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CASE REPORT

The usefulness of magnetic resonance imaging (MRI) for disseminated trichosporosis of the gastrocnemius muscles

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KEYWORDS

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Summary A 30-year-old man with acute myeloid leukemia who was pancytopenic after undergoing intensive chemotherapy developed pyrexia and severe pain of both lower legs. We immediately started empiric therapy with cefepime, vancomycin, and fluconazole for febrile neutropenia. However, symptoms progressed. After 4 days, Trichosporon was isolated from venous blood cultures. MRI showed hyperintense lesions within both gastrocnemius muscles and demonstrated reactive vasodilatation and interstitial tissue edema, thought to be induced by hyperpermeability of vessel membranes due to the local fungal infection. Amphotericin B was very effective against this organism. Trichosporosis is a rare infectious disease generally occurring in immunocompromised hosts. To the best of our knowledge, this is first reported case of bilateral Trichosporon infection of lower leg muscles. Severe leg pain was one of the most important signs of fungal infection in this patient with hematologic malignancy.

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Case report

Fungal infection is one of the most important complications of leukemia, and is not uncommon in patients with this disease. We recently encountered a patient who exhibited the unusual

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manifestation of bilateral gastrocnemius fungal infection. MRI findings paralleled the clinical course.

A 30-year-old Japanese man was diagnosed as having acute myeloid leukemia, M2 type (FAB classification), in April 2003. The patient failed to achieve complete remission despite receiving 4 different induction therapies, including high-dose Ara-C and anthracycline agents at another Tokyo hospital. He was therefore referred to our hospital for further chemotherapy regimens including stem cell transplantation. On August 20th, 2003, FLAGM (Fludarabine, Ara-C, Mitoxantrone, G-CSF) was administered. On August 25th (day 6) grade 4 (NCI-CTC) pancytopenia developed. On September 14th (day 26), the patient became febrile (around 38 °C) and developed severe spontaneous pain in both lower legs which was gripping in nature. At this point, blood pressure was 126/74 mmHg, and pulse rate was 90/min. No pharyngeal erythema was present and pulmonary sounds were clear. Other than the pain, the lower limbs were normal, with no sign of erythema, swelling, injury, desquamation or neurological disorder.

On September 14th (day 26), laboratory investigations revealed the followings: white cell count, 100/ μ l; hemoglobin, 8.4 g/dl; and platelet count, 1.7×10^4 / μ l; and C-reactive protein (CRP), 8.95 mg/dl. Hepatic and renal functions were within the normal range: AST, 10 mU/ml; ALT, 30 mU/ml; LDH, 166 mU/ml; ALP, 142 mU/ml; BUN, 8 mg/dl; creatinine, 0.61 mg/dl; and CPK, 37 mU/ml. Serum cryptococcal antigen and aspergillus antigen were negative, and serum β -D-glucan levels were within the normal range (16.8 pg/ml).

On September 14th (day 26), empiric therapy with cefepime, vancomycin and fluconazole was started for febrile neutropenia. However, the bilateral lower leg pain progressed. On September 15th (day 27), *Trichosporon* was isolated from a venous blood culture. We immediately changed the antifungal agent from fluconazole to amphotericin B (AMPB), which has high sensitivity for *Trichosporon*. This resulted in resolution of lower leg pain and fever after a few days.

On September 19th (day 31), the first MRI examination was performed for differential diagnosis of the lower leg pain. Gadolinium-enhanced hyperintense lesions were observed in the fibers of the gastrocnemius muscles bilaterally (Fig. 1A). On T2-weighting, cross-sectional images showed vasodilatation (Fig. 1B). This was not a specific finding; such changes can be seen in fungal infection, bacterial infection, thrombosis, and leukemic cell infiltration. Reactive vasodilatation and interstitial tissue edema appeared to have been

induced by hyperpermeability of the vessel membrane secondary to local inflammation. The other parts were normal images such as quadriceps, knee joints, and ankle joints of the MRI. On the next day, any other fungal infected region and micro abscess were not detected through thorax to abdomen by using computed tomography (CT).

After 14 days of AMPB treatment, the MRI appearance of the muscle fibers and blood vessels had improved markedly (Fig. 1C, D). In the present case, MRI findings paralleled the clinical manifestations, indicating that MRI was useful in the differential diagnosis and monitoring of fungal myositis.

Discussion

Trichosporon asahii is the main pathogen responsible for trichosporosis, which tends to develop in neutropenia.¹ All organs are targeted, including the lungs, digestive tract, kidney, spleen, central nervous system, eye, skin, and muscle.² However, to the best of our knowledge, this is the first reported case of the initial and only infected region being the bilateral lower leg muscles. In this case, the central vein catheter appeared a plausible route of infection; bilateral muscle lesions suggested hematogenous dissemination.

In febrile neutropenia, empiric therapy with broad spectrum antibiotics should be administered immediately. If fever is persistent or recurrent after the first few days of initial therapy, it is important to consider the possibility of fungal infection. However, despite antifungal therapy with fluconazole being commenced early in the present case, fungal infection of the lower legs could not be prevented.

If symptoms related to a specific organ occur in febrile neutropenia, tissue biopsy is necessary in order to definitively diagnose fungal infection. However, tissue biopsy is difficult in many cases and is particularly risky in immunocompromised patients with neutropenia or thrombocytopenia after intensive chemotherapy.

