厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋に対する AAV ベクターの安全性の検討

分担研究者　鈴木　友子
国立精神・神経センター神経研究所
遺伝子疾患治療研究部　室長

研究要旨

Duchenne 型筋ジストロフィー（DMD）に対する遺伝子治療法を確立するために，AAV ベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。*mdx* マウスにおいて得られた良好な治療成績を臨床に結び付ける為には，筋ジス犬を用いた研究を進める一方で，ヒトに近い動物を用いて，AAV ベクターの有効性と安全性を詳細に検討する必要がある。本研究では，多くの AAV の血清型を自然宿主とするサル骨格筋へ AAV ベクターを用いて LacZ 遺伝子又はマイクロジストロフィン遺伝子を導入し，骨格筋組織と血清を経時的に解析することにより，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAV ベクターの骨格筋に対する局所注入がヒトへ応用できるか検討する。

A．研究目的

アデノ随伴ウイルス（AAV）ベクターは非病原性で安全性が高く，骨格筋などの非分裂細胞において，導入遺伝子の長期発現が可能である。我々は，Duchenne 型筋ジストロフィー（DMD）に対する遺伝子治療法を確立するために，AAV ベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。これまでに，筋ジストロフィー病態モデルの一つである *mdx* マウスにおいて，良好な治療成績を得た。しかし，中型の筋ジストロフィー病態モデルを持つイヌの骨格筋へ AAV ベクターを導入したところ，免疫応答を伴った強い細胞浸潤が誘導された。AAV ベクターとマイクロジストロフィン遺伝子を用いる方法論を将来ヒトへ臨床応用することを考えると，イヌ骨格筋で認められた細胞浸潤が AAV ベクターの導入に伴う細胞毒性／免疫応答であるのか，イヌ固有の問題であるのか検討する一方で，ヒトに近い動物を用いて，AAV ベクターの有効性と安全性を詳細に検討する必要がある。そこで，多くの AAV 血清型を自然宿主（natural host）とするサルを用いて評価を行うことが重要と考えた。本研究では，サル骨格筋へ AAV ベクターを用いて LacZ 遺伝子又はマイクロジストロフィン遺伝子を導入し，骨格筋組織と血清を経時的に解析することにより，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAV ベクターの骨格筋に対する局所注入がヒトへ応用できるか検討する。

B．研究方法

カニクイザルの左右の上腕筋及び前脛骨筋の計 4 箇所に AAV ベクター（3 箇所）と PBS（1 箇所）を直接注入する。コントロールとしては，導入遺伝子を発現しない promoter-less AAV vector を投与する。LacZ 遺伝子組換え AAV ベクター投与群及びマイクロジストロフィン遺伝子組換

え AAV ベクター投与群の 2 群は 3 頭ずつを設け，コントロール群は 2 頭を用いる。なお，使用個体の雌雄は問わない。導入 1 及び 2 週後に筋組織の生検を，4 週後に安楽死後のサンプリングをそれぞれ行い，同時に各時点で採血も実施する。ベクターの投与及び採血は塩酸ケタミン，生検はイソフルランによる麻酔下で行う。サンプリングは，ペントバルビタールナトリウム深麻酔下に放血死させた後に実施する。ジストロフィンの発現をウエスタンブロット法及び免疫組織化学染色法を用いて解析し，β-ガラクトシダーゼの発現を組織化学染色法を用いて解析する。β-ガラクトシダーゼ及びマイクロジストロフィンに対する血清抗体価の測定を ELISA またはウェスタンブロット法で行う。

C．研究成果

カニクイザル（2 頭）の両側の上腕二頭筋と全脛骨筋の計 4 カ所に，LacZ 遺伝子組み換え AAV ベクターを投与し，それぞれをバイオプシーした。切片を作成 HE 染色を行い，β-ガラクトシダーゼ染色を施し，injection の有無とβ-gal の発現を確認した。

D．考察

今後，筋生検による導入遺伝子の発現解析，ウイルスベクターゲノムの有無（感染効率）を定量的かつシステマチックに解析する方法の確立が重要である。

E．結論

霊長類による AAV ベクターの安全性の検討は，AAV ベクターによる遺伝子治療の開発に重要である。今後も個体数を増やし，効率と安全性に関して検討を進める必要がある

F．健康危険情報

なし

G．研究発表

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H．知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

筋ジストロフィー心合併症の発症機序解明とその治療に関する研究
－ユートロフィンKO *mdx* マウスとエメリンKO マウスの心筋障害－

分担研究者　　埜中 征哉
国立精神・神経センター　武蔵病院

研究要旨

1. 重症の筋ジストロフィーモデルであるジストロフィン・ユートロフィン二重欠損マウスについて系統維持に成功した。
2. 二重欠損マウスは，骨格筋には変性が強いが，生後 20 週までは明らかな心筋障害は認められなかった。
3. エメリン KO マウスは，心伝導障害と心筋細胞の核膜の空胞化などの変性所見を認めた。

A．研究目的

mdx マウスはジストロフィンが欠損していて，病因はデュシェンヌ型筋ジストロフィーと同じである。しかし臨床的には筋力低下がほとんどみられないことが，遺伝子治療などの治療実験を行う妨げとなっていた[1]。この *mdx* マウスにさらに utrophin をノックアウト（KO）したマウスが Davies らにより作成され[2]，それは進行性の筋力低下をみる。心筋に障害があるかどうかが，問題として残された。

エメリンは核膜蛋白で，その欠損は心伝導障害を主症状とする Emery-Dreifuss 型筋ジストロフィーを起こす[3]。エメリン遺伝子をKOしたマウスの作成に成功したので，両モデルマウスでの心筋障害について検討した。

B．研究方法

対象：*mdx* マウスで *utrophin* 遺伝子が KO されたもの 10 匹，*mdx* マウスで *utrophin* があるもの（通常の *mdx* マウス）*mdx/utro* -/+10 匹を対象とした。すべてのマウスは生後 14-20 週齢のものであった。実験に使用した全てのマウスは実験動物中央研究所の日置研究員らから供与された。

エメリン KO マウスは神経研究所疾病研究第一部で neomycin resistance gene をエクソン６に挿入して作成された。

方法：両モデル動物とも，体重測定，筋力測定（金網保持時間，水中遊泳時間など），血清ＣＫ値の測定を行った。骨格筋はひらめ筋（赤筋），前頸骨筋，長指伸筋（白筋）に組織学的，組織化学的染色を行った。

心筋は通常の組織化学的染色のほかに，電子顕微鏡的に検討した。

C．研究成果

①成長：通常の *mdx* マウス（-/+）では生後 14-20 週の体重は 22.0-28.0（25.0g）あったのに，*utrophin* KO *mdx*（-/-マウス）では 15.3-18.0（平均 16.4g）と優位に体重が軽かった。さらに脊柱の変形もみられた。20 週以降にはるいそうのため死亡するものが出現した。エメリン KO マウスではコントロールの B10 マウスと成長はなんら変わらず，早期に死亡するものはなかった。

