Molecular Therapy
Methods & Clinical Development
Original Article

Improved transduction of canine X-linked muscular dystrophy with rAAV9-microdystrophin via multipotent MSC pretreatment

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Duchenne muscular dystrophy (DMD) is a severe congenital disease associated with mutation of the dystrophin gene. Supplementation of dystrophin using recombinant adeno-associated virus (rAAV) has promise as a treatment for DMD, although vector-related general toxicities, such as liver injury, neurotoxicity, and germline transmission, have been suggested in association with the systemic delivery of high doses of rAAV. Here, we treated normal or dystrophic dogs with rAAV9 transduction in conjunction with multipotent mesenchymal stromal cell (MSC) injection to investigate the therapeutic effects of an rAAV expressing microdystrophin (μDys) under conditions of immune modulation. Bone-marrow-derived MSCs, rAAV-CMV-μDys, and a rAAV-CAG-luciferase (Luc) were injected into the jugular vein of a young dystrophic dog to induce systemic expression of μDys. One week after the first injection, the dog received a second intravenous injection of MSCs, and on the following day, rAAV was intravenously injected into the same dog. Systemic injection of rAAV9 with MSCs pretreatment improves gene transfer into normal and dystrophic dogs. Dystrophic phenotypes significantly improved in the rAAV-μDys-injected dystrophic dog, suggesting that an improved rAAV-μDys treatment including immune modulation induces successful long-term transgene expression to improve dystrophic phenotypes.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder associated with mutations in the dystrophin gene, which causes progressive deterioration of skeletal and cardiac muscles. Progressive deterioration in striated muscle function in affected individuals ultimately results in early death due to cardio-pulmonary failure. Although there is currently no cure for DMD, several novel strategies for replacing or repairing the dystrophin gene are being developed, including gene replacement therapies mediated by viral vectors. Recombinant adeno-associated virus (rAAV) vectors have considerable promise for gene therapy because of their safety, low toxicity, and longevity of transgene expression, and there have been a number of gene therapy clinical trials using rAAV.[1] Recently, however, vector-related general toxicities, such as liver injury, neurotoxicity,[2] germline transmission,[3,4] and replication escape,[5] have been suggested to be associated with the systemic delivery of high doses of rAAV.

Supplementation of dystrophin using rAAV has been shown to be effective in alleviating pathogenesis in animal models of DMD. We and others have previously reported dystrophin supplementation strategies in which the local injection of rAAV-microdystrophin (μDys) into the skeletal muscle of *mdx* mice[6–8] and the systemic injection of rAAV9-microdystrophin into *mdx* mice[9] resulted in extensive expression of microdystrophin and improvement in myocardial dysfunction. However, immune responses elicited against AAV capsid proteins or transgene products can prevent efficient gene therapy. To induce effective long-term expression of a transgene, it is important to prevent immune-mediated destruction of the transduced muscle fibers by regulating the host immune response. Several groups, including ours, have reported that rAAV-mediated transfer of genes into the skeletal muscle of dogs evokes immune responses to rAAV or the transgene products.[10–12] The relevance of immune effects during gene transfer is emphasized by the results of a recent clinical gene therapy trial of rAAV2.5-minidystrophin in six DMD patients, in which T cell responses to transduced dystrophin were detected.[13] To overcome these responses against vectors or transgenes, we previously reported that a combination of tolerance induction against rAAV followed by a single intravenous injection of rAAV-microdystrophin into the fetus of dystrophic dogs achieved successful long-term transgene expression with an improved dystrophic phenotype.[14]

Received 23 October 2020; accepted 11 November 2020;
https://doi.org/10.1016/j.omtm.2020.11.003.

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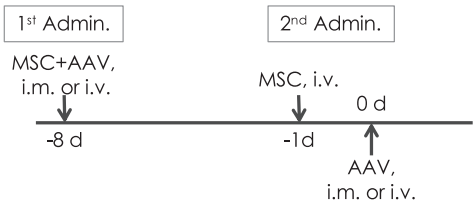

			1st administration		2nd administration	
Dog ID	Name	Sex	MSCs	AAV	MSCs	AAV
N1	11202FN	F	(-)	9-Luc, i.v.	(-)	9-Luc, i.m.
N2	11205MN	M	(+)	9-Luc, i.v.	(+)	9-Luc, i.m.
N3	11206FN	F	(+)	PBS, i.v.	(+)	PBS, i.m.
N4	11806MN	M	(-)	9-Luc, i.v.	(-)	9-Luc, i.v.
N5	11803MN	M	(+)	9-Luc, i.v.	(+)	9-Luc, i.v.
A0	6105MA	M	(-)	(-)	(-)	(-)
A1	11805MA	M	(+)	9-μD, i.v.	(+)	9-μD, i.v.

Figure 1. Genotypes and gender of the rAAV-treated dogs

112 series dogs (11202FN, 11205MN, and 11206FN) were injected with rAAV at 8 weeks old; 118 series dogs (11806MN, 11803MN and 11805MA) were injected with rAAV at 11 weeks old. For the first administration, MSCs 1.0–2.28 × 10^6 cells/kg BW with or without rAAV9-CAG-Luc-WPRE and rAAV9-CMV-microdystrophin 5.0 × 10^5 v.g./cell were injected. For the second administration, MSCs 1.8–2.0 × 10^6 cells/kg BW were injected. For local injection, rAAV9-CAG-Luc-WPRE 1.0 × 10^{12} v.g./muscle were injected. For systemic injection, rAAV9-CAG-Luc-WPRE and rAAV9-CMV-microdystrophin 1.0 × 10^{12} v.g./kg BW were injected. F, female; M, male; N, normal; A, affected.

