

- 準的な症状評価の指標が十分に確立していない
- ・ 有効性評価の代替指標についても十分に確立していない
 - ・ 国内外で既承認のアンチセンス・モルフォリノが存在しないため、作用機序が類似する化合物からも、安全性の推測が困難である

以上の検討結果を踏まえて、アンチセンス・モルフォリノの開発対象候補の国内での治験の導入にむけて、現在は以下の対策を行っている。

① 非臨床試験成績の充足性評価

臨床試験の開始時期は、ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) M3 ガイドライン¹⁾²⁾に従い、非臨床試験成績を充足させ、開発対象の化合物の安全性を評価しなければならない。現在、アンチセンス・モルフォリノの開発対象候補の開発企業（海外企業）が実施済みである非臨床試験成績の充足性を評価すべく、当該領域の専門家確保に努めている。

② 試験計画の企画・立案

神経筋疾患の世界的研究組織である The Cooperative International Neuromuscular Research Group

(CINRG) に参画し、平成 21 年 11 月に同組織により開催された年次総会に参加した。これにより、筋ジストロフィー研究の最近の動向を把握するとともに、各種症状評価尺度の主要評価項目としての妥当性についても検討した。国内試験での利用のために、症状評価尺度の信頼性及び妥当性に関する公表論文の内容についても、引き続き評価

していく。

Duchenne 型筋ジストロフィー患者の母集団の数は、他領域の疾患と比較しても極めて小さい。このため、臨床試験の規模に大きな制限が発生する。今後も、承認申請時の臨床データパッケージを見据えた開発ストラテジーを十分に検討していく。

③ 治験実施のための体制整備

Duchenne 型筋ジストロフィーが希少疾患であることより、治験を医師主導で行わざるを得ない可能性もある。この可能性を考慮して、当センター内での事務局機能を向上させることの整備にも着手している。

E. 結論

承認申請を目的とした Duchenne 型筋ジストロフィーに対する臨床試験については、その臨床評価方法も十分に確立していない状況である。その方法を検討するとともに、希少疾病であるため臨床試験の規模と数にも制限があるため、海外の開発状況を絶えず把握し、開発ストラテジーの立案を慎重に検討していく必要がある。

F. 研究発表

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疾患編

17. 筋ジストロフィー，多発筋炎

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Key words Duchenne 型筋ジストロフィー，多発筋炎，皮膚筋炎**要 点**

- ① Duchenne 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。
- ② DMD 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。
- ③ DMD 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 경우가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 recruit-

ment) 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/治療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), 必要に応じてシーケンス解析, 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SpO₂、%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SpO₂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、PaCO₂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1g/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

