### *Measurement of cellular cholesterol*

HepG2 cells (human hepatocytes) were maintained in a 12-well plate with 1 ml of MEM containing 10% fetal bovine serum, 1% nonessential amino acids (NEAA; ICN), and 1 mmol/l sodium pyruvate at 37°C in a 5% $CO_2$ incubator for 5 days. The medium was then replaced with 1 ml of MEM containing 10% lipoprotein-deficient serum (LPDS; ICN), 1% NEAA, and 1 mmol/l sodium pyruvate. After 2 days later, the cells were incubated with 1 ml of MEM containing 10% LPDS, 1% NEAA and 1 mmol/l sodium pyruvate, and various concentrations (0–100 ng/ml) of recombinant human IL-10 (PeproTech) or $10^{-5}$ mol/l fluvastatin (Tanabe) were added to the culture. At 2 days after the treatment, the cells were incubated with 0.5 ml of MEM containing 10% LPDS, 1% NEAA, 1 mmol/l sodium pyruvate, and 2 mmol/l acetic acid for 16 h. The cellular cholesterol level was measured by an enzymatic method using Determiner TC-555 (Kyowa Medics).

### *mRNA analysis of HMG-CoA reductase*

HepG2 cells were incubated with either recombinant human IL-10 (10 ng/ml) or fluvastatin ($10^{-5}$ mol/l) in 10% LPDS medium for 24 h. Total RNA was isolated from the cell culture using an RNeasy Mini kit (QIAGEN) and reverse-transcribed into a single-stranded cDNA using SuperScript Preamplification System (GIBCO BRL). To estimate the expression of HMG-CoA reductase in HepG2, quantitative PCR analysis was conducted by using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reaction was performed using the primer pairs specific for the *HMG-CoA reductase* (HMGCR-5′: GGCCCA GTTGTGCGTCTTCC and HMGCR-3′: GTTTGCTGC ATGGGCGTTGTAG) and *GAPDH* (GAPDH-5′: CGCG GGGCTCTCCAGAACATCAT and GAPDH-3′: CCAGCC CCAGCGTCAAAGGTG). Quantitative values were obtained from the threshold cycle ($C_t$) number that indicated exponential amplification of the PCR product. To normalize each sample, we also quantified the expression of the *GAPDH* gene. The relative target gene expression was also normalized with a calibrator (HepG2 cells without treatment of IL-10 or fluvastatin). The final result, expressed as *N*-fold differences in target gene expression relative to the *GAPDH* gene and the calibrator, was determined by the following formula: $N_{target} = 2^{correctedAC_t\,(GAPDH-target\,gene)}$. $C_t$ values of the sample were determined by subtracting the average $C_t$ value of the target gene from the average $C_t$ value of the *GAPDH* gene.

### *Statistical analysis*

Student's *t*-test or ANOVA combined with Scheffe's test was used to compare individual groups, and the Pearson's correlation test was employed to measure the linear association between two variables by using StatView (Abacus Concepts, Inc). Data are presented as means ± s.e.m. A value of $P<0.05$ was considered significant.

## *Acknowledgements*

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# Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes

**Research Article**

**Shinji Mochizuki[1,5], Hiroaki Mizukami[1*], Akihiro Kume[1], Shin-ichi Muramatsu[2], Koichi Takeuchi[4], Takashi Matsushita[1], Takashi Okada[1], Eiji Kobayashi[3], Akinori Hoshika[5], Keiya Ozawa[1]**

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School
[2]Department of Neurology, Jichi Medical School
[3]Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical School
[4]Department of Anatomy, Jichi Medical School
[5]Department of Pediatrics, Tokyo Medical University

***Correspondence:** Hiroaki Mizukami, M.D., Ph.D., Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-0498, Japan; TEL: +81-285-58-7402; FAX: +81-285-44-8675; E-mail: miz@ jichi.ac.jp

**Key words:** dependovirus, serotype, erythropoietin, promoters, muscle, liver

**Abbreviations:** Adeno-Associated Virus, (AAV); murine erythropoietin, (mEpo);

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## Summary

**AAV vectors have become a practical choice for gene transfer into a variety of tissues *in vivo*. For the purpose of supplemental gene therapy, liver and skeletal muscles are the major targets of transduction. Recently, vector serotypes and their tissue specificity is an important issue for successful gene transfer. Although the potential utility of each serotype has been announced, a systematic comparison has not been fully made. In addition, the choice of a suitable promoter is also critical. In this study, we investigated the level of the transgene product by comparing different serotypes as well as promoters. We utilized murine erythropoietin (*mEpo*)-expressing or *LacZ*-expressing AAV vectors and transferred them into immune-competent mice. For the muscle-directed expression, serotype 1 showed the highest Epo expression followed by 5, 2, 3 and 4. As for the promoter analysis in the liver, the CAG promoter achieved the highest Epo concentration, followed by CMV, PGK, and EF-1α. Further comparison in the liver with different serotypes utilizing the CAG promoter revealed that serotype 5 showed the highest level of expression. These results will aid in the design of an optimal AAV vector structure, especially for the systemic delivery of transgene products.**

## I. Introduction

AAV vectors are non-pathogenic, can transduce a wide range of tissues, and hold promise as a choice for efficient gene transfer toward a variety of applications including therapeutic approaches in humans. Among a panel of candidate diseases, hemophilia B is considered one of the best-suited models, and results of a human trial has already been documented (Kay et al., 2000). Although the potential usefulness of the AAV vector has been indicated, there is a need to improve the level of transgene expression, especially for the approaches which require the release of transgene products into the systemic circulation. For this purpose, muscle and liver are the preferred targets of transgene expression. Therefore, we made several improvements to optimize the expression profiles in these tissues. One approach is to find a choice of promoters and enhancer elements. The other issue is the use of appropriate serotypes. As for the human concern, AAV consists of 5 naturally occurring serotypes and there are possibilities that other serotype-based vectors possess higher utilities in certain applications than the 'classic' type 2. Recent progress has made it possible to develop these serotypes as vectors (Muramatsu et al., 1996; Chiorini et al., 1997; Chiorini et al., 1999; Xiao et al.,

1999). Recently, two novel serotypes were added to the list (Gao et al., 2002). At the same time, differences in tropism have gradually been substantiated by the discovery of molecules involved in the attachment to and entry of cells (Kaludov et al., 2001; Walters et al., 2001; Pasquale et al., 2003). Although the utilities of each serotype-based vector were suggested by the developers, there are few studies to compare usefulness on the same platform. For this purpose, we designed vectors encoding murine erythropoietin with various promoters and available serotypes and quantitated the level of transgene expression and biological consequences following injection.

## II. Materials and methods

### A. Cell lines and construction of the plasmids

HEK293 cells were maintained as described previously (Ogasawara et al., 1999). Plasmid pW1, which harbors the bacterial LacZ gene under the control of the CMV promoter (**Figure 1**), was utilized as a marker for transgene expression. Common molecular biology reagents e.g. restriction enzymes, were purchased through Takara Bio Inc., (Ohtsu, Japan). Plasmid p3.3.1c, which harbors a CMV promoter followed by a multiple cloning site and a poly A sequence, was a gift from Avigen, Inc. Plasmids encoding various promoters for comparison in this study were constructed by replacing the sequences of the CMV promoter of p3.3.1c with the corresponding sequence; Plasmid p3CAG was constructed by removing the Spe I - Cla I fragment from p3.3.1c, filled-in and ligated with a 1.6 kb EcoR I – Hind III fragment of pCAGmcs. Plasmid p3GCAG containing a Gst enhancer was constructed with the Cla I – Hind III fragment of pGST-E-CAG (a gift from Dr. T. Naruse, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) into a blunted Cla I- Spe I backbone of p3.3.1c. Plasmid p3EF was constructed in the same way in that the Spe I - Cla I fragment of p3.3.1 was replaced by the Hind III-EcoR I fragment of pEF-BOS (a gift from Dr. Stuart H. Orkin, Boston, MA). p3PGK was also generated by replacing Mlu I-BstB I sequences with the EcoR I-Hind III fragment of pUC/PGK (Lim et al., 1987) provided by Dr. D Williams (Indiana University, Indianapolis, Indiana) by blunt-end ligation. For the p3PGKG plasmid, Mlu I-Sac II sequences were replaced with PGK sequences.

### B. Murine erythropoietin sequence

Murine erythropoietin cDNA was constructed as follows. Total RNA was extracted from kidneys of an 8 week-old C57BL/6 mouse with Isogen (Nippongene, Tokyo, Japan), and reverse transcribed with Superscript II (Invitrogen Corp., Carlsbad, CA) using an oligo (dT) primer and the manufacturer's instruction. Then PCR was performed using a cDNA library as a template. Primers for PCR were MEPO5 : 5'-CGG AAT TCA TGG GGG TGC CCG AAC GTC CCA-3', MEPO3-2 : 5'-TAG GAT CCT GGC AGC AGC ATG TCA CCT GTC-3', and the conditions were 3min at 92°C, followed by 25 cycles of 98°C for 30 sec, 58°C for 1min, 72°C for 1min using pfu polymerase and the standard buffer (Stratagene, La Jolla, CA). Following the reactions, the product was digested with EcoR I and BamH I and inserted into the corresponding sites of pBluescript II sk (+). Murine Epo sequences was inserted into the plasmids containing the promoter sequences. Finally, the Not I fragments of the expression cassettes were subcloned into the Not I sites of the ITR-containing plasmid.

