Background

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

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

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

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

Methods

Animals

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

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

Histological and immunohistochemical analysis

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

Myosin extraction and gel separation

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

Results

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

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

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

MHC expression and fiber size distribution

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

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

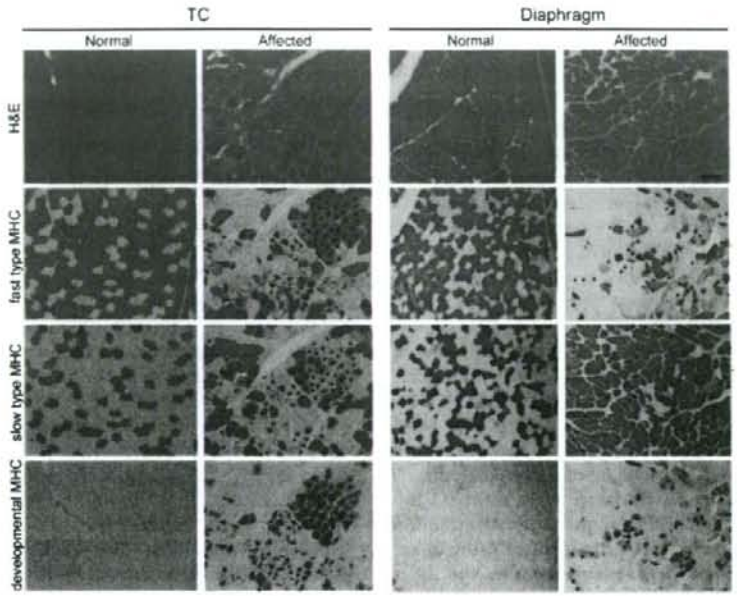

Figure 1
Representative images of histology (H&E) and expression of fast type, slow type, or developmental myosin heavy chain (MHC) in tibialis cranialis (TC) muscle and diaphragm of a normal (10 months old) or a CXMD$_J$ dog (11 months old). Identical parts of serial cross-sections are shown in longitudinal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