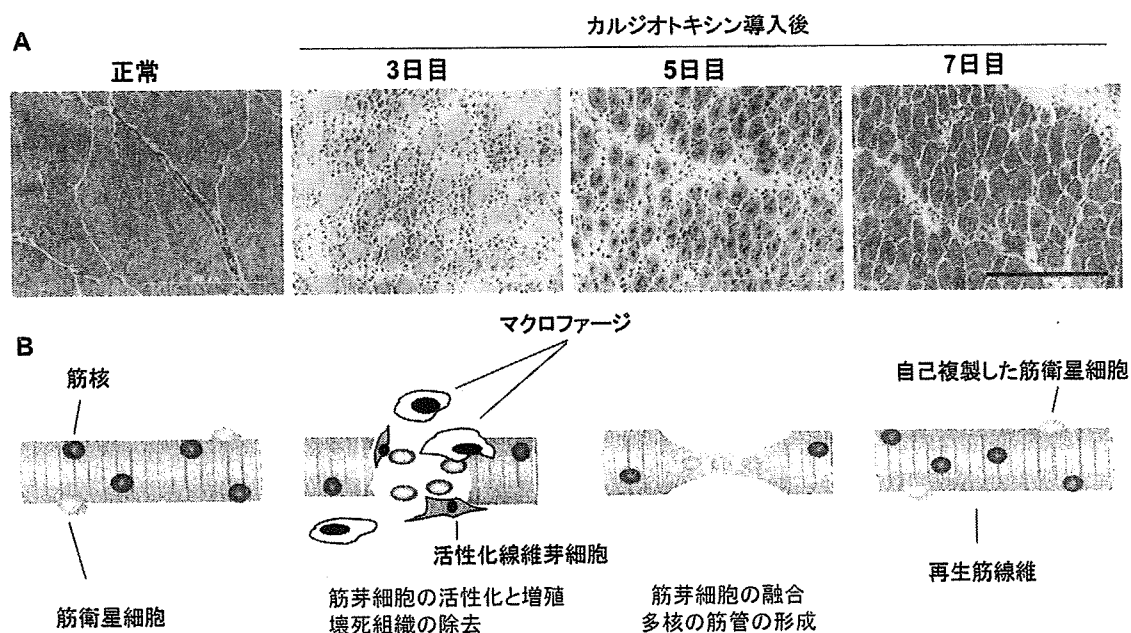


図1 骨格筋の再生

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）。

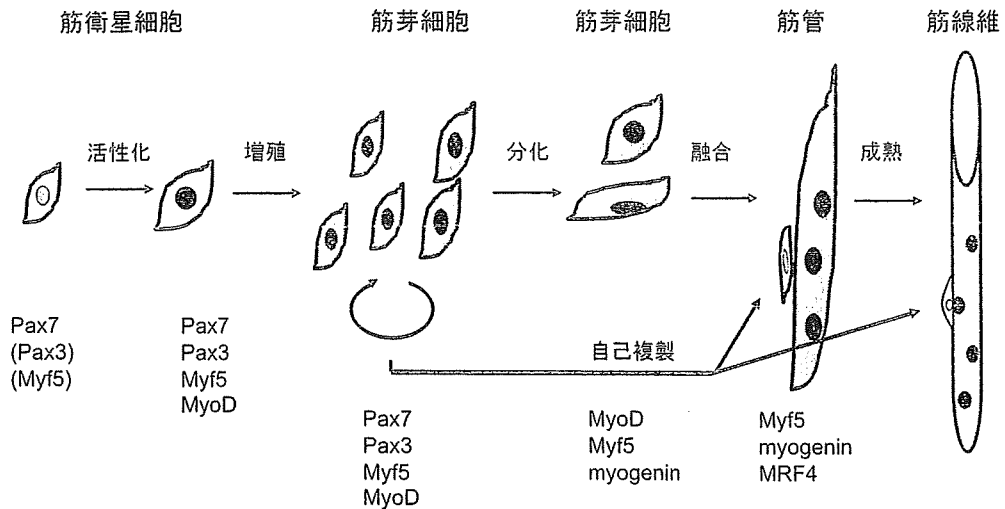


図2 筋衛星細胞の分化過程とその制御因子

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因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

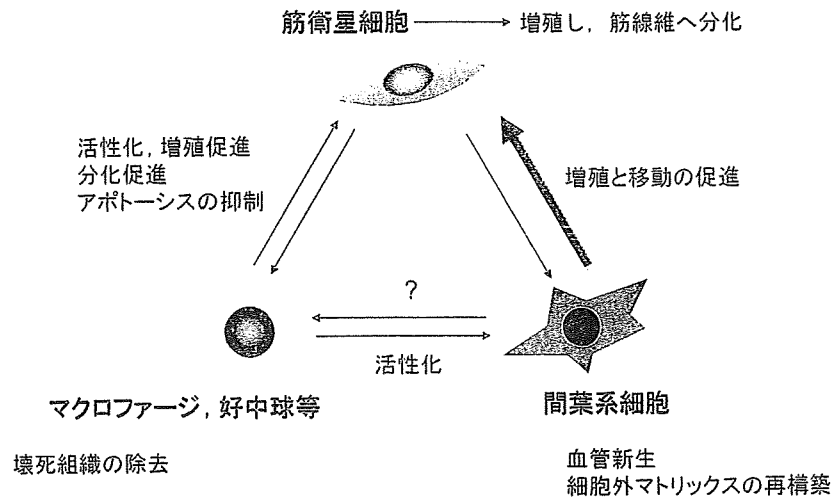


図3 筋再生を制御する細胞とそのネットワーク

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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Expression Pattern of *WWP1* in Muscular Dystrophic and Normal Chickens

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The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*) is classified into one of ubiquitin ligases which play an important role in ubiquitin-proteasome pathway. Previously, we identified the *WWP1* gene as a candidate gene of chicken muscular dystrophy by linkage analysis and sequence comparison. However, the mechanism causing pathological changes and underlying gene function remains elucidated. In the present study, we analyzed the *WWP1* gene expression in various muscles and tissues of normal chickens, and compared with those from muscular dystrophic chickens. Two mRNA isoforms were detected in all tissues examined and revealed almost equal expression level. The *WWP1* expression of dystrophic chickens was decreased in almost all skeletal muscles including unaffected muscles. These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy.

Key words: chicken, expression analysis, fast twitch muscle fiber, muscular dystrophy, *WWP1*

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Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*) is classified into an ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). E3 recognizes and catalyzes ubiquitin (Ub) conjugation to specific protein substrates (Liu, 2004). Comparative genome analysis reveals few genes encoding E1, tens of E2 encoding genes and hundreds of E3 encoding genes (Semple *et al.*, 2003).

