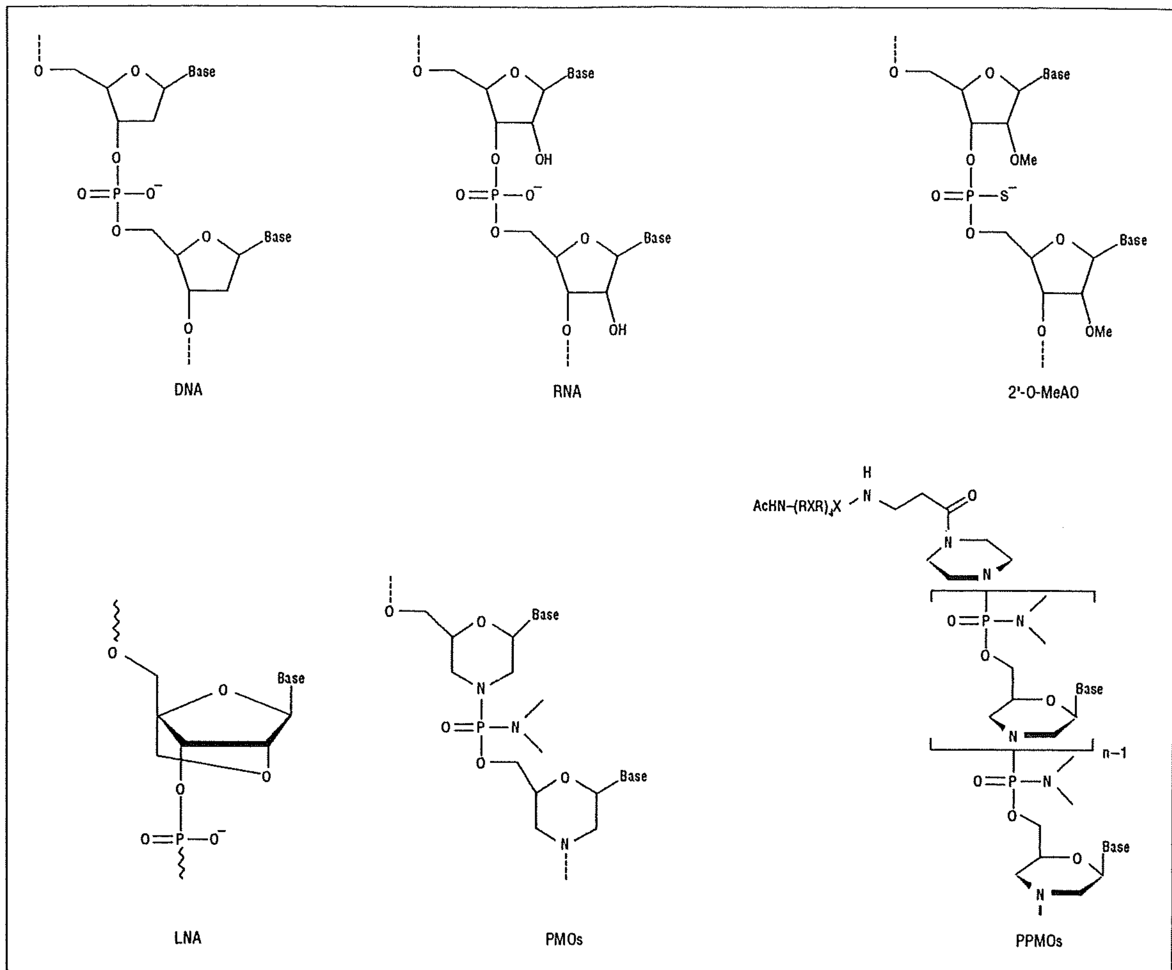

Figure 1. Comparison of chemistries used for the exon-skipping approach. Examples of artificially developed antisense oligomers such as 2′-O-methylated antisense oligonucleotides (2′-O-MeAO) (phosphorothioate), locked nucleic acid (LNA), phosphorodiamidate morpholino oligomers (PMOs), and peptide-tagged PMOs (PPMOs) are shown for comparison with DNA and RNA.

Administration (FDA), fomivirsen sodium (Vitravene; Isis Pharmaceuticals, Inc) delivered by intravitreous injection to inhibit cytomegalovirus retinitis in AIDS. Vitravene was approved in 1998 and there have been no subsequent successful approvals in the ensuing 10 years.

What has slowed the progress of AO drugs into the clinical arena, and why may this be changing?

There have been 2 major hurdles: off-target toxic effects and potency or delivery. Regarding toxic effects, most organisms do not take kindly to covert infiltration by foreign DNA or RNA. Indeed, all species have quite effective mechanisms to destroy foreign DNA and RNA as they are more likely than not to be viruses or other undesirable organisms. In addition, many of the clinical trials testing AO drugs have seen evidence of activation of the complement cascade, and this has been a key concern of the FDA. Delivery has also been a consistent problem. Because the target RNAs are always intracellular, it is imperative for the AO drug to achieve intracellular concentrations sufficient to enable it to bind and modulate the target RNA to a significant extent. The fact that AOs typically do not easily cross the lipid bilayers that bound the cell so as to achieve sufficient intracellular potency via systemic (intravenous) delivery has been problematic.

Recent developments are achieving success in overcoming both hurdles. Analogues of nucleic acid have been designed and synthesized in which the ribose backbone of RNA and DNA is replaced with different chemistries (**Figure 1**). Two are particularly promising: one uses a morpholino backbone (phosphorodiamidate morpholino oligomer [PMO]; AVI BioPharma, Portland, Oregon), and the second uses a locked nucleic acid backbone (Enzon Pharmaceuticals, Inc, Bridgewater, New Jersey). These new backbones are designed to maintain the molecular distance between bases (G, A, T/U, and C), enabling highly sequence-specific base pairing to the target RNA that is stronger in the case of PMO and locked nucleic acid drugs than DNA or RNA AOs. Equally important, these backbones are so dissimilar from the DNA and RNA ribose phosphodiester backbone that they are not recognized by most or any DNA and RNA binding proteins or degrading enzymes, thereby enhancing their stability and avoiding many or all off-target toxic effects.

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The second major barrier has been achieving sufficient intracellular concentrations (delivery). One successful approach is to take advantage of preexisting holes in the plasma membrane of the target cell. Infecting viruses breach the cell membrane during the process of infection and appear to bring along AO drugs in the process. As such, AOs have been quite successful in blocking downstream viral replication within cells, and PMO drugs are showing impressive promise as antiviral antidotes.[5] Another preexisting hole is found in muscle cells lacking dystrophin (Duchenne muscular dystrophy [DMD]).[6] The unstable plasma membrane of myofibers appears to allow the AO to leak into the cell.[7] An additional approach is to modify the AO drugs with cell delivery moieties, chemical adducts that penetrate the cell membrane. One example is the addition of arginine-rich peptides to one end of the AO drug (peptide-tagged PMO) (Figure 1).

From these advances has sprung a resurgence of interest in AO drugs for treatment of genetic disease, cancer, and infectious disease. The purpose of most applications is to knock down a target RNA so that it makes less of the deleterious protein product (eg, tumor growth factor β or hypoxia-inducible factor 1α in cancer cells, viral mRNAs, or dominant gain-of-function toxic proteins in inherited neurological disease). However, the disorder that may be most advanced in such applications is DMD. Here the AOs are used for a quite different objective than for previous applications; explicitly, AOs in DMD are designed to restore function to the target mRNA and protein rather than block it. The remainder of this review focuses on this application.

RATIONALE AND PROOF OF PRINCIPLE OF EXON-SKIPPING THERAPY

The principle of exon-skipping therapy for dystrophinopathies was initially demonstrated by Dunckley et al[8] in cultured mouse muscle cells in vitro. The rationale is as follows. Duchenne muscular dystrophy is caused by mutations of the 79-exon gene (commonly deletions of ≥1 exon). Within the myofiber, the remainder of the gene will be transcribed and spliced together. However, if the triplet codon reading frame of the mRNA is not preserved, the resulting frame shift will lead to the failure of dystrophin protein production. Becker muscular dystrophy (BMD) is a clinically milder and more variable disease in which mutations of the dystrophin gene are commonly such as to preserve the translational open reading frame; thus, after splicing together, the remainder of the gene retains some ability to synthesize the dystrophin protein. The goal of exon-skipping therapies is to force the dysfunctional mRNA with out-of-frame mutations in a patient with DMD to skip (exclude) some additional exons. The loss of additional material directed by AO drugs restores the reading frame, changing a Duchenne out-of-frame transcript to a Becker in-frame transcript. Fortunately, most mutations in the dystrophin gene occur in parts that do not code for functionally essential regions of the protein.

This AO-mediated exon-skipping method has been developed and extensively tested on the dystrophic *mdx* mouse model of DMD. The *mdx* mouse harbors a nonsense mutation in exon 23 that prevents translation beyond this point in the transcript. Both local intramuscular injection and systemic delivery of a single AO targeted against exon 23 in the primary transcript excludes this exon from the mRNA, leaving an in-frame transcript that generates dystrophin expression and produces a degree of functional recovery. Intramuscular and systemic injections of AOs for exon splicing of a dog model of DMD have also been demonstrated with a novel cocktail AO strategy (T.Y., S.T., Q.-L.L., T.A.P., A.N., E.P.H., and Masanori Kobayashi, DVM, unpublished data, 2006-2008). The principle is similarly illustrated in humans; van Deutekom et al[9] reported single-site intramuscular injections of 2′-O-methyl AO chemistry in 4 boys with DMD, showing evidence of de novo dystrophin production at the injection site.

These data demonstrate that the key hurdles of achieving intracellular delivery and avoiding toxic effects can be cleared. A similar strategy is being explored in other diseases such as myotonia, human immunodeficiency virus, and spinal muscular atrophy.[10-12]

HURDLES IN BRINGING EXON SKIPPING TO STANDARD OF CARE

Exon skipping using AO drugs has rapidly emerged as the frontline therapeutic approach for DMD. How soon can we expect exon skipping to reach the neuromuscular clinic and standard of care? This approach is breaking new ground and raising challenges not encountered previously in drug development. Different patients have different mutations, and many AO sequences will need to be designed, tested, and FDA approved. Also, current genotype and phenotype data suggest that there may be good in-frame deletions and not-so-good in-frame deletions; simply restoring the reading frame may not be synonymous with restoring dystrophin protein function. The optimization of dystrophin function will likely require deletions of multiple exons, and this will require mixtures of different AOs—new territory for drug development and the FDA. The approach will require regular injections of large amounts of AO drug; what are the long-term toxic effects? Moreover, are the standard toxicity tests appropriate for sequence-specific drugs? Each of these hurdles is discussed briefly in the remainder of this review.

