厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋に対するAAVベクターの安全性の検討

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研究要旨

Duchenne 型筋ジストロフィー（DMD）はジストロフィン欠損によって起こる進行性の筋変性疾患である。その遺伝子治療法を確立するために，我々は AAV ベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。*mdx* マウスでは良好な治療成績を得たので，霊長類を用いて，AAV ベクターの有効性と安全性をさらに詳細に検討した。本研究では，従来よく用いられてきた AAV-type 2 ベクターに LacZ 遺伝子又はマイクロジストロフィン遺伝子を組み込んだものをカニクイザル骨格筋へ導入し，その骨格筋組織と血清を経時的にサンプリングし，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAV ベクターによる治療が安全であるか検討した。

A．研究目的

アデノ随伴ウイルス（AAV）ベクターは骨格筋への遺伝子導入に適した特性を備えている。我々は，Duchenne 型筋ジストロフィー（DMD）に対する遺伝子治療法を確立するために，AAV ベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。ジストロフィンを欠損する *mdx* マウスにおいてマイクロジストロフィンは長期に発現し，張力も回復していた。しかし，AAV ベクターとマイクロジストロフィン遺伝子を臨床応用する前に，よりヒトに近い動物で AAV ベクターの導入に伴う細胞毒性／免疫応答を検討し，AAV ベクターの有効性と安全性を詳細に検討する必要がある。本研究では，サル骨格筋へ AAV ベクターを用いて LacZ 遺伝子又はマイクロジストロフィン遺伝子を導入し，骨格筋組織と血清を経時的に解析することにより，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAV ベクターが DMD 治療に応用できるか検討する。

B．研究方法

カニクイザルの左右の上腕筋及び前脛骨筋の計 4 箇所に AAV ベクター（3 箇所）と PBS（1 箇所）を直接注入する。コントロールとしては，導入遺伝子を発現しない promoter-less AAV vector を投与する。LacZ 遺伝子組換え AAV ベクター投与群及びマイクロジストロフィン遺伝子組換え AAV ベクター投与群の 2 群は 3 頭ずつを設け，コントロール群は 2 頭を用いる。なお，使用個体の雌雄は問わない。導入 1 及び 2 週後に筋組織の生検を，4 週後に安楽死後のサンプリングをそれぞれ行い，同時に各時点で採血も実施する。ベクターの投与及び採血は塩酸ケタミン，生検はイソフルランによる麻酔下で行う。サンプリングは，ペントバルビタールナトリウム深麻酔下に放血死させた後に実施する。ジストロフィンの発現をウエスタンブロット法及び免疫組織化学染色法を用いて解析し，β-ガラクトシダーゼの発現について組織化学染色法を用いて解析する。β-ガラクトシダーゼ及びマイクロジストロフィンに対する血清抗体価の測定を ELISA またはウエスタンブ

ロット法で行う。

C．研究成果

前年度に行った予備実験により明らかにした至適投与量（10^{12} vg）の LacZ 遺伝子組換え AAV ベクター及びマイクロジストロフィン遺伝子組換え AAV ベクターを，カニクイザルの両側の上腕二頭筋と前脛骨筋の計 4 カ所に投与し，1，2，4 週後に生検を行った。切片作成後 HE 染色を行い，LacZ 遺伝子組換え AAV ベクター投与群ではβ-gal 染色を施し，injection の有無とβ-gal の発現を確認した。マイクロジストロフィン遺伝子組換え AAV ベクター投与群についてはマイクロジストロフィンの発現を確認するため，ジストロフィン染色，ウエスタンブロットおよび PCR 法を行った。β-gal，AAV ベクターに対する血清抗体価の測定は ELISA 法を用いて行った。

その結果，LacZ 遺伝子組換え AAV ベクター投与群では 3 頭中 1 頭にβ-gal の発現が 1 週後，2 週後に軽度認められたのみで，予備実験と比較し発現効率が低かった。また，細胞浸潤などの免疫反応も強く認められた。ELISA 法では AAV-type2 ベクターに対する抗体が 1 週間後から認められ，IgM 抗体の上昇も認められず，本実験で用いたサルは AAV-type2 既感染であったと考えられた。
次に，マイクロジストロフィン遺伝子組換え AAV ベクターを 3 頭のカニクイザル骨格筋に投与した。1，2，4 週後ともウエスタンブロットでジストロフィンの発現は認められず，HE 染色では筋注部位に一致して細胞浸潤を認めた。PCR 法では AAV-type2 ベクターは検出された。ELISA 法では 3 頭中 2 頭に AAV-type2 ベクターに対する IgG 抗体の上昇が投与 1 週目から認められ，IgM 抗体の上昇はなく，AAV-type2 既感染であると考えられた。

今回の実験により，AAV ベクターの遺伝子導入効率は血清中の中和抗体の有無により左右されることが明らかとなった。また，ELISA 法では投与前血清における AAV-type2 に対する中和抗体の有無の判定は困難であった。

実験中に血液生化学的検査を定期的に施行したが，肝・腎障害，貧血など検査値の異常や他の全身状他の悪化などは認められなかった。

D．考察

現在の ELISA 法では投与前に中和抗体の有無を確認できないため，中和抗体のスクリーニング法の確立が重要である。さらに，筋生検による導入遺伝子の発現解析，ウイルスベクターゲノムの有無（感染効率）を定量的かつシステマチックに解析する方法の確立も重要である。また，最近開発された新しい血清型 AAV ベクターには，免疫反応を惹起しにくいもの，血流にのって，広範な骨格筋組織に遺伝子導入可能なものが報告されている。今回は AAV type2 を用いたが，新しい血清型のベクターで同様の検討を行い，遺伝子導入効率及び安全性を検討する事も意義深いと思われる。

E．結論

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

F．健康危険情報

なし

G．研究発表

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H. 知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

縁取り空胞型筋変性の発生機序に関する研究(第２報)