The *WWP1* gene is classified into HECT (homologous to the E6-AP carboxyl terminus)-type E3 which possesses one C2 domain, multiple WW domains and one HECT domain (Pirozzi *et al.*, 1997; Flaszka *et al.*, 2002). The C2 domain binds to the cellular membranes in a Ca²⁺-dependent manner (Plant *et al.*, 1997) and mediates interactions with other proteins (Plant *et al.*, 2000; von

Poser *et al.*, 2000; Augustine, 2001). The WW domain has two conserved tryptophan residues and binds proline-rich region (Sudol *et al.*, 1985). HECT domain, similar to E2s structurally, has a cysteine residue as an active center that transfers the activated Ub from E2 onto first itself, and then onto its substrates (Jackson *et al.*, 2000).

The muscular dystrophies are the group of inherited diseases with progressive weakness and degeneration of skeletal muscle (Partridge, 1991). It is well known that abnormalities of muscle proteins linking sarcolemma and basal lamina lead to cause muscular dystrophies (Lisi and Cohn, 2007), but there are a number of muscular dystrophies and related diseases of which causes are still unknown. We identified *WWP1* gene as a candidate responsible for the chicken muscular dystrophy by the linkage analysis (Matsumoto *et al.*, 2007) and the sequence comparison between normal and dystrophic chickens (Matsumoto *et al.*, 2008). The R441Q missense mutation was found in *WWP1* gene to cause the phenotype of muscular dystrophy.

The *WWP1*s of human (Flaszka *et al.*, 2002; Komuro *et al.*, 2004), mouse (Dallas *et al.*, 2006) and *C. elegans* (Huang *et al.*, 2000) were intensively studied and known

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that the *WWPI* gene is expressed ubiquitously, but strongly in liver, bone marrow, testis and skeletal muscles (Flasza *et al.*, 2002; Komuro *et al.*, 2004). In chicken, however, the *WWPI* expression has not been studied. The expression analysis of *WWPI* gene is important since it was reported that altered expression of known responsible gene could lead dystrophic phenotype (Smythe and Rando, 2006).

In this study, we analyzed the mRNA expression of *WWPI* in various skeletal muscles and other tissues of normal and dystrophic chickens by using Northern blotting and reverse transcription (RT)-PCR analysis to know the differences in the general expression pattern between them.

Materials and Methods

Chickens

A two-month-old dystrophic chicken (New Hampshire: NH-413) and an age-matched normal chicken (White Leghorn: WL-F) were used in this study. The New Hampshire (NH-413) strain is a homozygous dystrophic line introduced from University of California, Davis to Japan in 1976 (Kondo *et al.*, 1982). The disease in this strain is transmitted co-dominantly by a single gene, but the phenotype is modified by other background genes (Kikuchi *et al.*, 1981, 1987; Wilson *et al.*, 1979). The White Leghorn (WL-F) strain was established in 1970s, and maintained as closed colony in the Nippon Institute of Biological Science in Yamanashi, Japan. This study was carried out according to the guidelines of Animal Experimentation of Kobe University.

Expression analysis

For Northern blotting, mRNAs were isolated from *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps femoris* (BF), *M. triceps surae* (TS), *M. peroneus longus* (PL), heart (H), brain (B), liver (L), kidney (K) and whole embryo (E) with PolyATtract mRNA Isolation kit (Promega, Madison, WI, USA). The 2 µg of mRNAs, which were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), were resolved by 1.2% agarose gel electrophoresis in the presence of formaldehyde and blotted on to Hybond-N+ membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mRNAs were visualized using digoxigenin (DIG) reagents, and kits for non-radioactive nucleic acid labeling and detection system (Roche Diagnostics, Basel, Switzerland) according to the procedure specified by the manufacturer excepting that the washing was done with 4×SCC 0.1% SDS at room temperature for 10 min, 4×SCC 0.1% SDS at 40°C for 8 min and then 2×SCC 0.1% SDS at 40°C for 8 min twice. The DIG-labeled DNA probes were prepared by PCR using DIG-dUTP using pectorals cDNA sample of a WL-F strain female as a template. The primers applied in this procedure were 5'-tccctcataaatgttgaaagcagaca-3' (WWP1p-F), 5'-gtaataacccaagtaatatgtaaac-3' (WWP1p-R) (NM_001012554), 5'-ccgtgtgccaaccccaatgtctctg-3'

(GAPDHp-F) and 5'-cagtttctatcagcctctcccacctc-3' (GAPDHp-R) (NM_204305). The PCR was done for 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec (*WWPI*) and for 35 cycles at 94°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec (*GAPDH*) using TaKaRa Ex Taq[®] Hot Start Version (Takara Bio Inc., Tokyo, Japan). Quantitative analysis was performed with Scion Image (Scion Corporation, Frederick, MD, USA).

