12. Sachiko Ohshima, Jin-Hong Shin, Akiyo Nishiyama, Katsutoshi Yuasa, Hiroyuki Nakai, Akinori Nakamura, Takashi Okada, Shin'ichi Takeda:
Transduction profile and immune response in the dystrophic dogs with rAAV serotype 8.
15th Annual Congress of the European Society of Gene and Cell Therapy, Rotterdam, Netherlands, October 27-30, 2007.

13. Akiyo Nishiyama, Beryl N. Ampong, Jin-Hong Shin, Hiroyuki Nakai, Takashi Okada, Shin'ichi Takeda:
Extensive alpha-sarcoglycan expression by intramuscular and intravenous administration of rAAV type 8.
15th Annual Congress of the European Society of Gene and Cell Therapy, Rotterdam, Netherlands, October 27-30, 2007.

＜国内＞

1. 内堀亮介、岡田尚巳、松下卓、卜部匡司、水上浩明、久米晃啓、小澤敬也.
ベクター産生型間葉系幹細胞の開発と癌治療への応用.
第 5 回幹細胞シンポジウム 2007 年 5 月 17 日-19 日, 淡路

2. 内堀亮介、岡田尚巳、伊藤孝幸、卜部匡司、水上浩明、久米晃啓、小澤敬也.
ベクター産生型間葉系幹細胞の開発と自殺遺伝子療法への応用.
第 66 回日本癌学会学術総会 2007 年 10 月 3 日-5 日, 横浜

H．知的所有権の出願・登録状況

1. 特許取得
岡田尚巳、小澤敬也
ベクター産生型腫瘍標的細胞
PCT/JP2007/50013、平成 19 年 1 月 5 日出願

伊藤章、花園豊、岡田尚巳、小澤敬也
アデノウイルス吸着架橋剤
Agents for adsorption and bridging for adenovirus
米国特許 US 7238777 B2, Jul. 3, 2007 成立

2. 実用新案登録なし

3. その他，特記事項なし.

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

骨髄間質細胞からの筋細胞の誘導

分担研究者　出澤　真理
京都大学大学院医学研究科　准教授

研究要旨
骨髄間葉系細胞は患者本人からの採取が可能であり、旺盛な増殖力を有するので細胞移植治療に必要な細胞数確保が可能である。骨髄バンクの利用も展望できることから、再生医療の細胞ソースとして最適である。我々は、ヒトおよびげっ歯類の骨髄間葉系細胞から骨格筋系細胞を、他の要素を含まず特異的に効率よく誘導する方法を開発した。この誘導方法を用いて大型哺乳類での筋変性モデルでの有効性と安全性の検証を行なう。

A．研究目的

筋ジストロフィーなど遺伝性筋変性疾患の細胞移植治療法開発の一環として、筋肉に含まれている増殖可能な筋衛星細胞の分離や ES 細胞、あるいは胎児の細胞から筋肉細胞を誘導する方法が検討されてきた。しかし、得られる細胞数が僅かであること、死亡胎児を必要とすること、あるいは細胞の安全性を巡って問題点が指摘されてきた。

骨髄間質細胞は容易に採取できる骨髄液から培養可能であり、増殖力が旺盛で移植治療に必要な細胞数の確保が可能である。また、患者本人の細胞を用いる自己細胞移植が可能である。よって、骨髄間質細胞は移植治療の候補としてはポテンシャルの高い細胞であり、効率の良い分化誘導系の確立が期待されていた。今回、我々は骨髄間質細胞から効率よく骨格筋細胞を誘導する方法を見出した。この方法が安全であるか、また有効であるかを高等哺乳類であるイヌをモデルとして検証する。

B．研究方法

骨髄間質細胞は、ヒトおよびビーグル犬の骨髄穿刺液から樹立した接着性細胞を用いた。

（1）　骨髄間質細胞からの骨格筋誘導：
骨髄間葉系細胞を特定の密度で経代培養した後、サイトカイン投与（bFGF (10ng/ml), forskloin (FSK) (5 μ M), neuregulin (200ng/ml) および PDGF (5ng/ml) を含んだ培地（10% FBS, alpha-MEM)）を行い、その後に Notch 細胞質ドメイン (Notch intracellular domain; NICD) plasmid 導入を lipofection 用いて行い、G418 にて選択する。その後、100% confluent に達したところで分化培地を投与することによって多核の骨格筋を誘導することが可能となる。

誘導細胞の性質を調べるために FACS において CD34, CD45, c-Kit 陰性の細胞を採取し同様に筋誘導を来ない解析した。

さらに誘導した細胞における骨格筋マーカーの発現を real-time PCR, 免疫染色等で検証を行なった。また誘導骨格筋の安全性を確認するために、核型解析によって染色体の変異、欠損等の有無を調べ、さらに、ヌードマウスの大腿筋にヒトからの誘導細胞を注入し、６ヵ月後に全身状態と病理検査を行い、腫瘍形成などの有無を確認した。

骨格筋が誘導されるメカニズムの解析として、ラットおよびヒトでの骨髄間葉系細胞に bFGF, FSK, PDGF, Neureguli を投与し NICD-GFP plasmid を導入して２４時間後に細胞を採取し、GFP の免疫沈降を行い、Notch

に特異的に結合する蛋白の解析を行なった。

（倫理面への配慮）本プロジェクト全般において組み換えDNA実験とモデル作成・移植における動物実験は各所属機関の組み換えDNA実験委員会と動物実験委員会の指針に従って研究計画書を提出し、医学部長、研究所長、委員会長等の機関承認を得た後に実施した。

骨髄間質細胞の分化誘導並びに分子生物学的解析は京都大学を中心に行うが、すでに京都大学大学院医学研究科・医学部動物実験委員会の承認を得ている(「骨髄間質細胞の骨格筋細胞への分化誘導と移植応用」承認番号：MedKyo04245）。ヒト骨髄間質細胞はBioWhittaker社の細胞を用いるので倫理上問題はない。Notchの遺伝子導入実験は京大の組み替えDNA実験委員会の了承を得て行なっている（「「体細胞の神経系および骨格筋細胞への分化転換の遺伝子機構について」(承認番号：研研２第２２４−2号)。

Ｃ．研究成果

ヒトおよびラット骨髄間葉系細胞にbFGF, フォルスコリン, neuregulinおよびPDGFを含んだ培地で培養すると、この段階で筋肉発生の初期に認められるPax7が発現しており、筋前駆細胞様に分化したと考えられた。この細胞にNICDを導入すると骨格筋細胞へと分化転換し、MyoD, myogeninなどの骨格筋特有のマーカーの発現が認められる。さらに分化培地（２％ウマ血清を含むDMEM培地、あるいはITS medium (Insulin-Transferrin-Selenate)）に切り替えることにより、一部の細胞が融合を開始し、成熟した多核の筋管細胞に分化し、Myosin-heavy chain, skeletal myosin, troponinなどの細胞骨格蛋白と共に成熟マーカーとして知られているMRF4/Myf6も発現するようになる。これらのことから、本誘導は筋肉発生と類似した機構によって骨髄間葉系細胞から骨格筋が誘導されているものと思われた。

この分化した最終産物には増殖可能な①単核の筋芽細胞（MyoD陽性)、②骨格筋の幹細胞である筋衛生細胞（Pax7陽性)、そして③成熟した多核の骨格筋細胞の３種類の骨格筋系譜の細胞が含まれていた。

骨髄には造血系幹細胞の中には一部、筋系の幹細胞の存在が示唆されている。我々は本誘導がこれらの極く少数の幹細胞から分化したのか、あるいは接着性の大量に増殖している骨髄間葉系細胞から分化したのかを調べるためにCD34, CD45, c-kit陰性の細胞をFACSによって分離し、同様の誘導操作をおこなったところ、同様の時間経過で多核の成熟した骨格筋を誘導することが出来た。従って、本誘導は骨髄間葉系細胞に含まれる極一部の筋系幹細胞が分化したものではなく、大多数を占める接着性の骨髄間葉系細胞から誘導されるものと考えられた。