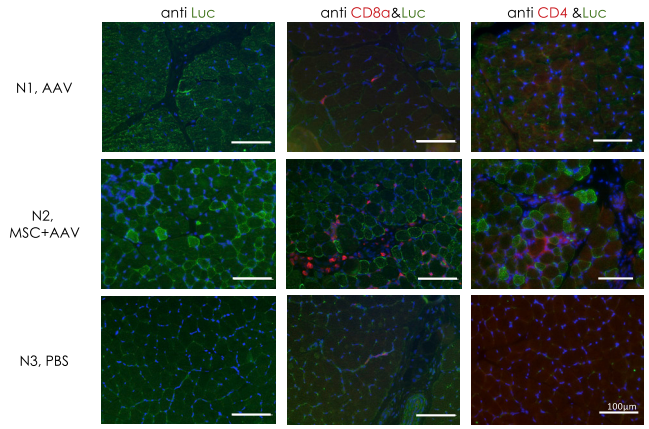

Figure 2. Immunohistochemical detection of the transgene in the skeletal muscle of rAAV-transduced dogs

Tibialis anterior muscles of the wild-type dogs were injected with rAAV-luciferase (N1), MSC pretreatment with rAAV-luciferase (N2), and PBS (N3), and analyzed 4 weeks after injection. Luciferase, CD8a, and CD4 were stained immunohistochemically. Scale bar, 100 μm.

Mesenchymal stromal cells (MSCs) are mesoderm-derived multipotent stromal cells that have self-renewal potential. These cells were initially evaluated as a cellular drug in humans and have recently emerged as promising candidates for cell-based immunotherapy in solid organ transplantation.[15,16] In addition to immune modulation, MSCs have also been reported to regulate various inflammatory diseases, including graft-versus-host disease (GvHD), by virtue of their immune-modulating effects. Furthermore, in Canada, New Zealand, and Japan, the use of MSCs has secured conditional approval.

In the present study, we hypothesized that by using bone-marrow-derived MSCs, we could identify effective strategies for inducing immune tolerance to the rAAV9 vector and transgene expression. Here, we provide evidence that rAAV injection with MSCs pretreatment can enhance the expression of an rAAV-derived transgene in dystrophic dogs.

RESULTS

Intramuscular injection of rAAV9-Luc with MSCs pretreatment improves gene transfer into normal dogs

We hypothesized that immune tolerance to the rAAV vector and/or its transgene in juvenile dystrophic dogs with Canine X-linked muscular dystrophy in Japan ($CXMD_J$) could be induced by pretreatment with MSCs prior to injection with rAAV. Initially, we evaluated the effects of the intramuscular injection of rAAV-luciferase after MSC pretreatment in normal dogs. Human MSCs (hMSCs) (2 × 10^6 cells/kg body weight [BW]) mixed with rAAV-luciferase (5 × 10^5 v.g./cell), were intravenously injected into normal beagles at 8 weeks of age. Seven days after the first injection, the dogs received a second intravenous injection of hMSCs, and the following day, rAAV-luciferase (1 × 10^{12} v.g./muscle) was injected into the tibialis anterior muscles of the same dogs (second rAAV injection) (Figure 1).

To determine the efficiency of vector gene transfer, we examined biopsy samples of the injected muscle obtained 4 weeks after the second rAAV injection, and thereafter rAAV-derived luciferase expression was confirmed by immunohistochemistry. As shown in Figure 2, compared with rAAV transduction alone, the administration of rAAV-luciferase following MSC treatment resulted in a higher expression of the transgene (luciferase) in the skeletal muscle. 4′, 6′-diamidino-2-phenylindole (DAPI) and hematoxylin and eosin (H&E) staining confirmed cell infiltration around the transduced muscle. Moreover, CD4 and CD8 were expressed in these infiltrated cells.

To analyze the T cell responses to rAAV in transduced dogs, we examined the induction of interferon-γ (IFN-γ) in peripheral blood mononuclear cells (PBMCs) after rAAV re-stimulation. PBMCs were purified from the transduced dogs during each week (~4 weeks) after the second rAAV injection. In neither the MSC plus rAAV-treated dog (N2) nor the PBS-injected dog (N3) was IFN-γ expression upregulated, whereas the rAAV-injected dogs (N1) showed increased IFN-γ expression at 3 weeks after the second rAAV administration. Therefore, MSC pretreatment prior to rAAV transduction led to a

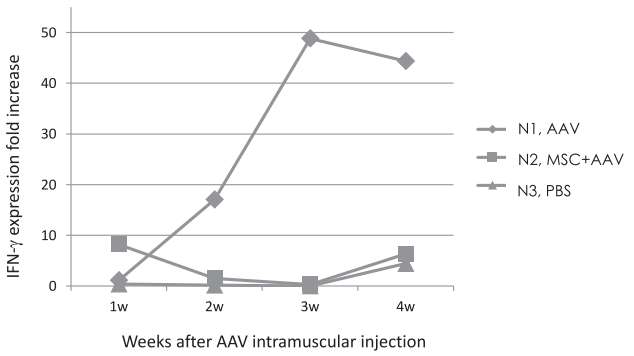

Figure 3. T cell responses to the rAAV and transgene product in transduced dogs

IFN-γ mRNA expression in PBMCs stimulated with rAAV. PBMCs from transduced dogs at each time point were stimulated overnight with rAAV9-luciferase or rAAV-microdystrophin. IFN-γ mRNA expression was determined by quantitative RT-PCR (ΔΔCt method). Expression levels of IFN-γ in the untreated normal dog are shown (N3). The RNA transcripts were normalized to endogenous 18S rRNA.

suppression of the induction of IFN-γ expression in the transduced dogs (Figure 3). Taken together, these results suggest that MSC pretreatment provided prior to rAAV intramuscular administration might have a certain immune modulating effect.

