Analysis of the epigenetic factors for the transduction efficiency of hepatoma cell lines in gene therapy with recombinant adeno-associated virus

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Analysis of the epigenetic factors for the transduction efficiency of hepatoma cell lines in gene therapy with recombinant adeno-associated virus

Directed by Professor Sung Hoon Noh

A Master's Thesis Submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment the requirements for the degree of Master of Medical Science

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December 2002

This certifies that the master's thesis of

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December 2002

Acknowledgements

언제나 내 안에 계신 하나님께 감사 드립니다.

저를 학생으로 받아 주시고 늘 아낌없는 격려와 유머로써 이끌어 주신 노성훈 교수님께 깊은 감사를 드립니다. 5년 뒤의 자신의 모습을 생각하며 현재 상황에서 최선을 다하라고 말씀하신대로 늘 최선을 다하겠습니다.

부족한 저를 끝까지 인내하시며 참 과학자, 의료인의 모습과 사랑으로 이끌어 주신 최승호 교수님께 진심으로 머리 숙여 감사를 드립니다. 무엇보다도 이 논문의 모든 실험을 함에 있어 조언을 아끼지 아니하시며 관심과 열의로 지도해 주신데 대해 깊은 감사를 드립니다.

또한 부족한 저의 논문을 심사하며 조언을 해 주신 김주항 교수님께 특별히 감사를 드립니다. 아울러 본 연구기간 중에 도움을 주신 김경섭 교수님, 오덕재 교수님, 이우정 교수님, 형우진 선생님께도 감사를 드립니다.

또한 순홍, 정연, 정안, 경화, 허민규, 이면희, 증가교회 고등부 선생님께 미안한 마음과 감사의 마음을 드립니다. 특별히 담임으로써 부족한 저를 오히려 격려 해준 증가교회 사랑하는 우리 고등부 3학년 학생들, 관심과 사랑으로 기도 해 주셨던 모든 분께 감사를 드립니다. 그리고 조언을 아끼지 아니하시고 많은 도움을 주신 이재훈 선생님께 고마운 마음을 전합니다.

끝으로, 언제나 저를 위해 헌신적인 기도와 사랑으로 보살펴 주신 나의 사랑하는 부모님, 그리고 가족들에게 깊은 감사의 마음과 함께 이 논문을 바칩니다.

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Analysis of the epigenetic factors for the transduction efficiency of hepatoma cell lines in gene therapy with recombinant adeno-associated virus

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Gene therapy is one of the promising treatment modalities to overcome incurable diseases. However, there are many problems to be solved, such as transient expression or not sufficient level of therapeutic protein. rAAV has been widely used as a gene delivery

vehicle in gene therapy, not only because it is less likely to induce inflammatory or immune response which result in prolonged expression, but because it is effective at transducing non-dividing cells such as neurons, myocytes, and hepatocytes. The expression by AAV varies in different tissues and organs. The liver is the good factory for protein synthesis, so liver-targeted gene therapy would be more effective and rAAV can be used as a vehicle to transfer the target gene to the liver. Still, it is difficult to reach therapeutic range of target protein in liver-targeted gene therapy with rAAV.

It has been reported that DNA-damaging agents like UV irradiation, heparin, or topoisomerase inhibitor facilitate the transduction of organ with rAAV. These prompted us to study the epigenetic factors that affect the transduction efficiency of liver in rAAV gene therapy. We generated rAAV by transfecting 293 cells with three plasmids namely, pAAV-LacZ, which has ITR of wild type2 upstream of CMV promoter and LacZ gene, pAAV-RC, which allows the expression of cap gene, and pHelper, which is required for AAV replication. We then examined the possible effects of several chemicals, i.e., heparin, chloroquine, butyrate, hydroxyurea, 5-FU, etoposide, cisplatin or camptothecin on the

transduction of hepatoma cell lines after rAAV infection. Also, we investigated whether hepatoma cells infected with rAAV, after being exposed to gamma-irradiation, affects their transduction efficiency, and if there is effect when cells are treated with gamma-irradiation and various chemical agents.

We found that pre-treatment of hepatoma cell lines with permissive growth medium or 4mM of hydroxyurea increased the transduction efficiencies of rAAV significantly ($p<0.05$). There was no decrease in the viability of cells in the concentration of 4mM of hydroxyurea or permissive medium. However, heparin, sodium butyrate and chloroquine did not affect the transduction efficiency of hepa1c1c7 cell lines with rAAV. Still more, high dose of hydroxyurea (100mM) or chloroquine (50μM) showed the cytotoxicity and induced the downregulation of Lac-Z expression. Pretreatment of hepatoma cell lines with camptothecin, etoposide, 5-FU or cisplatin increased the transduction efficiency of rAAV-LacZ and etoposide, one of topoisomerase inhibitor, was the most effective for increasing the transduction of hepatoma cell lines with rAAV. When the cells were exposed with γ-irradiation and infected with AAV-LacZ alone, the effect was similar to that

obtained with permissive growth medium. The results of combinations with chemotherapeutic agents (i.e., 1 μM of etoposide, 3 μM of 5-FU, 1 μM of cisplatin, 30 μM of camptothecin) and γ-irradiation were confusing. Combinations of chemotherapeutic agents(1 μM of etoposide, 3 μM of 5-FU, 1 μM of cisplatin, 30 μM of camptothecin) and γ-irradiation increased the transduction efficiency of H4IIE cells significantly. ($p<0.05$)

In contrast, combination of permissive medium and γ-irradiation or 3 μM of etoposide and γ-irradiation in hepa1c1c7 cells, and combination of permissive medium and γ-irradiation in H4IIE cells showed the downregulation of LacZ expression with cytotoxicity.($p<0.05$) Taken together, the preteatment of hepatoma cell lines with some chemicals such as hydroxyurea, chemotherapeutic drugs such as camptothecin, etoposide, 5-FU, cisplatin, and γ-irradiation resulted in higher transduction efficiencies in hepatoma cell lines compared to no pretreatment. Some combinations of γ-irradiation and chemotherapeutic drugs increased transduction efficiency in hepatoma cell lines by rAAV-LacZ. However, most combinations of chemotherapeutic agent and γ-irradiation induced the downregulation of LacZ expression.

These suggested us that chemotherapeutic agents or γ-irradiation might increase the effect of rAAV gene therapy, but the circumstances that show the cell cytotoxicity induce the down regulation of target gene expression in hepatoma cell lines with rAAV gene therapy.

Key Word : transduction, rAAV, β-galactosidase, liver, gene therapy

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I. Introduction

Currently, the majority of the approved clinical trials of gene therapy involve cancer patients even though the initial goal of gene therapy was to replace missing gene products in inherited diseases.[1]

Identification of genetic abnormalities that cause the development of malignant tumors presents an opportunity to use such genes as therapeutic targets. Most clinical gene therapy protocols use viral vectors for gene transfer, while only a minority uses non-viral delivery systems, such as plasmid DNA alone or in association with carrier proteins or liposomes. The most commonly used viral vectors such as retrovirus or adenovirus have disadvantages which is pathogenic to human and transient expression. The non-viral vectors are easier to produce, have low immunogenicity, and capable of delivering large gene-expression cassettes. Although they are safer than recombinant viruses, their gene transfer efficiency is much lower and the transgene expression is transient. AAV is a single-strand, non-enveloped DNA virus with a 4.7-kb genome.[2-4] The wild-type AAV is not pathogenic to human and has a broad range of infectivity. The AAV type 2 receptor is a membrane-associated heparin sulfate proteoglycan, which is present in many cell surfaces, thus explaining the broad host infectivity of this virus.[3, 5-9] However, the level of therapeutic protein is not frequently sufficient in AAV gene therapy.

