疾　患　編

17. 筋ジストロフィー，多発筋炎

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要　点

①Duchenne型筋ジストロフィー（DMD）の確定診断は，遺伝学的検査あるいは筋生検により行う。
②DMDの骨格筋および呼吸機能の改善を目的にステロイド投与を行う。
③DMDでは，心不全と呼吸障害を中心とした全身管理が重要である。
④多発筋炎・皮膚筋炎は，ステロイド，免疫抑制剤，免疫グロブリンにより加療を行う。
⑤多発筋炎・皮膚筋炎は，悪性腫瘍や間質性肺炎の合併に注意する。

重要ポイント

①筋ジストロフィーは，骨格筋の壊死・変性を主病変とし，臨床的には進行性の筋力低下をみる遺伝性の疾患である。筋ジストロフィーのうち，もっとも頻度が高く重症の経過をとるDuchenne型筋ジストロフィーを中心に，呼吸および循環管理に加えて，ステロイド，アンジオテンシン変換酵素阻害薬，交感神経β受容体遮断薬などにより筋障害の改善が試みられている。
②後天性筋疾患の代表である特発性炎症性ミオパチーは，多発筋炎，皮膚筋炎，封入体筋炎に分類される。特に多発筋炎は横紋筋のびまん性炎症性筋疾患であり，特徴的な皮疹を呈するものは皮膚筋炎という。成人に比較的多くみられ，ステロイド，免疫抑制剤，免疫グロブリンが筋力低下に奏効する場合が多いが，悪性腫瘍や間質性肺炎の合併例は予後不良である。

A Duchenne型筋ジストロフィー（Duchenne muscular dystrophy：DMD）

筋ジストロフィーは「骨格筋の壊死・変性を主病変とし，臨床的には進行性の筋力低下をみる遺伝性の疾患である」と定義される。筋ジストロフィーのうち，もっとも頻度が高く重症の経過をとるDuchenne型筋ジストロフィー（DMD）は，ジストロフィン遺伝子（Xp21.2）の変異により，骨格筋膜の安定性に重要なジストロフィンが欠損す

ることで発症する。ジストロフィンの欠損が不完全な場合はベッカー型筋ジストロフィー（Becker muscular dystrophy：BMD）の表現型をとる。DMDは，X染色体連鎖遺伝形式をとり，新生男児3,500人に1人の割合で発症する。

1．臨床像

2～5歳前後で転びやすい，歩行が遅いなどの症状で気づかれることが多いが，高CK血症を偶発的に指摘され診断に至ることもある。特徴的な登はん性起立（Gowers徴候）を呈する。病初期には明らかな筋萎縮は認めず，腓腹部や舌などの筋肥大を示す場合が多いが，徐々に近位筋優位の筋力低下が進行して歩行は動揺性となり，12歳までに歩行不能となり車椅子生活に移行する。前後して脊柱側彎や関節拘縮の出現をみる。13歳前後で座位の保持も困難となる。呼吸筋の筋力低下のため10歳後半に呼吸不全が生じ，次第に心機能の低下も出現する。主として呼吸管理の進歩により，平均死亡年齢は過去20年で10年程度延長し，30歳前後になった。現在の死因は主に心不全および呼吸不全である。

2．検査所見

1）血液生化学検査

乳児期より著明な高CK血症（20,000～40,000 U/L），アルドラーゼなどの筋原性酵素の上昇をみるが，筋萎縮の進行とともに低下する。AST，ALT，LDHも上昇し，肝機能障害と誤る場合がある。

2）筋電図

随意収縮時に，低振幅・短持続時間の運動単位，運動単位の早期動員（early recruitment）がみられる。

3）画像検査

骨格筋CT，MRIでは5歳頃から大殿筋の脂肪置換を認める。10歳以降では大腿四頭筋（特に大腿直筋），大内転筋と大腿二頭筋，傍脊柱筋を中心に近位筋優位に筋容積の減少や脂肪置換が顕著となるが，薄筋と縫工筋は比較的保たれる（selectivity pattern）。

4）遺伝学的検査

他の検査所見からDMD/BMDの可能性が疑われ，臨床的および遺伝医学的に有用と考えられる場合に実施を検討する。遺伝学的検査は，生涯変化しない個人の重要な遺伝学的情報を扱うため，担当医師から被験者（保護者）に対して，検査を行う意義，利点と限界，その結果が家族や親族に及ぼす影響について十分説明し，書面による同意を得た上で，遺伝子異常が診断されたときの支援まで準備して実施されるべきである。検査実施前後に遺伝カウンセラーが遺伝カウンセリングを実施することが望ましい。

最近，遺伝子変異が確立した男性のDMD/BMD患者を対象に，臨床試験/治験の実施を目的とした筋ジストロフィー患者登録サイトの運用が開始された（Remudy：registry of muscular dystrophy. http://www.remudy.jp/index.html）。登録に際しては，全例にmultiplex ligation-dependent probe amplification（MLPA）法によるスクリーニング検査を行い（保険診療），必要に応じてシークエンス解析，筋生検を実施する。

5）筋生検

筋病理では，筋の壊死・変性，再生線維の混在，筋線維の大小不同，結合織の増生

がみられる。免疫組織化学染色では，DMDの筋細胞膜はジストロフィンをほぼ完全に欠損するが，BMDの細胞膜はまだら状(faint and patchy）に染色される。

3．治療方針

DMDに対するステロイド投与の有効性に関して，筋力の増強あるいは維持と呼吸機能の改善がランダム化比較対照試験により証明されている。5～15歳の症例ではプレドニゾロン（プレドニン®）0.75 mg/kg/日の連続投与が治療の第一選択である。体重増加などの副作用の面から投与量の減量が望ましい場合には，0.5 mg/kg/日に減量し，3～4ヵ月でさらに0.3 mg/kg/日へと減量する。

【心不全】

定期的に脳性ナトリウム利尿ペプチド(brain natriuretic peptide：BNP）の測定や心エコーを施行し，左室駆出率40％以下(BNP 20～60 pg/mL）で，アンジオテンシン変換酵素阻害薬（レニベース®，2.5 mg/日から開始し漸増），交感神経β受容体遮断薬（アーチスト®，1.25 mg/日から開始し漸増。上限は10 mg/日）を開始する。心筋障害が進行した際は拡張型心筋症の心不全に準じ，強心薬，利尿薬も加える。

【呼吸障害】

定期的なSpO_2，％VC，ピークカフフロー，終末呼気炭酸ガス濃度の測定が重要である。開始時期は，低酸素に基づく症状がある場合，睡眠時にSpO_2低下がある場合，VCが1L（あるいは％VCが20％）を下回る時期の前後，$PaCO_2$が55 Torr以上であれば夜間に非侵襲的陽圧換気療法（Noninvasive Positive Pressure Ventilation：NPPV）を開始する。病状，病態に応じて昼間にもNPPVを追加する。排痰障害にはカフレーター(Mechanical In-Exsufflator：MI-E）や肺内パーカッション換気療法（IPV）も有効である。

4．患者指導とリハビリテーション

早期より側彎と関節拘縮の予防に努め，必要に応じて装具，コルセットを作製する。最大強制吸気量維持のため呼吸訓練を行い，舌咽頭呼吸の習得を試みる。側彎は外科的治療も含め積極的に治療する。過度の痩せは消化管機能を低下させるため栄養指導が大切である。

5．根本的治療開発の動向

現在DMDに対して，PTC124によるリード・スルー療法，ES/iPS細胞や筋芽細胞の移植治療，ウイルスベクターによる遺伝子治療などの開発が進められている。当研究部では，これまでアンチセンス・モルフォリノを用いたエクソン・スキッピング療法の前臨床試験を行ってきた。この成果を踏まえて，DMDを対象にしたエクソン51スキッピングの臨床治験を実施する準備を進めている。

B 多発筋炎（polymyositis：PM），皮膚筋炎（dermatomyositis：DM）

後天性筋疾患の代表である特発性炎症性ミオパチーは，多発筋炎，皮膚筋炎，封入体筋炎に分類される。特に多発筋炎は横紋筋のびまん性炎症性筋疾患であり，特徴的な皮疹を呈するものは皮膚筋炎という。多発筋炎・皮膚筋炎の有病率は人口10万あた

り約6人と推定される。男女比は女性が約2倍と多い。発症年齢の約半数は40～60歳である。

1. 病因

発症には自己免疫機序が関与する。多発筋炎では，筋線維・間質・血管周囲にマクロファージ，$CD8^+$T細胞が浸潤し，筋線維内のカルパインなどのタンパク分解酵素を活性化する結果，筋線維は壊死する（細胞性免疫）。皮膚筋炎では，主に筋周膜の血管周囲や間質にB細胞，$CD4^+$T細胞（ヘルパーT細胞）が浸潤する（液性免疫）。筋内微小血管の内皮細胞が傷害される結果，循環障害による筋束周囲萎縮が生じる。

2. 臨床像

急性ないし亜急性（数週～数ヵ月）に進行する。初発症状は，四肢近位筋・頸筋・体幹の筋力低下，筋痛，関節痛がみられることが多い。遠位筋力の低下は遅れて生ずる。進行例では筋萎縮を認めることがある。嚥下障害が生じることがあるが，構音障害を伴うことは少ない。びまん性間質性肺炎，肺線維症，心筋炎をしばしば合併する。自然寛解や増悪を繰り返しつつ徐々に進行し，5年生存率は約75％である。皮膚筋炎は多発筋炎と類似した臨床像を呈するが，ゴットロン徴候（指関節伸側で肥厚した紅斑），ヘリオトロープ疹（上眼瞼の紫紅色の浮腫性紅斑）を伴うことを特徴とする。皮膚筋炎の悪性腫瘍合併頻度は約20％であり，多発筋炎と比べて2.1～6.5倍高い。女性では，乳癌・卵巣腫瘍，男性では肺癌・消化器癌・前立腺癌が多い。女性の悪性腫瘍合併率は男性の約2倍で，50歳以上は高い。

3. 検査所見

1) 血液生化学検査

活動期には血清CK値は正常値の約10倍に上昇し，ミオグロビン値も上昇する。アルドラーゼ，AST，ALT，LDH，％クレアチン尿（尿中クレアチン/尿中クレアチン＋尿中クレアチニン）が上昇し，活動性の指標判定に有用である。