Systemic injection of rAAV9 with MSCs pretreatment improves gene transfer into normal and dystrophic dogs

On the basis of the results of local rAAV injection with MSCs pretreatment, we performed a systemic injection of rAAV with this protocol using normal and dystrophic ($CXMD_J$) dogs. As described above, MSCs mixed with rAAV (5×10^5 v.g./cell) were intravenously injected into the jugular vein of the dogs at 11 weeks of age. One week after the first administration, the dogs received a second intravenous injection of MSCs, and on the following day, rAAV was injected systemically (2×10^{12} v.g./kg BW). rAAV-luciferase with or without MSCs was administrated to normal beagles (dogs N4 and N5), and rAAV-microdystrophin with MSCs was administered to a dystrophic dog (dog A1). All the transduced dogs were littermates. Muscle biopsies were performed 4 weeks after the last rAAV transduction in all dogs. We opted to sample the temporal muscle by punch biopsy to minimize the invasive interventions to the skeletal muscle of the extremities. rAAV-derived luciferase expression was observed only in the rAAV-transduced dog with MSC pretreatment (dog N5; Figure 4). In addition, rAAV-derived microdystrophin expression was detected in the rAAV-microdystrophin transduced $CXMD_J$ dog with MSC pretreatment (dog A1; Figure 4; Figure S1). The skeletal muscles (tibialis anterior) of the transduced dogs were analyzed by immunohistochemical (IHC) staining using anti-dystrophin antibodies (Figure 4; Figure S1). Most DMD patients and animal models, including *mdx* mice and canine models, display so-called revertant fibers, which are dystrophin-positive muscle fibers that can be detected sporadically in the affected tissue. In the immunohistochemical study, we used two antibodies (i.e., DysB and Dys2), which, respectively, recognize domains from the N terminus of the full-length dystrophin

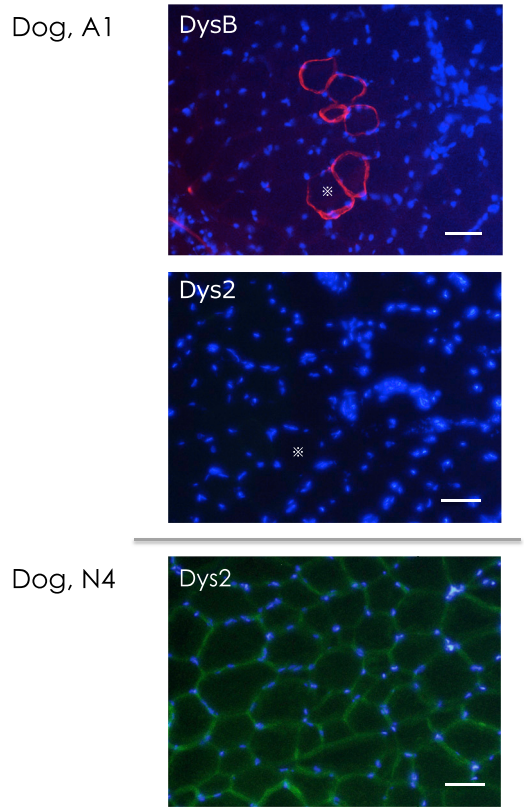

Figure 4. Immunohistochemical detection of the transgene in the rAAV-transduced dogs

Transgene expression in the skeletal muscle at 134 weeks old (121 weeks after systemic rAAV-microdystrophin delivery). Skeletal muscle (tibialis anterior muscle) from transduced (A1) and untransduced normal dogs (N3) were cryo-sectioned and stained with anti-N-terminal of dystrophin antibody (DysB) and anti-C-terminal of dystrophin antibody (Dys2). × shows same muscle fiber in adjacent sections. Scale bar, 100 μm.

and peptides from the C-terminal region that are deleted in the rAAV-derived microdystrophin. Whereas dog A1 (transduced with the rAAV-microdystrophin and MSCs) showed N-terminal dystrophin staining using DysB antibodies, we were unable to detect C-terminal dystrophin after staining with anti-Dys2 antibodies. Thus, the DysB-positive fibers found in dog A1 are unlikely to be revertant fibers.

