—Review—

Serotype-independent Method of Recombinant Adeno-associated Virus (AAV) Vector Production and Purification

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Abstract

A variety of gene transfer strategies have been developed to treat inherited, degenerative, and acquired diseases. Among the different vector systems developed so far, recombinant adeno-associated viral (AAV) vectors have shown notable benefits, including prolonged gene expression, transduction of both dividing and nondividing cells, and a lack of pathogenicity caused by wild-type infections. Thanks to these features, the use of AAV vectors as a gene transfer tool has increased dramatically during the past several years, and several recent clinical trials have used AAV vectors. However, AAV vectors are more complicated to produce than are other viral vectors. With steady advances toward clinical application, much effort has been made to isolate novel AAV serotypes and to develop methods for their efficient, scalable, and versatile production and purification. Here we review state of the art methods for AAV vector production and purification, which we have refined in our laboratory.

(J Nippon Med Sch 2012; 79: 394–402)

Key words: AAV vector, Serotype, Self-complementary AAV, Purification

I. Introduction

Recombinant adeno-associated viral (AAV) vectors are a promising tool for gene transfer. They have been widely used for gene delivery in animal models and are being evaluated for use in human gene therapies[1–3]. The increasing interest in AAV vectors is justified by their unique features, which distinguish them from many other viral vector systems, such as the retro/lentiviral and adenoviral vectors, and make them an attractive tool for gene therapy. The benefits of using AAV vectors for gene delivery include: 1) safety due to their lack of pathogenicity; 2) broad ranges of host and cell-type tropism; 3) ability to transduce both dividing and nondividing cells, in vitro and in vivo; 4) heat stability and resistance to solvents and changes in pH and temperature; 5) prolonged high-level gene expression in vivo; and 6) absence of a significant immune response[4]. Furthermore, the recent discovery of novel AAV serotypes[5,6] will further expand the universe of potential target organs, tissues and cells, enabling in vivo vector

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Table 1 AAV serotypes and their receptors

Serotype	Host/Origin	Receptor	Co-receptor
AAV1	Monkey	2,3N/2,6N-sialicAcid	Unknown
AAV2	Human	HSPG	FGFR-1, integrin, HGFR, LamR
AAV3	Human	HSPG	FGFR-1, HGFR, LamR
AAV4	Monkey	2,3O-sialic acid	Unknown
AAV5	Human	2,3N-sialic acid	PDGFR
AAV6	AAV1xAAV2	2,3N/2,6N-sialicAcid	EGFR
AAV7	Monkey	N-sialic acid	PDGFR
AAV8	Monkey	Unknown	LamR
AAV9	Human	N-galactose	LamR
AAVrh.10	Monkey	Unknown	Unknown
AAV10	Monkey	Unknown	Unknown
AAV11	Monkey	Unknown	Unknown
AAV12	Monkey	Unknown	Unknown

HSPG: heparan sulfate proteoglycan, FGFR: fibroblast growth factor receptor 1, HGFR: hepatic growth factor receptor, PDGFR: platelet derived growth factor receptor, LamR: 37/67 KD laminin receptor, EGFR: epidermal growth factor receptor

transduction to be expanded substantially and offering alternatives to the more studied AAV serotype 2.

2. AAV Vector

2-1. AAV Vector Serotypes

Among the more than 100 nonredundant AAV genotypes that have been identified[6], 12 AAV serotypes with unique properties have been used to produce most expression vectors[7]. **Table 1** shows the primary receptors of these 12 vectors. Because the surface of the AAV capsid is an essential component in the binding of the virus to the target cell and its subsequent internalization and intracellular trafficking, the serotype should be carefully considered in the context of the target organ and the level and duration of transgene expression, among other factors. We used an in vivo imaging system (IVIS 100; Xenogen, Alameda, CA, USA) to assess the luminescence obtained after intravenous injection of several AAV vectors encoding the luciferase reporter gene (**Fig. 1**). With the exception of the serotype 4 vector, which primarily transduced lung, all of the AAV vectors transduced mainly liver and muscle. On the basis of the levels of luciferase expression, the vectors were divided into 3 groups: low expression (serotypes 2 and 4), medium expression (serotypes 1, 5, and 10), and high expression (serotypes 7, 8, and 9). **Table 2** lists the AAV serotypes, their expression level, and appropriate target organs. On the basis of the results, serotype 9 is recommended for the transduction of the central nervous system and heart, serotype 6 for transduction of the spinal cord, serotype 8 for transduction of muscle and the retina, and serotype 4 for transduction of the lung.