健常犬のビーグル犬骨髄液から骨髄間葉系細胞を誘導したところ、ヒトおよびラットと同様にPax7, MyoD、Myogenin陽性の骨格筋系細胞群を得ることが出来た。骨格筋マーカーの発現を定量的に調べるためにヒトおよびビーグル犬から誘導した細胞におけるMyoD, Pax7, Myogenin, MEF2Aの発現を検討した。その結果、誘導前の無処理の細胞では、ヒト、イヌいずれにおいてもMyoD, Pax7, Myogeninの発現は見られず（ただしMEF2Aは発現が認められる)、誘導に伴って顕著な定量的上昇が確認された。免疫染色においてもほぼ同様の傾向が見られた。

安全性の確認としてヒトおよびイヌから誘導した細胞の核型検査を行なった。その結果、いずれのサンプルにおいても染色体の欠損、転座などの変異は認められないことが確認された。

また腫瘍形成の有無をみるために、ヒトから誘導した細胞（donar #1 (n=10), #2 (n=7) いずれも１０～５０万細胞)、positive controlとしてヒト腫瘍性細胞であるrhabdomyosarcoma（１０～５０万細胞, n=10), negative controlとしてPBS (n=10) を注入し、６ヶ月間体重推移、全身状態の観察および移植６ヵ月後の全身および移植した大腿筋の病理検査を行なった。その結果、rhabdomyosarcomaでは２匹で死亡、２匹で腫瘍形成が認められたがPBSでは全匹異常がなかった。さらにdonar #1, #2から誘導したヒトの細胞でも全く異常がみられず、病理変化でも腫瘍形成は認められなかった。

D．考察

骨髄間葉系細胞は再生医療に用いる候補として多くの利点があるために、本研究で見出された方法は筋ジストロフィーの治療方法につながる可能性が期待される。我々の方法ではヒト、ラットのみならず、イヌなどの高等哺乳類の骨髄間葉系細胞からも誘導されていることから、本誘導法は汎用性の高い方法であると同時に「自己細胞移植治療」への発展が期待される。ただし、筋ジストロフィーは遺伝子変異があるために、おそらく近親者や同じ HLA サブタイプの骨髄間葉系細胞を利用した同種移植が現実的であろうと思われる。
安全性はヒトへの応用において重要な要点であるが、誘導細胞の核型検査やヌードマウスでの腫瘍化試験の結果、際立った危険性は無いと推察される。さらに犬での有効性・安全性の確認はヒトへの応用に向けて非常に大きな意義があると考えられ、今後の推進すべき課題として認識している。

E．結論

実用性の高い骨髄間質細胞から、骨格筋を効率よく誘導する方法を見出した。特に Pax7 陽性の筋衛星細胞様細胞が含まれており、生体由来の骨格筋幹細胞（筋衛星細胞）はこれまで培養が困難とされていたので本研究は特に大きな意義がある。

F．健康危険情報

なし

G．研究発表

I．論文発表

＜英文＞

【欧文原著】

1. Shimizu S, Kitada M, Ishikawa H, Itokazu Y, Wakao S, Dezawa M.
Peripheral nerve regeneration by the in vitro differentiated-human bone marrow stromal cells with Schwann cell property.
Biochem Biophys Res Commun. 359: 915-920, 2007.

2. Yoshihara T, Ohta M, Itokazu Y, Matsumoto N, Dezawa M, Suzuki Y, Taguchi A, Watanabe Y, Adachi Y, Ikehara S, Sugimoto H,Ide C. Administration of Bone Marrow-Drived Mononuclear Cells After Spinal Cord Injury Suppresses Cavity Formation and is Followed by Functional Recovery.
J Neurotrauma 24: 1026-1036, 2007.

3. Ishikawa N, Suzuki Y, Ohta M, Cho H, Suzuki S, Dezawa M, Ide C.
Peripheral nerve regeneration through the space formed by a chitosan gel sponge.
J Biomed Mater Res A. (in press) 2007

【欧文総説】

1. Dezawa M, Nabeshima Y-I.
Transdifferentiation systems in bone marrow stromal cells and its application to muscle dystrophy: Insights into cell-based therapy.
"Recent Advances of Skeletal Muscle Differentiation" in Research Signpost (in press).

2. Dezawa M
Induction system of neuronal and muscle cells from bone marrow stromal cells and applications for degenerative diseases.
Inflammation and Regeneration, 27(2), 96-101, 2007.

3. Dezawa M.
The unexpected discovery of neuronal and muscle induction systems in bone marrow

stromal cells; insights into auto-transplantation therapy.
Medical Molecular Morphology (in press)

4. Kidata M & Dezawa M.
Differentiation system of neural and muscle lineage cells from bone marrow stromal cells;
a new strategy for tissue reconstruction in degenerative diseases Histology and Histopathology (in press)

＜和文＞

【和文著書】

1. 出澤真理：
骨髄間葉系細胞からの骨格筋細胞の誘導
田畑泰彦編、遺伝子医学MOOK (in press)

2. 出澤真理：
骨髄間葉系細胞からの神経細胞の誘導
田畑泰彦編、遺伝子医学MOOK (in press)

【和文総説】

1. 国府田正雄、出澤真理：
骨髄間葉系細胞を用いた脊髄損傷修復の試み　脳２１　10(2)；144-147, 2007

2. 石川裕人、出澤真理：
視神経移植とは「視覚と眼球運動のすべて」メディカルビュー社p. 216-221, 2007

3. 出澤真理：
骨髄間葉系細胞を用いた筋ジストロフィーへの再生医療の可能性　BRAIN and NERVE-神経研究の進歩 59; 503-508, 2007

4. 出澤真理
胚葉をこえた多分化能と自己細胞移植治療への可能性　蛋白質核酸酵素　シリーズ「幹細胞技術の現状と展望」52(2): 158-165, 2007.

5. 出澤真理
骨髄間葉系細胞からの神経・骨格筋系細胞の誘導　再生医療 16: 19-25 2007

6. 北田容章　出澤真理
神経・筋変性疾患における細胞移植治療；骨髄間葉系細胞を用いた自己細胞移植への可能性　Clin Neurosci, (in press)

Ⅱ．学会発表

【シンポジウム・特別講演 】

1. 出澤真理　骨髄間葉系細胞の分化誘導システムを用いた自己細胞移植治療への挑戦　第２８回日本炎症・再生学会　東京、2007.

2. 出澤真理　胚葉を越えた多分化能と自己細胞移植治療への挑戦　Translational Medicine Seminar,　箱根、2007.

3. 出澤真理　骨髄間葉系細胞を用いた神経・筋疾患への自己細胞移植治療の可能性　第２３回日本DDS学会　熊本、2007.

4. Dezawa M. Insights into auto-cell transplantation: the unexpected discovery of specific induction systems in bone marrow stromal cells. The 5th Annual Congress of IDDST, Shanghai, China, 2007.

5. 出澤真理　骨髄間葉系細胞を用いた脊髄再生治療の可能性　日本脊髄基金講演会　東京、2007.

6. 出澤真理　思わぬ発見のもたらした分化

誘導システム：骨髄間葉系細胞の分化誘導システムの確立と神経・筋疾患への応用の可能性。発生工学・疾患モデル研究会 東京, 2007.

7. 出澤真理　胚葉を越えた多分化能と自己細胞移植治療への可能性　第９６回日本病理学会総会　大阪、2007.

8. 出澤真理　自己細胞移植治療法の開発：思わぬ発見のもたらした分化誘導システム。　第１３回阪神小児神経筋疾患研究会、大阪、2007.