Clinical manifestations

We developed a scoring system for $CXMD_J$ pathology based on the monthly inspection of limbs for temporal muscle atrophy and mobility disturbance, for which the dogs were given scores between 0 (normal) and 30 (severely affected). The clinical score of dog A1 (MSCs with rAAV-microdystrophin treated) was significantly lower than that of non-treated $CXMD_J$ dogs in all categories throughout the experimental period (Figure 5; Videos S1 and S2). Routine evaluation of standard serum protein levels indicated that rAAV transduction with MSCs pretreatment induced no significant side effects

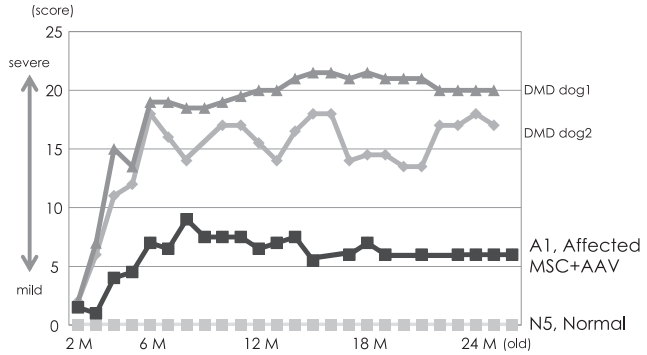

Figure 5. Clinical manifestations in the rAAV-transduced dogs

Clinical grading scale used to assess the transduced dogs. Clinical scores used to measure gait disturbance, mobility disturbance, limb or temporal muscle atrophy, drooling, macroglossia, and dysphagia are shown. N5, normal dog, A1, dystrophic dog, treated with MSCs and rAAV-microdystrophin. Scores from DMD dog 1 and 2 are from non-treated $CXMD_J$ in our dystrophic dog colony from the same age.

(Table 1). Furthermore, there were no significant differences in mean serum creatine kinase levels (Table 1).

Cardiac phenotype of the transduced $CXMD_J$ dog

Holter electrocardiogram (ECG) recordings were obtained for the treated $CXMD_J$ dog (dog A1) at 102 weeks of age to monitor the effects of rAAV-derived microdystrophin on the cardiac phenotype. The episodes of premature ventricular contraction and premature atrial contraction decreased significantly in the transduced affected dog (dog A1) compared with the untreated affected dog (dog A0), thereby indicating improved cardiac function upon transduction/systemic rAAV-microdystrophin injection.

The myocardial echogenicity of the transduced dogs (dog A1) was evaluated at 134 weeks of age. The average thickness of the left ventricular end-diastolic internal diameter, interventricular septal thickness, and the posterior wall in the transduced $CXMD_J$ dog did not differ from those in its normal littermate (dog N5). Furthermore, there were no significant differences in the left ventricular fractional shortening or the ejection fraction ratio of these dogs. The echocardiographic and biochemical data indicated that cardiac function was well maintained in the rAAV-microdystrophin-treated dog (Figure 6).

Transgene expression in rAAV-injected dogs (macroscopic and histopathologic analysis)

We next investigated the efficacy of the rAAV-microdystrophin injections with MSCs and systemic rAAV transduction. To evaluate the long-term transduction efficacy, we performed necropsies 121 weeks after systemic rAAV-microdystrophin delivery (at 134 weeks of age). Macroscopic observations of the heart of the transduced dog revealed that the cardiac pathology of the DMD was alleviated by MSCs with rAAV-microdystrophin treatment (Figure 7A). Interestingly, recombinant AAV-derived dystrophin expression was still present at 2 years and 3 months following the second rAAV systemic injection (Figures 7B and 7C; immunohistochemical analysis using DysB antibody). H&E staining revealed that, in comparison with the non-treated $CXMD_J$ dog, the AAV-microdystrophin and MSC-treated $CXMD_J$ dog showed histopathological features compatible with recovery, with a significant decrease in centrally nucleated fibers. However, we did observe some infiltrated cells (possibly macrophages) adjacent to the rAAV-derived Dys-positive muscle fibers. Therefore, our immune modulating conditions might benefit from further optimization.

DISCUSSION

In this study, we hypothesized that induction of immune tolerance by using an AAV administration method in combination with MSCs can be useful in reducing the dose of vector necessary to produce therapeutic effects. To confirm this assumption, we used a dog model of DMD, $CXMD_J$, to investigate whether we could induce immune tolerance against rAAV-microdystrophin in young dogs via pretreatment with MSCs prior to systemic injection of rAAV-microdystrophin.

The current study was designed to determine the effect of MSC pretreatment with rAAV (as a foreign, non-self antigen). As shown in Figure 1, our MSC pretreatment protocol is based on that described by Casiraghi et al.,[16,17] where MSCs and an antigen protein were administered when performing kidney transplants on mice of different strains. Merino et al.[18] also considered the timing for infusion of MSCs in a rat model of allogeneic kidney transplantation and found that a group with infusion of the MSCs at 7 days pre-transplantation showed better results than the other infusion timing groups.

We initially examined the effects of intramuscular injection of rAAV-luciferase with MSC pretreatment and observed higher levels of luciferase than in the dog injected with rAAV-luciferase only 4 weeks after rAAV administration. Expression of IFN-γ in the purified peripheral blood leukocytes after the rAAV9 exposure was not enhanced in the dog treated with rAAV9 and MSCs (Figure 3), thereby indicating the immune suppressive effects of the MSCs. Furthermore, intravenous administration of rAAV9 following MSC treatment resulted in a higher expression of the transgene (luciferase or microdystrophin) in the skeletal muscles (Figures 2 and 4; Figure S1), compared with rAAV9 transduction alone, and the $CXMD_J$ dog treated with MSCs and rAAV9-microdystrophin showed higher functional improvement than other DMD dogs of the same age (Figure 7).