In order to analyze mRNA expression of *WWPI* gene in the PS, *M. anterior latissimus dorsi* (ALD) and H, RT-PCR method was applied. The concentration of cDNA derived from these muscles was calculated by NanoDrop ND-1000 (NanoDrop Technologies) and measurable cDNAs were used as template. The primers applied were 5'-attaggaagagccactgtagact-3' (WWP1r-F) and 5'-tctgttgattgaggttctgtctg-3' (WWP1r-R) (NM_001012554). The PCR was done for 35 and 40 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec using TaKaRa Ex Taq[®] Hot Start Version (Takara Bio Inc.).

Histology

The PS, ALD and H were snap-frozen in liquid nitrogen-cooled isopentane and sectioned in a cryostat (Leica Microsystems Japan, Tokyo, Japan). The histopathology was made by hematoxylin-eosin staining (HE) method (Kikuchi *et al.*, 1981).

Results

The mRNA expression of *WWPI* gene was detected by Northern blotting in various muscles and other tissues of normal and muscular dystrophic chickens (Fig. 1). Two bands were detected in all tissues examined, and revealed almost equally expression level in any muscles and tissues observed.

In the PS, BF, TS, PL, B and K, *WWPI* gene was strongly expressed in normal than in dystrophic chickens (Fig. 1). *GAPDH* was used as an internal control of *WWPI* expression analysis. In TFL, L and E, similar *WWPI* expression level was observed between two phenotypes (Fig. 1).

RT-PCR analysis indicated that *WWPI* gene was expressed in slow tonic ALD, not only in PS and H of both phenotypes (Fig. 2A). Figure 2B shows histopathological changes in PS, ALD and H of normal and dystrophic chickens. The pathological findings in dystrophic PS were characterized by the degenerating fibers with many vacuoles in cytoplasm, the fatty infiltration into connective tissue, and the proliferation of nuclei within muscle fibers with large variation in sizes. However, no such lesions were observed in ALD and H from age-matched dystrophic chickens (Fig. 2B).

Discussion

Northern blotting with *WWPI* specific probe detected two bands in all tissues and muscles examined (Fig. 1). Northern blot analysis of *WWPI* expression in human tissues also exhibited two bands (Mosser *et al.*, 1998), and RT-PCR analysis showed that human *WWPI* gene had at

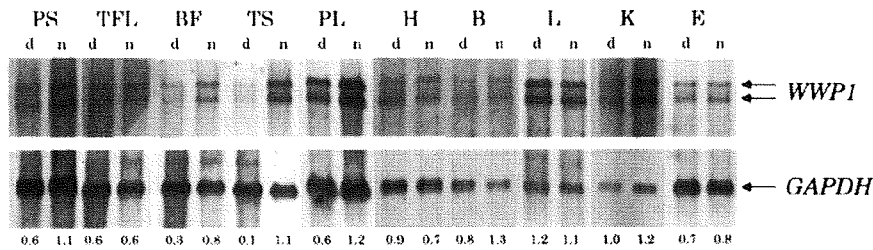


Fig. 1. Expression of chicken *WWP1* in various tissues.
 A *WWP1* cDNA probe was used to detect *WWP1* mRNA transcripts by Northern blotting using blots containing 2 μg of mRNAs from chicken muscles or various other tissues. *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps femoris* (BF), *M. triceps surae* (TS), *M. peroneus longus* (PL), heart (H), brain (B), liver (L), kidney (K) and embryo (E) were analyzed. A doublet band is detected at variable levels in all tissues. "d" indicates mRNAs from dystrophic chickens. "n" indicates mRNAs from normal chickens. The numbers below the *GAPDH* bands represent the relative ratios of *WWP1*/*GAPDH*.

