Review

Dysferlin and Animal Models for Dysferlinopathy

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Abstract: Dysferlin (DYSF) is involved in the membrane-repair process, in the intracellular vesicle system and in T-tubule development in skeletal muscle. It interacts with mitsugumin 53, annexins, caveolin-3, AHNAK, affixin, S100A10, calpain-3, tubulin and dihydropyridine receptor. Limb-girdle muscular dystrophy 2B (LGMD2B) and Miyoshi myopathy (MM) are muscular dystrophies associated with recessively inherited mutations in the *DYSF* gene. The diseases are characterized by weakness and muscle atrophy that progress slowly and symmetrically in the proximal muscles of the limb girdles. LGMD2B and MM, which are collectively termed "dysferlinopathy", both lead to abnormalities in vesicle traffic and membrane repair at the plasma membrane in muscle fibers. SJL/J (SJL) and A/J mice are naturally occurring animal models for dysferlinopathy. Since there has been no an approach to therapy for dysferlinopathy, the immediate development of a therapeutic method for this genetic disorder is desirable. The murine models are useful in verification experiments for new therapies and they are valuable tools for identifying factors that accelerate dystrophic changes in skeletal muscle. It could be possible that the genetic or immunological background in SJL or A/J mice could modify muscle damage in experiments involving these models, because SJL and A/J mice show differences in the progress and prevalent sites of skeletal muscle lesions as well as in the gene-expression profiles of their skeletal muscle. In this review, we provide up-to-date information on the function of dysferlin, the development of possible therapies for muscle dystrophies (including dysferlinopathy) and the detection of new therapeutic targets for dysferlinopathy by means of experiments using animal models for dysferlinopathy. (DOI: 10.1293/tox.25.135; J Toxicol Pathol 2012; 25: 135–147)

Key words : SJL/J mouse, A/J mouse, dysferlin, complement, dysferlinopathy

Introduction

Muscular dystrophy is a generic term that is used to refer to a group of hereditary muscular disorders characterized clinically by progressive muscular weakness and muscle atrophy and histopathologically by degeneration/necrosis and regeneration of skeletal muscle fibers. This group of inherited muscular disorders includes Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Emery–Dreifuss muscular dystrophy, limb-girdle muscular dystrophy (LGMD), distal muscular dystrophy including

Miyoshi myopathy (MM) and Fukuyama/non-Fukuyama congenital muscular dystrophy among others[1]. Of these disorders, LGMD is a muscular disorder in which weakness and atrophy of muscles progress slowly and symmetrically in the proximal muscles of the limb girdle. Because LGMD includes many types of unclassified muscular dystrophy and is etiologically heterogeneous, a locus-based classification has been proposed by a consortium that met under the auspices of the European Neuromuscular Centre. In this classification, the dominant LGMD loci are designated LGMD1A, LGMD1B, LGMD1C, etc., and the recessive loci are designated as LGMD2A, LGMD2B, LGMD2C, etc., in the order that they were identified[2].

LGMD2B and MM are both caused by recessively inherited mutations in the *dysferlin* (*DYSF*) gene[1]. LGMD2B is characterized by progressive wasting and weakness of the muscles of the proximal lower limb girdle, whereas MM mostly affects the distal muscle groups of the limb girdle. Both disorders are considered to result from a loss of dysferlin (DYSF) protein from the plasma membrane of muscle fibers, leading to abnormalities in vesicle traffic and membrane repair[3,4]; this process is collectively referred to as "dysferlinopathy".

The prevalence of progressive muscular dystrophy among the general population is about four cases in 100,000 people[5]. The relative frequency by the type of disease is 60% DMD, 30% LGMD and 10% facioscapulohumeral dystrophy. Among the 1420 patients diagnosed with muscular dystrophies at the National Center of Neurology and Psychiatry (Japan) in 2004, dystrophinopathy (DMD/BMD) (56%) was the most common condition followed by LGMD (19%). Of these LGMD patients, 18% were diagnosed with LGMD2B, which occurs at a relatively higher frequency in Japan than elsewhere[6]. At the same facility in 2010, dysferlinopathy was identified as the most frequent type of LGMD present among the Japanese population[7].

The progression of muscular dystrophy confines patients to a wheelchair or a ventilator, and it detracts from their quality of life. Various approaches to definitive therapy of muscular dystrophy have been attempted. These include gene therapies, such as gene transfer using plasmids or adeno-associated virus (AAV) vectors, or exon skipping using antisense oligonucleotides (AVI-4658 or GSK-2402968 [PRO-051])[8–10]; cell-based therapies[11]; therapies using small-molecule drugs that act through a read-through mechanism, such as aminoglycosides[12–14], ataluren (PTC124)[15] or TG003[16]; and an antibody therapy using anti-myostatin antibody[17]. Clinical studies for some of these therapies are ongoing in DMD/BMD patients or others. Since there has been no an approach to therapy for dysferlinopathy, the immediate development of a therapeutic method for this genetic disorder is desirable.