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研究要旨
縁取り空胞変性は、筋原線維の変性、それに続く自己貪食を主病変とする疾患であり、その代表的な疾患に縁取り空胞型遠位型ミオパチー(distal myopathy with rimmed vacuoles: DMRV)がある。われわれは、その疾患のモデルマウス *hmutGNE*Tg-*GNE*(-/-)マウスの作成に成功した。このマウスでは生後３２週以降に後肢の筋力低下をきたし、DMRV患者で見られる筋病理所見（縁取り空胞の出現、アミロイドの沈着）を再現してい、さらに電子顕微鏡的には筋原線維の変性、アミロイドの沈着、自己貪食空胞の形成を認めた。今後このマウスを使用して治療実験が可能になると期待されている。

A. 研究目的

筋線維変性過程の代表的なものは、筋ジストロフィーに代表される筋線維壊死である。次に多くみられるのが縁取り空胞(rimmed vacuoles: RV)型筋変性である。両者の筋変性過程、変性後の再生は全く異なっている。両者を比較、検討することは、筋疾患の病因・病態を考える上にきわめて重要である。

RV は遠位型ミオパチー(DMRV)、封入体筋炎、眼咽頭型筋ジストロフィー、Marinesco-Sjögren (MSS)症候群など数多くの疾患で認められる[1]。RV 形成について、まず上記各疾患での変性過程の相違を過去２年間検討してきた。本年度は遺伝子操作によるモデルマウスの作成に成功したので、その解析を中心に研究を進めた。

DMRV はシアル酸生合成の律速段階酵素 UDP-GlcNAc 2-epimerase/ManNAc kinase をコードする *GNE* 遺伝子の機能喪失型変異による疾患である[2]。罹患筋の病理観察ではリソソーム酵素活性を示す縁取り空胞の形成に加え、筋線維の大小不同、核内封入体形成、βアミロイドタンパク質の沈着などの特徴が見られる。しかし、*GNE* 変異からこれらの病理像や筋萎縮に至るプロセスは全く不明である。本疾患の病態解析と治療法開発を目的に、DMRV のモデル動物として変異 *GNE* のみを発現するマウスを作製し、その表現型を解析した。

B. 研究方法

①胎生致死である *GNE* 遺伝子ノックアウトマウス遺伝形質(*GNE*-/-)と、本邦 DMRV 患者で最も頻度の高いヒト V572L 変異 *GNE* を発現するトランスジェニックマウス(*hmutGNETg*)の掛け合わせにより、ヒト V572L 変異 *GNE* のみを発現するマウス（hmutGNETg-GNE-/-）を作製した。動物の作成、解析は神経研究所疾病研究第一部（西野一三部長、May C. V. Malicdan 研究員）の協同研究によって行われた。

②組織化学的・電子顕微鏡的検討

筋力低下をみたモデルマウスで、主に侵される腓腹筋と心筋をヘマトキシリン・エオジン染色、Gomori トリクローム変法染色、酸フォスファターゼ染色、コンゴーレッド（アミロイドの検出）、などを中心に染色した。電子顕微鏡的には腓腹筋をグルタールアルデヒド固定、オスミウム酸にて後固定、

エポンに包埋した。

C. 研究結果

①モデル動物の臨床症状

*hmutGNETg-GNE-/-*は、やや出生率が劣るが、生下時の外観および発達は、野生型とほぼ同様であった。前肢筋力測定では、30週齢以降で、野生型より低値を示した。また、30週齢以降、血清CK値が上昇していた。筋力低下と血清CK値上昇は、進行性であった。30週齢マウスでは、血清・骨格筋ともに、シアル酸量が低下していた。

②骨格筋の病理解析

38週齢以降に筋線維の大小不同が観察され、筋線維内にβアミロイドタンパク質の蓄積が観察された。さらに、50週齢以上のマウスの腓腹筋、大腿四頭筋では酸フォスファターゼ陽性の縁取り空胞が観察された。また、リソソーム膜タンパク質、ポリユビキチン、筋鞘膜タンパク質の筋線維内での強い免疫反応も観察された。

電子顕微鏡的にはRVの特徴とされる筋原線維の変性、自己貪食空胞、ミエリン小体の出現とともに、アミロイド構造の出現も確認できた。核内封入体は確認できなかったし、核の変かも確認できなかった。

D 結論

RV型筋変性も一様ではなく、種々のタイプがあることが分かった。いずれも、核の変化を伴っており、今後核の変化が何を意味するのか、注目されるであろう。とくにDMRVではシアル酸生合成の律速段階酵素UDP-GlcNAc 2-epimerase/ManNAc kinaseをコードする*GNE*遺伝子の機能喪失型変異による疾患であることが分かっている。この*GNE*遺伝子産物は核内にも存在することが明らかにされている。酵素異常がどのようにして、核に変化をきたし、筋原線維の変性、自己貪食機転、RV形成に関与するのか、不明な点が多い。まだ解析は十分でないが、*hmutGNETg-GNE-/-*マウスは前述の疑問に答えを与えてくれるモデルマウスと評価出来る。このマウスではRV形成と同じく、むしろ先んじてアミロイド形成がみられる。RVのアミロイド沈着は、タンパク変性の二次的な結果であると簡単に処理できない。MSSは著明な核の変化と、筋原線維の変化をみるがアミロイド沈着はみられない。MSSでは*SIL1*という小胞体機能の調節因子をコードする遺伝子に変異がみられている[3)]。この遺伝子変異とRV形成の関係も今後明らかにされねばならない。

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なし

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G. 知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋再生時の炎症・免疫学的反応に関する研究

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研究要旨
骨格筋の再生時には、炎症性細胞以外に線維芽細胞が増殖することを見出した。線維芽細胞は、線維の産生以外に骨格筋線維の安定化に必須のラミニンα２鎖を産生していることがわかり、常染色体性筋ジストロフィーの治療のための新規な方策を提供できた。また骨格筋の再生は、炎症性細胞、筋衛星細胞、線維芽細胞など、複数の細胞群が生み出す秩序だった相互作用でバランスがとられており、それぞれの機能分担の一端が明らかになった。

A．研究目的

骨格筋が再生するとき、種々の炎症性細胞の浸潤が認められる。筋障害初期（~24 時間）では顆粒球が、続いてマクロファージ（48 時間以降）が浸潤し、マクロファージの浸潤と同期して筋衛星細胞の増殖が始まる。これまで本研究者らは、マクロファージ減少下の条件で骨格筋再生を誘導したとき、筋再生不全とともに線維産生の亢進を認めたが、今回 CD90 陽性の線維芽細胞が線維産生に働いていることを明らかにした。本研究は、CD90 陽性線維芽細胞の機能についてさらに検討し、遺伝性筋疾患の進行予防や治療法の開発に役立つ機構を明らかにすることを目的とする。