②筋力低下：*Utrophin* KO *mdx* -/-マウスでは筋力低下は歴然としていた。閉鎖箱の中の動きは少なく，-/+の 1/5 以下であった。

また金網よじ登り試験でも，金網にしがみついても移動はほとんどなく落下した。-/+のものでは金網をつたっての移動が可能で，筋力低下による落下は 30 秒以内にはみられなかった。エメリン KO マウスでは，金網よじ登り試験でも，遊泳試験でもコントロールの B10 マウスと異常はなかった。

③心電図所見：エメリン KO マウスでは生後３０週以降にＰＲ間隔が優位に延長するようになった（41.6±4.9 n=51）（コントロール：39.1±3.5 n=15）（$p<0.01$）。その延長は成長とともにより顕著となった。

④電子顕微鏡所見：*utrophin/mdx* -/-マウスでは，電子顕微鏡的にも心筋に顕著な変化はみられなかった。一方，エメリン欠損マウスでは生後 21 週目頃から心筋細胞の核膜の離開，空胞化がみられるようになり，それは加齢とともに顕著となった。空胞は必ずしも核膜の離開によるとは限らず，核膜に接する小胞体の空胞化もみられた。障害の強いマウスでは核の 1/10 は変化していた。

D．考察

mdx マウスでは筋線維にジストロフィンが欠損し，筋線維は壊死する。そのプロセスはデュシェンヌ型筋ジストロフィーと同じであるが，*mdx* マウスでは再生が壊死を代償するために筋力低下がみられない。ジストロフィンを代償している *utrophin* 遺伝子を KO すると明らかに進行性の筋力低下をきたす。治療実験で，症状の改善の有無をみるのには最適な動物モデルである。このマウスを今年度から全国の実験者に供給できる体制を整えたことは大きな成果であった。

筋ジストロフィーには心筋障害が合併する。*Utrophin* KO *mdx* -/-マウスでは四肢筋の進行性の筋力低下をみて，マウスはるいそうで死亡する。本モデル動物で心筋障害が証明されれば，デュシェンヌ型の心合併の機序の解明と治療法の開発に大いに役立つことが期待できる。しかし，20 週までは心筋障害は証明されなかった。もっと年齢が高いマウスを検索する必要があるだろう。

エメリン KO マウスは期待通りの心合併症を来たした。このマウスは Emery-Dreifuss 型の心伝導障害病態解明に役立つだけでなく，治療実験にも利用されるであろう。現在系統維持に成功しているので，このマウスを使用しての治療（遺伝子治療を含む）実験を進めることができると期待される。

E．結論

Utrophin ノックアウト *mdx* マウスは，進行性の筋力低下，強い筋ジストロフィーの病理変化をみるので，デュシェンヌ型筋ジストロフィーのモデル動物としてきわめて有用性が高いことが確認できた。系統維持ができ，大量に研究者に供給できるようになった。しかし，人にみられる心筋障害は生後 20 週まではみられなかった。

エメリン KO マウスでは加齢とともに Emery-Dreifuss 型にみられる心伝導障害が認められるようになった。本症の心合併症の病態解明と治療法開発に役立つモデル動物として価値あると評価できる。

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F．健康危険情報

なし

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H. 知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋再生時の炎症・免疫学的解析

分担研究者　山元　弘
大阪大学大学院薬学研究科　教授

研究要旨
骨格筋の再生における細胞性・分子性メカニズムを明らかにするために，筋衛星細胞の増殖・分化時に発現増強する遺伝子群の同定と，筋再生時に浸潤するマクロファージが発現増強する遺伝子群の同定を試みた。

A．研究目的

骨格筋が再生する際，筋肉内に種々の炎症性細胞の浸潤が認められる。本研究では，炎症性細胞のなかでも，マクロファージの筋再生に及ぼす影響について検討し，その役割を明らかにすることにより筋再生の効率上昇のための新しい手法を確立することを目的とする。

B．研究方法

1. 正常マウス前脛骨筋にカルジオトキシン（CTX）を投与し，経時的に筋を組織化学的に解析した。
2. マクロファージを欠損するマウスでの筋再生の様子を調べた。
3. 新規モノクロナル抗体を用いて筋衛星細胞を特異的に濃縮することを試みた。
4. 再生筋からマクロファージを高純度に精製することを試みた。

C．研究成果

マウス筋衛星細胞特異的モノクロナル抗体，SM/C2.6 を樹立し，これを用いてマウス骨格筋から筋衛星細胞を単離精製することに成功した（Exp. Cell Research, 2004）。さらに武田らとの共同研究で，静止期筋衛星細胞が発現する遺伝子について，遺伝子チップを用いた網羅的解析を進め，多数の遺伝子を同定した（深田ら，2005）。一方これまでに，再生が始まる時期と同期して，再生筋組織にマクロファージが多量に浸潤すること，さらにマクロファージが欠損した条件下では筋再生はほとんど進行せず，むしろ線維の増生が盛んになることがわかっている（瀬川ら，2004）。

そこでマクロファージが産生する何らかの因子が筋再生に働いている可能性があると考え，再生途中にある骨格筋のサイトカイン mRNA 解析（ノザンブロット法によるアレイ解析）を進めたが，TGFβ以外には特徴的な遺伝子発現の変化は認められなかった。TGFβは，筋再生過程で線維芽細胞に働き，膠原線維の増生を促進していると考えられ，筋衛星細胞に働き，筋衛星細胞の増殖分化に働くサイトカインとは考えられないことがわかった。

マクロファージが産生し，筋衛星細胞に働く生理活性分子を同定するためには，再生途中の骨格筋から浸潤しているマクロファージのみを単離精製し，それが発現している遺伝子を同定しなければならない。この目的のために，カルジオトキシンにより再生を誘導したマウス骨格筋から，マグネットビーズ法でマクロファ

ージの精製を試み，９０％以上の純度で精製することができた。
現在，精製マクロファージの網羅的遺伝子チップ解析を進めており，正常マクロファージもしくは活性化マクロファージと比較して，再生途中の骨格筋のマクロファージに特徴的に認められる遺伝子を調べている。

D．考察

筋再生時には，多数の炎症性細胞の浸潤が認められる。本研究では，炎症性細胞のうち特にマクロファージに着目し，その役割を調べた。マクロファージは炎症後期に浸潤が盛んになり，特にマクロファージ浸潤時期に一致して筋再生が始まる。そこでマクロファージ機能が低下したマウスを作出し，その筋組織を調べたところ著明な筋再生の遅延が観察され，筋再生にはマクロファージが不可欠の機能を果たしていることが明らかになった。このことは，マクロファージが産生する何らかの生理活性分子が筋衛星細胞の増殖・分化に働いていると考えられる。筋衛星細胞上に，マクロファージで発現上昇する遺伝子産物に対する受容体遺伝子が発現上昇しておれば，筋再生に働く分子の同定につながると考えられ，今後，筋再生の効率を上昇させる手法の開発に役立つものと考えている。

E．結論

筋再生過程におけるマクロファージの役割を調べた。マクロファージの筋再生への役割を分子レベルで明らかにすることで，筋再生の効率を向上させる新しい方法が開発できることが期待される。

F．健康危険情報

なし

G．研究発表

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H．知的所有権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