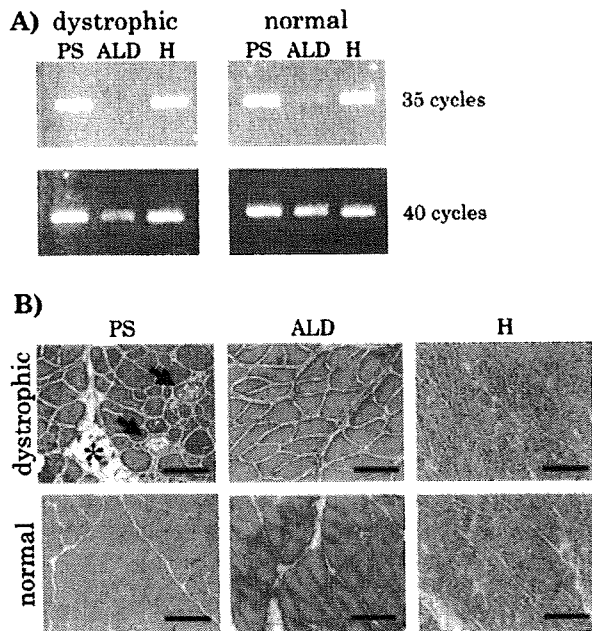


Fig. 2. RT-PCR detection of *WWP1* gene and histological analysis for three representative muscle types. *M. pectoralis superficialis* (PS), *M. anterior latissimus dorsi* (ALD) and heart (H) expressed *WWP1* less in muscular dystrophic chicken, but only dystrophic PS was severely harmed. A) Expression of *WWP1* in PS, ALD and H was analyzed by RT-PCR method. PCR was performed for 35 or 40 cycles. B) The PS, ALD and H of dystrophic (NH-413) and normal (WL-F) chickens were analyzed with HE staining. Vacuoles (arrows) and fatty infiltration (asterisk) are observed in PS of dystrophic chickens. It is also remarkable that, in dystrophic PS, many muscle fibers have many nuclei in cytoplasm and vary widely in size. These pathological features are not observed in ALD and H of dystrophic chicken. Scale bar=120 μm.

least six mRNA isoforms synthesized through the alternative splicing, two of which were strongly expressed and commonly observed in various tissues (Flasza *et al.*, 2002). The mRNA doublet bands of chicken *WWP1* by Northern blot analysis might be equivalent to two bands of human tissues, while a single band was observed by RT-PCR analysis in chicken (Fig. 2A), suggesting that the amplified region does not include alternative spliced site. Flasza *et al.* (2002) also mentioned that the relative ratio of these isoforms from human *WWP1* varied in a tissue-specific manner, but the doublet bands of chicken *WWP1* were expressed almost equally in all tissues examined.

The *WWP1* gene expression in *M. pectoralis superficialis* (PS) of dystrophic chicken was less than that of normal chicken (Fig. 1). The PS of chicken is a fast twitch muscle composed of two types of fast twitch fibers (aW and bW). TFL, BF, TS and PL muscles from wing and leg are mixed muscles co-existing fast twitch (aW and bW) with slow twitch fibers (bR) in a mosaic pattern (Ashmore and Doerr, 1971a), except that the ALD and *M. adductor magnus* are composed of slow tonic fibers (ST) innervated multiply (Ashmore *et al.*, 1978; Kikuchi *et al.*, 1986). In chicken muscular dystrophy, fast twitch fibers are initially and most severely affected, while slow twitch and slow tonic muscles persist relatively harmless throughout the life span (Ashmore and Doerr, 1971b; Barnard *et al.*, 1982). The *WWP1* expression in dystrophic BF, TS and PL showed a similar downward trend as observed in dystrophic PS (Fig. 1). These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy, since not only affected muscles but unaffected ones exhibited the same pattern. Moreover, the alteration of *WWP1* expression level was observed in other unaffected tissues, such as B and K, which reinforces our hypothesis that the alteration of *WWP1* expression levels

does not link directly to the dystrophic phenotype (Fig. 1).

To assess the genetic influence of mutant *WWPI* upon chicken muscular dystrophy, we examined *WWPI* gene expression and histological changes in three distinct muscle types, PS as a fast twitch type, ALD as a slow tonic type, and H as a different type of muscle. RT-PCR was applied to this study since ALD was not enough quantity of mRNA for Northern blotting. The *WWPI* mRNA expression was confirmed in all muscles examined (Fig. 2 A).

Figure 2B shows HE stained sections of PS, ALD and H from normal and dystrophic chicken. The dystrophic PS was severely affected, while ALD and heart of dystrophic chicken remained relatively intact (Fig. 2B) as described in a previous study (Kikuchi *et al.*, 1981). The *WWPI* was expressed even in unaffected muscles and the downward alteration of *WWPI* expression was observed commonly in almost all dystrophic muscles examined (Figs. 1, 2). The observation suggests that the alteration of *WWPI* might not be the cause of the pathological change in chicken muscular dystrophy. Hence, the mutation identified previously (Matsumoto *et al.*, 2008) might play a crucial role in leading the onset of chicken muscular dystrophy. The detected mutation lay between WW domains, highly conserved region among tetrapods (Matsumoto *et al.*, 2008), which has been predicted as substrate binding region (Pirozzi *et al.*, 1997; Flaszka *et al.*, 2002). This suggests that mutated *WWPI* could not recognize its substrates.

Many HECT-type E3s with WW domains including *WWPI* regulate membrane proteins (Chen and Matesic, 2007). Therefore, aberrant regulation of membrane protein may lead the onset of chicken muscular dystrophy. For example, *WWPI* could bind to β -dystroglycan, which is one of important muscle proteins consisting of membrane (Pirozzi *et al.*, 1997). Abnormal glycosylation of α -dystroglycan in chicken muscular dystrophy has been reported (Saito *et al.*, 2005). Furthermore, the fact that some E3s can recognize sugar chain (Yoshida *et al.*, 2002, 2003; Lederkremer and Gliskman, 2005) leads to the hypothesis that mutated *WWPI* might not be able to recognize the sugar chain of α -dystroglycan to regulate the glycosylated molecules, and that insufficiently glycosylated α -dystroglycan accumulates and causes the disease.