2-2. Self-complementary AAV Vector

Because the AAV genome is packaged as linear single-stranded DNA (ssDNA), to express a transgene in target cells transduced by an AAV vector, the ssDNA genome must first be converted to double-stranded DNA (dsDNA). This critical step is a key determinant of the level of transgene expression. Notably, this step can be entirely circumvented through the use of self-complementary AAV (scAAV) vectors, which package an inverted repeat genome that can fold into dsDNA without the need for DNA synthesis or base-pairing between multiple vector genomes[8,9]. **Figure 2** shows green fluorescent protein (GFP) expression in several organs transduced with an ssAAV or scAAV vector encoding GFP. The scAAV vector was able to transduce all organs more efficiently than was the ssAAV. Thus, an scAAV vector is advantageous for obtaining high levels of transgene expression.

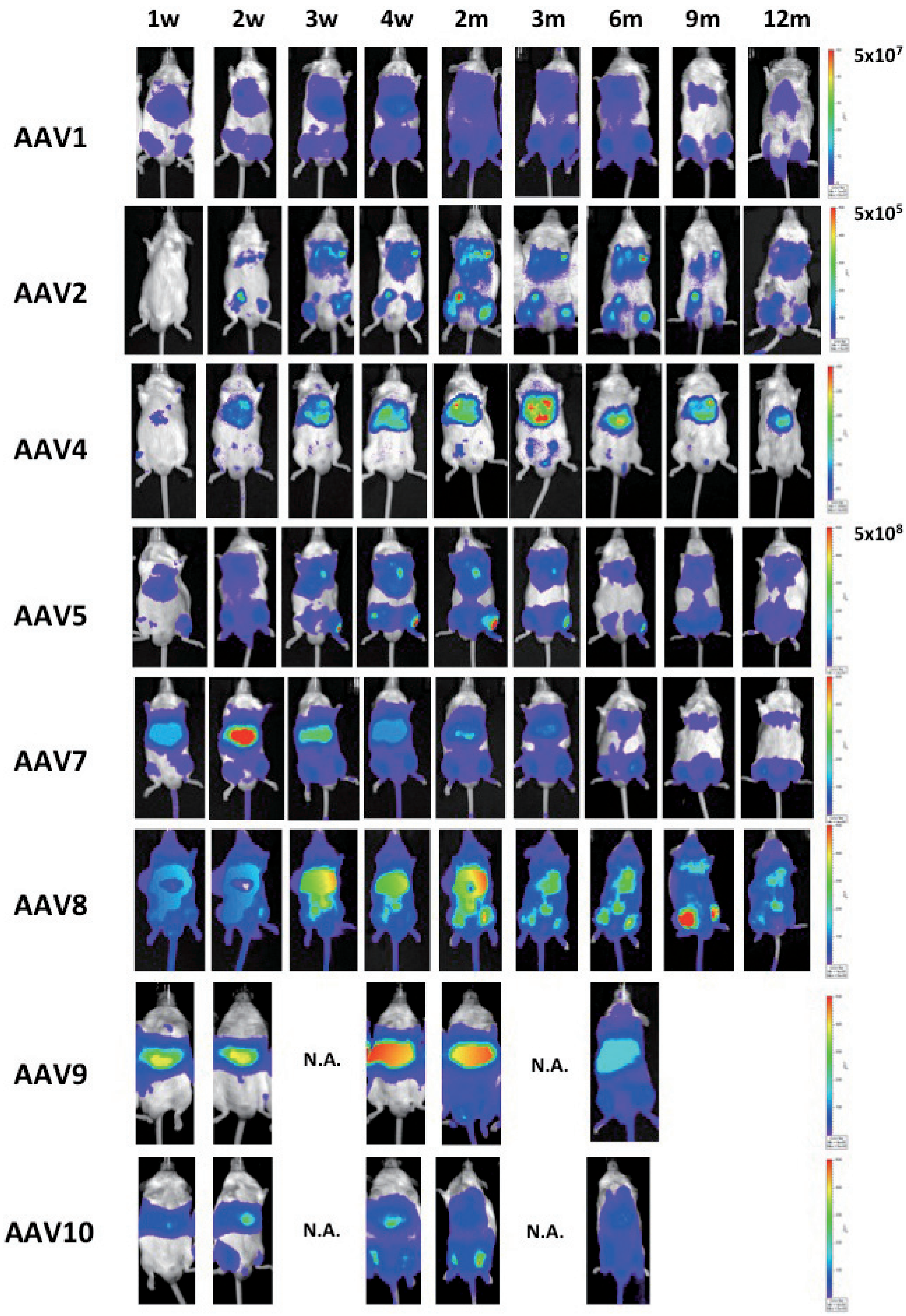

Fig. 1 Direct comparison of AAV serotypes using the IVIS in vivo imaging system