Cultures of fluids such as blood, cerebrospinal fluid, and pleural effusion are very important in the differential diagnosis. Indeed, *Trichosporon* was isolated from venous blood cultures in the present case. This is often a serious finding, as disseminated fungal infection is reported to have a high mortality rate.^{2,3,6} Antemortem diagnosis can be very difficult, as clinical manifestations are similar to those of candidiasis. However, the recently developed nested polymerase chain reaction has higher sensitivity than blood culture; this

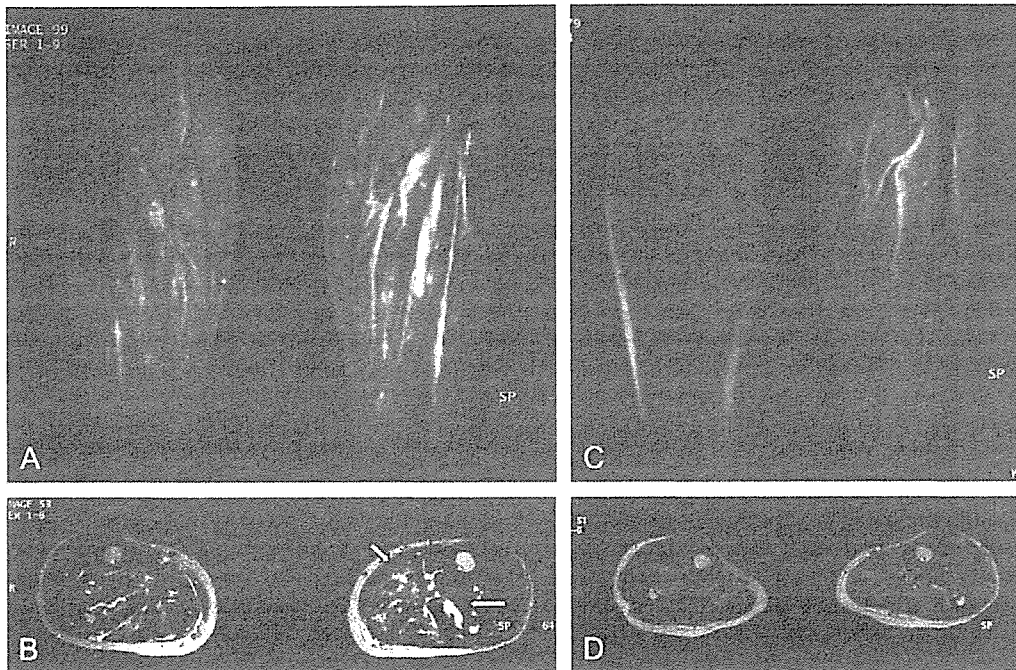


Figure 1 MRI findings of the lower legs. On September 19th, bilateral irregular gadolinium enhancement of the gastrocnemius muscle fibers was evident (A), and cross sections (B) revealed vasodilatation (arrows). After AMPB treatment, the appearance of the muscle improved (C, D).

technique can detect and predict such fungal infections several weeks before blood cultures become positive.⁴

AMPB is recommended as first choice regimen against trichosporosis. While high-dose miconazole and fluconazole therapy is more effective than AMPB *in vitro*, it has not been demonstrated superior *in vivo*.^{2,3,5,7,8} Voriconazole is another candidate in the treatment of trichosporosis.

In this case we were able to obtain MR images of gastrocnemius inflammation consistent with a lesion of disseminated trichosporosis before and after treatment. Fungal infection of skeletal muscle is rare in itself and the great majority of cases are caused by *Aspergillus* or *Candida*. Hence, the literature contains few reports of *Trichosporon* muscle infection.

In the present case, MRI reflected inflammation including that of soft tissue surrounding the muscle. This soft tissue demonstrated high signal intensity on T2 and T1 weighting. Differential diagnosis of these MRI findings included leukemic cell infiltration of soft tissues; however, this could be confirmed or refuted by muscle biopsy, as the patient had severe thrombocytopenia. Nevertheless, the possibility of leukemic invasion could be excluded for the following reasons. (1) The clinical course indicated that bone marrow suppression

occurred just after chemotherapy, hence even leukemic cells would not have been recovered in the peripheral blood. (2) Antifungal therapy resulted in obvious improvement of clinical manifestations and MRI findings.

In the present case, nonspecific inflammation of lower leg muscles was evident bilaterally, accompanied by clinical features of systemic dissemination. *Trichosporon* infection was diagnosed from blood culture. We have demonstrated that MRI can be used for early detection of fungal myositis. Early commencement of AMPB treatment resulted in successful treatment of disseminated trichosporosis, a condition reported to have high mortality.

Finally, the present case suggests that MRI is a very useful examination for the differential diagnosis and monitoring of severe leg pain in patients with hematological malignancy.