In the present study, we analyzed the mRNA expression of *WWPI* in various skeletal muscles and other tissues of normal and dystrophic chickens. The results suggest that *WWPI* expression level lowered in dystrophic phenotype is not directly related to the cause of disease in chicken muscular dystrophy, whereas mutated *WWPI* does not function normally to cause the onset of chicken muscular dystrophy.

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ABSTRACT: Duchenne muscular dystrophy (DMD) is a devastating muscle disorder that is characterized by progressive muscle necrosis, fibrosis, and fatty infiltration. To examine the temporospatial pathological changes, a noninvasive evaluation method such as magnetic resonance imaging (MRI) is needed. The aim of this study was to precisely assess muscle necrosis and inflammation based on a sequence of T2-weighted imaging (T2WI), gadolinium-enhanced imaging, and selective fat suppression, chemical shift selective T2-weighted imaging (CHESS-T2WI), on a 3.0-Tesla MRI unit in 3-month-old and 7-year-old dogs with canine X-linked muscular dystrophy (CXMD_J), a suitable animal model for DMD. The results show that CHESS-T2WI was more sensitive and useful from the early to late stages of CXMD_J than T2WI or contrast enhancement imaging in the evaluation of muscle necrosis, because these latter sequences can be influenced by fatty infiltration or interstitial connective tissues.

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EVALUATION OF DYSTROPHIC DOG PATHOLOGY BY FAT-SUPPRESSED T2-WEIGHTED IMAGING

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Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disease characterized by progressive skeletal muscle atrophy and weakness.¹ DMD is caused by mutations in the *dystrophin* gene, which encodes the cytoskeletal protein dystrophin.² A loss of dystrophin accompanied by a deficiency of dystrophin–glycoprotein complex (DGC) from the sarcolemma leads to progressive degeneration of striated muscle.^{3,4} In dystrophic skeletal muscles, muscle fiber necrosis with inflammation is followed by muscle regeneration, but the muscle is

finally replaced by fibrous or fatty tissue.^{5,6} For this devastating disorder, various therapeutic approaches, such as gene therapy, stem cell–based cell therapy, or pharmaceutical agents have been proposed and explored using various DMD animal models.

The X-linked muscular dystrophy (*mdx*) mouse and Golden Retriever muscular dystrophy (GRMD) dog are the most commonly used DMD animal models.^{7,8} *mdx* mice show extensive necrosis followed by regeneration, but their phenotypes are milder than those of DMD due to the absence of apparent fibrosis and fatty infiltration.^{7,9,10} The phenotypes of striated muscle in the GRMD dog are clinically and pathologically more similar to that of DMD,^{8,11,12} but it is very difficult to maintain this animal model due to the severe phenotype. We have therefore established a Beagle-based colony of canine X-linked muscular dystrophy in Japan (CXMD_J).¹³ We have found that the clinical and pathological findings in CXMD_J are similar to but milder than those in GRMD.^{14,15}

A method of noninvasive temporospatial assessment is required to investigate muscle involvement and, especially, to evaluate therapeutic

Abbreviations: ANOVA, analysis of variance; CE, contrast enhancement ratio; CHESS, chemical shift selective; CT, computed tomography; CXMD_J, canine X-linked muscular dystrophy in Japan; DGC, dystrophin–glycoprotein complex; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; FDS, flexor digitorum superficialis; FITC, fluorescein isothiocyanate; GC, gastrocnemius; Gd-DTPA, gadolinium diethylenetriamine pentaacetic acid; GRMD, Golden Retriever muscular dystrophy; MRI, magnetic resonance imaging; PCr, phosphocreatine; Pi, inorganic phosphate; ROI, region of interest; SNR, signal-to-noise ratio; STIR, short-tau inversion recovery; SI, signal intensity; TC, tibialis cranialis

Key words: chemical shift selective fat-suppressed T2-weighted imaging; Duchenne muscular dystrophy; dystrophic dog; magnetic resonance imaging; myopathy