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ARTICLE

AAV Vector-Mediated Microdystrophin Expression in a Relatively Small Percentage of *mdx* Myofibers Improved the *mdx* Phenotype

Madoka Yoshimura,[1,2] Miki Sakamoto,[1] Madoka Ikemoto,[1] Yasushi Mochizuki,[1] Katsutoshi Yuasa,[1] Yuko Miyagoe-Suzuki,[1] and Shin'ichi Takeda[1,*]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan*
[2]*Department of Neurology, Division of Neuroscience, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Tokyo 113-8655, Japan*

**To whom correspondence and reprint requests should be addressed. Fax: +81 42 346 1750. E-mail: takeda@ncnp.go.jp.*

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Duchenne muscular dystrophy (DMD) is a lethal disorder of skeletal muscle caused by mutations in the *dystrophin* gene. Adeno-associated virus (AAV) vector-mediated gene therapy is a promising approach to the disease. Although a rod-truncated microdystrophin gene has been proven to ameliorate dystrophic phenotypes, the level of microdystrophin expression required for effective gene therapy by an AAV vector has not been determined yet. Here, we constructed a recombinant AAV type 2 vector, AAV2-MCKΔCS1, expressing microdystrophin (ΔCS1) under the control of a muscle-specific MCK promoter and injected it into TA muscles of 10-day-old and 5-week-old *mdx* mice. AAV2-MCKΔCS1-mediated gene transfer into 5-week-old *mdx* muscle resulted in extensive and long-term expression of microdystrophin and significantly improved force generation. Interestingly, 10-day-old injected muscle expressed microdystrophin in a limited number of myofibers but showed hypertrophy of microdystrophin-positive muscle fibers and considerable recovery of contractile force. Thus, we concluded that AAV2-MCKΔCS1 could be a powerful tool for gene therapy of DMD.

Key Words: Duchenne muscular dystrophy, gene therapy, adeno-associated virus vector, dystrophin, microdystrophin, skeletal muscle, *mdx* mouse, hypertrophy

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by mutations in the *dystrophin* gene. There is no effective treatment for the disease at present, although gene therapy could be an attractive approach to the disease.

Several methods of gene transfer have been tried for the treatment of dystrophin-deficient muscular dystrophy: naked plasmid injection [1], full-length dystrophin cDNA transfer via a gutted adenovirus vector [2,3], forced splicing using oligonucleotides [4], and gene repair by a chimeric RNA/DNA oligonucleotide [5]. Among several gene transfer methods, an adeno-associated virus (AAV) vector-mediated gene transfer is one of the most promising approaches to DMD because AAV vectors have been shown to evoke minimal immune responses and mediate long-term transgene expression in skeletal muscle [6–8]. Since the capacity of an AAV vector to incorporate an exogenous gene is limited to 4.9 kb, several groups have attempted to truncate the 14-kb dystrophin cDNA to obtain functional microdystrophins to be inserted into AAV vectors [9–13]. We previously constructed three rod-truncated microdystrophins and generated transgenic *mdx* mice expressing these microdystrophins. Among the three microdystrophins tested, only the 4.9-kb microdystrophin CS1 completely prevented muscle degeneration of dystrophin-deficient *mdx* mice [14]. Based on this result, we generated an AAV vector carrying ΔCS1 microdystrophin cDNA, a modified version of CS1 cDNA, and injected the vectors (designated AAV2-MCKΔCS1) directly into both 10-day-old and 5-week-old *mdx* muscles. In this study, we demonstrate that AAV vector-injected *mdx* muscles showed functional recovery even 24 weeks after treatment. Surprisingly, when introduced into neonatal muscle, microdystrophin expression in a relatively small percentage of

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muscle fibers dramatically improved the contractile properties of dystrophic muscle. This improvement in contractile force was thought to be achieved by hypertrophied microdystrophin-positive muscle fibers.

RESULTS

Construction of an AAV-2 Vector Carrying Microdystrophin ΔCS1

We constructed an AAV-2 vector encoding microdystrophin ΔCS1 under the control of a truncated, muscle-specific creatine kinase (MCK) promoter [15]. We designated this recombinant AAV vector AAV2-MCKΔCS1. CS1, which has the N-terminal, actin-binding domain, four rod repeats and three hinges, the cysteine-rich domain, and the C-terminal domain, effectively rescued dystrophic phenotypes when introduced as a transgene [14]. To shorten CS1 cDNA (4.9 kb) further, we deleted the 5′ and 3′ untranslated regions (UTRs) and exons 71–78 (alternative splicing regions of dystrophin mRNA) by PCR techniques. We named the resultant 3.8-kb cDNA ΔCS1 (Fig. 1).

Expression of ΔCS1 Microdystrophin at the Sarcolemma after AAV2-MCKΔCS1-Mediated Gene Transfer into *mdx* Muscle

We injected AAV2-MCKΔCS1 into tibialis anterior (TA) muscles of 10-day-old and 5-week-old dystrophin-deficient *mdx* mice (7.5×10^{10} vector genomes (vg) for neonatal muscle; 2.5×10^{11} vg for young muscle). In a natural course, 10-day-old *mdx* muscle shows no obvious dystrophic changes, while 5-week-old *mdx* muscle shows active cycles of the degeneration–regeneration process. We also analyzed contralateral TA muscles from the treated *mdx* mice and TA muscles from age-matched C57BL/10 (B10) mice as controls. When examined at 8 and 24 weeks after AAV2-MCKΔCS1 injection, ΔCS1 had correctly localized at the sarcolemma (Figs. 2A and 2C). Western blot using a dystrophin antibody showed a band of the expected size (138 kDa) in the AAV vector-injected *mdx* muscles (Fig. 2G). Most of the ΔCS1-positive fibers were peripherally nucleated when AAV2-MCKΔCS1 was injected into 10-day-old *mdx* muscles (Figs. 2A and 2B). In contrast, we observed both centrally and peripherally nucleated fibers when the vectors were injected into 5-week-old *mdx* muscle (Figs. 2C and 2D). The mean percentages of ΔCS1-positive fibers were 22.2 ± 11.4% at 8 weeks and 16.5 ± 7.0% at 24 weeks after injection at 10 days of age (Fig. 2E). When injected at 5 weeks of age, the mean percentages of dystrophin-positive fibers were 39.2 ± 15.8% at 8 weeks and 51.5 ± 17.3% at 24 weeks after vector injection (Fig. 2F). Next, we quantified the amount of ΔCS1 protein by immunoblotting. The amount of microdystrophin protein in the AAV-injected *mdx* muscles at 10 days of age was 12.7 ± 8.4% of that of full-length dystrophin in B10 muscle at 8 weeks (data not shown) and 9.2 ± 1.4% at 24 weeks after injection (Fig. 2G). When we injected AAV2-MCKΔCS1 into 5-week-old *mdx* muscles, the amount was 32.6 ± 8.0% of that of B10 muscle at 8 weeks and 39.8 ± 7.0% at 24 weeks after injection (data not shown).