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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

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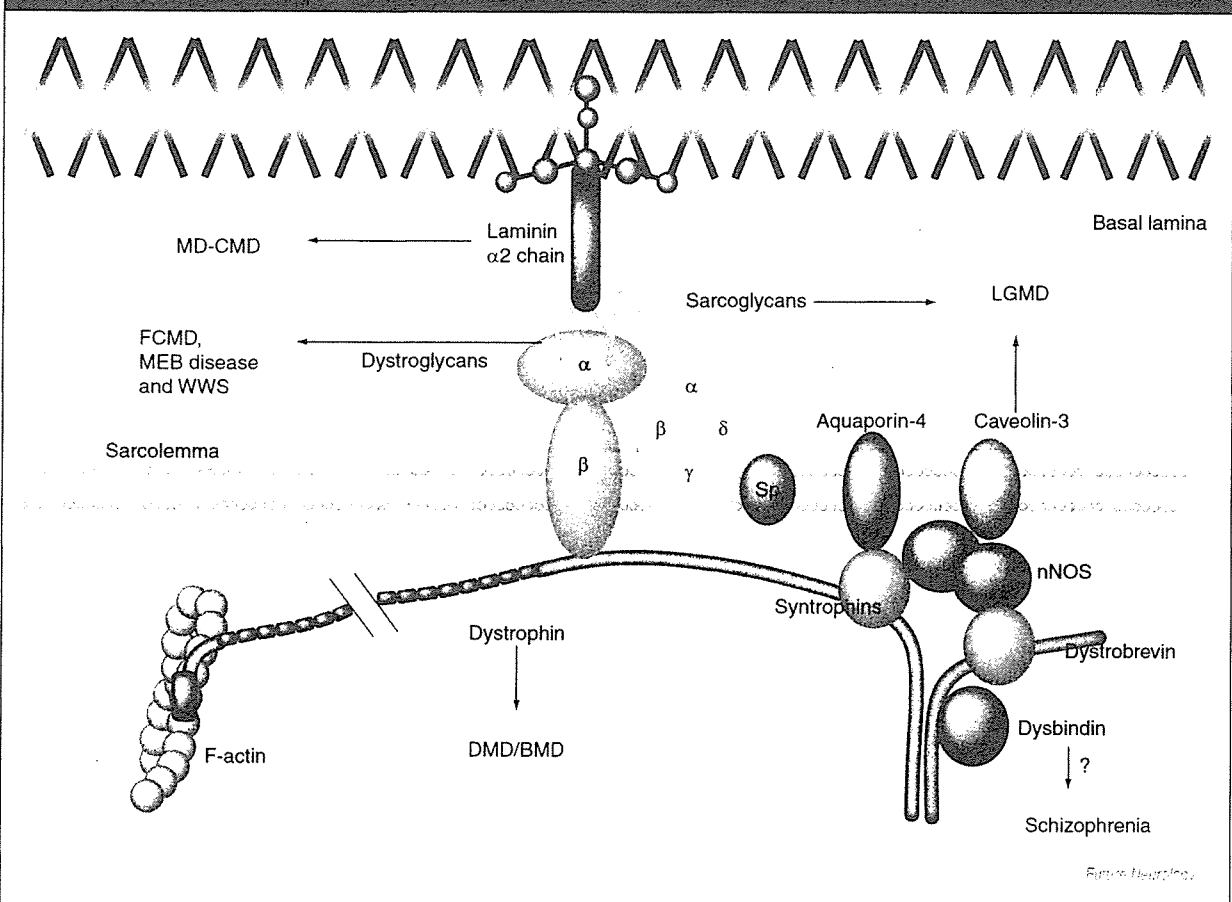
degrees of similarity in their capsid proteins and show diverse tissue tropisms. More than nine AAV vectors have already been developed and evaluated in animal models as a tool for gene transfer *in vivo* (Table 1) [10]. Although the molecular mechanisms of tissue and cell tropisms of AAV vectors are 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 $\alpha 2$ 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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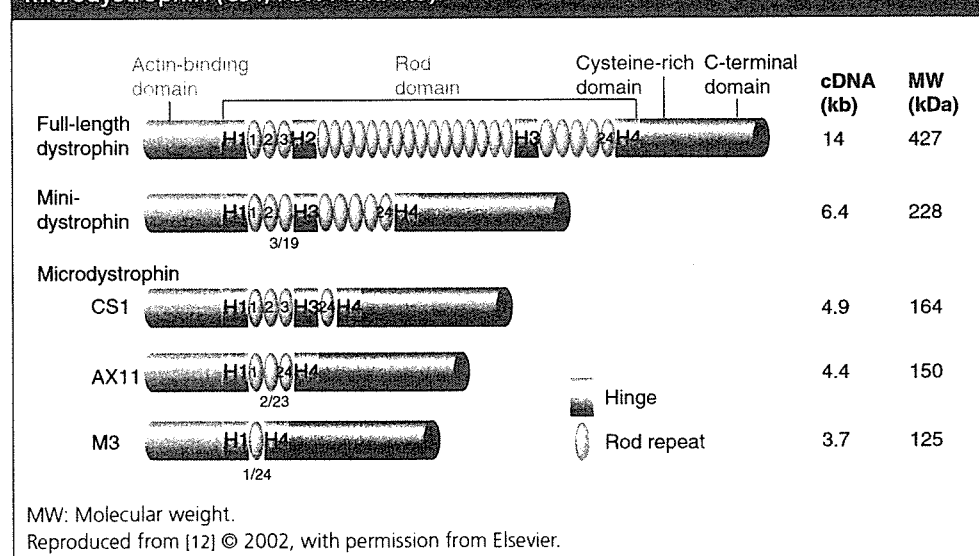
a full-length dystrophin gene (14-kb mRNA, 11-kb open reading frame). To overcome this drawback, several groups have designed small dystrophins in which the long, central rod domains are largely deleted, and tested their functions in dystrophin-deficient *mdx* mice [11]. The functions of three types 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).