The liver is a huge factory for protein synthesis and the liver-

targeted gene therapy is one of good strategies. AAV vectors infect non-dividing cells, which extends their usefulness to the treatment of slow growing tumors or CNS disease, although the rate of transduction of non-dividing cells is much lower than that of dividing cells in culture.[1, 11, 16-19] The liver is mostly consisted of non-dividing cell, so in liver-targeted gene therapy rAAV is effective vehicle to deliver the target gene. As mentioned above, for practical issues, the transduction efficiency should be increased in liver-targeted gene therapy with AAV.

The AAV genome consists of two open reading frames flanked by inverted terminal repeats.[10-12] All viral-encoded genes, approximately 96% of the viral genome, can be replaced with foreign DNA of choice and packaged into an AAV virion. The cis-acting AAV ITR's do not appear to contain dominant enhancer/promoter activity. The expression of the transgene is determined by the transcriptional regulatory elements inserted in the expression cassette.[13-15] AAV integrates into the host genome and is transmitted to the progeny of the cells. The virus is incapable of autonomous replication and spread. A productive infection requires co-infection of a non-AAV helper virus(adenovirus or

herpes virus), which rescues the integrated AAV sequences, which can replicate and cause a lytic infection. A variety of genotoxic stimuli, such as heat shock, hydroxyurea, UV light and irradiation are known as the stimulator of transduction efficiency of AAV.[10-13] AAV vectors infect non-dividing cells, which extends their usefulness to the treatment of slow growing tumors or CNS disease, although the rate of transduction of non-dividing cells is much lower than that of dividing cells in culture.[1,11,16-19] In addition, agents that affect DNA metabolism, including irradiation and topoisomerase inhibitors, improve the AAV transduction of both dividing and non-dividing cells.[5, 20, 21] However, the study for hepatoma cell lines are rare and the systemic research that compare several agents are limited.

In this report, we tested several agents in an attempt to increase transduction efficiencies of AAV in hepatoma cell lines.

II. Materials and methods

1. Cells and culture conditions

Mouse hepatoma cell line Hepa1c1c7 (ATCC CRL 2026), rat hepatoma cell line H4IIE (ATCC CRL 1548), human fibrosarcoma cell line HT1080 (ATCC CRL12011) and 293-EBNA cells (transformed human embryonic kidney, ATCC R620-07) were grown in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Gaithersburg, MD, USA) with 10% heat inactivated (30min at 56℃) fetal bovine serum (FBS), 2mM L-glutamine, 100units/ml penicillin, and 100mg/ml streptomycin at 37℃ in 5% CO_2. 293-EBNA cell line was maintained in medium containing Geneticin (G418, 250 μg/ml).

2. Vector production

The production of rAAV was followed by manual of the AAV Helper-free system (Stratagene, La Jolla, CA, USA). pAAV-LacZ contains lacZ report gene under control of CMV promoter. The rep

and cap proteins were provided by pHelper and pAAV-RC. 1ug of each plasmid DNA was transformed by XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA, USA) and purified with Qiagen plasmid kit (Qiagen, Valencia, CA, USA). The recombinant expression plasmid (pAAV-LacZ) was co-transfected into 293-EBNA cells with pHelper (carrying adenovirus-derived genes), and pAAV-RC (carrying capsid genes) which together supplied all of the trans-acting factors required for AAV replication and packaging in 293-EBNA cells.

A. Transient transfection

293-EBNA cells used in this experiment were maintained as monolayer cultures and grown in appropriate media. Plasmid DNAs were purified using Qiagen plasmid kit (Qiagen, Valencia, CA, USA). Cells were plated in 10cm tissue culture dishes at a density of 5 X 10^6cells/plate. After 20 hours attachment period, transfections were performed with lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's

instructions. Briefly, 16μg of each plasmid was diluted with 1ml of Opti-MEM I Medium(Life Technologies, Gaithersburg, MD, USA) lacking serum.. 40μl of lipofectamine 2000 reagent was diluted 1ml of Opti-MEM I Medium and then incubated for 5 min at room temperature. The diluted DNA and the diluted lipofectamine 2000 reagent were mixed and incubated at room temperature for 20 min to allow the DNA-lipofectamine 2000 reagent complexes to form. Monolayer of 293-EBNA cells grown to 90% confluence in 10cm culture dish were co-transfected with the DNA- lipofectamine 2000 reagent complexes (2ml) directly to each dish and mixed gently by rocking the plate back and forth. Cells were incubated at 37℃ in 5% CO_2 incubator for 66-72hrs.

B. rAAV production

At 66-72hrs after transfection, the transfected cells plus DMEM growth medium were transferred to a 50ml conical tube. To collect the cells from the plate, the cells scraped into the pool of growth medium with a cell lifter while the plate was held at an

angle. The cell suspensions were then subjected to four rounds of freeze/thaw by alternating the tubes between a dry ice-ethanol bath and a 37℃ water bath, and vortexing briefly after each thaw. Cell debris was collected by centrifugation at 10000 X g of Sorvall RC 5C Plus, SS-34 Rotor for 10 min at room temperature.

3. Titration of rAAV-LacZ

HT1080 cell line was purchased from ATCC and maintained in DMEM, supplemented with 10%(v/v) FBS, 100units/ml penicillin, 100mg/ml streptomycin, and 2mM L-glutamine. To determine viral titers, HT1080 cells were plated in 12-well tissue culture plates at a density of 1.5 X 10^5 cells/well in 1ml of DMEM growth medium. After 16hrs incubation, 0.2ml of AAV permissive medium was added per well, without removing the original medium and incubation continued at 37℃ in an incubator for 5-6 hours. The medium was then aspirated and cells were washed with 1ml of pre-warmed L-DMEM. Viral stocks were diluted in 2ml volumes over a 10-fold series from $10^{-1}$to 10^{-4} in L-DMEM. The plates were

incubated at 37℃ for 1-2hours, 0.5ml of pre-warmed H-DMEM was added and cells reincubated at 37℃ for 40-48 hours. Then, β-galactosidase expression in infected cells were analysed with X-gal staining. Blue-stained cells were counted and the number of transduction unit per ml of stock calculated.

4. Transduction of hepatoma cell lines with rAAV-LacZ

Hepa1c1c7 (ATCC CRL 2026), H4IIE (ATCC CRL1548) cells were plated in 24-well tissue culture plates at a density of 1.5 X 10^5 cells/well in 1ml of medium. After 16h incubation, 0.2ml of AAV permissive medium or chemical treatments per well were added, without removing the original medium and mixed well by swirling, and the plates were incubated at 37℃ for 5-6h.

The medium was removed the medium and the cells washed once with 1ml of pre-warmed L-DMEM (2%FBS, 2mM L-glutamine). 100μℓ of viral stocks (1.6 X 10^6 transduction units) in FACS-gal assay and 100μℓ of 2,000 dilution of stocks (0.8 X 10^3 transduction units) in X-gal staining were mixed L-DMEM, of

which total volume were 400μℓ, and added to the cells, which were then incubated at 37℃ for 10-2h in an incubator. The plates were swirled gently at 30min intervals during incubation and 0.5ml of pre-warmed H-DMEM (18%FBS, 2mM L-glutamine) was added to each well, incubation was continued for 40-48h at 37℃. After 48 hours incubation, β-galactosidase expression was evaluated.

