell death after 5-FC administration of lower concentration. The use of CD/5-FC gene system in cancer therapy have been described by Huber et al. [2] and Yoshimura et al.[3].

Glioma, especially malignant glioma (eg. glioblastoma multiform), is the most common primary tumor in the central nervous system (CNS), carrying a poor prognosis. In addition, malignant gliomas frequently produce profound and progressive disability

and lead to death in most cases. Fewer than 5% of patients survive longer than 5 years after diagnosis[4]. Surgery, chemotherapy, radiotherapy, immunotherapy, and combined treatments are common modalities in each section[5]. With the development of molecular biology and accomplishment of human genome project, many kinds of therapeutic methods such as gene therapy[6] and antiangiogenesis therapy[7] are being intensively investigated in various laboratories of worldwide. The potentials of gene therapy include: suicide gene therapy, immunological gene therapy, synergistic gene therapies, and so on. Significant antitumor effects have been reported in suicide gene therapy in vitro and/or in vivo. In our laboratory, CD/5-FC suicide gene therapy system is used to treat human malignant glioma in vitro.

1 Materials and Methods

1.1 Materials

1.1.1 Bacteria and cell line Host bacteria Escherichia coli DH5α and U251 human glioblastoma multiform cell line (abbreviated U251 cells) were obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences.

1.1.2 Reagents Restriction endonucleases BamHI and NotI were purchased from New England BioLabs Inc (USA). pcDNA3.0 was from Invitrogen (USA). Lambda DNA / HindIII Marker was from Huamei Biological Company (China). E.Z.N.A. plasmid miniprep kit I was from Omega (USA). LipofectAMINE2000 and G418 were from Gibco/BRL (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Hyclone (USA). 5-FU, 5-FC, MTT were from Sigma (USA). The pCMVCD plasmid was constructed by Dr. Yoshimura I.

1.2 Methods

1.2.1 Cell culture The U251 human glioblastoma multiform cells were cultured in DMEM containing 10% FBS at 37 ° C and 5% CO2, passaged weekly.

1.2.2 Plasmid construction, amplification and purification The CD gene was subcloned into the multicloning site of eukaryotic expression vector pcDNA3.0 to generate the pCMVCD plasmid (finished by Dr. Ichiro Yoshimura, Japan). The plasmid was amplified in Escherichia coli DH5α, and purified using E.Z.N.A. plasmid miniprep kit I.

1.2.3 Plasmid identification and gene sequencing The pCMVCD plasmid was cleaved by restriction endonucleases Bam HI and NotI digestion, and identified by electrophoresis through 1% agarose-formaldehyde gels. The 1.5 kb inserted fragment was further confirmed by auto-sequencing. The primer was: 5′-GCA ATG CCG TAA TCC TG-3′.

1.2.4 Transfection of the U251 cells The pCMVCD plasmid was used to transfect the U251 cells in vitro using Lipofectamine2000 cationic lipid reagent[3]. Briefly, 4 μg of DNA were mixed with 10 μL of LipofectAMINE2000 reagent and added to the medium in 6-well dishes. After gene transfection, the U251 cells were screened by 200 mg/L with G418 presence. G418-resistant clones (named U251/CD cells) were isolated after 2—3 weeks.

1.2.5 Cytotoxicity assay (or cells viability ratios assay) The U251/CD and U251 cells were seeded into the 96-well dishes (2000 cells / well, 6 wells / group). The U251/CD and U251 cells were incubated with 5-FC or 5-FU of different concentrations next day. Cytotoxicity assay was measured by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 7 days of drug treatment. Absorbance at a wavelength of 490 nm was measured to calculate the percentage of live cells against the control group (more than 3 times of assays).

1.2.6 5-FU concentration in cell medium The U251/CD cells were seeded into 24-well dishes (200 000 cells / well, 6 wells / group). Mediums with 5-FC of different concentrations (100, 1000, 5000 μmol/L) were added into 24-well dishes. Mediums

were collected at 24, 48, 96, 192 h after 5-FC incubation. 5-FU concentrations in cell mediums were detected by high-performance liquid chromatography (HPLC) method at a wavelength of 270 nm. Meanwhile, the U251 cells were used to be control groups.

1.3 Statistical analysis

Data were expressed as x±s. Statistical analysis was performed with One-Way ANOVA followed by LSD s post hoc tests, which was provided by SPSS 10.0 statistical software. Statistical significance was accepted at the level of $P<0.05$.

2 Results

2.1 pCMVCD plasmid construction and identification

The eukaryotic expression plasmid pCMVCD was successfully constructed. Restriction endonucleases BamHI and NotI digestion showed that CD gene was correctly inserted into the multicloning site. The CD fragment and pcDNA3.0 vector were about 1.5 kb and 5.4 kb, respectively (Fig.1).