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interventions. Computed tomography (CT), which shows high temporal and spatial resolution, has been used to detect selective muscle involvement, such as atrophy or fatty tissue replacement, in patients suffering from DMD,^{16,17} but it requires ionizing radiation and has limited sensitivity for soft tissues.¹⁸ Magnetic resonance imaging (MRI) produces high-resolution images with good contrast among soft tissues,¹⁹ and therefore it has been used to evaluate skeletal muscle involvement in DMD²⁰ and in *mdx* mice.²¹ In the early stages of dystrophy, the T1 relaxation time is prolonged due to muscle degeneration and regeneration together with an increase in muscle water concentration, and it is decreased owing to fat infiltration in the advanced stage.²² As the main magnetic field increases, however, the capacity to differentiate tissues on the basis of T1 relaxation time may decrease.²³ On the other hand, the T2 relaxation time is prolonged in necrotic as well as fatty and connective tissue¹⁹; therefore, it can hardly distinguish necrosis from fat replacement or fibrosis during the dystrophic process. To selectively detect necrotic changes, MR contrast agents, such as gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA), have been used extensively,²⁴⁻²⁶ but these agents may also enhance blood vessels and the interstitium,²⁷ and may cause severe adverse effects, such as anaphylaxis,^{28,29} which are critical for DMD patients. Thus, a safer imaging protocol is needed to distinguish necrotic lesions from fatty degeneration or fibrosis in the dystrophic skeletal muscle of DMD and CXMD_J.

To discriminate necrosis from fatty infiltration, one of the fat suppression sequences may be useful. As a fat suppression sequence, short-tau inversion recovery (STIR) MR imaging was used to detect muscle edema in DMD.⁶ However, STIR suppresses the signal from any tissue or fluid that has a short T1 relaxation time, and therefore it does not selectively suppress the fat signal.^{30,31} In contrast, chemical shift selective (CHESS) imaging, another fat suppression sequence, is a technique that selectively saturates fat magnetization by applying a 90° pulse matching with the fat resonance frequency and therefore leads to a highly selective suppression of fat signals. Moreover, the signal-to-noise ratio (SNR) of CHESS is better than that of STIR at a higher magnetic field. The sequence of CHESS combined with T2-weighted imaging (CHESS-T2WI) has been used to diagnose disorders such as lipomatous tumor or temporomandibular arthrosis.³²⁻³⁴ The method, however, has not been applied to evaluation of the dystrophic

changes seen in DMD or the animal models to date.

We, therefore, examined dystrophic dog muscle by CHESS-T2WI to determine whether this sequence is more useful for finding necrosis and inflammatory change than the conventional sequences of T2WI or contrast imaging.

METHODS

Animals. We used three 3-month-old normal male dogs (II-2308MN, II/III-3911MN, and II-4202MN), three littermate CXMD_J male dogs (II-2302MA, II/III-3903MA, and II-4204MA), one 7-year-old normal male dog (00-174MN), and two 7-year-old CXMD_J male dogs (II-C04MA and II-C12MA). II-2308MN, II-4202MN, II-2302MA, and II-4204MA were produced by mating a second-generation (G2) carrier female¹³ and G2 affected male. II/III-3911MN and II/III-3903MA were the offspring of a G2 carrier female and a third-generation (G3) affected male. We obtained II-C04MA and II-C12MA by mating first-generation (G1) carrier female dogs and pure-bred normal male Beagles. 00-174MN was a pure-bred normal Beagle. All dogs were part of the breeding colony at the General Animal Research Facility, National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan), or the Chugai Research Institute for Medical Science, Inc. (Nagano, Japan). Ages, body weights, and serum creatine kinase values at the time of MRI of each dog are shown in Table 1. This study was carried out according to the guidelines provided by the Ethics Committee for the Treatment of Middle-sized Laboratory Animals of the National Center of Neurology and Psychiatry (Approval Nos. 18-02, 19-02, and 20-02).

MR Scanning and Image Analysis. General anesthesia was induced by an intravenous injection of thio-pental sodium (20 mg/kg) before MRI scanning and was maintained by inhalation of isoflurane (2.0-3.0%). We examined lower leg muscles of these dogs by superconducting 3.0-Tesla MRI (Magnetom Trio; Siemens Medical Solutions, Erlangen, Germany) with a human extremity coil 18 cm in diameter. The MRI pulse sequences used were T1-weighted imaging (T1WI), T2WI, chemical shift selective T1-weighted imaging (CHESS-T1WI), CHESS-T2WI, gadolinium-enhanced T1-weighted imaging (Gd-T1WI), chemical shift selective gadolinium-enhanced T1-weighted imaging (CHESS-

Table 1. Clinical profiles of normal and dystrophic male dogs used in this study.

	Age (mo)	BW (kg)	Serum CK (IU/L)
Normal dogs			
II-2308MN	3	6.8	197
II/III-3911MN	3	7.7	318
II-4202MN	3	5.8	274
00-174MN	87	13.7	83
CXMDJ dogs			
II-2302MA	3	7.2	30,200
II/III-3903MA	3	6.6	22,300
II-4204MA	3	6.0	28,800
II-C04MA	85	11.5	6500
II-C12MA	94	11.6	1602

Body weight (BW) and serum creatine kinase (CK) values were measured on the day of MRI examination.

