

아데노 바이러스 Cytosine Deaminase/Thymidine Kinase 융합 유전자의 항 종양효과*

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= Abstract =

Antitumor Effect of an Adenoviral Cytosine Deaminase/Thymidine Kinase Fusion Gene in C6 Glioma Cells

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Objective : We investigated the feasibility of a double suicide gene/prodrug therapy, involving direct introduction of the herpes simplex virus Type 1 thymidine kinase(TK) gene and the Escherichia coli cytosine deaminase(CD) gene, via a recombinant adenoviral vector and ganciclovir(GCV) and/or 5 - fluorocytosine(5 - FC) treatment, in C6 glioma cells.

Methods : Efficient gene transfer and transduction of C6 glioma cells via a recombinant adenovirus were evaluated by infecting cells with adenovirus bearing the - galactosidase gene and then staining cells with 5 - bromo - 4 - chloro - 3 - indolyl - 13 - D - galactoside. CD/TK expression in cells infected with adenovirus bearing the CD/TK gene(ad - CD/TK) was examined by immunoblotting analysis. For *in vitro* cytotoxicity experiments, the cells were infected with ad - CD/TK or ad - E1(as a control). After addition of a variety of concentrations of GCV and 5 - FU, either separately or in combination, cell viability was determined by staining the cells with crystal violet solution 6 days after infection.

Result : C6 glioma cells were efficiently transduced with recombinant adenoviral vector at multiplicities of infection of 200 or more. *In vitro* cytotoxicity of GCV and/or 5 - FC, either alone or in combination, was exclusively observed in the cells transduced with ad - CD/TK. Obvious cytotoxicity(>50% inhibition) was observed in the presence of 5 - FC at concentrations greater than 30ug/ml or GCV at concentrations greater than 0.3ug/ml at a multiplicity of infection of 100. Additionally, cytotoxicity in the presence of both GCV and 5 - FC was greater than that after single - prodrug treatments, indicating additive effects of the prodrug treatments.

Conclusion : The administration of a double - suicide gene/prodrug therapy might have great potential in the treatment of brain tumors.

KEY WORDS : Adenovirus · - Galactosidase · Glioma · Suicide gene therapy.

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가 5).

1998
: 1998 - 3)

가

Cytosine Deaminase/Thymidine Kinase

가²⁾¹¹⁾²⁶⁾³⁰⁾
 가 suicide gene HSV - 1 thymidine kinase
 midine kinase . HSV - 1 thymidine kinase
 ganciclovir(GCV)
 thymidine
 monophosphate thymidine
 cell kinase
 DNA
¹⁸⁾ suicide gene E.coli
 cytosine deaminase가 . cytosine deaminase 5 -
 fluorocytosine(5 - FC) 5 - fluorouracil
 (deamination) ¹⁾⁶⁾⁹⁾¹⁸⁾ 5 - Fluorouracil
 5 - fluorouridine triphosphate 5 - fluoro - deoxyuri-
 dine monophosphate . 5 - Fluorouridine triph
 osphate . 5 - Fluorodeo-
 xyridine monophosphate thymidine 가
 DNA
 . TK CD transduction ganciclovir
 5 - FC
¹¹⁾¹²⁾ 가
 (bystander effect) . transduction
⁸⁾²⁶⁾²⁸⁾
 Rogulski ²²⁾ CD/TK
 (retroviral vector)
 9L gliosarcoma suicide gene
 (CD/TK)/
 가 transduction CD, TK
 CD/TK
 rat C6 glioma multiple
 suicide gene .