2) 筋電図

随意収縮時には，低振幅・短持続時間の運動単位，運動単位の早期動員（early recruitment）がみられる。刺入電位は亢進していることが多い。安静時には線維性収縮電位，陽性鋭波を認める。

3) 画像検査

急性期の骨格筋MRIは，STIR（Short TI Inversion Recovery）法および脂肪抑制T2強調画像では，病変は多巣性あるいはびまん性の高信号を示す。進行例は，筋萎縮およびT1強調画像で高信号を示す。

4) 筋生検

筋束内の周辺・筋線維の内部・血管周囲に$CD8^+$T細胞やマクロファージの浸潤像，筋線維の変性と再生，結合織の増生を認める。特に皮膚筋炎では血管周囲の細胞浸潤が主体であり，筋束周囲萎縮が認められることが多い。

5) 自己抗体

抗Jo-1抗体は肺線維症の合併のある多発筋炎の50％，皮膚筋炎の20％に認められる。抗シグナル認識粒子（SRP）抗体は筋炎と心障害を伴う急性発症の重症皮膚筋炎および多発筋炎の5％に検出される。皮膚筋炎に特異的な抗Mi-2抗体は35％で検出され，抗

p155抗体は悪性腫瘍合併例で高率とされる。その他の膠原病を合併するオーバーラップ（重複）症候群では，抗PM-1抗体（強皮症），抗Ku抗体（強皮症・全身性エリテマトーデス），抗nRNP抗体（混合性結合組織病）が陽性となることがある。

4．診断

BohanとPeterの診断基準や皮膚筋炎・多発筋炎の改訂診断基準（厚生省特定疾患自己免疫疾患調査研究班平成4年度研究報告，pp25-28，1993）が汎用される。

5．治療方針

多発筋炎あるいは皮膚筋炎の確定診断後は，プレドニン®1～1.5 mg/kg/日を1～2ヵ月間連日投与する。筋力の改善，血清CK値の減少がみられれば2週間に10％の割合でプレドニン®を減量し，2～3年程度は維持療法を行う。筋症状は早期治療例ほど回復がよい。改善がないときは同量を1～2ヵ月間投与するか，ステロイドパルス療法を2～3クール行う（メチルプレドニゾロン1 g/回の3日間連続投与）。効果がない場合はメトトレキサート（メソトレキセート®，5～25 mg/週，経口あるいは筋肉内投与），アザチオプリン（イムラン®，50～100 mg/日，経口投与）などを併用する。ステロイド，免疫抑制薬の無効例では，追加療法として免疫グロブリン療法を併用する。

6．治療のポイント

筋症状増悪時には筋炎の再燃か，ステロイドミオパチーの合併かの鑑別が重要である。血清CK値上昇，筋電図で線維性収縮電位や陽性鋭波の出現頻度が上昇した場合には再燃を疑う。ステロイドミオパチーは，プレドニン内服を4週間以上続けた場合に発症し，下肢近位筋優位の筋力低下・筋萎縮を呈するが，顔面筋および頸部伸展筋は保たれ，血清CK値は低下する。

7．患者指導とリハビリテーション

急性期は等尺性収縮以外の運動は避ける。安定後は誤嚥性肺炎，廃用性筋萎縮，関節拘縮予防のための理学療法を早期に開始する。

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5. 骨格筋

骨格筋は傷害を受けると再生する（図1）．筋傷害シグナルにより骨格筋特異的幹細胞である筋衛星細胞（muscle satellite cells）が活性化され，分裂・増殖し，やがてお互いに融合，あるいは既存の筋線維と融合して筋線維を再生する．デュシェンヌ型筋ジストロフィー（Duchenne muscular dystrophy；DMD）等の重篤な遺伝性筋疾患に対して筋・幹細胞を移植する再生医療が期待されているが，その確立のためには，筋組織の再生がどのように制御されているかを理解することが重要である．

a. 骨格筋衛星細胞

筋衛星細胞は筋基底膜と筋線維の間にある単核の細胞で1961年にAlexander Mauroによって初めてその存在を記載された．通常，細胞周期のG0の状態にあるが，筋傷害時に活性化され，増殖して筋線維を再生する．生直後は骨格筋組織の中の核の30％程度が筋衛星細胞の核であるが，成体になると5％程度とほぼ一定になる．体幹と四肢の骨格筋の発生学的な起源は沿軸中胚葉由来の体節であり，その中に形成されるdermomyotomeに出現するPax3，Pax7陽性の筋前駆細胞（muscle progenitor cells）が増殖し，やがてMyf5，MyoD等の筋分化制御遺伝子を発現して筋芽細胞（myoblast）となり，次に増殖を止め，融合して，筋線維を形成する．筋衛星細胞はその過程で派生してく

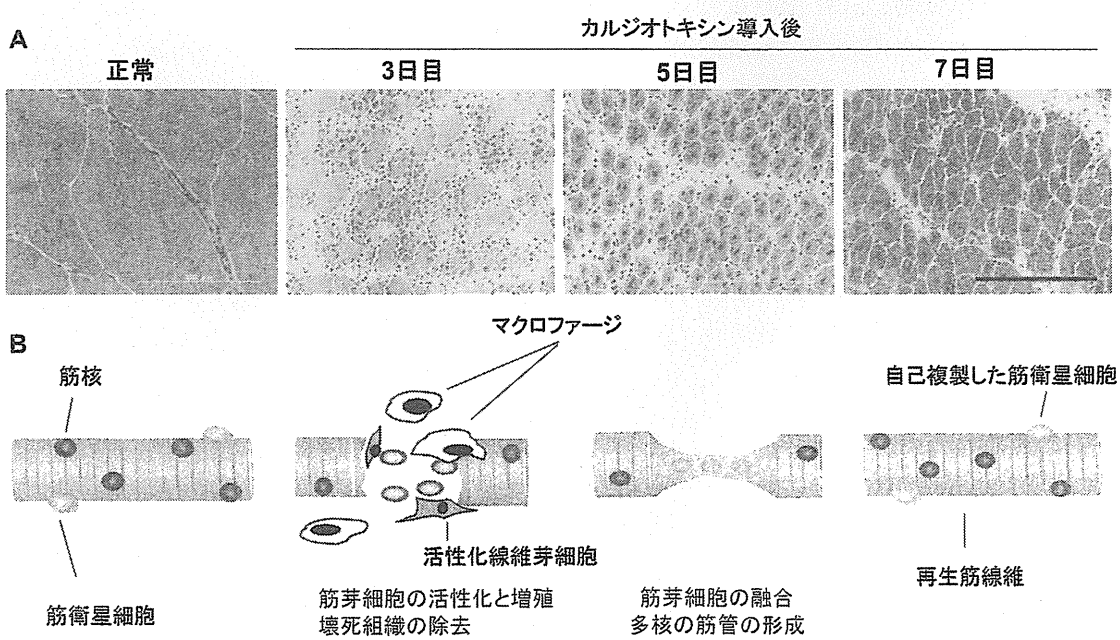

図1 骨格筋の再生

A） C57Bl/6マウス骨格筋に蛇毒であるカルジオトキシンを導入して筋傷害を引き起こした後の組織修復過程を示す．ヘマトキシリン・エオジン染色．スケールバー：200ミクロン．

B） 骨格筋特異的幹細胞である筋衛星細胞は，静止期の状態では筋基底膜と筋線維の間に存在するが，筋傷害時には活性化し，増殖する（筋芽細胞）．やがてお互いに融合し，あるいは既存の筋線維と融合して筋再生が完了する．この過程には好中球やマクロファージ等による壊死組織の貪食機能が重要である．活性化した筋衛星細胞の一部は，元の筋衛星細胞の状態に戻る（自己複製）．

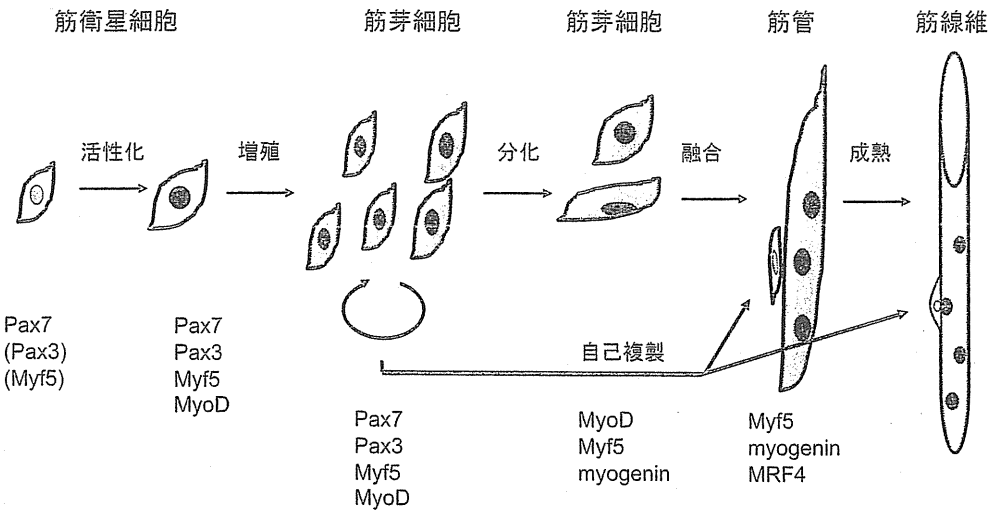

図2　筋衛星細胞の分化過程とその制御因子
筋衛星細胞の筋分化は，発生過程の筋分化と類似しているが，その維持にはPax7が重要な働きを持つ．筋衛星細胞が不均等分裂により自己複製すると考えられているが，そのタイミングや制御分子に関しては不明な点が多い．