9. Dezawa M. The discovery of specific induction system in bone marrow stromal cells: Insights into auto-transplantation in neuro- and muscle-degenerative diseases. 17th Lake Shirakaba Conference, Aurora in Regeneration, Vedbaek, Denmark, October, 2007

10. Dezawa M. The unexpected discovery of specific induction systems in bone marrow stromal cells and application for degenerative diseases. The International Stem Cells Conference, Agrigento, Italy, October, 2007

11. Dezawa M. Bone marrow stromal cells from auto-cell transplantation therapy: applications for neuro- and muscle-degenerative diseases. The 9th US-Japan symposium on Drug Delivery Systems. Hawaii, 2007 December

Ｈ．知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

骨髄間質細胞から誘導した筋細胞の移植法の確立

分担研究者　　鍋島　陽一
京都大学大学院医学研究科・教授

研究要旨
筋ジストロフィなどの筋変成疾患の原因については解析が飛躍的に進み、その治療法の開発が次の課題として浮かび上がり、薬物の開発、遺伝子治療、細胞移植治療などの多面的な試みが進められている。このような中で、細胞治療に活用できる細胞の樹立とその機能評価、安全性の確認について研究を進めてきた。我々は大量に培養可能な骨髄間質細胞から、骨格筋を極めて高い効率で誘導する画期的な方法を見出し、引き続いてラット、ヒト、サル、イヌの骨髄間質細胞より筋芽細胞、筋衛生細胞を誘導する方法を確立し、その分化転換機構の解析を進めた。

A．研究目的

骨髄間葉系細胞は患者の骨髄液から容易に採取可能な接着性の間葉系細胞であり、旺盛な増殖能力を備えているので、細胞移植治療に必要な細胞数確保が可能である。また、免疫拒絶や倫理問題のハードルが低く、更に骨髄バンクがすでに稼動しており、何らかの理由で本人の細胞が利用できない場合でも、骨髄バンクを用いることによって、供給源を広げることができる。

細胞移植治療の目的は、変性過程で失われた細胞を補充することであり、目的とする細胞への分化制御機構の解析と実用化に向けた方法を検討する必要がある。本研究では骨髄間葉系細胞から効率よく安全に骨格筋細胞を誘導する方法の開発を行うことを目的として、分化転換、誘導システムの分子機構を検討する。

B．研究方法

骨髄間質細胞はラット、ヒトの骨髄穿刺液から樹立した接着性細胞を用いた。

（1）骨髄間質細胞からの骨格筋誘導：
骨髄間葉系細胞を特定の密度(1700~2000 cells/cm2)でまいた後、サイトカイン投与（bFGF, Forskolin, PDGF, Neuregulin）を行い、その後にNotch細胞質ドメイン(Notch intracellular domain; NICD)および、ラムドメイン，アンキリンドメイン、転写活性化ドメインを持つ plasmid を lipofection を用いて導入し、G418 にて選択する。その後、confluent に達したところで分化培地（ウマ血清２％）に移すことによって多核の格筋を誘導した。

（2）Notch結合蛋白の検索
筋細胞屁の分化転換の鍵は Notch の役割を解明することであり、その一環としてNotchに結合する蛋白の解析を進めた。骨格筋誘導に必要な領域がアンキリンドメインであったことから、このドメインと GFP キメラ蛋白を発現させ、結合蛋白の候補を免疫沈降により分離し、質量分析により解析を進めている。

（倫理面への配慮）本プロジェクト全般において組み換え DNA 実験とモデル作成・移植に

おける動物実験は各所属機関の組み換え DNA 実験委員会と動物実験委員会の指針に従って研究計画書を提出し、医学部長、研究所長、委員会長等の機関承認を得た後に実施した。

骨髄間質細胞の分化誘導並びに分子生物学的解析は京都大学を中心に行うが、すでに京都大学大学院医学研究科・医学部動物実験委員会の承認を得ている(「骨髄間質細胞の骨格筋細胞への分化誘導と移植応用」承認番号：MedKyo04245)。ヒト骨髄間質細胞は BioWhittaker 社の細胞を用いるので倫理上問題はない。Notch の遺伝子導入実験は京大の組み替え DNA 実験委員会の了承を得て行なっている(「体細胞の神経系および骨格筋細胞への分化転換の遺伝子機構について」(承認番号：研研２第２２４−2号)。

C．研究成果

（1）骨髄間質細胞からの骨格筋誘導：

ラット、ヒト、サル、イヌから骨格筋細胞を誘導することに成功し、ヒト骨格筋細胞の安全性試験を行い、高い安全性を確認した。

骨髄間質細胞にサイトカイン投与後、; NICD、ラムドメイン, アンキリンドメ イン,転写活性化ドメインを個別に発現させ、筋細胞分化誘導活性を測定したところ、アンキリンドメインに基本的な活性があることが明らかとなった。そこで、NICD-GFP、アンキリン-GFP からなるキメラ蛋白を発現させ、抗 GFP 抗体により免疫沈降を行った。次いで、得られた沈降物を SDS ゲル電気泳動で分離し、コントールと比較し、特異的に見いだされるバンドを切り出し、質量分析にて解析した。多数の候補蛋白がノミネートされているが、その中のどれが中心的な役割を担っているか結論が得られていない。また、アンキリン-GFP キメラ蛋白を多量に合成し、アフィニティカラムを作成し、細胞抽出液をかけて結合する蛋白を回収し、質量分析を進めている。

骨髄間質細胞から分化転換によって誘導された筋細胞分画には多核の筋管細胞、筋芽細胞、筋衛生細胞が含まれており、筋衛生細胞を分離増殖させることが重要と考えており、効率的に精製する方法を検討し、新しい方法の確立にとって重要な点が明らかになりつつあり、２００８年度中には確立できると考えている。

D．考察

Notch は Hes の発現を誘導して分化を抑えると考えられてきたが、本誘導システムでは Notch が筋細胞、神経細胞への分化を誘導するシグナルとなっており、しかもアンキリンドメインのみでもゆうどうできる事実は Notch にはこれまで知られていない未知の機能があることを示唆しており、極めて興味深い。

E．結論

我々は大量に培養可能な骨髄間質細胞から、骨格筋を極めて高い効率で誘導する画期的な方法を見出し、引き続いてラット、ヒト、サル、イヌの骨髄間質細胞より筋芽細胞、筋衛生細胞を誘導する方法を確立し、その分化転換機構の解析を進めた。特に Notch に結合する蛋白の解析を進めた。

F．健康危険情報

なし

G．研究発表

I．論文発表

＜英文＞

【欧文原著】

1. Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S, Ide C, Nabeshima Y.

Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* 309(5732): 314-317, 2005.

＜和文＞

【和文総説】

1. 出澤真理、鍋島陽一, 骨髄間質細胞からの筋細胞の誘導:自己細胞移植治療の実現化に向けて、医学のあゆみ 216(2): 188-190, 2006

2. 出澤真理、鍋島陽一, 自己細胞移植治療の実現化に向けて:骨髄間質細胞を用いた筋細胞の誘導、難病と在宅ケア 11(11):56-59, 2006.

3. 出澤真理、鍋島陽一 自己細胞移植治療に向けて：骨髄間質細胞を用いた筋細胞の誘導、実験医学 23: 2811-2814, 2005.

H. 知的所有権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名，巻号：ページ，出版年
Yuasa K, Nakamura A, Hijikata T, Takeda S.	Dystrophin deficiency in canine X-linked muscular dystrophy in Japan (CXMDJ) alters myosin heavy chain expression profiles in the diaphragm more markedly than in the tibialis cranialis muscle	***BMC Musculoskeletal Disorders,*** 9; 9(1): 1, 2008
Fukada SI, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y, Takeda S.	Molecular Signature of Quiescent Satellite Cells In Adult Skeletal Muscle	***Stem Cells.,*** 25(10): 2448-59, 2007
Yuasa K, Yoshimura M, Urasawa N, Ohshima S, Howell JM, Nakamura A, Hijikata T, Miyagoe-Suzuki Y, Takeda S.	Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products	***Gene Ther.,*** 14(17): 1249-1260, 2007
Suzuki N, Motohashi N, Uezumi A, Fukada SI, Yoshimura T, Itoyama Y, Aoki M, Miyagoe-Suzuki Y, Takeda S.	NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS	***J Clin Invest.,*** 4; 117(9): 2468-2476, 2007
Ikemoto M, Fukada SI, Uezumi A, Masuda S, Miyoshi H, Yamamoto H, Wada MR, Masubuchi N, Miyagoe-Suzuki Y, Takeda S.	Autologous Transplantation of SM/C-2.6 (+) Satellite Cells Transduced with Micro-dystrophin CS1 cDNA by Lentiviral Vector into mdx Mice	***Mol Ther.,*** 28; 15(12): 2178-85, 2007
Miyagoe-Suzuki Y, Uezumi A, Takeda S.	Side population (SP) cells and skeletal muscle differentiation	***Recent Advances of Skeletal Muscle Differentiation,*** in press