AAV Vector-Mediated ΔCS1 Expression Ameliorated Dystrophic Phenotypes at 24 Weeks after Injection

When we analyzed *mdx* muscles treated at 10 days of age at 24 weeks after vector injection, only a small percentage of the ΔCS1-positive fibers (12.5 ± 7.8%) had central nuclei compared with untreated *mdx* muscle fibers (Fig. 3A), suggesting the protective function of ΔCS1 against muscle degeneration. In contrast, the percentage of

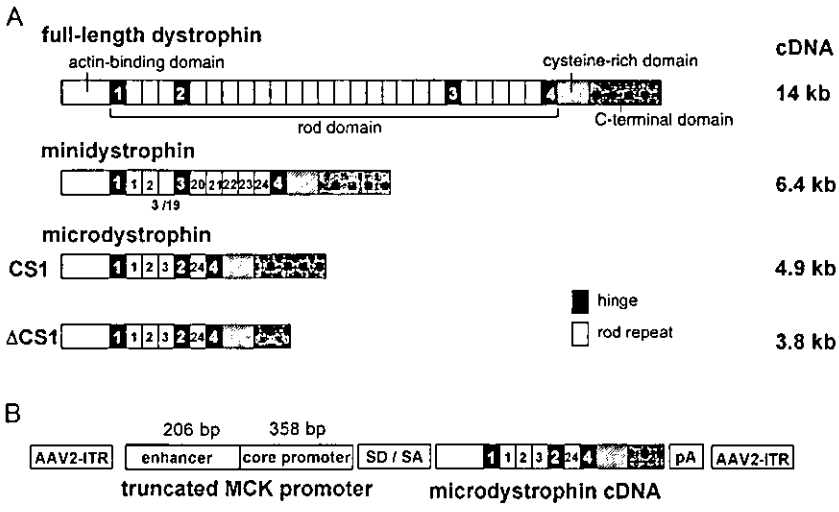

FIG. 1. Diagrams of human full-length dystrophin, minidystrophin, and microdystrophin cDNAs (CS1, ΔCS1) and AAV2-MCKΔCS1. (A) Full-length dystrophin has the N-terminal, actin-binding domain, central rod domain with 24 rod repeats and four hinges, cysteine-rich domain, and C-terminal domain. Minidystrophin, which was cloned from a mild Becker patient and reported previously, is shown as a reference [28]. CS1 has the N-terminal domain, a shortened version of the central rod domain with 4 rod repeats and three hinges, the cysteine-rich domain, and the C-terminal domain [14]. To incorporate microdystrophin CS1 cDNA into the AAV2 vector plasmid, we deleted the 3′ and 5′ untranslated regions and exons 71–78 from CS1 cDNA. The resultant ΔCS1 cDNA is 3.8 kb long. The number of the rod repeats or hinges is shown inside the squares. (B) Structure of the AAV2 vector expressing ΔCS1. ΔCS1 cDNA was incorporated into the AAV2 vector plasmid downstream of the truncated muscle-specific MCK promoter [15].

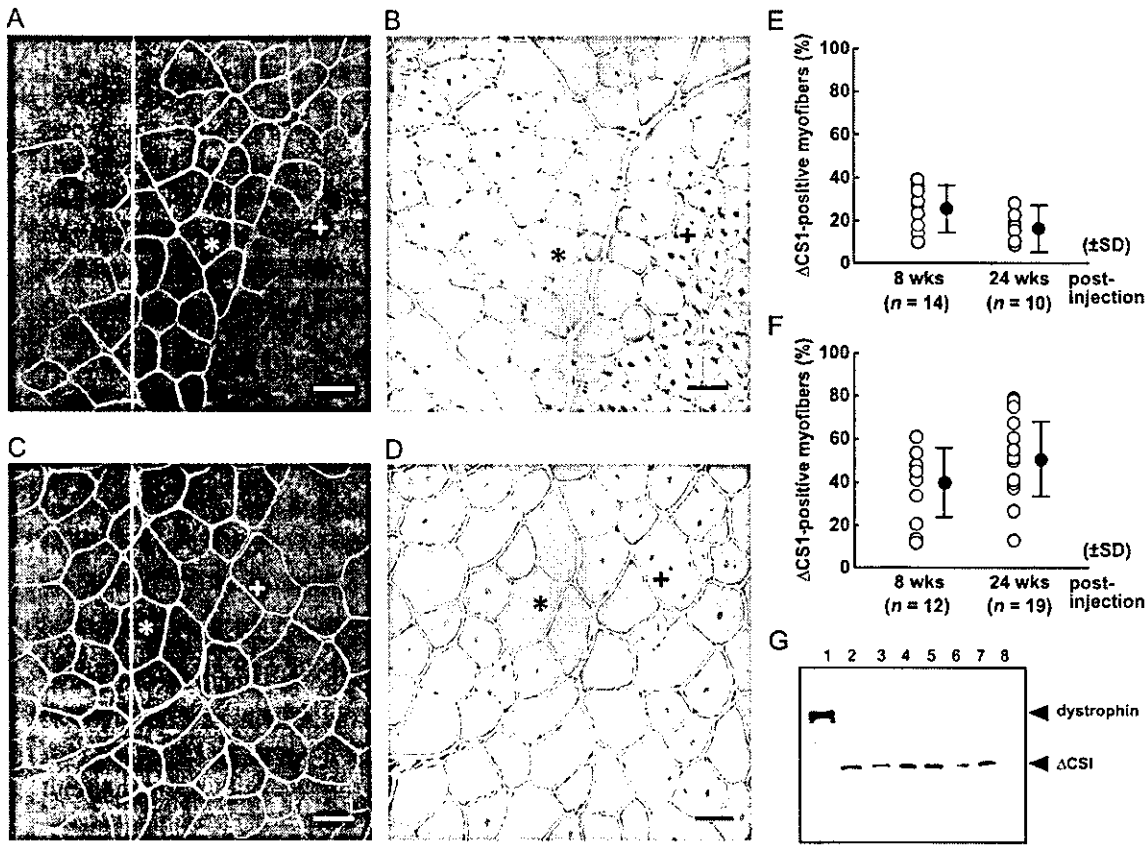

FIG. 2. ΔCS1 expression after AAV vector-mediated gene transfer into skeletal muscles of dystrophin-deficient *mdx* mice. AAV2-MCKΔCS1 was injected into TA muscles of *mdx* mice at (A, B, E) 10 days or at (C, D, F) 5 weeks of age and the muscles were analyzed at 8 or 24 weeks after injection. (A–D) Histological analysis of AAV-injected muscles 24 weeks after AAV injection. Immunofluorescence using a dystrophin antibody (A, C) and H&E staining of serial sections (B, D) is shown. ΔCS1 is correctly localized at the sarcolemma (A, C). Most ΔCS1-positive fibers (•) showed peripherally located nuclei, and their fiber diameters were relatively larger than ΔCS1-negative fibers (+) in the muscles injected at 10 days of age (A, B). In contrast to the muscles injected at the neonatal stage, many centrally nucleated fibers (+) coexist with peripherally nucleated fibers (•) in the muscles injected with the AAV vectors at 5 weeks of age (D). In (A and C), nuclei were stained with TOTO-3 (blue). Bar, 50 μm. (E, F) The percentage of ΔCS1-positive fibers among all fibers of the injected *mdx* muscle. The means (black circles) are indicated ± SD (bars). The percentage of ΔCS1-positive fibers in muscles injected at 5 weeks of age (F) was higher than in the muscles injected at 10 days of age (E). (G) Western blot analysis using a dystrophin antibody of AAV-injected *mdx* muscles. Muscles treated at 10 days of age were analyzed at 24 weeks after injection. Lane 1, C57BL/10 muscle; lanes 2–7, AAV vector-injected *mdx* muscles; lane 8, uninjected *mdx* muscle. Full-length dystrophin (lane 1) and ΔCS1 (lanes 2–7) were detected at the predicted sizes (427 or 138 kDa, respectively).

centrally nucleated fibers among ΔCS1-positive fibers in *mdx* muscles treated at 5 weeks of age (51.5 ± 11.0%) was higher than that of *mdx* muscles treated at 10 days of age (Fig. 3B).