As described above, although dysferlinopathy is a type of muscular dystrophy that has a relatively high prevalence in Japan, the development of a standard therapy has been slow, and therefore, animal models of dysferlinopathy remain useful for verification experiments on novel therapies. Additionally, because the histopathological lesions are localized in the limb-girdle muscles, despite a systemic deficiency in dysferlin protein, accelerating factors that produce the muscular damage in only the limb-girdle muscles must be present in patients with dysferlinopathy, and animal models of dysferlinopathy are useful as tools for exploring these accelerating factors.

This review provides up-to-date information on the function of dysferlin, the development of possible therapies for muscle dystrophies (including dysferlinopathy) and the detection of new therapeutic targets for dysferlinopathy by means of experiments using animal models for dysferlinopathy. It also discusses the effectiveness and the problems of using animal models for dysferlinopathy.

The Function of Dysferlin

The role of dysferlin in membrane-repair processes and in the intracellular vesicular system

DYSF is a 230-kDa transmembrane protein that has a C-terminal transmembrane domain. The *DYSF* gene is abundantly expressed in skeletal and cardiac muscle[18], and its gene product is distinct from the dystrophin–glycoprotein complex (Fig. 1)[19]. DYSF belongs to the ferlin family, which includes otoferlin, myoferlin and Fer-1 (identified in *Caenorhabditis elegans*[4]). The proteins of the ferlin family have several calcium-binding C2 domains associated with calcium-dependent membrane fusion and repair[4] (Fig. 2). Fer-1 mediates calcium-dependent membrane fusion of multiple intracellular vesicles (called "membranous organellas") to the spermatid plasma membrane during spermatogenesis in *C. elegans*[4]. DYSF has seven C2 domains and is implicated in Ca-dependent resealing after disruption of the sarcolemma. It is known that the membrane-repair process requires intracellular vesicles[20] that deliver excess membrane to form a "membrane patch" through calcium-triggered vesicular exocytosis[21,22]. It has been suggested that these intracellular vesicles are initially transported to the site of damage by sequential actions of motor proteins, including kinesin and nonmuscle myosin IIA and IIB[23,24]. It has also been suggested that DYSF is distributed to these intracellular vesicles together with caveolin-3[25], and it has been reported that DYSF is also associated with annexins A1 and A2 in a Ca^{2+}- and membrane injury-dependent manner[26].

Some studies have suggested that mitsugumin 53 (MG53) plays a role in facilitated vesicle translocation for muscle membrane repair[27–32]. The interactions between DYSF, MG53 and caveolin-3 have been examined *in vitro* by means of immunoprecipitation experiments[27]. Accordingly, DYSF is considered to act with MG53, annexins and other proteins in the accumulation of vesicles at the site of damage following membrane disruption (Fig. 3). In addition to MG53, annexins and caveolin-3, human neuroblast differentiation-associated protein (AHNAK)[33], affixin[34], S100A10[35], calpain-3[36], tubulin[37], and dihydropyridine receptor (DHPR)[38] have been reported to interact with DYSF. Figure 4 shows a schema of the proteins that interact with DYSF at the sarcolemma in skeletal muscle.

The role of dysferlin in T-tubule development

Some recent studies have shown that DYSF is active in with the development of T-tubules[39,40]. DYSF has been observed to be associated with developing T-tubules and also to interact with DHPR at the sarcolemma of T-tubules[40]. Because a deficiency in DYSF induces ultrastructural abnormalities in primary T-tubules in skeletal muscle, it has been suggested that DYSF is required for the maintenance or development of T-tubules.