B．研究方法

1. 抗c-fms抗体（抗M-CSF受容体抗体）を投与したマウス前脛骨筋にカルジオトキシンを投与し、骨格筋の再生を誘導した。
2. 再生時期の骨格筋からSM/C-2.6並びにCD90の発現に基づいて細胞を分画した。
3. 宿主はラミニンα２鎖(LAMA2)欠損マウス(dy^{3k})、ドナーはGFP-tgマウスを用いた。
4. C57BL/6マウスに6Gyのγ線を照射し、直後にGFP-tgマウス由来骨髄細胞を移植した。このマウスについて、CD90陽性細胞を調べた。

（倫理面への配慮：すべてマウスでの実験であり、大阪大学動物実験指針に沿って実施した。）

C．研究成果

1. SM/C-2.6抗体を用いて筋再生時の単核細胞を分画しLAMA2発現をRT-PCRで調べたところ、精製SM/C-2.6陽性細胞では弱いバンドしか見えないのに反し、SM/C-2.6陰性細胞では強い発現が認められた。
2. SM/C-2.6陰性細胞分画で認められたLAMA2発現は、筋再生時に増加するCD90陽性細胞が担っていることをRT-PCRで確認した。
3. CD90陽性細胞は、培養すると線維芽細胞のマーカー分子の発現と形態を示した。また正常な骨格筋では基底膜の間隙に認められた。
4. 骨髄キメラマウスでは、CD90陽性細胞は骨髄に由来する細胞ではなく、骨格筋に常在している細胞であることが示唆された。
5. CD90陽性細胞をdy^{3k}に移植すると、GFP陰

性の筋線維の周辺にLAMA2陽性の基底膜が観察できた。

D. 考察

骨格筋の再生は、浸潤した炎症性細胞と筋衛星細胞との相互作用で進行すると考えられてきた。

しかしこれら以外にも、マーカーが不明な細胞が多数認められ、特にマクロファージ不在下で多く存在する。その一つがCD90陽性細胞であり、これが線維化の原因であることを報告してきた。

従来マウスCD90は、胸腺細胞やT細胞のみならず、神経細胞や線維芽細胞にも発現されていることが知られている。そこで本研究では、CD90陽性細胞が筋再生の場で果す役割を検討し線維関連成分以外に、LAMA2の主要な産生細胞であることを示した。またLAMA2産生能は、胸腺上皮細胞や皮膚線維芽細胞にも認められた。

この成果は、骨格筋の再生に新たな細胞集団が働いていることを示し得たと同時に、将来、LAMA2欠損型の常染色体性筋ジストロフィーの細胞治療法の研究に資すると考えられる。

E. 結論

1. 筋再生時にCD90陽性細胞を検出した。
2. CD90陽性細胞にLAMA2産生能を見出した。
3. CD90陽性細胞は線維芽細胞であり、類似のLAMA2産生能は皮膚にも認められた。
4. CD90陽性細胞を利用することで、先天性筋ジストロフィーの治療への可能性を示した。

F. 健康危険情報

なし

G. 研究発表

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II. 学会発表

Fukada,S. et al. Molecular regulation of Quiescent Satellite Cells Revealed by Gene Expression Profiling. Frontiers in Myogenesis.2006.4, (Pine Mountain, GA, USA)

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
Suzuki N, Miyagoe-Suzuki Y, Takeda S	Gene Therapy for Duchenne Muscular Dystrophy	***Future Neurology***, Vol 2(1), 87-96, 2007
Yugeta N, Urasawa N, Fujii Y, Yoshimura M, Yuasa K, Nakamura A, Wada M, Nakura M, Shimatsu Y, Tomohiro M,Takahashi A, Machida N, Wakao Y, Takeda S	Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan ($CXMD_J$): electrocardiographic, echocardiographic, and morphologic studies	***BMC Cardiovasc Disord*** 4;6:47, 2006
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Gene therapy for Duchenne muscular dystrophy

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Gene therapy has great potential to treat Duchenne muscular dystrophy. Among many proposed strategies to deliver a therapeutic gene to muscle, recombinant adeno-associated virus-mediated gene transfer is the most promising. The recent isolation of new adeno-associated virus serotypes from human and nonhuman primates provides the opportunity to develop vectors that can achieve the long-term expression of a therapeutic gene in muscles of the entire body without detrimental effects. To translate the results from small animal models to clinical trials in humans, further work using larger animal models, such as dystrophic dogs or nonhuman primates, is required. This review also discusses recent progress in other gene transfer-related therapeutic approaches, including targeted exon skipping and gene correction.

Duchenne muscular dystrophy (DMD), which affects one in 3300 males, is a devastating, progressive, muscle-wasting disease caused by mutations in the dystrophin gene [1,2]. Skeletal muscles in DMD are characterized by myofiber degeneration and progressive fibrous and fatty changes. There is, currently, no way to prevent muscle fiber necrosis and patients suffer severely from respiratory and cardiac complications in the second decade of life. The *DMD* gene is among the largest genes known, spanning 2.4 Mb at Xp21 and encoding a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin, and several shorter isoforms (Dp260, Dp140, Dp116 and Dp71). The full-length dystrophin protein is composed of four domains: an N-terminal actin-binding domain, a central rod domain consisting of 24 spectrin-like repeats, a cysteine-rich domain and a C-terminal domain. Dystrophin binds actin at the N-terminal domain, β-dystroglycan at the cysteine-rich domain and dystrobrevin and syntrophins at the C-terminal domain, forming the dystrophin–glycoprotein complex (DGC) at the sarcolemma (Figure 1) [3]. A lack of dystrophin at the sarcolemma causes secondary loss of the DGC and other functional molecules, such as neuronal nitric oxide synthase (nNOS) [4] and aquaporin-4 [5]. Importantly, mutations in the genes encoding other members of the DGC cause several different types of muscular dystrophy. The mechanism of the degeneration and death of dystrophin-deficient myofibers is not yet fully understood, but it is believed that myofibers lacking dystrophin and the DGC at the cytoplasmic membrane are mechanically weak and highly susceptible to contraction-induced injury. As a result, the affected muscle experiences continuous cycles of myofiber death and regeneration, resulting in the gradual loss of myofibers and contractile force. In addition to mechanical weakness, abnormalities in calcium handling and changes in mitogen-activated protein (MAP) kinase and GTPase signaling in dystrophin-deficient muscle have been reported and proposed as underlying processes of muscular dystrophy [6,7].