CERTAIN EXON DELETIONS MAY RETAIN MORE DYSTROPHIN FUNCTION THAN OTHERS

The molecular diagnostics of DMD and BMD frequently refer to the reading frame rule, where out-of-frame deletions are given a DMD diagnosis and in-frame deletions are given a BMD diagnosis. However, as many as 30% of patients with BMD do not adhere to this rule.[13] A thorough understanding of reading frames is critical for appropriate design of exon-skipping therapies, both so that the best AO can be given to the patient and so that an optimally functional dystrophin protein is produced as a result of the expected exon skipping. Currently, the best information from which to predict the capabilities of partially deleted dystrophins to rescue the DMD phenotype comes from analysis of the thousands of geno-

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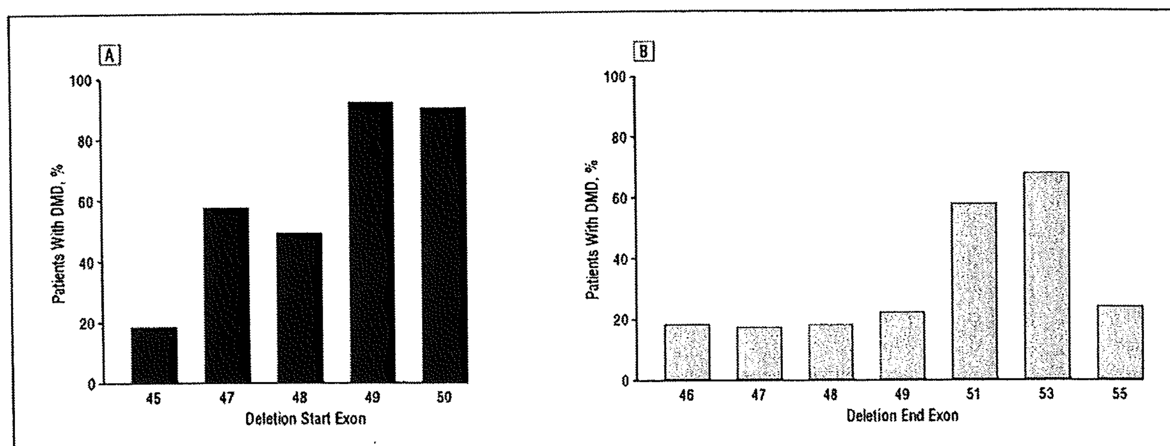

Figure 2. Clinical phenotypes associated with specific start (A) and end (B) sites for in-frame deletions. Percentages of patients with Duchenne muscular dystrophy (DMD) out of patients with DMD or Becker muscular dystrophy with specific start and end exons are shown. Combined muscular dystrophy databases of 14 countries (from Argentina, Belgium, Brazil, Bulgaria, Canada, China, Denmark, France, India, Italy, Japan, The Netherlands, the United Kingdom, and the United States) at Leiden University (http://www.dmd.nl), where diagnoses were performed using multiplex ligation-dependent probe amplification/multiplex amplification and probe hybridization, Southern blotting, or polymerase chain reaction primer sets that allow deletion boundaries to be assigned accurately to a specific exon, are used (deletion start sites: n=288 for exon 45, n=23 for exon 47, n=9 for exon 48, n=12 for exon 49, and n=10 for exon 50; deletion end sites: n=11 for exon 46, n=115 for exon 47, n=95 for exon 48, n=51 for exon 49, n=53 for exon 51, n=40 for exon 53, and n=21 for exon 55).

type and phenotype correlations in patients with DMD and BMD that have been published in the literature and on the Internet. We examined all in-frame deletions and determined the proportion of observed cases that showed mild or severe phenotypes. This was gleaned from combined muscular dystrophy databases of 14 countries (from Argentina, Belgium, Brazil, Bulgaria, Canada, China, Denmark, France, India, Italy, Japan, The Netherlands, the United Kingdom, and the United States) at Leiden University (http://www.dmd.nl), excluding diagnoses that did not allow deletion boundaries to be assigned accurately to a specific exon.[14] Of all observed in-frame deletion patterns on genomic DNA in the central rod domain hotspot region (exons 42-57; 28 distinct patterns), 57% (16 of 28 patterns) were associated with DMD rather than BMD. This analysis showed that there are considerable discrepancies between population-based ratios and pattern-based proportions of severe DMD vs mild BMD phenotypes, and interestingly, the ratio of DMD to BMD remarkably varies between specific deletion patterns. For example, in-frame deletions starting or ending around exon 50 or 51 that encode the hinge region were most commonly associated with severe phenotypes (**Figure 2**) (eg, deletions at exons 47-51, 48-51, and 49-53 are all reported to be associated with a severe DMD phenotype rather than BMD).[15,16]

Two questions arise. First, why do specific patterns of in-frame mutations tend to result in a severe DMD phenotype in contradiction to the reading frame rule? Second, why do different individuals with the same exonic deletion pattern exhibit such different clinical phenotypes? Likely contributory factors include the following: the effect of the specific deletion breakpoints on mRNA splicing efficiency and/or patterns; translation or transcription efficiency after genome rearrangement; and stability or function of the truncated protein structure. The mechanisms controlling accurate splicing of the 79-exon, 2.4 million–base pair dystrophin gene are clearly complex. Introns of the dystrophin gene are highly variable in size, and it is likely that exonic splicing does not take place in an ordered 5′ to 3′ sequence. A complication in interpreting genotype and phenotype correlations is that the deletion in genomic DNA does not always correspond to the material missing from the resulting mRNA. We and others have shown that even in the absence of AOs, a patient may produce 1 or more transcripts that skip additional exons present in the genomic DNA, in effect performing their own private exon skipping.[13] Disruption of splice site information (such as an intervening sequence) in some patients with in-frame gene deletions may cause skipping of additional exons at mRNA splicing, thus leading to out-of-frame transcripts from an in-frame genomic DNA deletion as Kesari et al[13] have recently described. As Menhart[17] has pointed out, it is also likely that quasi-dystrophin variants in the rod domain may show different stability or function because of different types of derangement of spectrinlike repeat domains. Not enough is known about dystrophin structure and function, and the relative importance of the protein sequence within the rod domain remains entirely a matter of speculation. Historically, lack of dystrophin expression has been used as the key criterion for DMD diagnosis. This together with the presence of the DMD clinical picture with such in-frame mutations argues that other confounding variables such as imprecisely defined mutation or aberrant splicing may explain these "exceptions to the reading frame rule." Thus, it is anticipated that most or all patients with mutations in the central rod domain would benefit from the production of truncated dystrophin.

PARALLELING AO TRIALS: TESTING NEW EXONS AND MIXTURES

Clinical proof-of-concept trials testing limited intramuscular injection with a 2′-O-methyl AO against exon 51

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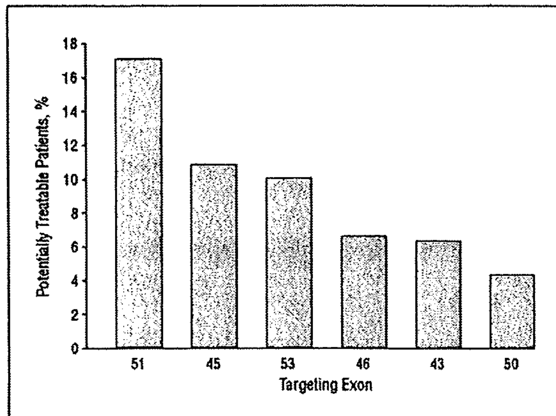

Figure 3. Targets of exon skipping and population of potentially treatable patients. Percentage of patients with the dystrophin deletion who are potentially treatable by targeting specific exons for Duchenne muscular dystrophy. For example, 17% of patients with Duchenne muscular dystrophy who have the dystrophin deletion can be potentially treated by targeting exon 51 using antisense oligonucleotides.

have been published,[9] and similar studies with PMO chemistry are under way in the United Kingdom. Given the many questions concerning the sequence specificity of toxic effects and the large number of AO sequences that will need to be developed as drugs to treat most patients with DMD, it is critical to parallel studies on many more AOs for DMD (**Figure 3**).

It should be noted that about 30% of patients with DMD have nondeletion mutations (duplication, nonsense mutations, small rearrangement, or splice site mutations). Most mutations are theoretically amenable to exon skipping; however, there are no hot spots for point mutations, so relatively few patients would be treatable with each targeted exon by comparison with deletion mutations. Moreover, if skipped to remove a nonsense mutation, the exons that are candidates to restore the reading frame in patients with deletions (eg, exons 43, 45, 46, 50, 51, and 53) will require additional deletion of at least 1 further exon to restore the reading frame because these are frame-shifting exons. Thus, only 35% of nonsense mutations are potentially treatable by single-exon targeting, but the combined data of the Leiden DMD mutation database imply that more than 90% could be responsive to multiskipping.[14]

Development of exonic cocktails (mixtures) could resolve a number of problems, including optimization of dystrophin function and covering relatively high proportions of patients with DMD with a single mixture. The mixture approach has clear advantages and disadvantages. As an example, an 11-exon AO cocktail skipping exons 45 through 55 is predicted to result in a particularly mild BMD phenotype (94% of reported patients).[14] Encouragingly, this large deletion is regularly associated with clinically milder phenotypes than any of the smaller in-frame deletions within the same range of exons 45 to 55.[18] A second advantage is that the cocktail could conceivably be approved as a single drug for most patients with DMD who have dystrophin deletions, independent of their precise deletion, eg, an 11-exon AO cocktail targeting exons 45 through 55 is potentially applicable to more than 60% of patients with a dystrophin deletion (**Figure 4**).[18,19] In total, more than 90% of patients with DMD could potentially be treated by multiskipping, whereas single-exon skipping could treat around half of the patients with dystrophin deletions and point mutations. Systemic studies in the large dog model of DMD have been done using a 3-exon PMO cocktail, and this has clearly been shown to be efficacious by multiple clinical, imaging, histological, and biochemical or molecular end points (T.Y., S.T., Q.-L.L., T.A.P., A.N., E.P.H., and Masanori Kobayashi, DVM, unpublished data, 2006-2008).

A disadvantage of the cocktail approach is the addition of novel hurdles for FDA or regulatory approval. Current FDA regulations require each component of a drug mixture to undergo toxicological and clinical testing and then require the mixture to similarly undergo toxicological and clinical testing. In the context of an ideal 11-exon AO cocktail, the regulatory barriers become truly intimidating. In addition, the 11-exon cocktail PMO approach would lead to delivery of some AOs that may not have a target in a specific patient (eg, the patient already has a deletion of ≥1 exon in the AO mix). Thus, some parts of the mixture will have no possible potential molecular or clinical benefit to individual patients. This would again be uncharted territory for the FDA. While clinical development of the 11-exon mixture is likely ambitious at present, it will be important to initiate toxicological and clinical trials of exon mixtures for subsets of patients who cannot be treated with a single AO. Also, for future trials on multiskipping such as with exons 45 through 55, we should have as many AOs in hand as possible because they can be used as part of multiskipping AOs.

PERSONALIZED MEDICINE AND THE FDA: ARE EXISTING GUIDELINES APPROPRIATE?

Personalized medicine has many definitions, but most share the concept of optimizing a treatment for a particular patient. Designing and using AO drugs targeted for a patient's specific gene mutation would seem to fit well within this rubric. As such, the promising AO exon-skipping approach may bring neuromuscular disease to the frontline in development of drugs for personalized medicine. It is important to examine the existing FDA guidelines for drug development and reinterpret these guidelines in the context of AO and DMD. For example, the drug development pipeline includes phase 1 studies of the drug in healthy volunteers. However, successful on-target exon skipping of the dystrophin gene in healthy volunteers would give them DMD, a clear adverse effect that is entirely irrelevant to toxic effects in the target patient population (boys with DMD). Toxicity tests are currently done in animal models (typically 2 species), but one of the major concerns regarding toxic effects of AO drugs is binding to off-target RNAs. For example, if an AO drug designed for exon skipping of the dystrophin mRNA also binds to the closely related utrophin mRNA, then exon skipping of utrophin might occur and could result in off-target adverse effects. The utrophin sequence of mice or rats is different from the utrophin sequence of humans, so the standard rodent toxicity tests