In the present study, we used AAV9 due to the aim of delivering the therapeutic gene (microdystrophin) to the myocardium, but AAV9 has been reported to be rather low in terms of infectivity of AAV serotypes and MSCs. Zhang et al. (H. Zhang et al., 2018, Ann. Rheum. Dis., abstract) investigated the infectivity of AAV1, 2, 5, 6, 8, 9, PHP, and DJ against MSCs and found that the infectivity of AAV9 is rather

Table 1. $CXMD_J$ serum biochemistry data

Dog	Age (months)	AST (IU/L)	ALT (IU/L)	LDH (mg/dL)	BUN (mg/dL)	CRE (mg/dL)	TBIL (mg/dL)	TP (g/dL)	CK (IU/L)	CRP (mg/dL)
11803MN	15	42.0	35.0	352.0	10.30	0.70	0.30	5.50	173.0	ND
	18	41.0	49.0	254.0	11.50	0.70	0.30	5.40	140.0	ND
11805MA	15	447.0	637.0	317.0	8.10	0.2	0.40	5.8	13,700	0.45
	18	497.0	450.0	337.0	8.20	0.2	0.20	6.0	18,100	0.30
$CXMD_J$ (n = 9)	15	616.63 ± 55.76	522.13 ± 36.48	406.25 ± 84.84	9.95 ± 0.58	0.265 ± 0.03	0.36 ± 0.03	6.01 ± 0.14	33,574 ± 6,952	1.17 ± 0.31
	18	601.38 ± 77.43	444.63 ± 33.42	355.5 ± 90.57	9.725 ± 0.94	0.26 ± 0.03	0.33 ± 0.04	5.89 ± 0.12	28,509 ± 5,384	0.6 ± 0.13

$CXMD_J$ serum biochemistry data (mean ± SD) for age-matched muscular dystrophy dogs (n = 9 each age) housed at the same facility. AST, aspartate amino transferase; ALT, alanine aminotransaminase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; CRE, creatinine; TBIL, total bilirubin; TP, total protein; CK, creatine kinase; CRP, C-reactive protein; .

low in bone-marrow-derived MSCs. In the current study, we mixed both MSCs and AAVs prior to administration of MSCs, but the mixing time was less than 30 min, and we believe that the effect of mixing on both is minimal.

Recently, Le Guiner et al.[20] have reported that loco-regional and systemic delivery of a rAAV-microdystrophin is effective in restoring dystrophin expression and improving clinical symptoms in studies performed on 12 treated Golden Retriever muscular dystrophy (GRMD) dogs. Intravenous administration without immunosuppressive drugs resulted in significant and sustained levels of microdystrophin in skeletal muscles and reduced dystrophic symptoms for over 2 years. These authors observed dose-dependent improvements of clinical parameters in response to treatment with 1×10^{14} v.g./kg BW rAAV-microdystrophin, although they still found heterogeneity among the treated dogs. Furthermore, they observed anti-canine microdystrophin (rAAV-derived)-specific circulating immunoglobulin G (IgG) antibodies in some GRMD dogs of the treated group. The heterogeneous efficacy observed in the rAAV-microdystrophin treatment may be attributable to this immune response. Our dystrophic dog data show that low titers, 2×10^{12} v.g./kg, of rAAV9-microdystrophin with MSC pretreatment can compensate the microdystrophin expression in a cost-effective and scalable way. Using our MSC pretreatment method with AAV administration, it may at least be possible to reduce the virus titer necessary for treatment.

rAAV vectors are one of the safest and most widely used vectors in the field of gene therapy and are currently approved by the European Union (EU; Glybera, treatment for lipoprotein lipase deficiency) and the US Food and Drug Administration (FDA; Luxturna for inherited retinal disease and Zolgensma for spinal muscular atrophy [SMA]). Zolgensma is a rAAV9 that contains human SMN protein-encoding gene, administered intravenously or intrathecally. The distribution of rAAV9 after intravenous administration has been reported in numerous studies in mice, dogs, and monkeys.[14,21–25] Clinical trials using rAAV vectors are also being conducted for other autosomal recessive diseases. In 2019, the FDA approved Zolgensma for the treatment of SMA. However, this drug requires large doses of vectors to be administered for it to be effective.[1] Large-dose administration is not only expensive but also potentially associated with the appearance of side effects.[1,2] Furthermore, very recently, two deaths have been reported in patients treated with AAV9 in a trial for X-linked myotubular myopathy (XLMTM).[26] The cause of these deaths may have been hepatotoxicity associated with pre-existing liver disease, but no deaths have occurred in the low-dose group (1×10^{14} v.g./kg BW), suggesting that the deaths were due to high doses (3×10^{14} v.g./kg BW) of AAV. When AAV is administered in a clinical trial, it is common to administer immunosuppressive drugs at the same time. The recently approved Luxturna (rAAV-RPE) also assumes the prior administration of corticosteroid, and it is believed that an alternative immunosuppressive agent or method would be particularly useful for the practical application of gene therapies. The corticosteroids may decrease the possible immune response to either vector capsids or transgene products. Samelson-Jones et al.[27] reported that anti-capsid cellular immune response can be controlled by adjusting the timing of administration of T cell-directed immunosuppressants with AAV administration using non-human primates.