At present, only corticosteroids are reported to effectively attenuate the progress of the disease [8], and current treatment options focus on respiratory and cardiac assistance and improvement of quality of life. Many research groups are still attempting to develop an effective therapy for DMD. In this review, we describe recent progress in gene and related therapies for DMD.

Recombinant adeno-associated virus vector: a promising tool for delivery of dystrophin gene to skeletal muscles

Among several gene transfer vectors and methods developed to date, the adeno-associated virus (AAV) vector is the most suitable to introduce the exogenous gene into postmitotic, nondividing myofibers. An AAV is a tiny, nonpathological, replication-defective virus, with a 4.7-kb single-stranded DNA genome, belonging to the parvovirus family. AAV vectors induce fewer immunological and inflammatory responses *in vivo* than adenovirus vectors [9]. Although the virus genome persists predominantly in episomal form, expression of the transferred gene lasts months to years in adult skeletal muscle. To date, more than 100 AAVs with distinct virus genome sequences have been isolated from humans, nonhuman primates and other species [10]. They display varying

Keywords: adeno-associated virus vector, Duchenne muscular dystrophy, dystrophin, exon skipping, gene therapy

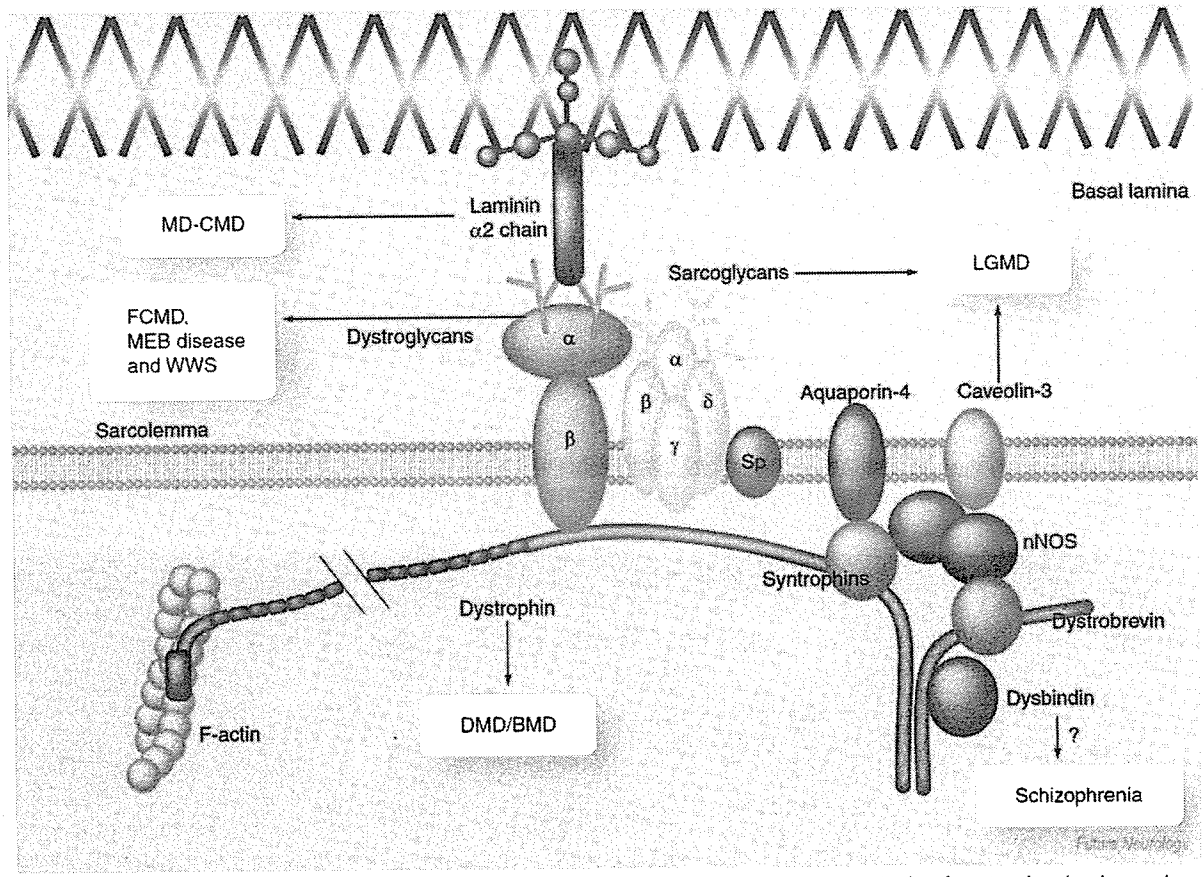
10.2217/14796708.2.1.87 © 2007 Future Medicine Ltd ISSN 1479-6708

degrees of similarity in their capsid proteins and show diverse tissue tropisms. More than nine AAV vectors have already been developed and evaluated in animal models as a tool for gene transfer *in vivo* (Table 1) [10]. Although the molecular mechanisms of tissue and cell tropisms of AAV vectors are not fully explained, they are likely to use different cellular receptors for entry into and binding to the host cells. The expression of the therapeutic genes is not permanent, mainly because recombinant (r)AAV does not replicate in the host and is barely incorporated into the genome of satellite cells. They are diluted out with the turnover of myofibers and, therefore, repeated administrations are required. New AAV serotypes would provide good options for follow-up treatments because they have the potential to evade pre-existing neutralizing antibodies against the previously used AAV serotype. However, to avoid the risks of *in vivo* vector delivery, it is important to better understand the vectors and the natural infection with the corresponding virus.

Generation of microdystrophin suitable for use in rAAV vectors

The rAAV vector is a promising tool for gene transfer to DMD muscle, but the limitation of the insertion size to 4.9 kb excludes incorporation of

Figure 1. Dystrophin forms the large dystrophin–glycoprotein complex at the sarcolemma, linking the basal lamina to the cytoskeletal actin.