대상 및 방법

1. 세포주 및 재조합 아데노 바이러스
 C6 glioma 293
⁵ American Type Culture Collection
 (Manassas, VA) . 10% heat - in-
 activated fetal bovine serum, 2 mmol/L L - glutamine

100U/ml penicillin/50ug/ml streptomycin Dul-
 becco 's modified Eagle 's medium 5% CO₂
 . 5~6 가 C6 glioma
 . 가 ad - CD/
 TK cloning sequence . CD/TK
 plasmid pWZLneoCDglyTK
 Bam HI EcoRI pWZL neoCDglyTK gene
 pcDNA3
 cloning (Invitrogen, Carlsbad,
 California). ad - CD/TK CMV
 , CD/TK , bovine growth hor-
 mone polyadenylation sequence
 Bgl , Pvu
 (homologous recombination) adenoviral
 shuttle vector p E1sp1A(Microbox, Toronto, Canada)
 Bgl EcoRV cloning p E1sp1A/
 CD/TK . plasmid p E1sp1A/CD/TK Ad -
 E1 homologous recombination
 E1 gene CD/TK gene cloning
 ad - CD/TK ³⁾
 (ad - E1) sh-
 uttle vector viral plasmid pJM17 293
¹²⁾¹³⁾ (ad - E1,
 ad - - galactosidase) shuttle vector
 (pCA14, p E1sp1A/ - galactosidase)
 .
 293 plaque
¹²⁾¹³⁾
 1) ad-CD/TK
 PWZLneoCDglyTK(plasmid) adenovirus E1
 gene p E1sp1A CD/TK gene
 adenovirus.
 2) ad-ΔE1
 adenovirus E1 가 ade-
 novirus ade-
 novirus¹²⁾¹³⁾.

3) pΔE1sp1A, PWZLneoCDglyTK
 plasmid PWZLneoCDglyTK p E1sp1A
 (homologous recombination) p
 E1sp1A/CD/TK plasmid ad - E1 ad -
 CD/TK .

4) Bgl II, EcoRV (restriction enzyme).

2. X-5-Bromo-4-chloro-3-indolyl-13-D-galactoside(X-Gal) staining
 - galactosidase X - Gal staining
 . ad - - galactosidase C6 glioma
 (1.0% formaldehyde, 0.2% glutaraldehyde in distilled water) 5~10
 phosphate - buffered saline(PBS) 2
 (0.4mg/ml X - Gal, 4mmol/L K₄Fe, 2mmol/L MgCl₂ in PBS, pH 7.2) 37 4~24
 - galactosidase C6 glioma cell 4 2~10
 phosphate - buffered saline 3 37 24
 (0.4mg/ml X - Gal, 4mmol/L K₄Fe and 2mmol/L MgCl₂ in PBS, pH 7.2 containing 0.02% Nonidet P - 40 and 0.01% sodium deoxycholate)

3. Immunoblotting analysis
 25cm² 2 x 10⁶ 1ml Dulbecco 's modified Eagle 's (multiplicity of infection, MOI) ad - E1 ad - CD/TK 3~4
 가 가 3~4
 phosphate buffered saline 300ul
 (lysis buffer, 50mmol/l N - 2 - hydroxy - ethylpiperazine - N ' - 2 - ethanesulfonic acid, pH 7.5, containing 0.15mol/L NaCl and 0.5% Nonidet p - 40)
 (30ug of lysate, 20ug of protein/ lane) (lysate)
 sodium dodecyl sulfate - polyacrylamide gel electrophoresis polyvinylidene fluoride membrane (RPN 303F ; Amersham, Arlington Heights, IL).
 TK(4C8 ; Dr. W.C. Summers, Yale University) - actin(Oncogene, Cambridge, MA)
 1~5ug

4. CD/TK 융합유전자와 관련된 세포독성의 실험연구
 C6 glioma 25cm² ad - E1 ad - CD/TK

ad - CD/TK 100 200 Dulbecco 's modified Eagle 's medium 3~4
 가 96 - well plate
 10, 20, 30, 40, 50, 100ug/ml 5 - FC 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1ug/ml GCV 가 가
 4
 50% methanol 1% crystal violet staining solution microplate reader 540nm

% cell survival = (absorbance for the experimental group - absorbance of the blank)/(absorbance for the control group - absorbance of the blank) x 100

5. 통계분석 (ANOVA)

±
 p<0.05
 결 과

1. C6 glioma 세포에서 재조합 아데노 바이러스의 감염 효율과 CD/TK 융합 유전자의 발현

C6 glioma
 ad - - galactosidase X - Gal - galactosidase
 Fig. 1 C6 MOI
 MOI 500 가
 가 plate
 MOI 1000
 Immunoblotting CD/TK
 C6 293
 ad - CD/TK TK
 immunoblotting