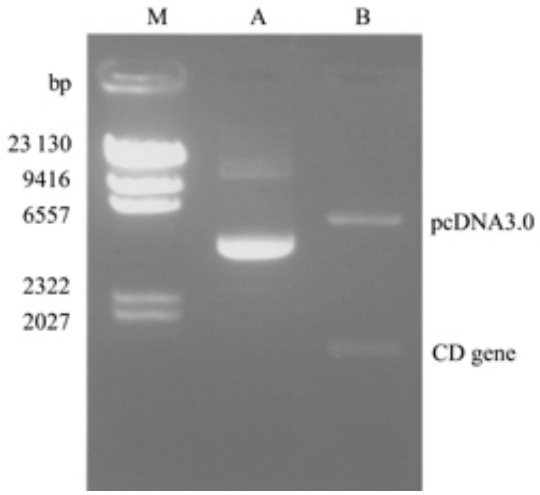

Fig.1 Plasmid identification by electrophoresis through 1% agarose-formaldehyde gels

M, Lambda DNA/HindIII marker; A, pCMVCD plasmid; B, fragments by BamHI and NotI digestion.

2.2 CD gene sequence

DNA sequencing data showed that the coding region of CD gene was 1284 bp, which was identical to the sequence published by Austin EA[8] in GenBank(document not shown).

2.3 Cell culture and gene transfection of U251 cells

The U251 cells were harvested in DMEM/10% FBS medium. The U251 cells could express CD gene using LipofectAMINE2000-mediated gene transfection. G418-resistant clones (named U251/CD cells) were obtained for later use after 2—3 weeks.

2.4 5-FC or 5-FU-induced growth inhibition and cell death in vitro

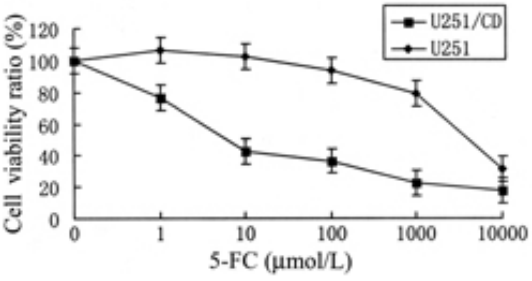

Fig.2 Effects of 5-FC administration on U251 and U251/CD cell viability ratios

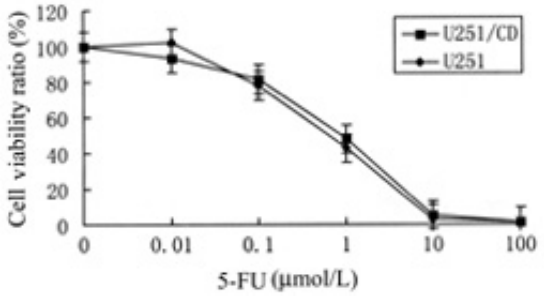

Fig.3 Effects of 5-FU administration on U251 and U251/CD cell viability ratios

Viability of the U251 cells at the 7th day after transfection with CD gene and administration of 5-FC were remarkably decreased from the MTT assay. As shown in Fig.2 and Fig.3, both U251 and U251/CD cells were highly sensitive to 5-FU, with IC50 (calculated using Bliss and Finney methods) less than 1 μmol/L. However, the U251 cells were insensitive to 5-FC, with the IC50 about 6500 μmol/L. After gene transfection, the IC50 was about 10 μmol/L. In addition, significantly morphological changes between U251 and U251/CD cells were observed after 1000 μmol/L 5-FC administration at the 7th day [Fig.4(A) and (B)]. Viability ratios of U251 and U251/CD cells were about 78.9% and 22.8%, respectively. The typical features of U251/CD cells were growth inhibition and cell death after 5-FC administration at lower concentration.

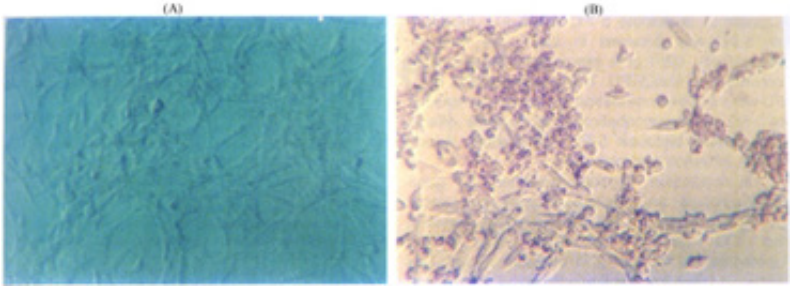

Fig.4 Morphological analysis by phase-contrast microscope (×100)
(A) The U251 cells without CD gene expression displayed well-growth at 1000 μmol/L 5-FC incubation after 7 days. The cell viability ratio was about 78.9% (MTT assay); (B) The U251/CD cells displayed growth inhibition and cell death at 1000 μmol/L 5-FC incubation after 7 days. The cell viability ratio was about 22.8% (MTT assay).

2.5 5-FU concentrations in cell medium

The data are listed in the Table 1. From the table, we found that 5-FU was detected in cell medium after different concentration of 5-FC incubation by HPLC analysis (Fig.5). The concentrations of 5-FU were correlation to the concentrations and the incubation time. No 5-FU was detected in U251 cell mediums after different concentration of 5-FC incubation.

