

Amount of Green Fluorescent Protein in the Anterior Chamber after Intravitreal Injection of Triple-Mutated Self-Complementary AAV2 Vectors is Not Affected by Previous Vitrectomy Surgery

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Background: The adeno-associated virus (AAV) vector is a promising vector for ocular gene therapy. Surgical internal limiting membrane peeling before AAV vector administration is useful for efficient retinal transduction. However, no report has investigated localization of AAV vectors after administration into a post-vitrectomy eye. This study investigated the effects of vitrectomy surgery on intravitreal-injected AAV vector-mediated gene expression in the anterior segment and examined the presence of neutralizing antibodies (NAbs) in serum before and after AAV vector administration.

Methods: Of six eyes from three female cynomolgus monkeys, four were vitrectomized (Group VIT) and two were non-vitrectomized (Group IV). All eyes were injected with 50 μ L of triple-mutated self-complementary AAV2 vector (1.9×10^{13} v.g./mL) encoding green fluorescent protein (GFP). NAbs in the serum were examined before administration and at 2 and 6 weeks after administration. GFP expression was analyzed at 19 weeks after administration.

Results: Immunohistological analysis showed no GFP expression in the trabecular meshwork in any eye. The GFP genome copy in two slices of the anterior segment was 2.417 (vector genome copies/diploid genome) in Group VIT and 4.316 (vector genome copies/diploid genome) in group IV. The NAb titer was 1:15.9 (geometric mean) before administration, 1:310.7 at 2 weeks after administration, and 1:669.4 at 6 weeks after administration.

Conclusion: Previous vitrectomy surgery did not affect gene expression in the anterior segment after intravitreal injection of AAV vectors. (J Nippon Med Sch 2021; 88: 103–108)

Key words: AAV vector, intravitreal, injection, vitrectomy, neutralizing antibodies (NAbs), trabecular meshwork

Introduction

The adeno-associated virus (AAV) vector is a promising vector for ocular gene therapy. Gene therapy for eye diseases was initially proposed, in 2008, in a clinical study of Leber's congenital amaurosis by Bainbridge *et al.*¹

Many ongoing clinical trials include patients with Leber's congenital amaurosis²⁻⁵, age-related macular degeneration⁶, and retinoschisis⁷. The two strategies used to deliver the vector to the retina are subretinal and intravitreal injection. Because the efficiency of transduction

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Table 1 Animal characteristics and surgical treatment

No.	ID	Sex	BW (kg)	Age (years)	Eye	pre-treatment	Group
1	1310102013	female	2.90	13	1R	VIT	Group VIT
					1 L	VIT+ILM	Group VIT
2	1310308088	female	3.56	11	2R	VIT	Group VIT
					2 L	NO	Group IV
3	1310412153	female	2.92	10	3R	VIT+ILM	Group VIT
					3 L	NO	Group IV

VIT: vitrectomy, ILM: internal limiting membrane peeling, NO: no operation, IV: intravitreal injection, BW: body weight

by injection of subretinal AAV vectors has been previously demonstrated, we performed AAV experiments by using subretinal AAV vector injections⁸⁻¹⁰. However, subretinal injections require creation of iatrogenic retinal detachment, which could lead to retinal dysfunction¹¹. In contrast, intravitreal injections are safe and advantageous for gene transduction to the inner retina. The transduction efficiency by intravitreal injection of AAV vectors is high in rodents^{12,13}. In contrast, the transduction efficiency for simple intravitreal injection of AAV vectors remains low when used in non-human primate retinas, such as those of monkeys¹⁴. The internal limiting membrane (ILM) may act as a barrier against gene transduction by intravitreal injection¹⁵. Boye *et al.* reported that although sub-ILM injection of the AAV vector improved transduction efficiency¹⁶, pressure from sub-ILM injection caused retinal damage; thus, adopting this method for glaucoma would be difficult. Takahashi *et al.* previously reported that surgical ILM peeling before intravitreal administration of AAV vectors was safe and useful for efficient transduction of non-human primate retina¹⁷. Leberherz *et al.* reported that intravitreal AAV7 vector injection was able to transduce anterior segment structures¹⁸. In addition, because the vitreous is a gel-like collagen, it may prevent widespread exposure of a vector solution in the retina¹⁹. Vitrectomy can cause spreading of AAV vectors, which are injected intravitreally, to the anterior chamber. However, no study has investigated localization of AAV vectors when administered into the vitreous of a vitrectomy eye. In the present study, we compared transduction efficiency in the anterior segment of vitrectomized and non-vitrectomized eyes.