る．c-Met，Pax7，Myf5，M-cadherin，CD34等が筋衛星細胞特異的マーカーとして知られているが，昨今の網羅的遺伝子発現研究等の結果，新しいマーカー（カルシトニン・レセプター，odz4等）がリストに加わってきた．筋衛星細胞研究には実験動物の骨格筋から筋衛星細胞を高い純度で分離する方法が有用だが，従来は，線維芽細胞との培養皿への接着性の違いを利用したpreplating法，現在は各種細胞表面マーカーで染色しセル・ソーターで分離する方法が用いられている．筋衛星細胞は自己複製することで，一生涯にわたって筋再生能を維持する（図1，図2）．その機構として不均等分裂が提唱されているが，その分裂様式，制御因子等，不明な点が残されている．

b．筋衛星細胞の活性化，増殖，分化

骨格筋が傷害されるとnitric oxide synthase（NOS）が活性化され，nitric oxide（NO）が産生され，hepatocyte growth factor（HGF；肝細胞増殖因子）を活性化し，c-Metレセプターへ結合する．c-Metを介したシグナルが筋衛星細胞を活性化し，筋衛星細胞は活発に増殖する．筋衛星細胞の増殖能は分裂を繰り返すと徐々に低下する．とくに筋ジストロフィー等の，筋変性・壊死，再生を繰り返す筋疾患では，筋衛星細胞の増殖能は徐々に低下し，筋再生が筋壊死に追いつかなくなり，筋線維が脱落し，筋力が低下していく．筋衛星細胞は筋細胞の他に脂肪細胞，骨細胞にも分化することが報告されているので，筋疾患の進行した段階で認められる脂肪変性や，徐々に筋組織の骨化が進行する進行性骨化性線維異形成症等の遺伝性の疾患の発症に関与する可能性がある．

c．筋・幹細胞と再生医療

1990年代前半，近親者から得た筋衛星細胞を培養後，DMD患者の骨格筋へ移植する筋芽細胞移植が行われたが，その効率は低かった．移植直後に多くの筋芽細胞が死んでしまうこと，移植後筋芽細胞があまり移動しないこと，免疫抑制が不十分であったこと等が原因であったと推察されている．1998年，骨髄細胞が筋線維へ分化し，さらに筋衛星細胞へ分化することが示され[1)]，造血幹細胞の可塑性との関連で，DMDへの治療応用が期待されたが，その筋線維再生への寄与率はわずかであり，またその分化機序は依然不明で，大部分は細胞融合に

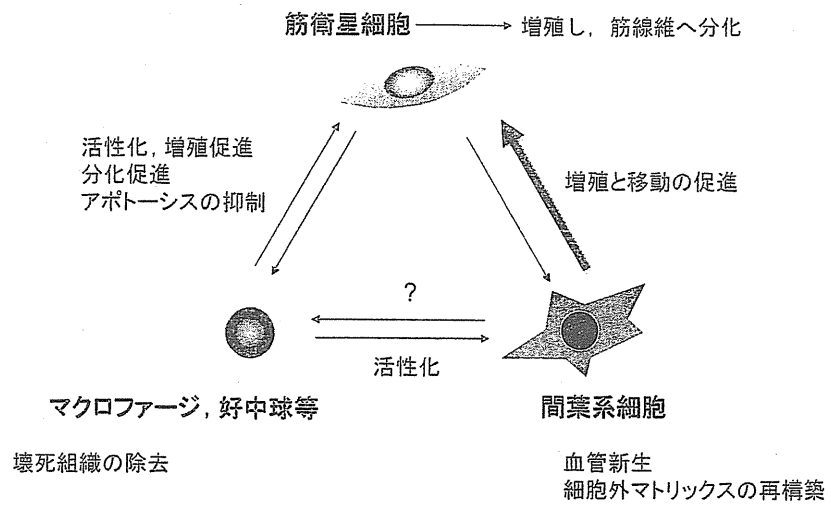

図3　筋再生を制御する細胞とそのネットワーク
筋再生過程では，筋前駆細胞である筋衛星細胞が中心的な役割を果たすが，その他に，壊死組織の除去を担うマクロファージ，好中球等が重要な細胞である．さらに間葉系細胞が間質に存在し，筋傷害時に活性化し，細胞外マトリックスの分解と再構築を促進し，血管新生を制御することで筋再生を制御している．これらの細胞は，直接相互作用する他に，サイトカイン等を介してお互いの活性化，増殖や移動，生存，分化を制御している．

よると思われた．しかし血中の AC133 陽性細胞は移植すると効率よく筋線維に分化するという報告もあり，循環している細胞の中に筋分化能を持つ特別な細胞が存在する可能性は否定できない．一方，骨格筋組織の間質や血管周囲にも，多能性を持ち，筋細胞へも分化する細胞が数多く報告されているが，これらの細胞の相互関係ははっきりしない．それらは，ヘキスト色素を排出する能力に富む side population 細胞（SP cells），血管周囲に存在するペリサイト（pericyte），同じく血管組織に由来するメソアンギオブラスト（mesoangioblast），muscle-derived stem cells，myo-endothelial cells 等である．数量的には筋衛星細胞が筋線維再生に最も寄与していることは広く認められているが，筋変性疾患に対する移植治療という観点では，移植後の生存率が低く，局所にしか生着しない筋衛星細胞に対して，経動脈的，あるいは経静脈的に移植可能なこれらの多能性幹細胞の利用が期待されている[2]．

d．筋再生におけるマクロファージや線維芽細胞の役割

筋再生はさまざまな細胞間の相互作用によって完了する．なかでもとくに重要な細胞はマクロファージと間質の線維芽細胞様の間葉系細胞であろう（図3）．マクロファージは壊死組織の除去の他に，筋衛星細胞の活性化やアポトーシスの抑制，筋分化の促進等の機能があると考えられており[3]，その機能不全で筋再生は障害される．間葉系細胞も筋再生時に活性化され，増殖し，MMPs 等のプロテアーゼを分泌し，細胞移動の促進，細胞外マトリックスの分解・再構築，血管新生，各種成長因子の活性化に関わっている．また，各種ケモカインを分泌しており，炎症細胞，免疫担当細胞の制御にも関与していると思われる．間葉系細胞は in vitro でも脂肪細胞へ分化しやすい傾向を持ち，この細胞の機能低下や異常な活性化が，筋再生の遅延，筋組織の線維化，脂肪変性に関わっていると考えられるので，再生医療の良き標的である．

［鈴木友子，武田伸一］

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Long-term Engraftment of Multipotent Mesenchymal Stromal Cells That Differentiate to Form Myogenic Cells in Dogs With Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an incurable genetic disease with early mortality. Multipotent mesenchymal stromal cells (MSCs) are of interest because of their ability to differentiate to form myogenic cells *in situ*. In the present study, methods were developed to expand cultures of MSCs and to promote the myogenic differentiation of these cells, which were then used in a new approach for the treatment of DMD. MSC cultures enriched in CD271$^+$ cells grew better than CD271-depleted cultures. The transduction of CD271$^+$ MSCs with MyoD caused myogenic differentiation *in vitro* and the formation of myotubes expressing late myogenic markers. CD271$^+$ MSCs in the myogenic cell lineage transplanted into dog leukocyte antigen (DLA)-identical dogs formed clusters of muscle-like tissue. Intra-arterial injection of the CD271$^+$ MSCs resulted in engraftment at the site of the cardiotoxin (CTX)-injured muscle. Dogs affected by X-linked muscular dystrophy in Japan (CXMD$_J$) treated with an intramuscular injection of CD271$^+$ MSCs similarly developed muscle-like tissue within 8–12 weeks in the absence of immunosuppression. In the newly formed tissues, developmental myosin heavy chain (dMyHC) and dystrophin were upregulated. These findings demonstrate that a cell transplantation strategy using CD271$^+$ MSCs may offer a promising treatment approach for patients with DMD.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disease in which mutations in the gene encoding the cytoskeletal protein dystrophin result in a deficiency of the dystrophin–glycoprotein complex of the sarcolemma.[1,2] The deficiency causes the progressive degeneration of striated muscle, manifesting as muscle weakness and eventual skeletal muscle atrophy.[3,4]

Cell-based therapy for DMD has the potential to restore dystrophin expression and therefore also the muscle parenchyma. The transplantation of normal stem cells, such as hematopoietic stem cells, myoblasts, and muscle-derived stem cells, has been examined as a possible treatment strategy for DMD and as a system to deliver therapeutic recombinant proteins to target muscle tissues.[5–7] Indeed, the intramuscular or intravenous injection of these cells was shown to improve the disease phenotype in the *mdx* mouse model.[8]

Bone marrow stem cells have been shown to participate in the regeneration of injured muscle.[9,10] Bone marrow mesenchymal stem cells (MSCs) are conventionally defined as adherent nonhematopoietic (NH) cells expressing several common cell-surface antigenic markers, such as CD44, CD73, CD90, and CD105, but not the hematopoietic markers CD34 and CD45.[11] After treatment with inducing agents,[12] MSCs can differentiate *in vitro* into adipocytes, chondrocytes, and osteocytes, but also into neurons, hepatocytes, pancreatic islets, and muscle.[13] In *mdx* mice, transplanted adult human MSCs were incorporated into myofibers with the subsequent restoration of dystrophin expression.[14–16] However, no therapeutic strategy using MSCs has proven successful for models of DMD in mid-sized animals, such as the dog. Moreover, while successful cell therapy in canine muscular dystrophy has been demonstrated using heterologous mesoangioblasts,[17] the development of an analogous approach for clinical use in humans has been hindered by the inability to overcome several obstacles, including poor cell survival rates, limited dissemination of injected cells, immune responses to allogeneic cells, the presence of the neotransgene product in dystrophic muscle, and the inability to specifically target the cells, such as to cardiac tissue.[18]

Clinical interest in MSCs in cell therapy applications is based on their anti-inflammatory properties and ability to release cytokines into the surrounding environment, thereby modifying the developmental fate of neighboring cells. Thus, not only can MSCs themselves be induced to differentiate along, *e.g.*, a myogenic pathway, in which they fuse with myotubes and promote the formation of new muscle fibers,[16] but in skeletal muscle, for