Mutations in the dystrophin gene end in the secondary loss of dystrophin–glycoprotein complex and other functional molecules, such as nNOS and aquaporin-4. Mutation in the laminin α2 chain gene causes congenital MD. Abnormal glycosylation of α-dystroglycan is commonly observed in FCMD, MEB disease and WWS. Abnormal glycosylation of α-dystroglycan also causes abnormalities in the eye and the CNS. Mutations in any of four sarcoglycan genes (α, β, γ and δ) result in LGMD. These observations emphasize the importance of dystrophin and associated molecules for muscle integrity. The dysbindin (*DTNBP1*) gene is one of the several putative susceptibility genes for schizophrenia.
BMD: Becker MD; DMD: Duchenne MD; FCMD: Fukuyama-type congenital MD; LGMD: Limb girdle MD; MD: Muscular dystrophy; MD-CMD: Merosin-deficient congenital MD; MEB: Muscle–eye–brain; nNOS: Neuronal nitric oxide synthase; WWS: Walker–Warburg syndrome.

Table 1. Characterization of nine serotypes of AAV vectors.

Serotype	Amino acid homology to AAV2 (%)	Isolated from	Tissue tropism				Delivery system
			Skeletal muscle	*Heart*	*Liver*	*CNS*	
1	84	NHP	+++	++	+	+	Local
2	100	Human	+	+	+	+	Local
3	88	Human		+	±	+	Local
4	64	NHP		+	±	+	Local
5	61	Human	+	+	++	++	Local
6	84	Human	++	++	+	+	Local, systemic
7	83	NHP	+++	+++	++	++	Local, systemic
8	84	NHP	+++	+++	+++	++	Local, systemic
9	83	Human	+++	+++	+++	++	Local, systemic

AAV: Adeno-associated virus; NHP: Nonhuman primate.

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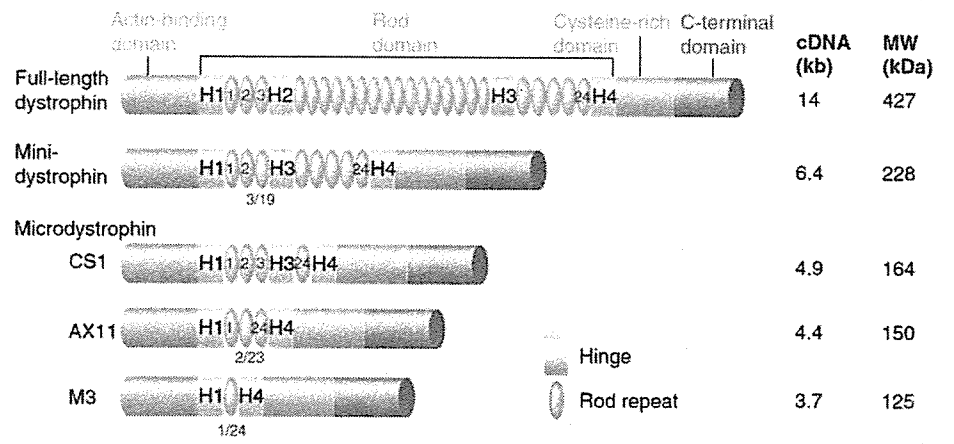
a full-length dystrophin gene (14-kb mRNA, 11-kb open reading frame). To overcome this drawback, several groups have designed small dystrophins in which the long, central rod domains are largely deleted, and tested their functions in dystrophin-deficient *mdx* mice [11]. The functions of three types of microdystrophins (CS1, AX11 and M3; Figure 2) have been tested on microdystrophin-transgenic, dystrophin-deficient *mdx* mice, and it was observed that over-expressed CS1 with four repeats and three hinges almost completely ameliorated dystrophic phenotypes [12]. Therefore, a rAAV2 vector was constructed expressing CS1, driven by a skeletal muscle-specific muscle creatine kinase promoter [13], and was injected it into the anterior tibialis muscles of immunocompetent adult *mdx* mice. A total of 24 weeks after injection, 50% of myofibers, on average, expressed microdystrophin and the treated muscles demonstrated improved contractile force [14].

Systemic delivery of rAAV-serotype 6, 8 & 9 vectors

Systemic delivery systems for the treatment of DMD require improvement to enable transfer of the therapeutic genes to the complete musculature of the body, especially to the heart and diaphragm. Gregorevic and colleagues reported that intravenous injection of rAAV6 vectors efficiently delivered a microdystrophin gene to the

Figure 2. Structure of full-length dystrophin and constructs of mini- and microdystrophin (CS1, AX11 and M3).



MW: Molecular weight.

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muscles of an adult mouse and the ratio of microdystrophin-positive fibers was increased when co-injected with vascular endothelial growth factor [15]. The widespread expression of microdystrophin was sufficient to correct susceptibility to contraction-induced injury and to lower serum creatine kinase levels [15]. Wang and colleagues, and Nakai and colleagues, demonstrated that AAV8 was more efficient than AAV6 or AAV1 at attaining systemic gene transfer, especially to the cardiac muscles of mice or hamsters, without pharmacological intervention [16,17]. More recently, Inagaki and colleagues reported that AAV9 vectors demonstrated robust systemic transduction in mice [18]. Remarkably, rAAV9 is superior to rAAV8 for gene delivery to cardiac muscle by systemic vector administration [18]. The molecular basis of the high transduction efficiency via the bloodstream is not fully understood, but these results are encouraging for researchers who are developing gene therapies for DMD patients. On the other hand, however, AAV8 or 9 vectors also increase transduction of nonmuscle tissues, such as liver (Table 1), which may be deleterious.

AAV vectors for human muscle

Animal models are indispensable for the evaluation of the efficacy and safety of AAV-mediated gene therapy of DMD, but a recent report on clinical gene transfer studies for hemophilia B demonstrated that the data obtained in preclinical studies in animals are not always predictive of vector efficacy in humans [19]. Certain human populations are exposed to AAVs in daily life: 50–96% are seropositive for AAV2 and at least a third have a neutralizing antibody to AAV2 [10]. Therefore, prior exposure to AAV2 explains the unsatisfactory results of clinical trials using rAAV2-factor IX gene transfer on hemophilia B patients [19]. The new serotypes of AAVs are reported to be prevalent in human and nonhuman primates. Prescreening of patients for neutralizing antibodies against the vector serotype and transient immune suppression would be required to avoid the elimination of rAAV particles by neutralizing antibodies.