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

Time courses of histology and MHC expression

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

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

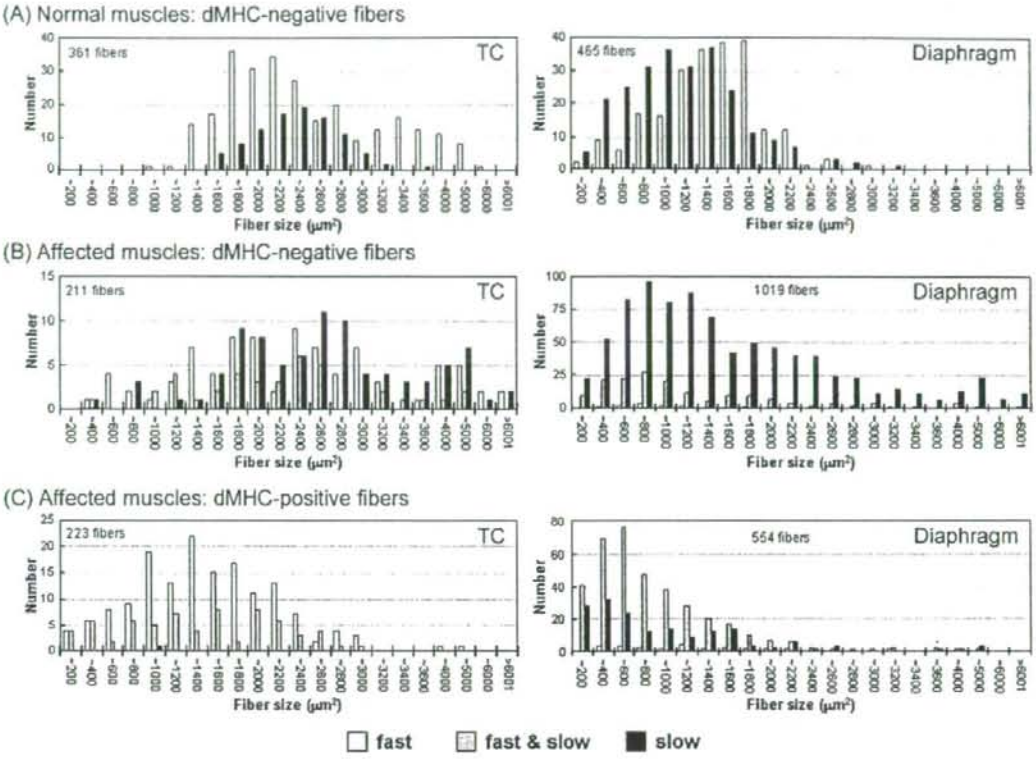

Figure 2

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

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

Temporal changes of MHC isoforms

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

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

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

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

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

Discussion

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

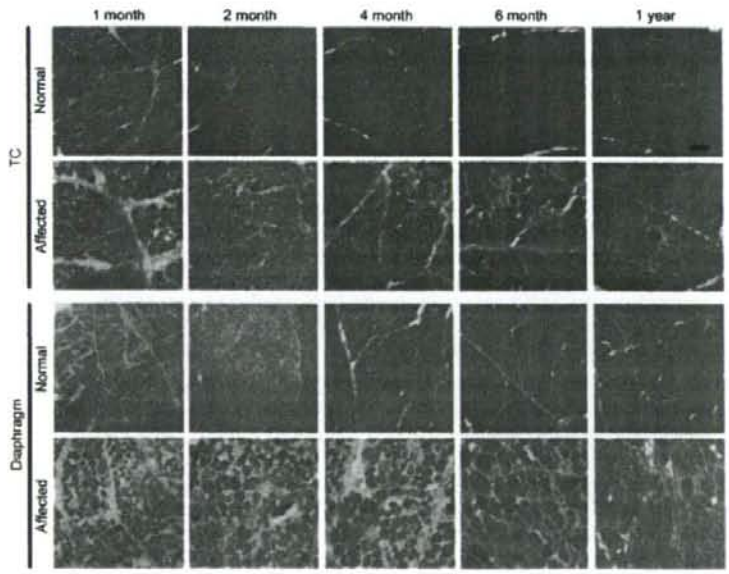

Figure 3
Representative histological findings in TC muscles and diaphragms of a normal or a $CXMD_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that severe degenerative lesions were observed from early ages in affected diaphragms, as compared with affected TC muscles. Bar: 200 μm.

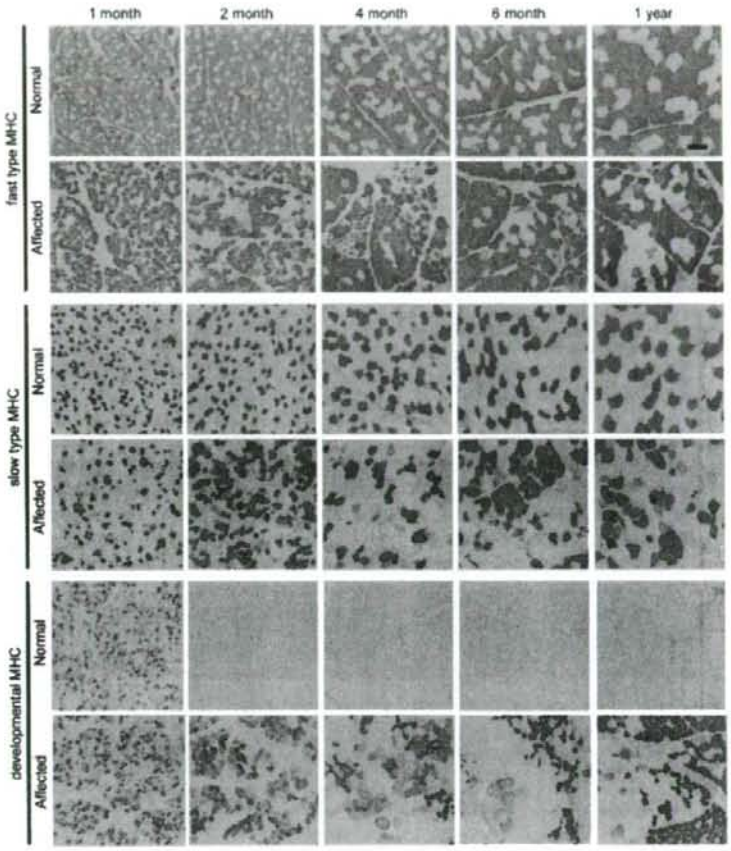

Figure 4
Expression of fast type, slow type, and developmental MHCs in TC muscles of a normal or a CXMD$_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that there were no notable differences between expression levels of fast and slow type MHCs in normal and affected TC muscles. Bar: 200 μm.