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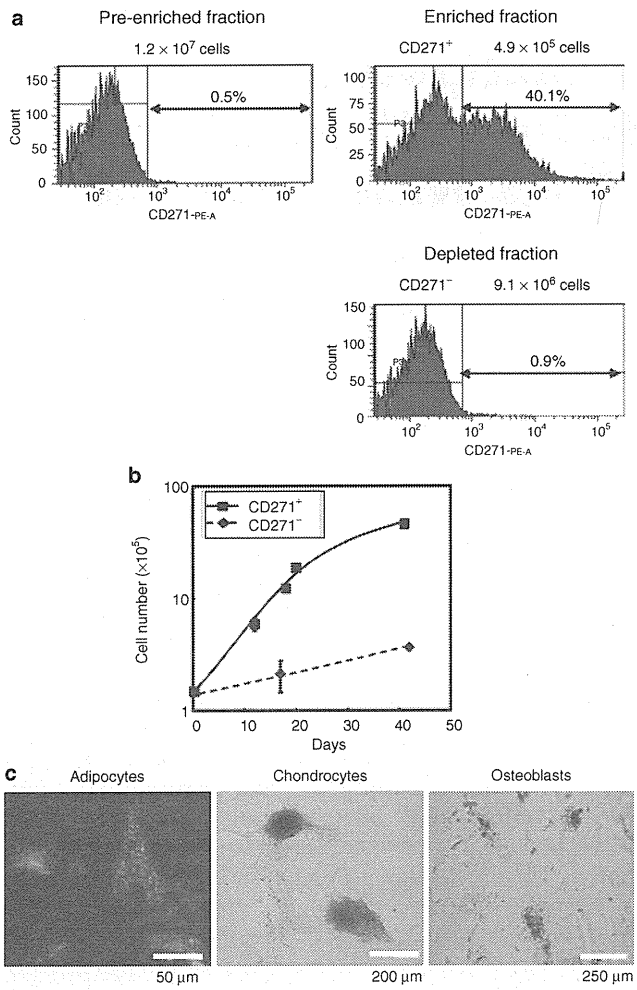

Figure 1 Isolation and expansion of canine CD271$^+$ mesenchymal stromal cells (MSCs) using immunomagnetic isolation. (**a**) CD271$^+$ cells from bone marrow mononuclear cells were enriched by immunomagnetic isolation using CD271 microbeads. Flow cytometric analysis of the pre-enriched cells and the CD271-enriched (+) and -depleted (−) fractions after separation. The percentages of CD271$^+$ cells are indicated. (**b**) Growth curve of CD271$^+$ and CD271$^-$ cells derived from wild-type dogs. (**c**) The differentiation of CD271$^+$ MSCs obtained from wild-type dogs into adipocytes, chondrocytes, and osteoblasts. Differentiation was confirmed *in vitro* using Nile red staining after culture in adipogenic medium (left panel), toluidine blue staining after chondrogenic induction (middle panel), and alkaline phosphatase activity measurements after osteogenic induction (right panel).

example, they can induce the myogenic differentiation of neighboring satellite cells in the interstitial tissue.[19] Nonetheless, the transplantation of cells already induced to differentiate along the pathway of interest would seem to offer a more direct and readily controllable therapeutic approach than conventional strategies that rely on the innate properties of MSCs to target undifferentiated cell populations. Here, we demonstrate that CD271-enriched (+) MSCs isolated from bone marrow showed improved growth expansion in culture and, as evidenced by MyoD expression, myogenic conversion. CD271 is a marker of progenitor cells and a specific marker for bone-marrow–derived MSCs.[20] Our study describes the development of CD271$^+$ MSC expanded cultures, the myogenic differentiation of these cells, and the methods used to achieve their delivery and effective cell transplantation. Successful engraftment was achieved with CD271-enriched (+) MSCs injected by either the intramuscular or the systemic, intra-arterial route in littermate donor-recipient pairs of dogs with dog leukocyte antigen (DLA) identity[21] (wild-type dogs) as well as in a Beagle with canine X-linked muscular dystrophy in Japan ($CXMD_J$),[22] in which a splice-acceptor site mutation in intron 6 of the *DMD* gene leads to the failed expression of dystrophin.[23] This is the first report of a protocol for the preparation of dog MSCs and the successful long-term engraftment of CD271$^+$ MSCs with a myogenic lineage. This cell-based therapeutic strategy merits further investigation for clinical use in DMD patients.

RESULTS

Isolation and characterization of CD271$^+$ MSCs from canine bone marrow

Canine bone marrow cells were isolated from biopsy specimens from donor Beagle dogs. Since bone marrow MSCs are difficult to expand in culture, they were enriched for CD271$^+$ cells using an immunomagnetic column system (**Figure 1a**) to obtain a subpopulation of cells with better growth potential, which are thus more readily expandable for use in transplantation (**Figure 1b**). Both CD271$^+$ and CD271$^-$ MSCs demonstrated a fibroblast-like morphology after monolayer growth expansion *in vitro* (data not shown). Flow cytometry analysis showed that CD44$^+$ and CD90$^+$ cells were present in the CD271$^+$ fraction, whereas CD45$^+$ cells were limited to the CD271$^-$ fraction (**Supplementary Figure S1a**). We then tested whether CD271$^+$ MSCs were able to differentiate into adipogenic, chondrogenic, and osteogenic cell lineages (**Figure 1c**). Adipogenesis was evidenced by the accumulation of neutral lipid vacuoles that stained with Nile red. Characteristic morphological changes and toluidine blue staining were indicative of chondrogenesis. Osteogenesis was confirmed by an increase in alkaline phosphatase activity. Whereas the CD271 expression on MSCs decreased after 4 weeks in culture (**Supplementary Figure S1b**), these cells retained their ability to differentiate into adipocytes, chondrocytes, and osteoblasts. Similar results were also obtained for dystrophic CD271$^+$ MSCs (**Supplementary Figure S2**), which suggested that CD271-enriched canine MSCs are capable of growth expansion as well as differentiation into multiple cell lineages.

MyoD-mediated myogenic differentiation of canine CD271$^+$ MSCs

CD271$^+$ MSCs from wild-type or DMD dogs were tested for myogenic conversion following their *in vitro* transduction with MyoD, which was transiently expressed from an adenovirus vector (Ad-MyoD). Efficient adenovirus infection requires coxsackie-adenovirus receptor and α(v) integrin. The efficiency of adenovirus-mediated transgene expression[24] is improved by increasing coxsackie-adenovirus receptor expression, which is achieved by treating the transduced cells with the histone deacetylase inhibitor FK228 24 hours before infection.[24] As shown in **Figure 2a**, this resulted in a dose-dependent enhancement of infection, as confirmed in CD271$^+$ MSCs transduced with an adenovirus vector expressing enhanced green fluorescent

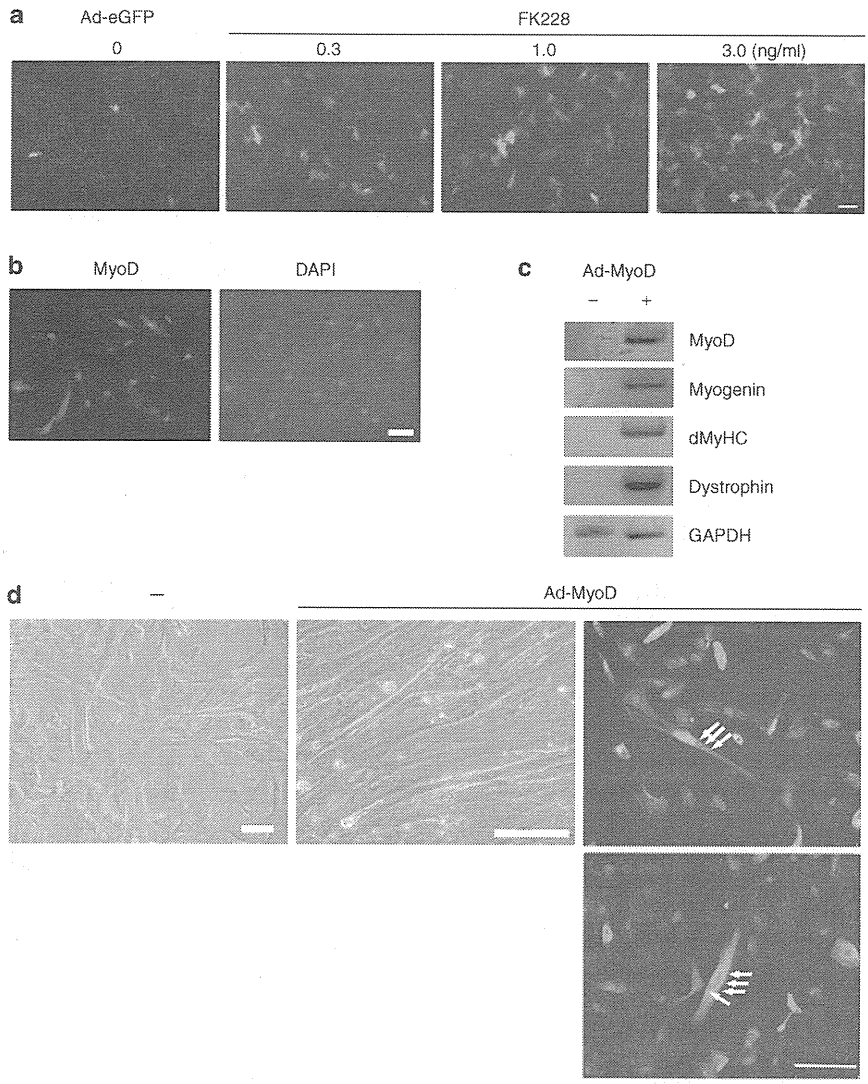

Figure 2 MyoD induces the myogenic differentiation of CD271$^+$ mesenchymal stromal cells (MSCs). (**a**) Canine CD271$^+$ MSCs were pretreated with FK228 (0.3, 1.0, or 3.0 ng/ml) and then transduced with adenovirus vector expressing enhanced green fluorescent protein (Ad-eGFP). After 7 days of incubation, eGFP$^+$ cells were detected. Bar = 200 µm. (**b**) CD271$^+$ MSCs were transduced with Ad-MyoD (multiplicity of infection (MOI) = 50), cultured for 3 days and examined for immunofluorescence after staining for MyoD (red); nuclei of the corresponding cells were labeled with 4′, 6′-diamidino-2-phenylindole (DAPI). Bar = 100 µm. (**c**) CD271$^+$ MSCs transduced with control Ad-null or Ad-MyoD were cultured for 14 days and then analyzed by reverse transcriptase (RT)-PCR using primer-specific MyoD, myogenin, developmental myosin heavy chain (dMyHC), dystrophin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**d**) Bright-field images show control Ad-null (left panel) or Ad-MyoD-treated wild-type CD271$^+$ MSCs (middle panel). Images of cells stained for α-actinin immunofluorescence (green) were compared with those of DAPI-stained nuclei (blue) from the same microscopic field (right panels, arrow). Bar = 100 µm.

protein (Ad-eGFP). Indeed, 2 days after Ad-MyoD transduction of CD271$^+$ MSCs in the presence of FK228, >90% of the CD271$^+$ MSCs expressed MyoD in the nucleus, as shown with an anti-MyoD antibody (**Figure 2b**), whereas no MyoD signal was observed in the cells transduced with the control Ad-eGFP vector (data not shown). Several markers of myogenesis, such as myogenin, developmental myosin heavy chain (dMyHC) and dystrophin, were detected in the MyoD-transduced CD271$^+$ MSCs using reverse transcriptase-PCR (**Figure 2c**). After 14 days of culture in differentiation medium, multinucleated myotube formation was noted. These results demonstrated that Ad-MyoD–transduced CD271$^+$ MSCs had undergone myogenic differentiation (**Figure 2d**), suggesting that MyoD is able to initiate myogenesis of CD271$^+$ MSCs.