5. Chemical agents or γ-irradiation treatments of hepatoma cells

Chemical stocks solutions of hydroxyurea (1M) and sodium butyrate (0.5M) in phosphate buffered saline were stored at –20℃, and heparin 50mg/ml in distilled water was stored at 4℃. Stocks solutions were diluted into DMEM for use in the experiments. Anti-cancer agents such as etoposide (20mg/ml), camptothecin (20mg/ml), 5-FU (50mg/ml), and cisplatin (0.5mg/ml) were stored at 4℃. The AAV permissive growth media contained 10% (v/v) heat-inactivated FBS, 2mML-glutamine, 240mM hydroxyurea (HU) and 6mM sodium butyrate. Chemical treatments were incubated for 6-

7h. After treatments, cultures were washed twice with DMEM. rAAV-LacZ was added for transduction, and the cells were reincubated for 48h at 37℃.

Hepatoma cells were exposed to γ-irradiation at 40Gy. Immediately after γ-irradiation, the cells were infected with rAAV-LacZ

6. Analysis of β-galactosidase expression with X-gal staining

Histochemical staining for β-galactosidase expression was performed in cells. Briefly, cells were washed once with PBS, fixed in 0.5% glutaldehyde and stained with 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM magnesium chloride, 10% dimethyl sulfoxide, 1mg of 5-bromo-4-chloro-3-indolyl-D- β-galactopyranoside (X-gal) per ml in PBS at 37℃ overnight. β-galactosidase positive cells were determined by counting the number of blue cells under an inverted microscope.

7. FACS-gal assay

FACS-gal(FACS Calibur, Becton Drive, Franklin Lakes, NJ, USA), a highly sensitive method, was used to detect the rate and intensity of β-galactosidase expression in hepatoma cell lines. Cells were harvested from exponentially growing cultures prior to staining. Cell suspensions were resuspended in 4-ml FACS tubes in staining media (PBS containing 0.1M HEPES, PH 7.3, 4%FBS) and were incubated in a water bath at 37℃ for 10 min. Twenty-five micro liters of 2mM fluorescein-di-β-galactosidase (FDG) (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells with rapidly and thoroughly mixing. Cells were then incubated at 37℃ for 1min. Cells were kept on ice before performing the FACS analysis. In these experiments, we calculated the optical density of 520nm(OD_{520}) by analyzing fluorescence inside visible cells due to the hydrolysis of FDG by the β-galactosidase expressed in the cells.

8. Statistics

All transduction studies were carried out using three separate experiments. Data are expressed as means ± S.D. Statistical analysis was carried out using Microsoft Excel (Microsoft, Redmond, WA, USA) and Student's T-test.

The transduction efficiency of rAAV-LacZ alone was defined as 1. The relative transduction efficiency was calculated as the number of blue cells after pretreatment followed by rAAV-LacZ infection divided by the number of that in rAAV-LacZ alone without pretreatment.

III. Results

1. Titration of rAAV-LacZ vectors

Primary AAV stocks were prepared for the biological titers of rAAV vectors expressing the reporter gene beta-galactosidase (rAAV-LacZ). Titers of infectious rAAV-LacZ particles were determined as follows: HT1080 cells were transduced with serial dilutions of rAAV-LacZ. 48 hours later, cells were assayed LacZ activity by X-gal staining. As a result, rAAV vectors were well prepared and staining was dependent on virus dose. As shown in Fig. 1, a 10 fold series of rAAV-LacZ was diluted from 10 to 10^4. Blue stained cells were counted and the number of viral particles per ml of stock was calculated. Infectious rAAV-LacZ titers were 1.6 X 10^7/ml transduction units (Fig. 1).

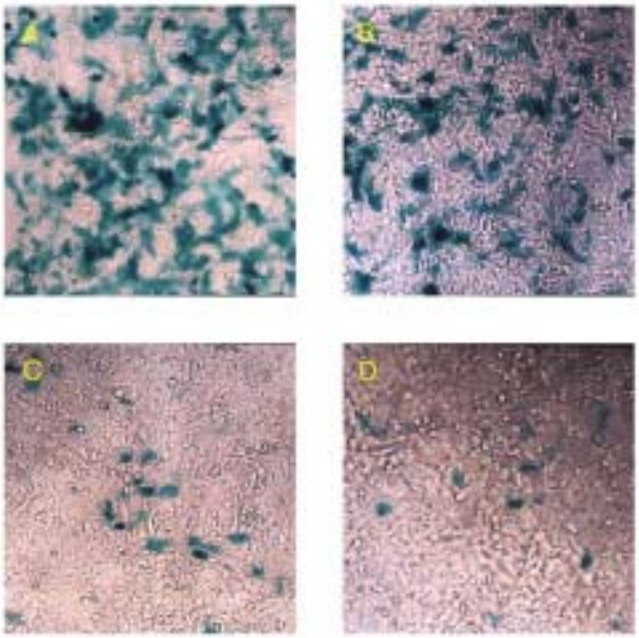

Fig. 1. Titration of rAAV-LacZ. HT1080 cells were plated at a density of 1.5 X 10^5 per well in 1ml of DMEM in 24well tissue culture plates. After 16 hours of incubation, 0.2ml of Permissive Growth Medium was added without removing the original medium. The plate was reincubated for 5-6 hours, and the medium aspirated, viral stocks were diluted over 10-fold series from 10 to 10^4. The plate was incubated again at 37°C for 40-48 hours, cells were fixed and stained using the β- galactosidase staining assay. Blue stained cells in the wells were counted and the number of stained cells per ml of stock was calculated. A : 10 fold, B : 10^2 fold , C: 10^3 fold, and D : 10^4 fold diluted stock.

2. The effects of the transduction of hepatoma cells by only rAAV-LacZ and permissive growth medium

Hepatoma cell lines (Hepa1c1c7, H4IIE) were employed to study transduction efficiency by rAAV vector. Hepatoma cells were plated at a density of 1.5 X 10^5 per well in 1ml of DMEM in 24well tissue culture plates. After incubation for 16 hours, 0.2ml of Permissive Growth Medium was added without removing the original medium. The plates were incubated for 5-6 hours and then 100 μl of adjusted viral particles were infected. After 40-48 hours of incubation, to quantify lacZ gene expression accurately, FACS-gal analysis and X-gal staining were conducted(Fig. 2). Very low transduction efficiencies were observed in these hepatoma cells infected with rAAV-LacZ alone. In hepa1c1c7 cell line and H4IIE cell line, 2.0 ± 0.5 blue cells per 24well plate were counted by Lac-Z staining in rAAV-LacZ alone group. Also, FACS-gal assay in rAAV-LacZ alone group showed that OD_{520} was 12.0 ± 0.6 in Hepa1c1c7 cell line and 11.1 ± 1.4 in H4IIE cell line. In contrast, combined infection with permissive growth medium produced a dramatic increased in rAAV transduction($p<0.05$). Pretreatment

with permissive medium demonstrated 462.0 ± 11.1 of OD_{520} in Hepa1c1c7 cell line, 462.4 ±10.7 in H4IIE cell line by FACS analysis, and 66.5 ± 5.2 in Hepa1c1c7, 66.5 ±3.8 in H4IIE cell line by X-gal staining.