MSCs have immunosuppressive properties and are being used in allogeneic transplantation, with market authorization for GvHD treatment. The application of MSCs in solid organ transplantations, such as those for kidney, skin, and heart, is being evaluated in clinical trials. In the case of solid organ transplantation, the recipient's immune system recognizes the graft as foreign or non-self and attempts to reject it. In some cases of composite tissue allotransplantation, MSC treatment could prolong transplanted tissue survival. In patients with monogenic disorders such as DMD, the defective protein that the therapeutic transgene product is designed to replace may be either altered or completely absent. The newly expressed therapeutic protein derived from rAAV is then recognized by the immune system as non-self, resulting in the activation of both humoral and cell-mediated immune responses. Taking this into account, and similar to their use in tissue transplantation, MSC pre-administration with AAV-therapeutic genes (non-self) could prolong therapeutic gene expression. Recently, Ho et al.[28] have reported that a single rAAV-microdystrophin systemic injection and weekly administration of DNA vaccine (plasmid) over a 32-week period reduced antibody reactivity to

A

	N5	A1
LVIDd	2.32	1.70
LVIDs	1.36	1.00
%FS	41.60	41.83
EF	75.00	76.00
IVS	1.14	1.25
LVPWd	1.44	1.05

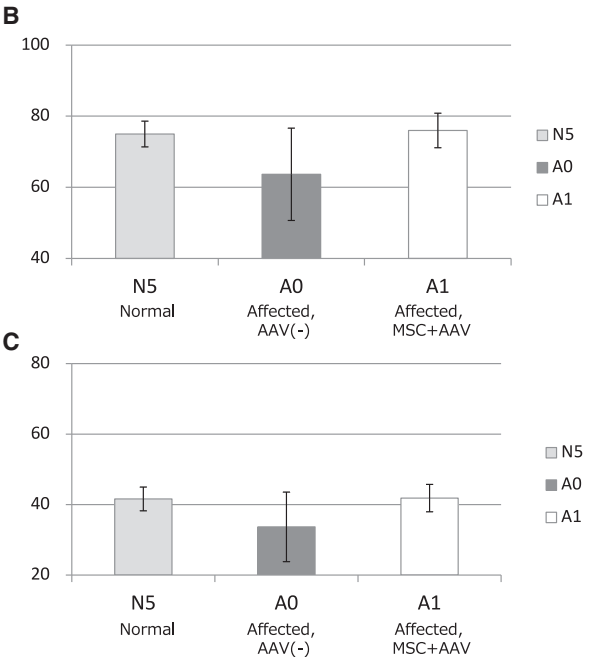

Figure 6. Summary of the echocardiography at 1 year old and 24-h Holter ECG recordings for the transduced dogs

Echocardiography at 1 year old. (A–C) Summary (A), fractional shortening (%, B), and ejection fraction (%, C) are shown. Dog N5 is a normal beagle administered AAV9-luciferase and MSCs. Dog A0 is $CXMD_J$ without any treatment. Dog A1 is $CXMD_J$, treated with AAV9-microdystrophin and MSCs.

both dystrophin and AAV capsid. However, their vaccination protocol is not applicable for clinical use.

Our results show that after treatment there is still infiltration around the damaged muscle fibers and that although the dystrophic phenotype improved, it did not completely revert to normal. Nevertheless, we believe that our protocol can be further refined, and we expect to obtain better results after optimization. Previous studies have noted that several types of stem cells (including MSCs, dental pulp stem cells, and amniotic stem cells) can effectively suppress the immune response. Mesenchymal stem cells, in particular, exhibit immunosuppressive properties that are being studied for use in the treatment of autoimmune diseases. Some reports demonstrate that MSC-derived extracellular vesicles exhibit immunosuppressive effects.[29,30] A further study with more focus on an optimized protocol using somatic stem cell pretreatment with AAV administration is therefore suggested.

Our results indicate that rAAV injection with MSCs pretreatment enhanced the expression of the rAAV-derived transgene in dogs. This strategy would be a practical approach for analyzing the expression and function of the transgene *in vivo*. These findings also support the future feasibilities of rAAV-mediated protein supplementation strategies for the treatment of DMD.

MATERIALS AND METHODS

Construction of a proviral plasmid and recombinant rAAV production

A previously constructed rAAV proviral plasmid harboring the luciferase gene and a CAG promoter was used as a reporter construct. As a therapeutic gene for DMD, canine microdystrophin was placed under the control of the CMV promoter. The vector genome was packaged into the pseudotyped rAAV9 capsid in HEK293EB cells.[31] A large-scale cell culture method with an active gassing system was used for transfection. The vector production process involved triple transfection with a proviral plasmid, the rAAV helper plasmid pAAV2/9 (a gift from Dr. James M. Wilson), and the adenovirus helper plasmid pAdeno. The rAAV particles were purified using a dual ion-exchange procedure based on high-performance membrane absorption, as previously described.[31] Viral titers were determined by quantitative real-time PCR using the MyiQ single-color detection system (Bio-Rad, Hercules, CA, USA) and the following primer pairs: forward primer 5′-GATACGCTGCTTTAATGCCTTT-3′ and reverse primer 5′-GTTGCGTCAGCAAACACAGT-3′ for rAAV9-CAG-luciferase; and forward primer 5′-TCGAGGAACTGAAAAACCAGAAA-3′ and reverse primer 5′-CACTTCCGTACAGGCCTAGAAGT-3′ for rAAV-CMV-microdystrophin.