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 non-human 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.

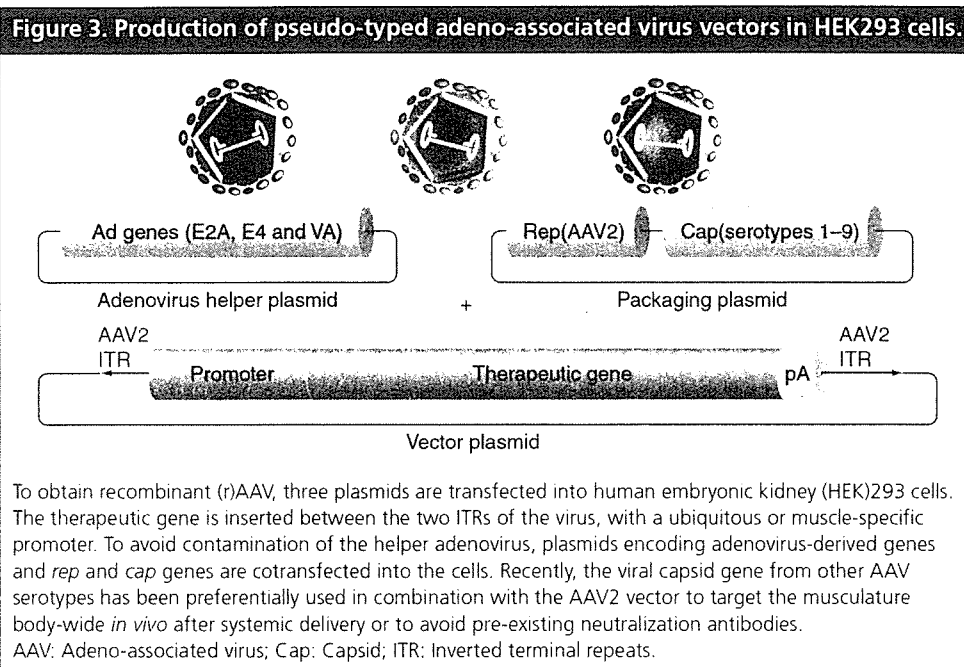
In an attempt to introduce a therapeutic gene larger than 4.7 kb into target cells, the trans-splicing approach, in which the gene is split between two rAAV vectors, each containing part of an intron with either a splice-acceptor or a splice-donor sequence, has been developed. After formation of head-to-tail 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 $2\text{--}5 \times 10^{13}$ 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 non-mammalian 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.



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 micro-dystrophin 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 dystrophin 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 over-expressing 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.

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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, AC 133-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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Research article

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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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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.

Conclusion: Cardiac involvement in CXMD₁ dogs is milder and has slower progression than that described in GRMD dogs. The distinct deep Q-waves have been ascribed to myocardial fibrosis in the posterobasal region of the left ventricle, but our data showed that they precede the lesion on echocardiogram and histopathology. These findings imply that studies of CXMD₁ may reveal not only another causative mechanism of the deep Q-waves but also more information on the pathogenesis in the dystrophin-deficient heart.

Background

Duchenne muscular dystrophy (DMD) is a common and lethal genetic disease characterized by progressive muscle wasting. It is an X-linked recessive disorder caused by mutations in the dystrophin gene, which encodes a cytoskeletal protein, dystrophin [1]. The absence of dystrophin is accompanied by a loss of dystrophin-glycoprotein complex at the sarcolemma and results in progressive degeneration of skeletal and cardiac muscle with fibrotic tissue replacement and fatty infiltration [2,3]. The onset of the disease occurs between 2 and 5 years of age, and most patients die of respiratory or cardiac failure [4,5]. Cardiac involvement, which occurs commonly in DMD patients, has increasingly become an important cause of death because recent clinical progress has reduced the risk of death due to respiratory failure [6,7]

Like dystrophin-deficient skeletal muscle, dystrophin-deficient cardiac muscle is replaced by fibrotic or fatty tissue, especially in the left ventricular posterobasal wall region [8-11]. Atrophic changes with loss of striation, vacuolation, fragmentation, or nuclear degeneration in the myocardium have also been reported [12]. Progressive involvement of the left ventricle leads to wall motion abnormality and results in dilated cardiomyopathy. In DMD patients, the electrocardiogram (ECG) may show tall R-waves in the right precordial leads, deep Q-waves in leads I, aVL, V5-6 or II, III, and aVF [8-13], as well as an increased heart rate, shortened PQ (PR) interval, conduction abnormalities or arrhythmias such as sinus arrhythmia, atrial ectopic beats, and ventricular premature complexes in DMD patients [13-16]. One of the electrocardiographic abnormalities, deep Q-waves, has been considered to be attributable to myocardial fibrosis [8,9,17]. Echocardiography indicates myocardial thickening, wall motion abnormalities, enlargement of the left ventricle, and left ventricular systolic and diastolic dysfunction. Hypokinesis of the posterobasal wall is consistent with the spreading fibrosis and significant decrease in the internal dimensions of the ventricles [14,15]. There are, however, many unresolved issues in cardiac involvement, such as the reason why the posterobasilar segment of the left ventricle is consistently the first lesion, whether extensive fibrosis involves the conduction system, the pathogenesis of inappropriate tachycardia or electrocardiographic abnormalities, and whether abnormal smooth muscle regulation affects the cardiomyopathy [18]. One

way to clarify these problems is to study suitable animal models.