Dezawa M, Nabeshima Y-I.	Transdifferentiation systems in bone marrow stromal cells and its application to muscle dystrophy: Insights into cell-based therapy	***Recent Advances of Skeletal Muscle Differentiation,*** in press
Dezawa M.	Induction system of neuronal and muscle cells from bone marrow stromal cells and applications for degenerative diseases	***Inflammation and Regeneration,*** 27(2), 96-101, 2007

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Dystrophin deficiency in canine X-linked muscular dystrophy in Japan ($CXMD_J$) alters myosin heavy chain expression profiles in the diaphragm more markedly than in the tibialis cranialis muscle

Katsutoshi Yuasa*[1,2], Akinori Nakamura[2], Takao Hijikata[1] and Shinichi Takeda[2]

Address: [1]Department of Anatomy and Cell Biology, Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Musashino University, Nishi-tokyo, Tokyo 202-8585, Japan and [2]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan

Email: Katsutoshi Yuasa* - k_yuasa@musashino-u.ac.jp; Akinori Nakamura - anakamu@ncnp.go.jp; Takao Hijikata - hijikata@musashino-u.ac.jp; Shinichi Takeda - takeda@ncnp.go.jp

* Corresponding author

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Abstract

Background: Skeletal muscles are composed of heterogeneous collections of muscle fiber types, the arrangement of which contributes to a variety of functional capabilities in many muscle types. Furthermore, skeletal muscles can adapt individual myofibers under various circumstances, such as disease and exercise, by changing fiber types. This study was performed to examine the influence of dystrophin deficiency on fiber type composition of skeletal muscles in canine X-linked muscular dystrophy in Japan ($CXMD_J$), a large animal model for Duchenne muscular dystrophy.

Methods: We used tibialis cranialis (TC) muscles and diaphragms of normal dogs and those with $CXMD_J$ at various ages from 1 month to 3 years old. For classification of fiber types, muscle sections were immunostained with antibodies against fast, slow, or developmental myosin heavy chain (MHC), and the number and size of these fibers were analyzed. In addition, MHC isoforms were detected by gel electrophoresis.

Results: In comparison with TC muscles of $CXMD_J$, the number of fibers expressing slow MHC increased markedly and the number of fibers expressing fast MHC decreased with growth in the affected diaphragm. In populations of muscle fibers expressing fast and/or slow MHC(s) but not developmental MHC of $CXMD_J$ muscles, slow MHC fibers were predominant in number and showed selective enlargement. Especially, in $CXMD_J$ diaphragms, the proportions of slow MHC fibers were significantly larger in populations of myofibers with non-expression of developmental MHC. Analyses of MHC isoforms also indicated a marked increase of type I and decrease of type IIA isoforms in the affected diaphragm at ages over 6 months. In addition, expression of developmental (embryonic and/or neonatal) MHC decreased in the $CXMD_J$ diaphragm in adults, in contrast to continuous high-level expression in affected TC muscle.

Conclusion: The $CXMD_J$ diaphragm showed marked changes in fiber type composition unlike TC muscles, suggesting that the affected diaphragm may be effectively adapted toward dystrophic stress by switching to predominantly slow fibers. Furthermore, the MHC expression profile in the $CXMD_J$ diaphragm was markedly different from that in *mdx* mice, indicating that the dystrophic dog is a more appropriate model than a murine one, to investigate the mechanisms of respiratory failure in DMD.

Background

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by mutations in the dystrophin gene, which encodes a large sub-sarcolemmal cytoskeletal protein, dystrophin. DMD is characterized by a high incidence (1 in 3,500 boys) and a high frequency of *de novo* mutation [1]. The absence of dystrophin is accompanied by the loss of dystrophin-associated glycoprotein complex from the sarcolemma, leading to reduce membrane stability of myofibers. This dysfunction results in progressive muscle weakness, cardiomyopathy, and subsequent early death by respiratory or heart failure in DMD patients.

For basic and therapeutic studies of DMD, it is very important to perform analysis and evaluation using dystrophin-deficient animal models, such as the *mdx* mouse and dystrophic dog. The *mdx* mouse has been well utilized in many DMD studies, but the murine model shows moderate dystrophic changes unlike severe human DMD [2]. In contrast, golden retriever muscular dystrophy (GRMD) shows similar dystrophic phenotypes to those of human patients: elevated serum CK level, gross muscle atrophy with joint contracture, cardiomyopathy, prominent muscle necrosis, degeneration with mineralization and concurrent regeneration, and endomysial and perimysial fibrosis [3]. Therefore, the dystrophic dog is more suitable than the *mdx* mouse for studies to gain insight into the pathogenic and molecular biological mechanisms of human DMD, as well as for pre-clinical trials [4]. Therefore, we have recently established a colony of beagle-based canine X-linked muscular dystrophy in Japan ($CXMD_J$) [5], and have demonstrated that $CXMD_J$ also exhibited severe symptoms similar to GRMD. To date, we have utilized the littermates of the $CXMD_J$ colony for pathological [6,7], molecular biological [8], and therapeutic examinations [9] of DMD.

Skeletal muscles are composed of heterogeneous populations of muscle fiber types, which contribute to a variety of functional capabilities. In addition, muscle fibers can adapt to diverse situations, such as aging, exercise, and muscular diseases, by changing fiber size or fiber type composition. Therefore, it is important to analyze fiber types to evaluate the condition of skeletal muscle with disease. Fiber types can be distinguished by biochemical, metabolic, morphological, and physiological properties. One of the most informative methods for identification of fiber types is detection of myosin heavy chain (MHC) [10,11]. Myofibers express various MHC isoforms containing slow (type I), fast (types IIA, IIX, IIB), embryonic, and neonatal forms. MHC expression, however, seems to differ between animal species and muscle types. Three MHC isoforms (types I, IIA, and IIX) have been identified in limb skeletal muscles of human and dog, while the fourth isoform, MHC IIB, is abundantly present in small mammals including mouse [10,11]. In addition, expression profiles of MHCs in dystrophin-deficient muscles have been widely examined in limb skeletal muscles of DMD patients [12] and animal models, such as the *mdx* mouse [13] and GRMD [14], but it has not been fully analyzed in skeletal muscles of a canine model. Furthermore, expanded studies of the diaphragm were restricted to that of the *mdx* mouse [13,15]. Therefore, it is important to perform detailed evaluation of fiber types and fiber sizes in limb skeletal muscles and the diaphragm of $CXMD_J$ to understand adaptations toward disease by changes in fiber type composition in the skeletal muscles of human DMD.

In this study, to investigate fiber types of myofibers in dystrophin-deficient skeletal muscles of dystrophic dogs, we evaluated the expression profiles of MHCs in tibialis cranialis (TC) muscles and diaphragms of $CXMD_J$ at various ages, by immunohistochemical and electrophoretic techniques. Briefly, we detected myofibers expressing fast type, slow type, and/or developmental MHCs. In addition, the numbers of fast or slow MHC fibers and the size distribution of these myofibers were analyzed among populations of muscle fibers with or without developmental MHC. The composition of MHC isoforms was also examined in pairs of normal and affected dogs at various ages. This is the first report of evaluation of the detailed distribution of fiber types in TC muscles and diaphragms of dystrophic dogs.

Methods

Animals

Experimental dogs were wild-type and dystrophic littermates at ages from 1 month to 3 years, from the beagle-based $CXMD_J$ breeding colony at National Center of Neurology and Psychiatry (Tokyo, Japan) [5,6]. Within a few days after birth, the genotypes (wild-type, carrier, or dystrophy) of the littermates were determined by a snapback method of single-strand conformation polymorphism (SSCP) analysis [16], and the phenotypes were also confirmed by measuring serum CK level [5]. All animals were cared for and treated in accordance with the guidelines approved by Ethics Committee for Treatment of Laboratory Animals at NCNP, where three fundamental principles (replacement, reduction, and refinement) were also considered. Adult control and $CXMD_J$ dogs (10 months to 3 years) were analyzed in early experiments (three to six animals). Series consisting of a pair of a normal dog and an affected littermate at ages of 1, 2, 4, 6 months, or 1 year old were examined in subsequent experiments. TC muscles and diaphragms were removed from the dogs after necropsy, in which euthanasia was performed by exsanguination under anesthesia with isoflurane taken to prevent unnecessary pain. TC muscle was used as a

representative limb skeletal muscle, and it corresponds to the tibialis anterior muscle in mice and humans. The muscle blocks were divided into pieces and frozen immediately in isopentane pre-cooled with liquid nitrogen.