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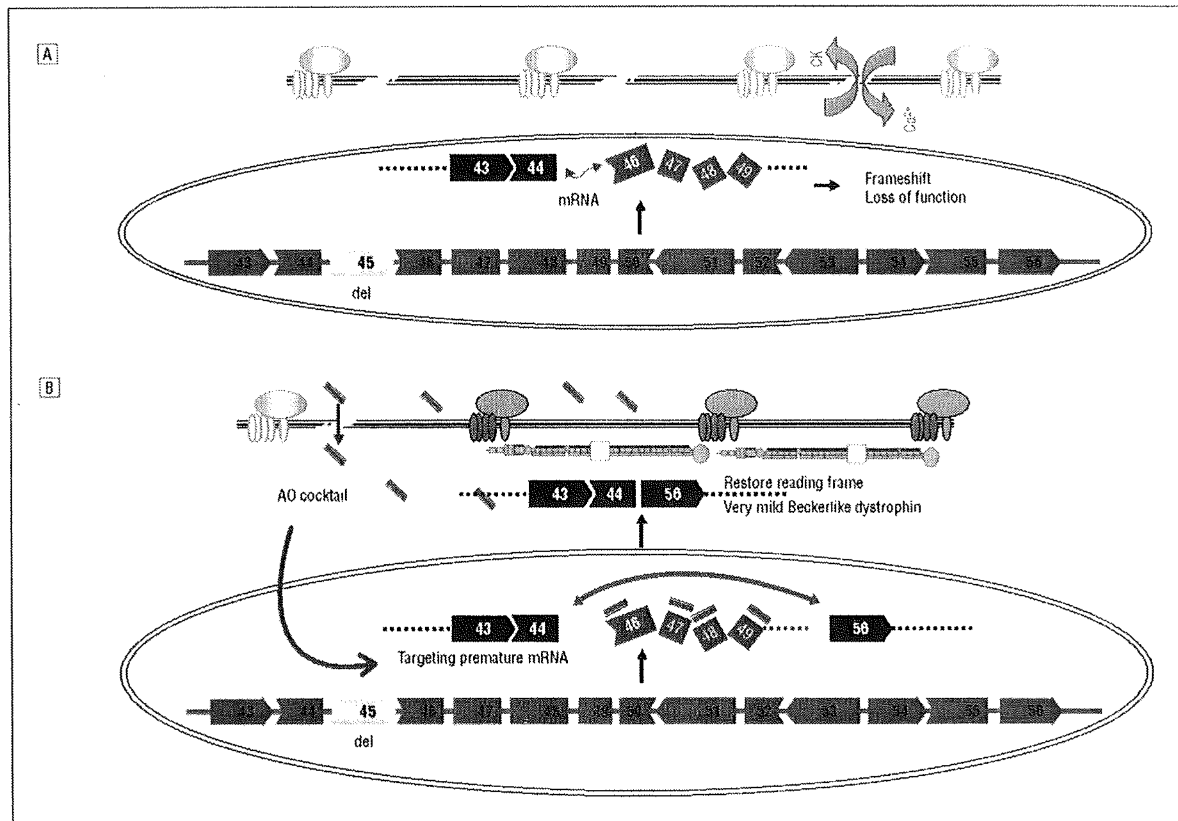

Figure 4. Mechanism of multiexon skipping of exons 45 through 55 to rescue 60% of patients with Duchenne muscular dystrophy with dystrophin deletions. A, More than 60% of deletion mutations of the dystrophin gene occur within the hot-spot range of exons 45 through 55 (exon 45 is deleted in this schematic [del]) in Duchenne muscular dystrophy muscles. The messenger RNA (mRNA) of remaining exons is spliced together but the reading frame is disrupted, resulting in failure of the production of functional dystrophin protein. CK indicates creatine kinase; Ca^{2+}, calcium ions. B, An antisense oligonucleotide (AO) cocktail targeting exons 45 through 55 likely enters the Duchenne muscular dystrophy muscle through its leaky membranes, then binds to the dystrophin mRNA in a sequence-specific manner. The AOs block the splicing machinery and prevent inclusion of all exons between exons 45 and 55. Skipping these exons restores the reading frame of mRNA, allowing production of quasi-dystrophin containing exons 1 through 44 and exons 56 through 79, which is not normal but likely retains considerable function as evidenced by patients with clinically milder Becker muscular dystrophy with identical partial dystrophin.

may not accurately assess off-target toxic effects of AOs for human use.

Perhaps the largest challenge facing implementation of exon-skipping therapy for DMD is in developing new approaches to toxicity testing and clinical trial regulatory procedures that are relevant and appropriate for sequence-specific drugs. The pharmaceutical industry often quotes a price tag of $500 million to bring any new drug to the market. Given the discussion earlier, implementation of AO drugs in DMD will require many exon-specific drugs. If the $500 million is assessed for each individual AO sequence, then both time and money become insuperable barriers to helping the existing generation of boys with DMD. The silver lining in this cloud is the lack of any detectable toxic effects with PMO AO drugs to date. If multiple AOs all show a lack of long-term toxic effects, then there is hope that specific AO drugs could be approved with more limited toxicological and phase 1 testing.

A practical resolution of this problem is to consider each component of the potential toxic effects of these highly targeted drugs individually. Tests of the generic toxic effects of morpholinos at the doses at which they are likely to be functionally effective could be conducted quite straightforwardly with either a scrambled or arbitrary sequence of a particular molecular weight. It is the notion of individual sequence-specific toxic effects that raises problems. The argument that any specific sequence may have off-target effects (eg, binding to utrophin transcripts) cannot be properly tested in other species because they may have different potential off-target sequences as compared with those in humans. This carries the dire implication that a lack of sequence-specific toxic effects in a test species can provide no assurance, indeed no information at all, as to the sequence's safety in humans. Tests in healthy human volunteers are also problematic. Ethical issues arise from the possible generation of a pathogenic frameshift in healthy muscle by successful suppression of the targeted exon. Moreover, the lack of innate pathological abnormalities in healthy human muscle would stifle access of the AO to its intended intramuscular target while at the same time providing a different spectrum of potential off-target molecules (eg, utrophin transcripts). A further complication arises from the individualistic nature of the entire rationale, for it precludes the possibil-

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ity of learning from experience; the probability that any given sequence may be toxic is independent of the number of safe experiences with other sequences. In effect, for safety, we can test for sequence-specific toxic effects only in human volunteers with DMD by progressive dose escalation. Only in this way would the reagents have access to their intended targets as well as any unintended targets in a physiological context that is inappropriately modeled both in other species and in healthy human volunteers.

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Correspondence: Eric P. Hoffman, PhD, Research Center for Genetic Medicine, Children's National Medical Center, 111 Michigan Ave NW, Washington, DC 20010 (ehoffman@cnmcresearch.org).
Author Contributions: *Study concept and design:* Yokota, Takeda, Partridge, and Hoffman. *Acquisition of data:* Yokota and Lu. *Analysis and interpretation of data:* Yokota, Nakamura, and Hoffman. *Drafting of the manuscript:* Yokota, Partridge, Nakamura, and Hoffman. *Critical revision of the manuscript for important intellectual content:* Takeda, Lu, and Partridge. *Obtained funding:* Hoffman. *Administrative, technical, and material support:* Lu. *Study supervision:* Yokota, Takeda, Partridge, Nakamura, and Hoffman.
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Transduction Efficiency and Immune Response Associated With the Administration of AAV8 Vector Into Dog Skeletal Muscle

Sachiko Ohshima[1,2], Jin-Hong Shin[1,3], Katsutoshi Yuasa[1,4], Akiyo Nishiyama[1], Junichi Kira[2], Takashi Okada[1] and Shin'ichi Takeda[1]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan;* [2]*Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan;* [3]*Department of Neurology, Graduate School of Medicine, Pusan National University, Busan, Republic of Korea;* [4]*Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Musashino University, Tokyo, Japan*

Recombinant adeno-associated virus (rAAV)-mediated gene transfer is an attractive approach to the treatment of Duchenne muscular dystrophy (DMD). We investigated the muscle transduction profiles and immune responses associated with the administration of rAAV2 and rAAV8 in normal and canine X-linked muscular dystrophy in Japan ($CXMD_J$) dogs. rAAV2 or rAAV8 encoding the *lacZ* gene was injected into the skeletal muscles of normal dogs. Two weeks after the injection, we detected a larger number of β-galactosidase-positive fibers in rAAV8-transduced canine skeletal muscle than in rAAV2-transduced muscle. Although immunohistochemical analysis using anti-CD4 and anti-CD8 antibodies revealed less T-cell response to rAAV8 than to rAAV2, β-galactosidase expression in rAAV8-injected muscle lasted for <4 weeks with intramuscular transduction. Canine bone marrow-derived dendritic cells (DCs) were activated by both rAAV2 and rAAV8, implying that innate immunity might be involved in both cases. Intravenous administration of rAAV8-*lacZ* into the hind limb in normal dogs and rAAV8-*microdystrophin* into the hind limb in $CXMD_J$ dogs resulted in improved transgene expression in the skeletal muscles lasting over a period of 8 weeks, but with a declining trend. The limb perfusion transduction protocol with adequate immune modulation would further enhance the rAAV8-mediated transduction strategy and lead to therapeutic benefits in DMD gene therapy.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited disorder causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. No effective treatment has been established despite the development of various novel therapeutic strategies including pharmacologic and gene therapies. Dystrophin-deficient *mdx* mice and dystrophin-utrophin double-knockout mice are the animal models most widely used to evaluate therapeutic efficacy, although the symptoms of *mdx* mice are not comparable to those of human DMD patients. Dystrophin-deficient canine X-linked muscular dystrophy was found in a golden retriever,[1,2] and we have established a Beagle-based model of canine X-linked muscular dystrophy in Japan ($CXMD_J$) dogs.[3] The clinical and pathological characteristics of the dystrophic dogs are more similar to those of DMD patients than murine models.[3]

The recombinant adeno-associated virus (rAAV) can be used for delivering genes to muscle fibers. Several serotypes of rAAV exhibit a tropism for striated muscles.[4,5] Intramuscular or intravenous administration of rAAV carrying the microdystrophin gene was reported to restore specific muscle force and extend the lifespan in dystrophic mice.[6,7] In contrast to the success of transgene delivery in mice, rAAV2 or rAAV6 delivery to canine striated muscles without immunosuppression resulted in insufficient transgene expression, and rAAV evoked strong immune responses.[8,9] An assay of interferon-γ released from murine and canine splenocytes suggested that the immune responses against rAAV and transgene products in mice and in dogs are dissimilar.[8] Uptake of rAAV2 by human dendritic cells (DCs) and T-cell activation in response to the AAV2 capsid have been reported,[10] indicating that DCs play key roles in the immune response against rAAV-mediated transduction. On the other hand, other serotypes, including rAAV8, that are capable of whole-body skeletal muscle expression after intravenous administration,[4,5]induce less T-cell activation.[11] We hypothesized that the level of activation of canine DCs by rAAV8 might be lower than that achieved by rAAV2. However, the transduction profile and immune response in the rAAV8-injected dog skeletal muscle have not been elucidated.,

In this study, we chose to use intramuscular injections under ultrasonographic guidance so as to minimize the inflammatory reaction caused by incisional intramuscular injection.[8] In

Correspondence: Shin'ichi Takeda or Takashi Okada, Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan. E-mail: takeda@ncnp.go.jp or t-okada@ncnp.go.jp

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addition, intravascular delivery was performed as a form of limb perfusion, in an attempt to bypass the immune activation of DCs in the injected muscle.[12] We investigated the transgene expression and host immune response to two distinct serotypes of rAAV in normal and dystrophic dogs after direct intramuscular injection and after limb perfusion.

RESULTS

Extensive expression of β-galactosidase in rAAV8-transduced muscles in wild-type dogs

We administered nonincisional intramuscular injections under ultrasonographic guidance so as to minimize injury. With incisional injection, the ordinary method of intramuscular viral administration in dogs,[8] the skin is opened to identify the individual muscles. This may enhance the immune reaction by recruiting inflammatory cells for wound healing. After nonincisional injection of rAAV2-*lacZ*, faint β-galactosidase (β-gal) expression was detected, whereas lymphocyte infiltration still occurred (**Supplementary Figure S1**). To investigate the transduction efficiency of rAAV8 in canine skeletal muscle, normal dogs were transduced with rAAV-*lacZ* serotypes 2 and 8 (**Table 1**). Prominent expression of β-gal was observed in the rAAV8-*lacZ*-injected muscles, whereas the rAAV2-*lacZ*-injected muscles showed minimal transgene expression (**Figure 1**). While β-gal expression in the rAAV8-injected muscle was correlated with the viral dose, β-gal expression in the rAAV2-injected muscle was not augmented with viral dose escalation. However, rAAV8-*lacZ*-injected muscles, which showed extensive β-gal expression at 2 weeks, also exhibited reduced expression at 4 weeks after the injection, thereby suggesting that the transgene product had immunogenicity (**Supplementary Figure S2**).