Minidystrophin coded by two AAV vectors (dual vector system)

Microdystrophin proteins, with 3–4 spectrin-like repeats in the rod domain, do not completely compensate for the lack of full-length dystrophin. Among the constituents of DGC and its binding proteins, the expression of nNOS cannot be recovered through the introduction of microdystrophin. In an attempt to introduce a therapeutic gene larger than 4.7 kb into target cells, the trans-splicing approach, in which the gene is split between two rAAV vectors, each containing part of an intron with either a splice-acceptor or a splice-donor sequence, has been developed. After formation of head-to-tail concatamers, trans-splicing of the two RNA transcripts from the two different expression cassettes removes the intervening sequence, producing a functional mRNA larger than could be delivered in a single vector. This approach was employed to deliver a minidystrophin to *mdx* muscle [20], however, the coordinated nature of transcription and splicing makes this strategy highly inefficient *in vivo*.

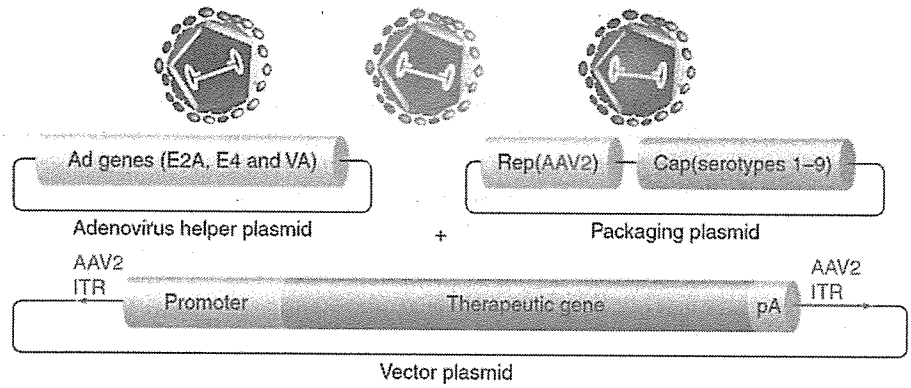
Production of AAV vectors on a large scale

rAAV vector plasmids are generated by deleting the viral genome except for the inverted terminal repeats. To obtain recombinant AAV particles, double [21] or triple [22] transfection of the plasmids into human embryonic kidney (HEK)293 cells is performed to provide rep and capsid proteins and adenoviral helper functions (Figure 3). The obtained AAV vectors are further purified by CsCl gradient sedimentation or ion-exchange chromatography. In the case of rAAV2, it is estimated that at least 1×10^{13} vg/kg is required to treat humans with hemophilia, whereas the titer of the vectors prepared by this standard method is approximately $2–5 \times 10^{13}$ genome copies from 1×10^{9} HEK293 cells [13]. In clinical trials, an inexpensive, safe, large-scale system must be developed for the production of AAV. For example, Urabe and colleagues described a highly powerful production of rAAV using nonmammalian cell culture [23]. Okada and colleagues described a large-scale AAV vector production with active gassing [24].

Safety issues

Currently, most research on AAV-mediated gene transfer focuses on the systemic delivery of therapeutic genes via the blood circulation. Some have demonstrated the effectiveness of high-pressure arterial [25] or venous [26] infusion. These procedures seem to be powerful in transducing the therapeutic genes into targeted muscle groups, but the safety should be tested carefully in larger animal models. In particular, the mutagenic and carcinogenic potentials of recombinant genomes should be investigated, in addition to their potential for germline transfer after systemic delivery.

Figure 3. Production of pseudo-typed adeno-associated virus vectors in HEK293 cells.



To obtain recombinant (r)AAV, three plasmids are transfected into human embryonic kidney (HEK)293 cells. The therapeutic gene is inserted between the two ITRs of the virus, with a ubiquitous or muscle-specific promoter. To avoid contamination of the helper adenovirus, plasmids encoding adenovirus-derived genes and *rep* and *cap* genes are cotransfected into the cells. Recently, the viral capsid gene from other AAV serotypes has been preferentially used in combination with the AAV2 vector to target the musculature body-wide *in vivo* after systemic delivery or to avoid pre-existing neutralization antibodies.
AAV: Adeno-associated virus; Cap: Capsid; ITR: Inverted terminal repeats.

Gutted adenoviral vectors expressing full-length dystrophin

Adenoviral vectors infect both dividing myoblasts and terminally differentiated muscle fibers, and possess a large insert capacity. However, early generations of adenoviral vectors, however, elicited substantial immune reactions in immunocompetent *mdx* mice and, hence, a rapid loss of transgene expression [27,28]. To circumvent this problem, a 'gutted' adenoviral vector, from which most viral DNA sequences are deleted, has been developed. Gutted adenoviral vectors are capable of carrying the large dystrophin gene together with regulatory sequences, and show reduced immunotoxicity compared with conventional adenoviral vectors [29–31]. Preparation of the gutted adenovirus vector requires a conventional adenovirus to supply replication and packaging functions *in trans*, and therefore has a high risk of helper virus contamination that may elicit immunological reactions upon delivery to tissues. In addition, recombinant adenoviral vectors remain comparatively toxic, especially in the liver, when administered systemically and have yet to achieve comparable transduction efficiency compared with AAV vectors.

Other vectors

A lentiviral vector is an alternative option for *in vivo* gene transfer into skeletal muscle. Kobinger and colleagues demonstrated that a lentiviral vector encoding minidystrophin targeted both satellite cells and myofibers of *mdx* mice and provided functional correction *in vivo* [32].