Histological and immunohistochemical analysis

Serial transverse cryosections (10 μm thick) were stained with hematoxylin and eosin (H&E), and immunostained using anti-MHC antibodies. Immunohistochemistry was performed as described previously [17]. Cryosections were incubated with the following primary antibodies: mouse monoclonal antibodies against fast type MHC (NCL-MHCf; Novocastra), slow type MHC (NCL-MHCs), and developmental MHC (NCL-MHCd). The primary antibodies were detected using a Vectastain® ABC kit (Vector Laboratories) and then visualized with diaminobenzidine. Images were recorded using a microscope (Eclipse E600; Nikon) equipped with a CCD camera (HV-D28S; Hitachi), and fiber types of individual myofibers from 400 to 1200 per muscle were identified, based on serial sections immunostained with three types of MHC antibodies. Subsequently, the fiber number of each group was counted, and fiber sizes were also measured using Image-Pro Plus (Media Cybernetics). Furthermore, the differences in MHC expression between two groups (normal, dMHC (-) *vs* affected, dMHC (-); affected, dMHC (-) *vs* affected, dMHC (+)), between muscles (TC muscle *vs* diaphragm), or among ages (1, 2, 4, 6 months, and 1 year) were evaluated by Yates's chi-square test.

Myosin extraction and gel separation

Myosin was extracted on ice for 60 min from cryosections, as described previously [18,19]. MHC isoforms were separated on 8% SDS-polyacrylamide gels containing 30% glycerol, according to the methods described previously [19,20] with some modifications. Briefly, aliquots of 0.4 μg of total protein were loaded in each well of mini-gels (Bio-Rad). Electrophoresis was carried out at 60 V at 5°C for 48 h using upper buffer containing additional 10 mM 2-mercaptoethanol. The gels were stained with silver, and the image was scanned and analyzed using NIH image.

Results

MHC expression in TC muscle and diaphragm of adult CXMD$_J$

To investigate the relationship between the pathology and fiber types in dystrophic skeletal muscles of CXMD$_J$, we first examined histological features and MHC expression in TC muscles and diaphragms of normal and affected dogs at adult stages (10 months to 3 years old) (Fig. 1). In H&E-stained sections, affected muscles exhibited some dystrophic characteristics, such as necrosis, regeneration, cellular infiltration, fibrosis, fiber splitting, and fiber size variation. Especially, clusters of infiltrating cells were prominently observed in TC muscles, while endomysial fibrosis was predominant in diaphragms.

We next detected expression of fast and slow type MHCs for fiber type identification, and further examined developmental MHC, which means neonatal and/or embryonic MHC, as a marker of regenerating fibers (Fig. 1). In TC muscles and diaphragms of adult normal dogs, individual myofibers showed expression of either fast or slow type MHC. In affected TC muscles, the proportions of fast or slow MHC fibers were similar between normal and affected muscles. In addition, large numbers of developmental MHC-expressing fibers were observed in clusters, and many of these fibers co-expressed fast type MHC. In the affected diaphragms, the numbers of fast MHC fibers were much lower than in the normal counterparts, and slow type MHC was expressed in almost all fibers. Furthermore, the numbers of developmental MHC fibers were less than in affected TC muscle, and almost all of these fibers co-expressed slow type MHC, unlike TC muscle. These results indicated that the influences of dystrophin deficiency on MHC expression are significantly different between TC muscle and the diaphragm of CXMD$_J$, suggesting that the diaphragm would be more greatly influenced with regard to the composition of fiber types and muscle regeneration than TC muscle.

MHC expression and fiber size distribution

To further evaluate the size distribution of individual myofibers related to MHC expression, we measured transverse areas of all muscle fibers within one area in TC muscle or diaphragm of adult CXMD$_J$ (Fig. 2 and Table 1). We then analyzed three types of MHC-positive fibers (fast, slow, and hybrid) among populations of myofibers expressing fast and/or slow type MHC(s) together with or without developmental MHC, which were defined as regenerating or non-regenerating fibers, respectively. In non-regenerating fibers of affected TC muscle and diaphragm, the proportion of slow MHC fibers increased and these fibers showed a larger size distribution than those in the normal counterparts, indicating increased number and enlarged fiber size of slow fibers (Fig. 2B and Table 1). Interestingly, fast MHC fibers disappeared in the adult CXMD$_J$ diaphragm.

In regenerating fibers of both affected muscles, the distributions of all three populations shifted to smaller sizes than those in the normal counterparts, and a large number of hybrid fibers co-expressing fast and slow type MHCs were observed at a high rate (Fig. 2C and Table 1). In addition, fast MHC fibers were predominant in a regenerating population in TC muscle, while slow MHC fibers were predominant in the diaphragm except for hybrid fibers. These observations suggested that fast fibers could be more susceptible to dystrophic stress than slow fibers, and

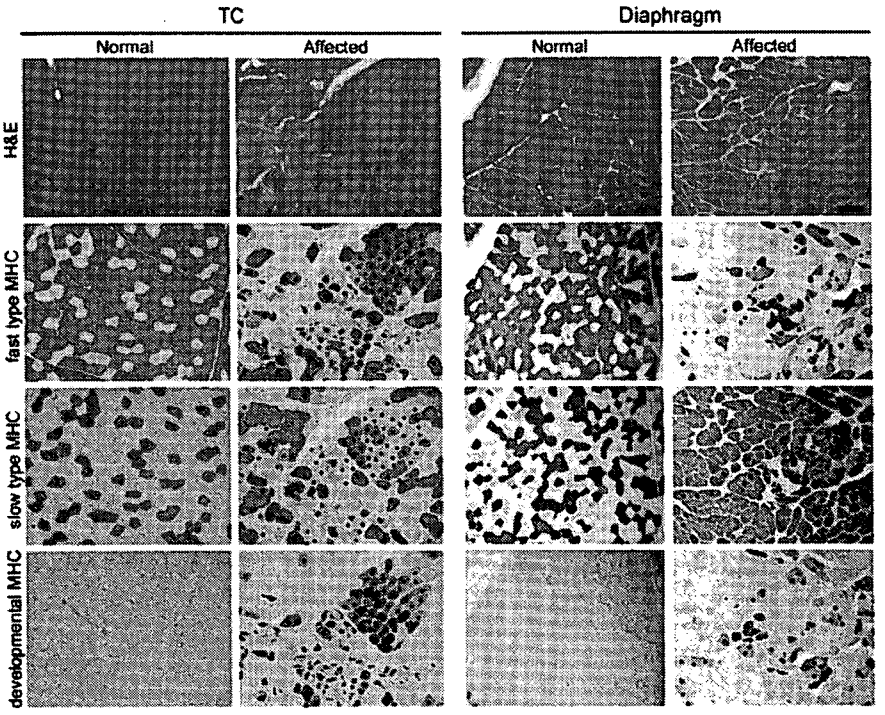

Figure 1
Representative images of histology (H&E) and expression of fast type, slow type, or developmental myosin heavy chain (MHC) in tibialis cranialis (TC) muscle and diaphragm of a normal (10 months old) or a CXMD$_J$ dog (11 months old). Identical parts of serial cross-sections are shown in longitudinal panels. In panels of affected muscles, dots show the fibers expressing developmental MHC. Bar: 200 μm.

alteration of MHC expression and regeneration of muscle fibers would be different between TC muscle and the diaphragm.

Time courses of histology and MHC expression

To investigate how MHC expression alters together with growth of CXMD$_J$, we examined MHC expression in TC muscles and diaphragms of a normal or an affected littermate at various ages from neonatal to adult stages (1 month to 1 year old) in relation to histopathological features. Affected TC muscles showed mild lesions at 1 and 2 months old, but severe degenerative lesions were evident at over 4 months old (Fig. 3). Expression of fast or slow type MHC did not alter much with aging, and developmental MHC was expressed continuously (Fig. 4). In contrast, degenerative lesions were severe in the affected diaphragm at all ages examined (from 1 month old onward), and endomysial fibrosis was dominantly present over 6 months old (Fig. 3). Fast MHC fiber number decreased markedly, while the number of slow MHC fibers increased significantly in affected diaphragms after 6 months old (Fig. 5). In addition, expression of developmental MHC decreased at 6 months and 1 year old. These observations indicated that MHC expression is altered greatly in the affected diaphragms after 6 months old, unlike TC muscles.