To evaluate the difference in transduction efficiency between rAAV2 and rAAV8 at 2 weeks after the injection, relative quantifications of the vector genome and mRNA were performed. The result demonstrated higher transduction rates in the rAAV8-injected muscles as increasing amounts of the vector were administered (**Figure 2a,b**). The amount of protein expression was also well correlated with that of transgenic DNA (**Figure 2c, Supplementary Table S1**). Immunohistochemical analysis revealed that the rAAV2-injected muscles showed much more infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the endomysial space than the rAAV8-injected muscles did (**Figure 3a**). mRNA levels of TGF-β1 and IL-6 (representative markers of inflammation) in the rAAV-injected muscles were standardized with the β-gal expression. rAAV2-injected muscles had higher TGF-β1 and IL-6 expression than rAAV8-transduced muscles (**Supplementary Figure S3**). We also examined humoral immune responses against the rAAV particles in the sera of rAAV-injected dogs. The levels of serum IgG in reaction to rAAV2 or rAAV8 gradually increased with time in both serotypes (**Figure 3b**). These results suggest

Table 1 Summary of gene transduction experiments

Dog ID	Sex	Age[a]	BW[b]	rAAV serotype	Transgene	Route	Muscle	Vector dose[c]	Transgene expression[d] 2 weeks	Transgene expression[d] 4 weeks	Transgene expression[d] 8 weeks	Cellular infiltration[e] 2 weeks	Cellular infiltration[e] 4 weeks	Cellular infiltration[e] 8 weeks
2201MN	M	10	4.5	2	lacZ	i.m.	TA, ECR	1×10^{11}	−	−		−	++	
3004MN	M	5	2.8	2	lacZ	i.m.	TA, ECR	1×10^{11}	±	−		+	±	
3007FN	F	5	2.5	2	lacZ	i.m.	TA, ECR	1×10^{11}	±	±		++	++	
2204FN	F	10	2.5	2	lacZ	i.m.	TA, ECR	1×10^{12}	−	−		+	++	
2801FN	F	10	5.2	2	lacZ	i.m.	TA, ECR	1×10^{12}	+	−		+	++	
2901MN	M	6	2.8	2	lacZ	i.m.	TA, ECR	1×10^{12}	−			±		
7M48	M	7	3.3	2	lacZ	i.m.	TA, ECR	1×10^{12}	−			+		
2206FN	F	10	3.0	2	lacZ	i.m.	TA, ECR	1×10^{13}	±	±		+	++	
2205MN	M	10	4.2	8	lacZ	i.m.	TA, ECR	1×10^{11}	++	±		−	++	
2905MN	M	6	2.8	8	lacZ	i.m.	TA, ECR	1×10^{11}	±			−		
NL52F	F	10	3.5	8	lacZ	i.m.	TA, ECR	1×10^{12}	+++			±		
2106FN	F	6	3.2	8	lacZ	i.m.	TA, ECR	1×10^{12}	+++	−		−	++	
7M49	F	6	3.2	8	lacZ	i.m.	TA, ECR	1×10^{12}		±	−		++	±
2109FMN	M	7	3.3	8	lacZ	i.m.	TA, ECR	1×10^{12}	+++			−		
2903MN	M	6	3.2	8	lacZ	i.m.	TA, ECR	1×10^{12}	+++			±		
2209MN	M	10	4.3	8	lacZ	i.m.	TA, ECR	1×10^{13}	+++	±		±	+++	
2309FA	F	6	3.2	8	M3	i.m.	TA, ECR	1×10^{12}	±	+				
LH49F	F	8	3.3	8	lacZ	i.v.		1×10^{14}	+++			+		
3805MN	M	6	3.5	8	lacZ	i.v.		1×10^{14}		+++	+		+	+
2704FA	F	8	3.6	8	M3	i.v.		1×10^{14}	+	+++				
4001MA	M	6	3.2	8	M3	i.v.		1×10^{14}		+++	+			

BW, body weight; F, female; M, male.

[a]Age at injection (weeks). [b]BW at injection (kg). [c]Vectors (vg/ml) were intramuscularly (i.m.) injected into extensor carpi radiolis (ECR) (1 ml) and tibialis anterior (TA) (2 ml) on both sides. Vectors were also intravenously (i.v.) injected into the lateral saphenous vein (vg/kg/limb) by using limb perfusion method. [d]β-Gal or microdystrophin-positive fibers per 3,000 fibers: −, 0; ±, <100; +, <300; ++, <1,000; +++, >1,000. [e]Infiltrating cells: −, not detected; ±, a few; +, moderate; ++, extensive.

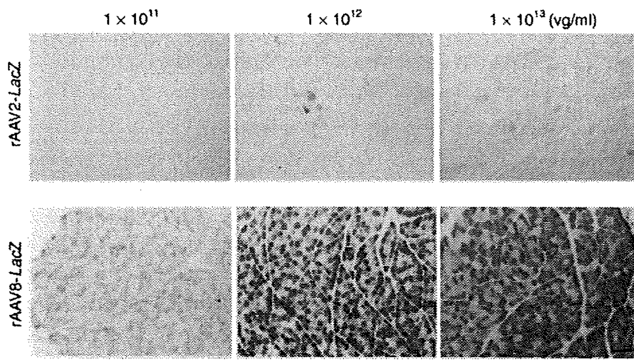

Figure 1 Canine skeletal muscles stained for β-galactosidase. Two milliliters of rAAV2-*lacZ* or rAAV8-*lacZ* (1 × 10^{11}–10^{13}vg/ml) were injected intramuscularly into the tibialis anterior (TA) muscle of normal dogs ($n = 16$) under ultrasonographic guidance. The muscles were biopsied 2 weeks after the injection. Upper: rAAV2-*lacZ*-injected TA muscles, Lower: rAAV8-*lacZ*-injected TA muscles. Bar = 200 μm.

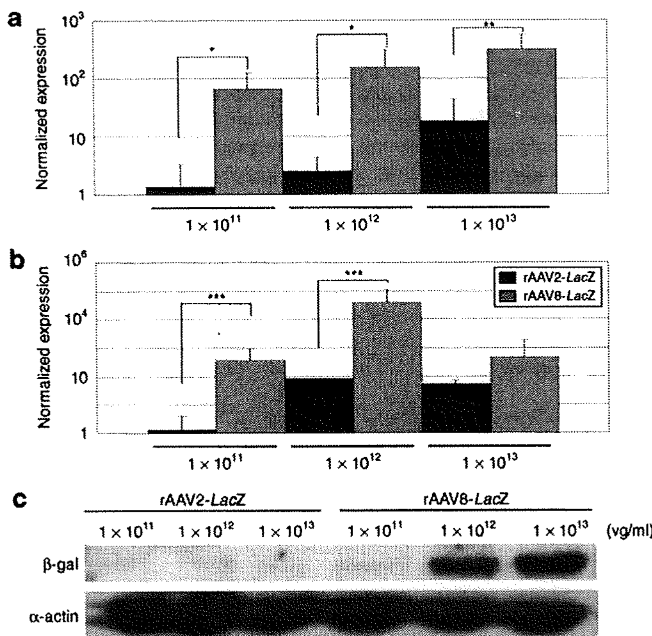

Figure 2 Quantification of viral vector genome, mRNA, and transgene expression. (a) Relative quantification of genomic PCR for rAAV2-*lacZ*-injected muscle (black bars) or rAAV8-*lacZ*-injected muscle (gray bars). DNA samples were extracted from the TA muscles. $*P < 0.05$. $**P < 0.01$. Error bars represent 2 SD. **(b)** Relative quantification showed more extensive β-gal mRNA expression caused by rAAV8-*lacZ* (gray bars) as compared to that caused by rAAV2-*lacZ* (black bars). 18S rRNA was used for an internal control. $***P < 0.05$. Error bars represent 2 SD. **(c)** Western blots of β-gal protein (120 kDa) and α-actin (42 kDa); the β-gal signal was normalized to α-actin for comparison.

that cellular and humoral immune responses are elicited in both rAAV2- and rAAV8-transduced muscles.

Bone marrow-derived DC reactions to rAAV2 and rAAV8

We next cultured bone marrow-derived DCs to investigate their response to rAAV injection in dogs. Flow cytometric analyses of these cells at 7 days of culture revealed marked expressions of CD11c and MHC class II molecules on the surface (**Figure 4a,b**). The DCs were cultured with the rAAV-*luciferase* of either serotype 2 or 8 for 48 hours to evaluate transduction efficiency, or cultured with rAAV-*lacZ* for 4 hours to investigate kinetic changes in mRNA. The luciferase assay showed that the transduction efficiency of rAAV2-*luciferase* in DCs was approximately two times that of rAAV8-*luciferase* (**Figure 4c**). mRNA levels of MyD88 and costimulating factors, such as CD80, CD86, and type I interferon (interferon-β, IFN-β) were elevated in both conditions (**Figure 4d**), suggesting that rAAV8 also induces a considerable degree of innate immune response in dog skeletal muscles. Although rAAV2-transduced DCs showed higher IFN-β expression than rAAV8-transduced DCs, the differences between the effects of rAAV2 and rAAV8 on the mRNA levels of MyD88, CD80, CD86, and IFN-β were not statistically significant.

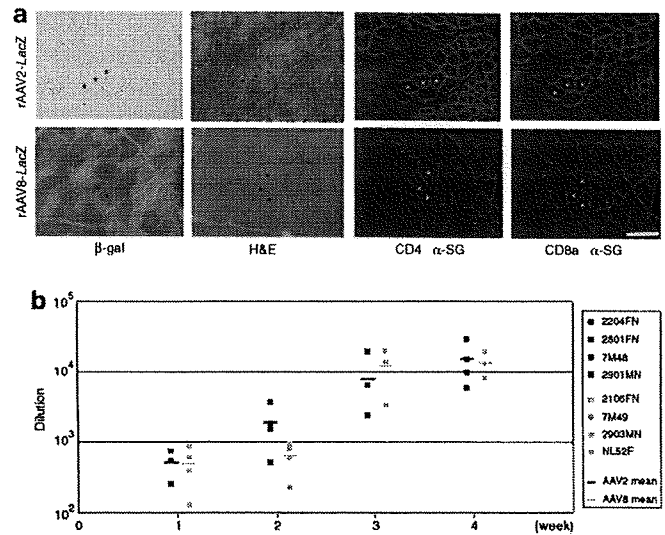

Figure 3 Immune response to rAAV. (a) Lymphocyte infiltration after rAAV transduction. Muscles were biopsied 2 weeks after rAAV2- or rAAV8-*lacZ* injection (2 × 10^{12}vg/muscle). Serial cross-sections were stained with β-gal and H&E, and were immunohistochemically stained with antibodies against canine CD4, CD8a (Alexa 568, red), and α-sarcoglycan (α-SG, Alexa 488, green). Upper: rAAV2-*lacZ*-injected TA muscle; lower: rAAV8-*lacZ*-injected TA muscle. Bar = 100 μm. **(b)** Humoral immune responses to rAAV capsid in dogs. Serum was collected weekly from rAAV2- or rAAV8-*lacZ*-injected dogs and analyzed for the presence of IgG antibody against the rAAV2 or rAAV8 capsid. The data represent dilution rates with 50% reactivity of anti-rAAV2 (black boxes) and anti-rAAV8 (gray boxes) capsid antibodies. The mean reconstitution values are shown as straight lines. Each symbol represents an individual dog that was injected with rAAV at 2 × 10^{12}vg/muscle.

