

Recombinant Adeno-associated Virus Vectors Efficiently Transduce Foreign Gene Into Bovine Aortic Endothelial Cells: Comparison With Adenovirus Vectors

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ABSTRACT—Because the features and kinetics of adeno-associated virus (AAV)-mediated gene transfer to endothelial cells (EC) are yet to be ultimately determined, we tested variables pertinent to the efficiency of AAV-mediated gene transfer to bovine aortic endothelial cells (BAEC). The variables with AAV vectors were compared with the better characterized adenovirus (Ad) vectors. There is a dose-response relationship between multiplicity of infection (moi) of AAV or Ad vectors and transduction efficiency in BAEC. The higher moi of AAV vectors achieved more than 80% of transduction efficiency in cultured BAEC. AAV and Ad vectors showed an incubation-time-dependent increase in transduction efficiency of LacZ gene to the BAEC up to 12 h of vector exposure. Although the similar kinetics of transduction efficiency of LacZ gene to BAEC was found in both vectors, the duration of gene expression was longer in AAV vector than that in Ad vectors *in vitro*. These results indicate that AAV-vector is efficient for gene transfer to EC, and higher moi of vectors or a longer period exposure of vectors to EC can facilitate efficient transduction of a foreign gene into cultured EC. For the duration of gene expression, the AAV vectors may be better than Ad vectors.

Keywords: Endothelial cell, Gene transfer, Adeno-associated virus vector, Adenovirus vector

Endothelial cell (EC) damage participates in the pathogenesis of many vascular diseases through endothelial migration, oxidant production and modulation of lipoprotein lipase transportation (1–3). EC may be a target cell for gene therapy of various vascular diseases. Several investigators have reported that successful adenovirus (Ad)-mediated gene transfer to EC (4–7), however, the adeno-associated virus (AAV), the other DNA virus vector, mediated gene transfer to EC has not been comprehensively studied.

Although Ad vectors are the widely-tested method for gene delivery to target cells, several hurdles should be considered for controlling vascular disease with gene therapy *in vivo* using Ad vectors. Ad vectors are known to cause extensive host immune and inflammatory responses (8), and the immune response may be responsible for the loss

of transgene expression (9). Furthermore, Ad vector infection affects target cell proliferation and causes cell death, *i.e.*, apoptosis (10–12). Compared with Ad vectors, AAV vectors so far appear to be safe, and the wild-type virus is not known to cause human disease (13). In addition, the long-term expression of therapeutic gene by AAV vectors has been reported up to 180 days in rodents and primates (14, 15), whereas the gene expression by first generation Ad vectors in airway epithelia are more transient *in vivo*. Such comparisons support the feasibility of the AAV vectors for gene therapy in patients with cardiorespiratory diseases (16).

However, many features of AAV vector-mediated gene transfer to endothelial cells have not been elucidated. It has been reported that variables of vector moi, the concentration of vector-containing medium, the duration of vector incubation time, are known to affect the transduction efficiency of AAV vectors in several cells (17, 18). However, effects of the variables of AAV vectors on gene transfer

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efficiency have not yet been conclusively determined in EC.

The purpose of the present study is to determine the variables related to the efficiency of AAV-mediated gene transfer to EC. We examined kinetics of transduction efficiency of the LacZ gene to endothelial cells. In addition, the effects of the concentration and volume of vector-containing medium on transduction efficiency of recombinant AAV (rAAV) vectors are elucidated. We also determined the relationship between the duration of incubation time of rAAV vectors with cells and gene transfer efficiency in EC. Because the Ad vectors are the most widely evaluated as vectors for many gene therapy protocols, these results with rAAV vectors were compared with those with recombinant Ad vectors (rAd vectors).