Next, we evaluated the contractile properties of AAV2-MCKΔCS1-injected *mdx* muscle. Untreated *mdx* muscle showed remarkable hypertrophy, but its specific force was much lower than in control B10 muscle (Table 1). Similarly, the wet weight of *mdx* TA muscles treated with AAV2-MCKΔCS1 at 10 days of age was much heavier than that of control B10 TA muscles, but importantly, the maximal force was also increased (Table 1). As a result, there was no statistical difference in specific tetanic force between AAV2-MCKΔCS1-treated *mdx* muscles and age-matched B10 muscles (Table 1).

The transduction efficiency of AAV2 vector-mediated gene transfer into 10-day-old *mdx* mice was relatively low (only up to 20% of positive fibers) (Fig. 2E) compared to gene transfer into 5-week-old *mdx* mice (around 50%) (Fig. 2F), but, surprisingly, the specific tetanic force at 24 weeks after vector injection was almost equivalent to that of age-matched B10 mice and much higher than that of untreated *mdx* muscle (Table 1). To clarify the mechanism of force generation recovery by small percentages of ΔCS1-positive fibers, we next examined the relationship between muscle hypertrophy and force generation. We found a positive correlation ($r = 0.779$, $P < 0.05$) between the wet weight of the AAV2-MCKΔCS1-injected TA muscle and the force generation (Fig. 4A), but not between the muscle weight and the relative interstitial

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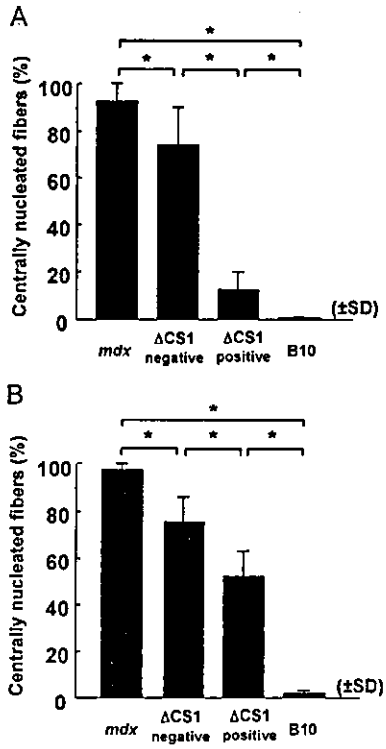

FIG. 3. Microdystrophin ΔCS1 prevents muscle degeneration. Quantitative analysis of centrally nucleated fibers among ΔCS1-positive and -negative fibers in AAV-injected *mdx* muscle. AAV2-MCKΔCS1 was injected into TA muscles of *mdx* mice at (A) 10 days or at (B) 5 weeks of age and analyzed at 24 weeks after injection. Uninjected *mdx* and age-matched control B10 muscles were also examined. (A, B) Most of the ΔCS1-positive fibers in muscles injected at 10 days of age have peripherally located nuclei (A). The percentage of centrally nucleated fibers in ΔCS1-positive fibers in muscles treated at 5 weeks of age (51.5 ± 11.0%) (B) was higher than that in muscles treated at 10 days of age (12.5 ± 7.8%) (A). Note that the ratio of centrally nucleated fibers was significantly reduced even in ΔCS1-negative fibers in muscles injected at both ages. *$P < 0.01$.

area ($r = -0.596$, $P > 0.05$) (Fig. 4B). To confirm whether increased muscle weight reflected myofiber hypertrophy, we measured individual cross-sectional areas (CSAs) of ΔCS1-positive or -negative myofibers in AAV-injected *mdx* muscles. The mean value of fiber CSAs was remarkably larger in ΔCS1-positive *mdx* fibers than in B10 fibers (Fig. 4C). Histogram analysis further demonstrated that the fiber CSA distribution of ΔCS1-positive *mdx* fibers was shifted to the right compared to that of B10 muscle; larger caliber fibers were dominant, reflecting hypertrophy of ΔCS1-expressing fibers (Fig. 4E). Thus, hypertrophied ΔCS1-positive fibers seemed to greatly improve contractile force generation. Some of the untreated and ΔCS1-negative *mdx* fibers were also hypertrophied, but the hypertrophied fibers seemed not to improve the specific force (Fig. 4E).

When we injected 5-week-old mice, force generation was recovered in AAV-injected *mdx* muscles, as when we treated 10-day-old mice, but there was no statistically significant difference in muscle weight between AAV-treated *mdx* muscles and B10 muscles (Table 1). The muscle weight had no correlation with the force generation ($r = -0.512$, $P > 0.05$) (data not shown). In addition, hypertrophy of ΔCS1-positive fibers was not obvious (Fig. 4D). Therefore, we concluded that improved specific force of the *mdx* muscles treated at 5 weeks of age was achieved without hypertrophy, possibly because approximately 50% of the muscle fibers were fully functional for ΔCS1 expression.

DISCUSSION

The level of dystrophin or minidystrophin expression required for effective gene therapy has not been determined yet, although estimates based on either transgenic *mdx* mouse studies [16–18] or the analysis of asymptomatic carriers of dystrophin-deficiency have been reported [19]. Clerk et al. reported that immunostaining showed very few dystrophin-negative fibers in muscles of asymptomatic DMD carriers, while immunoblot analysis revealed a considerable reduction in dystrophin [19]. Furthermore, it was reported that transgenic *mdx* mice expressing a minidystrophin at only 20–30% of endogenous dystrophin levels showed significantly reduced myopathic phenotypes [17]. Phelps et al. suggested that uniform expression of dystrophin is much more beneficial than the variable pattern when the overall levels of dystrophin expression were the same [18]. These findings suggest that the percentage of dystrophin-expressing fibers is more critical than the total amount of the protein. On the other hand, Rafael *et al.* showed that the expression of minidystrophin in only half the *mdx* muscle fibers resulted in a markedly milder phenotype than *mdx* mice showed [16], suggesting that dystrophin-positive fibers rescue surrounding dystrophin-negative fibers from degenerative changes. In this study, we administrated a recombinant AAV vector containing a human microdystrophin gene to the *mdx* muscle and analyzed the relationship between the level or extent of microdystrophin expression and the recovery of contractile force. Importantly, relatively small percentages of muscle fibers (less than 20%) dramatically improved the specific contractile force of dystrophic muscle. Although the molecular mechanisms by which microdystrophin recovers the specific contractile force remain to be shown, this result is encouraging in that the function of dystrophin-deficient muscle might be greatly improved by fewer dystrophin-positive myofibers than previously estimated. As shown in Fig. 3, however, there is a significant reduction in the percentage of centrally nucleated fibers among ΔCS1-negative *mdx* fibers compared to untreated *mdx* muscle, suggesting that microdystrophin expression at levels below the detection limits of immunostaining might be partially protective. There-