Transplantation of CD271$^+$ MSCs into DLA-identical dogs by intramuscular injection

To test whether the MyoD-transduced CD271$^+$ MSCs contributed to muscle regeneration, allogeneic CD271$^+$ MSCs were transplanted into DLA-identical donor-recipient pairs of dogs, described in **Table 1**. The donor was a wild-type Beagle and the recipients were wild-type or DMD phenotype CXMD$_J$ littermates. Transplantation was carried out as follows: DLA-identical wild-type dogs were allogeneically transplanted with wild-type CD271$^+$

Table 1 Summary of transplantation experiments

Dog ID	Sex	Age[a]	BW[b]	Transgene	Route[c]	Muscle	Cell number	Engraftment of MSCs	Immunosuppression (mg/kg/day)	Experimental period
3403MN	M	2	7.6	GFP	i.m.	ECU	1×10^6	+	–	10 days
3404MN	M	5	8.5	GFP	i.m.	TA, ECR	2×10^6, 1×10^6	+	–	10 days
3403MN	M	6	9.5	Luciferase	i.m.	TA	2×10^6	+	MMF (30), CSP (25)	4 weeks
3407MA	M	6	8.7	GFP	i.m.	ECR	2×10^6	+	CSP (25)[d]	4 weeks
3407MA	M	9	10.4	Luciferase	i.m.	TA	$4 \times 10^6 \times 2$	++	–	12 weeks
4203FC	F	0.5	0.5	GFP	i.a.	TA	2×10^6	–	–	1 week
3405MN	M	16	11.0	GFP	i.a.	TA	5×10^6	+	MMF (10), CSP (8.3)	4 weeks
5606MN	M	2	4.0	GFP	i.a.	TA	1×10^6	++	MMF (10), CSP (8.3)	8 weeks

Abbreviations: BW, body weight; CSP, cyclosporine; F, female; GFP, green fluorescent protein; M, male; MMF, mycophenolate mofetil; MSC, mesenchymal stromal cell. [a]Age at injection (months). [b]BW at injection (kg). [c]MSCs were administered by intramuscular (i.m.) or intra-arterial (i.a.) injection; the latter was preceded by transient avascularization. MSC-positive fibers: –, not detected; ±, <10; +, >10; ++, >100. [d]Before transplantation, cyclosporine administration was terminated because of the side effects.

MSCs transduced with MyoD and luciferase into the cardiotoxin (CTX)-injured tibialis anterior (TA) muscle (2×10^6 cells/injection). Eight weeks after transplantation, CD271$^+$ MSC clusters were observed at the injection site of the muscle, as determined using fluorescence microscopy and anti-luciferase antibody. In addition, CD271$^+$ MSCs gave rise to dystrophin-positive fibers (21/4,872 fibers, 0.4%) in the CTX-injured TA muscle (**Figure 3**).

Widespread engraftment of CD271$^+$ MSCs by intra-arterial injection

Next, we examined whether systemic arterial injection of CD271$^+$ MSCs resulted in their ultimate distribution in muscle. As described in **Materials and Methods**, the dogs used in these experiments were administered immunosuppressants, which did not cause any side effects either during or after treatment. The TA muscles of three wild-type dogs were treated with CTX. Five days later, 5×10^8 CD271$^+$ MSCs transduced with MyoD and eGFP were injected into the dogs' femoral artery. For more effective cell engraftment, prior to the injection the artery was treated with papaverine, a clinically used blood vessel relaxant that increases blood flow to the heart and throughout the body. Eight weeks after the transplantation, eGFP$^+$ myofibers (7/216 fibers, 3.2%) derived from the donor were distributed in the TA muscle (**Figure 4a**) and in the heart (**Supplementary Figure S3**). Most of these eGFP$^+$ fibers expressed dMyHC, which is normally transiently re-expressed in regenerated muscle after injury or in response to disorders involving skeletal muscle.[25] This result suggested that the muscle fibers had regenerated following injury. PCR analysis of *eGFP* gene expression in various dog tissues detected donor cells at the injected hindlimb, the right TA and right extensor digitorum longus muscles, and in the heart, but not in the diaphragm, liver, kidney, or lung (**Figure 4b**). In addition, eGFP expression was higher in dogs whose femoral arteries were pretreated with papaverine hydrochloride than in dogs not treated with the drug. Neither ectopic tissue formation nor tumor development occurred after transplantation. There was no increase in acute-phase C-reactive protein levels in the recipient dogs during the experiments, suggesting the absence of a severe inflammatory response (data not shown). Other biochemical tests confirmed normal liver and kidney function and that there was no reaction to the intra-arterial injection of CD271$^+$ MSCs (data not shown). These

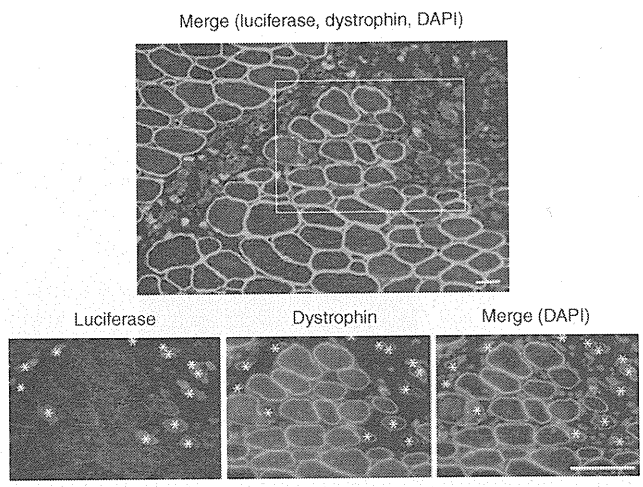

Figure 3 Engraftment of CD271$^+$ mesenchymal stromal cells (MSCs) after intramuscular injection into dog leukocyte antigen (DLA)-identical dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dogs were transduced with Ad-MyoD and RV-Luc and then injected into cardiotoxin (CTX)-treated tibialis anterior (TA) muscle. Eight weeks after transplantation, the muscles were biopsied. Microscopy cross-sections of the muscles were prepared and then analyzed by immunofluorescence staining with luciferase (red), dystrophin (green), and 4′, 6′-diamidino-2-phenylindole (DAPI) (blue), which revealed luciferase and dystrophin double-positive muscle fibers (arrow and *). High-magnification images (lower panels) were from the boxed region in the upper panel.

results indicate that the intra-arterially injected CD271$^+$ MSCs are capable of long-term survival and widespread tissue engraftment, without adverse consequences for the transplanted animal. Furthermore, successful engraftment of CD271$^+$ MSCs in DLA-identical dogs was achieved not only by the intramuscular but also by the intra-arterial route of injection.

Long-term engraftment and myogenic lineage of CD271$^+$ MSCs in dystrophic dogs without immunosuppression

Based on the encouraging results obtained in wild-type dogs, we asked whether the same results, i.e., MyoD expression, could be obtained in DMD dogs. In addition, due to the well-known side

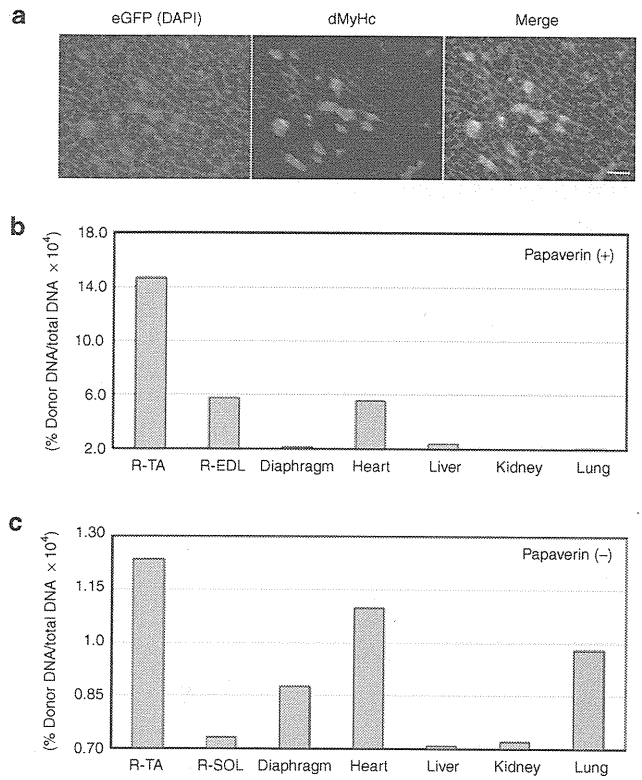

Figure 4 Survival and distribution of CD271$^+$ mesenchymal stromal cells (MSCs) after intra-arterial injection in dog leukocyte antigen (DLA)-identical dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dogs were transduced with Ad-MyoD and a retroviral vector expressing enhanced green fluorescent protein (eGFP). The femoral artery was transiently avascularized using a tourniquet and then injected with the transduced CD271$^+$ MSCs. (**a**) Cryosections from recipient cardiotoxin (CTX)-treated tibialis anterior (TA) muscle 8 weeks after injection with CD271$^+$ MSCs were stained using antibodies specific for eGFP (red) or developmental myosin heavy chain (dMyHC) (green), and the nuclear stain 4′, 6′-diamidino-2-phenylindole (DAPI) (blue). Bar = 100 μm. (**b**) Two independent experiments were performed in DLA-identical dogs. Cell-transplanted right tibialis anterior (TA) muscle (R-TA), right extensor digitorum longus muscle (R-EDL), right soleus muscle (R-SOL), diaphragm, heart, liver, kidney, and lung were analyzed by reverse transcriptase (RT)-PCR using primers specific for eGFP.

effects associated with the use of immunosuppressants (cyclosporine and mycophenolate mofetil), we decided to transplant the cells into DMD dogs without immunosuppression. CD271$^+$ MSCs transduced with luciferase and MyoD (4×10^6 cells) were injected into the TA muscles of $CXMD_J$ in the absence of immunosuppression. Eight weeks later, numerous clusters of luciferase-positive cells (188/9,408 fibers, 2.0%) with several centrally located nuclei were detected in the TA muscle by immunohistochemistry (**Figure 5a**). The mean diameters of the luciferase-positive cells (8 weeks after transplantation, 33.6 ± 10.7 μm; 12 weeks after transplantation, 47.6 ± 12.0 μm) were smaller than those of the untreated muscle fibers (95.4 ± 23.3 μm). Twelve weeks later, the diameters of the luciferase-positive cells had increased and were larger than observed 8 weeks after transplantation (**Figure 5b**).