After the same amount of AAV vectors expressing the luciferase gene (serotypes 1, 2, 4, 5, 7, 8, 9, and 10: 1×10^{12} vector genome/mouse) were injected into DDY mice via the tail vein, luciferase activities were analyzed with the IVIS in vivo imaging system for 1 year.

Table 2 AAV serotypes, their expression levels, and appropriate target organs

Serotypes	Expression level	Target organs
AAV1	medium	muscle, liver, CNS
AAV2	low	CNS, liver
AAV3	low	liver (cancer cells)
AAV4	low	lung, ependymal cell
AAV5	medium	muscle, liver, CNS, retina
AAV6	medium	muscle, liver, spinal cord
AAV7	high	muscle, liver
AAV8	high	muscle, liver, retina
AAV9	high	CNS, heart, muscle, liver
AAV10	medium	CNS, muscle, liver

CNS: central nervous system

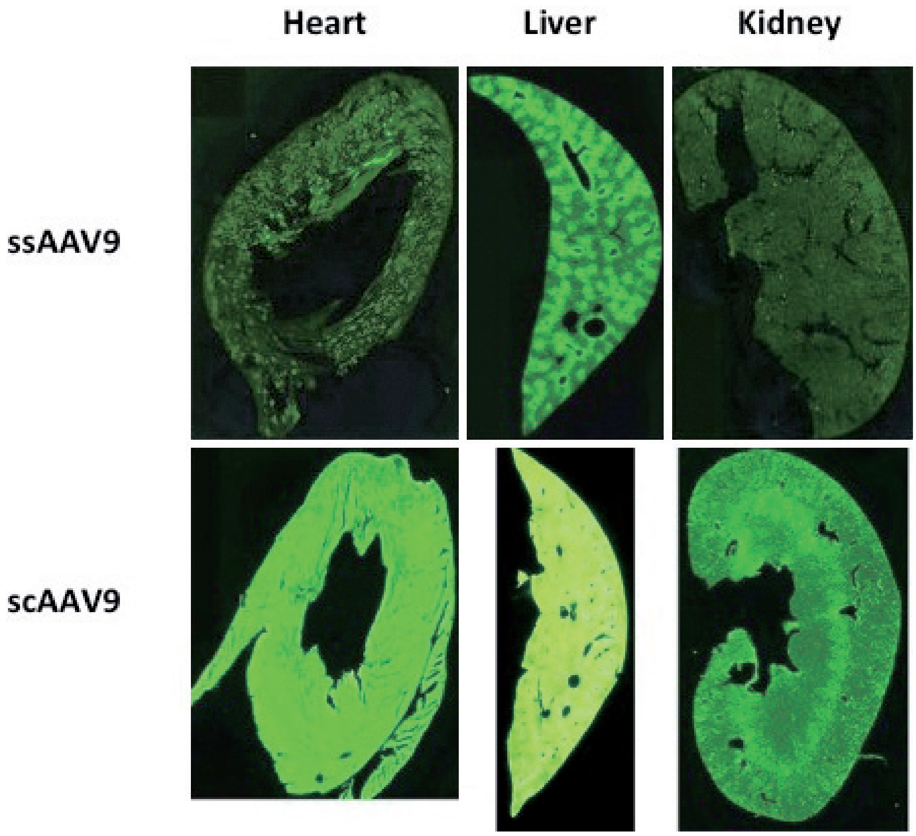

Fig. 2 Direct comparison of ssAAV and scAAV
After the same amounts of ssAAV or scAAV vectors expressing the GFP gene (serotype 9: 7×10^{12} vector genome/mouse) were injected into C57BL/6 mice via the tail vein, GFP expression was analyzed with immunohistochemical staining using an anti-GFP antibody.