### C. AAV vector production system

We used production systems for AAV serotypes 1-5 based on the following materials and information. The AAV vector production system for serotype 2 was obtained from Avigen Inc. The production system for serotype 3 was already developed (Muramatsu et al., 1996), and the system for serotype 4 and 5 production (Chiorini et al., 1997; Chiorini et al., 1999) was a gift from Dr. Chiorini (NIDCR, NIH). For the serotype 1-derived vector production system, we modified the helper plasmid of serotype 2 by replacing the capsid sequence for serotype 1. Wild type AAV serotype 1 virus was obtained from ATCC (Manassass, VA). Following the extraction of viral DNA by heat denaturation at 100 °C for 5 minutes, the sequences corresponding to the serotype 1 capsid were amplified by standard PCR, utilizing the following primers for sense and

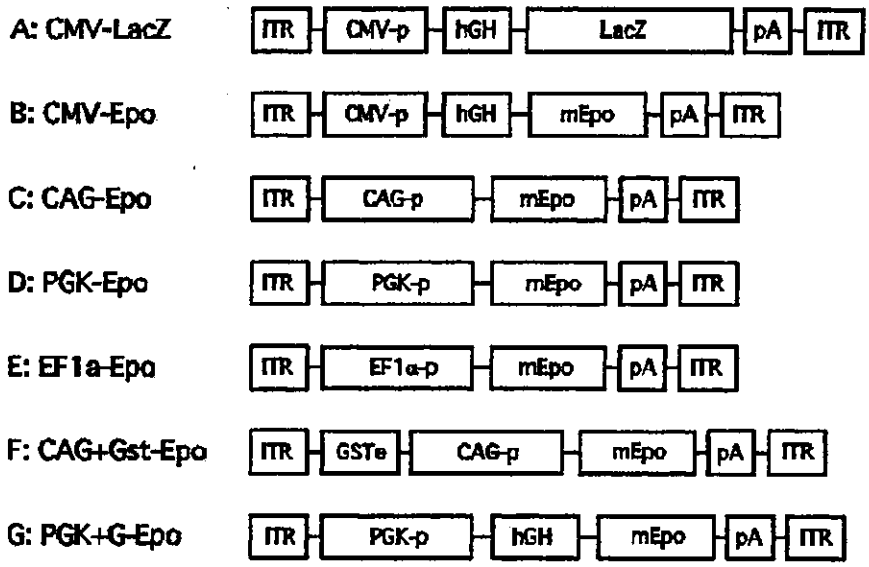



**Figure 1.** The structures of the helper plasmids and the vectors. The promoters used in this study were CAG, CMV, EF-1α, and PGK and tested for the capability of expression in the liver. As for the LacZ expression, only the CMV promoter was used due to the size restriction of the transgene cassette for AAV vectors.

antisense; 5'- agc ttt gtT TAA ACC AGG TAT GGC TGC CGA TG and 3'- agc ttt gtt tAA ACG AAT CAA CCG GTT TAT TGA T, respectively using pfu polymerase and the standard buffer (Stratagene, La Jolla, CA). The 2.3kb PCR fragment was digested by Pme I, and ligated into the Swa I-SnaB I sites of the HLP19 plasmid. As for the vector production, we utilized the transfection of triple plasmids into HEK293 cells as described previously (Matsushita et al., 1998).

### D. Vector purification

To avoid potential interference of CsCl with the specific serotype-derived vectors (Auricchio et al., 2001), we utilized Iodixanol as a separation medium based on the reports (Hermens et al., 1999; Zolotukhin et al., 1999). In order to improve the purity, we utilized high salt conditions during the separation period of ultracentrifugation (Arella et al., 1990). The separation medium consists of 2M NaCl, 33% Iodixanol and 50mM Hepes. The separation was performed twice for 3hours, with the use of a vertical rotor VTI50 (Beckman, CA). The fractions corresponding to the full capsid were recovered, dialyzed against 50mM Hepes-150mM NaCl, and finally concentrated to around 1 x $10^{10}$ vg/μl. The titers of the vector stocks were determined by dot blot analysis utilizing BAS 1500. The integrity of the vectors was tested by infecting 293 cells with LacZ-encoding vectors, and the results were similar to those obtained with the CsCl purified vectors (data not shown).

### E. Animal experiments

All of the animal experiments were performed according to the guidelines of the Jichi Medical School. C57BL/6J mice were purchased from CLEA Japan, Inc., and were subjected to vector injection at 6 weeks of age. For the intramuscular injection, 6 x $10^{10}$ genome copies of each serotype vector, dissolved in 50 μl of saline, was injected into bilateral tibialis anterior muscle. For the portal vein injection, 1 x $10^{11}$ genome copies of each serotype vector, dissolved in 400 μl of saline, was injected under laparotomy. For the promoter analysis, AAV2-based vectors encoding Epo were injected at 1 x $10^{10}$ genome copies to analyze the difference in more detail. Whole blood was collected from these animals every 2 weeks and analyzed for complete blood counts including hemoglobin content and plasma Epo concentrations. Complete blood counts were performed using a PC-608 particle counter (Erma, Tokyo, Japan). Plasma Epo concentrations were quantified with an ELISA kit purchased from Roche Diagnostics Inc. (Mannheim, Germany). Error bars indicate standard deviations throughout the study and the statistical significances were analyzed by Welch's t-test.

### F. Tissue preparation and X-Gal staining and immunohistochemistry

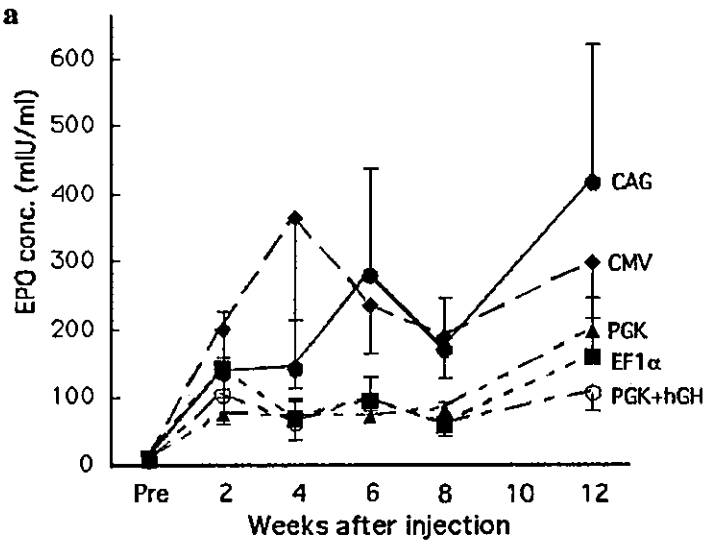
Following the observation period, the mice were sacrificed, and the target organs were recovered. These sections were frozen fresh, and sliced with the microstat. The sections were stained with X-Gal. At the same time, these sections were collected, and the β-galactosidase activity was measured using a β-Gal Assay kit (Pierce, Redford, Illinois), then adjusted according to the protein content of the sample determined with BCA kit (Pierce, Redford, Illinois).

The immunohistochemistry was performed with these sections. A goat polyclonal antibody against human Epo (sc-1310, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was utilized at a concentration of 10 μg/ml. Following a reaction with biotinylated anti-goat IgG (Vector laboratories, Burlingame, CA), horseradish peroxidase-conjugated streptavidin (Vector laboratories, Burlingame, CA), was used following the manufacturer's instructions. The overall reactions were visualized using the DAB-$H_2O_2$ system.

## III. Results

### A. Effect of promoter selection on liver-directed transduction

In our series of experiments to compare the promoter activity, CAG and CMV promoters showed the highest expression of Epo. For the first 4 weeks, CMV showed the strongest expression; thereafter, CAG showed the highest level of Epo expression. No differences were observed between these groups. EF-1α and PGK promoters showed significantly lower levels of Epo expression relative to the former promoters (**Figure 2a**). As for the effects upon red blood cell counts, no significant differences were observed among the groups with different promoters (**Figure 2b**). In order to analyze the difference in more detail, the promoter activities were tested at the lower dose of the vectors.



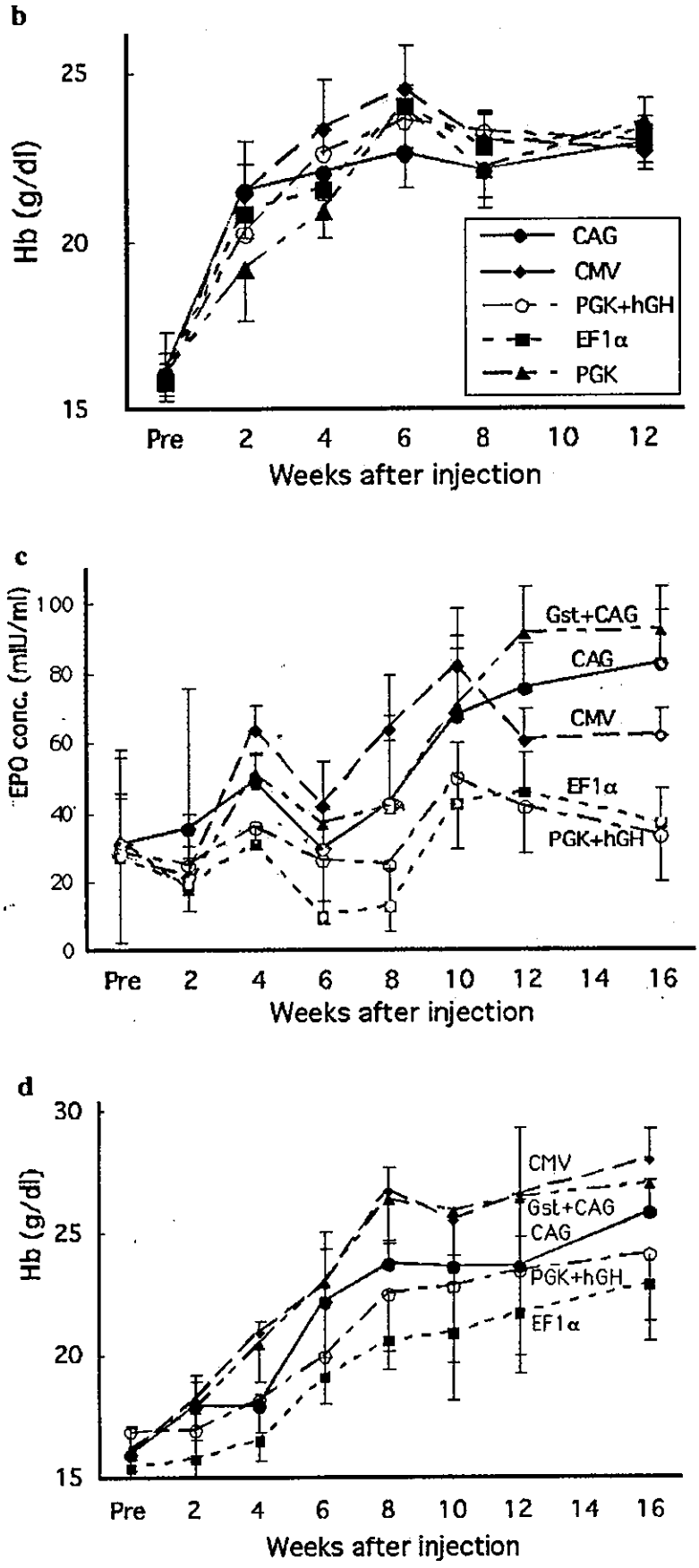



**Figure 2.** Expression profiles for Epo according to the choice of the promoter following intraportal injection of the vectors. The vectors utilized various promoters to drive Epo with the serotype 2 capsid. A higher dose (1 x $10^{11}$ vector genomes per body) was used in the upper panels showing **a)** plasma Epo concentration, **b)** Hemoglobin content of the blood. **c)** and **d)** corresponds to these profiles with the lower dose (1 x $10^{10}$ vector genomes per body). CMV and CAG promoters showed higher expression levels than the rest of the promoters..