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

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

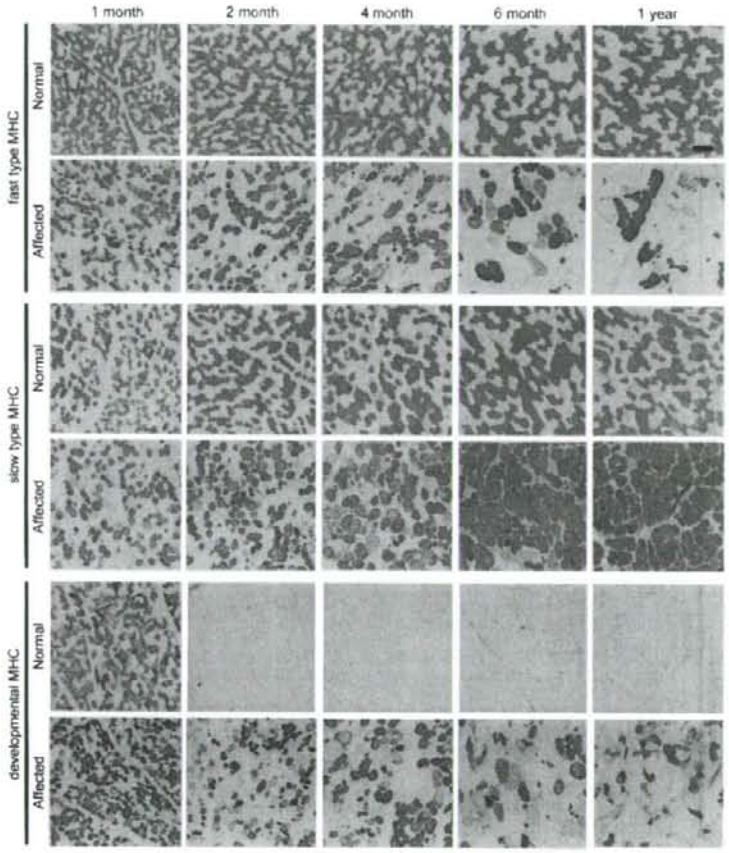

Figure 5
Expression of fast type, slow type, and developmental MHCs in diaphragms of a normal or a $CXMD_J$ dog at 1, 2, 4, 6 months, and 1 year old. Note that slow MHC fibers were increased markedly in the affected diaphragms after 6 months old, while fast MHC fibers were decreased. Bar: 200 μm.