3. Production of AAV Vectors

As was mentioned earlier, AAV vectors have emerged as an attractive means of gene transfer, especially in vivo. However, AAV vectors are more complicated to produce than are other viral vectors. Extensive study of these vectors for various applications has resulted in a wide variety of approaches to their production[10–13]. Most established

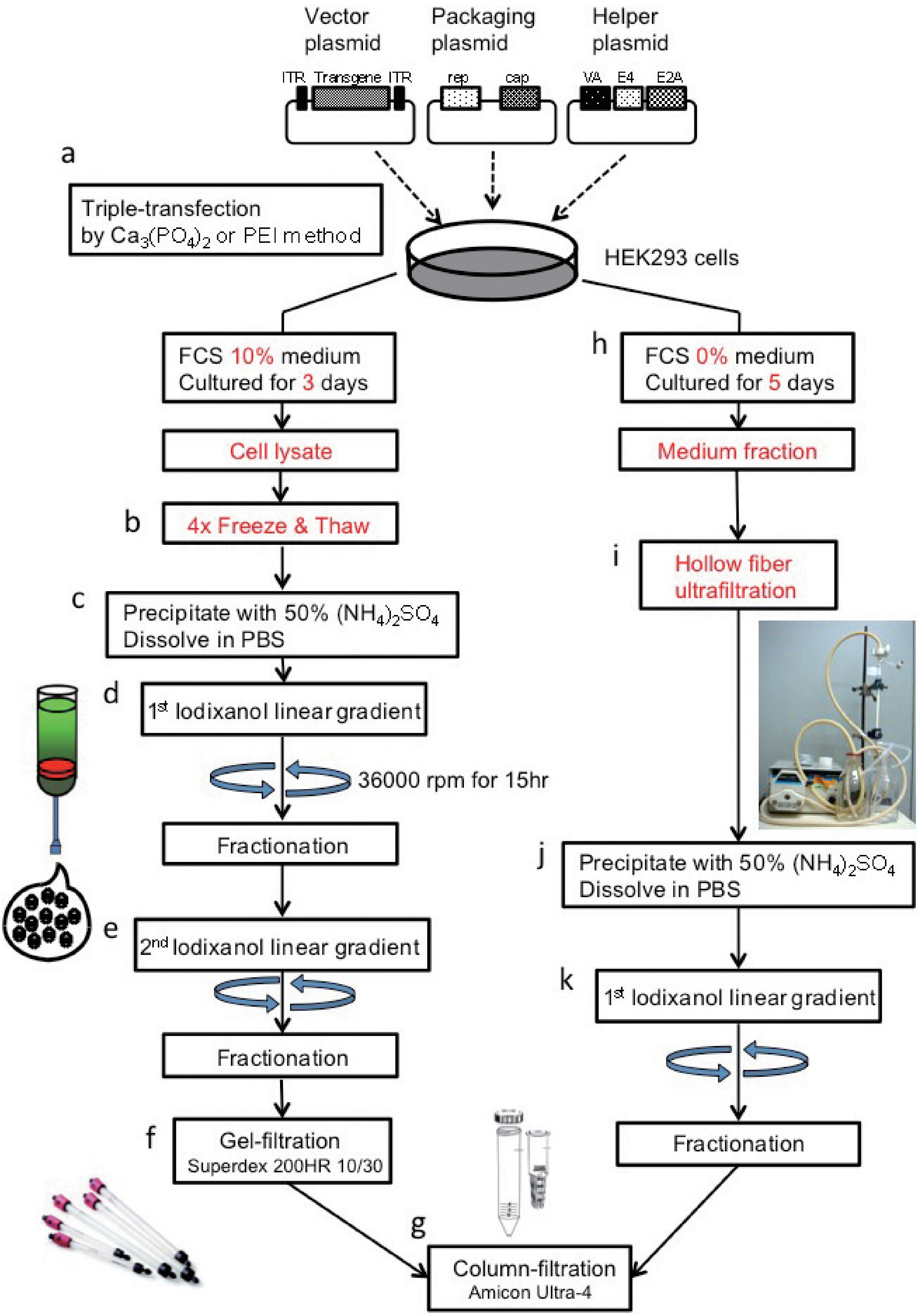
Vector plasmid
Packaging plasmid
Helper plasmid
ITR Transgene ITR
rep cap
VA E4 E2A
a
Triple-transfection by $Ca_3(PO_4)_2$ or PEI method
HEK293 cells
FCS 10% medium Cultured for 3 days
Cell lysate
b
4x Freeze & Thaw
c
Precipitate with 50% $(NH_4)_2SO_4$ Dissolve in PBS
d
1st Iodixanol linear gradient
36000 rpm for 15hr
Fractionation
e
2nd Iodixanol linear gradient
Fractionation
f
Gel-filtration Superdex 200HR 10/30
g
Column-filtration Amicon Ultra-4
h
FCS 0% medium Cultured for 5 days
Medium fraction
i
Hollow fiber ultrafiltration
j
Precipitate with 50% $(NH_4)_2SO_4$ Dissolve in PBS
k
1st Iodixanol linear gradient
Fractionation