To identify whether the smaller cells found in the treated muscles were of a myogenic lineage, dMyHC expression was analyzed. Merged images showed that the luciferase-positive fibers (95% positivity) also expressed the skeletal muscle isoform of dMyHC (**Figure 5a**). Reverse transcriptase-PCR analysis and immunohistochemical experiments demonstrated the expression of myogenin, dMyHC, and dystrophin (**Figure 5c**). Dystrophin expression was also confirmed in wild-type donor-derived cells at the engrafted site, although the number of dystrophin-positive cells was relatively small (**Figure 5d**).

Hematoxylin and eosin staining of the CD271$^+$ MSCs-injected muscles showed cellular infiltrates around degenerating fibers (**Figure 5a**). The infiltrates were also detected using antibodies directed against CD8 and CD11b, indicative of lymphocytes and macrophages, respectively. As shown in **Figure 5e**, small CD8$^+$ and CD11$^+$ T cells accumulated close to the luciferase-positive myofibers. Twelve weeks after engraftment, these accumulations decreased in size compared to those observed in muscle biopsied 8 weeks after transplantation (**Supplementary Figure S4**). Immunoblot analysis failed to detect anti-dystrophin antibodies (**Supplementary Figure S5** and **Supplementary Materials and Methods**), implying that they were not produced in the recipient dogs. Dog sera were also tested for the presence of neutralizing antibody to adenovirus type 5.[26] Transplanted dogs initially negative for neutralizing antibody levels remained negative, with titers <1:4. Mean serum creatine kinase, and C-reactive protein also did not increase during the experiment.

These results demonstrated that transplanted CD271$^+$ MSCs expressing MyoD were converted to myogenic cells and fused with existing muscle fibers, and then formed myotubes in muscle tissue. Moreover, engraftment, differentiation, and the long-term survival of myogenic cells were accomplished by the transplantation of CD271$^+$ MSCs into dystrophic skeletal muscle in the absence of immunosuppression.

DISCUSSION

To develop a new strategy for the cell-based therapy of DMD, we examined methods for the *in vitro* expansion and myogenic differentiation of CD271$^+$ MSCs, using these cells in the allogeneic transplantation of dogs. The enrichment procedure yielded a large population of canine CD271$^+$ MSCs that could be induced to differentiate into myogenic cells following the transient expression of MyoD. Long-term engraftment of CD271$^+$ MSCs within muscle tissues was successfully accomplished by either intramuscular or intra-arterial injection of myogenic cells into DLA-identical dogs.

The effective therapeutic use of MSCs requires their relatively simple large-scale production and a method to induce their differentiation along a myogenic lineage. CD271 provides a marker for the enrichment of non-HSCs from bone marrow aspirates.[20,26] Canine MSCs were obtained from the bone marrow of wild-type or $CXMD_J$ in amounts sufficient for transplantation experiments by enriching the subpopulation of CD271$^+$ cells, as previously reported for humans,[27] under conditions in which the capacity of these cells for multipotent differentiation was maintained. We hypothesized that the CD271-enriched fraction of MSCs would promote allogeneic as well as autologous cells transplantation. CD271 is also known as the low-affinity nerve growth factor receptor/p75, which plays a key role in satellite cell function.[28] Nerve growth factor acts through its low-affinity CD271

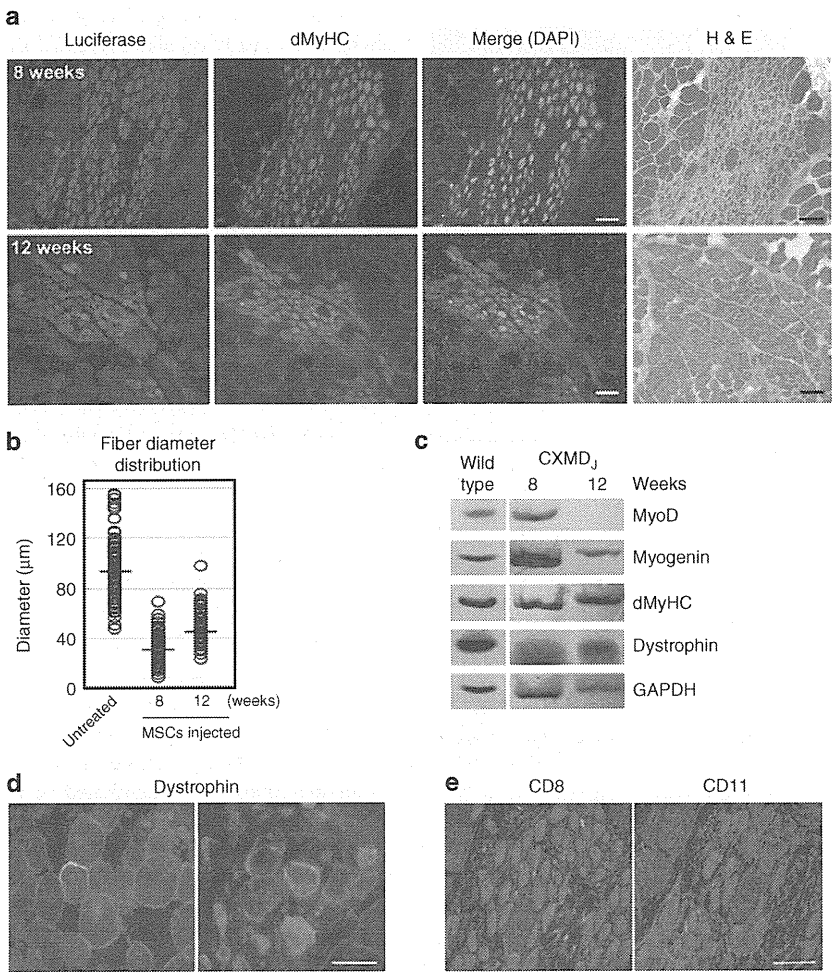

Figure 5 Successful long-term engraftment and myogenic differentiation of CD271$^+$ mesenchymal stromal cells (MSCs) in dog leukocyte antigen (DLA)-identical dystrophic dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dog were transduced with Ad-MyoD and RV-Luc and then injected into the left and right tibialis anterior (TA) muscles of the nonimmunosuppressed recipient CXMD$_J$ dog. (**a**) Cryosections from the recipient muscle at 8 and 12 weeks after CD271$^+$ MSCs injection were stained using antibodies specific for luciferase (red) and the late myogenic marker developmental myosin heavy chain (dMyHC) (green), or the nuclear stain 4′, 6′-diamidino-2-phenylindole (DAPI) (blue); the images were then merged. Hematoxylin and eosin (H&E) staining of the same field is shown for comparison (right panels). Bar = 100 μm. (**b**) The mean values ± SD of the diameters of CD271$^+$ MSC$^+$ fibers in the recipient muscle 8 and 12 weeks after injection. (**c**) Cell-transplanted muscles were analyzed by reverse transcriptase (RT)-PCR using primers specific for MyoD, myogenin, dMyHC, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and dystrophin. (**d**) Immunofluorescence staining of dystrophin (red) and corresponding DAPI staining are shown. (**e**) Merged images of cells stained for luciferase (red), CD8$^+$ or CD11$^+$ (green), and DAPI (blue) 8 weeks after transplantation.

receptor to induce a developmentally regulated signaling pathway necessary for myogenic differentiation and muscle repair *in vivo*. Therefore, it was presumed that CD271 would enhance the *in vivo* myogenic differentiation of MSCs to form muscle fibers.

Recombinant adenovirus transduction of MSCs usually occurs with low efficiency, which limits its utility. Therefore, improved adenovirus-mediated transduction *in vitro* was achieved by pretreating CD271$^+$ MSCs with the histone deacetylase inhibitor (FK228),[29] which resulted in the efficient expression of MyoD and, subsequently, myogenic differentiation of the cells.

Other agents that induce the differentiation of MSCs, such as 5-azacytidine[30] and Notch I intracellular domain,[16] are known to be injurious to primary cultured cells. Here, we showed that MyoD expression triggers the myogenic differentiation of CD271$^+$ MSCs. These cells maintained their multipotent characteristics, including their myogenic potential, and were successfully used for transplantation in dogs. Furthermore, MyoD transduction of CD271$^+$ MSCs was shown to cause their myogenic differentiation with the induced expression of the late myogenic markers myogenin, dMyHC and dystrophin, although multinucleated myotube formation was less efficient. These results demonstrate that MyoD expression is able to trigger myogenic differentiation of CD271$^+$ MSCs and that, after cell transplantation in muscle tissue, MyoD-transduced MSCs fuse with myoblasts to form the final differentiated myofibers.