MATERIALS AND METHODS

rAAV vectors

rAAV vectors are prepared as follows (17): The AAV plasmid vector *pAd11*, which encodes ITRs and β -galactosidase gene, was transfected with the AAV helper plasmid *pAAV/Ad*, which encodes AAV structural proteins (19), via the calcium phosphate method into 293 cells. The next day, cells were infected with an adenovirus DNA followed by deletion of the left-sided terminal of *dl 309* using *XbaI* and *ClaI*. After 48–72 h, viruses were harvested from 293 cells by multiple freeze/thaw cycles. rAAV vectors were purified by double CsCl gradient ultracentrifugation, separated serially into small volume fractions (200 μ l/tube) using a fraction collector (Model 2110; BioRad, Hercules, CA, USA), and stored at -70°C until used for infection of endothelial cells. Vectors titer [transducing unit (TU/ml)] was determined from the number 293 cells expressing LacZ measured histochemically per milliliter of vector in the presence of wild type adenovirus [multiplicity of infection (moi)=2]. This procedure typically produced vector lots with titers of $4-10 \times 10^8$ transducing units per milliliter (TU/ml). The ratio of TU to genome-based viral particle number was approximately 1:100. Particle numbers were determined by Southern blot analysis of purified vector DNA (19). The AAV vector lots were not heat inactivated, because this rAAV vector preparation method using left-sided deleted adenovirus DNA theoretically produces AAV free of wild type adenovirus (20).

Adenoviral vectors

Replication defective adenoviral vectors based on human Ad 5 serotype (hAd5) were used for the study. In the hAd5-CMV-LacZ, E1 and E3 sequences were deleted and replaced with a gene containing the CMV promoter and a cytoplasmic LacZ gene inserted at the site of the E1 deletion of Ad (10–12). The Ad vectors were propagated in 293 cells, purified by CsCl gradient ultracentrifugation and

stored at -70°C until used for infection of endothelial cells. The rAd vectors titers (TU/ml) were determined by the number of LacZ gene expressing 293 cells per milliliter of vectors by histochemical X-gal staining (16–20). The experiments used rAd vectors produced with titers of $1-4 \times 10^{11}$ (TU/ml). The viral particle number was estimated by the equation that 1.0 A as measured by OD at 260 nm is equal to 1×10^{12} particles (21). The ratio of TU to viral particle number was approximately 1:20. The moi was determined by TU/cell.

Cell cultures

Bovine aortic endothelial cells (BAEC) were obtained from the American Type Culture Collection (Rockville, MD, USA) (22). All experiments using BAEC were performed on a single clone, between passage 5 to 7. The cells were fed on alternate days with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

rAAV vector or rAd vector infection of bovine aortic endothelial cells

BAEC were plated at 1×10^5 cells per dish in 12-well supports (Costar, Cambridge, MA, USA), allowed to attach for 12 h, and then non-adherent cells were removed by gentle washing with phosphate-buffered saline (PBS). One day after plating, the total cell number/dish equaled approximately 2×10^5 ($n=5$). Dishes were then randomly assigned to one of seven experimental groups: moi equal to 0.1, 1, 10, 100, 1000 or vehicle control (DMEM + 0.4% FBS media). rAAV vectors or rAd vectors were added to the dishes in 0.5 ml of DMEM with 0.4% FBS, exposed to the cells for predetermined times at 37°C in air plus 5% CO_2 , following which the cells were washed with PBS and fed with fresh DMEM with 0.4% FBS. The control group was exposed to 0.5 ml of vehicle (DMEM + 0.4% FBS) alone for comparable time periods and washed as above.

Effect of the concentration and volume of vector-containing medium on transduction efficiency of rAAV vectors or rAd vectors

In order to elucidate the effect of the concentration of vector-containing medium on transduction efficiency of rAAV vectors or rAd vectors in BAEC, the adherent cells in 12-well supports one day after plating were exposed to a different concentration of rAAV vectors or rAd vectors in a volume of medium for 1 h. We next tested the effect of the volume of vector-containing medium on transduction efficiency of each vector in BAEC. The cells were exposed to a concentration of each vector in different volumes of media ranging from 0.3 ml to 2.0 ml in 12-well supports. The use of 0.5 ml of the media is necessary to cover the surface of a well of 12-well supports. One day after infection, the transduction efficiency was quantitated as the

percentage of LacZ expressing cells using X-gal staining (23–25).