As shown in **Figure 2c**, CAG promoters showed the highest expression, followed by CMV, EF-1α and PGK promoters. No significant differences were observed among the data with CAG, CMV and Gst+CAG promoters, whereas the results with EF-1α and PGK promoters were significantly lower than the rest. At this vector dose, plasma hemoglobin content showed differences among groups, mostly consistant with the degree of Epo expression (**Figure 2d**). Statistic analysis revealed that the results with EF-1α were significantly lower than the rest of the groups.

## B. Serotype-related difference in expression profiles in the liver

For the liver-directed transgene expression, we compared serotype-based vectors encoding LacZ. Serotype 5-based vector showed the highest number of cells as assessed by the X-Gal staining of tissue sections. A typical micrograph of X-Gal staining in the case of AAV-5 is shown in **Figure 3a**. The overall results with the X-Gal-positive cells *in vivo* are summarized in **Figure 3b**. The majority of the X-Gal-positive cells were hepatocytes, regardless of the serotype.

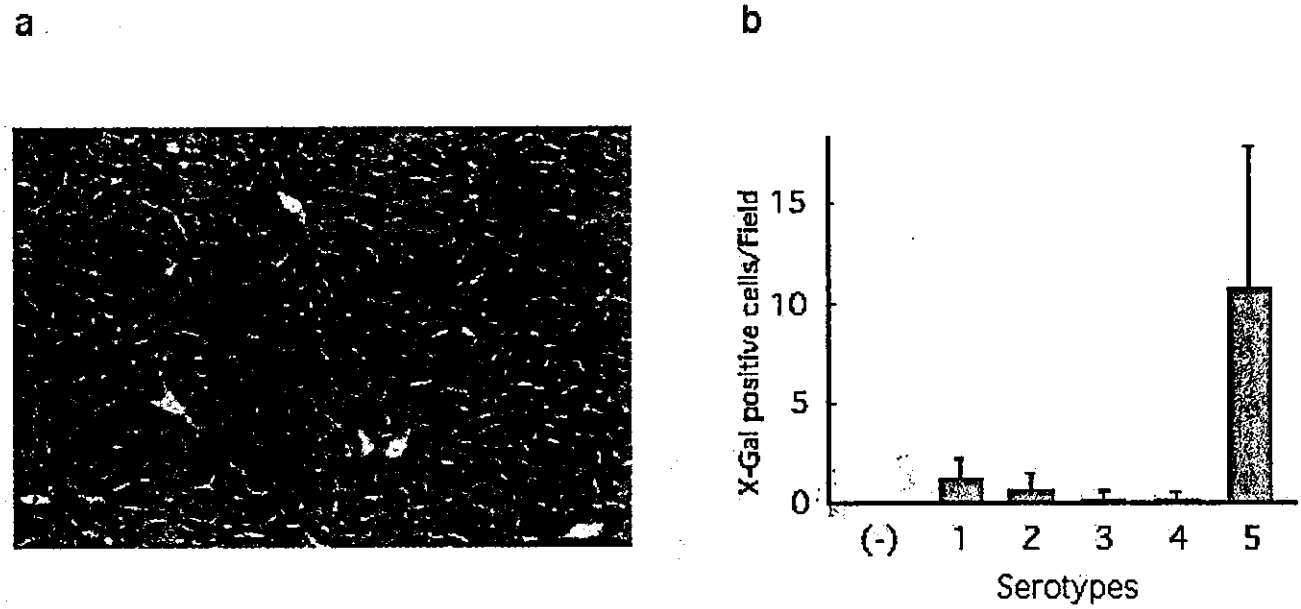
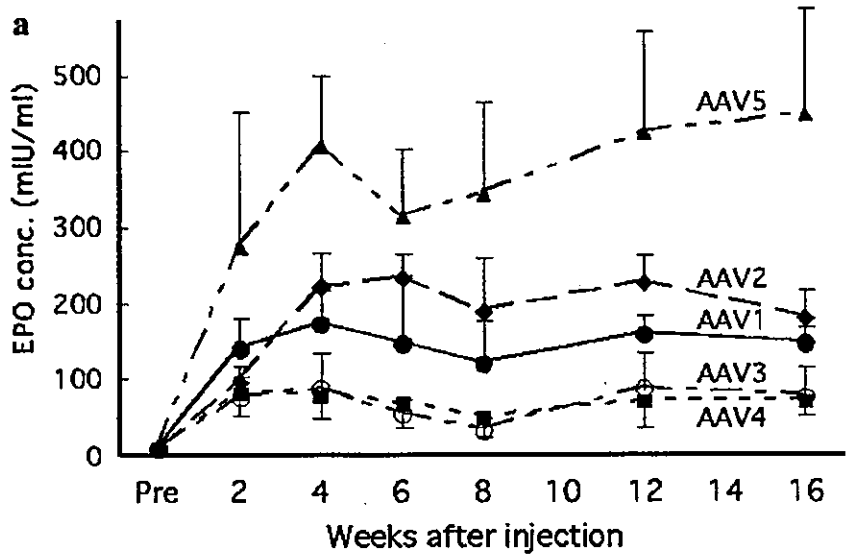



**Figure 3.** Histological analysis of liver tissue following intraportal injection (1 x $10^{11}$ vector genomes) of the serotype-derived vectors. LacZ-encoding vectors with CMV promoter utilizing serotype-derived capsid were injected, and the mice were sacrificed 2 weeks after the injection. The liver was sliced and X-Gal staining was performed. **a)** a representative photograph following injection with serotype 5-derived vectors. Original magnification was x 4. The slides were counterstained with eosin. **b)** the number of X-Gal-positive cells within a field under low magnification are shown. At least 5 different fields were observed, and the numbers were statistically analyzed. Serotype 5- based vectors showed a higher transduction rate than the rest of the groups ($p < 0.05$).



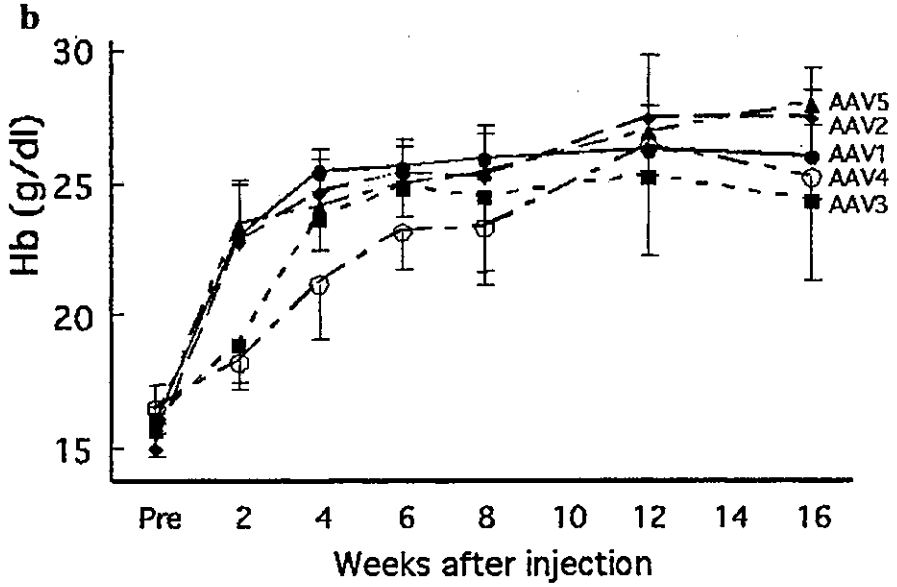



**Figure 4.** Expression profiles of Epo following intraportal vector injection. Serotype-derived vectors using the CAG promoter were produced, and the concentrations of Epo were compared. **a)** among the serotypes, AAV-5 showed the highest Epo concentration. **b)** the difference in hemoglobin content and other red blood cell parameters tended to be similar.

In experiments with Epo genes, the results were almost comparable to that with LacZ; AAV-5 showed the highest expression, followed by AAV-1 and 2. In the cases of AAV-3 and 4, the expression was the lowest (**Figure 4a**). All animals became polycythemic regardless of the degree of Epo expression (**Figure 4b**).

### C. Serotype-related difference in expression profiles in the muscle

In the experiments with LacZ-encoding vectors, AAV-1 and 5 showed the highest levels among all the serotypes. The rest showed a similar degree of expression by X-Gal staining (**Figure 5a-e**). The activity of β-galactosidase reflected the color of the X-Gal staining (**Figure 5f**). In terms of Epo expression, AAV-1 had the highest level, followed by 5, 4, 3 and 2, respectively (**Figure 6a**). Every group was significantly different from others. A prompt increase in the blood cell count was observed in every serotype, without significant difference among serotypes in this regard, as in the case of liver mediated gene transfer (**Figure 6b**).

### D. Histological examination of the tissues

Specific staining in the tibialis anterior muscle was observed in animals transduced with vectors encoding Epo (**Figure 7a**). A reaction without the use of primary antibody was performed as a negative control and is shown in **Figure 7b.**

## IV. Discussion

In this study, we compared the level of transgene expression using serotype-based vectors as well as promoters. Theoretically, the use of a capsid relates to the gene transfer efficiency, and the choice of the promoter / enhancer element affects steps for expression of the transferred gene. In order to search for the optimum combination, it is ideal to use a transgene whose product can be quantitated, and the biological consequences of which can be estimated without triggering immune reactions. For this purpose, we chose murine erythropoietin cDNA as a transgene to focus on the differences within the vector structure *per se*. Our results indicate that for the muscle-mediated expression, AAV-1 has unparalleled capacity among the serotypes tested. This result is consistent with the data reported by other groups (Chao et al., 2000; Rabinowitz et al., 2002), using different transgenes. As for the liver, the highest expression was attained by the use of AAV5-based vectors; which was also reported recently using other transgenes (Mingozzi et al., 2002). The molecular basis of these differences is still mostly unknown, despite steady progress in finding the steps in the transduction route (Walters et al., 2001).