Successful *microdystrophin* gene transfer with rAAV8 into dystrophic dogs

Dystrophin expression in normal skeletal muscle is localized on the sarcolemma, whereas it is totally absent in $CXMD_J$ dogs (**Supplementary Figure S4a,b**). Microdystrophin expression in the rAAV8-injected skeletal muscle of $CXMD_J$ dogs was maintained, even in the absence of any immunosuppressive therapy, for at least 4 weeks after the injection (**Table 1**). Previously, we had shown that microdystrophin expression of ca 20% was sufficient to achieve functional recovery in mdx mice[6]. However, the amount of the expression in intramuscularly injected muscles

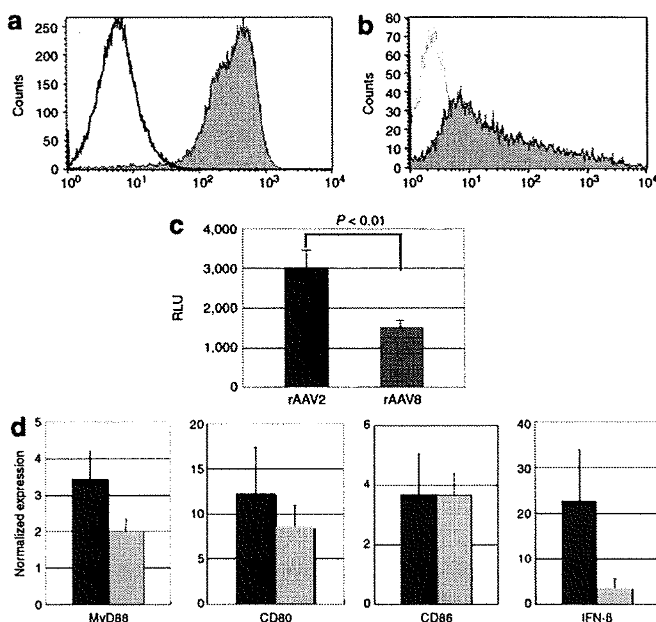

Figure 4 Responses of dendritic cells (DCs) to rAAV in dogs. Bone marrow-derived DCs were obtained from the humerus bones of dogs and cultured in RPMI (10% FCS, p/s) for 7 days with canine GM-CSF and IL-4. **(a)** Flow cytometric analysis of cell surface molecules on day 7. The cells were stained with PE-conjugated CD11c antibody and isotype control. **(b)** DCs were stained with FITC-conjugated MHC Class II antibody and isotype control. **(c)** DCs were transduced with rAAV-*luciferase* (1×10^6vg/cell) for 48 hours. To analyze luciferase expression relating to the use of rAAV2 or rAAV8, relative light unit (RLU) ratios were measured. *$P < 0.01$. Error bars represent s.e.m., $n = 8$. **(d)** DCs were transduced with 1×10^6vg/cell of rAAV2 (black bars) or rAAV8-*lacZ* (gray bars) for 4 hours, and mRNA levels of MyD88, CD80, CD86, and IFN-β were analyzed. Untransduced cells were used as a control to demonstrate the relative value of expression. The results are representative of two independent experiments. Error bars represent s.e.m., $n = 3$.

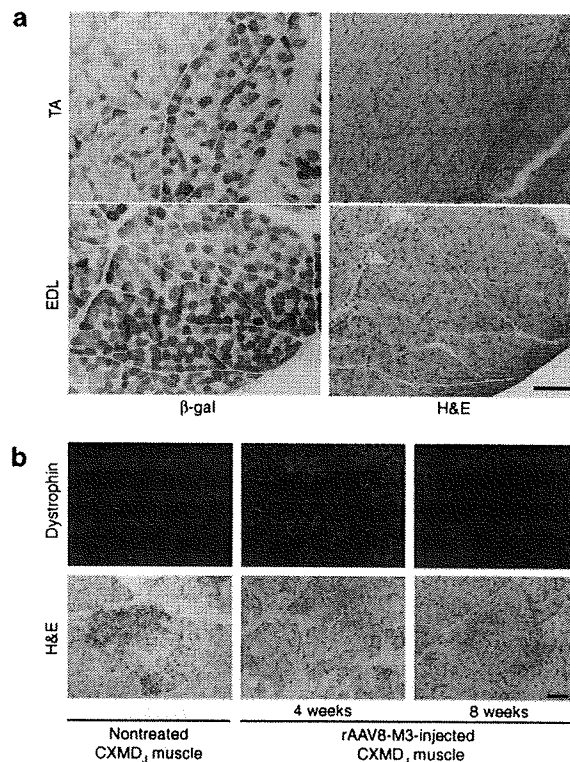

Figure 5 rAAV8-mediated muscle transduction using the limb perfusion method. (a) Transduction of normal dog with rAAV8-*lacZ*, using the limb perfusion method. Muscles were biopsied 2 weeks after the injection and stained with β-gal and H&E. TA, tibialis anterior, EDL, extensor digitorum longus. Bar = 200 µm. **(b)** Transduction of canine X-linked muscular dystrophy in Japan (CXMD$_J$) dog with rAAV8-*M3*. Muscles of CXMD$_J$ dogs were biopsied 4 and 8 weeks after limb perfusion with rAAV8-*M3*. Samples were immunohistochemically stained with anti-dystrophin antibody (dys2, NCL). Left: nontreated CXMD$_J$ muscle. Middle and right: muscles injected with rAAV-*M3* using limb perfusion method, examined at 4 or 8 weeks after the transduction. Bar = 100 µm.

seemed to be insufficient to produce the expected functional recovery (**Supplementary Figure S4c**).

For more efficient gene delivery by rAAV8, we tried a limb perfusion method in the hind limb through the lateral saphenous vein, in an attempt to prevent muscle damage due to direct injection and to bypass immune activation through DCs in the injected muscle. We had observed highly efficient β-gal expression in nearly all the muscles of the distal hind limb at 2 weeks after a single injection (**Table 1, Figure 5a**). We then injected rAAV8-*M3* into the hind limbs of CXMD$_J$ dogs, using the same method (**Table 1**). The induction of microdystrophin expression in the muscle at 4 weeks after intravascular injection was more efficient and free of noticeable immune response as compared to intramuscularly injected muscle (**Figure 5b, Supplementary Figure S4d**). These results suggest that the intravascular method is superior to the intramuscular method of administration. Although microdystrophin expression persisted at 8 weeks after injection of rAAV8-*M3*, the number of microdystrophin-positive cells at this time point was lower than in the muscles that were sampled at 4 weeks after injection. It is clear, therefore, that long-term microdystrophin expression can be obtained by the limb perfusion method, but that the expression does not last at the same level over a period of weeks. The same phenomenon was observed in rAAV8-*lacZ*-transduced muscles (**Supplementary Figure S5**).

DISCUSSION

In this article, we present evidence that the transfer of rAAV8-*lacZ* to canine skeletal muscles produces higher transgene expression with less lymphocyte proliferation than rAAV2-*lacZ* does, at 2 weeks after injection. Given the advantages of rAAV8, the administration of rAAV8-*M3* by limb perfusion produced extensive transgene expression in the distal limb muscles of CXMD$_J$ dogs without obvious immune responses for as long as 8 weeks after injection. However, transgene expression in the rAAV8-transduced muscles attenuated in the absence of an immunosuppressive regimen over the course of observation. In addition, humoral immune responses were elicited by both rAAV2 and rAAV8. mRNA levels of MyD88 and costimulating factors such as CD80, CD86, and type I interferon (interferon-β) were elevated in both rAAV2- and rAAV8-transduced DCs *in vitro*.

In our previous study, we had demonstrated extensive lymphocyte-mediated immune responses to rAAV2-*lacZ* after direct intramuscular injection into dogs, in contrast to the reported successful delivery of the same viral construct into mouse skeletal

muscle.[8] The fact that the promoter-deleted rAAV2 caused fewer cytotoxic cellular responses suggested that the massive destruction of transduced muscle cells might be the result of cellular immunity against the transgene product. In this study, there was extensive expression of β-gal in rAAV8-*lacZ*-injected canine muscles even in the absence of any immunosuppressive treatments (**Figure 1**), while the rAAV2-*lacZ*-injected muscles showed minimal β-gal expression with considerable inflammatory infiltration. If the transgene product were the main inducer of immune responses, lymphocyte activation would be correlated with transduction efficiency; however, this is not the case based on our results relating to the vector genome, mRNA expression level, and protein delivered through either rAAV2 or rAAV8 (**Figure 2**). These data suggested that the rAAV particle is associated with potent immunogenicity. Besides, β-gal expression disappeared 4 weeks after injection in the rAAV8-injected muscle as in the rAAV2-transduced muscles (**Supplementary Figure S2**). To investigate whether AAV itself has immunogenicity properties, we further characterized the immune responses caused by rAAV2 or rAAV8.

Immunohistochemical analysis revealed that the rAAV2-injected muscles showed higher rates of infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the endomysium than rAAV8-injected muscles did (**Figure 3a**). Considering the stringent immunogenicity of *lacZ* gene expression, we normalized the activity of TGF-β1 and IL-6 by lacZ expression to exclude the effect of transgene products (**Supplementary Figure S3a**). The total activity of TGF-β1 and IL-6 in the rAAV8-injected muscles was higher than that in rAAV2-injected muscles (**Supplementary Figure S3b**). As a result, rAAV2 induced a stronger cellular immune response than rAAV8 did. To investigate the humoral immune response, we quantitated neutralizing antibodies against rAAV particles in the sera of rAAV-injected dogs (**Figure 3b**). Antibodies against AAV2 and AAV8 capsids were below the detectable level before the injection and were elevated with time after the injection. Because the dogs were bred in a specific pathogen-free facility and not vaccinated, we assume that the elevation of antibody levels was not caused by anamnestic reaction.

Recently, Li *et al.*[10] reported that the AAV2 capsid can induce a cellular immune response through MHC class I antigen presentation with a cross-presentation pathway, and the effects of rAAV2 on human DCs have been described.[10,13] In contrast, other serotypes such as rAAV8 induced less T-cell activation.[11,14] Plasmacytoid DCs are critically important in innate immunity because of their unsurpassed ability to present adenoviral antigens to T-cells for the generation of primary cellular and humoral immune responses.[15–17] The response of DCs against rAAV in dogs was yet to be elucidated. We prepared bone marrow-derived DCs to investigate rAAV-mediated transduction of DCs. The difference between rAAV2 and rAAV8 in respect of the transduction rate of DCs *in vitro* was no greater than the difference in distinct β-gal expressions *in vivo* (**Figure 2,4c**). Quantitative analysis of mRNA of the transduced DCs by RT-PCR revealed that both rAAV2 and rAAV8 upregulated the expression of costimulating factors, with no significant difference between mRNA levels in rAAV2- and rAAV8-transduced cells. Therefore, both rAAV2 and rAAV8 may activate innate immunity in the context of extensive muscle transduction. Whereas AAV capsids cause immune response, transgene products may play adjuvant roles in the immunity to the AAV capsids.[18]

rAAV8 encoding the human *microdystrophin* gene was also intramuscularly injected into the skeletal muscles of $CXMD_J$ dogs. rAAV8-mediated gene expression without any immunosuppression was confirmed over a period of 8 weeks after the injection, whereas there was much less transduction with the use of rAAV2 (data not shown). rAAV8-mediated transduction was also expected to provide effective intravenous delivery.[12] In this context, the venous system is an attractive route for limb perfusion administration because it is a direct channel to multiple muscles of the limb. Moreover, veins are easier to access through the skin and there is less potential for muscle damage during injection. By using the limb perfusion method, we could reach nearly all the muscles of the lower limb, held transiently isolated by a tourniquet around the thigh. Limb perfusion administration could possibly have the potential to bypass the DC recognition caused by intramuscular injection. We intravenously injected rAAV8-*lacZ* into the hind limbs of normal dogs and rAAV8-*M3* into the hind limbs of $CXMD_J$ dogs, and obtained more extensive expression of β-gal or microdystrophin than by intramuscular injection. Interestingly, the inflammatory response was not significant in the intravenously injected muscles, although no immune suppression was attempted. We think that one reason rAAV8-*M3* resulted in better expression than rAAV8-*lacZ* is that the immunogenicity of *M3* is lower than that of *lacZ*. Although microdystrophin expression was lower at 8 weeks after the transduction with the limb perfusion, cellular infiltration was not significant.