Effect of the cell density on transduction efficiency of rAAV vectors or rAd vectors

The first series of experiments were done on 2×10^5 cells of BAEC, which were approximately 50% of confluence in 12-well supports. Because the cell density seems to affect efficiency of several transduction methods, we examined the transduction efficiency of both vectors in confluent cells, whose density is 100% in 12-well supports.

BAEC were plated at 1×10^5 cells per dish in 12-well supports, allowed to attach for 12 h, and then non-adherent cells removed by gentle washing with PBS. Seven days after plating, the cells were confluent in the 12-well supports. The total cell number/dish equaled approximately 1×10^6 . Dishes were then randomly assigned to one of seven experimental groups: moi equal to 0.1, 1, 10, 100, 1000 or vehicle control (DMEM + 0.4% FBS media). rAAV vectors or rAd vectors were added to the dishes in 0.5 ml of DMEM with 0.4% FBS and exposed to the cells for 1 h at 37°C in air plus 5% CO₂, following which the cells were washed with PBS and fed with fresh DMEM with 0.4% FBS. The control group was exposed to 0.5 ml of vehicle (DMEM + 0.4% FBS) alone for comparable time periods and washed as above.

One day after infection, the transduction efficiency was quantitated as the percentage of LacZ expressing cells using X-gal staining (23–25).

Assessment of the duration of transduction efficiency of rAAV vectors or rAd vectors in bovine aortic endothelial cells

To assess the duration of transgene expression after vector infection, X-gal staining was performed on every day up to 4 weeks after rAAV vector or vehicle exposure. Because the cells were almost confluent 7 days after the infection with each vector, the cells were trypsinized and sub-cultivated every week. In all experiments, at least three wells for each group were stained for LacZ gene expression and more than 500 cells/well were counted to determine the percentage of LacZ expressing cells.

Statistical analyses

Data were presented as the mean \pm S.E.M. Analysis of variance (ANOVA) with Fisher's protected least significant difference method was used for comparing the data in the same group. The analyses were performed by Stat View 4.0 (Abacus Concepts, Inc., Berkeley, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Transduction efficiency of rAAV vectors or rAd vectors in BAEC

The dose-effect relationships between each vector moi and transduction efficiency in BAEC was investigated. Although 1 moi of vectors minimally transduces a foreign gene into the cells, 1000 moi of each vector transduce nearly 100% of EC cells in culture (Fig. 1).

Effect of the concentration and volume of vector-containing medium on transduction efficiency of rAAV vectors or rAd vectors in BAEC

In rAAV vectors, the LacZ transduced cells were clearly increased in a vector concentration-dependent manner. A concentration of 10^{10} particles per ml of rAAV vectors achieved more than 80% transduction efficiency of rAAV vectors in BAEC in 12-well supports. This may be consistent with the relationship between the vector moi and the transduction efficiency in BAEC. There is a similar relationship between rAd vector concentration and the transduction efficiency. However, 10^{10} particles per ml of rAd vectors achieved nearly 100% transduction efficiency of rAd vectors in BAEC. Because the ratio of virus particles to transducing units was 100 in rAAV vectors and 20 in rAd vectors, the transduction efficiency of rAd vectors was better than that of rAAV vectors at the same particle numbers per ml.