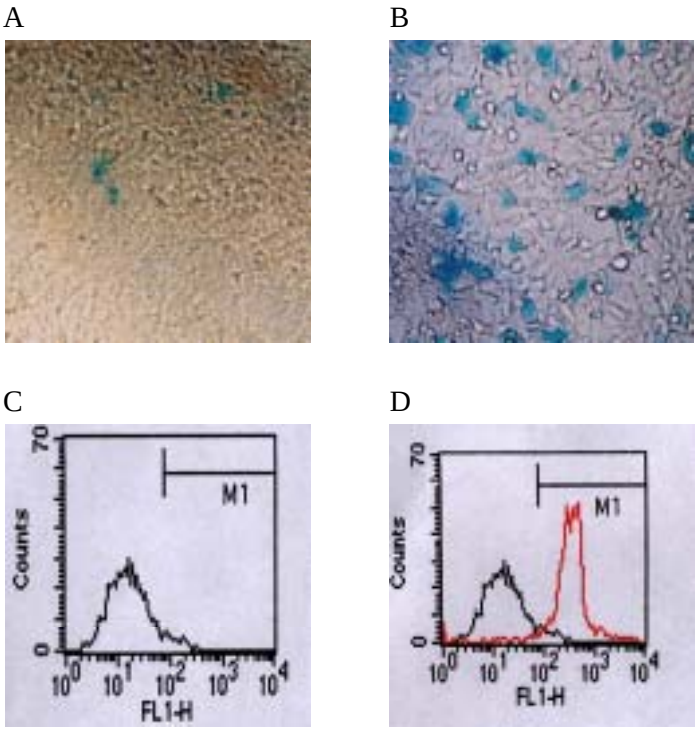

Fig. 2. Transduction of hepatoma cells by rAAV-LacZ alone and permissive growth medium. Hepatoma cell lines were infected to study transduction efficiency by rAAV vector. Cells were plated at a density of 1.5 X 10^5 per well in 1ml of DMEM in 24well tissue culture plates. After 16 hours of incubation, 0.2ml of Permissive Growth Medium was added without removing the original medium. The plates were incubated for 5-6 hours. X-gal staining (A, B) and FACS-gal assay (C,D) were then performed. A and C: Hepatoma cell lines infected with rAAV-LacZ alone. B and D: Hepatoma cell lines pre-treated with permissive growth medium and subsequently infected with rAAV-LacZ.

3. The effects of hydroxyurea on transduction by AAV vectors.

Transduction efficiencies were measured in hepatoma cell cultures after 6 hours exposure to hydroxyurea (HU). At the concentration of 4mM of hydroxyurea , OD_{520} was 480.0 ± 12.8 in Hepa1c1c7 by FACS-gal assay (Fig. 3A), and the number of blue stained cells was 102.0 ± 11.2 by X-gal staining (Fig. 3B) in Hepa1c1c7. The relative transduction efficiencies of hepatoma cells were increased to levels higher than those of the permissive growth medium. There was no decrease of the cell viability in the concentration of 4mM but high dose of HU (100mM) induced cytotoxicity (Fig. 3B). Unexpectedly, at the concentration of 100mM of HU, LacZ expression was markedly decreased ($p<0.05$). the number of blue stained cells was 27.4 ± 3.7 by X-gal staining and OD_{520} was 47.1 ± 2.9 by FACS-gal assay in Hepa1c1c7. These data suggest that high dose of HU induce the down regulation of LacZ expression.

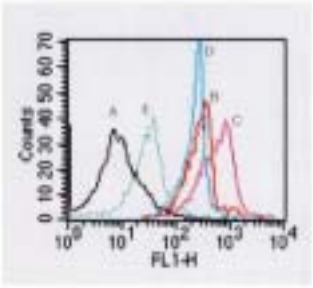

Fig. 3A. The effects of hydroxyurea on transduction by FACS-gal assay. Hepatoma cells were exposured with (A) AAV-LacZ alone, (B) permissive growth medium, (C) 4mM of hydroxyurea, (D) 10mM of hydroxyurea, (E) 40mM of hydroxyurea, and (F) 100mM of hydroxyurea. After incubation for 6 hours, rAAV-LacZ was added. 48hours later a FACS-gal assay was performed.

A B

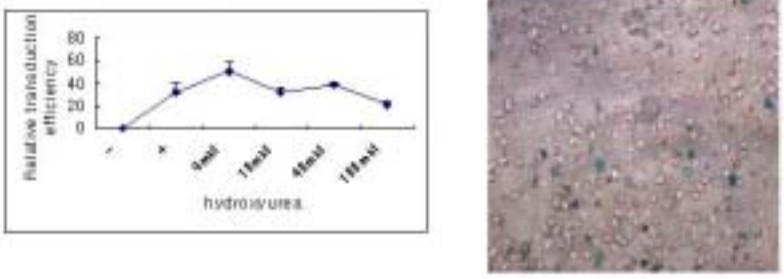

Fig. 3B. The effects of hydroxyurea on transduction by X-gal staining. (A) Hepatoma cell lines were exposed with (-) AAV-LacZ alone, (+)permissive growth medium and 4-100mM of hydroxyurea. After 6 hours incubation, rAAV-LacZ were infected. 48hours later, X-gal staining was performed. (B) Hepatoma cell lines were exposed with 100mM high dose of hydroxyurea, incubated for 48 hours, and then X-gal stained. Cytotoxic effects were seen and the relative transduction efficiency was decreased. The relative transduction efficiency of rAAV-LacZ alone was defined as 1. The relative transduction efficiency was defined as the number of rAAV-LacZ per 100ul of vector stock produced after each drug treatment divided by the number in rAAV-LacZ alone. Results are the means ± S. D. of three independent experiments performed in triplicate.

4. The effects of heparin, sodium butyrate and chloroquine on transduction of hepatoma cell lines by rAAV.

Hepa1c1c7 cell lines were treated with various chemical agents such as heparin, sodium butyrate, and chloroquine for 6 hours and then infected with rAAV-LacZ (Fig. 4). Pretreatment with 250 μg/ml of heparin, 100 μM of sodium butyrate, and 25 μM of chloroquine showed one or two blue cells per 24 well in X-gal staining. In these concentrations of each chemical, there were no effects on cell viability. High concentration (50 μM) of chloroquine was observed cytotoxicity and the number of blue cell were very scanty.

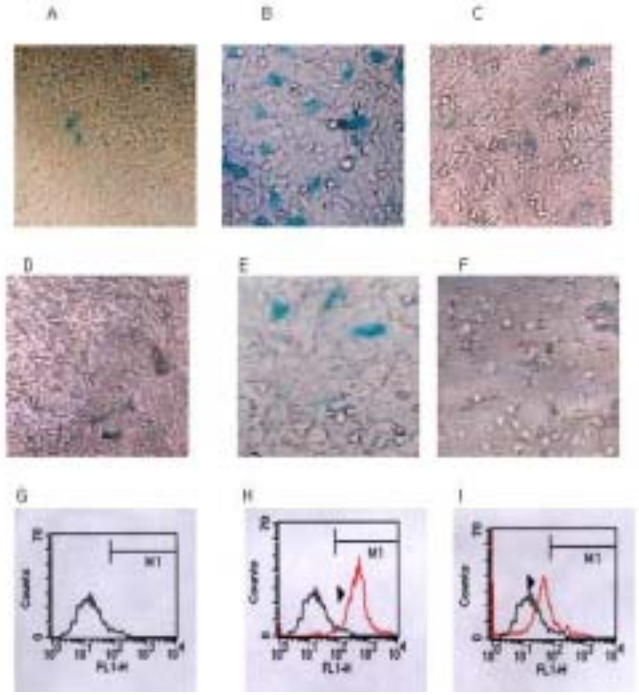
A
B
C
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Fig. 4. The effects of chemical agents on transduction. Hepatoma cells were treated chemical agents for 6 hours, and then infected with rAAV-LacZ. 48 hours later, X-gal staining (A~F) and FACS-gal assay (G~I) were used. (A and G) hepatoma cell lines were infected with rAAV-LacZ alone. (B and H) Hepatoma cells were pre-treated with permissive growth medium, (C) heparin 250 μg/μl, (D) sodium butyrate 10mM, (E and I) chloroquine 25 μM, and (F) chloroquine 50 μM, and then infected with rAAV-LacZ.