As for the choice of the promoters for liver, CAG and CMV promoters were superior to the rest in experiments with various vector titers. No significant differences between the results using the CAG and CMV promoters were observed at the vector doses, although CAG tended to be higher at most of the data points. For the liver-directed gene therapy, there seems to be a discrepancy among the reports. The CBA promoter was reported superior to the CMV promoter in liver-mediated gene transfer (Xiao et al., 1998; Xu et al., 2001). The CBA promoter used in these studies is essentially the same as the CAG promoter used in this study (Niwa et al., 1991). The reasons for these differences are not clear, but may relate to the difference in transgene, strain of mouse, or technical details concerning the vector injection. In the present study, we utilized an immunocompetent strain of mouse and as the CAG promoter is about 2kb in length, the application of this promoter might be relatively limited in the case of AAV vector-mediated gene transfer. Adding the Gst enhancer sequence to CAG promoter was not

beneficial in this system. In addition, appending the intron sequences from human growth hormone did not affect the activity of PGK promoter (**Figure 2a**). It is interesting that in liver-mediated gene transfer, approximately 5-fold difference was observed in expression in most cases by the 10-fold difference in vector dose indicating non-linear response (**Figures 2a** and **2c**). In our series of experiments, we focused on the strongest promoter to

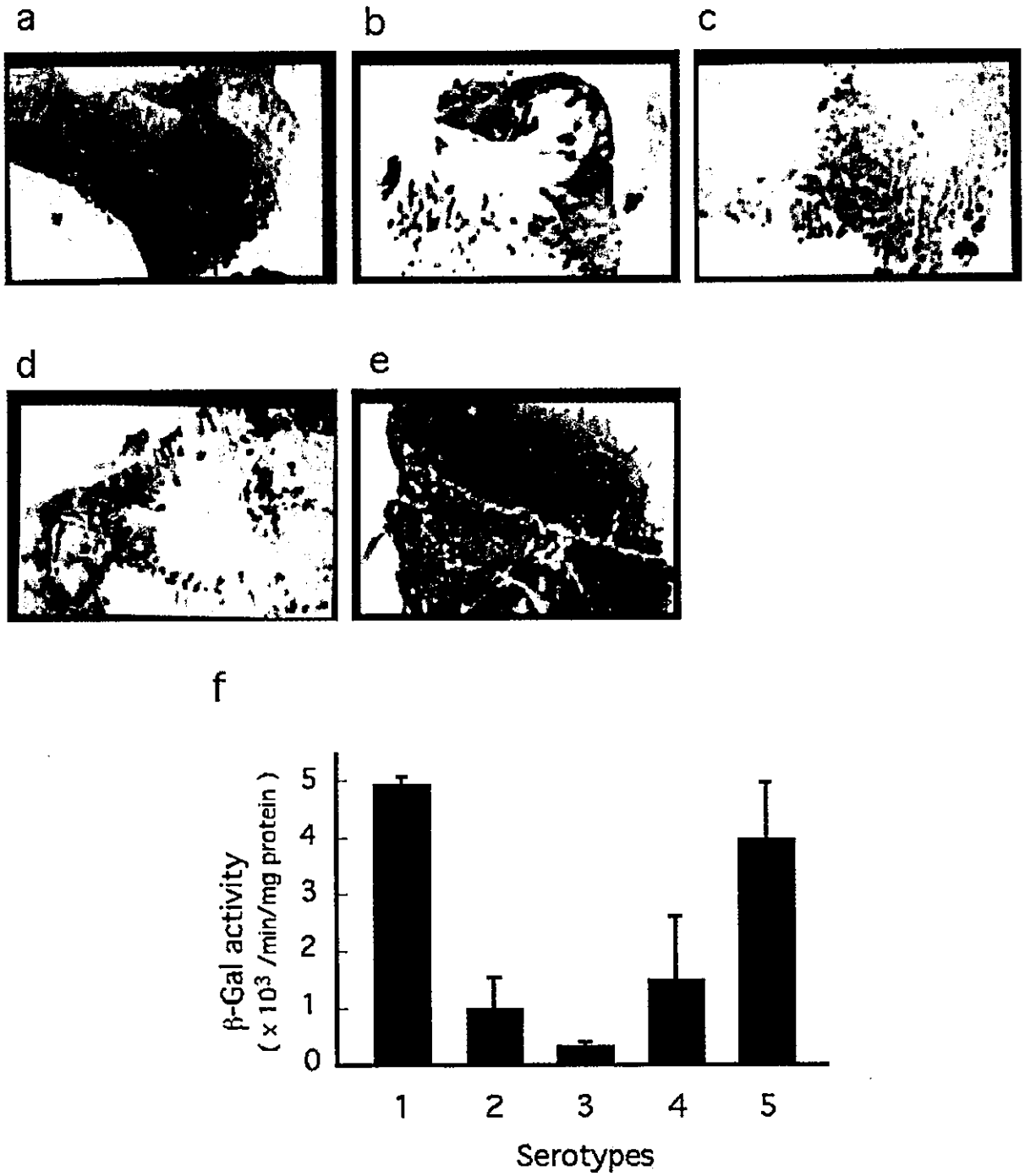



**Figure 5.** Expression of LacZ following intramuscular injection of the serotype-derived vectors. Each serotype-derived vector with the CMV promoter (3 x $10^{10}$ vector genomes per muscle) was injected into the tibialis anterior muscle of mice. Two weeks following the injection, mice were sacrificed and the muscle was histologically analyzed. X-Gal staining was performed, and the best image was chosen. **a)-e)** corresponds to the serotypes 1-5. **f)** The activity of β-Galactosidase was analyzed using the sections of muscle. Ten slices of the muscle specimen (10 μm of thickness) were collected into tube and the activity of the enzyme was determined based on the ONPG assay (Invitrogen Corp., Carlsbad, CA). The protein content was also determined by the BCA method (Pierce, Rockford, IL) to adjust the enzyme activity.

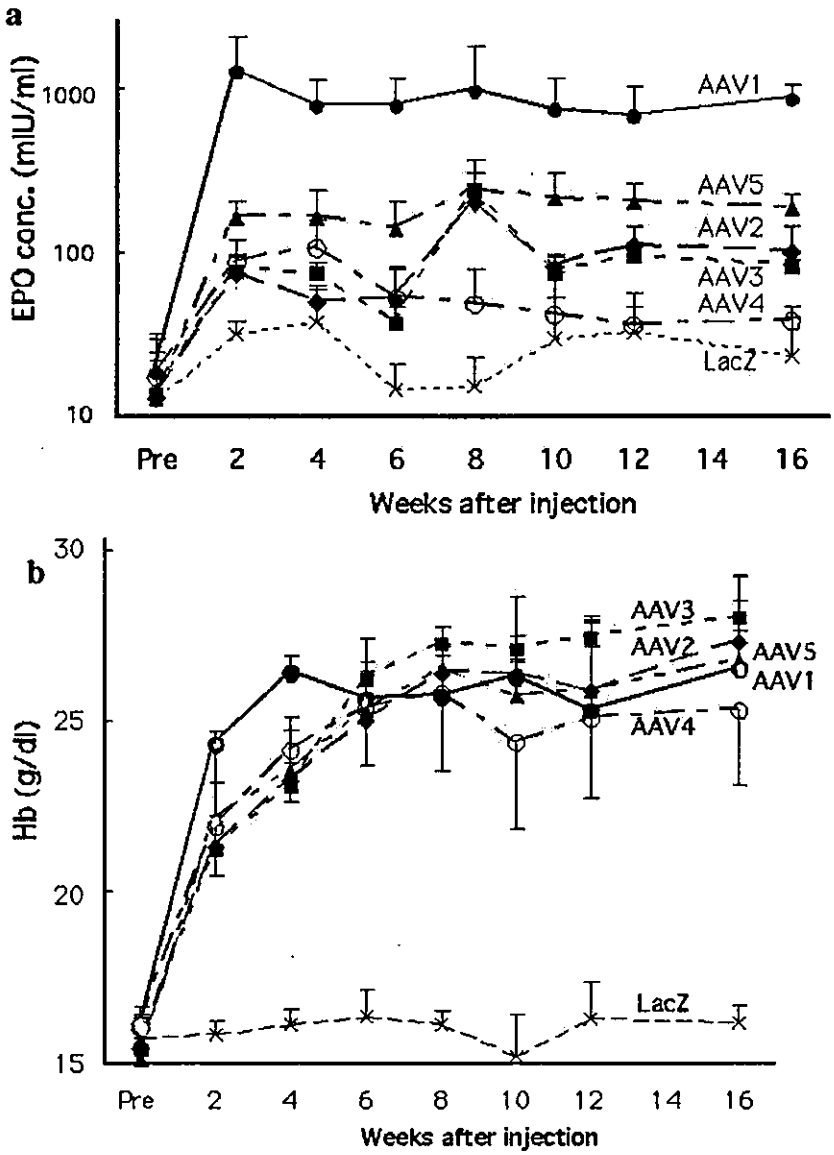



**Figure 6.** Muscle-directed Epo expression using serotype-derived vectors under the control of the CMV promoter. Bilateral gastrocunemius muscles were transduced by injecting 3 x $10^{10}$ vector genomes per muscle. a) Serum Epo concentrations were monitored. Animals injected with serotype 1- derived vectors showed the highest concentration throughout the observation period. b) Hemoglobin content of the blood showed a significant increase in every serotype.

drive the transgene. In clinical applications, tissue-specific promoters would be preferred for safety, to make sure that the expression of the transgene is limited to the target tissues. However, tissue-specific promoters are generally much weaker than the 'ubiquitous' promoters used in this study. Therefore, the tissue-specific promoters will become a practical choice if a more efficient method to transfer genes into the target tissue is developed. In the case of promoters for muscle, the CMV promoter seems to be the best match with a long history of expression of therapeutic genes (Song et al., 1998). The situation is similar to the liver in that several tissue-specific promoters are expected for future use especially the MCK promoter (Hauser et al., 2000). Approaches to develop synthetic promoters are also promising (Li et al., 1999).