Compared with the effect of the concentration of both vectors, the effect of the depth of vector-containing medi-

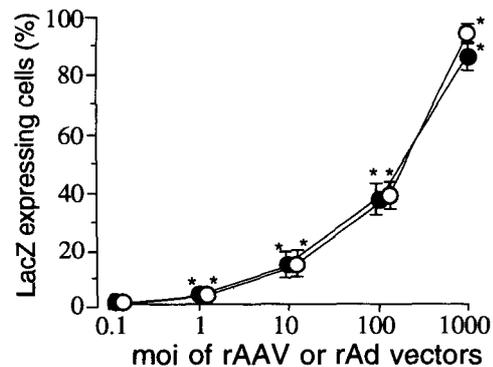


Fig. 1. Transduction efficiency of rAAV and rAd vectors in bovine aortic endothelial cells. The sub-confluent (2×10^5) BAEC cells were exposed to rAAV or rAd vectors for 1 h. The volume of vector-containing medium is 0.5 ml. Values represent the mean \pm S.E.M., $n = 5$, moi = multiplicity of infection; * $P < 0.05$, compared with the corresponding value at a moi of 0.1. The moi was determined as transducing unit (TU)/cell. The ratio of TU to rAAV particle number was approximately 1:100. The lot of rAAV vectors was 4×10^{10} particles/ml. The ratio of TU to rAd particle number (as measured by OD at 260 nm) was approximately 1:20. The lot of rAd vectors was 2×10^{12} particles/ml. ● rAAV vector, ○ rAd vector.

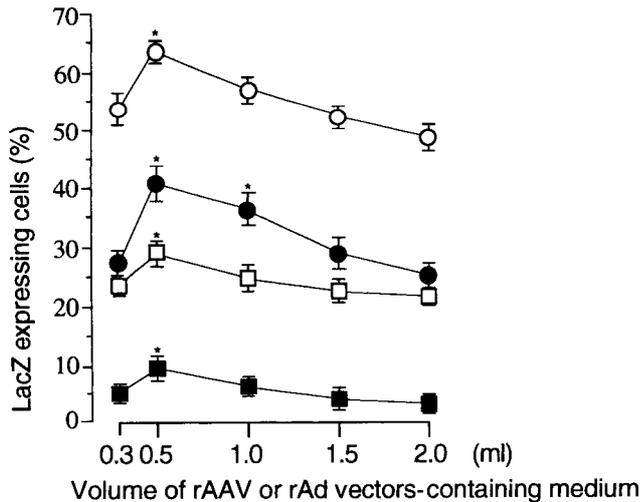


Fig. 2. Effect of the volume of vector-containing medium on transduction efficiency of rAAV and rAd vectors in bovine aortic endothelial cells. Values represent the mean \pm S.E.M., $n=5$; the concentration of rAAV or rAd vector in medium was kept constant; 2×10^5 BAEC cells were exposed to rAAV or rAd vectors for 1 h. * $P < 0.05$, compared with the corresponding value at a volume of 0.3 ml. rAAV vector: ● 10^9 (particles/ml), ■ 10^8 (particles/ml); rAd vector: ○ 10^9 (particles/ml), □ 10^8 (particles/ml).

um on transduction efficiency of the vectors in BAEC was not obvious (Fig. 2). The minimum volume, such as 0.5 ml, of the vector-containing media, which covered a well of the 12-well supports, was better for transduction of LacZ gene in BAEC than too much or less volume of the media.

Effect of prolonged incubation time on the transduction efficiency of rAAV and rAd vectors in BAEC

Both vectors showed an incubation time dependent increase in transduction efficiency of LacZ gene to the BAEC up to 12 h of vector exposure and the percentage of LacZ expressing cells plateaued at 24 h of vector exposure (Fig. 3). The increased efficiency of vector transduction with longer periods of incubation with rAAV or rAd vectors occurred with relatively similar kinetics in BAEC. No greater further effects was detected in more than 24-h incubations with rAAV or rAd vectors.