The role of dysferlin in ATP release and intracellular Ca^{2+} signaling

Another study has indicated that DYSF might be involved in ATP release and intercellular Ca^{2+} signaling[41]. It has been suggested that DYSF mediates Ca^{2+}-triggered intercellular signaling in response to membrane wounding in sea urchin embryos, as it has been shown that morpholino knockdown of DYSF mRNA expression in sea urchin embryos effectively blocks the release of ATP after membrane damage and thereby suppresses the consequent intercellular
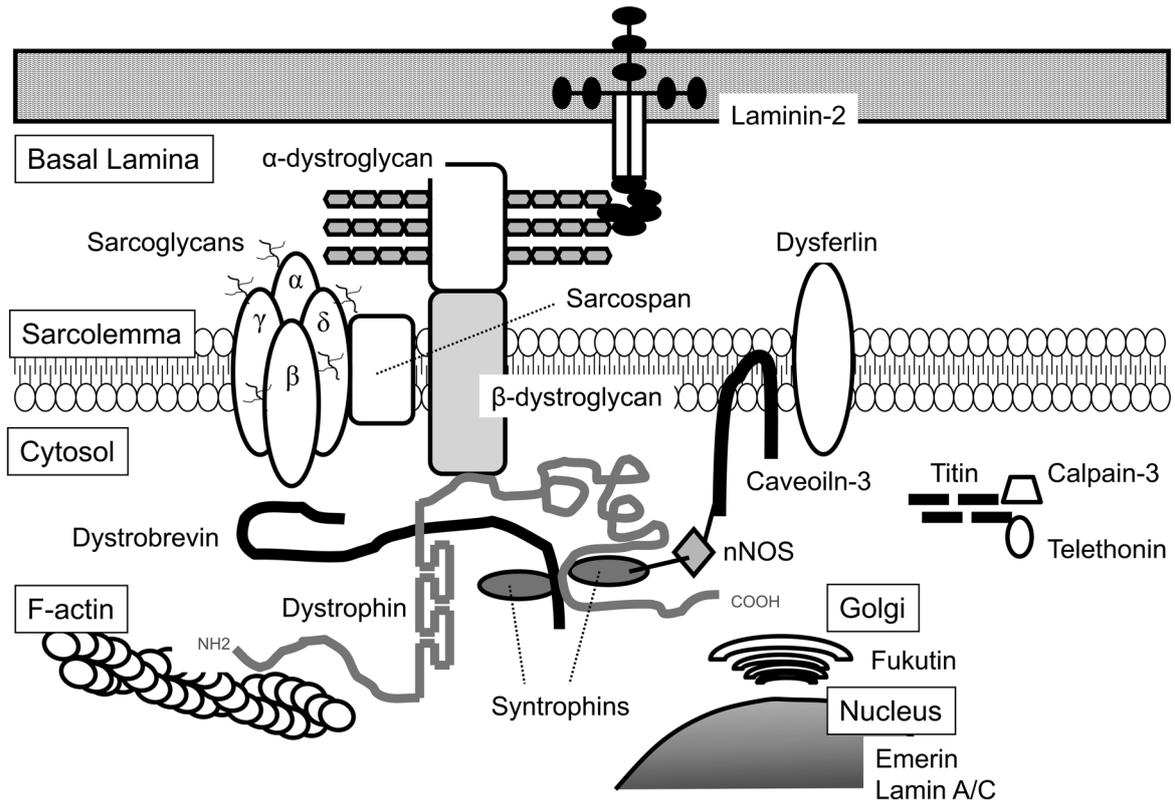
Fig. 1. Schematic showing the organization of some of the integral and peripheral components involved in muscular dystrophies in skeletal muscles.
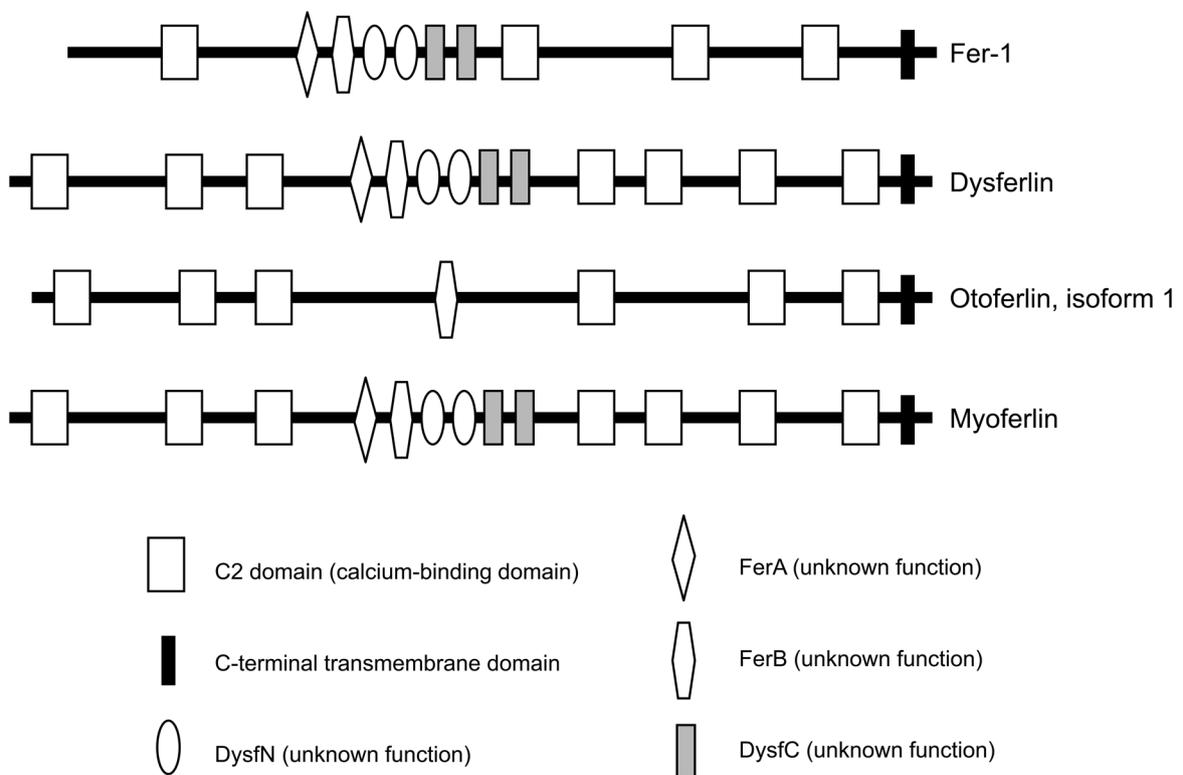
Fig. 2. Conserved structure of the ferlin family. Proteins of the ferlin family have highly homologous structures. The proteins have a variable number of tandem C2 domains and a C-terminal transmembrane domain.
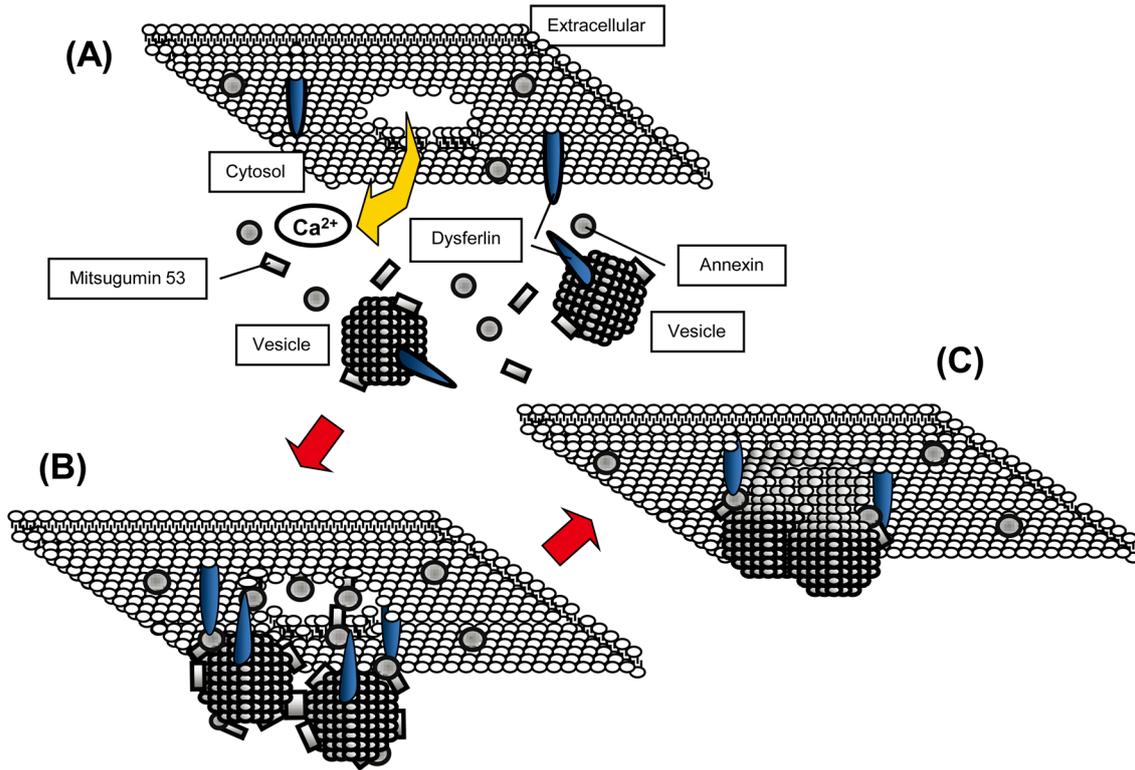