Animals, cells, and intramuscular and intravenous injection with MSCs pretreatment

Seven- to 10-week-old wild-type dogs or dogs with $CXMD_J$ obtained from the beagle-based $CXMD_J$ breeding colony at the National Center of Neurology and Psychiatry Middle Animal Faculty (Tokyo, Japan) were used for gene transduction studies. All the animals were cared for and treated following the guidelines approved by the bioethics committees of the National Center of Neurology Middle Animal Faculty. For tolerance induction using MSCs, rAAV9-CAG-luciferase or rAAV9-microdystrophin (2×10^{12} v.g./kg BW) mixed with human MSCs (2×10^6 cells/kg BW; JCR, Japan), the mixing time of MSCs and rAAV9 was no longer than 30 min. The mixture of MSCs and rAAV9 was slowly injected directly into the cephalic vein. Seven days after the first injection, human MSCs were again injected systemically. Prior to intravenous injection of the MSCs, the dogs were sedated with thiopental and received chlorpheniramine maleate (0.15 mg/kg BW) half an hour before the injection for prophylactic purposes. One day after MSC injection, 8-week-old wild-type dogs were injected intramuscularly with rAAV-luciferase. Dogs were sedated and anesthetized with isoflurane by mask inhalation. rAAV9-CAG-luciferase (750 μL; 1×10^{12} v.g./muscle) mixed with MSCs (2×10^6 cells/kg BW) was injected intramuscularly into both tibialis anterior muscles of the wild-type dogs. For rAAV intravenous injection, 1 day after the second MSC administration, wild-type dogs or dogs with $CXMD_J$

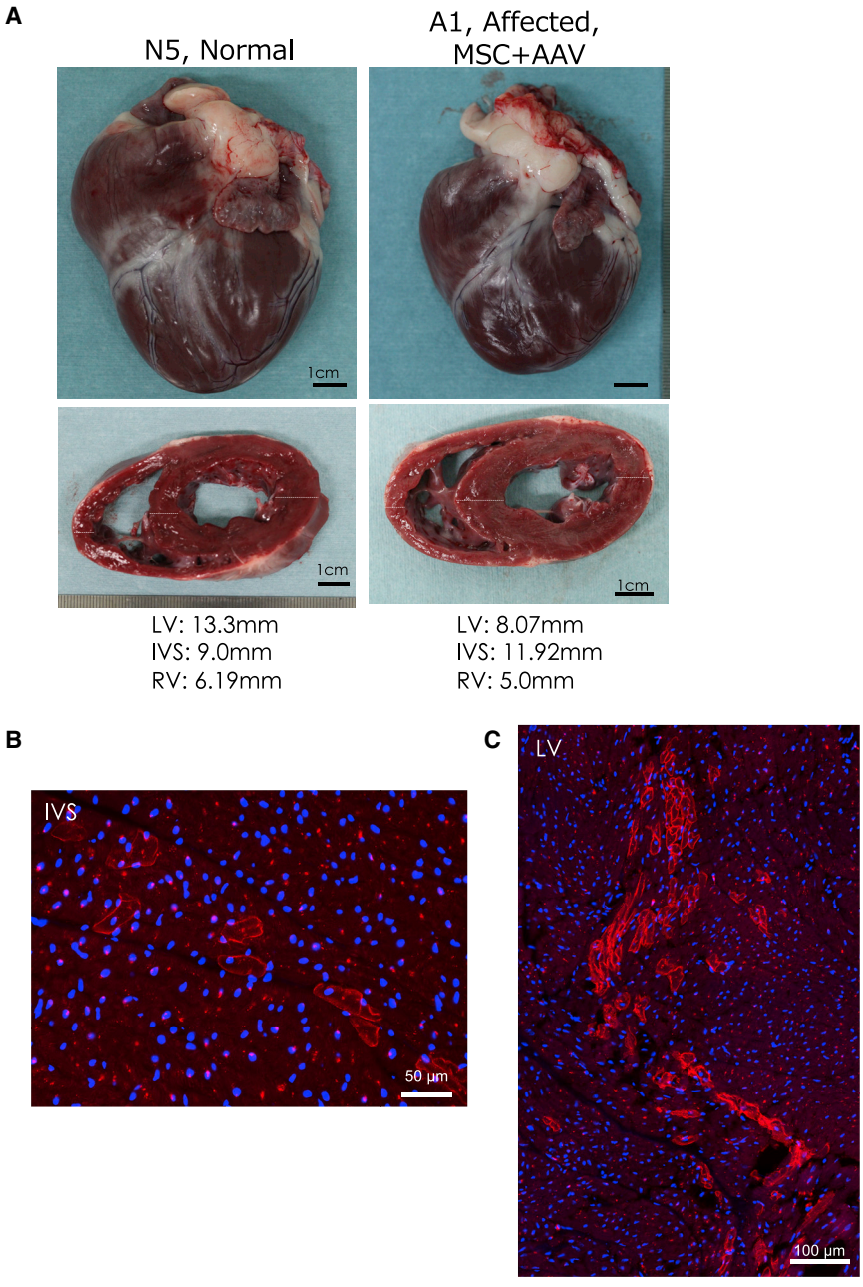

Figure 7. Macroscopic findings of the transduced dogs

(A) Gross morphology of the heart from each dog at the autopsy. The bottom panels show a cross-sectional view. Scale bar, 1 cm. White dotted lines show the position of cross dissection. LV, thickness of left ventricular wall; IVS, thickness of the intraventricular septum; RV, thickness of the right ventricular wall. (B and C) Immunohistochemical detection of the transgene in the heart of the rAAV-transduced dogs. Cardiac muscle (B, IVS; C, LV) from a transduced (A1) DMD dog were cryo-sectioned and stained with DysB. Scale bar, 50 μm (B) and 100 μm (C).

were injected with rAAV-luciferase or rAAV-microdystrophin via the cephalic vein at 10 weeks of age. None of the $CXMD_J$ dogs receiving rAAV showed any adverse effects. All the protocols were performed without immunosuppressants.