Effect of the cell density on transduction efficiency of rAAV vectors or rAd vectors

Both vectors showed a vector moi-dependent increase in transduction efficiency of LacZ gene to the BAEC in post-confluent-cultures (Fig. 4). The transduction efficiency of rAAV and rAd vectors in confluent cells, whose cell density was 100%, was similar to that in the sub-confluent and/or growing cells (Figs. 1 and 4). Transduction efficiency of rAAV or rAd vectors at a moi of 0.1, 1, 10, 100 and 1000

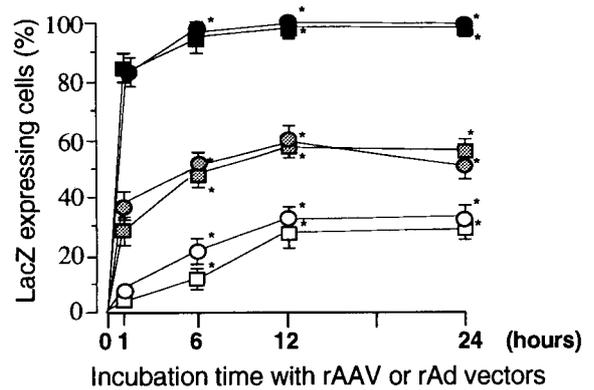


Fig. 3. Effect of prolonged incubation time on transduction efficiency of rAAV and rAd vectors in BAEC cells. Values represent the mean \pm S.E.M., $n=5$; moi = multiplicity of infection; * $P < 0.05$, compared with the corresponding value at 1-h incubation of rAAV vectors with cells. The lot of rAAV vectors was 10×10^{10} particles/ml. The lot of rAd vectors was 8×10^{12} particles/ml. rAAV vector: ■ moi = 1000, □ moi = 100, ○ moi = 10; rAd vector: ● moi = 1000, ○ moi = 100, ○ moi = 10.

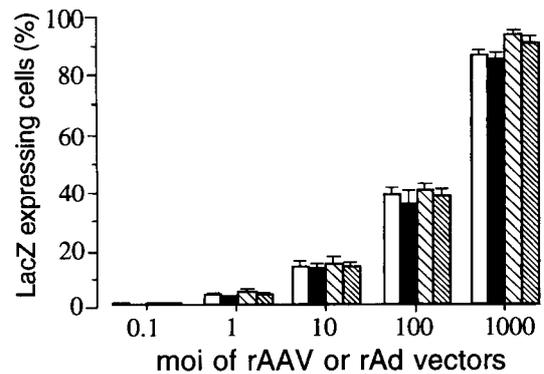


Fig. 4. Transduction efficiency of rAAV and rAd vectors in BAEC on post-confluent-culture. The confluent ($1 - 2 \times 10^6$) BAEC cells were exposed to rAAV or rAd vectors for 1 h. Values represent the mean \pm S.E.M., $n=5$; moi = multiplicity of infection. The mean value of the particles/ml of the lots of rAAV vectors was 10×10^{10} . The mean value of the particles/ml of the lots was 8×10^{12} . rAAV vector: □ subconfluent cells, ■ post-confluent cells; rAd vector: ▨ subconfluent cells, ▩ post-confluent cells.

was not different between the sub-confluent cells and post-confluent cells in vitro.

The duration of rAAV-mediated transgene expression in BAEC

The kinetics of transgene expression after rAAV or rAd vector infection is depicted in Fig. 5. The maximal gene expression by rAAV vectors was observed three days after infection in BAEC, whereas the maximal gene expression by rAd vectors was obtained one day after the administration. For rAAV vectors, the percentage of LacZ transduced cells