In the future, systemic delivery of rAAV8-*microdystrophin* could ameliorate the symptoms of DMD patients. Even though portal vein injection of rAAV2-*FIX* into hemophilia B dogs produced long-term expression, a clinical study failed to demonstrate long-term expression in humans.[19,20] In advance of future clinical trials, several studies are required to confirm safety. Sequential peripheral blood monitoring showed no severe adverse events, including liver dysfunction, during 8 weeks (data not shown). We are now developing a systemic delivery strategy with a muscle-specific promoter. It is also necessary to improve vector constructs or regulate immune reaction against transgene products. Recently, Wang *et al.* reported sustained AAV6-mediated human microdystrophin expression in dystrophic dogs for 30 weeks, using combined immunosuppressive therapy of Ciclosporin, Mycophenolate Mofetil, and anti-thymocyte globulin.[9] In this study with rAAV8-*M3*, we confirmed effective transduction into dog skeletal muscle for 4 weeks without immunosuppressive therapy. However, considering the fact that not only rAAV2 but also rAAV8 induced activation of DCs *in vitro*, immunological modulation would be required for sufficient long-term expression. A novel protocol with systemic or localized immunosuppression using immunosuppressive drugs or local immunosuppression with an IFN-α or -β blockade could help avoid host immune reaction.

In summary, we achieved successful rAAV8-mediated muscle transduction in wild-type dogs as well as in dystrophic dogs by using the limb perfusion method of administration. Also, by manipulating bone marrow-derived DCs, we observed the probable contribution of antigen-presenting cells to the immune response against rAAV8-mediated gene therapy. Although the

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cellular responses against rAAV8 were not significant *in vivo*, this DC activation may possibly be involved in limiting long-term transduction when the limb perfusion method is used. The limb perfusion transduction protocol with improved AAV constructs or immune modulation would further enhance rAAV8-mediated transduction strategy and lead to therapeutic benefits.

MATERIALS AND METHODS

Animals. Five- to ten-week-old male and female wild-type dogs obtained from the Beagle-based $CXMD_J$ breeding colony at the National Center of Neurology and Psychiatry (Tokyo, Japan) were used for the *lacZ* gene transduction.[3] Six- to eight-week-old $CXMD_J$ dogs were used for *microdystrophin* gene transduction. All the animals were cared for and treated in accordance with the guidelines approved by the Ethics Committee for Treatment of Laboratory Animals at National Center of Neurology and Psychiatry, where the three fundamental principles of replacement, reduction, and refinement are also considered. Dogs were not vaccinated to avoid the immune responses to vaccination.

Construction of proviral plasmid and recombinant AAV vector production. The AAV2 vector proviral plasmids harboring the *lacZ* or *luciferase* gene with a CMV promoter and SV40 late-gene polyadenylation sequence were propagated.[8] As a therapeutic gene for DMD, the human *microdystrophin* gene, *M3*, was used under the control of the CMV promoter and a bovine growth hormone polyadenylation sequence.[21] The vector genome was packaged into the AAV2 capsid or pseudotyped AAV8 capsid in HEK293 cells. A large-scale cell culture method with an active gassing system was used for transfection.[22] The vector production process involved triple transfection of a proviral plasmid, an AAV helper plasmid pAAV-RC (Stratagene, La Jolla, CA) or p5E18-VD2/8, and an adenovirus helper plasmid pHelper (Stratagene).[21] All the viral particles were purified by CsCl gradient centrifugation. The viral titers were determined by quantitative PCR using SYBR-green detection of PCR products in real time with the MyiQ single-color detection system (Bio-Rad, Hercules, CA) and the following primer sets: for AAV-*lacZ*, lacZ-Q60: forward primer 5′-TTATCAGCCGGAAAACCTACCG-3′, and reverse primer 5′-AGCCAGTTTACCCGCTCTGCTA-3′; for AAV-*microdystrophin*: forward primer 5′-CCAAAAGAAAAAGGATCCACAA-3′, and reverse primer 5′-TTCCAAATCAAACCAAGAGTCA-3′; and for AAV-*luciferase*: forward primer 5′-GATACGCTGCTTTAATGCCTTT-3′, and reverse primer 5′-GTTGCGTCAGCAAACACAGT-3′.

Direct administration of rAAVs into normal and dystrophic skeletal muscle. Experimental dogs ($n = 16$) were sedated with isoflurane by mask inhalation and intubated. Anesthesia was maintained with 2–4% isoflurane. Two milliliters of rAAV2-*lacZ* or rAAV8-*lacZ* (1×10^{11}–10^{13} vg/ml) were injected intramuscularly into the tibialis anterior muscles and 1 ml into the extensor carpi radialis muscles of the normal dogs under ultrasonographic guidance. rAAV8-*M3* (1×10^{12} vg/ml) was intramuscularly injected at a volume of 2 ml into the tibialis anterior muscles and 1 ml into the extensor carpi radialis muscles of a $CXMD_J$ dog.

Intravenous delivery of rAAVs into the limb veins of dogs. Intravenous injection was administered as described elsewhere.[12] Briefly, a blood pressure cuff was applied just above the knee of an anesthetized normal dog. A 24-gauge intravenous catheter was inserted into the lateral saphenous vein, connected to a three-way stopcock, and flushed with saline. With the blood pressure cuff inflated to over 300 mm Hg, saline (2.6 ml/kg) containing papaverine (0.44 mg/kg, Sigma-Aldrich, St Louis, MO) and heparin (16 U/kg) was injected by hand over 10 seconds. The three-way stopcock was connected to a syringe containing rAAV8-*lacZ* (1×10^{14} vg/kg, 3.8 ml/kg). The syringe was placed in a PHD 2000 syringe pump (Harvard Apparatus, Edenbridge, UK). Five minutes after the papaverine/heparin injection, the rAAV8-*lacZ* was injected at a rate of 0.6 ml/second. Two minutes after the rAAV injection, the blood pressure cuff was released and the catheter was removed. The $CXMD_J$ dogs were injected with rAAV8-*M3* using the same method.

Sampling of transduced muscles. Either the muscles of the transduced dogs were biopsied or the animals were killed at 2, 4, and 8 weeks after the injection. We sampled tibialis anterior and extensor carpi radialis muscles on both sides in the intramuscularly transduced dog. In the case of the limb perfusion study, tibialis anterior or extensor digitorum longus muscle of the injected side of the leg was sampled. For biopsy and necropsy, the individual muscle was cropped tendon-to-tendon, divided into several pieces, and immediately frozen in liquid nitrogen-cooled isopentane. Two to eight blocks were sampled from the transduced muscle. We analyzed at least 30 sections from the blocks to observe the general representation.

Histological analysis. Transverse cryosections (10 μm) from the rAAV-*lacZ*-injected muscles were stained with hematoxylin and eosin or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.[23] Eight-micrometer-thick cryosections from the rAAV-*M3*-injected muscles were immunohistochemically stained as described.[24] Briefly, the cryosections were fixed by immersion in cold acetone at −20 °C. Fixed frozen sections were blocked in 5% goat serum in phosphate-buffered saline at room temperature and incubated with mouse monoclonal anti-dystrophin C-terminal antibody (NCL-dys2, Novocastra, Newcastle upon Tyne, UK). The signal was visualized with an Alexa 568-conjugated anti-mouse IgG. Fluorescent signals were observed using a confocal laser scanning microscope (Leica TCS SP, Leica, Heidelberg, Germany). Immunohistochemical analyses were performed with mouse monoclonal antibodies against canine CD4 (CA13.1E4, Serotec, Oxford, UK), canine CD8a (CA9. JD3, Serotec), and double-stained with rabbit polyclonal antibody against α-sarcoglycan.[25] The signal was visualized with an Alexa 568-conjugated anti-mouse IgG, and 488-conjugated anti-rabbit IgG.

Detection of AAV genomes. Total DNA was extracted from muscle cryosections. Cryosections were homogenized using a Multi-beads shocker (Yasui Kikai, Osaka, Japan), and extracted using a Wizard SV Genomic DNA purification system (Promega, Madison, WI). The rAAV genome was detected by relative quantitative PCR using SYBR-green detection of PCR products in real time with a primer set of *lacZ*-Q60. For an internal control, forward primer, 5′-GAACACGCGTTAATAAGGCAATCA-3′, and reverse primer, 5′-CTGACATTCATCGCATCTTTGACA-3′, directed to an ultra-conserved region, were used.[26]

Real-time RT-PCR. Total RNA was isolated from cryosections using a Multi-beads shocker (Yasui Kikai), and RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen). mRNA was detected using primer sets of lacZ-Q60, forward primer 5′-TGATGGCTA CTGCTTTCCCTAC-3′ and reverse primer 5′-GAGATTTTGCCGA GGATGTACT-3′ for IL-6, and forward primer 5′-CAAGGATCTGGGC TGGAAGTGGA-3′ and reverse primer, 5′-CCAGGACCTTGCTGTA CTGCGGT-3′ for TGF-β1. For an internal control, a primer set of 18S rRNA (Ambion, Foster City, CA) was used.

Western blot analysis. Muscle cryosections were homogenized with four volumes of sample buffer (10% SDS, 70 mmol/l Tris-HCl, 10 mmol/l EDTA, and 5% β-mercaptoethanol). The samples were boiled for 5 minutes and centrifuged at 14,500 rpm for 15 minutes. Protein samples (30 μg per lane) were electrophoresed on a 7.5% polyacrylamide gel (Bio-Rad). The membranes were incubated with a 1:1,000 dilution of the primary antibody for detecting 120 kDa lacZ protein (rabbit anti-β-galactosidase IgG fraction, Molecular Probes, Eugene, OR) or 42 kDa α-actin (mouse anti α-sarcomeric actin IgM, Sigma-Aldrich). Anti-rabbit IgG peroxidase F(ab′)

(GE Healthcare, Buckinghamshire, UK), or peroxidase-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for ECL immunodetection (GE Healthcare). Quantification of LacZ protein was performed using a specialized software (ImageJ, US National Institutes of Health, Bethesda, MD).

ELISA for anti-canine AAV IgG. A microtiter plate (MS-8596F, Sumitomo Bakelite, Tokyo, Japan) was precoated with promoter-deleted rAAV2 or rAAV8 (2×10^9 genomes/well) and blocked with a blocking buffer (Block Ace, DS Pharma Biomedical, Osaka, Japan). The plate was incubated for 2 hours at room temperature with the sera from rAAV-transduced dogs, followed by a 1:5,000 dilution of peroxidase-conjugated rabbit anti-dog IgG (Sigma-Aldrich) for 1 hour. Color was visualized using a peroxidase substrate system (TMBZ, ML-1120T, Sumitomo Bakelite). Reactivity was detected at a wave-length of 450 nm with a reference at 570 nm, using an APPLISKAN Multimode Reader (Thermo Fisher Scientific, East Greenbush, NY).

Bone marrow aspiration and preparation of DCs. After the dogs were anesthetized with thiopental and isoflurane, ~0.5 ml of bone marrow was obtained from each humerus by aspiration with a syringe containing 2 ml of 16 mmol/l EDTA-2Na PBS. Bone marrow-derived DCs were generated as described.[15] Mononuclear cells were isolated by density centrifugation using Histopaque-1077 (Sigma-Aldrich). Cells were suspended in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (MP Biomedicals, Aurora, OH) and 1% penicillin-streptomycin (Sigma-Aldrich), and cultured at 37 °C in a humidified 5% CO_2-containing atmosphere. Recombinant canine GM-CSF (25 ng/ml, R&D Systems, Minneapolis, MN) and canine IL-4 (12.5 ng/ml, R&D Systems) were added to the culture medium. On days 3 and 5 of the culture, 60% of the medium volume was changed. On day 7 of the culture, loosely adherent cells were collected and used for fluorescence-activated cell analysis. A FACS Vantage system (Becton Dickinson, Franklin Lakes, NJ) was used for flow cytometry event collection. For the purpose of examining the infectious rate of rAAV, cells were cultured for 48 hours with rAAV2- or 8-*luciferase*. The luciferase activity of rAAV2- or rAAV8-*luciferase* co-cultured cells was estimated using an APPLISKAN Multimode Reader (Thermo Fisher Scientific). Total RNA was isolated using an RNeasy Fibrous Tissue Mini kit (Qiagen), and QuantiTect Reverse Transcription kit (Qiagen). mRNA of cytokines were analyzed using the primer set, forward primer 5′-GAGGAGATGGGCTTCGAGTA-3′ and reverse primer 5′-GTTCCACCAACACGTCGTC-3′ for MyD88; forward primer 5′-GCATCATCCAGGTGAACAAG-3′ and reverse primer 5′-AAGTCAGCAAAGGTGCGATT-3′ for CD80; forward primer 5′-AGGTTACCCAGAACCCAAGG-3′ and reverse primer, 5′-TTGC AGGACACAGAAGATGC-3′ for CD86; and forward primer 5′-ATT GCCTCAAGGACAGGATAAA-3′ and reverse primer 5′-TTGACG TCCTCCAGGATTATCT-3′ for IFN-β. mRNA levels of MyD88, CD80, CD86, and IFN-β in DCs were normalized with a house keeping gene, 18s rRNA. The mRNA levels in the transduced cells were presented as ratios relative to the sample obtained from the untransduced DCs.