For quantitative evaluation of MHC expression in individual myofibers, we counted three types of MHC-expressing fibers among non-regenerating or regenerating populations within an area in the TC muscle or diaphragm of a normal or an affected littermate (Fig. 6). As normal muscles still expressed developmental MHC at 1 month old (Fig. 4 and 5), we performed the examinations at both adolescent (2 and 4 months old) and adult stages (10 or 11 months old). In normal dogs, the number of fast MHC fibers in TC muscle was three times greater than that of slow MHC fibers throughout aging, while the proportions in the diaphragms remained constant and equivalent between the two types (Fig. 6A). In non-regenerating fibers, the proportions of fiber types were not constant in affected TC muscles at the ages examined, but the majority of these fibers consisted of slow MHC fibers in the affected diaphragms (Fig. 6B). These observations indicated that slow fibers were already predominant in non-regenerating populations of CXMDJ diaphragms at younger ages. In

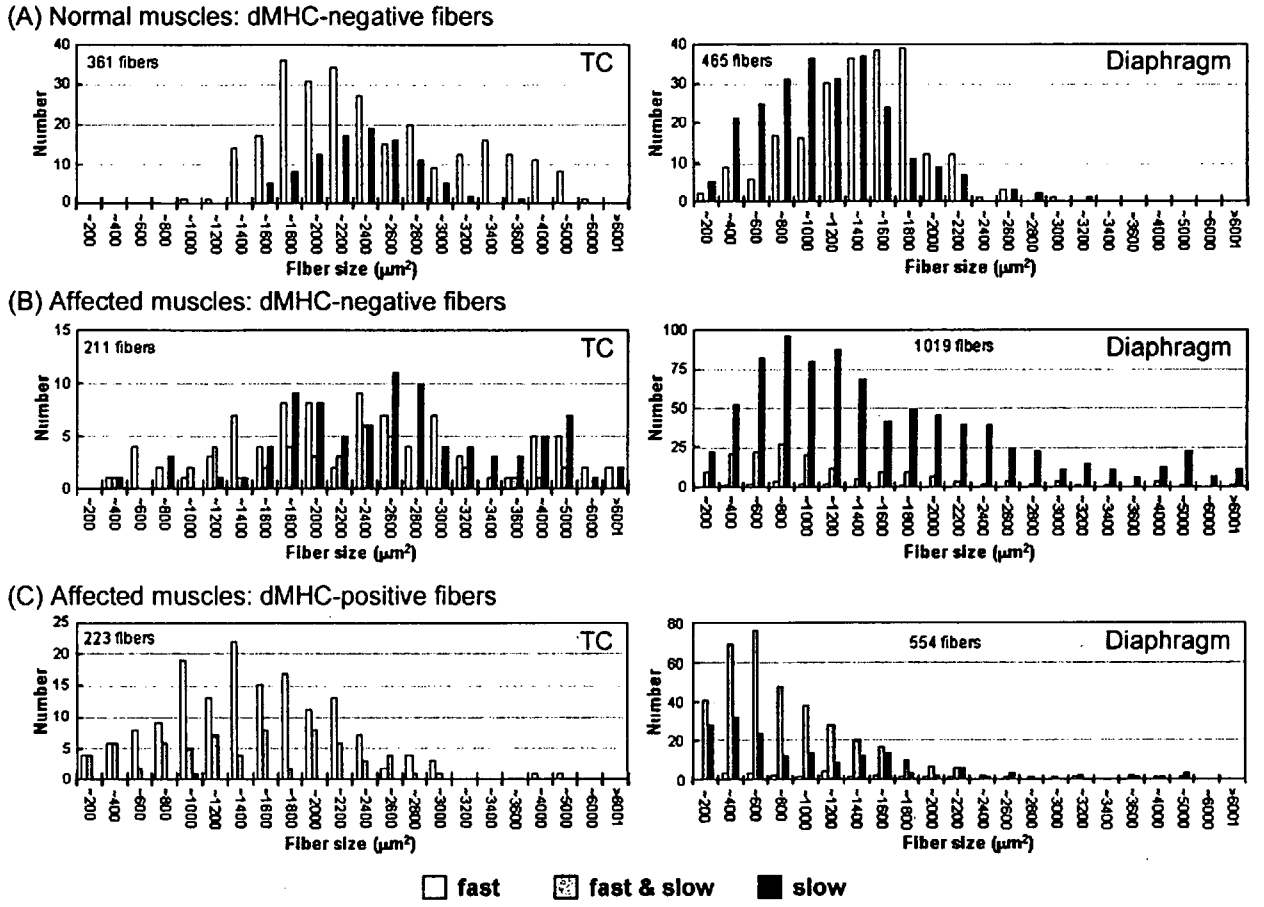

Figure 2

The size distribution of myofibers expressing fast and/or slow type MHCs in skeletal muscles of a normal (10 months old) or a CXMD$_J$ dog (11 months old). On the basis of expression of fast and slow type MHCs, all fibers within an area of TC muscle or diaphragm of a normal (A) or an affected dog (B, C) were classified into three types of MHC-positive fiber. Furthermore, fast (white), hybrid (gray), or slow MHC myofibers (black) were analyzed among populations of muscle fibers with non-expression of developmental MHC (A, B) or with expression of developmental MHC (C) in terms of fiber numbers (see Table 1) and fiber sizes (A-C). Note that larger sizes of slow MHC fibers were noticeable in populations of muscle fibers expressing fast and/or slow MHC(s) but not developmental MHC of affected muscles (B).

regenerating fibers, in contrast to the observation that fast MHC fibers consistently accounted for the majority of fibers in affected TC muscles, the affected diaphragms were mainly composed of hybrid and slow MHC fibers and the proportion increased gradually with age (Fig. 6C). These observations indicated that MHC expression in regenerating fibers was also different between affected TC muscle and diaphragm after 4 months old, although it was relatively similar in the two at 2 months old.

Temporal changes of MHC isoforms

To examine how progressive degeneration alters the composition of fiber types in affected skeletal muscles, we detected myosin isoforms in TC muscles and diaphragms of CXMD$_J$ at various ages by electrophoretic gel separation (Fig. 7). Four MHC isoforms (I, IIA, IIX, and embryonic), which migrated on electrophoresis as IIA-embryonic-IIX-I from slowest to fastest [11,12], were detected in canine skeletal muscles (Fig. 7A). In affected TC muscles, type I, IIA, and embryonic isoforms were consistently detected at similar levels, but the level of type IIX MHC was lower than those in normal TC muscles after 2 months old. In contrast, type IIA MHC level decreased gradually in affected diaphragms with growth, and type I accounted for the majority of MHC components in animals over 6 months old. In addition, the embryonic isoform

Table 1: The numbers of myofibers co-expressing fast type, slow type, and/or developmental MHCs in skeletal muscles of a normal (10 months old) or a CXMD$_J$ dog (11 months old).

	TC			Diaphragm		
	Normal	Affected		Normal	Affected	
Developmental	-	-	+	-	-	+
Fast	265 (73%)	85 (40%)	155 (70%)	222 (48%)	12 (1%)	20 (3.6%)
Fast & slow	0 (0%)	38 (18%)	67 (30%)	0 (0%)	160 (16%)	370 (66.8%)
Slow	96 (27%)	88 (42%)	1 (0%)	243 (52%)	847 (83%)	164 (29.6%)
Total	361 (100%)	211 (49%)	223 (51%)	465 (100%)	1019 (65%)	554 (35%)

The numbers of fibers analyzed were results from a normal or an affected dog. MHC expression between two groups (normal, dMHC (-) *vs* affected, dMHC (-); affected, dMHC (-) *vs* affected, dMHC (+)), or between muscles (TC muscle *vs* diaphragm) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected in all tests.

decreased in affected diaphragms after 6 months old. These results were consistent with those of immunohistochemical analyses (Figs. 4 and 5). These observations suggested that type IIX and IIA fast fibers may be preferentially affected in TC muscle and diaphragm of CXMD$_J$, respectively. Furthermore, these observations suggested that muscle regeneration may deteriorate from relatively younger age in the affected diaphragm, unlike TC muscle.