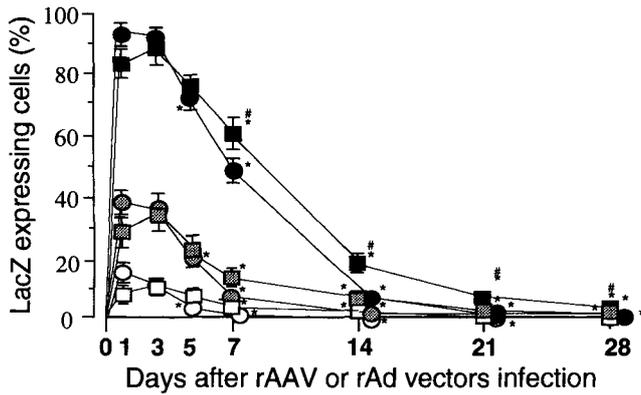


Fig. 5. Changes in the percentage of LacZ gene expressing cells at various days after rAAV and rAd vectors infection in BAEC cells. Values represent the mean \pm S.E.M., $n = 5$; moi = multiplicity of infection; * $P < 0.05$, compared with the corresponding value at day 1; # $P < 0.05$, compared with the corresponding value at the same moi of rAd vectors. The lot of rAAV vectors was 4×10^{10} particles/ml. The lot of rAd vectors was 2×10^{12} particles/ml. rAAV vector: ■ moi = 1000, ▨ moi = 100, □ moi = 10; rAd vector: ● moi = 1000, ⊙ moi = 100, ○ moi = 10.

slightly decreased with days in culture, but the gene expression could be found 4 days after the infection at a higher moi. For rAd vectors, the percentage of LacZ transduced cells consistently decreased with time in culture one day after rAd vector administration for BAEC. Three weeks after the cells were infected with rAd vectors, no LacZ gene expression could be observed in BAEC in culture. Because of the differences in kinetics of gene expression, significant differences in transduction efficiency in BAEC between rAAV vectors and rAd vectors appeared seven days after vector administration.

DISCUSSION

Several investigators have implied that recombinant AAV-mediated gene transfer to endothelial cells is potentially a powerful tool for in vivo delivery of a foreign gene to the endothelium (26–29). Because the endothelial cells play major roles in various pathologic processes of vascular disease (1–3), a therapeutic gene transfer to endothelial cells is a candidate means for the treatment of many vascular disorders. Although several studies have revealed that a certain gene transfer to endothelial cells is completed by rAAV vectors (25–28), the variables pertinent to the maximum efficiency of rAAV-mediated gene transfer to EC have not yet been fully elucidated. This study was conducted to investigate variables affecting optimal transduction efficiencies of AAV vectors in endothelial cells. We examined transduction efficiencies of rAAV vectors in BAEC as a function of moi (Fig. 1). A dose-dependent relationship between the moi of rAAV vectors and the percentage

of LacZ expressing cells was investigated. More than 80% transduction efficiency was achieved with higher vector moi (>1000) of vectors. These results suggest that rAAV vectors are effective for transducing a foreign gene into EC in a dose-dependent manner.

To date, replication-defective adenoviruses, i.e., Ad vectors, are being evaluated as vectors for many human gene therapy clinical or preclinical trials and are the most advanced method for gene delivery for cardiovascular diseases (30, 31). We compared the efficiency and kinetics of AAV-mediated gene transfer to endothelial cells with those of Ad vectors. A immediate transduction efficiency of rAAV vectors in EC are not different from that of rAd vectors. There is a similar dose-dependent relationship between the vector moi and LacZ expressing cells in each vector.

We next examined the influences of the concentration and volume of vector containing media on gene transfer efficiency of rAAV and rAd vectors in BAEC. The LacZ expressing cells after rAAV or rAd vector infection were markedly increased in a higher concentration of rAAV vectors. More than 10^9 particles per ml of rAAV and rAd vectors could achieve approximately 50% and 70% transduction efficiency in BAEC in 12-well supports, respectively. Because the ratio of virus particles to transducing units in rAAV vectors was fivefold greater than that in rAd vectors, the transduction efficiency of rAd vectors was better than that of rAAV vectors at the same particle numbers per ml. However, there was no difference in gene transfer efficiency between rAAV and rAd vectors at the same transducing unit/cell, i.e., moi. Although the vector concentration significantly affects the transduction efficiency of both vectors, there was a small effect of the volume of vector-containing medium on transduction efficiency of rAAV or rAd vectors in BAEC (Fig. 2). The 0.5 ml of vector-containing media in a well of the culture dish transduced LacZ gene into EC better than the larger volume of media. However, the volume of 0.3 ml was not sufficient to overlay the well of the culture dish, resulting in less transduction efficiency of rAAV or rAd vectors in EC. These results indicate that the transduction efficiency of rAAV or rAd vectors in BAEC may be optimal when higher concentrations or lower volumes of vector containing medium were utilized in culture.