Statistical analysis. Statistical significance was determined on the basis of an unpaired, two-tailed Student's *t*-test using specialized software (Statview; SAS Institute, Cary, NC). A *P* value of <0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Figure S1. Histological findings with incisional and nonincisional injection under ultrasonographic guidance.
Figure S2. β-gal expression 4 weeks after injection.
Figure S3. Levels of mRNA were investigated using rAAV-injected muscles.
Figure S4. Intramuscular injection of rAAV8-*M3* into $CXMD_J$
Figure S5. Long-term β-gal expression using limb perfusion injection.
Table S1. Protein expression analyzed with ImageJ.

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Levodopa in the early treatment of Parkinson's disease

Miho Murata *

Department of Neurology, National Center Hospital of Neurology & Psychiatry, Kodaira, Japan

Abstract

L-dopa has many advantages as initial therapy for Parkinson's disease (PD). It is safer, more efficacious, associated with fewer adverse effects, few interactions, easier for patients to use and for clinicians to prescribe, and cheaper than dopamine (DA) agonists. Although L-dopa is more likely than DA agonists to introduce motor fluctuations and dyskinesia, L-dopa is also more effective in improving motor function. Furthermore, there is no long-term benefit from delaying L-dopa based on the risk of motor complications or psychiatric symptoms. Many investigations have shown that L-dopa does not accelerate disease progression. Now is the time to re-evaluate L-dopa for initial treatment of PD.
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Keywords: Parkinson's disease; L-dopa; DA agonist; Motor fluctuation, Dyskinesia

1. Introduction

Although it is recommended that dopamine (DA) agonists should be chosen as initial treatment for Parkinson's disease (PD), it is time to re-evaluate the use of levodopa for initial treatment of PD.

While many dopaminergic drugs have been introduced, L-dopa therapy has remained the gold standard for symptomatic treatment of PD. L-dopa is safer, more efficacious, associated with fewer adverse effects, has few interactions, is easier for patients to use and for clinicians to prescribe, and it is cheaper than DA agonists. Despite these advantages, many previous guidelines (Fig. 1) [1,2] have stated that for early stage patients with PD it is appropriate to start treatment with a DA agonist unless the patient is either older than 75 years or has dementia. The rationale for these recommendations has been that (1) L-dopa may accelerate disease progression, and (2) DA agonists are less likely to induce motor fluctuation.

2. Is L-dopa really neurotoxic?

Concern that exogenous L-dopa may be neurotoxic and contribute to the progression of PD arises from the oxidative stress hypothesis of PD [3]. Many *in vitro* studies have demonstrated that the addition of L-dopa or DA to cultured dopaminergic neurons increased cell death [4,5]. However, these experiments were performed under non-physiologic conditions; the concentrations of L-dopa and DA in these experiments were high (>5 μM) and exceeded what would be expected in the brains of patients treated with therapeutic doses (<2 μM). Furthermore, some *in vitro* studies have shown that L-dopa and DA are neuroprotective when neurons are co-cultured with glial cells [6,7].

The ELLDOPA study, conducted to show whether L-dopa is toxic for PD patients, was a large, double-blind, randomized, controlled clinical trial comparing three different doses (150, 300, 600 mg per day) of L-dopa with placebo in patients with early PD [8]. At the end of the trial (after a 2-week washout period), the mean Unified Parkinson's Disease Rating Scale (UPDRS) score of patients treated with L-dopa was better than that of the placebo group. The mean UPDRS score of the highest dosage group was the best, even after the washout period. These results suggest that L-dopa is not toxic, and may even be neuroprotective. This study also included evaluation with β-CIT SPECT

* Address for correspondence: Miho Murata, MD, PhD. National Center Hospital of Neurology & Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo, Japan 187-8551. Tel.: 81-42-341-2711; Fax: 81-42-346-1735.
E-mail address: mihom@ncnp.go.jp

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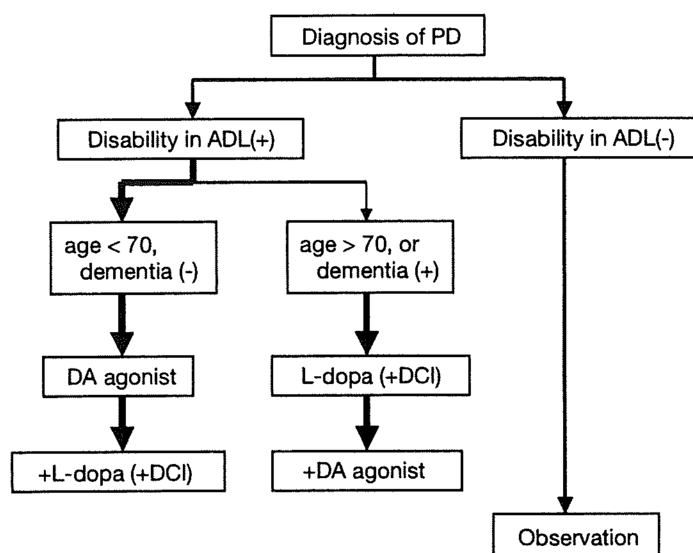

Fig. 1. Guideline for Parkinson's disease (early stage) made by Japanese Society for Neurology, 2002. ADL, activities of daily living; DCI, decarboxylase inhibitor.

imaging, as a marker for intact nigrostriatal dopaminergic neuronal functioning. The imaging studies showed that there was a larger decrease in β-CIT uptake in patients treated with L-dopa in a dose–response manner. These clinical and imaging results suggested that β-CIT SPECT imaging is not appropriate for evaluating the neuroprotective ability of the drugs. Up to that time, several studies had shown that DA agonists are more protective than L-dopa using β-CIT SPECT [9,10] or F-DOPA PET [11]. The results of the ELLDOPA study permitted re-evaluation of the results of these studies, and there is now a consensus that L-dopa does not accelerate disease progression [12].

3. L-dopa is more likely than DA agonists to induce motor fluctuations and dyskinesia

Several clinical studies [9,11] have shown that treatment with DA agonists is less likely to induce motor complications than treatment with L-dopa. Theses studies also showed that L-dopa monotherapy improves activities of daily living and motor function to a greater degree than DA agonists (plus later optional L-dopa). What is of most importance to our patients? The severity of both motor fluctuation and dyskinesia in these studies was low. For example, in the CALM-PD study (4 years) the percentage of disabling dyskinesia was 4.4% in the pramipexole group and 6.9% in the L-dopa group [13]. Furthermore, retrospective investigations failed to show any long-term benefit from delaying L-dopa based on the risk of motor complications, dementia, or psychiatric symptoms [13–16].

Apart from motor complications, the frequency of other common side effects is less with L-dopa than with DA agonists. For example, hallucinations are about three times more likely to occur with ropinirole or pramipexole than with L-dopa [17]. Somnolence, edema, and cardiac valvular fibrosis (pergolide, cabergoline) are also more frequent with DA agonist treatment than with L-dopa [17]. In Japan, those who are taking ropinirole or pramipexole are prohibited from driving because of the risk of sudden onset sleep. Can the incidence of motor fluctuation be reduced only at the expense of improvements in motor function and activities of daily living, and of other side effects such as hallucination, sudden onset sleep, and fibrosis?

4. Mechanism of wearing-off

Disease progression is associated with "wearing off" of therapeutic benefit and the appearance of unpredictable treatment responses, resulting in complex "on-off" response fluctuations. These arise in between doses of L-dopa because the patient no longer has the ability to store dopamine. The other factors driving development of response fluctuations are changes in peripheral L-dopa pharmacokinetics and in post-synaptic function that accompany large-dose and long-term L-dopa therapy [18]. Jenner and colleagues reported on the relation between the amount of lesion and the development of dyskinesia and motor fluctuation by using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model [19]. In primates with 50% lesions (model of early PD), L-dopa produced an antiparkinsonian response with no induction of dyskinesia. In the group with 75% lesions, L-dopa produced an antiparkinsonian response with a gradual development of dyskinesia. In the >90% lesion group, L-dopa produced a pulsatile anitiparkinsonian response and rapid induction of severe dyskinesia.

Pathological examination in patients with PD demonstrates an exponential loss of nigral pigmented neurons [20]. At 5 years from symptom onset, about 50% of the pigmented neurons remain, compared with age-adjusted controls, and at 10 years 30% remain. The presymptomatic phase of PD, dating from the onset of neuronal loss, was estimated to be about 5 years. Therefore, at the initial symptomatic stage

of the disease there is little possibility of developing motor fluctuation and dyskinesia as long as the appropriate dose of L-dopa is used. In fact, the ELLDOPA study showed the incidence of motor complications in the L-dopa 150 mg and 300 mg groups to be almost equal to that in the placebo group [8].

5. The advantage of L-dopa as initial therapy for Parkinson's disease

The initial use of L-dopa for the symptomatic treatment of PD has many advantages over DA agonists: (1) L-dopa has significantly greater efficacy than DA agonists in alleviating the motor symptoms of PD and improving activities of daily living. (2) Titration of L-dopa to therapeutic levels is much easier and faster than that of DA agonists. (3) L-dopa is much less likely to induce hallucinations, somnolence, edema, or constipation compared to DA agonists. (4) There is a clear cost benefit to using L-dopa. (In Japan, L-dopa + decarboxylase inhibitor 300 mg/day costs about 1 US dollar per day; pramipexole 4.5 mg/day about 16 US dollars; and ropinirole 15 mg/day 26 US dollars.) However, late-developing motor fluctuation and dyskinesia deserve consideration, and the potential for other common side effects such as hallucinations and sleep attacks must also be factored into the treatment decision. Furthermore, early in the course of the disease, L-dopa provides an enduring response that can last several days [21].

There is much evidence to show that DA agonists are efficacious in controlling L-dopa motor fluctuations (as later adjunctive therapy). However, another option is to initiate treatment with levodopa, adding a DA agonist after the first sign of developing motor complications has appeared (early combination).

6. Continuous stimulation by using L-dopa

Continuous daytime intraintestinal infusion of L-dopa can diminish motor complications [22]. This shows that motor complications can be improved by changing the pharmacokinetics of L-dopa. For example, catechol-*O*-methyltransferase (COMT) inhibitors can extend the half-life of serum L-dopa concentration. The duration of L-dopa efficacy can also be extended by taking L-dopa after a meal (Fig. 2). Long-term L-dopa therapy increases the peak L-dopa concentration (C_{max}) and decreases its half-life ($T_{1/2}$) [23]. C_{max} is decreased and $T_{1/2}$ is increased by taking L-dopa after a meal compared to taking it before meals. Therefore, taking L-dopa after meals not only extends effective time but also decreases dyskinesia. By taking L-dopa after meals, the dose of L-dopa can be increased, but the risk of dyskinesia can be decreased. If needed, a low dose of L-dopa may be taken before meals for immediate improvements, with the remainder of the dose taken after meals.

There is much evidence for the efficacy of DA agonists as adjunctive therapy in controlling L-dopa motor fluctuations and dyskinesia [24]. Monoamine oxidase (MAO) B inhibitors [24] and zonisamide [25] may also be used as adjunctive therapy to improve motor fluctuations.

7. Initial symptomatic treatment for early Parkinson's disease

It is appropriate to start treatment of PD with either L-dopa or DA agonists. As highlighted by the American Academy of Neurology practice parameter [17], the choice of initial treatment depends on the relative importance for the patient of improving motor disability and limiting adverse events versus the possibility of lowering the risk of developing long-term motor complications.