Intravitreal administration of AAV vectors generates neutralizing antibodies (NAbs) against the AAV capsid that block AAV vector-mediated gene expression upon re-administration via an intravitreal route into the other eye²⁰. Kotterman *et al.* reported that NAb levels in serum

are correlated with those in vitreal fluid²¹. Moreover, after intravitreal administration of AAV, they found that the presence of pre-existing NAb titers in serum from monkeys was associated with weak, decaying, or no transgene expression²¹. These previous findings suggest that when performing intravitreal administration of AAV vectors, monitoring NAb titers in the serum may be important. Thus, this study compared serum NAb titers before, and at 2 and 6 weeks after, intravitreal administration of AAV vectors.

Materials and Methods

Animals

Three adult female cynomolgus monkeys from the Tsukuba Primate Research Center were used for this study. Each animal weighed approximately 3 kg and was 10-13 years of age (Table 1). All animal procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and the Animal Experimental Ethical Review Committee of Nippon Medical School (approval number 27-055). The Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation approved the protocols and experimental procedures.

Production and purification of AAV vectors

Dr. Arun Srivastava of the University of Florida kindly provided an AAV packaging plasmid (pACG2-3M) generated by introducing a triple tyrosine-to-phenylalanine (Y444+500+730F) mutation into the VP3 region of the AAV serotype 2 capsid and a self-complementary AAV vector plasmid carrying cDNA encoding enhanced green fluorescent protein (EGFP) (pdsCBA-GFP)²². A recombinant scAAV vector (tm-scAAV2/GFP) was produced with a previously described method¹⁷. We determined the genome titers of the AAV vector by using quantitative polymerase chain reaction (qPCR) analysis with a

7500 Fast Real-Time PCR Instrument (Applied Biosystems, Carlsbad, CA, USA) and the following primers for *EGFP*: forward, 5'-AGCAGCACGACTTCTTCAAGTCC-3' and reverse, 5'-TGTAGTTGACTCCAGCTTGTGCC-3'.

The total vector genome (v.g.) number for tm-scAAV2/EGFP was 1.9×10^{13} v.g./mL.

Surgical procedures

We used mixed anesthesia (ketamine and xylazine) to anesthetize all animals. Of the six eyes from three monkeys, four received a standard 3-port vitrectomy (VIT) before the AAV injection, as previously described¹⁷ (Group VIT). Two eyes in the Group VIT underwent ILM peeling in addition to VIT, for use in another study¹⁷. No pretreatment was performed for the remaining two eyes (Group IV)¹⁷ (Table 1). The VIT and ILM peeling were performed as described previously¹⁷.

Intravitreal injection

At 1 month after pretreatment, all six eyes were injected with AAV. We chose 1 month for the injection time to avoid any adverse effects of pretreatment. All animals were anesthetized with mixed anesthesia (ketamine and xylazine), after which we penetrated the vitreous at the pars plana and administered 50 μ L of tm-scAAV2/GFP with a 30-gauge needle, as previously described¹⁷.

Histological analysis

At 19 weeks after the AAV vector injection, animals were euthanized for histological analysis, as previously described¹⁷. Eyes were enucleated and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. The cornea and lens were then collected at the pars plana, followed by removal of the lens. The posterior segments, which contained the retina and optic nerve, were examined and GFP expression was confirmed, as previously described¹⁷. The anterior segments, which contained the ciliary body and iris, were again fixed in 4% paraformaldehyde in PBS overnight at 4 °C. The eyes were subsequently fixed in 10% sucrose for 4 h, in 20% sucrose overnight, and in 30% sucrose overnight, after which they were frozen in O.C.T. compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan) on dry ice/ethanol. We cut 10- μ m-thick sections with a CM1950 cryostat (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