5. The effects of the chemotherapeutic agents on rAAV transduction of hepatoma cell lines

Hepa1c1c7 and H4IIE cells were treated with different concentrations of chemotherapeutic agents and then infected with rAAV-LacZ. Transduction efficiency was analyzed by FACS-gal assay (Fig. 5). Incubation period of chemotherapeutic treatments were 6 hours. After treatment, the cultures were washed with DMEM, rAAV-LacZ was added, and the cultures were maintaining 10% DMEM. As shown in Fig. 5A, no treatment of hepa1c1c7 cells and pretreatment of hepa1c1c7 cells with permissive medium showed 12 ± 1.3 of OD_{520} and 462.4 ± 10.7 of OD_{520} in each. Pretreatment of hepa1c1c7 cells with 10 μM of camptothecin, 3 μM of etoposide, 1 μM of cisplatin, and 1 μM of 5-FU showed 600.0 ± 10.9, 756 ± 13.6, 165.0 ± 2.2, and 212.0 ± 3.2 of OD_{520} in each. In H4IIE cells, OD_{520} of no treatment group was 11.4 ± 0.5 and that of pretreatment with permissive medium was 462 ± 12.1. However, pretreatment with 10 μM of camptothecin, 3 μM of etoposide, 1 μM of cisplatin, and 1 μM of 5-FU demonstrated 249.5 ± 10.8, 639.8± 13.6, 513.1± 8.2, and 186.5±5.8 of

OD_{520} of OD_{520} in each.. In these experiment, chemotherapeutic agents increased the transduction efficiency of hepatoma cell lines and etoposide was the most efficient agent to increase the transduction of hepatoma cell lines with rAAV.

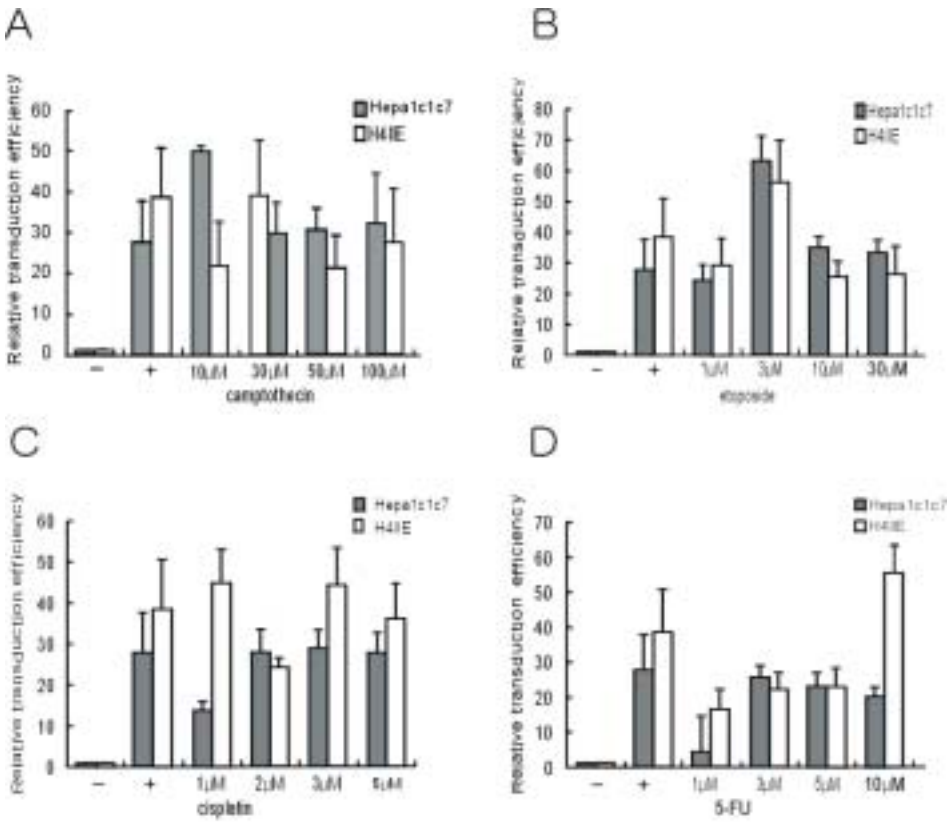
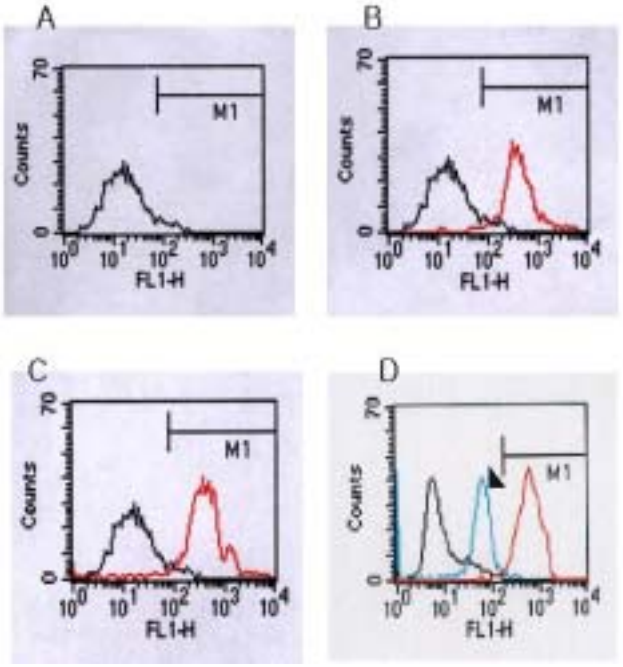


Fig. 5. The effect of chemotherapeutic agents on rAAV-LacZ transduction. Hepatoma cells were performed FACS-gal assay. Hepa1c1c7 and H4IIE cells were pretreated with (A)camptothecin, (B)etoposide, (C)cisplatin and (D)5-FU, and subsequently infected with rAAV-LacZ. (-): Infected with rAAV-LacZ alone indicate. (+): pre-treated with permissive growth medium and subsequently infected with rAAV-LacZ indicate. The relative transduction efficiency of rAAV-LacZ alone was defined as 1. The relative transduction efficiency was defined as the number of rAAV-LacZ per 100ul of vector stock produced after each drug treatment divided by the number in rAAV-LacZ alone. Results are the means ± S. D. of three independent experiments performed in triplicate.

6. Comparison of the effect of γ-irradiated with non-irradiated of hepatoma cells transduced by rAAV

The transduction efficiency of γ-irradiated hepatoma cells was observed by FACS-gal analysis. (Fig. 6) or by counting the number of cells stained with X-Gal (data not shown). For γ-irradiation, hepatoma cell lines were treated prior to infection with 10Gy, 20Gy, and 40Gy. The cells were then infected with rAAV-LacZ. All cells were infected with rAAV-LacZ. After 40-48 hours, FACS-gal assays and X-gal staining were performed. The transduction efficiency after 10Gy and 20Gy exposures did not increase significantly (data not shown) but in the case of 40Gy, the transduction efficiency increased significantly(389.4±15.8 of OD_{520} in hepa1c1c7 cell and 446.7±10.2 in H4IIE cell). However, when γ-irradiated cells were added permissive growth medium, the effects of transduction were decreased significantly (57.15±1.5 of OD_{520} in H4IIE cell and 228±3.2 of OD_{520} in hepa1c1c7) and observed cytotoxicity.