Fig. 3 Flow diagram of the production of an AAV vector
(a) Transfection: Human embryonic kidney (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum with 1% penicillin/streptomycin. HEK293 cells at approximately 70% confluence were transfected with the AAV vector plasmid (with AAV inverted terminal repeats [ITRs]), the packaging plasmid (with AAV rep and cap), and adenovirus helper plasmid (pHelper; Agilent Technology, Santa Clara, CA, USA), at a ratio of 2 : 1 : 1. Six hours after transfection, the medium was replaced with fresh culture medium, and the cells were cultured for 48 hours at 37℃. (b) Freeze and thaw: After incubation, the cells were harvested and pelleted by means of centrifugation at 3,000*g* for 20 minutes at 4℃. The pellets were then resuspended in PBS (pH 8.5), and the cells were subjected to 4 freeze-thaw cycles (Dry-ice in ethanol/37℃ in water bath). (c) Precipitaion with ammonium sulfate: The cell suspension was treated with Benzonase (Merck KGaA) at 37℃ for 20 minutes to digest the cellular genomic DNA and plasmids, followed by the process of the half-saturated ammonium sulfate precipitation (pH 8.5) on ice for 20 minutes. The resultant pellets were separated with centrifugation at 12,000 rpm for 30 minutes at 4℃. (d) First iodixanol linear gradient centrifugation: The AAV vector contained cell pellets that were dissolved in PBS, and the viral solution was layered with Optiprep (iodixanol; Axis-Shield plc, Oslo, Norway); after iodixanol continuous gradient centrifugation at 36,000 rpm for 15 hours at 16℃, the viral fractions were collected from the bottom of the gradient. Then the physiological or biological viral titer of each fraction was measured with real-time quantitative polymerase chain reaction or direct-transduction of the cultured cell lines. (e) Second iodixanol linear gradient centrifugation: The vector-containing fractions of the first iodixanol linear gradient centrifugation were mixed and reloaded onto an iodixanol continuous gradient for further purification with the same conditions as for the first one. (f) Gel filtration: Size-exclusion chromatography was performed with an AKTA Explorer 100 HPLC system (GE Healthcare) equipped with a 2-mL sample loop. A Superdex 200 HR 10/30 GL column (GE Healthcare) was equilibrated with MHA buffer (3.3 mM MES, 3.3 mM HEPES, 3.3 mM NaOAc, 50 mM NaCl, pH 6.5). The vector-containing fractions were loaded onto the column at a flow rate of 0.5 mL/min, and the eluate was collected as 0.5-mL fractions over the duration of 1 column volume (23 mL). The AAV peak fractions were identified with absorbance at 280/260 nm and real-time quantitative polymerase chain reaction with vector-specific primers. (g) Concentration by column filtration: The purified AAV vectors were then concentrated with Amicon Ultra-4 tubes (Ultracel-30k, Millipore) with centrifugation at 1,500 rpm for 15 minutes at 25℃. (h) Transfection: After transfection, cells ware cultured with fetal calf serum (FCS) 0% medium for 5 days. (i) Hollow-fiber ultrafiltration: About 900 mL of the cultured medium from twelve 25-cm square dishes was clarified through a 0.45-μm Bottle top filter (#295-4545, Nalgene, Rochester, NY, USA). The clarified medium was then concentrated by means of tangential flow filtration (TFF) with Hollow Fiber Cartridges (UFP-750-E-3MA, GE Healthcare; 750-kDa molecular weight cut-off). A 20-fold concentration to 40 mL was performed with the hand-made system. (j) Precipitaion with ammonium sulfate: The concentrated medium fraction was also treated with Benzonase at 37℃ for 20 minutes, followed by the process of the half-saturated ammonium sulfate precipitation (pH 8.5) on ice for 20 minutes. (k) Iodixanol linear gradient centrifugation: The AAV-containing-pellets were dissolved in PBS, and the viral solution was also layered with Optiprep and purified with iodixanol continuous gradient centrifugation at 36,000 rpm for 15 hours at 16℃.