The sections from each eye were immunohistochemically analyzed, as previously described^{10,17}. The primary antibody was a rabbit anti-GFP IgG antibody (1:1,000; Invitrogen, Carlsbad, CA, USA) and the secondary antibody was Alexa 488-conjugated goat anti-rabbit IgG (1:

500; Invitrogen). The stained sections were washed with PBS with Triton X (PBST), mounted using medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA), and examined under a fluorescence microscope (DP-70, Olympus Fluorescence Microscope, Tokyo, Japan). We examined three slices per eye.

qPCR

Using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), we extracted DNA from two 10- μ m slices cut from the frozen block of the anterior segment. We used vector plasmid (pdsAAV-CB-EGFP) as the standard. The GFP titer was determined with qPCR using a 7500 Fast Real-Time PCR Instrument (Applied Biosystems) and the following primers for *EGFP*: forward, 5'-AGCAGCACGACTTCTTCAAGTCC-3' and reverse, 5'-TGTAGTTGACTCCAGCTTGTGCC-3'. PCR conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s.

NAb assay

To detect NABs to AAV, human embryonic kidney (HEK) 293 cells were used for the assay. Cells were plated at a density of 5×10^4 cells in each well of a 96-well plate and incubated for 6 h at 37 °C in 5% CO₂. Serum samples were diluted in 50 μ L of culture medium at ratios of 1:4, 1:10, 1:20, 1:50, 1:100, 1:300, 1:1,000, 1:2,500, 1:5,000, or 1:10,000. The diluted serum samples were then mixed with an equal volume of culture medium containing tmAAV2-GFP (2×10^9 v.g./well). A mixture of the serum sample and tmAAV2-GFP was added to each well of the HEK 293 cells. After 72 h of additional incubation, cells were washed three times with PBS, followed by the addition of 100 μ L of PBS to each well. GFP expression was measured with a fluorescence plate reader at 485 nm/535 nm (Wallac 1420 ARVO; PerkinElmer). Serum NABs were scored as positive if the fluorescence intensity was $\leq 50\%$ of that observed when tmAAV-GFP-infected HEK 293 cells were pre-incubated without sera²³. This test was performed once in duplicate.

Results

GFP expression in the anterior chamber

Transduction efficiency was assessed immunohistochemically with an anti-GFP antibody at 19 weeks after intravitreal injection of 50 μ L of tm-scAAV2/GFP vector. No GFP expression was detected in Group VIT or Group IV (Fig. 1).

AAV genome copy number in the anterior chamber

GFP DNA was determined with qPCR analysis. As-

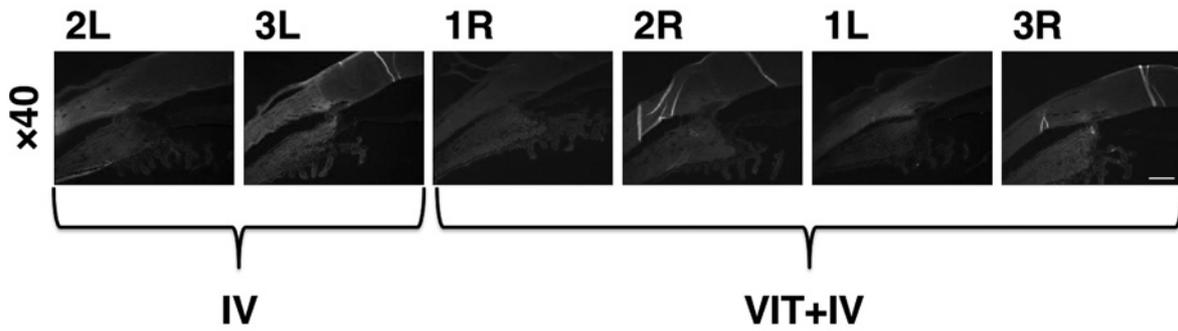


Fig. 1 Histological analysis for the detection of GFP

Sections from each eye were immunohistochemically analyzed. The primary antibody was rabbit anti-GFP IgG antibody and the secondary antibody was Alexa 488-conjugated goat anti-rabbit IgG.