The frequency of the development of wearing-off depends on the age of disease onset. Younger-onset patients (younger than 50 years at onset) are more prone to severe dyskinesia and motor fluctuation, while patients older than 70 years at symptom-onset rarely develop disabling dyskinesia and motor fluctuation. Older patients may be more prone to develop hallucinations and other common adverse effects. Therefore, L-dopa is preferred for elderly patients as initial treatment. In younger patients, DA agonists are preferred, but if the patient is at risk of losing his or her job owing to motor disability, L-dopa should be started. Patients whose age of onset is between 50 and 70 years can be prescribed

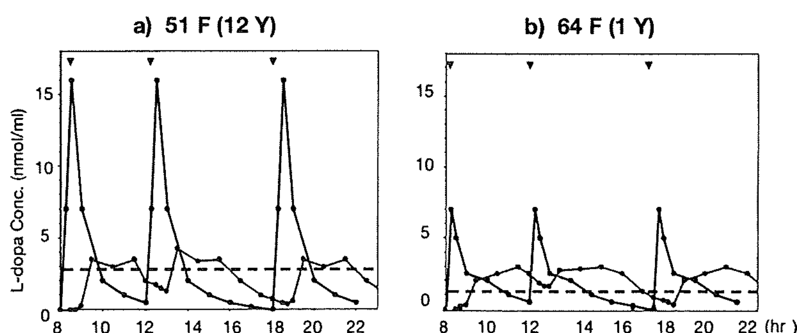

Fig. 2. Effects of a meal on L-dopa pharmacokinetics. (a) 51-year-old woman with 12 years' duration of Parkinson's disease. (b) 64-year-old woman with 1 year duration of Parkinson's disease. A tablet of L-dopa 100 mg + benserazide 20 mg was taken. Black curve: just before meal, red curve: after meal 3 times a day. ▼: meals (at 08.00, 12.00, 18.00 hours). Dashed line: the concentration of effective threshold estimated by clinical symptoms. C_{max} is 2–3 times higher when L-dopa was taken before meals than after meals. L-dopa pharmacokinetics are more stable when taken after meals than before meals.

either L-dopa or a DA agonist. Treatment selection should reflect the patient's needs and under-treatment should be avoided.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

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Musculoskeletal Pathology

Muscle CD31(−) CD45(−) Side Population Cells Promote Muscle Regeneration by Stimulating Proliferation and Migration of Myoblasts

Norio Motohashi,*† Akiyoshi Uezumi,*
Erica Yada,* So-ichiro Fukada,*
Kazuhiro Fukushima,*¶ Kazuhiko Imaizumi,†
Yuko Miyagoe-Suzuki,* and Shin'ichi Takeda*

From the Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo; the Division for Therapies against Intractable Diseases,‡ Institute for Comprehensive Medical Science, Fujita Health University, Aichi; the Department of Immunology,‡ Graduate School of Pharmaceutical Sciences, Osaka University, Osaka; the Laboratory of Physiological Sciences,† Faculty of Human Sciences, Waseda University, Saitama; and the Third Department of Medicine, Neurology, and Rheumatology,¶ Shinshu University School of Medicine, Matsumoto, Japan*

CD31(−) CD45(−) side population (SP) cells are a minor SP subfraction that have mesenchymal stem cell-like properties in uninjured skeletal muscle but that can expand on muscle injury. To clarify the role of these SP cells in muscle regeneration, we injected green fluorescent protein (GFP)-positive myoblasts with or without CD31(−) CD45(−) SP cells into the tibialis anterior muscles of immunodeficient *NOD/scid* mice or dystrophin-deficient *mdx* mice. More GFP-positive fibers were formed after co-transplantation than after transplantation of GFP-positive myoblasts alone in both *mdx* and *NOD/scid* muscles. Moreover, grafted myoblasts were more widely distributed after co-transplantation than after transplantation of myoblasts alone. Immunohistochemistry with anti-phosphorylated histone H3 antibody revealed that CD31(−) CD45(−) SP cells stimulated cell division of co-grafted myoblasts. Genome-wide gene expression analyses showed that these SP cells specifically express a variety of extracellular matrix proteins, membrane proteins, and cytokines. We also found that they express high levels of matrix metalloproteinase-2 mRNA and gelatinase activity. Furthermore, matrix metalloproteinase-2 derived from CD31(−) CD45(−) SP cells promoted migration of myoblasts *in vivo*. Our results suggest that CD31(−) CD45(−) SP cells support muscle regeneration by promoting proliferation and migration of myoblasts. Future studies to further define the molecular and cellular mechanisms of muscle regeneration will aid in the development of cell therapies for muscular dystrophy. ***(Am J Pathol 2008, 173:781–791; DOI: 10.2353/ajpath.2008.070902)***

Regeneration of skeletal muscle is a complex but well-organized process involving activation, proliferation, and differentiation of myogenic precursor cells, infiltration of macrophages to remove necrotic tissues, and remodeling of the extracellular matrix.[1–3] Muscle satellite cells are myogenic precursor cells that are located between the basal lamina and the sarcolemma of myofibers in a quiescent state, and are primarily responsible for muscle fiber regeneration in adult muscle.[4] Recent studies also demonstrated that a fraction of satellite cells self-renew and behave as muscle stem cells *in vivo*.[5,6] On the other hand, several research groups reported multipotent stem cells derived from skeletal muscle. These include muscle-derived stem cells,[7] multipotent adult precursor cells,[8] myogenic-endothelial progenitors,[9] CD34(+) Sca-1(+) cells,[10] CD45(+) Sca-1 (+) cells,[11] mesoangioblasts,[12] and pericytes,[13] and all were demonstrated to contribute to muscle regeneration as myogenic progenitor cells.

Side population (SP) cells are defined as the cell fraction that efficiently effluxes Hoechst 33342 dye and therefore shows a unique pattern on fluorescence-activated cell sorting (FACS) analysis.[14] Muscle SP cells are proposed to be multipotent[15,16] and are clearly distinguished from satellite

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Address reprint requests to Yuko Miyagoe-Suzuki, M.D., Ph.D., Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan. E-mail: miyagoe@ncnp.go.jp.

cells.[17] Previous reports showed that muscle SP cells participated in regeneration of dystrophic myofibers after systemic delivery[15] and gave rise to muscle satellite cells after intramuscular injection into cardiotoxin (CTX)-treated muscle.[17] Muscle SP cells adapted to myogenic characteristics after co-culture with proliferating satellite cells/myoblasts *in vitro*,[17] and expressed a satellite cell-specific transcription factor, Pax7, after intra-arterial transplantation.[18] However, the extent to which muscle SP cells participate in muscle fiber regeneration as myogenic progenitor cells is still primarily unknown. Importantly, Frank and colleagues[19] recently showed that muscle SP cells secret BMP4 and regulate proliferation of BMP receptor1α (+) Myf5^{high} myogenic cells in human fetal skeletal muscle, raising the possibility that SP cells in adult muscle play regulatory roles during muscle regeneration.

Previously we showed that skeletal muscle-derived SP cell fraction are heterogeneous and contain at least three subpopulations: CD31(+) CD45(−) SP cells, CD31(−) CD45(+) SP cells, and CD31(−) CD45(−) SP cells.[20] These three SP subpopulations have distinct origins, gene expression profiles, and differentiation potentials.[20] CD31(+) CD45(−) SP cells account for more than 90% of all SP cells in normal skeletal muscle, take up Ac-LDL, and are associated with the vascular endothelium. CD31(+) CD45(−) SP cells did not proliferate after CTX-induced muscle injury. Bone marrow transplantation experiments demonstrated that CD31(−) CD45(+) SP cells are recruited from bone marrow into injured muscle. A few of them are thought to participate in fiber formation.[21] Cells of the third SP subfraction, CD31(−) CD45(−), constitute only 5 to 6% of all SP cells in adult normal skeletal muscle, but they actively expand in the early stages of muscle regeneration and return to normal levels when muscle regeneration is completed. Although CD31(−) CD45(−) SP cells are the only SP subset that exhibited the capacity to differentiate into myogenic, adipogenic, and osteogenic cells *in vitro*,[20] their myogenic potential *in vivo* is limited compared with satellite cells. Therefore, we hypothesized that CD31(−) CD45(−) SP cells might play critical roles during muscle regeneration other than as myogenic stem cells.

In the present study, we demonstrate that the efficacy of myoblast transfer is markedly improved by co-transplantation of CD31(−) CD45(−) SP cells in both regenerating immunodeficient *NOD/scid* and dystrophin-deficient *mdx* mice. We also show that CD31(−) CD45(−) SP cells increased the proliferation and migration of grafted myoblasts *in vivo* and *in vitro*. We further show that CD31(−) CD45(−) SP cell-derived matrix metalloproteinase (MMP)-2 greatly promotes the migration of myoblasts *in vivo*. Our findings would provide us insights into the molecular and cellular mechanisms of muscle regeneration, and also help us develop cell therapy for muscular dystrophy.

Materials and Methods

Animals

All experimental procedures were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to twelve-week-old C57BL/6 mice and *NOD/scid* mice were purchased from Nihon CLEA (Tokyo, Japan). MMP-2-null mice were obtained from Riken BioResource Center (Tsukuba, Japan).[22] GFP-transgenic mice (GFP-Tg) were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan). C57BL/6-background *mdx* mice were generously given by Dr. T. Sasaoka (National Institute for Basic Biology, Aichi, Japan) and maintained in our animal facility.

Isolation of Muscle SP Cells

To evoke muscle regeneration, CTX (10 μmol/L in saline; Sigma, St. Louis, MO) was injected into the tibialis anterior (TA) (50 μl), gastrocnemius (150 μl), and quadriceps femoris muscles (100 μl) of 8- to 12-week-old GFP-Tg mice, C57BL/6 mice, MMP-2-null mice, and their wild-type littermates; 3 days later, SP cells were isolated from the muscles as described by Uezumi and colleagues.[20] In brief, limb muscles were digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. After elimination of erythrocytes by treatment with 0.8% NH_4Cl in Tris-buffer (pH 7.15), mononucleated cells were suspended at 10^6 cells per ml in Dulbecco's modified Eagle's medium (Wako, Richmond, VA) containing 2% fetal bovine serum (JRH Biosciences, Inc., Kansas City, KS), 10 mmol/L Hepes, and 5 μg/ml Hoechst 33342 (Sigma), incubated for 90 minutes at 37°C in the presence or the absence of 50 μmol/L Verapamil (Sigma), and then incubated with phycoerythrin (PE)-conjugated anti-CD31 antibody (1:200, clone 390; Southern Biotechnology, Birmingham, AL) and PE-conjugated anti-CD45 (1:200, clone 30-F11; BD Pharmingen, Franklin Lakes, NJ) for 30 minutes on ice. Dead cells were eliminated by propidium iodide staining. Analysis and cell sorting were performed on an FACS VantageSE flow cytometer (BD Bioscience, Franklin Lakes, NJ). APC-conjugated anti-CD90, Sca-1, CD34, CD49b, CD14, CD124, c-kit, CD14 (BD Pharmingen), CD44 (Southern Biotechnology Associates), and CD133 (eBioscience, San Diego, CA) were used at 1:200 dilution.

Preparation of Satellite Cell-Derived Myoblasts and Macrophages

Satellite cells were isolated from GFP-Tg mice or C57BL/6 mice by using SM/C-2.6 monoclonal antibody[23] and expanded *in vitro* in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and 2.5 ng/ml of basic fibroblast growth factor (Invitrogen, Carlsbad, CA) for 4 days before transplantation. Macrophages were isolated from C57BL/6 mice 3 days after CTX injection. Mononucleated cells were stained with anti-Mac-1-PE (1:200, clone M1/70; BD PharMingen) and anti-F4/80-APC (1:200, clone CI, A3-1; Serotec, Oxford, UK). Mac-1(+) F4/80(+) cells were isolated by cell sorting as macrophages.

Cell Transplantation

To induce muscle regeneration, 100 μl of 10 μmol/L CTX was injected into the TA muscle of *NOD/scid* muscles,