A
B
C
D
M1
Counts
FL1-H
70
0

E

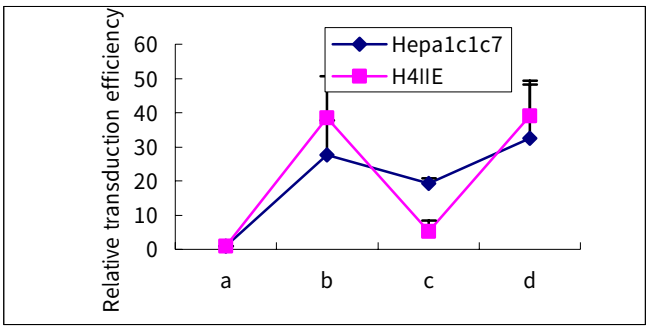

Relative transduction efficiency
60
50
40
30
20
10
0
a
b
c
d
Hepa1c1c7
H4IIE

Fig. 6. Comparison of the effect of γ-irradiated with non--irradiated of hepatoma cell lines transduced by rAAV. AAV-LacZ expression of γ-irradiated hepatoma cell lines was compared with non-irradiated cells. Cells were irradiated or not irradiated and then infected with AAV-LacZ. After 40-48 hours, FACS-gal assays were performed. (A) Hepatoma cells were infected with AAV-LacZ, (B) the cells were pretreated with permissive growth medium for 6 hours and then infected with AAV-LacZ, (C) the cells were exposed to γ-irradiation and infected with AAV-LacZ alone and (D) the cells were pretreated with permissive growth medium for 6 hours and exposed to γ-irradiation and then infected with AAV-LacZ. (E) The relative transduction efficiency of rAAV-LacZ alone was defined as 1.The relative transduction efficiency was defined as the number of rAAV-LacZ per 100ul of vector stock produced after each drug treatment divided by the number in rAAV-LacZ alone. a: Hepatoma cells were infected with AAV-LacZ, b: The cells were pretreated with permissive growth medium for 6 hours and infected with AAV-LacZ, c: the cells were pretreated with permissive growth medium for 6 hours and exposed to γ-irradiation and infected with AAV-LacZ, and d: the cells were exposed with γ-irradiation and infected with AAV-LacZ alone. Results are the means ± S. D. of three independent experiments in triplicate.

7. The effects of combinations of chemotherapeutic agents and γ-irradiation

In hepa1c1c7 cells, combinations of chemotherapeutic agents(camptothecin, etoposide, cisplatin, and 5-FU) and γ-irradiation did not show synergistic effects. ($p>0.05$)(Fig. 7A). However indeed, combination of permissive medium and γ-irradiation or 3 μM of etoposide and γ-irradiation in hepa1c1c7 cells showed the downregulation of LacZ expression($p<0.05$)(Fig. 7B). However, the transduction efficiency of H4IIE cells increased significantly when combinations of chemotherapeutic agents (1 μM of etoposide, 3 μM of 5-FU, 1 μM of cisplatin, 30 μM of camptothecin) and H4IIE cells were used ($p<0.05$) (Fig. 7C). In H4IIE cells, combinations of permissive medium and γ-irradiation showed the significant decrease of transduction with cytotoxicity.

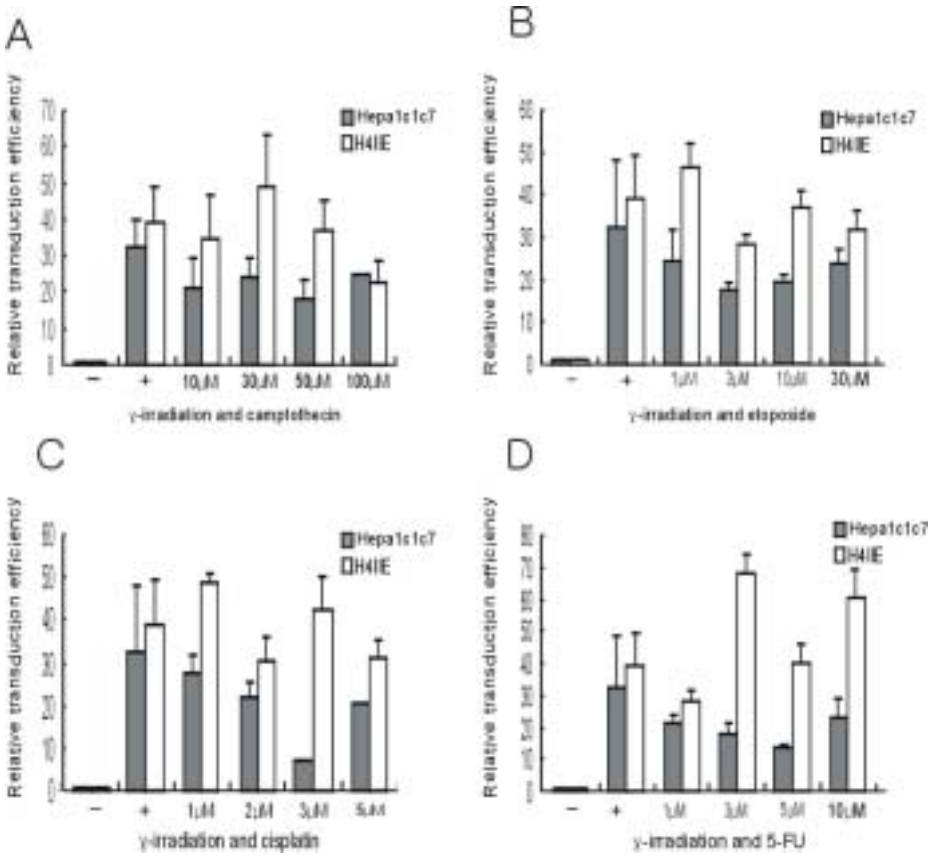

A
Relative transduction efficiency
Hepa1c1c7
H4IIE
−
+
10μM
30μM
50μM
100μM
γ-irradiation and camptothecin
B
Relative transduction efficiency
Hepa1c1c7
H4IIE
−
+
1μM
3μM
10μM
30μM
γ-irradiation and etoposide
C
Relative transduction efficiency
Hepa1c1c7
H4IIE
−
+
1μM
2μM
3μM
5μM
γ-irradiation and cisplatin
D
Relative transduction efficiency
Hepa1c1c7
H4IIE
−
+
1μM
3μM
5μM
10μM
γ-irradiation and 5-FU

Fig. 7A. The effects of combinations of chemotherapeutic agents and γ-irradiation. Hepatoma cells were treated chemotherapeutic drugs, and incubated for 6 hours prior to γ-irradiation(40Gy) treatment. (A) Hepa1c1c7 and H4IIE cells were pretreated with camptothecin for 6 hours and then exposed to γ-irradiation(40Gy). (B) Hepa1c1c7 and H4IIE cells were pretreated with etoposide for 6 hours and then exposed to γ-irradiation(40Gy). (C) Hepa1c1c7 and H4IIE cells were pretreated with cisplatin for 6 hours and then exposed to γ-irradiation(40Gy). (D) Hepa1c1c7 and H4IIE cells were pretreated with 5-FU for 6 hours and then exposed to γ-irradiation(40Gy).(-): not pretreated and unexposed by rAAV-LacZ alone. (+): pre-treated with permissive growth medium and then exposed to γ-irradiation and subsequently infected with rAAV-LacZ. After 48 hours incubation, the cells were performed FACS-gal assay. The relative transduction efficiency of rAAV-LacZ alone was defined as 1. The relative transduction efficiency was defined as the number of rAAV-LacZ per 100ul of vector stock produced after each drug treatment divided by the number in rAAV-LacZ alone. Results are the means ± S. D. of three independent experiments performed in triplicate.