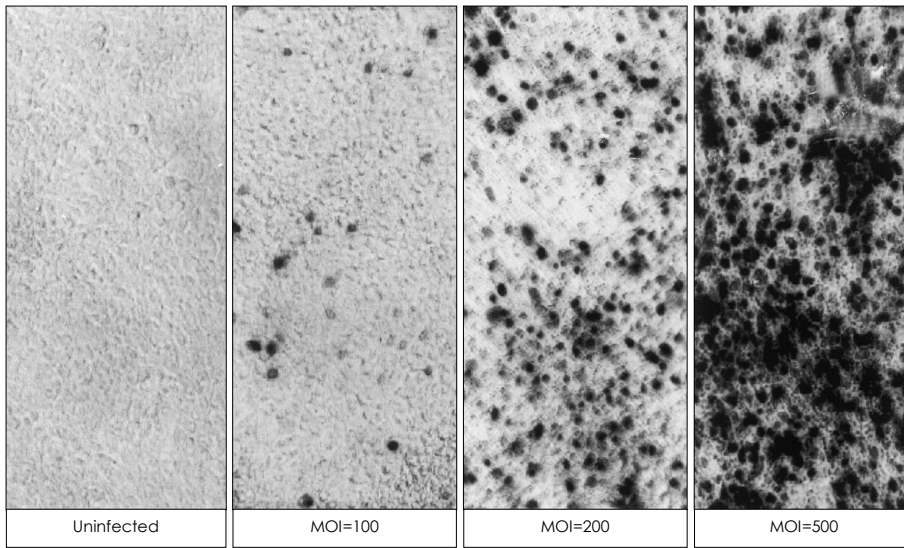


Fig. 1. The transduction efficiency of recombinant adenovirus on C6 glioma cells. Transduction efficiency was determined by examining expression of β -galactosidase (LacZ) after infecting the cells with ad- β -galactosidase. Dark staining represented successful β -galactosidase gene expression in the cells. The expression of β -galactosidase in cultured C6 glioma cells. The cells were infected with ad- β -galactosidase at MOI of 100, 200 or 500 and then stained with X-gal. Two days post-infection.

CD/TK
(Fig. 2). Fig. 2

ad - CD/TK

- actin

293

CD/TK 가

CD/TK가 가

C6 glioma CD/TK

(0.1 - 1 MOI 293 10 MOI C6) CD/TK

TK MOI 가

C6 glioma

CD/TK

(Fig. 2). 1000 MOI

100 MOI 가 500 MOI가

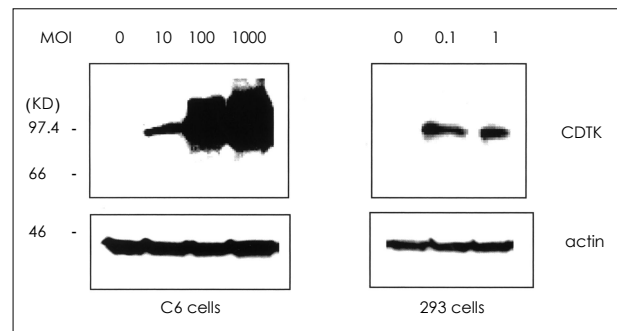


Fig. 2. The expression of CD/TK in C6 glioma or 293 cells. The cells were infected with ad-CD/TK at designated MOIs and the equal amount of proteins was subjected to 6% SDS-PAGE. The CD/TK fusion proteins were visualized by immunoblotting analysis with monoclonal antibody to TK. The position of fusion protein CD/TK was indicated by arrow.

2. ad-CD/TK 감염후 전구약물의 세포독성에 대한 실험 연구

10 MOI

CD/TK (5 -

FC, GCV)

C6 glioma MOI

100~200 ad - CD/TK ad - E1

5 - FC GCV 53 ± 8%

30ug/ml 5 - FC GCV

MOI 100 0.3ug/ml

GCV

MOI 200 5 - FC(10ug/ml) GCV(0.1mg/ml)

50ug/ml 5 - FC, 0.5ug/ml GCV

Fig. 3C

GCV

5 - FC

100 MOI 10ug/ml 5 - FC 0.1

ug/ml GCV (10

28 ± 10%가 가 .).