Common features between TC muscle and diaphragm of $CXMD_J$

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

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

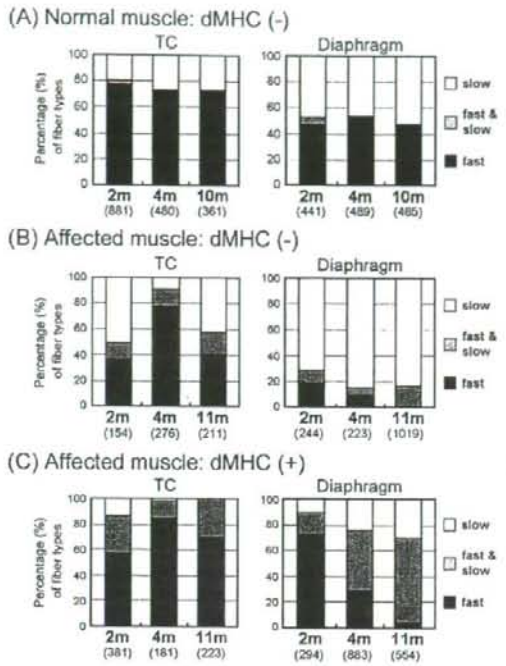

Figure 6

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

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

Differences between TC muscle and diaphragm of $CXMD_J$

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

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

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

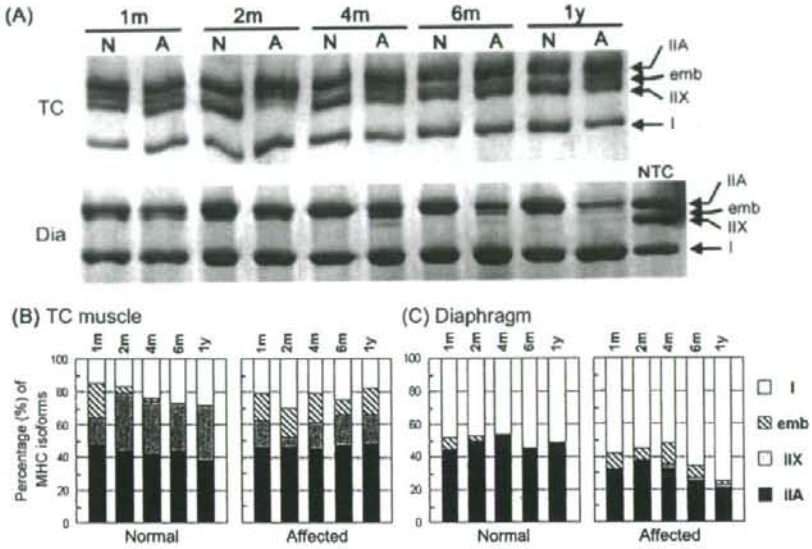

Figure 7

MHC isoforms in skeletal muscles of normal and CXMD$_J$ dogs. (A) Electrophoretic separation of MHC isoforms in TC muscle and diaphragm. Myosin was extracted from muscles at various ages (1, 2, 4, 6 months, and 1 year old), and aliquots of 0.4 μg of protein were separated on 8% SDS-polyacrylamide gels containing 30% glycerol. Four MHC isoforms (I, IIX, IIA, and embryonic) were detected. NTC: normal TC muscle at 1 year old. Note that MHC type I increased in the affected diaphragm after 6 months old. (B) Quantitative analysis of MHC isoforms. MHC expression between two groups (normal *vs* affected) or among ages (1, 2, 4, 6 months and 1 year) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected between normal and affected groups in TC muscles after 2 months old or in diaphragms after 4 months old, and between 1 and 2 months old in normal TC muscles.

regenerating fibers of the CXMD$_J$ diaphragm, the proportion of myofibers expressing slow type MHC increased markedly after 4 months old (Fig. 6C). These results suggested that MHC expression in TC muscle and the diaphragm of CXMD$_J$ would be influenced by different mechanisms after 4 months old. These age-dependent MHC expression might be related to body growth, particularly increasing of muscle mass. One possibility is participation of insulin-like growth factor (IGF)-1, which is important for postnatal growth of skeletal muscles [33] and can activate multiple Ca^{2+}-dependent signaling pathways, including the calcineurin/NFAT pathway [30]. When growth rate of body weight decreases after 4 months old [5], signaling activity of IGF-1 might reduce and MHC expression might be regulated predominantly by alternative signaling pathways.

Comparison among mdx, CXMD$_J$ and DMD diaphragms

MHC expression in normal skeletal muscle has been well studied in mice [15,34], dogs [11], and humans [35]. In normal dogs, the proportions of fiber types in TC muscle were relatively similar to those in the representative tibialis anterior muscles of mice and humans. In the diaphragm, however, the proportion of fiber types differed markedly among these species. The murine diaphragm is composed mainly of fast type IIA and IIX isoforms [15,34], but the canine diaphragm consists of equal populations of slow type MHC I and fast type MHC IIA [11], as also shown in our study. In normal human diaphragm, the distribution of myosin isoforms has been estimated that types I, IIA, and IIX account for approximately 45%, 40%, and 15%, respectively [35]. Thus, the proportions of MHC isoforms in the diaphragm of healthy dogs are much closer to those of humans than those of mice.