GFP expression was not detected in group IV or group VIT+IV.

IV: The AAV vector was injected intravitreally without first performing a vitrectomy.

VIT+IV: Vitrectomy was performed before the AAV vector was injected intravitreally. (magnification $\times 40$; scale bar, 500 μm)

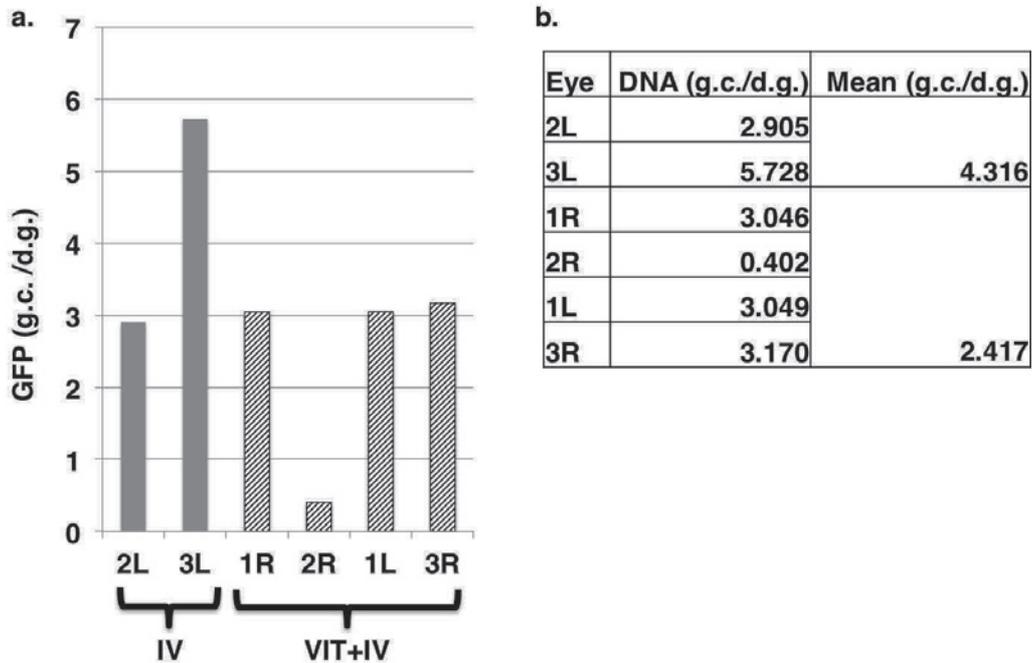


Fig. 2 Results of qPCR analysis

DNA was extracted from two 10- μm slices cut from a frozen block. The GFP titer was determined with qPCR.

a) Results of qPCR for GFP in each eye.

b) PCR analysis revealed the presence of the vector genome in the anterior chamber in the VIT group (2.417 vector genome copies (g.c.) /diploid genome (d.g.)). The amount did not exceed that in group IV (4.316 g.c./d.g.).

suming 6.181 pg genomic DNA per diploid genome (d. g.), qPCR results indicated that the vector genome copies (g.c.) of GFP were 2.417 g.c./d.g. in Group VIT and 4.316 g.c./d.g. in Group IV (Fig. 2). Only a small amount of GFP was detected with qPCR, and GFP detected in the VIT group did not exceed that in the IV group.

AAV NABs in the serum

We collected serum samples from each monkey before, and at 2 and 6 weeks after, AAV vector injection. The NAb titers were 1:10, 1:20, and 1:20 (Geometric mean; 15.9) before administration; 1:100, 1:300, and 1:1,000 (310.7) at 2 weeks post-administration; and 1:300, 1:1,000, and 1:1,000 (669.4) at 6 weeks post-administration (Fig.