10ug/ml 5 - FC 0.1ug/ml GCV

53 ± 8% 가 가

MOI 5 - FC

(Fig. 3C).

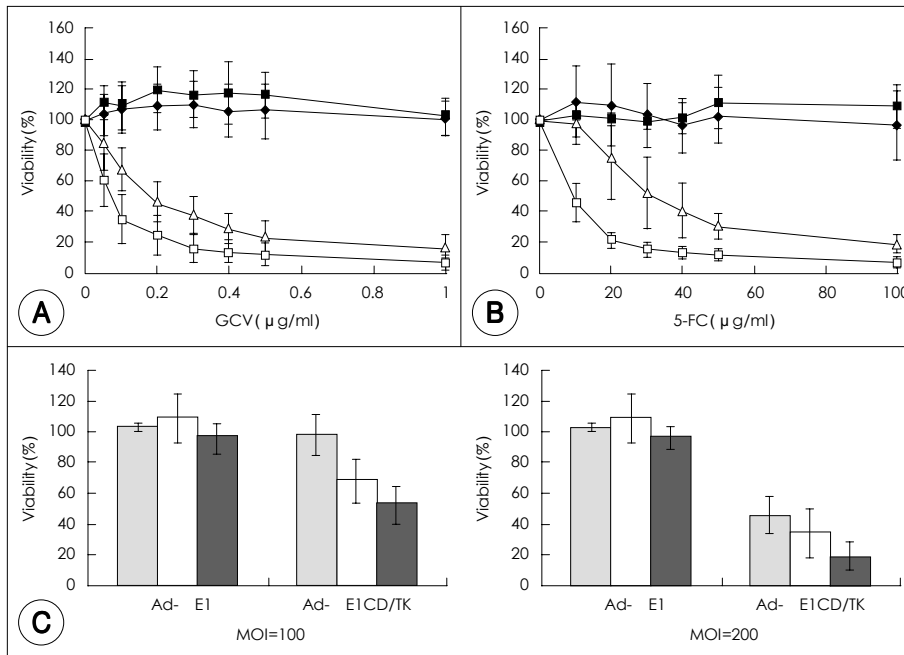


Fig. 3. *In vitro* cytotoxicity of ad-CD/TK combined with GCV or 5-FU treatment in C6 glioma cells. The cells infected with ad-CD/TK were exposed to GCV or 5-FU alone or in combined treatments after plating into 96 wells. The cell survival rate was determined by crystal violet staining six days after infection. The mean values of at least three independent experiments are represented and standard errors are represented by error bars. A: Sensitivity to GCV of the cells transduced with ad-CD/TK. The cells were infected with ad-CD/TK at MOI of 100 (□) or 200 (○). As a control group, the cells were mock-infected (○) or infected with ad-E1 at MOI of 200 (○). B: Sensitivity to 5-FU of the cells transduced with ad-CD/TK. The data were represented as Fig. 3A. C: Additive effect of GCV and 5-FU treatment. The cells were treated with 5-FU of 100 µg/ml (□), GCV of 0.1 µg/ml (○) or both (■) after transduction with ad-E1 or ad-CD/TK at MOI of 100 (left) or 200 (right).

고찰

HSV-1 TK/GCV가 CD/5-FU가 (bystander effect) (15)(20)(27)

CD/TK suicide gene가 (22)

CD/TK / 5-FU GCV CD/TK C6 glioma (Fig. 3)

suicide gene (CD/TK) Rogulski, HSV-1 TK CD (15) CD/TK C6가 GCV 5-FU가 (18) TK/GCV, CD/5-FU TK/ (8)(9)

5-FU human deoxycytidine kinase/cytosine arabinoside (8)(10)(19)(25) Nishihara (20)가

17)22)

CD/TK
C6 glioma

GCV

suicide gene

가

가

CD/TK
5 - FC/
가

CD/TK
rat C6 glioma
suicide gene

가

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