MyoD has also been shown to trigger myogenic conversion in other cell types.[31] For example, telomerase gene transfer was used to transduce MyoD into immortalized fibroblasts, which were then engrafted into regenerating muscle, demonstrating that dystrophin rescue is achieved *ex vivo* as well as *in vivo*.[32] However,

the potential for ectopic tissue formation and cell tumorigenicity remains a concern.

To avoid immune-mediated cell rejection, DLA-identical dogs were transplanted with allogeneic CD271$^+$ MSCs. These MyoD-transduced CD271$^+$ MSCs were able to efficiently engraft and then develop along a myogenic cell lineage in the muscle tissue of the recipient dog. The use of allogeneic cell types generally requires long-term immunosuppression to prevent immune-mediated rejection of the transplanted cells.[33,34] However, in this study, the long-term engraftment of CD271$^+$ MSCs was successful whether or not the DLA-identical dogs were treated with immunosuppressants after transplantation. Although immunohistochemistry revealed several inflammatory infiltrates, mainly containing macrophages, in the recipient muscle of the CXMD$_J$, the majority of CD271$^+$ MSCs migrated into only transient, mild inflammatory foci. The infiltrations were observed at 8 weeks after transplantation but were less abundant after 12 weeks. Our results contrast with those obtained following the transplantation of heterologous mesoangioblasts in golden retrievers with muscular dystrophy, in which the use of immunosuppressants, such as cyclosporine and rapamycin, was required for dystrophin expression.[17] In another study, the intramuscular injection of bone marrow cells in littermate recipients rendered tolerant to the cells did not lead to the production of new myofibers.[35]

The successful induction of dystrophin expression achieved in our experiments may have been due to the anti-inflammatory and immunosuppressive properties of MSCs, which inhibit T-cell proliferation. Mechanistically, MSCs are known to inhibit tumor necrosis factor-α secretion and to promote interleukin-10 secretion, which together may affect the maturation state and functional properties of dendritic cells and skew the immune response towards an anti-inflammatory/tolerant phenotype.[36] Alternatively, the presence of MSCs in an inflammatory microenvironment inhibits interferon-γ secretion from T_H1 and NK cells and increases interleukin-4 secretion from T_H2 cells, thereby promoting a T_H1–T_H2 shift and thus an increase in the proportion of regulatory T-cells. However, once MSCs differentiate into skeletal muscle, they may lose their immunosuppressive properties. Nonetheless, the differentiated cells might be invisible to the host's immune system because of their low-level expression of HLA class I. This would allow the long-term engraftment of CD271$^+$ MSCs without rejection by the DLA-identical CXMD$_J$ recipient following immunosuppressant-free transplantation.

The safety and utility of MSCs underlines their potential clinical benefits. Indeed, the infusion of MSCs has been shown to relieve immune-mediated complications, as reported in patients with severe acute graft versus host disease (GVHD).[37] However, in a phase III trial, a single infusion of MSCs at the time of transplantation did not prevent the development of GVHD in major histocompatibility complex-mismatched donor-recipient pairs. There was also no significant difference in the incidence of GVHD between patients receiving MSCs and the controls.[38] Therefore, further studies aimed at improving the immunosuppressive function of MSCs are needed. In a combined approach, a DMD patient was treated with MSC-like endometrial regenerative cells, CD34 umbilical cord blood, and mixed lymphocyte reaction-matched positive cells, which resulted in improvements in muscular strength and clinical respiratory function.[19] Although these improvements were ascribed to the anti-inflammatory properties of MSCs, our cell transplantation strategy with subsequent myogenic differentiation offers a more direct form of therapy and thus may well be more effective.

In the transplanted CXMD$_J$, most of the CD271$^+$ MSC-derived muscle fibers expressed dMyHC and myogenin. Indeed, MSCs can fuse with dystrophic muscle and produce trophic factors that augment the activity of endogenous myosatellite cells.[19,39] Because myogenin is deeply associated with myotube differentiation, our result suggested that the engrafted cells were committed to differentiate, and some of them were even dystrophin-positive. The infiltration of CD8$^+$ and CD11$^+$ T cells might explain the low number of dystrophin-positive fibers; however, it may be that dystrophin expression and fiber formation by MSCs-derived myotubes is a long-term event. Indeed, dystrophin expression was induced in MSC-like dental pulp stem cells one year after their transplantation in golden retrievers with muscular dystrophy littermates, whereas only a small number of fibers were detected 3 months after transplantation.[40] Thus, our method requires continued studies to determine the efficiency of post-transplantation dystrophin expression by MyoD-transduced CD271$^+$ MSCs. Future studies might also include the modification of the immunosuppressive function of MSCs to further improve engraftment efficiency and differentiation.

The low efficiency of widespread engraftment and differentiation of intramuscularly injected cells is clearly inadequate for the effective treatment of large muscles. To overcome this problem, we devised a strategy in which the intra-arterial injection of cells allowed the widespread engraftment of the CD271$^+$ MSCs in CTX-injured muscle and in the heart. It therefore seems likely that MSCs selectively accumulate in injured muscles,[41] perhaps in response to various chemokine signals, such as stromal cell-derived factor 1 or macrophage/monocyte chemotactic protein-3, both of which are associated with the migration of MSCs into injured tissues.[42] To our knowledge, this is the first report demonstrating the potential for systemic delivery of MSCs to injured tissues in dogs.

In conclusion, the work described herein offers a method to isolate and expand canine bone-marrow–derived CD271$^+$ MSCs *in vitro*. Since MSCs have anti-inflammatory properties, long-term engraftment in dog muscle was facilitated by the intramuscular or intra-arterial injection of CD271$^+$ MSCs into DLA-identical dogs, which obviated the need for immunosuppression. Finally, MyoD expression was able to direct the myogenic differentiation of CD271$^+$ MSCs in dog muscle. Further development of this approach is needed to obtain fully differentiated adult muscle cells from undifferentiated fetal cells.

In addition to their ability to regenerate muscle tissue, MSCs can be used to produce vectors for efficient gene delivery to target tissues.[43,44] For example, MSCs producing recombinant adeno-associated virus (rAAV) can effectively express the gene products at sites of inflammation. In fact, it was also reported that rAAV2 or rAAV6 delivery to the striated muscles of a nonimmunosuppressed canine recipient resulted in insufficient transgene expression but strong immune responses,[45] although Gregorevic *et al.* reported that the administration of rAAV carrying the microdystrophin

gene restored specific muscle force and extended the lifespan of dystrophic mice.[46] Also, dystrophin-specific T cells were detected after the treatment of DMD patients with functional rAAV that encoded dystrophin.[47] MSCs producing rAAV-microdystrophin would be beneficial in autologous cell transplantation for DMD treatment if the immune reactions were inhibited by specifically using immunosuppressive CD271$^+$ MSCs for transduction.

MATERIALS AND METHODS

Production and vector transduction. The canine MyoD genome or eGFP complementary DNA was inserted into the adenoviral vector AVC2.null DNA-protein complex through homologous recombination using an In-Fusion Dry-Down PCR cloning kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.[48] The resultant recombinant adenovirus vectors (Ad-cMyoD, Ad-eGFP, and Ad-null) were propagated in 293 cells and purified by two-tier CsCl gradient centrifugation. Viral titers were determined based on the 50% tissue culture infective dose ($TCID_{50}$). To generate the vesicular stomatitis virus-glycoprotein (VSV-G)-pseudotyped retroviral vector encoding firefly luciferase (RV-Luc) or eGFP (RV-eGFP), HEK-293 cells were transduced with pDNLuc or pDNeGFP, pGag-pol[49] and pVSV-G.[49] The culture supernatant was collected 48 hours later and centrifuged to enrich the recombinant retrovirus, which was further purified using an ion-exchange procedure.[44] MSCs were transduced with RV-eGFP or RV-Luc and Ad-cMyoD or control Ad-null at a multiplicity of infection of 10 or 50.

Animals. All animals were housed in the National Center of Neurology and Psychiatry (Tokyo, Japan). DLA-identical littermate donor/recipient pairs (n = 6) were matched at DLA class II (DRB-1, DQA-1, DQB-1). Male and female wild-type dogs, 2–16-months-old, were obtained from the Beagle-based $CXMD_J$ breeding colony at the National Center of Neurology and Psychiatry (Tokyo, Japan) and used as recipients or donors for cell transplantation. Age-matched $CXMD_J$ were used as donors for cell transplantation. All of the animals were cared for and treated in accordance with the guidelines approved by the Ethics Committee for the Treatment of Laboratory Animals at National Center of Neurology and Psychiatry which has adopted the three fundamental principles of replacement, reduction, and refinement.

Bone marrow aspiration and isolation of canine MSCs. Donor wild-type dogs were anesthetized with thiopental and isoflurane, and then 1.0 ml of bone marrow fluid was obtained from each humerus by aspiration with a syringe containing 2 ml of RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) with 16 mmol EDTA-2Na. Mononuclear cells were isolated by density centrifugation using Histopaque-1077 (Sigma-Aldrich, St Louis, MO). CD271$^+$ MSCs were enriched and cultivated by using the MSC Research Tool Box-CD271 (LNGFR) containing CD271 (LNGFR)-PE and Anti-PE Micro Beads for cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany). The percentage of CD271$^+$ cells in the enriched and depleted cell fractions was assessed by flow cytometry (FACS Canto II; BD Biosciences, San Jose, CA). Each of the cell fractions was cultured with NH expansion medium (Miltenyi Biotec) supplemented with 100 U penicillin/ml and 100 μg streptomycin (Sigma-Aldrich)/ml and cultured at 37 °C in a humidified 5% CO_2-containing atmosphere.

Differentiation of MSCs. The differentiation of CD271$^+$ MSCs into adipocytes, chondrocytes, and osteoblasts was analyzed *in vitro* after 2 or 4 weeks of culture in NH differentiation medium. To confirm adipogenesis and chondrogenesis, cells cultured for 4 weeks in differentiation medium in 24-well plates (IWAKI, Tokyo, Japan) were fixed in 1% formaldehyde-phosphate-buffered saline (PBS) for 1 hour at 4 °C, permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, and then washed twice with PBS. The cell monolayers were incubated with 100 ng Nile red (Invitrogen)/μl for 1 hour or toluidine blue for 30 minutes at room temperature, and washed twice with PBS. For osteogenesis, cells that had been cultured for 4 weeks in differentiation medium in 24-well plates were fixed in cold methanol for 5 minutes at −20 °C and then washed twice with dH_2O. The cell monolayers were incubated with 2 ml of alkaline phosphatase BCIP/NBT (Sigma-Aldrich) for 10 minutes at room temperature and washed twice with PBS.