Direct injection of naked plasmid into dystrophic animals

Direct injection of a naked plasmid containing a full-length dystrophin cDNA into the muscles of DMD patients has been proposed as a promising treatment to restore the expression of dystrophin. The efficiency was low in animal models [33,34] and in a Phase I gene therapy clinical trial [35], but the dystrophin expression is relatively stable and evoked no signs of humoral or cellular immune responses. Experiments using mouse models demonstrated that the efficiency of gene transfer can be enhanced by electroporation coupled with the intramuscular application of hyaluronidase [36,37]. However, combination of electroporation and hyaluronidase administration would act to damage the muscle. The application to DMD patients is questionable. Hydrodynamic delivery of naked plasmid DNA expressing full-length dystrophin into the *mdx* mice has been reported to be effective [38]. Dystrophin expression was seen in 1–5% of the myofibers of the targeted muscle group of the hind limb for an extended period. To protect dystrophin-deficient muscles from muscle degeneration, repeated administration of plasmids would be required.

Ex vivo gene transfer into myogenic cells

Cell-mediated therapy can be used to deliver the normal dystrophin gene to dystrophic muscle. In particular, *ex vivo* transfer of a functional dystrophin gene into patients' satellite cells (myogenic progenitor cells usually located between myofibers and muscle basal lamina in a dormant state) and their progeny (myoblasts) is an attractive option for cell-based therapies for DMD since several methods to freshly purify satellite cells from muscle have been established [39,40]. A lentivirus vector would be the first choice for *ex vivo* mini- or microdystrophin gene transfer into autologous myogenic cells because it can infect freshly isolated satellite cells without lowering their proliferation and differentiation potential [Ikemoto *et al.*, Unpublished Data]. Stem cells other than satellite cells, such as muscle side population (SP) cells [41–43], mesoangioblasts [44], and AC133-positive human stem cells [43], have been reported to participate in muscle regeneration. Muscle SP cells are isolated by their ability to efflux Hoechst dye. Bachrach and colleagues demonstrated that SP cells from *mdx* (5cv) mice transduced with microdystrophin *ex vivo* were transplanted successfully via the tail vein and delivered human microdystrophin to the skeletal muscle of nonirradiated *mdx* (5cv) mice [45]. Recently, Dezawa and colleagues reported a novel method to induce muscle progenitor cells from human bone marrow stromal cells with a high efficiency [46].

Correction of endogenous genes

Gene conversion using chimeraplasts attempts to correct point mutations of the *DMD* gene in the cell. The first generation of chimeraplasts comprises hybrid RNA/DNA molecules that are homologous to a targeted gene, yet include one mismatched base. These hybrid nucleotides trigger gene conversion from a mutant to a functional allele via intranuclear DNA mismatch repair mechanisms. Injection of chimeric oligonucleotides into *mdx* mice resulted in the expression of full-length dystrophin in muscle fibers at the site of injection [47]. Gene correction mediated by chimeraplasts has also been demonstrated in the dystrophic golden retriever dog [48]. A second generation gene editing tool is a linear DNA oligonucleotide, 25-mer or longer containing a single central mismatch. This tool repaired single point mutations in the dystrophin gene with efficiencies comparable to that seen with chimeric RNA/DNA oligonucleotides, but yielded more consistent results [49]. Approximately 20% of DMD patients have single point mutations and, therefore, are potential targets of this therapeutic approach. However, gene repair techniques may not work for all mutations. Further data on the efficacy of the correction *in vivo* are required, using a range of point mutations of the dystophin gene.

Targeted exon skipping

Antisense oligonucleotides

DMD and *mdx* muscles have a few revertant fibers that express functional dystrophin [50,51]. This phenomenon is explained by aberrant splicing, which omits one or more exons and, as a result, restores a disrupted reading frame and dystrophin expression. Based on this observation, forced exon skipping is being developed as a future treatment to restore dystrophin expression from the mutated DMD gene in humans. The main tools for targeted exon skipping are antisense oligonucleotides (AOs). 2′-*O*-methyl-modified RNA on a phosphorothioate backbone, endowed oligonucleotides with greater resistance to nuclease degradation and, therefore, additional increases in stability were achieved [52–54]. Direct intramuscular injection of 2′-*O*-methyl phosphorothioate AOs resulted in a significant increase in the number of dystrophin-positive fibers (20%) in *mdx* mouse muscle [55]. Phosphoro-amide morpholino oligonucleotides have also proven to be effective in producing functional dystrophin in dystrophin-deficient muscle [56]. Weekly intravenous injections of morpholino AOs induced the expression of functional levels of dystrophin body-wide in skeletal muscles of the dystrophic *mdx* mouse and improved muscle function [57]. Based on the successful results in animal models, a clinical trial using AOs has already started in Leiden and is about to commence in the UK. Theoretically, AO-based exon skipping is applicable to 80% of dystrophin gene mutations. Furthermore, it is estimated that targeting just 12 exons restores the open reading frame of 75% of all deletions responsible for DMD.

AAV-mediated exon skipping

AOs display a limited half-life *in vivo*, and administration of AOs to patients must be repeated weekly or monthly. To obtain a longer-term effect, rAAV1 vectors expressing a modified U7 small nuclear RNA gene were used to direct exon skipping in *mdx* mice [58]. Following a single, high-pressure injection of the rAAV1/U7 vector

into the femoral artery of mdx mice, normal levels of dystrophin expression were restored and sustained for over 6 months. Although the initial study was limited to delivery to a single limb, this technique could be coupled with systemic delivery of AAV vectors of new serotypes.

Insulin-like growth factor-1 & myostatin blockade rescue dystrophin-deficient muscle

Myostatin (also known as growth and differentiation factor [GDF]8) is a transforming growth factor (TGF)-β family member that negatively regulates skeletal muscle growth, as evidenced by the increased musculature of the mice with a null mutation in this gene [59]. Mutation of the myostatin gene has also been found in human [60]. The myostatin-null child was reported to be muscular without any health problems at 4.5 years of age [60]. Myostatin blockade in *mdx* mice results in increases in both muscle mass and muscle strength and reductions in muscle fiber degeneration and serum creatine kinase levels [61]. Based on this observation, the recombinant human antibody against myostatin (MYO-029) is now being tested on adult muscular dystrophy patients.

Increased insulin-like growth factor (IGF)-1 within *mdx* myofibers reduces the breakdown of dystrophic muscle during the acute onset of muscle degeneration [62]. This mechanism of action can partly account for the long-term reduced severity of the dystrophic pathology in *mdx* mice overexpressing mIGF-1 and provides opportunities for therapeutic strategies [63].