methods for producing AAV vectors use adherent HEK293 cells (human embryonic kidney cells encode the E1 region of adenovirus type 5 genome) chemically cotransfected with plasmids encoding the necessary viral proteins along with the vector transgene. **Figure 3** shows a flow diagram of the production of an AAV vector.

3-1. Transfection

Production of AAV vectors most often entails the transfection of HEK293 cells cultivated on 10- to 15-cm dishes. Mid- to large-scale production generally consists of 30 to 50 of these dishes. To avoid the complication of large numbers of dishes, twelve 24.5 × 24.5-cm square dishes (surface area, 500 cm^2; 240835, Nunc, Roskilde, Denmark) are used in our laboratory. To produce an AAV vector, the AAV vector plasmid (containing 2 inverted terminal repeats [ITRs], an appropriate promoter, and the transgene), the helper plasmid (containing helper virus genes encoding the E2A, E4, and VA regions of adenovirus type 5 genome), and the packaging plasmid (containing rep and cap) are cotransfected into HEK293 cells (**Fig. 3-a**). The serotype of the AAV can be manipulated by using different rep/cap constructs during packaging. The vector: helper: packaging plasmid ratio we use is 2 : 1 : 1, and the transfection method we use is calcium phosphate coprecipitation or polyethylenimine coprecipitation[14].

3-2. Concentration and Purification

Three days after transfection, the transfectants

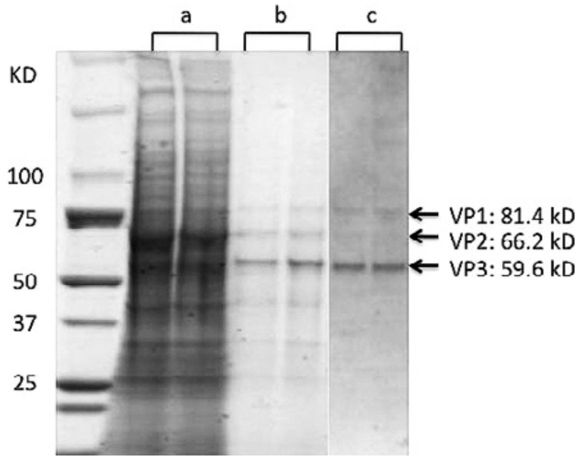

Fig. 4 Coomassie-Brilliant Blue R-250-stained sodium dodecylsulfate-polyacrylamide gel electrophoresis of the purified AAV serotype 1 vectors
After several purification steps (a: First iodixanol linear gradient centrifugation, b: Second iodixanol linear gradient centrifugation, c: 2 times iodixanol linear gradient centrifuge and gel filtration), the purified AAV vectors samples were separated under reducing conditions on a 12% sodium dodecylsulfate-polyacrylamide gel. Coomassie-Brilliant Blue R-250 staining was performed with a Quick-CBB staining kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The purified AAV serotype 1 capsid proteins (VP1, VP2, and VP3) were detected at 81.4, 66.2, and 59.6 kD, respectively.