TABLE 1: Contractile properties of AAV2-MCKΔCS1-injected *mdx* muscle

	Muscle length (L_0, mm)	Muscle weight (MW, mg)	Maximal force (P_0, mN)	Specific force[a] (mN/mm^2)
		Injection at 10 days of age		
B10 (n = 4)	16.1 ± 0.9	48.7 ± 2.8	91.3 ± 18.1	32.0 ± 5.9
AAV-*mdx* (n = 7)	16.1 ± 1.2	64.3 ± 4.7*,**	116.5 ± 29.9**	30.9 ± 7.9**
mdx (n = 7)	16.1 ± 1.0	70.9 ± 7.0*	79.8 ± 20.0	19.1 ± 3.8*
		Injection at 5 weeks of age		
B10 (n = 6)	15.8 ± 0.9	54.9 ± 5.7	69.0 ± 31.0	20.9 ± 9.0
AAV-*mdx* (n = 11)	15.3 ± 0.8	60.9 ± 6.8	81.7 ± 24.2**	22.3 ± 8.2**
mdx (n = 11)	15.7 ± 0.7	67.9 ± 7.6*	42.2 ± 26.1	10.9 ± 7.5*

Force generation was measured 24 weeks after injection. Data are expressed as means ± SD.

[a] Specific force = ($P_0 \times L_0 \times 1.06$)/MW.

* Significant difference ($P < 0.05$) compared to B10.

** Significant difference ($P < 0.05$) compared to *mdx* muscles.

fore, exact estimation of the percentage of microdystrophin-positive fibers required for the full amelioration is somewhat difficult.

Recovery of absolute maximal force and specific tetanic force is one of the barometers of amelioration. The difference between the contractile properties of ΔCS1-expressing hypertrophied muscle and those of hypertrophied *mdx* muscle by overexpression of IGF-1 [20,21] deserves attention: ΔCS1-positive *mdx* muscle showed considerable recovery of specific force, whereas IGF-mediated hypertrophy modestly restored specific force and the muscle remained susceptible to damage. Similarly, a myostatin blockade of treated *mdx* muscle reportedly improved specific force to some extent, but showed a decrease in ECC force to the same extent as control *mdx* muscle [22]. For comparison of the effects of these different approaches toward DMD therapy, the resistance of ΔCS1-treated muscle to eccentric contraction remains to be evaluated. Importantly, however, myostatin antibody-treated mice showed significantly decreased serum creatine kinase concentrations, suggesting that myostatin blockade endowed dystrophin-deficient fibers with membrane stability.

Positive correlation between muscle wet weight and specific tetanic force indicates that muscle hypertrophy is responsible for functional amelioration at 10-day-old injected *mdx* mice. However, lack of small-caliber, presumably regenerative fibers is the most prominent finding on histograms of ΔCS1-positive *mdx* muscles injected at 10 days of age. Therefore, not only a mild increase in hypertrophic fibers, but also a decrease in small-sized fibers due to inhibition of the cycle of degeneration/regeneration, could greatly contribute to normalization of specific tetanic force. Reduction in embryonic myosin heavy chain and increase in mature myosin heavy chain might contribute to the functional recovery.

Watchko *et al.* injected an AAV2 vector carrying microdystrophin into *mdx* mice and observed incomplete recovery of specific tetanic force with 30–60% of dystrophin-positive fibers [13]. Their microdystrophin was slightly longer than our ΔCS1, it had three hinges, and the C-terminal domain was also deleted. Subtle differences in the construction could affect the functional aspects, and therefore we think that a functional examination of transgenic *mdx* is inevitable.

We also injected AAV2-MCKΔCS1 into 5-week-old *mdx* muscles, which usually show active cycles of muscle degeneration and regeneration. Dystrophin staining revealed that approximately 50% of *mdx* myofibers expressed human-type ΔCS1 microdystrophin 24 weeks after injection. There was no obvious sign of an immune response (data not shown). In contrast to neonatal muscle, *mdx* muscles treated at 5 weeks of age were not hypertrophied but still generated improved contractile force, indicating that widely expressed ΔCS1 could recover muscle function of adult mice without compensatory hypertrophy. Importantly, the CSAs of ΔCS1-positive fibers treated at a neonatal stage were larger than those of ΔCS1-positive fibers treated at 5 weeks of age (Figs. 4C and 4D). One possible explanation for this is that neonatal muscle has a high potency of compensatory hypertrophy in response to force deficit.

Our ΔCS1 and R4-R23/delta71-78, previously reported by Harper et al. [11], have similar structures. R4-R23/delta71-78 robustly transformed centrally nucleated fibers into peripherally nucleated fibers [11]. In contrast, the expression of ΔCS1 in adult *mdx* myofibers resulted in a modest reduction of centrally nucleated fibers when introduced into 5-week-old *mdx* muscle (Fig. 3B). A possible explanation for this discrepancy is a difference in the promoters that drive the microdystrophin expression. Harper *et al.* used a muscle-specific, potent promoter, CK6, and the positions of the Kozak sequences or splicing units are different from ours [11]. These differences might greatly influence the timing and levels of microdystrophin expression. A second possible factor