Some groups have studied expression profiles of MHC isoforms in the diaphragm of the *mdx* mouse. The *mdx* diaphragm shows increases in MHC type I fibers and elimination of type IIX population at 2 years old, but not at young ages (3 to 6 months old) [13,15,34]. In contrast to the *mdx* diaphragm, that in CXMD$_J$ exhibited drastic changes even in younger animals (6 months old). On the

other hand, there is no direct information available regarding the changes in fiber type composition in the diaphragm in human DMD. In addition, there is an important difference of MHC expression even in limb skeletal muscles between large mammals (including dogs and humans) and mammals with smaller body mass, especially rodents. The former do not express the fastest MHC IIB isoform in limb muscles [10,11,36], while it is abundantly expressed in the latter [34]. Therefore, changes/adaptations in skeletal muscles of dogs with muscular dystrophy are likely to be more relevant to human DMD, than that in the *mdx* mouse. As it is difficult to examine the diaphragms of DMD patients, it would be important to investigate the differences between murine and canine models for understanding the mechanisms of respiratory failure in human DMD.

Conclusion

Based on fiber type classification using MHC expression, we demonstrated the predominant replacement with slow fibers and reduced muscle regeneration with progression of muscular dystrophy in the diaphragm of a canine DMD model, but these phenomena were much less strict in affected TC muscle. In addition, the expression profiles of MHC isoforms in the $CXMD_J$ diaphragm were evidently different from those of the *mdx* mouse. Our results indicated that dystrophic dog is a more appropriate model than a murine one for human DMD, and would be useful for investigation of the mechanisms of respiratory failure in DMD, as well as pathological and molecular biological backgrounds, and therapeutic effects in clinical trials.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KY designed the study, carried out the pathological and immunohistological examinations, and drafted the manuscript. AN participated in interpretation of data, and helped to draft the manuscript. TH participated in coordination of the study. ST participated in the design, planning, and coordination of the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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筋ジストロフィーと細胞移植治療
――骨髄間葉系細胞からの骨格筋細胞の誘導

Cell-based therapy for muscle degenerative diseases : induction of skeletal muscle cells from bone marrow stromal cells

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◎幹細胞を用いる細胞移植治療は，次世代の医療としてその発展が期待されている．とりわけ，重篤な疾患である筋ジストロフィーについてもその早い確立が切望されているが，移植細胞の候補として筋芽細胞や各種幹細胞，筋肉に含まれている増殖可能な幹細胞，ES 細胞，あるいは胎児の細胞から筋肉細胞を誘導する方法などが検討されている．今回，著者らは骨髄間葉系細胞から効率よく筋細胞を誘導する方法を開発したので紹介する．

Key word：間葉系幹細胞，分化転換，Notch，筋ジストロフィー，筋衛星細胞

筋ジストロフィーを代表とする筋肉変性疾患に対しては遺伝子治療や薬剤投与などをはじめ各種の方法が試みられており，そのひとつとして細胞移植治療の開発も取り組まれてきた．さまざまな種類の細胞が移植治療の対象として検証されてきており，筋芽細胞(myoblast)は，Duchenne muscular dystrophy(DMD)モデルマウスに移植するとdystrophin を発現する筋肉になるということで先駆的な研究として注目を浴びた[1]．しかし，1990年代に臨床試験が行われたが機能改善がみられないという結果に終わっており，それは免疫拒絶によって生着する細胞数が減少するからであろうとの結論であった[2]．筋芽細胞のほかに mesangioblasts(vessel-associated progenitor cell ともいわれている)[3]，血液あるいは筋肉由来の $CD133^+$細胞[4]，side population(SP)細胞[5]なども筋変性モデルへ移植され，その有効性が報告されており，細胞移植治療の候補として考えられている．

とりわけ重要なものに骨格筋幹細胞があげられるであろう．骨格筋組織に存在する幹細胞(muscle-derived stem cells：MDSC)は，筋線維とそれを取り巻く基底膜との間に存在する筋衛星細胞であることが知られており，当然ながらこの細胞は筋肉組織の再生の元となるため，細胞の分離と樹立が研究されている[6]．しかし，生体外では増殖力が低下するため，移植に必要な大量培養が困難であるなどの問題もある．同時に ES 細胞，あるいは胎児の細胞から筋肉細胞を誘導する方法が検討されてきており，細胞移植治療に大きな可能性が見出されつつあるが，倫理問題や安全性，細胞数確保などの問題もある．