Fig. 3. Schema for a model of the membrane-resealing processes associated with dysferlin *in vitro*. (A) shows the intact condition. Membrane disruption leads to an influx of calcium ions (B). Transport of intracellular vesicles toward the damaged site by the motor proteins kinesin and myosin may be facilitated by mitsugumin 53 (MG53) in an oxidation/cholesterol-dependent manner. The vesicles dock through oxidized MG53 and fuse with each other and the plasma membrane, possibly with mediation by annexin, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and dysferlin in the presence of the calcium ion. Dysferlin interacts with annexins A1 and A2 and mediates wound healing of the sarcolemma. A membrane patch is consequently formed and reseals the membrane lesion (C). Although the proteins dysferlin and MG53 are known to be involved in muscle repair, there is still no direct evidence of an *in vivo* interaction between them.
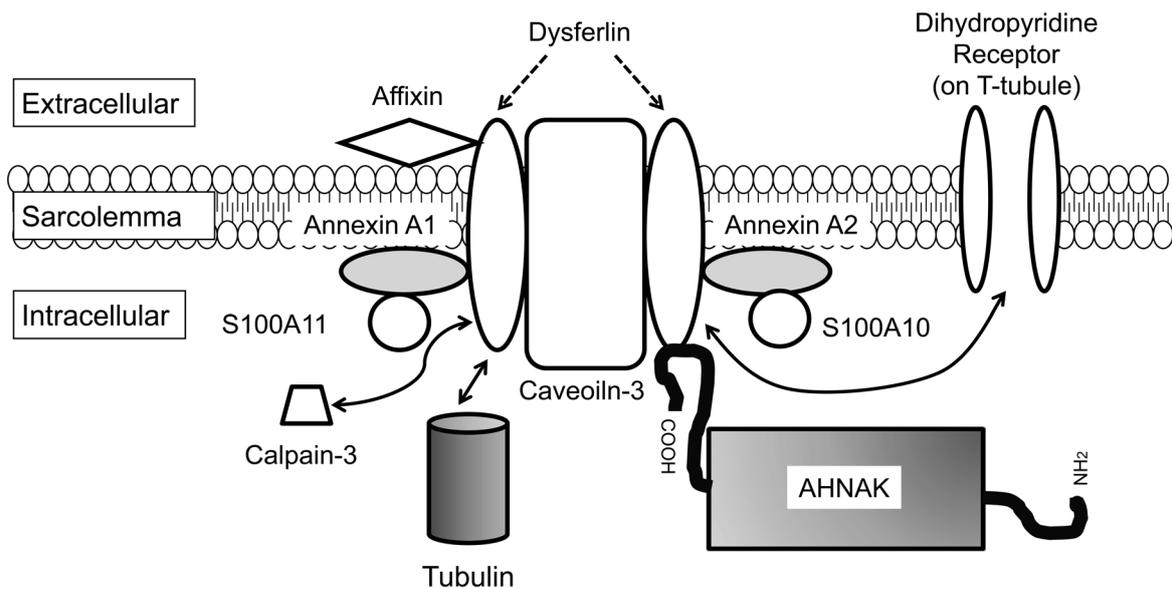
Fig. 4. Schema showing the interactions of proteins with dysferlin at the sarcolemma in skeletal muscle.