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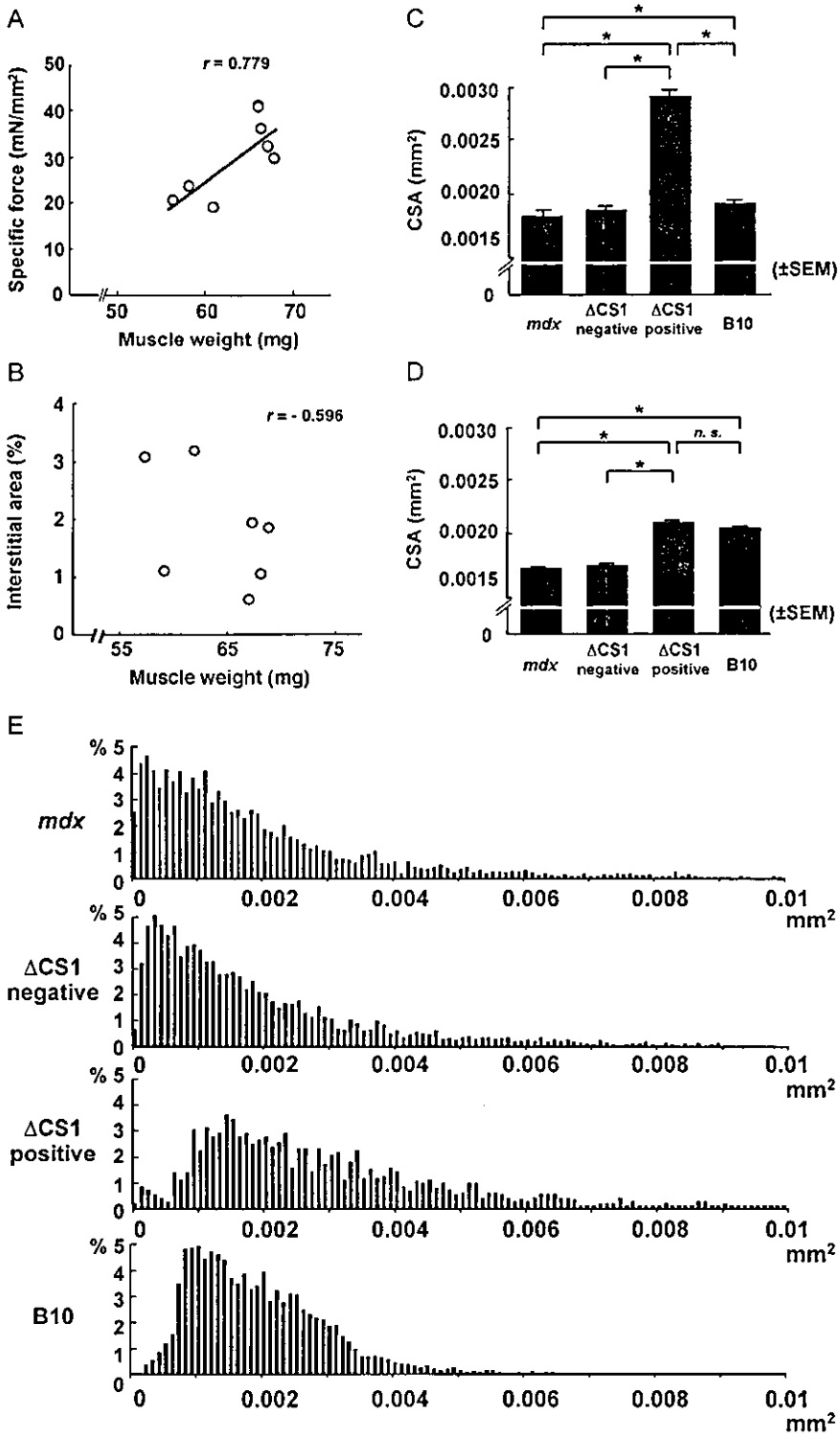

FIG. 4. ΔCS1-positive *mdx* fibers in AAV-treated muscle at 10 days of age show hypertrophy. TA muscles of *mdx* mice were injected with AAV2-MCKΔCS1 at 10 days of age (A–C, E) or at 5 weeks of age (D) and analyzed at 24 weeks after injection. (A) Correlation between muscle wet weight and tetanic force generation ($n = 7$). There was a significant positive correlation between muscle weight and force generation ($r = 0.779$, $P < 0.01$). (B) Relationship between muscle weight and relative interstitial area in AAV-injected muscles ($n = 7$). The increase in muscle weight is not proportional to the interstitial area. (C, D) Mean cross-sectional area (CSA) of uninjected *mdx* muscle fibers and ΔCS1-positive or -negative fibers in AAV-injected *mdx* muscles or age-matched B10 muscle fibers. Three TA muscles were examined for each group. The total numbers of fibers traced were 9674, 6347, 1525, and 5877 in (C) and 8077, 4075, 3479, and 5476 in (D) for *mdx*, ΔCS1-negative *mdx*, ΔCS1-positive *mdx*, and B10 fibers, respectively. When injected at 10 days of age, the CSA of ΔCS1-positive *mdx* fibers was definitely larger than that of normal B10, ΔCS1-negative *mdx*, or contralateral *mdx* fibers (C). In contrast, when mice were treated at 5 weeks of age, the mean CSA of ΔCS1-positive *mdx* fibers was similar to that of B10 muscle and slightly larger than that of *mdx* or ΔCS1-negative *mdx* fibers in treated muscle (D). $^*P < 0.01$. (E) Distribution of the fiber CSAs of untreated *mdx*, ΔCS1-positive, or ΔCS1-negative fibers in *mdx* muscles injected at 10 days of age and age-matched B10 fibers. Shown are the same data set presented in (C). In untreated *mdx* muscles or in ΔCS1-negative *mdx* fibers, small-caliber fibers are dominant, reflecting regeneration. The distribution pattern of ΔCS1-positive fibers deviated to the right, reflecting a larger size than that of B10 fibers. Similar to B10 muscles, small-caliber fibers were markedly reduced in ΔCS1-positive *mdx* fibers, indicating that ΔCS1 prevented muscle degeneration.

is the difference in muscle types used. Harper *et al.* injected AAV vectors into gastrocnemius muscles of 32-day-old mice. The gastrocnemius shows less centrally nucleated fibers than TA muscle in the natural course of the disease (Yuasa *et al.*, unpublished data). In our result, however, AAV-treated *mdx* muscle with a high

percentage of centrally nucleated fibers (50%) did not show dystrophic dysfunction, e.g., decreased force generation. Therefore, centrally nucleated fibers do not disprove the protective function of ΔCS1. The percentage of centrally nucleated fibers would reflect the state of muscles at the time of injection. Our results are important because it is sometimes difficult to start gene therapies before the onset of the clinical course of DMD, and AAV2-MCKΔCS1 is expected to show therapeutic effects even after the onset of disease.

Although we injected high titers of AAV-vector particles into the muscle, the transduction efficiency in 10-day-old *mdx* muscles was much lower than that in 5-week-old *mdx* muscle. This phenomenon was also noticed for the injection of AAV2-CMVLacZ into neonatal B10 muscle (unpublished results). This difference might be due to the preferential expression of receptors or coreceptors for an AAV-2 particle on adult muscle fibers. Several molecules, such as heparan sulfate proteoglycan [23], αVβ5 integrin [24], and dynamin I [25], are proposed to play certain roles in AAV type 2 infection, although the expression of these molecules in muscle fibers during development and aging has not been fully determined. Another possibility is dilution of AAV vectors by rapid growth of neonatal muscle. When we cultured satellite cells prepared from AAV2-CMVLacZ-injected *mdx* muscle, we observed no blue myoblasts or myotubes (data not shown). Therefore, proliferation and fusion of nontransduced satellite cells/myoblasts would greatly dilute the microdystrophin protein.

Our results are promising because AAV vector-mediated ΔCS1 gene transfer had a restorative function for dystrophin-deficient *mdx* muscle. However, demonstration of the benefits of this gene transfer strategy for human DMD patients requires careful testing. Differences between humans and mice, such as muscle size, life span, or biological properties (especially immune responses), should be taken into consideration. A bigger animal model, e.g., canine X-linked muscular dystrophy [26], will contribute to the preclinical study of gene therapy.