Discussion

To investigate the alterations in fiber types in skeletal muscles of a canine DMD model, we examined MHC expression in the TC muscle and diaphragm of CXMD$_J$ at various ages. Our results indicated that the influences of dys-

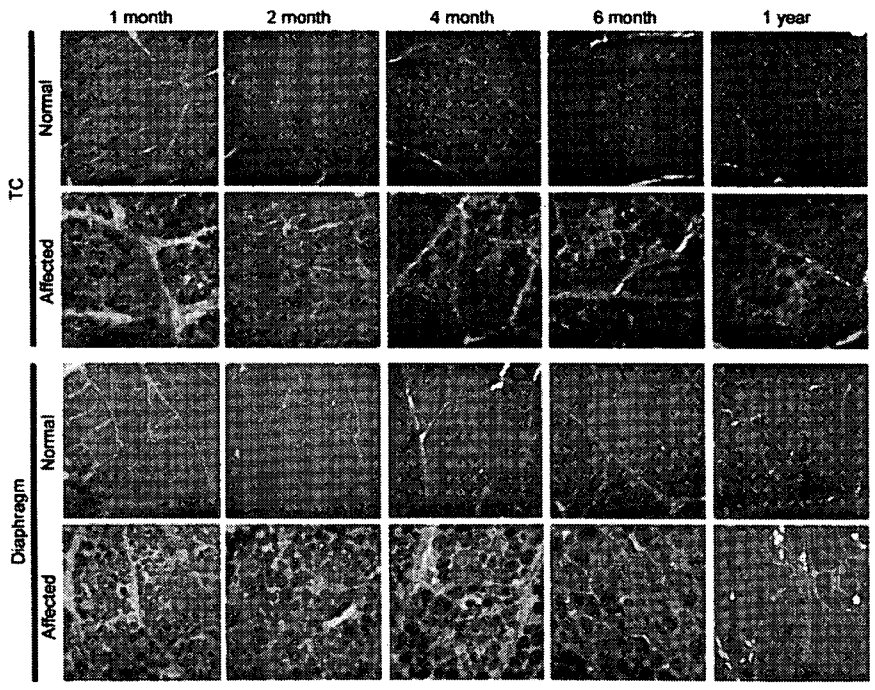

Figure 3
Representative histological findings in TC muscles and diaphragms of a normal or a CXMD$_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that severe degenerative lesions were observed from early ages in affected diaphragms, as compared with affected TC muscles. Bar: 200 μm.

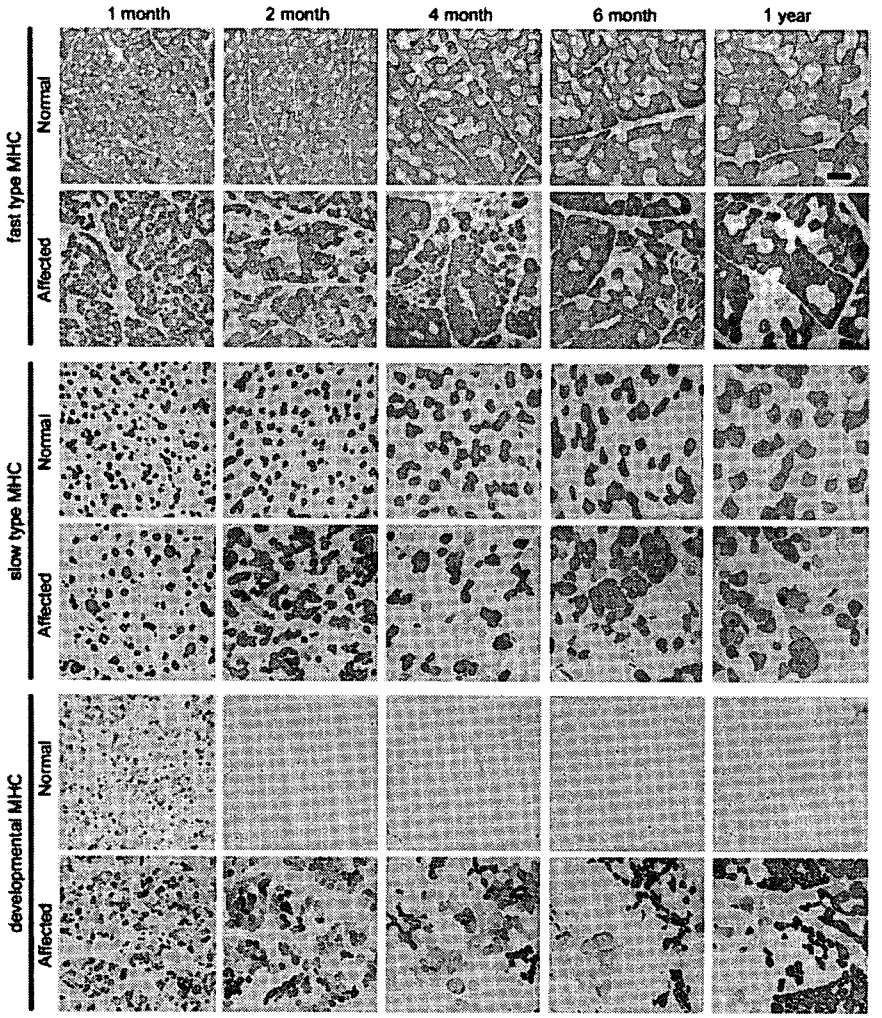

Figure 4
Expression of fast type, slow type, and developmental MHCs in TC muscles of a normal or a CXMD$_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that there were no notable differences between expression levels of fast and slow type MHCs in normal and affected TC muscles. Bar: 200 μm.

trophin deficiency on fiber type composition were significantly different between TC muscle and diaphragm.

To analyze MHC expression in details, we compared fiber type composition and fiber size distribution of MHC-expressing fibers between a normal dog (10 months old) and an affected dog (11 months old). In normal and affected dogs, body weight rapidly increased to approximately 9 kg at 4 months old, and then slightly increased to approximately 14 and 11 kg at 12 months old, respectively [5]. As body weight reflects muscle weight, muscle mass and fiber size would not extremely change in 1 month after 4 months old, especially in normal dogs. In fact, in TC muscles or diaphragms of normal dogs, there were no significant differences among compositions of fiber types and MHC isoforms after 4 months old (Fig 6 and 7). In addition, we examined normal dogs at 11, 12 and 14 months old, and affected dogs at 10, 12, 13 and 15 months old. Normal muscles of adult dogs showed similar expression of fast type, slow type, or developmental MHC at all adult ages, and affected muscles also showed similar MHC expression at examined ages (data not shown). These observations implied that there would be no significant difference in MHC expression between at 10 and 11 months old, in both of normal and affected dogs.