今回，著者らの研究グループは骨髄間葉系細胞から骨格筋系譜の細胞を誘導する方法を開発した．この細胞のもつメリットや問題点などに焦点を当て，筋細胞移植治療の可能性について考察したい．

骨髄間葉系細胞とは

骨髄には造血系細胞とは別に間葉系の細胞がある．造血系の細胞は浮遊細胞であるので，培養皿に接着しないが，骨髄間葉系細胞はよく接着するため，骨髄液を培養すると，この性質の違いによって接着性細胞として容易に採取することが可能である[7]．ここで念頭におかなくてはならないのは，

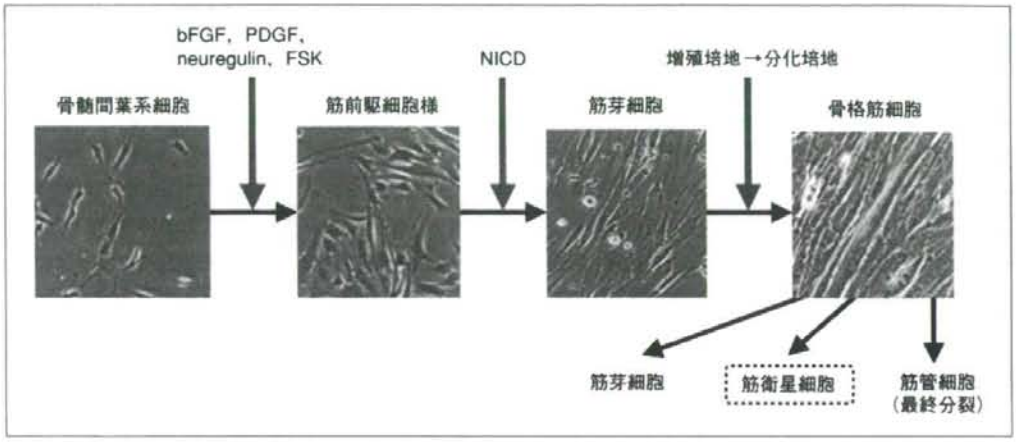

図 1　ヒト骨髄間葉系細胞からの骨格筋系細胞の誘導法

このようにして採取された細胞は間葉系細胞ではあるものの，遺伝子発現や細胞表面抗原などにおいてはけっして均質な細胞群ではなく，ヘテロな集団であるということである．実際に FACS 解析を行うと，間葉系のマーカーとされている CD29, CD90 などはほとんどの細胞に出ているが，一方，CD34，c-Kit など造血系マーカーは数パーセントであるとはいえ混在しており，けっしてゼロではない．おそらく分化転換能においてもヘテロな集団であり，ポテンシャルは細胞によって差があるものと推定される．

骨髄間葉系細胞は増殖力が非常に旺盛であり，短期間で移植にもっていけるだけの細胞数を確保することが可能である．たとえば，数十 ml の骨髄液を培養すれば数週間で約 1,000 万個の細胞を獲得することができる．なによりも患者本人の細胞を利用できるので，倫理問題のハードルが低く，また免疫拒絶の問題も回避できるなど，利点の多い細胞である．

また，骨髄間葉系細胞には骨・軟骨・脂肪・心筋などに分化する能力があることが以前より示唆されてきた[6-8]．静脈注射などの方法で生体に投与するとさまざまな臓器に分布し，肝，神経系細胞などをはじめ多様な細胞に分化転換すると報告されている．しかし，このような生体内での自発的な分化転換の効率はきわめて低いようである．一方で，培養下で成長因子や薬剤，メチル化剤などを投与し，特定の細胞に分化させ生体に移植しようという試みもされてきた[8]．

このように，骨髄間葉系細胞は移植治療の候補としてはポテンシャルの高い細胞であるが，効率のよい分化誘導系の確立が必要な段階にあった．

筋肉細胞の誘導法

骨髄液から接着性の骨髄間葉系細胞を培養し，数代の継代培養を経て，細胞がある程度安定して増殖するようになってから誘導を開始する．RT-PCR レベルでは，これら誘導前の骨髄間葉系細胞は胎児期での筋肉発生初期に認められる Pax7 や骨格筋に特有のマーカー MyoD, Myogenin などは発現していないが，筋発生初期でみられる Pax3 は弱いながら発現が認められている[9]．