We further tested the variable of incubation time on the transduction efficiency of rAAV or rAd vectors in BAEC. Both vectors exhibited a significant incubation time-dependent increase in transgene expression up to 12 h (Fig. 3). These observations suggested that the nature of rAAV vectors transduction of endothelial cells was similar to that of rAd vectors. The mechanism accounting for the phenomenon was not determined by the present study. However, the similar relationship between incubation time of vectors

with cells and transduction efficiency has been observed in airway cells (17).

Information on the effects of cell density may be important, since cell density or cell cycles are likely to affect efficiency of several transduction methods. We compared the transduction efficiency of rAAV or rAd vectors in BAEC between the sub-confluent cells and cells in post-confluent-cultures. Although the AAV preferentially transduces a foreign gene to airway cells in the S phase (32), there was no difference in transduction efficiency of AAV vectors between the sub-confluent/growing BAEC cells and post-confluent/slowly growing BAEC cells. The similar results in BAEC were obtained by rAd vectors. These observations indicated that both vectors could efficiently transduce foreign gene to confluent cells, which are not growing/dividing, *in vitro*. However, the efficiency of transduction in post-confluent-cultures may not always be feasible to predict results of transduction efficiency of rAAV and rAd vectors *in vivo*. Another mature culture model like freshly excised human endothelium *ex vivo* may be a better predictor of transduction efficiency of gene transfer vectors *in vivo*.

Because the transient gene expression of Ad vectors may be a limitation of gene therapy using Ad vectors (9), we finally examined the gene expression *in vitro* for a maximum period of 4 weeks after the vector administration. The results indicated that there was a different pattern of gene expression kinetics between rAAV vectors and rAd vectors. The maximal LacZ gene expression in BAEC occurred 3 days after infection of rAAV vectors, whereas the maximal LacZ gene expression in BAEC was investigated 1 day after infection of Ad vectors. As to transduction efficiency of rAAV, second strand DNA synthesis may be rate limiting (33). The differences in prolonged transgene expression between Ad vectors and rAAV vectors may reflect different effects of vectors on the biology of target cells (17). Because replication-deficient Ad exhibit untoward effects on infected cells including triggering apoptotic cell death (10–12), it is likely that the duration of expression from Ad vectors *in vitro* is limited in part by cell life span. rAAV vectors totally removed the inherit major genes of wt-AAV, *i.e.*, *rep* and *cap*, suggesting that rAAV infection does not affect the life-span of infected cells.

The relatively stable gene expression of rAAV vectors in EC is in agreement with the duration of rAAV-mediated foreign gene expression in other cells (17, 18). Because the gene delivery by AAV vector is rarely integrated into the host genome, the dilution of vectors during cell division may have occurred in BAEC.

The major limitation of the current study is the results were obtained from bovine, but not human, endothelial cells. There is no doubt that human endothelial cells are the best target cells *in vitro* system. However, the pheno-

type difference between the bovine endothelial cells and human endothelial cells must be smaller than the difference between any endothelial cell and other cell types including epithelial cells, muscle cells and neurons. In fact, the transduction efficiency of rAAV vectors in BAEC are better than those in human airway epithelial cells and similar to fibroblasts (17).

In summary, this study describes variables affecting the efficiency of AAV- or Ad- mediated gene transfer to endothelial cells in culture. A dose-dependent relationship between vector moi and transduction efficiency was investigated. Furthermore, the period of maximal gene expression by AAV vectors in BAEC was longer than that by Ad vectors. rAAV vectors may be useful to achieve efficient gene transfer to endothelial cells *in vitro*, and they may be a candidate means for delivering a therapeutic gene to the endothelium *in vivo*.

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