Table 1 5-FU concentration in cell medium after 5-FC incubation (μmol/L)

Group (n=6)	Concentration			
	24 h	48 h	96 h	192 h
Control	0	0	0	0
100	2.3±0.2	5.3±0.5	6.9±0.8	6.5±0.2
1000	23.1±3.6	39.0±6.0	92.2±10.4	111.8±11.9
5000	86.8±12.2	172.9±22.7	552.9±50.0	1127.8±140.3

Data were expressed as x±s.

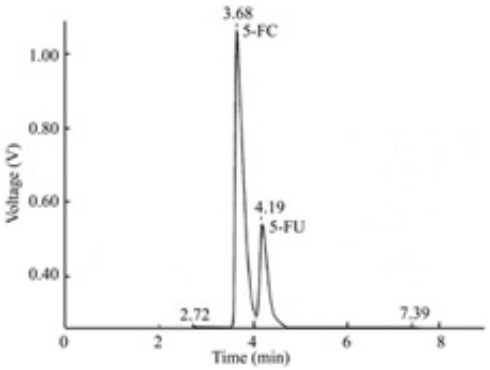

Fig.5 HPLC chromatogram of 5-FU concentration in the U251/CD cell medium after 5-FC incubation

3 Discussion

It is well known that 5-FU is an antimetabolite agent for cancer therapy in clinical trials. 5-FU exerts its toxic effect by interfering with DNA and protein synthesis due to substitution of uracil by 5-FU in RNA and inhibition of thymidilate synthetase by 5-fluorodeoxyuridine monophosphate, resulting in impaired DNA biosynthesis[9]. But the side effects are also reported, such as bone marrow inhibition and gastrointestinal effects, especially in longer 5-FU administration of high concentration. The aim of this study would be expressed as avoiding the side effects of 5-FU using gene transferring.

Herpes simplex virus thymidine kinase (HSV-tk)/ ganciclovir (GCV) system is widely used in suicide gene therapy[10]. The prodrug GCV is limited for lower penetration of blood-brain-barrier (BBB). The CD gene is another example of a suicide gene. Mammalian cells do not produce this enzyme, whereas a variety of bacteria and fungi do. CD enzyme has the ability to deaminate 5-FC to 5-FU. The pharmacokinetics of 5-FC and 5-FU are very clear to us. Unlike 5-FU, 5-FC is nontoxic to human being at therapeutic doses, and oral intake easier penetration of BBB. In our study, CD/5-FC system is used to treat malignant glioma. Penetration of BBB must be considered at first in CNS cancer therapy in vivo.

Many vector systems have been utilized to deliver target genes to target cells in many laboratories. In general, vector systems are categorized into two major groups:

viral vectors and non-viral vectors[11]. The present study was carried out using a non-viral vector, liposome-mediated transfection method. LipofectAMINE2000 is a proprietary formulation suitable for the transfection of nucleic acids into eukaryotic cells. The higher transfection efficiency can appear in many cell types and formats (eg. 6-well and 24-well dishes). DNA-LipofectAMINE2000 complexes can be added directly to cells in culture medium in presence or absence of serum. And it is a very convenient strategy to us to gene transfer. But it is less effective than the use of viral vectors, such as adenovirus (document not shown). In fact, it is safer to use in clinical applications and very rapid technical innovations in this field are occurring.

In this study, we demonstrated that transduction of CD gene into human malignant glioma made the U251 cells highly sensitively to 5-FC at lower concentration (10 μmol/L). The U251 cells without CD gene expression would induce growth inhibition and cell death at highly concentration (>6500 μmol/L). It was 650 times more than the U251/CD cells. In addition, significantly morphological changes between U251 and U251/CD cells were observed after 1000 μmol/L 5-FC administration at the 7th day. We found that the U251/CD cells appeared growth inhibition and cell death at 1000 μmol/L 5-FC by phase-contrast microscope. The MTT assay showed that cell viability ratios were 78.9% and 22.8%, respectively. But the U251 cells were harvested at the same concentration. Morphological analysis indicated that 5-FC-induced growth inhibition and cell death of the U251 cells should be aroused by gene transfection. In our study, bystander effect was also observed, which represented that only approximately 10% cells expressed CD gene could result in about 100% cells death in culture medium (in press).

HPLC analysis, a very common assay in pharmaceutical research[12], was used to determine the production of 5-FU in U251/CD cell medium after 5-FC incubation at different time. We observed significant differences in 5-FU production between U251 and U251/CD cell medium. Untransfected U251 cells were not capable to deaminate 5-FC into 5-FU, therefore no 5-FU was detected in U251 cell medium. Alternatively, U251 cells expressed CD gene could convert 5-FC into 5-FU. The concentrations of 5-FU were positive correlation to the concentrations of 5-FC and the incubation time. But their effects were limited because 5-FU itself would induce growth inhibition and cell death.

In conclusion, liposome-mediated CD gene transfection is an effective strategy for cancer gene therapy in vitro. We are planning to transfect the CD gene into nude mice using LipofectAMINE2000 agent in the near future. The relatively lower transfection efficiency of liposome and longer gene expression in vivo will be considered completely at first.

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