1,700～1,900cells/cm^{2}の密度で細胞をまき，細胞分化の誘導因子でもある線維芽細胞増殖因子(bFGF：10 ng/ml)のほかに，ニューレグリン(neuregulin：200 ng/ml)および血小板由来増殖因子(PDGF：5 ng/ml)，細胞内 cAMP 上昇作用をもつフォルスコリン(forskloin：5 μM)などを含んだ培地(10%仔ウシ血清の入ったα-MEM という培養液)で数日間培養する．この段階で Pax7 が発現されるようになり，骨髄間葉系細胞は筋肉の前駆細胞様(未分化な筋肉細胞)に分化したと考えられる(図 1)．

このように変化した細胞に Notch1 の細胞質ドメイン(Notch intracellular domain：NICD)(動物個体の発生過程で細胞の分化や増殖を制御する重要

な遺伝子)を含むプラスミドベクター(pCI-neo vector の CMV promoter の下流に組み込んだもの)をリポフェクションにて導入し，G418 選択を行う．この細胞を数日培養すると，細胞は骨格筋細胞へと分化転換し，MyoD，myogenin などの骨格筋特有のマーカーの発現が認められるようになる．さらに，培養液を成熟した骨格筋に分化させる培地(2%ウマ血清を含む DMEM 培地，あるいは無血清培地)に切り替えることによって，一部の細胞が融合を開始して成熟した多核の筋管細胞に分化し，Myosin-heavy chain などの細胞骨格蛋白とともに MRF4/Myf6 の成熟マーカーの発現も認められる．

最終的に誘導される細胞群には，増殖可能な，①単核の筋芽細胞(MyoD 陽性)，および②筋衛星細胞様細胞(Pax7 陽性)，そして③最終分裂を終えた成熟した多核の筋管細胞(Myosin-heavy chain などが陽性)，の 3 種類の細胞が含まれる(図 1)[9]．さらに，増殖可能な①筋芽細胞，および②筋衛生細胞様細胞をクローン培養することによって，誘導過程で混在していた骨格筋に分化する能力をもたない細胞を除去することができる[9]．

骨髄には，わずかではあるが骨格筋に分化する能力を有する幹細胞があることが報告されている[6]．それらの幹細胞は間葉系ではなくむしろ c-kit，CD45，CD34 陽性の細胞で造血系細胞に含まれると考えられている[6]．しかし，培養した骨髄間葉系細胞にごくわずかに骨格筋系の幹細胞が混在し，それらの細胞が上記の筋誘導を担っている可能性もある．著者らはこの点を検証するために，FACS を用いて c-kit，CD45，CD34 陰性の骨髄間葉系細胞だけを集め，上記の方法で骨格筋誘導を行った．その結果，同じ時間経過で 3 種の骨格筋系譜の細胞が誘導することができた．このことから，本現象は培養細胞に含まれるわずかな骨格筋幹細胞によるものではなく，間葉系細胞のシステム的な分化転換によるものであると推定している[9]．

骨格筋への移植

誘導細胞が実際に生体で機能することを確認するために，上記の①筋芽細胞，および②筋衛星細胞様細胞を，cardiotoxin によって変性させたラットの前脛骨筋に移植した．移植細胞は GFP で標識し，10^6の細胞を変性筋肉組織に局所注入，または尾静脈からの静脈注射により投与した．いずれの投与方法でも 2 週後には移植細胞は変性筋組織に生着し，ヒト dystrophin を発現しており，また局所注入のほうが静脈投与に比べやや高い生着率を示していた．筋ジストロフィーのモデルマウスである mdx-nude マウスに移植しても，ほぼ同様の結果が確認された[9]．

通常，筋肉が変性・崩壊すると，筋幹細胞である筋衛星細胞が刺激を受けて増殖を開始し，筋細胞に分化すると同時に一部が筋衛星細胞自身として残るので，繰り返し再生が可能となっている．このようにわれわれの筋肉は日々維持されており，筋衛星細胞はその筋再生の鍵である．