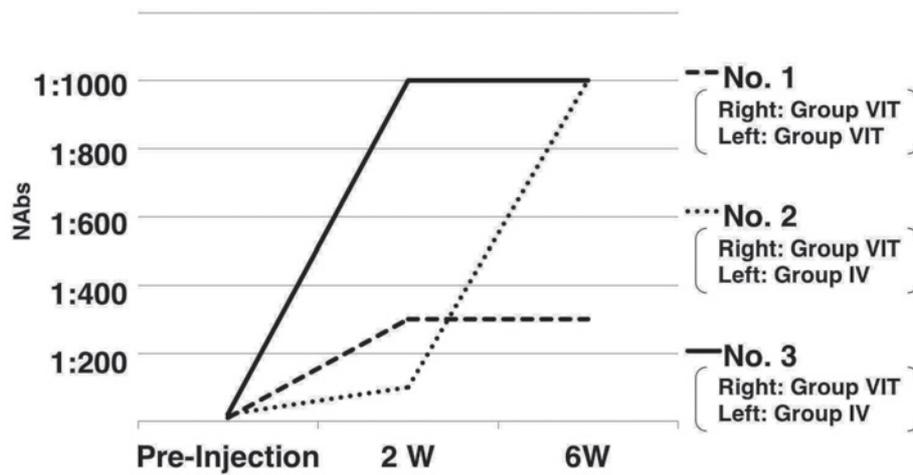


Fig. 3 AAV neutralizing antibodies (NAbs) in the serum

To detect NAbs to AAV, human embryonic kidney (HEK) 293 cells were used for the assay. Serum NAbs were scored as positive if fluorescence intensity was $\leq 50\%$ of that observed when tmAAV-GFP-infected HEK 293 cells were pre-incubated without sera. This test was performed once in duplicate.

The NAb titers were 1:10, 1:20, and 1:20 (geometric mean, 15.9) before administration; 1:100, 1:300, and 1:1,000 (310.7) at 2 weeks post-administration; and 1:300, 1:1,000, and 1:1,000 (669.4) at 6 weeks post-administration.

3).

Discussion

In our study, immunohistochemical analysis showed no GFP in the anterior chamber after vitreous administration of AAV vectors. PCR showed a miniscule amount of GFP in the anterior chamber; however, performing vitrectomy in advance did not increase the amount of GFP in the anterior chamber. Intravitreal administration of AAV vectors resulted in increased NAbs in serum.

The two strategies for delivering vectors to the retina are subretinal injection and intravitreal injection. The intravitreal route is considered safer, as it does not cause retinal detachment¹¹. However, transduction efficiency in the primate retina is low²⁴. Takahashi *et al.* previously reported that ILM peeling improved intravitreal AAV-mediated inner retinal gene transduction in cynomolgus monkeys¹⁷. When ILM peeling is performed, vitrectomy is required. Because the vitreous is a gel-like collagen, researchers have hypothesized that it can prevent widespread exposure of a vector solution to the retina¹⁹. After vitrectomy, AAV vectors injected intravitreally can spread to the anterior chamber. However, localization of AAV vectors after intravitreal injection has not been reported. In the present report, we compared transduction efficiency in the anterior segment in vitrectomized and non-vitrectomized eyes. Vitrectomy did not affect the amount of vector that was transduced in the anterior chamber,

which suggests that vitrectomy is not a risk factor for unintended gene transfer into the anterior chamber when performing gene therapy in the retina by vitreous administration. The present study aids our understanding of the local effect in the eye when gene therapy in the retina-for diseases such as retinitis pigmentosa, age-related macular degeneration, retinoschisis, and glaucoma-is performed with ILM peeling¹⁷.

Because of ocular immune privilege, researchers have believed that the entire body has little effect after intravitreal administration. However, we confirmed that intravitreal administration of AAV vectors generated NAbs against the AAV vector in monkeys. After intravitreal administration, the level of NAbs in the serum increased 40-fold. This result is similar to those reported by Li *et al.* for mice²⁰ and by Korrterman *et al.* for non-human primates (2-50-fold)²¹.

It was reported that presence of the AAV vector in the anterior chamber, Schlemm's canal, or collector channels could result in systemic exposure²⁵. In our study, PCR detected a miniscule amount of GFP in the anterior chamber in all eyes. This may be associated with systemic exposure and increased NAbs in serum. Clinical application of retinal AAV-mediated gene therapy will require monitoring of NAbs and consideration of possible systemic exposure. Future experiments should examine how AAV vectors are systemically exposed and how NAbs are generated.

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Conflict of Interest: None.

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