In our series of experiments, there was a discrepancy in the results with LacZ and erythropoietin. In the experiments for muscle transduction, results with LacZ showed a comparable expression using AAV-1 and 5, whereas in the erythropoietin experiments, results with AAV-1 showed much higher expression. In this case, the amount of X-Gal seems extremely high, so the β-galactosidase activity within the myocyte may reach a plateau level. As erythropoietin is likely to be released efficiently from the muscle, its serum concentration reflect the production rate within muscle tissue. Immunohistochemical analysis supports persistent expression from transduced tissue (**Figure 7**). In our results for liver-directed gene transfer, the number of X-Gal-positive cells is relatively small. On the other hand,

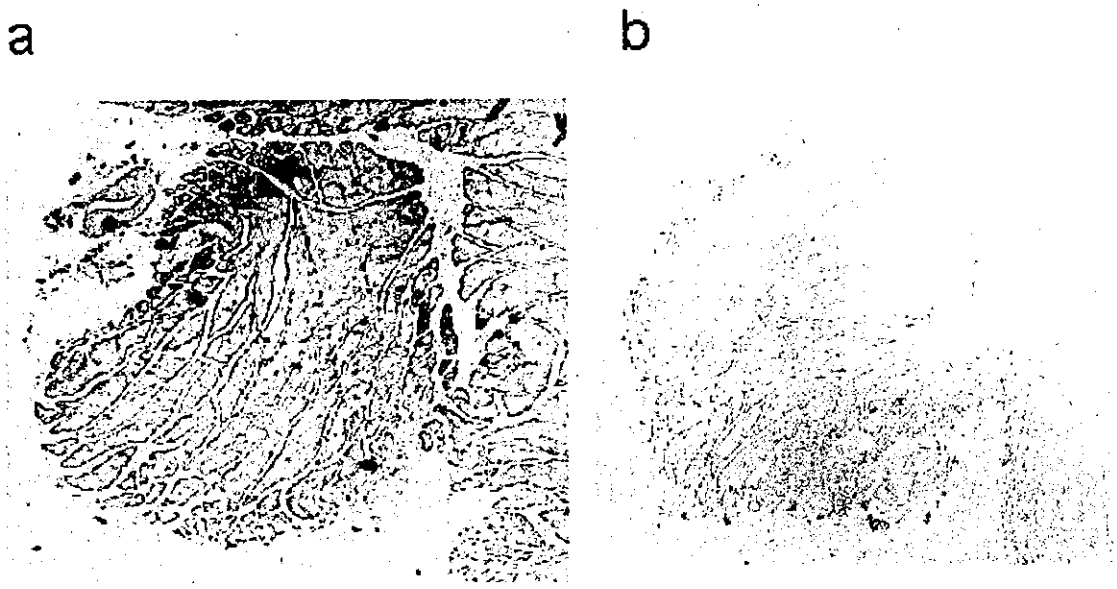



**Figure 7.** Immunostaining against Epo in the muscles injected with serotype 1-derived vectors. The muscle of the animal described in Figure 6 was analyzed. **a)** shows a muscle section stained with 1st and 2nd antibody. **b)** a control without the 1st antibody. Original magnification x 10.

the net expression was comparable to the levels obtained in the muscle transduction. Therefore, the difference may reflect the distribution of transduced cells as the liver is much larger than the tibialis anterior muscle. Although the expression levels of Epo differ significantly among groups, the difference in hemoglobin concentration was relatively modest. The reason seems that as the supraphysiological concentrations were achieved by Epo gene transfer, most animals became unanimously polycythemic. In the cases of lower Epo expression, increase in the hemoglobin levels tend to be slower, due to the presence of the steps to produce red blood cells (typically observed in Figure 2b). On the other hand, in the cases of high Epo expression, increase in the hemoglobin levels were prompt (observed in **Figure 4b**). In this study, we did not measure the functional titers of the vector stocks. It is ideal to quantify the functional titer of each vector stock, and confirm that there is not a discrepancy between these titers. However, there is not a good system to compare the functional titer in a sole system; notably, AAV-4 and –5 have a tropism distinct from the others. Therefore, it is practically impossible to compare functional titers with the same platform. In order to maintain the function of the vector, we paid special attention to the following points. We avoided the use of CsCl as this reagent may affect the functional aspects of the AAV vectors especially AAV-2 (Auricchio et al., 2001). Although there is an increasing number of reports on how to optimize purification strategies for a specific serotype, there is not a purification scheme equally applicable to all of the serotype-based vectors. For this reason, we needed to find a practical method equally applicable to all of the serotypes. In contrast to CsCl, Iodixanol is known to be safe, and can be administered directly to humans. Therefore, we decided to use this reagent in the current study as described (Hermens et al., 1999). Step gradient ultracentrifugation followed by an isopycnic stage resulted in greater purity. In addition, the use of high salt conditions contributes to the purity of the vector stocks, due to a chaotropic action of the chloride ions.

In our study design, we tried not to provoke the immune system in order to compare the relative utility. However, in the case of immunogenic products, there may be a difference in the effect of the immune system. If each serotype-based vector is equally safe, we can choose the optimal type as the vector for a specific target tissue. Our results will guide to design vectors for supplemental gene therapy.

## Acknowledgments

The authors are grateful to Avigen (Alameda, CA) and Dr. John A Chiorini (NIDCR, NIH, Bethesda, MD) for supplying vector production systems for AAV-2 and AAV-4 and 5, respectively. We also thank Mrs. Miyoko Mitsu, for excellent technical assistance. This work was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan, Grants-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Special Coordination Funds for promoting Science and Technology of the Science and Technology Agency of the Japanese Government.

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Dr. Shinji Mochizuki

# Topoisomerase inhibitors enhance the cytocidal effect of AAV-HSVtk/ganciclovir on head and neck cancer cells

TAKEHARU KANAZAWA[1,4], HIROAKI MIZUKAMI[1], HIROSHI NISHINO[2], TAKASHI OKADA[1], YUTAKA HANAZONO[1], AKIHIRO KUME[1], KEN KITAMURA[5], KEIICHI ICHIMURA[2] and KEIYA OZAWA[1,3]

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Departments of [2]Otolaryngology, and [3]Hematology, Jichi Medical School, 3311-1 Yakushiji Minamikawachi, Tochigi 329-0498; [4]Department of Otolaryngology, Faculty of Medicine, University of Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215; [5]Department of Otolaryngology, School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima Bunkyo, Tokyo 113-8519, Japan

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**Abstract.** Adeno-associated virus (AAV) is a non-pathogenic virus with a single-strand DNA genome. AAV vectors have several unique properties suited for gene therapy applications. However, an obstacle to their application is a low efficiency of transgene expression, mainly due to a limited second-strand synthesis. Previously, we reported that γ-rays enhanced the transduction efficiency and cytocidal effect of AAV vector harboring the herpes simplex virus-thymidine kinase (AAVtk) and ganciclovir (GCV) system. In the present study, we investigated whether topoisomerase inhibitors (etoposide and camptothecin) enhance the AAV vector-mediated transgene expression and the killing effect by AAVtk/GCV system. The enhancement of transgene expression was observed in a concentration-dependent manner on human laryngeal carcinoma cells (HEp-2 cells) and HeLa cells. Southern analysis confirmed that etoposide enhanced the double-strand synthesis of the AAV vector genome in HEp-2 cells and HeLa cells. The cells were efficiently killed by AAVtk/GCV system, as expected. More importantly, both etoposide and camptothecin augmented the cytocidal effect of the AAVtk/GCV system. These findings suggest that the combination of AAV-mediated suicide gene therapy and treatment with topoisomerase inhibitors may have synergistic therapeutic effects in the treatment of cancers.

## Introduction

Advanced head and neck cancers exhibit a high mortality rate despite aggressive treatments involving surgery, radiotherapy, and chemotherapy. Patients often present with locally advanced conditions and the long-term survival rates have not improved appreciably over the past several decades. Current treatments for the advanced stages have shown little success, because tumors cannot be eradicated with an acceptable toxicity. One alternative strategy that has shown promise in the treatment of cancer is a gene therapy. Several virus vectors have been explored for the experiments of head and neck cancers, including retroviral (1), adenoviral (2) and adeno-associated virus (AAV) vectors (3). AAV is a non-pathogenic virus with a single-strand DNA genome (4,5). AAV vectors have emerged as a useful alternative to other vectors (6), and AAV have been evaluated in preclinical and clinical models for cystic fibrosis (7), Parkinson's disease (8) and hemophilia B (9). AAV vectors have a broad host range and can transduce head and neck cancer cells (3). However, an obstacle to their application is a low transgene expression efficiency, mainly due to limited second-strand synthesis (10,11). Genotoxic stresses such as chemotherapeutic agents, UV, heat shock, γ-ray irradiation have been reported to enhance the second-strand synthesis of the AAV vector genome and improve the transgene expression (12-15). Thus, an AAV vector encoding a suicide gene would kill target cells more efficiently when combined with other therapeutic agents. One well-studied suicide gene is the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) (16,17). Although the HSVtk/GCV enzyme/prodrug system has been shown to be effective for controlling tumor growth in animal models, it is difficult to eradicate cancer cells by the HSVtk/GCV system alone and tumors may recur after termination of the prodrug therapy (18-21). Thus, other therapeutic modalities, such as combination therapies, are under development (22,23). In our previous study, we demonstrated that γ-ray irradiation enhance AAV-mediated transgene expression and augmented the antitumor activity of HSVtk/GCV system on human head and neck cancer xenografts (24). Although radiotherapy has been one of the most valuable treatments for advanced head and neck cancer, chemotherapy with topoisomerase inhibitors has also been utilized. In this study, we explored the possibility of combining suicide gene therapy using the AAV vector with topoisomerase inhibitors, frequently used as chemotherapeutic agents, to enhance cytotoxicity, thereby providing a more effective means to control tumor growth.