To date, the X-linked muscular dystrophy (*mdx*) mouse and the Golden retriever-based muscular dystrophy dog (GRMD) have been used for elucidation of the pathogenesis and development of therapy for DMD. The phenotypes of GRMD are more similar to DMD than that of the *mdx* mouse [19-21], and GRMD also shows similar electrocardiographic findings and progressive cardiomyopathy comparable to the cardiac involvement of DMD patients [20-23]. In this respect, GRMD is a useful model to explore cardiac involvement, but GRMD is very difficult to maintain because of their severe phenotypes. Mild phenotypes can be expected in small sized dogs such as Beagle, indicated by the cross-bred study by Valentine *et al.* [20]. Moreover, medium-sized Beagle is easy to handle or raise than GRMD, therefore they have definite advantages in animal housing or welfare. Therefore, we established a Beagle-based dog colony named canine X-linked muscular dystrophy in Japan (CXMD₁) [24]. In CXMD₁, involvement of the temporalis and limb muscles is observed from 2 months of age, and macroglossia, dysphagia, drooling, and joint contracture are apparent from 4 months of age; the phenotypes of CXMD₁ are thus almost comparable to GRMD [25]. In this study, we investigated the cardiac phenotypes in CXMD₁ using electrocardiography, echocardiography, and pathological examinations. Abnormalities on echocardiogram and cardiac pathology were detected from 12 months of age; however, the distinct deep Q-waves in leads II, III, and aVF on ECG were consistently observed by 6-7 months of age in all CXMD₁ dogs examined. The cardiac phenotypes of CXMD₁ were identical to but milder than those of GRMD described in the literature. Thus, CXMD₁ may also be a suitable animal model for elucidation of the above-mentioned problems.

Methods

Animals

We imported frozen GRMD semen and artificially inseminated a Beagle bitch. The carriers produced were mated with unrelated Beagles, and a Beagle-based canine X-linked muscular dystrophy (CXMD₁) breeding colony was established [24]. In this study, four normal male and eight affected male dogs of the third generation (G3) between 2 to 21 months of age were examined. All of the affected and normal dogs were descendents of a single affected

male, and were part of the CXMD₁ breeding colony at the General Animal Research Facility, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP) (Tokyo, Japan) or the Chugai Research Institute for Medical Science, Inc. (Nagano, Japan). The clinical and histopathological characteristics, except for cardiac involvement, of CXMD₁ dogs were recently described [25]. These dogs were cared for and treated in accordance with the guidelines provided by the Ethics Committee for the Treatment of Laboratory Middle-Sized Animals of the National Institute of Neuroscience, NCNP (Tokyo, Japan) or the Ethics Committee for Treatment of Laboratory Animal of Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). These studies were also approved by the Ethics Committee for the Treatment of Laboratory Middle-Sized Animals of NCNP (approved No. 13-03, 14-03, 15-03, 16-03, 17-03, and 18-03). All experiments were performed with consideration for preventing unnecessary pain.

Genotyping of CXMD₁ allele

Each affected or normal male dog was identified by genotyping. A snapback method of single-strand conformation polymorphism analysis was used to determine the GRMD allele as described previously [26].

Measurement of serum creatine kinase (CK)

Blood samples were obtained from the cephalic vein at sacrifice. Serum CK level was measured by colorimetric assay using a FDC3500 clinical biochemistry analyzer (FujiFilm Medical Co., Tokyo, Japan).

Electrocardiographic studies

Leads I, II, III, aVR, aVL and aVF were recorded in the right lateral recumbency using an ECG-922 electrocardiograph (Nihon Koden, Tokyo, Japan) [27]. All ECGs were obtained at a paper speed of 50 mm/sec and calibration of 10 mm/mV. First, the electrocardiography were performed in two CXMD₁ (III-302MA, III-303MA) and one normal littermate (III-301MN) dogs at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age, and the heart rate (HR), intervals of PQ and QRS, and Q/R ratios were measured. However, in normal control and in CXMD₁, Q waves were not prominent in leads aVR and aVL, therefore, we measured the Q/R ratios in leads I, II, III and aVF. Next, we compared the HR, intervals of PQ and QRS, or Q/R ratios in I, II, III and aVF in eight CXMD₁ and four normal dogs at 6–7 months of age.

Echocardiographic studies

M-mode and two-dimensional echocardiography was performed using an EUB-8000 echocardiograph (Hitachi Medical Corporation, Tokyo, Japan). The thickness of the interventricular septum (IVS) and left ventricular posterior wall (LVPW) at end-diastole, left ventricular internal

dimension at end-diastole (LVIDd) and systole (LVIDs), and fractional shortening (FS) were examined on normal and CXMD₁ dogs using M-mode echocardiography. We calculated the M-mode parameters based on multiple measurements of 5 consecutive heart cycles, or 3 or 5 representative heart cycles. We examined the parameters mentioned above and myocardial echogenicity in two CXMD₁ (III-302MA, III-303MA) and one normal littermate (III-301MN) dogs at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age. We also examined the parameters mentioned above and myocardial echogenicity in six CXMD₁ (III-D53MA, III-D55MA, III-1803MA, III-D38MA, III-D02MA, III-D08MA) and three normal dogs (III-D56MN, III-1804MN, III-D03MN) at the time point just before euthanasia. Among those dogs, myocardial echogenicity in one CXMD₁ (III-D02MA) and its normal littermate (III-D03MN), and another CXMD₁ (III-D08MA) dogs were also examined at various time points.