今回，著者らは骨髄間葉系細胞から Pax7 陽性の筋衛星細胞様細胞を誘導しているが，この細胞が本来の筋衛星細胞と同様の特性をもつかということを検討した．最初の移植 4 週後に移植細胞の生着を組織生検によって確認し(筋管細胞の多くは辺縁核で成熟した筋管であることを確認した)，ふたたび細胞を移植することなく cardiotoxin を投与し，2 度目の筋変性を誘導した．移植された細胞から形成された多核の筋管細胞は筋変性によって崩壊し，ふたたび筋細胞になることはできない．しかし，もし移植された細胞の一部が筋衛星細胞の性質を保持していれば，筋細胞変性に伴って増殖誘導が起こり，筋管細胞を形成することができる．そこで 2 度目の筋変性を誘導し，2 週後に筋組織を調べたところ，最初に移植したヒト由来の GFP 陽性の細胞から分化した“中心核の筋管細胞”の再生を確認することができた．筋衛星細胞から筋管細胞が形成される過程ではまず中心核の成熟過程にある筋管細胞がつくられ，ついで辺縁核の筋管(筋線維)へと成熟する．“中心核の筋管細胞”が観察されたことは，2 度目の筋変性後に筋衛星細胞からあらたに形成された筋管細胞であることを示している．すなわち，本方法で誘導された筋衛生細胞を移植すれば，2 度 3 度と筋肉が崩壊することがあっても生着した筋衛星細胞が増殖し，崩壊した筋肉を補うことが可能であ

る[9]．

筋ジストロフィーの治療においては，移植された正常な機能をもつ筋管細胞は長期にわたって生着し，機能することが期待できるが，1回の移植ですべての細胞を置換することは困難である．残念ながら残った患者本人の筋肉細胞は日々，変性・崩壊が進行すると推定される．ところが，本方法では移植された正常な筋衛星細胞が繰り返し増殖刺激を受け，増殖と分化を繰り返すと推定される．すなわち，徐々に異常な筋管細胞から正常な筋管細胞への置き換えが起こることが期待できる．この性質は筋ジストロフィーの治療においては利点であると考えられる．

骨格筋発生において，Notch は筋芽細胞の分化を抑制すると報告されている[10]．今回の研究結果では骨髄間葉系細胞への Notch 遺伝子が用いられているが，これまで知られている筋肉発生における Notch の作用からすれば，逆の作用をもたらしたように見受けられる．従来より Notch の下流で Hes1，Hes5 が機能し，細胞分化を抑制していると考えられているが，今回のシステムでは NICD の導入による Hes1，Hes5 の誘導は認められないなど，骨髄間葉系細胞に特有のシステムがあると推定している．

さらに安全面という観点からすれば，遺伝子導入ではなく蛋白導入などの方法を用いることによって汎用性が広がると思われる．

おわりに

骨髄間葉系細胞には，ES 細胞には匹敵しないまでもいろいろな細胞に分化するポテンシャルがあり，これまで骨，軟骨，脂肪のほかに，心筋，肝細胞，インスリン産生細胞，気道系上皮細胞が誘導されると報告されている[8,11,12]．重要なことは，骨髄間葉系細胞が生体内において仮に自発的に分化転換を起こすとしても，その効率はきわめて低く，細胞移植治療に活用できるものではないということである．胚葉を超えた分化転換を引き起こし，目的とする細胞を一定量以上得るためには，順序立てた分化誘導操作を step-by-step に行う必要があると考えている．

骨髄間葉系細胞は骨髄移植の際にすでに移植されている細胞であり，安全，かつ容易に採取可能で，旺盛な増殖力をもっている．患者本人や家族のほうからの採取も可能であり，既存の骨髄バンクの登録者から提供を受ける方法もある．とくに患者本人の細胞を用いた場合には，免疫拒絶のない自分の細胞を用いた細胞移植治療，すなわち“自己細胞移植治療”が可能となる．ただし筋ジストロフィーではジストロフィン遺伝子など機能を欠失している遺伝子を何らかの方法で補う必要がある．

実際に治療に応用するためには，安全性，有効性など解決しなければならない多くの課題が残されている．とくに Notch の遺伝子導入が操作に入っていること，また本来の間葉系細胞から大きく性質を転換させた骨格筋を移植するとなれば，腫瘍形成の有無などを大型哺乳類を用いて慎重に検証しなくてはならない．

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