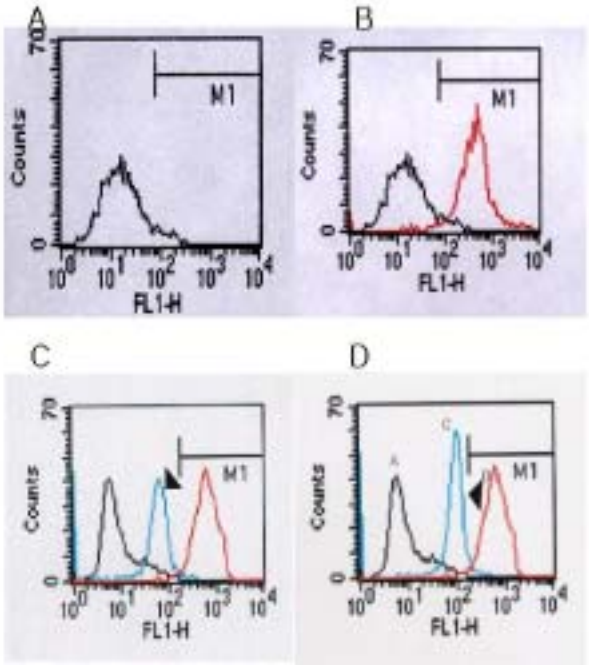


Fig. 7B. Down regulation of rAAV-LacZ with chemical agents and γ-irradiation. Hepa1c1c7 cells were treated combination of chemical agents and γ-irradiation. 48 hours laters, the cells were performed FACS-gal assay. (A) Hepa1c1c7 cells were infected with rAAV-LacZ alone. (B) Hepa1c1c7 cells were exposed to γ-irradiation and then infected with rAAV-LacZ. (C) Hepa1c1c7 cells were pretreated with permissive growth medium prior to γ-irradiation and then infected with rAAV-LacZ. (D) Hepa1c1c7 cells were pretreated with 3 μM of etoposide prior to γ-irradiation and then infected with rAAV-LacZ.

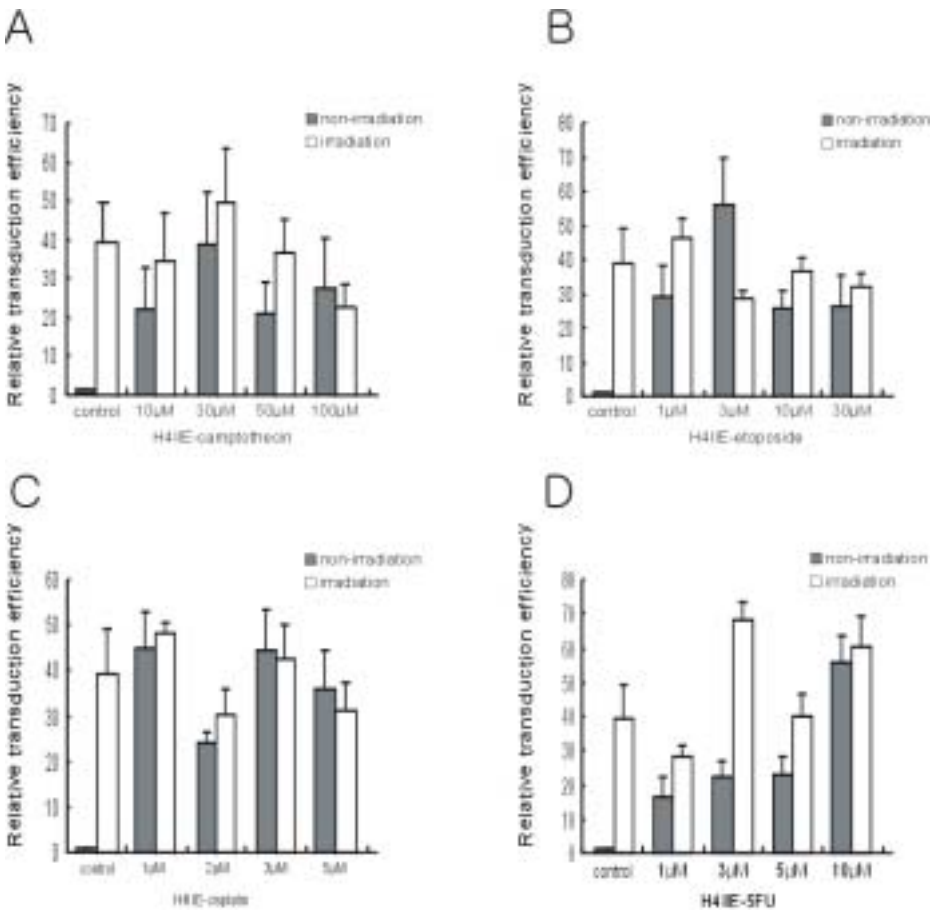


Fig. 7C. Comparison of chemotherapeutic agents with γ-irradiation in terms of the transduction of H4IIE by rAAV. H4IIE cells were treated with chemotherapeutic agents for 6 hours, and 48 hours later, a FACS-gal assay was performed. H4IIE were pretreated with chemotherapeutic agents for 6 hours prior to being γ-irradiated (40Gy). After 48 hours of incubation, the cells were performed FACS-gal assay. H4IIE cell treated with (A) camptothecin, (B) etoposide, (C) cisplatin and (D) 5-FU. Solid bars represent non-irradiation and chemotherapeutic agents, blank bars represent γ-irradiation and chemotherapeutic agents. Control is non-irradiated cell and infected rAAV-LacZ alone (solid bars) irradiated cell and

infected AAV-LacZ (blank bars). The relative transduction efficiency of rAAV-LacZ alone was defined as 1. The relative transduction efficiency was defined as the number of rAAV-LacZ per 100ul of vector stock produced after each drug treatment divided by the number in rAAV-LacZ alone. Results are the means ± S. D. of three independent experiments performed in triplicate.

Ⅳ. Discussion

Although rAAV vectors have been used successfully for gene transfer to the normal liver, the assessment of rAAV transduction efficiency has not been systematically investigated in hepatoma cells. In the present work, we aimed to evaluate the potential of rAAV for gene transfer to hepatoma cells in vitro and the effect of genotoxic agents on transduction efficiency. Our findings showed that gene transfer efficiency in vitro was very low in hepatoma cells. Even though hepatoma cell lines are dividing state, gene transfer efficiency was lower than 0.01%. These suggest that Lac-Z alone could not achieve the therapeutic range of target protein in liver-targeted AAV gene therapy

Sodium butyrate, heparin, hydroxyurea, and chloroquine are known as the stimulator of transduction of cells in AAV infection. Optimal combination is used to permit the transduction of cells. Our results showed that hydroxyurea and permissive media increased the transduction efficiency of AAV in hepatoma cell lines. In case of hydroxyurea, low dose (4mM) was the most effective

concentration and in high concentration (100mM) the cell tested was cytotoxic and decreased the transduction efficiency compared to low dose of hydroxyurea. Unexpectedly, sodium butyrate, heparin, and chloroquine did not affect the transduction efficiency of hepatoma cell lines in AAV infection. Also hepatoma cell lines are resistant to AAV transduction compared to HT1080 cell line that is used for the titration of AAV.