Macroscopic and histopathological analyses

All dogs in this study underwent cardiac histological analysis. After a dog was given an overdose of intravenous pentobarbital, the whole heart was removed and immediately fixed in 15% buffered formalin. Formalin-fixed hearts were cross-sectioned, and samples were taken from the right and left ventricles at the apical papillary muscle and basal levels (each level containing the interventricular septum, anterior wall, lateral wall, and posterior wall). The tissue blocks were embedded in paraffin, and 10 μm sections were stained with hematoxylin and eosin or Masson's trichrome stain. Photographs were taken with a DAS Mikroskop LEITZ DMRβ microscope (Leica, Wetzlar, Germany), using a digital still camera system HC-2500 (Fuji-Film, Tokyo, Japan).

Statistics

Data are expressed as means ± SE. Student's *t* test was used to evaluate differences between the two groups. A *p* value of less than 0.05 was considered to indicate statistical significance.

Results

Clinical profiles of CXMD₁

We recently reported the detailed clinical and histopathological characteristics of CXMD₁ except for cardiac phenotypes [25]. None of the dogs in the present study showed clinical signs of heart failure, and no murmur was present on auscultation in any CXMD₁ dog examined. We evaluated body and heart weight, the ratio of heart to body weight, and serum CK in eight CXMD₁ and four normal male dogs aged 6–21 months just before euthanasia (Table 1). There were no differences in body and heart weight and heart/body weight ratio between normal and CXMD₁ dogs. Serum CK levels in the CXMD₁ dogs ranged from 12,500 to 13,800 IU/l. These values were signifi-

cantly different from those in normal control dogs (60 to 515 IU/l). One 9-month-old CXMD₁ dog, III-D55MA, did not show any signs of respiratory or cardiac failure. When we tried to record a routine ECG of the dog, the dog struggled to escape from recording and then ceased moving. Immediately afterwards, we recorded ECG and the monitor showed an idioventricular rhythm. The dog died despite attempted cardiopulmonary resuscitation.

Electrocardiographic findings

The HR and PQ intervals of the affected littermates were no different from those recorded from a normal littermate at 12 months of age, but we detected an increase in HR and a decrease in PQ interval in the affected dogs after 15 months of age (Fig. 1A). The HR and PQ intervals were negatively correlated both in normal and affected dogs (data not shown). The QRS interval in the affected dogs did not differ from that in the normal littermate at any age (Fig. 1A). Prominent deep Q-waves were observed in limb leads II, III, and aVF in some CXMD₁ dogs, but not in the normal littermates, as shown in Fig. 1B. The Q/R ratios were definitely increased in the affected littermates at 6 months of age or older compared with the normal littermate (Fig. 1C). In all normal and CXMD₁ dogs at 6–7 months of age, the HR, and intervals of PQ and QRS were not different between the two groups of dogs (Fig. 2A), but the Q/R ratios in leads II, III, and aVF in the affected dogs were significantly higher than those in the normal dogs (Fig. 2B).

Echocardiographic findings

The thickness of LVIDd, IVS, and PW in two CXMD₁ (III-302MA, III-303MA) were not different from those in a normal littermate (III-301MN) by sequential analysis with advancing age (Fig. 3A). Those parameters were not different between other six CXMD₁ and three normal dogs,

when examined just before euthanasia (Table 2). FS in III-302MA decreased with advancing age, and the value (27.3%) at 21 months of age was lower than that of the normal littermate, but was within normal range reported previously [28,29]. FS in the other seven CXMD₁ were normal, even just before euthanasia (Table 2). A mild hypokinesis of the left ventricular wall was detected in III-302MA at 21 months of age (Fig. 3B), but any clinical signs had not been developed in the dog.

The sequential studies of myocardial echogenicity with advancing age in III-302MA and in III-303MA demonstrated that the hyperechoic lesions in the left ventricular posterior wall were seen at 12 months of age or older (Fig. 4A, Table 3). In the subsequent examinations of six CXMD₁, we found the hyperechoic lesion in a CXMD₁, III-D08MA, at 5 months of age (Fig. 4B, Table 3), however the hyperechoic lesion was not detected in other four CXMD₁ at 5 to 7 months old (Fig. 4B, Table 3). One CXMD₁, III-D38MA, did not reveal any hyperechoic lesions when examined at 12 months of age, but has not been examined at 5 to 7 months of age (Table 3). Taken these echocardiographic data, it is considered that the cardiac functions in CXMD₁ were basically maintained well by 21 months of age, despite showing hyperechoic lesions of the left ventricle in limited numbers of CXMD₁.