*Correspondence to*: Dr Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji Minamikawachi, Tochigi 329-0498, Japan
E-mail: kozawa@ms.jichi.ac.jp

*Key words*: adeno-associated virus vector, herpes simplex thymidine kinase, head and neck neoplasms, topoisomerase inhibitor

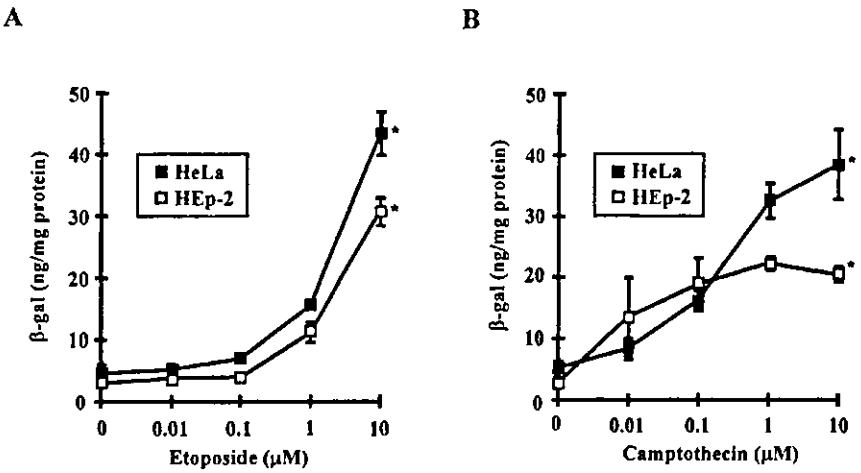


Figure 1. Effect of topoisomerase inhibitors on AAV vector-mediated transgene expression. HeLa cells (closed square) or HEp-2 cells (open square) were transduced with $1x10^3$ particles/cell of AAVLacZ 12-h pre-treatment with topoisomerase inhibitors. (A), The cells were pre-treated with etoposide at concentrations ranging from 0 to 10 μM. (B), The cells were pre-treated with camptothecin at concentrations ranging from 0 to 10 μM. Thirty-six hours after transduction, the expression levels of LacZ were assayed by using the ß-gal ELISA kit. Data were statistically analyzed by one-way ANOVA (*P<0.01).

## Materials and methods

*Cell lines.* HeLa cells, 293 cells and the human laryngeal carcinoma HEp-2 cell line (a gift from the Cell Resource Center for Biomedical Research, Tohoku University), were cultured in DMEM/F12 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Irvine Scientific, Santa Ana, CA) at 37°C in 5% $CO_2$.

*Plasmids.* The plasmid pAAVLacZ contains the CMV promoter, human growth hormone first intron, *Escherichia coli* LacZ gene, and SV40 early polyadenylation sequence between two inverted terminal repeats. A 1.8-kb DNA fragment encoding the HSVtk gene was obtained by double digestion with *Hin*cII and *Pvu*II of plasmid M2 (25) (a gift from Dr Y. Mishina, Yokohama City University, Japan) and subcloned into the pAAVLacZ in place of the LacZ gene (pAAVtk). pH19 is an AAV helper plasmid harbouring rep/cap sequences, and an adenovirus helper plasmid plAd5 contains the adenovirus early genes; E2a, E4, and VA.

*AAV vector production.* AAV vectors were produced based on the plasmid transfection (26). Briefly, subconfluent 293 cells were cotransfected with AAV vector plasmid, pH19, and plAd5 by a calcium phosphate-precipitation method. The cells were harvested and the recombinant AAV particles were released by three cycles of freeze/thaw. The vector solution was then purified through CsCl gradient twice as described previously (3). The vector titer was determined by a quantitative dot blot hybridization of DNase-treated stocks.

*Transduction efficiency of HeLa or HEp-2 cells with AAV vectors.* One day before transduction, $1x10^5$ cells were plated onto 3.5-cm dishes in triplicate. The cells were transduced with different amounts of AAVLacZ. Thirty-six hours after transduction with AAVLacZ, the amount of ß-galactosidase was quantitated by using the ß-gal ELISA kit (Boehringer-Mannheim, Hilden, Germany).

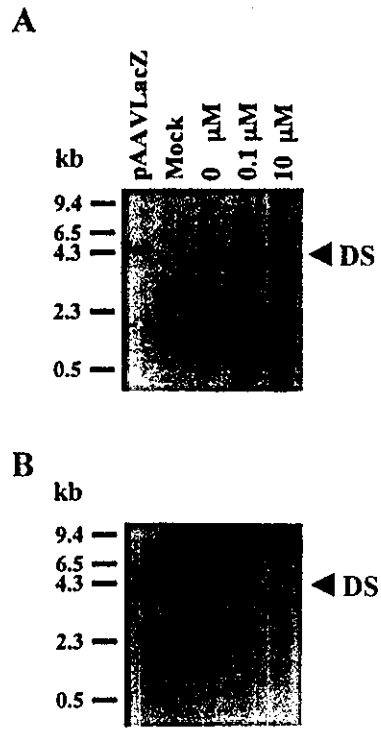


Figure 2. Second-strand synthesis of AAVLacZ genome after etoposide treatment in HeLa cells or HEp-2 cells. HeLa cells (A) or HEp-2 cells (B) were transduced with $1x10^4$ particles/cell of AAVLacZ following pre-treatment with 0, 0.1, 10 μM of etoposide. Two days later, total DNA was isolated. After mung bean nuclease treatment, the DNA samples were loaded on 1% agarose gels, transferred onto nylon membranes, and then hybridized with a radiolabeled CMV-specific probe. Signals were detected by using an imaging analyzer. Lane 1, a 4.7-kb fragment derived from pAAVLacZ; lane 2, mock-transduced; lanes 3-5, AAVLacZ-transduced following pre-treatment with etoposide (0, 0.1, 10 μM) treatment.

*The enhancement of transgene expression by topoisomerase inhibitor treatment.* The topoisomerase inhibitors used in this study were etoposide (Wako Pure Chemical, Osaka, Japan) and camptothecin (TopoGEN, Inc., Columbus, USA). Stock solutions of etoposide (10 mM) and camptothecin (10 mM)

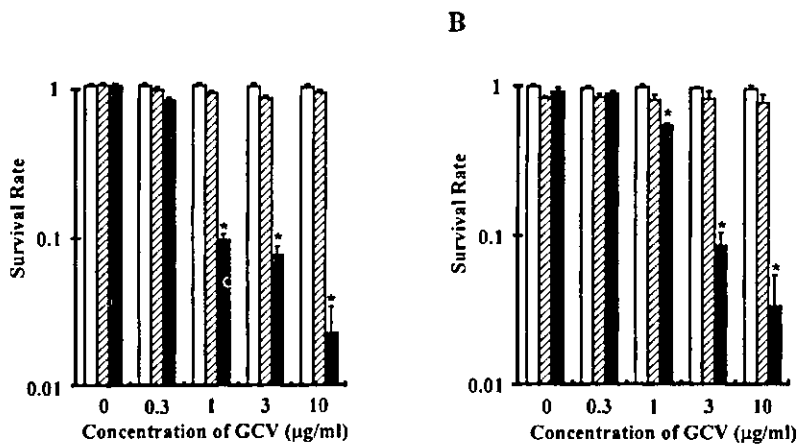


Figure 3. Survival of HeLa cells and HEp-2 cells upon AAVtk/GCV in terms of GCV concentration. HeLa cells (A) or HEp-2 cells (B) were mock-transduced (open bar), transduced with $3x10^5$ particles/cell of AAVLacZ (hatched bar), or AAVtk (closed bar). Twenty-four hours after transduction, the cells were exposed to different concentrations of GCV. Data were analyzed by two-way ANOVA. Asterisks mean that the data obtained for AAVtk-transduction were significantly different from those with or without transduction of AAVLacZ ($P<0.01$).

in dimethyl sulfoxide were stored at -20°C and diluted into PBS for use in experiments. HeLa cells and HEp-2 cells were treated with topoisomerase inhibitors for 12 h, washed twice with PBS, and then transduced with $1x10^3$ particles/cell of AAVLacZ. Thirty-six hours after transduction, we measured the amount of ß-galactosidase with the ß-gal ELISA kit (Boehringer-Mannheim).

*Analysis of the second-strand synthesis of the vector genome.* HeLa cells and HEp-2 cells grown in 10-cm dishes ($1x10^6$/dish) were transduced with $1x10^4$ particles/cell of AAVLacZ following pre-treatment with etoposide. Two days later, total DNA was isolated with the DNA Extractor WB kit (Wako Pure Chemical). Genomic DNA (40 μg) digested with 80 units of mung bean nuclease (Takara, Tokyo, Japan) were resolved on 1% agarose gels, transferred to nylon membranes (Hybond N+; Amersham, Buckinghamshire, UK) in 50% formamide, 6X SSC, 0.5% sodium dodecyl sulfate, 5X Denhart's solution, and 100 μg/ml of denatured salmon sperm DNA at 42°C overnight. The membranes were washed, and then analyzed by using an image analyzer (BAS-1500, Fuji, Tokyo, Japan).

*GCV treatment.* The cells were plated and transduced with AAVtk at the dose of $1x10^5$ particles/cell. Twenty-four hours after transduction with AAV vectors, culture media were replaced by fresh media containing various concentrations of GCV ranging from 0 to 10 μg/ml. After a 7-day incubation in the presence of GCV, surviving cells were counted. The survival rate was calculated from the ratio of the number of cells not treated with GCV. To evaluate the synergistic effect of the AAVtk/GCV system and topoisomerase inhibitor treatment, the cells were transduced with AAVtk following either etoposide (0.1 μM, 10 μM) or camptothecin (0.01 μM, 1 μM) treatment.

## Results

*Effect of topoisomerase inhibitor on AAV-mediated transgene expression.* The transduction efficiency of HEp-2 cells was almost as high as that of HeLa cells. The amount of ß-gal in HeLa cells were 220 μg/mg proteins, when the cells were transduced with $1x10^4$ particles/cell of AAVLacZ (24). Both etoposide and camptothecin have shown to increase the transduction efficiency with AAV vectors, mainly by accelerating the rate of leading strand synthesis of the AAV vector genome. In Fig. 1, etoposide and camptothecin treatment significantly increased LacZ expression in HeLa cells and HEp-2 cells in a concentration-dependent manner (one-way ANOVA: $P<0.01$).

*Topoisomerase inhibitors enhance the second-strand synthesis of the AAV genome in HeLa and HEp-2 cells.* To examine whether the second-strand synthesis of the AAV vector genome occurs more efficiently in the topoisomerase inhibitors treated-cells, HeLa cells and HEp-2 cells were treated with 0, 0.1, or 10 μM of etoposide, and then transduced with $1x10^4$ particles/cell of AAVLacZ. Forty-eight hours after transduction, total DNA was isolated, treated with mung bean nuclease, and then loaded on 1% agarose gels. After transfer to nylon membranes, signals corresponding to the AAVLacZ genomes were detected (Fig. 2). Mung bean nuclease was used to digest the single-strand DNA and to clearly visualize the double-stranded replicative form (DS) of the AAV vector genome. The DS was almost equal to 4.7-kb fragment derived from pAAVLacZ in size. At the concentration of 10 μM, in both HeLa and HEp-2 cells, the intensity of signal corresponding to the DS increased significantly, suggesting that the augmented transgene expression was associated with the conversion of the AAV vector genome to the double-stranded form.