Myogenic differentiation of MSCs. CD271$^+$ MSCs (5.0×10^5–1.0×10^6 cells/well) plated on collagen-coated coverslips (IWAKI) in 24-well plates were exposed to 3.3 ng of the histone deacetylase inhibitor FK228 (Astellas Pharma, Tokyo, Japan) per ml of NH expansion medium for 7 hours at 37 °C. CD271$^+$ MSCs obtained from wild-type dogs were pretreated with FK228 and then cultured for 2 days with the adenovirus vectors (Ad-cMyoD or Ad-eGFP, multiplicity of infection = 25, ref. 50). These cells were washed and maintained in NH expansion medium or DMEM (Invitrogen) containing 2% horse serum, 100 U penicillin/ml, and 100 μg streptomycin/ml for 7–10 days, with a medium change every 3 days.

Characterization of DLA genotyping. DNA from blood sampled from the wild-type, carrier and $CXMD_J$ was extracted using the DNeasy extraction kit (Qiagen, Crawley, UK). DLA class II typing was performed by means of PCR and sequencing for DLA-DRB1, DQA-1, and DQB-1 alleles.[21] DNA sequence-based typing was used to characterize the dogs.

MSCs transplantation experiments. Allogeneic MSCs were transplanted on day 0 of the experiment. Five days earlier, muscle degeneration and regeneration cycles were induced in the TA muscle of wild-type dogs by the injection of 10.5 nmol CTX (Sigma-Aldrich)/kg. Stable MSC transformants obtained from the DLA-identical recipient wild-type dog were established using RV-Luc expressing luciferase. MSCs were transduced with Ad-MyoD (multiplicity of infection = 50); 2 days later, these cells were injected into the extensor carpi ulnaris and TA muscles. Recipient dogs were given 25 or 8.3 mg oral cyclosporine (Sandimmune; Novartis, E. Hannover, NJ)/kg body weight daily from day 5 until the end of the experiments, and 30 or 10 mg mycophenolate mofetil (CellCept; Roche Laboratories, Nutley, NJ)/kg body weight daily from day 0 until the end of the experiments.[50] The same MSCs were also injected into the extensor carpi ulnaris (1×10^6 cells diluted in 1 ml PBS) and TA (2 or 4×10^6 cells diluted in 1 ml PBS) muscles of nonimmunosuppressed $CXMD_J$. The injected muscles were then biopsied at 10 days and 4, 8, and 12 weeks after transplantation, or the animals were sacrificed at the end of the experiment. MyoD and eGFP-transduced MSCs (5×10^6 cells) were also administered into the femoral artery. Five days after the injection of CTX into the TA muscle of recipient wild-type dogs, the femoral artery was subjected to transient avascularization using a tourniquet, followed by the injection of papaverine hydrochloride (0.44 mg/kg). Serum creatine kinase, C-reactive protein, and cyclosporine concentrations were estimated 1 week after initial treatment and at the time of necropsy. The transplanted muscles were biopsied at 2, 4, or 8 weeks after injection. For biopsy and necropsy, the individual muscle was dissected tendon-to-tendon, divided into several pieces and immediately frozen in liquid nitrogen-cooled isopentane. At least 30 sections from the blocks were analyzed. The dogs underwent periodic veterinary examinations during the experiments. Hematological and serum biochemical testing was performed using a semiautomated hematology analyzer (Sysmex Hematology Analyzer F-820; Sysmex, Hyogo, Japan). Serum alanine aminotransferase, alkaline phosphatase, and creatine kinase, C-reactive protein levels were measured using an automated analyzer (DRI-CHEM3506; Fujifilm, Tokyo, Japan).

Histopathology and immunohistochemistry. The biopsied muscles were fixed in 15% neutralized formaldehyde-PBS. Transverse cryosections (10-μm thick) prepared from the MSCs-injected muscles were stained with hematoxylin and eosin using standard procedures. For immunohistochemistry, cells cultured on glass coverslips in 24-well plates were fixed

in 1% formaldehyde-PBS for 1 hour at 4°C. Thick cryosections (8-μm thick) prepared from MSCs-injected muscles tissues were fixed in 1% paraformaldehyde-PBS for 30 minutes at 4°C. Cell monolayers and tissue sections were permeabilized for 5 minutes in PBS containing 0.1% Triton X-100 and then blocked with 3% bovine serum albumin in PBS. The following antibodies were used for antigen detection at 1:50–1:100 dilutions of rabbit anti-MyoD antibodies (Santa Cruz Biotechnology, Delaware, CA): mouse anti-actinin antibodies (EA-53; Sigma-Aldrich), rabbit anti-firefly luciferase antibodies (ab21176; Abcam, Cambridge, UK), chicken anti-GFP antibodies (AB16901; Millipore, Billerica, MA), mouse anti-dMyHC antibodies (NCL-MHCd; Leica, Heidelberg, Germany), or mouse antidystrophin (NCL-DYS3; Leica). All antibodies were diluted with 0.5% bovine serum albumin in PBS and incubated with the cells or tissue sections for either 1 hour at room temperature or overnight at 4°C. The cells or tissue sections were washed with PBS and then incubated with Alexa 488- or Alexa 568-conjugated anti-mouse IgG antibodies (Invitrogen), Alexa 488- or Alexa 568-conjugated anti-rabbit IgG antibodies (Invitrogen), or Cy3.5-conjugated anti-chicken IgY (IgG) antibodies (ab6962; Abcam) at a 1:500 dilution for 1 hour at 4°C. Coverslips or glass slides were washed with PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA) with 4′, 6′-diamidino-2-phenylindole. Immunofluorescence was visualized using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcriptase-PCR. Total RNA (1 μg) from cultured MSCs or muscle samples disrupted in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) was isolated using the RNeasy Micro kit (Qiagen). First-strand complementary DNA was synthesized using a Super Script III First-Strand Synthesis System for reverse transcriptase-PCR (Invitrogen). From the 20 μl complementary DNA reaction volume, 0.5–2 μl were used for each PCR assay. The primers used in this study were as follows: developmental myosin heavy chain: forward, 5′-gcatcgagtggaccttcattgac-3′, and reverse, 5′-acagtctcattgagagggt ccttg-3′; myogenin: forward, 5′-acgagcggactgagctcagcc-3′, and reverse, 5′-ggtagcggaggtcccgctcctcctggttgag-3′; MyoD: forward, 5′-atggagcttcta tcgccgccac-3′, and reverse, 5′-aggcctcattcactttgctcag-3′; dystrophin: forward, 5′-gattctcctgagctgggtccgac-3′, and reverse, 5′-gccttggcaacatttcca-cttcctg-3′; and eGFP: forward, 5′-gtgagcaagggcgaggag-3′, and reverse, 5′-gtggtgcagatgaacttcagg -3′. As an internal control, a primer set of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase or TATA-binding protein was used. complementary DNA was amplified using an Ampli Taq Gold 360 Master (Applied Biosystems, Tokyo, Japan) and primers, with a total of 40 cycles. PCR products were resolved by agarose gel electrophoresis. The eGFP gene was amplified using SYBR Premix Ex TaqII (Perfect Real Time; Takara Bio) for 60 cycles. Quantitative PCR was carried out using SYBR green detection of PCR products in real time with the MyiQ single-color detection system (Bio-Rad, Hercules, CA).

SUPPLEMENTARY MATERIAL

Figure S1. Flow cytometry analysis of fresh or cultured CD271$^+$ and CD271$^-$ cells.
Figure S2. Characterization of CD271$^+$ MSCs derived from CXMD$_j$.
Figure S3. Immunofluorescence staining of intra-arterially injected CD271$^+$ MSCs engrafted in the heart of a recipient dog.
Figure S4. Immunofluorescence staining of lymphocytes from muscle injected with CD271$^+$ MSCs.
Figure S5. Immunoblotting for the detection of dystrophin antibody.
Materials and Methods.

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RESEARCH ARTICLE

Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic *CXMDj* dog

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Abstract

Background Duchenne muscular dystrophy (DMD) is a severe, inherited, muscle-wasting disorder caused by mutations in the dystrophin gene. Preclinical studies of adeno-associated virus gene therapy for DMD have been described in mouse and dog models of this disease. However, low and transient expression of microdystrophin in dystrophic dogs and a lack of long-term microdystrophin expression associated with a $CD8^+$ T-cell response in DMD patients suggests that the development of improved microdystrophin genes and delivery strategies is essential for successful clinical trials in DMD patients.

Methods We have previously shown the efficiency of mRNA sequence optimization of mouse microdystrophin in ameliorating the pathology of dystrophic *mdx* mice. In the present study, we generated adeno-associated virus (AAV)2/8 vectors expressing an mRNA sequence-optimized canine microdystrophin under the control of a muscle-specific promoter and injected intramuscularly into a single canine X-linked muscular dystrophy (*CXMDj*) dog.

Results Expression of stable and high levels of microdystrophin was observed along with an association of the dystrophin-associated protein complex in intramuscularly injected muscles of a *CXMDj* dog for at least 8 weeks without immune responses. Treated muscles were highly protected from dystrophic damage, with reduced levels of myofiber permeability and central nucleation.

Conclusions The data obtained in the present study suggest that the use of canine-specific and mRNA sequence-optimized microdystrophin genes in conjunction with a muscle-specific promoter results in high and stable levels of microdystrophin expression in a canine model of DMD. This approach will potentially allow the reduction of dosage and contribute towards the development of a safe and effective AAV gene therapy clinical trial protocol for DMD.

Keywords AAV vector; CXMDj; DMD; dystrophic dog; microdystrophin

Introduction

Duchenne muscular dystrophy (DMD) is a severe progressive muscle wasting disease caused by mutations in the dystrophin gene [1,2]. A lack of dystrophin protein decreases the integrity of skeletal myofibers, resulting in increased calcium influx and myofiber necrosis [3]. To prevent muscle degeneration in DMD, gene transfer leading to approximately 30% of the normal expression