Conclusion

Almost 20 years have passed since the discovery of dystrophin. Unfortunately, we have yet to find an effective therapy that can mitigate the dystrophic process. Numerous approaches are currently being explored, but many suffer from a variety of drawbacks. Among the gene therapy approaches to DMD under investigation, rAAV-mediated gene transfer is the most promising but still faces several obstacles. Other therapeutic approaches, including cell therapy and pharmacological intervention, would be used in complement with AAV-microdystrophin gene transfer.

Future perspective

An important step towards the clinical use of gene therapy is the evaluation of the efficacy and safety of gene transfer methods and protocols using animals larger than mice. We have established a beagle-based canine X-linked muscular dystrophy (CXMD) colony at the National Institute of Neuroscience in Japan ($CXMD_J$) and reported their severe phenotypes [64]. Beagle-based $CXMD_J$ is smaller and easier to handle than golden retriever CXMD, and is, therefore, a useful model for DMD. Preclinical studies using nonhuman primates would also be informative before clinical trials. Importantly, there are so many variables, even in a single treatment, such as myostatin blockade with antibodies, that more trials will be needed.

At present, gene therapy trials and related strategies face various hurdles and difficulties. Effective treatment of DMD may be achieved through a combination of different therapeutic approaches; for example, a combination of AAV vector-mediated gene transfer plus corticosteroid administration or myostatin blockage.

Acknowledgements

The authors are grateful to A Nishiyama and T Yokota for discussion and E Takahashi for help in preparing this manuscript. Studies in the authors' laboratory are supported by a Grant for Research on Nervous and Mental Disorders (16B-2), and Health Science Research Grants for Research on the Human Genome and Gene Therapy (H16-genome-003), for Research on Brain Science (H15-Brain-021, H18-Brain-019) from the Ministry of Health, Labor and Welfare, and Grants-in-Aid for Scientific Research (16590333, 18590392) from the Ministry of Education, Culture, Sports, Science and Technology.

Executive summary

Introduction

- Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes a 427-kDa subsarcolemmal cytoskeletal protein, dystrophin.
- At present, there is no treatment to arrest the progression of DMD and patients generally suffer from respiratory and/or cardiac complications in the second decade of life.
- Among several therapeutic strategies for this disease, recombinant adeno-associated virus (rAAV)-mediated gene transfer is the most promising.

Executive summary

Viral vector-mediated gene therapy

- AAV vectors drive long-term expression of the therapeutic gene in skeletal muscle *in vivo*, but the insertion size is limited to 4.9 kb.
- Functional, rod domain-deleted dystrophin (microdystrophin) can be incorporated into AAV vectors.
- New serotypes of AAV vectors have been isolated and developed as gene-transfer vectors, some of which transport the therapeutic genes to all the muscles of the body after systemic delivery.

Ex vivo *gene transfer into myogenic stem cells*

- Cell-mediated therapy can be used to deliver a normal dystrophin gene to dystrophic muscle in the hope that the delivered cells will participate in muscle-fiber regeneration in dystrophic muscle, express dystrophin and improve muscle function.
- Muscle satellite cells, side population cells, mesangioblasts, AC133-positive cells and bone marrow stromal cells are expected to be potential cell sources for cell-mediated therapy.

Gene correction & exon skipping using antisense oligonucleotides

- Chimeraplasts, which are chimeric RNA/DNA oligonucleotides homologous to a targeted gene (except for the inclusion of one mismatched base) can be used to direct the correction of a mutation by inducing preferential gene conversion from a mutant to a functional allele.
- Exon skipping using antisense oligonucleotides (AOs) targets transcribed RNA molecules to omit a nonsense mutation and restore a disrupted reading frame.
- Weekly intravenous injections of morpholino phosphorodiamidate (morpholino) AOs induce the expression of functional levels of dystrophin in skeletal muscles body-wide in the dystrophic *mdx* mouse.

Myostatin & insulin-like growth factor-1

- Blockage of myostatin and delivery of insulin-like growth factor-1 are effective to improve dystrophic phenotypes and the contractile force of dystrophin-deficient muscle.

Future perspective

- Preclinical studies using dystrophic dogs and nonhuman primates would be informative before human clinical trials.
- To overcome this devastating disease, multiple, diverse therapeutic strategies should be combined.

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Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan (CXMD$_J$): electrocardiographic, echocardiographic, and morphologic studies

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Published: 04 December 2006

BMC Cardiovascular Disorders 2006, 6:47 doi:10.1186/1471-2261-6-47

Received: 14 July 2006
Accepted: 04 December 2006

This article is available from: http://www.biomedcentral.com/1471-2261/6/47

Abstract

Background: Cardiac mortality in Duchenne muscular dystrophy (DMD) has recently become important, because risk of respiratory failure has been reduced due to widespread use of the respirator. The cardiac involvement is characterized by distinctive electrocardiographic abnormalities or dilated cardiomyopathy, but the pathogenesis has remained obscure. In research on DMD, Golden retriever-based muscular dystrophy (GRMD) has attracted much attention as an animal model because it resembles DMD, but GRMD is very difficult to maintain because of their severe phenotypes. We therefore established a line of dogs with Beagle-based canine X-linked muscular dystrophy in Japan (CXMD$_J$) and examined the cardiac involvement.

Methods: The cardiac phenotypes of eight CXMD$_J$ and four normal male dogs 2 to 21 months of age were evaluated using electrocardiography, echocardiography, and histopathological examinations.

Results: Increases in the heart rate and decreases in PQ interval compared to a normal littermate were detected in two littermate CXMD$_J$ dogs at 15 months of age or older. Distinct deep Q-waves and increase in Q/R ratios in leads II, III, and aVF were detected by 6–7 months of age in all CXMD$_J$ dogs. In the echocardiogram, one of eight of CXMD$_J$ dogs showed a hyperechoic lesion in the left ventricular posterior wall at 5 months of age, but the rest had not by 6–7 months of age. The left ventricular function in the echocardiogram indicated no abnormality in all CXMD$_J$ dogs by 6–7 months of age. Histopathology revealed myocardial fibrosis, especially in the left ventricular posterobasal wall, in three of eight CXMD$_J$ dogs by 21 months of age.