Macroscopic and histopathological findings

The right and left ventricular walls were examined macroscopically and histopathologically in four normal and eight affected male dogs at the ages shown in Table 1. The base view of the formalin-fixed heart did not show any macroscopic lesions in III-1803MA at 7 months and III-302MA at 21 months of age (Fig. 5A) like other affected dogs (data not shown). No histopathological abnormality was found in the posterior wall of the left ventricle of

Table 1: Clinical profiles of normal and CXMD₁ male dogs

	Age (mo)	BW(g)	HW(g)	HW/BW (%)	Serum CK (IU/l)
Normal dogs					
III-D56MN	6	12.0	95.1	0.97	515
III-1804MN	7	13.6	110.0	0.81	215
III-D03MN	14	13.1	127.0	0.84	215
III-301 MN	21	14.4	120.0	0.96	60
CXMD₁ dogs					
III-D53MA	6	9.6	91.9	0.90	63,100
III-D55MA	7	10.0	80.0	0.80	42,000
III-1803MA	9	14.4	128.6	0.69	69,100
III-D38MA	12	11.4	78.8	1.01	138,000
III-D02MA	15	9.1	92.0	0.87	17,600
III-D08MA	15	12.0	104.3	0.97	40,700
III-302MA	21	12.4	120.0	0.86	12,500
III-303MA	21	13.9	120.0	0.80	23,000

BW, body weight; HW, heart weight; HW/BW, heart weight/body weight ratio; * $p < 0.01$ normal dogs vs CXMD₁ dogs

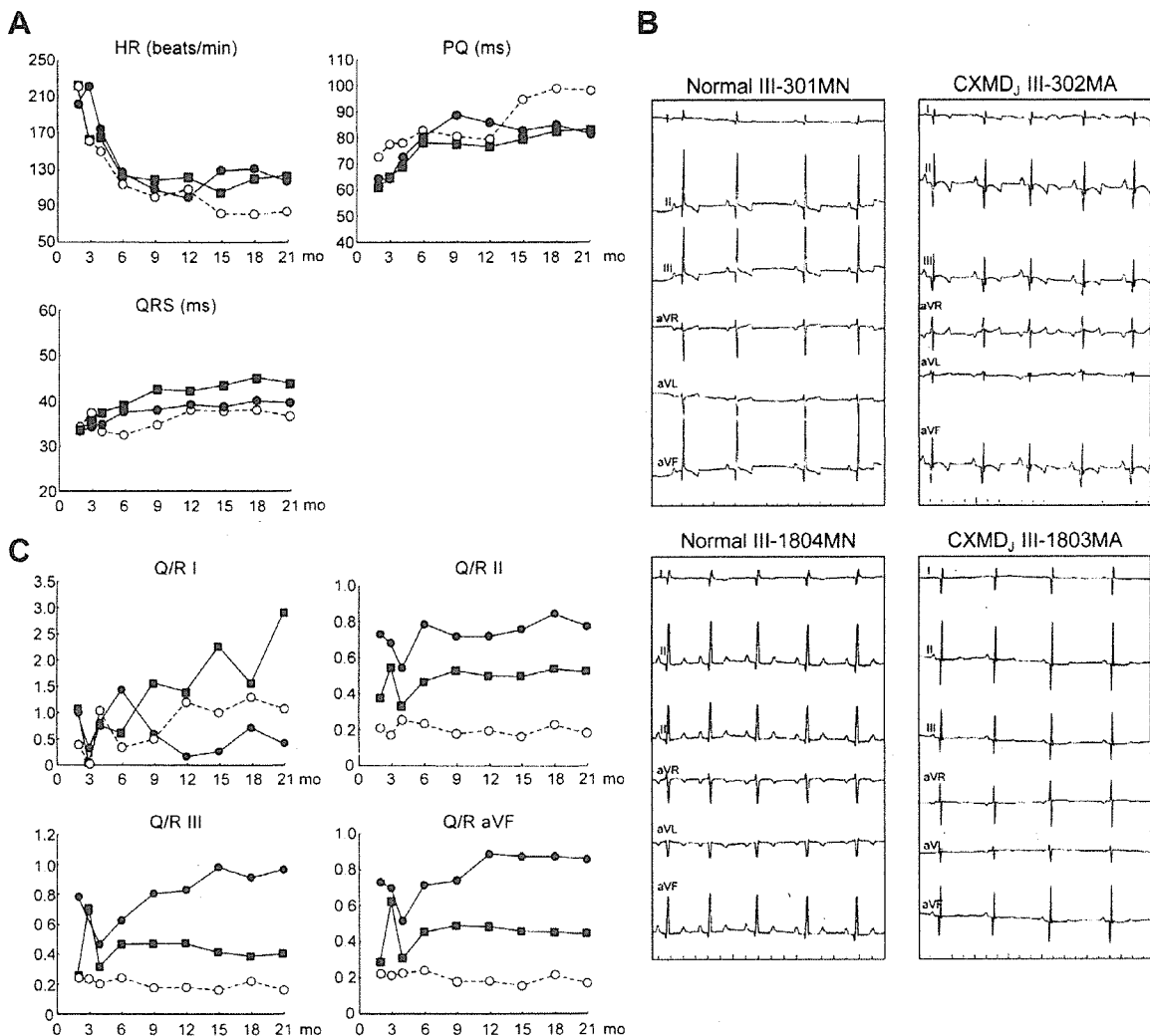


Figure 1
Electrocardiographic findings in CXMD_J. **A:** Sequential studies in electrocardiographic parameters with advancing age in normal and CXMD_J dogs. Heart rate (HR) (beats/min), PQ interval (ms), and duration (ms) of QRS complex on ECG in a normal littermate III-301MN (open circle), and CXMD_J dogs III-302MA (closed circle) and III-303MA (closed square) at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age. **B:** Representative ECGs in normal and CXMD_J male dogs. ECGs were recorded from normal dogs, III-301MN and III-1804MN, and CXMD_J dogs, III-302MA and III-1803MA, at 6 months of age. Distinct deep Q waves were present in the CXMD_J dogs. Leads were recorded at 50 mm/s, 1 cm/mV. **C:** Sequential studies in Q/R ratios with advancing age in limb leads I, II, III, and aVF in normal and CXMD_J dogs. Q/R ratios in limb leads I, II, III, and aVF in a normal littermate III-301MN (open circle), and the CXMD_J dogs III-302MA (closed circle) and III-303MA (closed square) at 2, 3, 4, 6, 9, 12, 15, 18, and 21 months of age.