Previous reports have also shown that X-ray irradiation and UV irradiation of tumor cells can increase the transduction efficiency of AAV vectors[23]. Peng et al.[11] et al also reported that very low transduction efficiency was observed in HCC cell lines both in vitro and in vivo, even at high doses of the vector (i.e., $2x10^4$ particles/cell) alone. In the presence of adenovirus, approximately 70-90% of HCC cells could be transduced in vitro. However, when the recombinant AAV was injected into the tumor, along with adenovirus, enhanced transduction occurred only around the injection needle tracks. In contrast, this transduction efficiency was enhanced by radiation therapy (18 Gy), leading to increased β-galactosidase expression throughout the tumor. In addition, RT appeared to accelerate transgene expression, so that high levels of

β-galactosidase expression were seen after 3 days. RT may augment the permeability of the tumor vasculature, and thereby decrease the interstitial fluid pressure inside the tumor, thus promoting diffusion of the vector. The synergism of RT with these viral vectors could be exploited in clinical trials when adjuvant viral gene therapy can be scheduled after RT.[25-28]

In this study, we did not try the combination of adenovirus and AAV in the transduction efficiency of hepatoma cell lines. Adenovirus is pathogenic and expected the side effect, if clinical protocol is included the adenovirus. We also found that radiation increased the transduction efficiency markedly in hepatoma cell line. In 40 Gy, the hepatoma cell lines did not show the cytotoxicity and the transduction efficiency was increased to over 50 times compared to no pretreatment. These data indicated that in radiation-sensitive cancer, combination of radiation and AAV gene therapy might be good strategy and radiation-resistant cancer should be tested by using the combination of radiation and AAV gene therapy protocol.

Transduction efficiency is the combined result of intracellular processing, including trafficking, promoter, phage un-coating, and

strand conversion. Presumably, the facilitation of any of these processes might improve the transduction efficiency considerably. In the case of the single-stranded viral vector, AAV, genotoxic treatments, such as gamma irradiation, the Topoisomerase I inhibitor, and anti-cancer agents have been shown to increase transduction .The mechanism of increased transgene expression is not fully understood but is thought to involve activation of the host cell repair machinery in response to DNA damage[24]. Transduction in response to chemotherapeutic agents is likely to be caused by differences in sensitivity to genotoxic stress. In these study, we tested the chemotherapeutic agent such as camptothecan, etoposide, cisplatin, and 5-FU. All these agents increased the transduction efficiency of AAV in hepatoma cell lines. Among the agents tested, etoposide was the most effective agent that increased the transduction efficiency. Weger et al demonstrated that Rep78 and Rep68, which are part of AAV gene product are interacting a p53 (p53BP3) and a topoisomerase I interacting protein (Topors).[31]

Still, the mechanism by which the transduction of AAV in hepatoma cell lines is increased is not clear, but topoisomerase inhibitor might be involved in the AAV gene therapy for cancer

patients.

Finally, combinations of chemotherapeutic agents and γ-irradiation in gene delivery, by targeted AAV, have significant implications for the use of the AAV vector in cancer gene therapy. Radiation and anti-cancer agents are cytotoxic to carcinoma cells and could lead to tumor regression in variety of tumor models[25, 26] Gene transfer efficiency in vitro was very low in all tested cells, even with a high rAAV dose. Combination of chemotherapeutic agents and γ-irradiation, however, was able to effectively transduce 70 to 90% of hepatoma cells.[11,15] In additions, pre-treatments of hepatoma cells with etoposide, cisplatin, camptothecin, γ-irradiation or combinations were able to increase gene transfer efficiency to hepatoma cells. In out study, the results were not consistent. However, at the range of cell cytotoxicity, the expression of Lac-Z was markedly decreased. These suggested that in clinical protocol, high dose of stimulator or combination pretreatment for the increase of transduction efficiency should be abandoned.

V. Conclusions

Our results indicate that the preteatment of hepatoma cell lines with some chemicals such as hydroxyurea, chemotherapeutic drugs such as camptothecin, etoposide, 5-FU, cisplatin, and γ-irradiation enhance tranduction of hepatoma cells by rAAV. Combinations of irradiation and chemotherapeutic agents show synergic effects or reversal down-regulation effects These suggested us that chemotherapeutic agents or γ-irradiation might increase the effect of rAAV gene therapy, but the circumstances that show the cell cytotoxicity induce the down regulation of target-gene expression in hepatoma cell lines with rAAV gene therapy. Under optimal condition and optimal dose, our results allow for gene transfer efficiency to be improved in liver-targeted cancer gene therapy.

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국문요약

recombinant adeno-associated virus를 이용한 유전자 치료에 있어서 hepatoma 세포주의 transduction efficiency를 높이기 위한 인자 분석

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유전자치료에서 gene delivery vehicle로써 rAAV(recombinant Adeno-associated virus)를 이용하는데, 이는 염증반응이나 면역반응이 거의 없고 neuron, myocyte, hepatocyte와 같은 non-dividing cell의 transduction에 효과가 있다. AAV는 다양한 장기에서 발현을 보이는데 각각의 장기마다 단백질의 발현이 다르게 나타나기에 유전자 치료를 함에 있어 단백질의 발현기관으로 간 (liver)을 대상으로 하여 단백질의 발현을 증가시킬 수 있다면 좀더 효과적인 유전자 치료가 되리라 예측된다. 일부의 연구에서 DNA-damaged agent인 irradiation이나 heparin, topoisomerse inhibitor(camptothecin)등이 간의 transduction efficiency을 높여준다는 보고가 있다 [3-5]. 따라서 본 연구에서는 rAAV를 여러 물리적 화학적 방법을 사용하여 transduction에 차이가 있는지를 살펴 보았고 heparin, chloroquine, butyrate, hydroxyurea 혹은 chemotherapeutic agents를 rAAV와 함께 처리했을 때 hepatoma cell에서 transduction에 어떠한 영향이 있는지를 살펴보았다. 또한 γ-irradiation을 hepatoma cell에 expose시켜서 rAAV를 infection하면 transduction efficiency에 영향을 주는지를 살

펴보았다. γ-irradiation과 함께 여러 화학적 agent를 처리하여 synergistic effect가 있는지를 살펴보고, hepatoma cell에서 효과가 있는지를 AAV-mediated β-galactosidase gene인 pAAV Lac Z를 이용한 β-galactosidase의 발현여부와 activity를 통한 transduction efficiency를 평가하였다. cell type에 따라 각각 다르게 나타났으나 chemotherapeutic agents인 camptothecin, etoposide를 rAAV와 함께 처리했을 때 transduction에 효과가 있었다. 또한 γ-irradiation만을 cell에 expose하여 rAAV를 infection해도 permissive environment가 되었다. 이를 좀더 세분화시켜 γ-irradiation과 chemotherapeutic agents인 camptothecin이나 5-FU를 combination했을 때 효과가 있었다. 이상의 결과를 토대로 rAAV를 이용한 permissive environment를 위한 적정 농도와 적정 조건이 향후 유전자 치료의 protocol을 결정하는 자료가 되리라 여겨진다. 이를 통해 앞으로 in vivo실험에서 좀더 체계적인 유전자 치료를 하는데 좋은 평가가 되리라 여겨진다.

핵심되는 말 : transduction, recombinant adeno-associated virus, camptothecin, etoposide.