Ca^{2+} signaling[41]. In contrast, it has been hypothesized that a deficiency of DYSF in mammalian skeletal muscles results in the release of ATP or other endogenous danger/alarm signals such as high-mobility group box-1 (HMGB1), S100 proteins or heat-shock proteins (HSPs), possibly through a compensatory vesicle-trafficking pathway mediated by the synaptotagmin-like protein Slp2a and the small GTPase Rab27A. It has also been suggested that the released factors activate an inflammatory pathway by means of toll-like receptors or a P2X7 receptor (a mammalian ATP-gated non-selective cation channel)[42]. In fact, P2 receptor antagonist lowered serum levels of CK and reduced muscle damage in dystrophin-deficient *mdx* mice and in sarcoglycan-deficient BIO 14.6 hamsters[43]. Further studies are necessary to elucidate the relationship between releases of danger/alarm molecules and DYSF deficiency.

The role of dysferlin in phagocytosis

Monocytes derived from DYSF-deficient mice and from human patients with dysferlinopathy have been shown to promote phagocytic activity[44]. Knockdown of DYSF mRNA expression by RNA interference in the J774 macrophage cell line significantly enhanced this phagocytosis. Therefore, the accumulation of macrophages in muscles showing dystrophic changes may be a primary lesion caused by DYSF deficiency rather than a secondary lesion that occurs after muscle degeneration/necrosis. However, because muscle-specific transgenic expression of DYSF at appropriate levels suppresses the progression of dystrophic changes in dysferlin-deficient mice[45,46], enhanced phagocytic activity alone in DYSF-deficient monocytes is considered to be insufficient to cause muscle damage.

Animal Models for Dysferlinopathy

Two naturally occurring animal models for LGMD2B have been identified: SJL/J (SJL) mice and A/J mice. These have been shown to have mutations in the *DYSF* gene associated with phenotypical features of progressive muscular dystrophy[18,47]. SJL mice have a splice site mutation in which part of the highly conserved C2E domain in *DYSF* is removed[47]. A/J mice bear a unique ETn retrotransposon insertion near the 5' end (intron 4) of the *DYSF* gene[47].

Characteristics of animal models for dysferlinopathy
(1) Histopathological findings: Distribution of histopathological lesions:

Histopathological characteristics of animal models of dysferlinopathy include degeneration/necrosis of muscle fibers, variations in the size of muscle fibers, atrophy of muscle fibers, inflammatory cell infiltration, centronuclear fibers, fatty infiltration and fibrosis in the limb girdle (mainly the rectus femoris and lateral longissimus muscles)[47–49].