*The killing of HeLa and HEp-2 cells in terms of GCV concentration.* Fig. 3 shows the killing effect of various concentration of GCV on HeLa cells (Fig. 3A) and HEp-2 cells (Fig. 3B) transduced with $3x10^5$ particles/cell of AAVtk (closed bar). When the AAVtk-transduced-cells were treated with 1 μg/ml of GCV, 90% of HeLa cells and 47% of HEp-2 cells were killed. As the concentration of GCV was increased, surviving cells were reduced and 98% of HeLa cells and 96% of HEp-2 cells were killed by the exposure to 10 μg/ml of GCV. This killing rate was significantly higher than that in

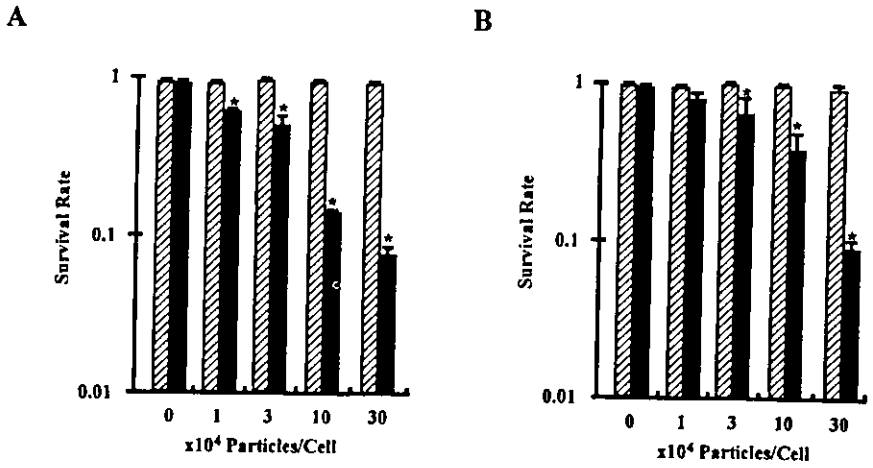


Figure 4. Survival of HeLa cells and HEp-2 cells upon AAVtk/GCV in terms of AAV doses. HeLa cells (A) or HEp-2 cells (B) were, transduced with various doses of AAVLacZ (hatched bar), or AAVtk (closed bar). Twenty-four hours after transduction, the cells were exposed to 3 μg/ml of GCV. Data were analyzed by two-way ANOVA. Asterisks mean that the data obtained for AAVtk-transduction were significantly different from those with or without transduction of AAVLacZ (P<0.01).

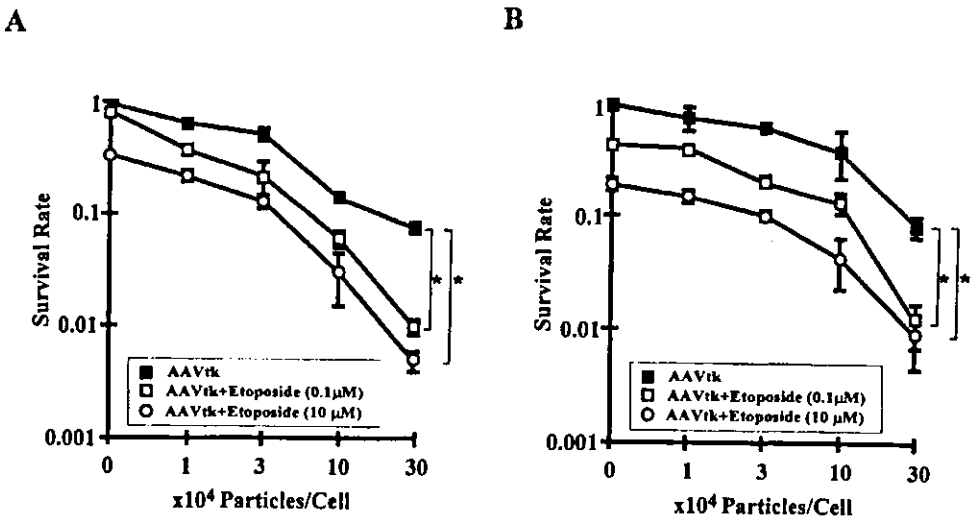


Figure 5. Enhancement of the cytocidal effect of AAVtk by etoposide. HeLa (A) or HEp-2 cells (B) were transduced with various concentrations of AAVtk with or without etoposide treatment. Twenty-four hours after transduction, the cells were treated with 3 μg/ml of GCV. Closed squares: AAVtk-transduced cells. Open squares: AAVtk-transduced with etoposide treatment (0.1 μM). Open circles: AAVtk-transduced cells with etoposide treatment (10 μM). Asterisks mean that the AAVtk-transduced and etoposide treated cells were significantly different from the AAVtk-transduced and non-treated cells (P<0.01).

AAVLacZ-transduced cells (hatched bar) or mock-transduced cells (open bar) (two-way ANOVA: P<0.01).

*The killing of HeLa and HEp-2 cells in terms of AAV vector dose.* Fig. 4 shows the killing effect of GCV (3 μg/ml) on HeLa cells and HEp-2 cells transduced with various doses of AAVtk. When HeLa cells were transduced with $3x10^4$ particles/cell of AAVtk, 50% of the cells were killed upon exposure to GCV. As the dose of AAVtk was increased, the number of surviving cells was reduced. This killing rate was significantly higher than that in the case of AAVLacZ-transduced cells, as expected (Fig. 4A). Similarly, AAVtk-transduced HEp-2 cells were killed by exposure to GCV, which was significantly higher than the killing rate in AAVLacZ-transduced cells (Fig. 4B) (two-way ANOVA: P<0.01).

*Enhanced cytocidal effect of the AAVtk/GCV system by topoisomerase inhibitors (etoposide or camptothecin).* To investigate whether etoposide treatment enhances the killing effect of AAVtk/GCV, HeLa (Fig. 5A) and HEp-2 cells (Fig. 5B) were transduced with various doses of AAVtk following pre-treatment with 0.1 μM or 10 μM etoposide, and then cultured in GCV (3 μg/ml). When HeLa cells were transduced with $3x10^4$ particles/cell of AAVtk, 53% of the 0.1 μM treated cells and 84% of the 10 μM treated cells were killed by the addition of GCV. When HeLa cells were transduced at $3x10^5$ particles/

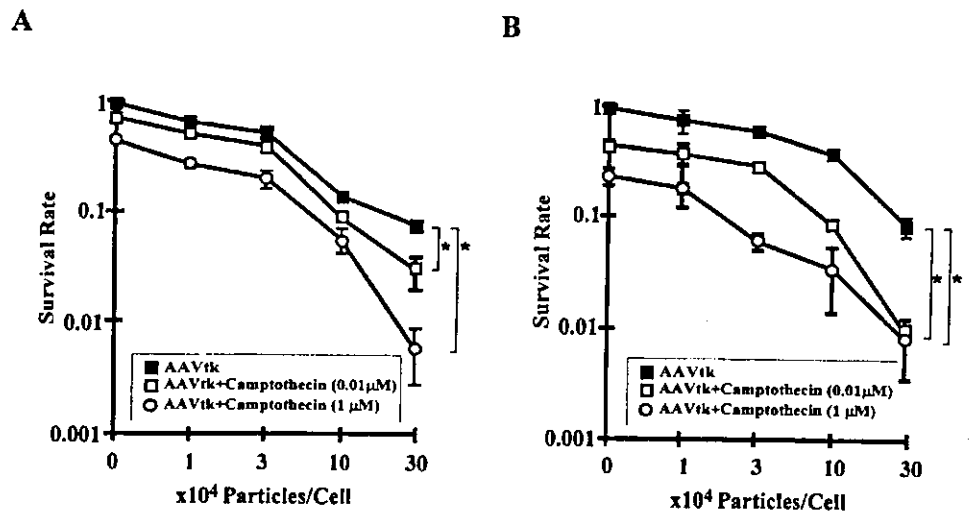


Figure 6. Enhancement of the cytocidal effect of AAVtk by camptothecin. HeLa (A) or HEp-2 cells (B) were transduced with various doses of AAVtk with or without camptothecin treatment. Twenty-four hours after transduction, the cells were treated with 3 μg/ml of GCV. Closed squares: AAVtk-transduced cells. Open squares: AAVtk-transduced with camptothecin treatment (0.01 μM). Open circles: AAVtk-transduced cells with camptothecin treatment (1 μM). Asterisks mean that the AAVtk-transduced and camptothecin treated cells were significantly different from the AAVtk-transduced and non-treated cells ($P<0.01$).

cell, etoposide enhanced the killing effects of AAVtk/GCV system by 21-fold. Etoposide treatment also enhanced the killing effects on HEp-2 cells by 5-fold. The enhancement by etoposide treatment was calculated from the ratio of 10 μM-treated survival rate to non-treated survival rate. These results show that etoposide treatment enhances the killing effects of AAVtk/GCV system significantly (two-way ANOVA: $P<0.01$). Similarly, we examined whether camptothecin treatment enhanced the killing effect of AAVtk/GCV. When HeLa cells were transduced at $3x10^5$ particles/cell, the combined therapy with camptothecin and AAVtk/GCV killed 12-fold HeLa cells (Fig. 6A) and 9-fold HEp-2 cells (Fig. 6B), compared with AAVtk/GCV system alone. These results show that camptothecin treatment also enhances the killing effects of AAVtk/GCV system significantly (two-way ANOVA: $P<0.01$).

## Discussion

The application of AAV vectors to gene therapy for head and neck cancer is limited by their low transduction efficiency. Our previous studies showed the enhancement of AAV vector-mediated transgene expression and killing effect of AAVtk/GCV on the head and neck cancer cells by γ-ray (3). In this study, we demonstrated that topoisomerase inhibitors enhanced AAV vector-mediated transgene expression and cytocidal effect of AAVtk/GCV on target cells. The enhanced transgene expression and killing effect can be explained by the higher conversion efficiency of AAV vector genome to double-stranded form. In fact, we demonstrated that topoisomerase inhibitors augmented the second-strand synthesis and transgene expression on the cancer cells. Topoisomerase inhibitors have been utilized as chemotherapeutic agents. Topoisomerases are known to catalyze the reversible breakage and rejoining of DNA to check the DNA unwind during replication (27). Although etoposide and camptothecin inhibit different enzymes, both can induce similar DNA repair function.