are lysed in a small, disposable vessel with 4 cycles of freeze-thaw lysis (**Fig. 3-b**; Dry ice-ethanol/37℃ in water bath). Recombinant endonuclease (Benzonase; Merck KGaA, Darmstadt, Germany) is added, and the transfectants are incubated at 37℃ for 20 minutes to digest the cellular genomic DNA and plasmids, after which the insoluble cell components are removed with low-speed centrifugation. Half-saturated ammonium sulfate [$(NH_4)_2SO_4$] is then used to efficiently precipitate the AAV vector from the clarified cell lysate (**Fig. 3-c**). Following low-speed centrifugation, the pellet containing the AAV is resuspended in phosphate-buffered saline (PBS) and further concentrated and purified with an iodixanol linear gradient[15] (**Fig. 3-d**). The purity of the AAV vector was analyzed with Coomassie-Brilliant Blue R-250 staining (**Fig. 4-a**). The resultant crude AAV vector fraction can be used for in vitro gene transfer and in vivo gene transfer into mice. However, when we injected this crude fraction into the brain of a nonhuman primate, some toxicity was observed due to contamination by cell-derived proteins (**Fig. 5-a**). Therefore, we purified this fraction further through a second iodixanol linear gradient (**Fig. 3-e**, **Fig. 4-b**), which was followed by gel filtration with a Superdex 200 HR 10/30 GL column (**Fig. 3-f**, **Fig. 4-c**) with the AKTA Explorer 100 HPLC system (GE Healthcare, Uppsala, Sweden). The AAV vector thus purified could be used to transduce nonhuman primate brain with no toxicity (**Fig. 5-b**). To obtain a more concentrated AAV vector, we performed a column filtration step through an Amicon Ultra-4 centrifugal filter unit (**Fig. 3-g**: Ultracel-30k, Millipore, Bedford, MA, USA). With this step, the AAV vector could be concentrated by a factor of 100.

3-3. Production of AAV Vectors from Supernatant

To obtain purified AAV vectors more easily, we developed a method for isolating AAV vector from the supernatant of transfected HEK293 cells[16]. A flow diagram of this production method is also presented in **Fig. 3**. To obtain a large amount of pure AAV, we cultured the transfected HEK293 cells in serum-free medium for 5 days (**Fig. 3-h**). The conditioned supernatants were then concentrated with Hollow Fiber Cartridges (UFP-750-E-3MA, GE Healthcare) and systems (**Fig. 3-i**), after which the AAV vector was precipitated with half-saturated ammonium sulfate precipitation (**Fig. 3-j**). With this fraction, a single iodixanol linear gradient was sufficient to obtain purified AAV vector (**Fig. 3-k**). This method has the potential to yield 10^{13} to 10^{14} AAV particles, which is comparable to yields from cell lysates.

4. Summary

Here we have described the methods we use to synthesize, concentrate, and purify AAV vectors in our laboratory. These methods can be used to produce several AAV vector serotypes as well as scAAV vectors. These AAV vectors could be used