Genotyping of $CXMD_J$

The phenotype was initially determined based on an estimation of serum creatine kinase and confirmed by genomic PCR. The umbilical cord was isolated at delivery and genomic DNA was purified with DNAzol (Invitrogen, Carlsbad, CA, USA) and used for real-time PCR to confirm genotyping (forward primer; 5′-GGGCATGGGTTGTCAA TTAAA-3′, and phosphorothioate-modified normal; 5′-CAATCAAACAGGTCTGGC*G*T-3′ or affected; 5′-CAATCAAACAGGTCT GGC*G*C-3′ reverse primers).[14]

Histological analysis

For histological and immunohistochemical evaluation, biopsied temporal muscle and other skeletal muscle samples were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C. Cryostat sections (7 μm thick) were processed. Routine H&E staining was used for histological analysis. For immunofluorescence staining, cryosections were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. The tissue sections were then permeabilized for 5 min in PBS containing 0.1% Triton X-100 and then treated with BlockAce (DS Pharma Biomedical, Osaka, Japan) in PBS. The following antibodies were used for antigen detection (at dilutions of 1:50–100): rabbit anti-firefly luciferase (ab21176, Abcam, Cambridge, UK) and mouse anti-dystrophin (NCL-DysB and NCL-DYS2, Leica). Antibodies were diluted in BlockAce/PBS and reacted with the tissue sections overnight at 4°C. After washing three times with PBS, the tissue sections were incubated with Alexa 488- or Alexa 568-conjugated anti-mouse IgG antibodies (Thermo Fisher Scientific), or anti-rabbit IgG antibodies (Thermo Fisher Scientific) diluted 1:500 for 1 h at room temperature. Coverslips or slide glasses were washed with PBS and mounted in DAPI-Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) containing DAPI.

T cell responses

Peripheral lymphocytes were isolated from 5 mL of whole blood using Lympholyte-Mammal (Cedarlane, ON, Canada) and SepMate-50 (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. Purified lymphocytes were incubated with rAAV9-CMV-microdystrophin (1.0×10^6 v.g./cell) overnight.

Total RNA was isolated from cultured lymphocytes using TRIzol (Thermo Fisher Scientific). First-strand cDNA was synthesized using a SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). The primers employed in this study were specific for canine IFN-γ, (F: 5′-TTTCATTCAACCCCTTCTCG-3′, R: 5′-TAAATGCACAACCCACAGGA-3′) and 18S rRNA primers in QuantumRNA Universal 18S Internal Standards (Thermo Fisher Scientific).

Clinical manifestations

The detailed clinical cardiac and histopathological characteristics associated with $CXMD_J$ have been reported previously.[11,32] Disturbances in gait and mobility, limb contracture, macroglossia, dysphagia, and drooling were regarded as clinical signs in the rAAV-transduced and untransduced dogs. These signs were monitored monthly. The severity of each sign was classified according to a new grading scale for $CXMD_J$ based on that outlined in a previous report.[32] At least one veterinarian with experience assessing symptoms in dystrophic dogs performed all the clinical examinations.

Holter monitoring

For electrocardiogram (ECG) monitoring, a jacket containing a Holter electrocardiograph (QR2100; Fukuda M-E Kogyo, Tokyo, Japan) was worn by the dogs at 102 weeks of age, and ECG was recorded using NASA and CM5 leads. After a recording period of at least 24 h, the Holter monitor was removed and the data were transferred to an ECG analyzer (HS1000; Fukuda M-E Kogyo) for analysis.[14,33]

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.11.003.

ACKNOWLEDGMENTS

We thank Dr. James M. Wilson for providing the helper plasmid pAAV2/9. We also thank JCR Pharmaceuticals (Hyogo, Japan), Kaneka Corporation (Tokyo Japan), and Daiichi Sankyo (Tokyo, Japan) for collaborative investigation; Drs. Naoko Yugeta, Yoko Fujii, Tetsuya Nagata, and Takashi Saitoh for technical advice, support, and helpful discussion; Kazue Kinoshita for rAAV preparation and purification; Ryoko Nakagawa and Satoru Masuda for technical assistance; and Hideki Kita, Shin-ichi Ichikawa, Yumiko Yahata-Kobayashi, Aya Kuriyama, Takayoshi Hikage, Akane Hanaoka, Natsumi Ogawa, and other staff members of JAC for their care of the dogs. This work was supported by Japan Agency for Medical Research and Development (AMED) under grant numbers 19ae0201005h0002, 19ae0201005h9902 and Intramural Research Grant (28-6) for Neurological and Psychiatric Disorders of NCNP, JAPAN.

AUTHOR CONTRIBUTIONS

H.H.-K., and T.O. conceived and designed the experiments and wrote the manuscript; Y.N.-K. recorded the echocardiograms and assisted in the dissection of dogs; M.K. provided advice on veterinary aspects, dissection, clinical grading of the dystrophic dogs, and data analysis; G.P.-H. marked up the manuscript; H.O. and T.C. prepared the rAAV; and S.T. participated in meaningful discussion. All authors reviewed, edited, and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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