It is unclear what kind of host DNA polymerases require for the conversion of single-stranded vector genomes to double-stranded molecules. According to the study that compared the transduction efficiency of dividing cells to non-dividing cells (13), DNA repair synthesis or other activities associated with DNA repair require transduction rather than replicative DNA synthesis. Some unspecified DNA repair mechanism activated by topoisomerase inhibitors may contribute to enhance the second-strand synthesis of AAV vector genome.

Since the mechanism of enhancement of transgene expression has vital significance for the use of AAV vectors, several studies have focused on this topic. Qing *et al* (28) reported that dephosphorylation of the single-stranded D sequence-binding protein facilitated second-strand synthesis of the AAV vector genome. Sanlioglu *et al* (29) reported that the enhancement of AAV vector transduction by UV and adenovirus E4orf6 correlated with induction of two distinct molecular conversion pathways, and UV led to increased abundance of circular AAV vector genome. However, the precise mechanisms by which genotoxic agents facilitate second-strand synthesis of AAV vector genome remains unknown.

Several studies reported that AAV vectors were useful for the treatment of cancers in model experiments. Kunke *et al* (30) showed that expressing the antisense of human papillomavirus early gene effectively killed the tumors derived from cervical cancer cells. In suicide gene therapy, HSVtk-expressing AAV vectors have been reported in the application to several kinds of cancer. The AAV vectors expressing HSVtk and interleukin 2 effectively killed glioma cells implanted into brains of nude mice (31). The expression of HSVtk driven by a liver-specific promoter via AAV vectors in tumors experimentally produced by implantation of hepatocellular carcinoma cells successfully retarded the tumor progression (32). We previously demonstrated the enhancement of the cytocidal effect of AAVtk/GCV system by γ-ray *in vitro*

and *in vivo* (3,24). As shown in Fig. 4, the AAVtk/GCV system effectively killed the cancer cells depending on the concentration of GCV. Furthermore, when the cancer cells were treated with topoisomerase inhibitors prior to transduction, they were killed more efficiently in a concentration-dependent manner (Figs. 5 and 6).

The combination therapy using other vectors and chemotherapy have been reported (15,33), but the augmentation of the second-strand synthesis by chemotherapeutic agent is unique to AAV vectors. Favorably, apoptosis induced by topoisomerase treatment enhance the bystander effects, which may allow nearby untransduced cells to take up the apoptotic vesicles containing phosphorylated toxic GCV metabolites. Thus, the combination therapy of AAV-mediated suicide gene therapy with chemotherapeutic agents or other genotoxic stress such as radiotherapy seems to be valuable for the treatment of cancers.

Recently, the HSVtk mutants with improved GCV mediated killing and bystander effect have been developed (34,35). Since GCV has side effects such as pancytopenia and acute renal failure, the concentration of GCV should be kept as low as possible. Our model would be another alternative to improve AAV-mediated suicide gene therapy of cancer. Although several studies were reported on combining chemotherapy and viral vector-mediated gene therapy (15,33), our therapeutic model made it with acceptable concentrations of topoisomerase inhibitors. AAV-mediated suicide gene therapy and chemotherapy may provide a more effective and safer alternative for the treatment of head and neck cancer.

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# Translocation and cleavage of myocardial dystrophin as a common pathway to advanced heart failure: A scheme for the progression of cardiac dysfunction

Teruhiko Toyo-Oka*†‡§, Tomie Kawada¶, Jumi Nakata*, Han Xie*, Masashi Urabe‖, Fujiko Masui*, Takashi Ebisawa*, Asaki Tezuka*, Kuniaki Iwasawa†‡, Toshiaki Nakajima‡, Yoshio Uehara†, Hiroyuki Kumagai**, Sawa Kostin††, Jutta Schaper††, Mikio Nakazawa‡‡, and Keiya Ozawa‖

*Department of Pathophysiology and Internal Medicine, †Health Service Center, and ‡Department of Cardiovascular Medicine, University of Tokyo, Tokyo 113-0033, Japan; ¶Division of Pharmacy and ‡‡Department of Medical Technology, Niigata University, Niigata 951-8520, Japan; ‖Division of Gene Therapy, Jichi Medical School, Tochigi 329-0498, Japan; **Department of Pharmacology, Gunma University, Maebashi 371-8511, Japan; and ††Department of Experimental Cardiology, Max Planck Institute, Bad Nauheim 61231, Germany

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**Advanced heart failure (HF) is the leading cause of death in developed countries. The mechanism underlying the progression of cardiac dysfunction needs to be clarified to establish approaches to prevention or treatment. Here, using TO-2 hamsters with hereditary dilated cardiomyopathy, we show age-dependent cleavage and translocation of myocardial dystrophin (Dys) from the sarcolemma (SL) to the myoplasm, increased SL permeability *in situ*, and a close relationship between the loss of Dys and hemodynamic indices. In addition, we observed a surprising correlation between the amount of Dys and the survival rate. Dys disruption is not an epiphenomenon but directly precedes progression to advanced HF, because long-lasting transfer of the missing *δ-SG* gene to degrading cardiomyocytes *in vivo* with biologically nontoxic recombinant adenoassociated virus (rAAV) vector ameliorated all of the pathological features and changed the disease prognosis. Furthermore, acute HF after isoproterenol toxicity and chronic HF after coronary ligation in rats both time-dependently cause Dys disruption in the degrading myocardium. Dys cleavage was also detected in human hearts from patients with dilated cardiomyopathy of unidentified etiology, supporting a scheme consisting of SL instability, Dys cleavage, and translocation of Dys from the SL to the myoplasm, irrespective of an acute or chronic disease course and a hereditary or acquired origin. Hereditary HF may be curable with gene therapy, once the responsible gene is identified and precisely corrected.**

Despite the steady progress of pharmaceutical therapy, it is still difficult to completely prevent heart failure (HF) from proceeding to an advanced stage. Cardiac transplantation is the last choice to save the patient at the end stage, and this treatment entails many sociomedical problems. An alternative strategy for therapy is urgently required (1, 2). Primary or secondary degradation of dystrophin (Dys) might be of great significance in determining the cause of HF. Muscular dystrophy results in HF, and poor outcome in patients and animal models is associated with genetic mutations of Dys or the sarcoglycan (SG) complex (1–6). In the present study, we examined the following phenomena: (*i*) the time course of the hemodynamics with biventricular catheterization under stable anesthesia (7) until the TO-2 animals started to show overt HF and cardiac death; (*ii*) *in situ* sarcolemma (SL) stability by double fluoromicroscopy for the entry of an SL-impermeable dye, Evans blue dye (EB), into cardiomyocytes (8) and immunostaining of Dys or δ-SG; (*iii*) Western blotting of Dys and protein quantification; (*iv*) the correlation between limited proteolysis of Dys and hemodynamics; and (*v*) *in vivo* gene transduction in TO-2 hamsters. We also evaluated pathological features in rats with acute and acquired HF due to isoproterenol (Isp) toxicity (9) and in humans with advanced dilated cardiomyopathy (DCM).

## Materials and Methods

**Experimental Animals, the *rAAV* Vector Gene Construct, and *in Vivo* Gene Delivery.** Male $F_1B$ (control) and TO-2 hamster strains were obtained from Bio Breeders (Watertown, MA), and rAAV/lacZ vector alone or a mixture of recombinant adenoassociated virus (rAAV)/lacZ and rAAV/δ-SG was intramurally injected into the cardiac apex of the 5-week-old hamsters (7). pW1, an rAAV plasmid containing lacZ or a 1.2-kb fragment of δ-SG cDNA flanked by inverted terminal repeats of the AAV genome, pHLP19, a helper plasmid with *rep* and *cap* genes, and pladeno-1, a plasmid harboring the adenovirus *E2A*, *E4*, and *VA* genes, were used for rAAV/lacZ or rAAV/δ-SG production. pWSG with a δ-SG expression cassette driven by a cytomegalovirus (CMV) promoter was used for rAAV/δ-SG production (7, 8). Under open chest surgery with constant-volume ventilation, rAAV/lacZ alone or a mixture of rAAV/lacZ and rAAV/δ-SG was intramurally injected into the cardiac apex twice (each injection was 15 μl, for a total of $8.4 \times 10^{10}$ and $6 \times 10^{10}$ copies for lacZ and δ-SG, respectively).

**Morphological and Immunological Analyses.** A polyclonal, site-directed antibody to δ-SG was prepared at a high titer, by using a synthetic peptide with a sequence deduced from the cloned cDNA as a specific epitope (4). Monoclonal antibodies to Dys and to the transgene of lacZ (β-galactosidase) were obtained from NovoCastra (Newcastle, U.K.) and Funakoshi (Tokyo). The density of antibody-specific bands for the rod domain of Dys was measured within a linear intensity range for the applied amount of protein, after Western blotting of whole-heart homogenates, by 5–15% SDS/PAGE. For the Isp study, 10–20% SDS/PAGE was used to detect degradation products of both Dys and δ-SG. To simultaneously monitor Dys disruption, SL fragility *in situ*, and expression of the δ-SG transgene, double fluoromicroscopy was used to detect immunostaining of Dys with a FITC-labeled antibody specific to the rod domain of Dys, the entry of membrane-impermeable EB into cardiomyocytes, and immunostaining of δ-SG with a rhodium isothiocyanate (RITC)-labeled specific antibody by using a Nikon Diaphot or a Leica (Heidelberg, Germany) TCS SL confocal microscope. Where indicated, the Dys immunoproduct in the SL and myoplasm was semiquantified on cardiomyocytes, with or without transduction of δ-SG in the same observation field.

Abbreviations: HF, heart failure; Dys, dystrophin; SG, sarcoglycan; SL, sarcolemma; EB, Evans blue dye; Isp, isoproterenol; DCM, dilated cardiomyopathy; rAAV, recombinant adenoassociated virus; LVP, left ventricular pressure; EDP, end diastolic pressure; CVP, central venous pressure.

§To whom correspondence should be addressed. E-mail: toyooka_3im@hotmail.com.