affected dogs III-1803MA, III-D55MA, and III-D02MA (Fig. 5B), and other affected dogs under the age of 12 months (III-D53MA, III-D38MA). On the other hand,

moderate fibrosis in the left ventricular wall, especially on the posterior side, was detected in an affected dog, III-302MA, at 21 months (Fig. 5B) as well as in III-D08MA at

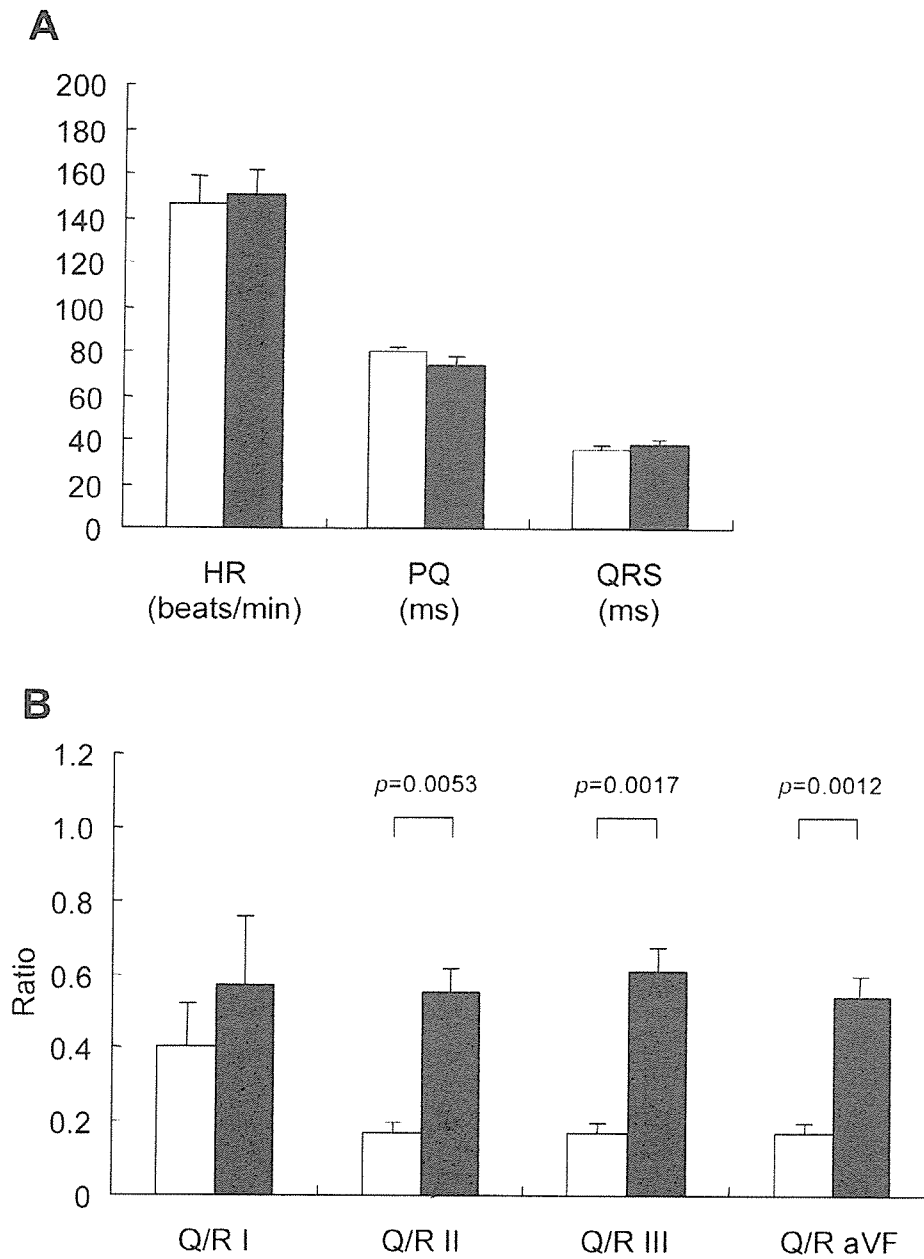


Figure 2
Comparison of electrocardiographic parameters and Q/R ratios between normal and CXMD₁ dogs at 6-7 months of age **A.** Heart rate (HR) (beats/min), PQ interval (ms), and duration of QRS complex (ms) on ECG in normal (n = 4) and CXMD₁ (n = 8) dogs at 6-7 months of age. White columns indicate normal dogs, and black columns represent CXMD₁ dogs. Bar shows mean +/- SE. **B.** Q/R ratios in limb leads I (Q/R I), II (Q/R II), III (Q/R III), and aVF (Q/R aVF) on ECG in normal (n = 4) and CXMD₁ (n = 8) dogs at 6-7 months of age. White columns indicate normal dogs, and black columns represent CXMD₁ dogs. Bar shows mean +/- SE.