Studies have also shown that there are differences between SJL and A/J mice in terms of the progress and prevalent sites of skeletal muscular lesions[47,48]. In particular, the difference between SJL and A/J mice in their sensitivity

to muscular dystrophic lesions was most apparent in their lumbar (longissimus and sublumbar) muscles. These findings support the hypothesis that, as well as a deficiency in DYSF, additional enhancers or modifiers might be involved in the progression of skeletal muscle lesions in dysferlinopathy. Figure 5 shows typical histopathological findings for the femoral muscles of SJL and A/J mice aged 10 and 30 weeks in comparison with normal BALB/c mice of the same age.

(2) Histochemical findings: Typing of damaged muscle fibers:

In dysferlin-deficient patients with an advanced-stage dystrophic pattern, a predominance (in excess of 80%) of Type 1 (slow-twitch) fibers has been observed, suggesting a selective loss of Type 2 (fast-twitch) fibers or a process of conversion of Type 1 fibers into Type 2 fibers[50]. Similarly, histochemical staining for NADH-TR and SDH enzymes revealed that degeneration of fast-twitch muscle fibers was a predominant characteristic of SJL mice[48]. However, the medial vastus and iliocostalis muscles, which originally consisted mainly of fast-twitch muscle fibers, showed little degeneration/necrosis of muscle fibers. These findings indicate that the sensitivity of Type 2 fibers to injury might be site-specific, as seen in the rectus femoris and lateral longissimus muscles of SJL mice.

(3) Immunohistochemical findings: Identification of infiltrating cells:

Mononuclear cells found in or around degenerative and/or necrotic muscle fibers show a positive reaction to mouse F4/80 antigen (widely used to identify mouse macrophages in lesions[51,52]) and must therefore be macrophages. A previous immunohistochemical analysis using an antibody to the Mac-1 α-chain (also known as CD11b or integrin $α_M$ chain) showed that macrophages were the predominant type of infiltrating cell in the muscles of SJL mice[49]. It is not known whether this macrophage infiltration at the muscle lesions occurs solely as a result of the uptake of cellular debris (such as necrotic muscle fibers) or is a consequence of enhanced phagocytosis of the target cells as a result of opsonization of the sarcolemma by C3b.

Gene expression profiling of animal models for dysferlinopathy
(1) Endoplasmic reticulum stress-associated gene:

It has been reported that a novel mutant (L1341P) DYSF spontaneously aggregates in the endoplasmic reticulum (ER), resulting in phosphorylation of the eukaryotic translation-initiation factor subunit eIF2α and conversion of microtubule-associated protein light chain 3 (LC3) until ER-stress-related cell death eventually occurs[53]. Moreover, it has been reported that ER dysfunction plays a significant role in the pathophysiology of several myopathies[54–56]. Therefore, to examine the relationship between ER stress and skeletal muscle lesions in SJL or A/J mice, we have conducted a semiquantitative analysis of expression levels of spliced XBP1 mRNA as an ER stress marker in SJL mice[48];
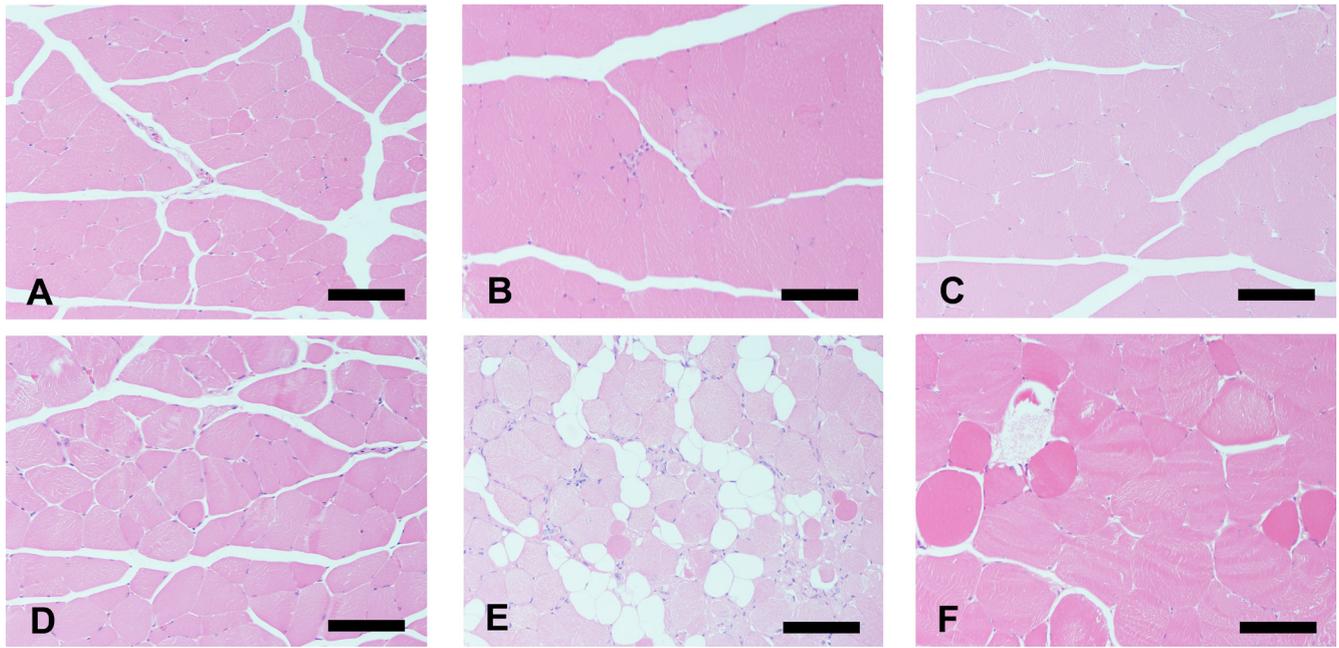
Fig. 5. Histopathology of the rectus femoris in BALB/c, SJL/J and A/J mice. At 10 weeks of age (upper figures), no significant changes were observed in the skeletal muscle fibers of BALB/c (A) and A/J mice (C), and a few muscle fibers showed minimal degeneration with mononuclear cell infiltration in SJL/J mice (B). At 30 weeks of age (lower figures), BALB/c mice did not exhibit histopathological changes in any skeletal muscles (D). The histopathological lesions of skeletal muscles in SJL/J mice progressed in severity with age and were characterized by the following findings: degenerative/necrotic muscle fibers, centronuclear muscle fibers, fatty infiltration and variations in the size of the muscle fibers (E). The muscle fibers in A/J mice showed only degenerative/necrotic features and variations in size (F). Hematoxylin and eosin (HE) staining. Bar: 100 μm.