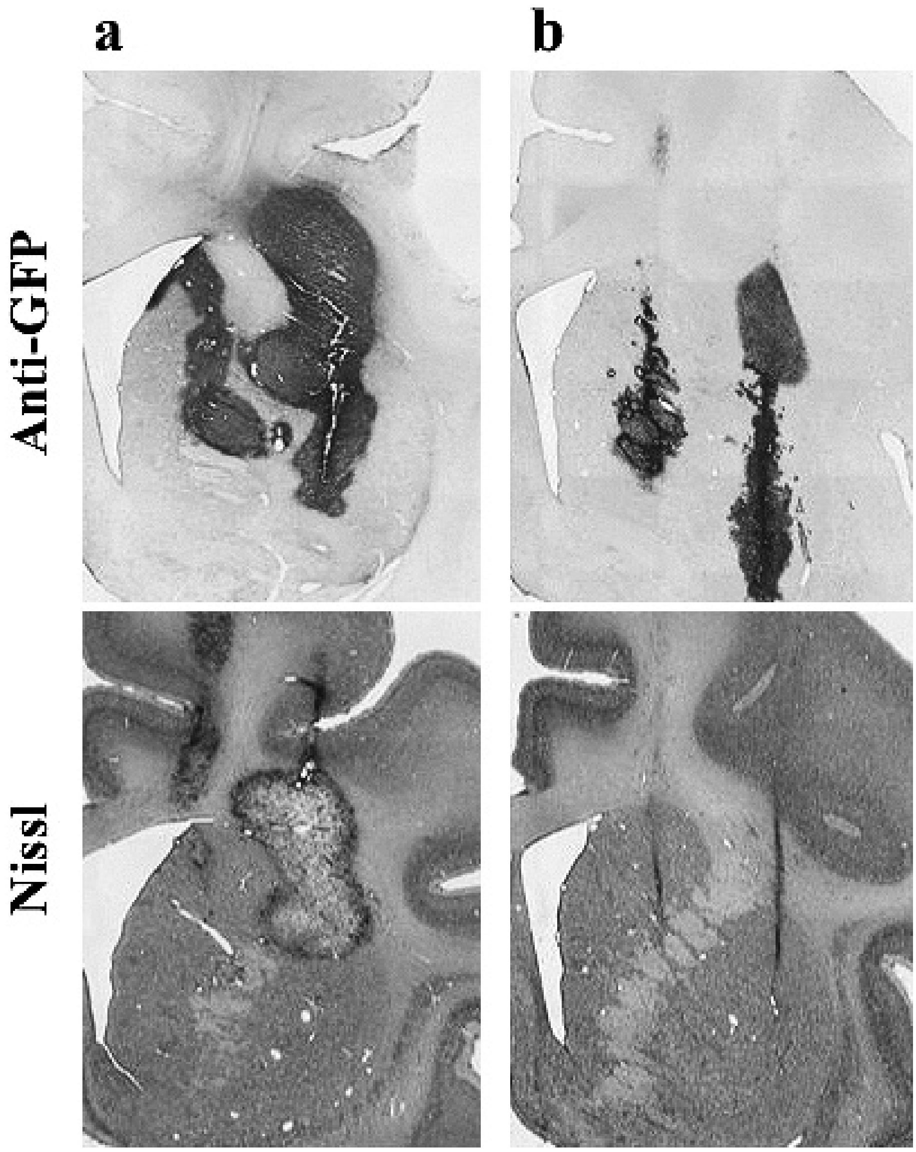

Fig. 5 Direct injection of the AAV serotype 1 vector into nonhuman primate brain
After direct injection of the AAV serotype 1 vector expressing GFP purified with (a) 1 time Iodixanol linear gradient centrifugation, or (b) 2 times Iodixanol linear gradient centrifugation and gel filtration into nonhuman primate brain, the toxicity and GFP expression were analyzed with Nissl staining and diaminobenzidine staining with an anti-GFP antibody.

to efficiently transduce a variety of organs in vivo. To obtain high levels of transgene expression, one must choose the appropriate AAV vector serotype, which would depend on the target tissue, cell type, and route of administration. In addition, developments in large-scale transient transfection methods with serum-free supernatant provide a vector production system that enables large amounts of purified AAV vector to be obtained much more easily. These methods should be useful for all investigators engaged in research requiring gene transfer.

Acknowlegements: We thank Dr. James Wilson (University of Pennsylvania), Dr. R. Jude Samulski (University of North Carolina), Dr. Robert M. Kotin (National Institutes of Health), Dr. David W. Russell (University of Washington), Dr. Arun Srivastava (University of Florida College of Medicine), and Dr. A. Dusty Miller (Fred Hunchins Cancer Research Center) for kindly providing AAV packaging plasmids. We also thank Dr. Kenichi Inoue and Dr. Masahiko Takada

(Primate Research Institute, Kyoto University) for joint research on nonhuman primates. This work was supported in part by grants from the Ministry of Health, Labour and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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(Received, October 26, 2012)
(Accepted, November 27, 2012)