MATERIALS AND METHODS

Constructs of human rod-truncated microdystrophin cDNAs and generation of AAV vectors expressing microdystrophin. To incorporate microdystrophin CS1 cDNA (4.9 kb) [14] into an AAV type 2 vector, we further deleted the 3′ and 5′ UTRs and exons 71–78 from CS1 cDNA. In brief, DNA fragments of the 5′-terminal and 3′-terminal regions were independently amplified by PCR to remove exons 71–78 and the 5′ and the 3′ UTRs and then replace them with corresponding sequences of CS1 cDNA. The resulting microdystrophin cDNA was 3.8 kb long and designated ΔCS1. Microdystrophin ΔCS1 cDNA was then cloned into an AAV type 2 vector plasmid [15]. The recombinant AAV vector expressing ΔCS1 under the control of the truncated muscle-specific MCK promoter, designated AAV2-MCKΔCS1, was then purified and titrated as previously described [15].

Administration of AAV vector to murine skeletal muscle. Fifteen microliters (7.5×10^{10} vg) or 50 μl (2.5×10^{11} vg) of AAV2-MCKΔCS1 was injected directly into the right TA muscles of dystrophin-deficient C57BL/10 *mdx* mice at 10 days or 5 weeks of age, respectively. AAV vector-injected and uninjected *mdx* muscles and normal muscles of age-matched C57BL/10 mice were isolated at 8 and 24 weeks after injection.

Contractile properties of AAV2-MCKΔCS1-injected TA muscles. Tetanic force generation was measured and analyzed as described previously with some modifications [14,27]. The entire TA muscle was removed with its tibial origin intact, and the distal portion of the TA tendon and its origin was secured with a 5-O silk suture. The TA was mounted in a vertical tissue chamber and connected to a force transducer, UL-10GR (Minerva, Nagano, Japan), and a length servosystem, MM-3 (Narishige, Tokyo, Japan). Electrical stimulation using a SEN3301 (Nihon Kohden, Tokyo, Japan) was applied through a pair of platinum wires placed on both sides of the muscle in physiological soft solution (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM Hepes, pH 7.4, and 0.02 mM D-tubocurarine). Muscle fiber length was adjusted incrementally by using a micropositioner until peak isometric twitch force responses were obtained (optimal fiber length (L_0)). Maximal tetanic force (P_0) was assessed by stimulation frequencies of 125 pulses/s delivered in 500-ms duration trains with 2 min intervening between each train. Following two measurements, the stimulated muscle was weighed after tendon and bone attachments were removed. All forces were normalized to the physiological cross-sectional area (pCSA), the latter estimated on the basis of the following formula: muscle wet weight (in mg)/(L_0 (in mm) × 1.06 (in mg/mm^3)). The estimated pCSA was used to determine specific tetanic force (P_0/pCSA) of the muscle. After measurement of contractile force, the muscle was quickly frozen in liquid nitrogen-cooled isopentane for histopathological analysis.

Histopathological analyses. Histological, immunohistochemical, and Western blot analyses were performed as described [14]. After blocking with an M.O.M. kit (Vector Laboratories, Burlingame, CA, USA), dystrophin was detected using a monoclonal anti-dystrophin antibody NCL-DysB (Novocastra, Newcastle, UK; 1:20 dilution) and visualized with Alexa 488-labeled goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA) (1:200 dilution). Nuclei were stained with TOTO-3 (Molecular Probes). In some cases, the signal was visualized with diaminobenzidine and counterstained with hematoxylin. We counted the number of centrally or peripherally nucleated fibers in dystrophin-positive or -negative fibers of whole cross sections of TA muscle. In addition, the CSA of each fiber was measured using an image analysis system, ImagePro-Plus (Media Cybernetics, Silver Spring, MD, USA). To evaluate the level of fibrosis, we performed modified Masson trichrome staining, and the blue-stained area was measured using Image Pro-Plus. The relative connective tissue area was calculated to the entire muscle cross-sectional area (%). The signals on immunoblotting were quantitated using NIH Image.

Statistical analysis. Data were expressed as means ± SD or ± SEM. If a significant F ratio was detected by analysis of variance, comparisons among each group were performed using Fisher's PLSD. A P value of <0.05 or <0.01 was considered statistically significant. The relation between the muscle weight and the specific tetanic force was analyzed with Pearson's correlation coefficient ($P < 0.05$).

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Mac-1^{low} early myeloid cells in the bone marrow-derived SP fraction migrate into injured skeletal muscle and participate in muscle regeneration ☆

Koichi Ojima [a,b], Akiyoshi Uezumi [a], Hiroyuki Miyoshi [c], Satoru Masuda [a], Yohei Morita [d,e], Akiko Fukase [a], Akihito Hattori [b], Hiromitsu Nakauchi [d,e], Yuko Miyagoe-Suzuki [a], Shin'ichi Takeda [a,*]

[a] *Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan*
[b] *Department of Animal Science, Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan*
[c] *BioResource Center, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan*
[d] *Laboratory of Stem Cell Therapy, Center for Experimental Medicine, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[e] *Department of Immunology, Institute of Basic Medical Sciences, The University of Tsukuba, and CREST (JST), 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*

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Abstract

Recent studies have shown that bone marrow (BM) cells, including the BM side population (BM-SP) cells that enrich hematopoietic stem cells (HSCs), are incorporated into skeletal muscle during regeneration, but it is not clear how and what kinds of BM cells contribute to muscle fiber regeneration. We found that a large number of SP cells migrated from BM to muscles following injury in BM-transplanted mice. These BM-derived SP cells in regenerating muscles expressed different surface markers from those of HSCs and could not reconstitute the mouse blood system. BM-derived SP/Mac-1^{low} cells increased in number in regenerating muscles following injury. Importantly, our co-culture studies with activated satellite cells revealed that this fraction carried significant potential for myogenic differentiation. By contrast, mature inflammatory (Mac-1^{high}) cells showed negligible myogenic activities. Further, these BM-derived SP/Mac-1^{low} cells gave rise to mononucleate myocytes, indicating that their myogenesis was not caused by stochastic fusion with host myogenic cells, although they required cell-to-cell contact with myogenic cells for muscle differentiation. Taken together, our data suggest that neither HSCs nor mature inflammatory cells, but Mac-1^{low} early myeloid cells in the BM-derived SP fraction, play an important role in regenerating skeletal muscles.

Keywords: Side population cells; Muscle regeneration; Myogenic differentiation; Bone marrow; Muscular dystrophy

Skeletal muscles have a remarkable capacity for regeneration in response to various types of damage, such as chemicals, stretching, exercise, injury, and diseases including inherited muscular dystrophies. Satellite cells are skeletal muscle-specific precursors and play an important role in muscle fiber regeneration [3]. They are located beneath the basement membrane and are mitotically

☆ *Abbreviations:* β-Gal, β-galactosidase; BM, bone marrow; CTX, cardiotoxin; FACS, fluorescence-activated cell sorting; GC, gastrocnemius; GFP, green fluorescence protein; HE, hematoxylin and eosin; HSC, hematopoietic stem cell; MP, main population; SP, side population; TA, tibialis anterior; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

* Corresponding author. Fax: +81 42 346 1750.
E-mail address: takeda@ncnp.go.jp (S. Takeda).

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