we have also conducted an analysis of ER stress-associated genes (*HSP5*, *Grp78*, *Atf6*, and *Chop*) in SJL and A/J mice by means of a quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan® Gene Expression Assays[57]. Because these ER stress-associated gene expression analyses did not show any increases in gene expression, we consider that ER stress does not affect the progression of skeletal muscle lesions in SJL or A/J mice in advanced stages of dysferlinopathy.

(2) Lipid metabolism associated gene:

A qRT-PCR study demonstrated that SJL mice tend to show increased expression of uncoupling protein 2 (Ucp2) in the rectus femoris and longissimus lumborum at 30 weeks of age, which is when dystrophic lesions become histopathologically pronounced [57].

Forced expression of Ucp2 in pancreatic islets resulted in a decreased content of ATP, and the islet cells of Ucp2 knockout mice showed increased levels of ATP[58]. Overexpression of UCP2 in primary cardiomyocytes led to a significant decline in the ATP level and an enhanced sensitivity to hypoxia–reoxygenation[59]. Ucp2-mediated energy loss may be related to muscle degeneration/necrosis in SJL mice. On the other hand, *Tbc1d1* gene-deficient cells exhibited inhibited trafficking of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane[60], suggesting a decrease in intracellular glucose levels and a subsequent enhancement of fatty acid oxidation. These results suggest that

the *Tbc1d1* gene-deficient skeletal muscles in SJL mice[61] are likely to show uncoupling.

(3) Anti-oxidative stress-associated genes and heat-shock protein genes:

Heme oxygenase 1 (Hmox1) was upregulated in the rectus femoris, longissimus lumborum and abdominal muscles at 30 weeks of age; dystrophic lesions occur more commonly in these muscles in SJL mice[57]. The gene expression levels of HSP70 in most muscles of A/J mice were lower than those in BALB/c mice used as controls.

Hmox1 provides the first line of defense against oxidative stress, because it responds rapidly to oxidants[62]. However, Txnrd1, which (together with Hmox1) is a part of the anti-oxidation system, was not upregulated in any muscles of SJL mice. Recently, Ca-dependent upregulation of Hsp70 and Hmox-1 in skeletal muscle cells and in hepatocytes has been reported[63,64]. Because DYSF-null muscle fibers are defective in Ca^{2+}-dependent resealing of disruptions of the sarcolemma[19], these muscle fibers may cause persistent influx of Ca into the cytoplasm after membrane injury. The gene expression levels of Hmox1 were correlated with the severity of histopathological lesions in femoral (rectus femoris), lumbar (longissimus lumborum) and abdominal muscles; therefore, Ca influx into the cytoplasm following muscle injury may induce Hmox1 gene expression. However, Hsp70 was also upregulated in the diaphragms of SJL mice, where few histopathological changes were observed at