Gd-T1WI), and multi-echo T2WI for calculation of T2 relaxation time. In contrast-enhanced images, we injected 0.2 ml/kg of the gadolinium-based MR contrast agent Gd-DTPA (Magnevist; Bayer Schering Pharma, Berlin, Germany) for each sequence. In 3-month-old dogs, we scanned the images for 26 minutes, about 5 minutes after the intravenous injection. On the other hand, we took the images for 13 minutes in 7-year-old dogs at 25 minutes after the injection in order to minimize the risk of anesthesia on the cardiac involvement seen in advanced CXMD_J.¹⁵ CHES was employed to assess necrotic and inflammatory changes more precisely. The acquisition parameters for T1WI, CHES-T1WI, Gd-T1WI, and CHES-Gd-T1WI were based on spin echo: repetition time (TR)/echo time (TE) = 500/7.4 ms; slice thickness = 4 mm; field of view = 18 × 18 cm; matrix = 256 × 256; and NEX = 3. The parameters for T2WI and CHES-T2WI were chosen based on fast spin echo: TR/TE = 4000/85 ms; slice thickness = 4 mm; field of view = 18 × 18 cm; matrix = 256 × 256; turbo-factor = 9; and NEX = 3. The parameters for multi-echo T2WI were selected based on spin echo: TR = 2000; TE = 11.8–118.0 (10 echoes); slice thickness = 4 mm; field of view = 28 × 28 cm; matrix = 256 × 256; and NEX = 2. We were able to clearly distinguish each lower leg muscle by each sequence. Representative cross-sectional images and anatomical locations of lower leg muscles by CHES-T1WI in a 7-year-old normal dog are shown in Figure 1.

For quantitative analysis of the images, the manufacturer's software (Syngo MR2004A; Siemens Medical Solutions, Erlangen, Germany) was used. Flow artifacts were slight, but regions of interest (ROIs) were selected to avoid flow artifacts and large vessels

as follows: three circular ROIs were picked in both right tibialis cranialis (Rt. TC) and extensor digitorum longus (Rt. EDL) muscles of the 3-month-old dogs. ROIs were also selected in the Rt. TC of the 7-year-old dogs and a normal dog. Then, T2 relaxation time or signal intensities (SIs) of CHES-T1WI, CHES-Gd-T1WI, and CHES-T2WI were measured in these ROIs. Signal-to-noise ratios (SNRs) of each ROI were calculated by the equation: $SNR = SI / SD_{air}$, where SD_{air} was the standard deviation (SD) of background noise.³⁵ The contrast enhancement (CE) ratio was calculated using the SNR of CHES-T1WI ($SNR_{precontrast}$) and SNR of CHES-Gd-T1WI ($SNR_{postcontrast}$) by the following equation: $CE = SNR_{postcontrast} / SNR_{precontrast}$. We used the means of the quantitative values at three points of ROIs for statistical analysis.

Statistical Analysis. The T2 relaxation time, CE ratio, and SNR of CHES-T2WI were evaluated using a one-way analysis of variance (ANOVA) to determine differences among the groups. When a significant difference was found with one-way ANOVA, intergroup comparisons were undertaken using Fisher's protected least significant difference test. All values are expressed as mean ± SE, and statistical significance was recognized at $P < 0.05$.

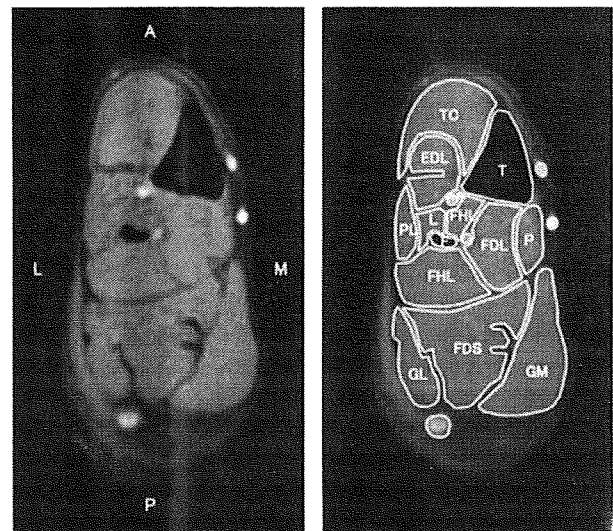


FIGURE 1. Cross-sectional images and anatomical orientation of right lower leg muscles of a 7-year-old normal dog in CHES-T1WI. A 7-year-old normal dog (00-174MN) was used for this study. T, tibia; F, fibula; TC, tibialis cranialis; EDL, extensor digitorum longus; FHL, flexor hallucis longus; FDL, flexor digitorum longus; FDS, flexor digitorum superficialis; GM, gastrocnemius medialis; GL, gastrocnemius lateralis. A, anterior; P, posterior; L, lateral side; M, medial side.