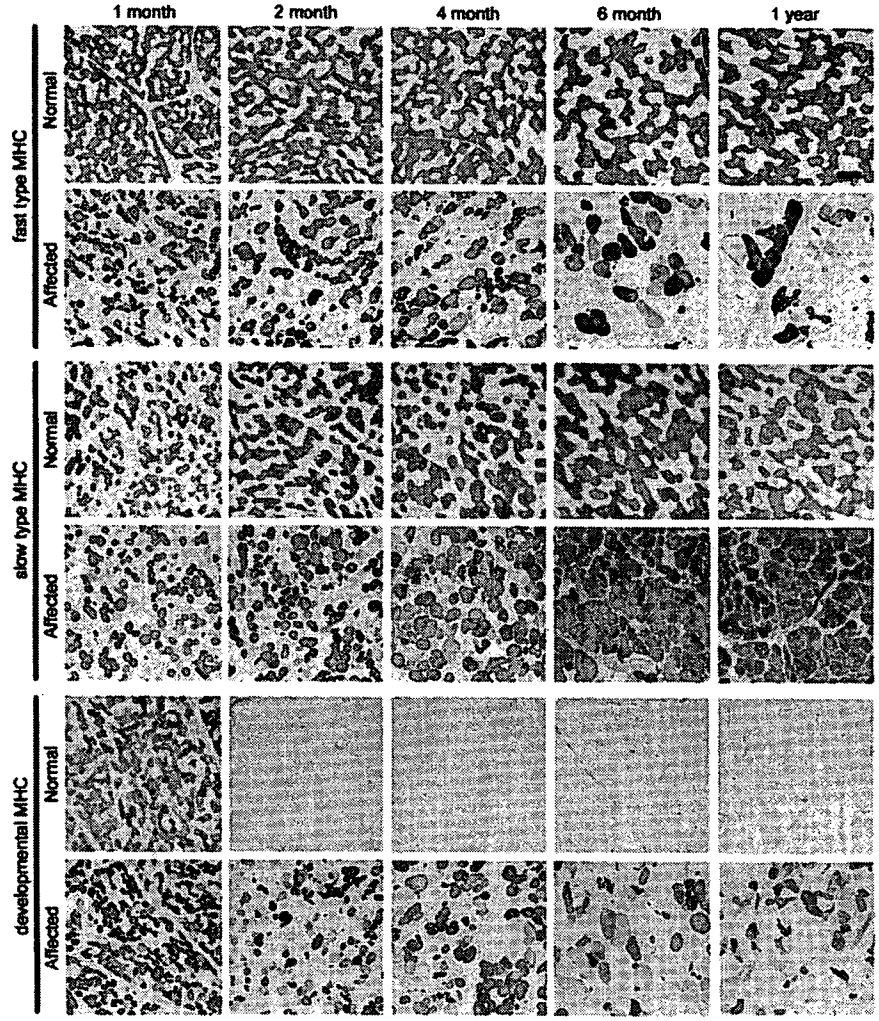

Figure 5
Expression of fast type, slow type, and developmental MHCs in diaphragms of a normal or a CXMD$_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that slow MHC fibers were increased markedly in the affected diaphragms after 6 months old, while fast MHC fibers were decreased. Bar: 200 μm.

Common features between TC muscle and diaphragm of CXMD$_J$

TC muscle and diaphragm of CXMD$_J$ shared the features that slow MHC fibers increased and enlarged selectively in non-regenerating populations, while fast type IIX or IIA MHC isoform decreased. Similar observations have been reported in skeletal muscles of the *mdx* mouse [13], GRMD [14], and human DMD [12,21]. In general, increasing and enlarging of slow fibers may be a consequence of adaptive responses by metabolic enzyme systems and energy consumption, because slow fibers have lower capacity for power output and consume less energy than fast fibers [22]. Our results also supported the hypothesis that slow fibers would be more adaptable to dystrophic stress than fast fibers, to compensate for the reduced abilities of muscle function.

Two mechanisms were considered to explain the selective increase in slow fibers during progressive muscle degeneration. One possibility is that slow fibers may be more resistant to dystrophic stress than fast fibers, leading to selective survival of slow fibers. This was supported by the observation that slower muscle fibers contained significantly more utrophin, a homolog of dystrophin, in comparison to faster counterparts [23,24]. Another is transition of MHC isoforms, where type IIA or IIX MHC

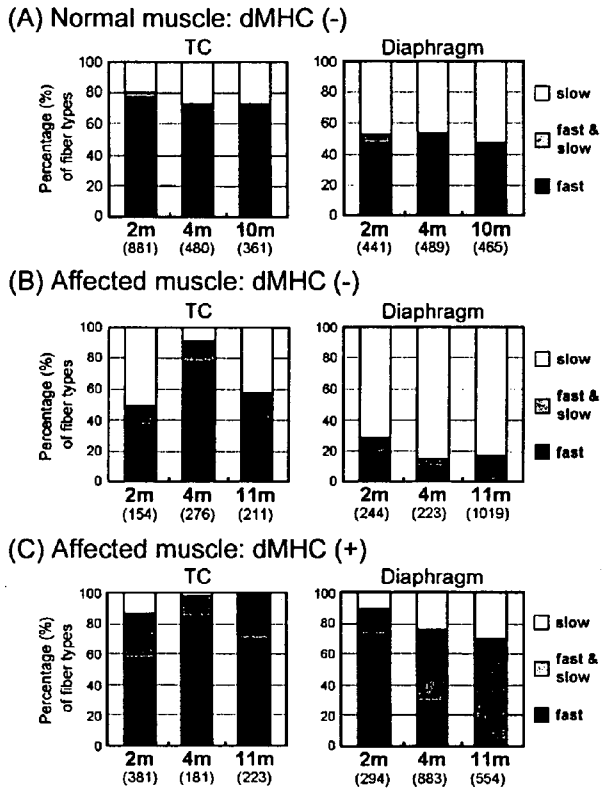

Figure 6

Proportions of fiber types in skeletal muscles of a normal or a CXMD$_J$ dog at various ages. The numbers of fast (black), hybrid (gray), and slow MHC myofibers (white) among populations of myofibers without developmental MHC (A, B) and with developmental MHC (C) were counted in TC muscle and diaphragm of a normal (A) or an affected dog (B, C) at adolescent (2 or 4 months old) or adult stages (10 or 11 months old). The numbers under the ages show total fibers examined. MHC expression between two groups (normal, dMHC (-) *vs* affected, dMHC (-); affected, dMHC (-) *vs* affected, dMHC (+)), between muscles (TC muscle *vs* diaphragm), or among ages (2, 4, and 10 or 11 months) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected in all tests, except for no significant differences between 4 and 10 months old in normal TC muscles or diaphragms. Note that slow MHC fibers were consistently larger than other fibers, in populations of muscle fibers without developmental MHC of affected diaphragms. In populations of muscle fibers co-expressing developmental MHC and other MHC isoform(s), slow MHC and hybrid fibers were increased markedly in the affected diaphragm at 4 and 11 months old, unlike TC muscles.

isoforms could be transited to type I, as seen in hypertrophy and exercise [25]. MHC I, IIa, IIx, and IIb gene expression are known to be regulated by the calcineurin pathway [26,27]. Dystrophin deficiency may accelerate MHC transition to slower types *via* calcineurin/NFAT signaling in skeletal muscles of CXMD$_J$, because calcineurin and activated NFATc1 protein content were higher in muscles from *mdx* than wild-type mice [28]. However, it remains possible that both mechanisms may be active at the same time, because the calcineurin/NFAT cascade can regulate not only the MHC promoters but also the utrophin A promoter [24,29,30].

Differences between TC muscle and diaphragm of CXMD$_J$

The CXMD$_J$ diaphragm developed severe degenerative lesions from earlier stages than TC muscle, which corresponded to previous reports [3,5,31]. In addition, dystrophic changes in the CXMD$_J$ diaphragm not only markedly altered the expression of fast and slow type MHCs but also decreased the amount of the developmental (embryonic and/or neonatal) MHC with growth, unlike affected TC muscle. Especially, fast MHC fibers disappeared and slow MHC fibers enlarged in the adult CXMD$_J$ diaphragm. The greater cross-sectional area of slow fibers in affected diaphragms might be due to hypertrophy in compensation for loss of fast fibers, relating to plasticity of muscle fibers, as mentioned above. The diaphragm keeps continuous contraction of muscle fibers without resting, while limb skeletal muscle regularly rests its movement. Therefore, replacement with slow fibers may be particularly enhanced in the diaphragm rather than TC muscle, depending on pathological severity and contractile activity of skeletal muscles.

Fiber type determination and fiber type-specific gene expression are regulated by multiple signaling pathways and transcription factors. As partially described above, a key mediator, calcineurin, plays an important role in acquisition of fiber phenotype [29,30] and may induce not only transition of MHC isoforms from faster to slower types but also transformation of myofiber phenotypes in mouse or rat muscles [26,27,32]. In addition, calcineurin signaling activity was greater in the diaphragm than in the tibialis anterior muscle of the *mdx* mouse [28]. Therefore, replacement with slow fibers may be up-regulated to a greater extent in the diaphragm than in the TC muscle of CXMD$_J$.

We also showed age-related changes of MHC expression in affected diaphragms after 6 months old, in contrast to TC muscles (Fig 4, 5 and 7). In addition, fiber type compositions in non-regenerating or regenerating fibers were also different between the TC muscle and the diaphragm, depending on age. In non-regenerating fibers of affected TC muscles, fast MHC fibers at 4 months old was higher than those at 2 and 11 months old (Fig. 6B). It might be partially involved in pathological changes that degenerative lesions appeared obviously in affected TC muscles after 4 months old, as described previously [3,5,31]. In