Table 1. Changes of Gene Expression and Prospective Events in SJL and A/J Mice

Strain	Cause	Change of gene expression	Prospective event
SJL	Gene mutation (frame shift deletion)	Dysferlin: ↓	Membrane repair function: ↓
	Fatty acid oxidation: ↑	Ucp2: ↑	ATP production: ↓
	Muscle fiber injury: ↑	Hmox1: ↑	Cellular protective activity: ↑
	Unknown	S100A4: ↑	Macrophage recruitment/chemotaxis: ↑
	Unknown	Daf1/CD55: ↓	Sensitivity to complement-dependent cytotoxicity: ↑
A/J	Gene mutation (transposon insertion)	Dysferlin: ↓	Membrane repair function: ↓
	Dysferlin: ↓ (?)	Hsp70: ↓	Cellular protective activity: ↓

any stage when this was examined. In addition, no change in the level of expression of the *Hsp70* gene was observed in abdominal muscles of SJL mice in where histopathological changes were observed at 30 weeks of age. The muscles and hearts in histopathologically normal BALB/c mice exhibited upregulation of the expression of this gene from 15 weeks of age[57]. It has been reported that significant increases in Hsp70 are observed at 12 weeks postpartum in normal rats[65]. The physiological mechanism of the expression of the *Hsp70* gene may develop somewhat later in noncardiac muscles of SJL mice; therefore, an unknown factor in addition to persistent Ca influx may cause Hmox1 induction in these muscles of SJL mice.

In contrast, the levels of expression of the *Hsp70* gene in most muscles of A/J mice were lower than those in the control. Loss of *fer-1*, a DYSF homolog, in *C. elegans* causes downregulation of *hsp-70*[66]. It is possible that the downregulation of *Hsp70* gene expression in the skeletal muscles of A/J mice is caused by a functional loss of DYSF.

(4) Ca-binding protein gene:

S100 calcium-binding protein A4 (S100A4) was upregulated in the rectus femoris, longissimus lumborum and abdominal muscles in SJL mice at 30 weeks of age[57]; these are the muscles in which dystrophic lesions occur most commonly in these mice[48]. These upregulations of S100A4 coincide approximately with the occurrence of dystrophic changes in the associated lesions. Most of the infiltrating cells in muscle lesions in SJL mice are F4/80 antigen-positive macrophages[48]. Recently, it has been demonstrated that S100A4 mediates macrophage recruitment and chemotaxis *in vivo*[67]. Upregulation of S100A4 in the rectus femoris and longissimus lumborum of SJL mice may be linked to the pathological characteristics of muscle in SJL mice.

(5) Complement control factor gene:

In comparison with BALB/c mice, SJL mice of all ages showed a marked lowering of the expression of *Daf1/CD55* gene in all studied muscles, except for the heart[57]. In contrast, there was no predominant difference in the levels of *Daf1/CD55* gene expression in A/J mice compared with those in BALB/c mice[57]. As previously indicated, it has been reported that gene expression of *Daf1/CD55* as a complement inhibitor is downregulated in the skeletal muscles of LGMD2B patients and in those of SJL mice[68]. Moreover, the serum concentration of C5 in SJL mice is known to be

significantly greater than that in other strains[69]. On the other hand, A/J mice are genetically deficient in C5[70]. At the time of the study, we understood that the difference in phenotype between the two DYSF-deficient mice was related to the presence or absence of C5; however, it was later revealed that genetic ablation of C5 had a minimal effect on muscle lesions in DYSF-deficient mice[45].

Table 1 lists gene expression and prospective events in SJL and A/J mice. It has also been reported that differential gene expression profiles of proximal and distal muscle groups are altered in prepathological C57BL/10.SJL-Dysf mice[71]. Furthermore, the expression profiles of 10,012 genes in the quadriceps femoris muscles of control and SJL mice have been established by means of a cDNA microarray analysis, with the aim of identifying genes that are involved in the degeneration and regeneration process and in the functional network of dysferlin[72]. However, it cannot be stated with confidence that common factors associated with muscular changes in dysferlinopathy were discovered in these studies (including our own), and further investigations are required to determine whether alterations in gene expression levels are the cause of dystrophic changes or they occur as a result of damage to the muscle.

Problems associated with animal models for dysferlinopathy

In general, murine models for muscular dystrophies, including SJL and A/J mice, do not show any significant muscle weakness. This makes it difficult to evaluate improved muscle strength, and therefore, the screening of therapies by using murine models depends exclusively on histopathological examination.

Because it is simple to induce autoimmune diseases in SJL mice, they have been used as animal models for autoimmunological diseases such as experimental autoimmune encephalomyelitis[73], experimental autoimmune myositis[74], and experimental autoimmune hypophysitis[75]. In addition, it has been reported that SJL mouse-derived monocytes increase phagocytic activity[76] and that dysferlin deficiency induces an upregulation of inflammasome[42]. However, a more recent study using C57BL/10-SJL.Dysf mice, which have a more controlled genetic background, did not find any change in the phagocytic activity of dysferlin-deficient monocytes[77]. A/J mice, on the other hand, show later onset of muscle lesions than do SJL mice or DYSF-deficient mice with the genetic background 129SvJ, and the lumbar mus-

cles of SJL and A/J mice showed a difference in their sensitivity to muscular dystrophic lesions. There is a concern therefore that differences in the genetic or immunological background of SJL and A/J mice could cause modifications in the nature of any muscle damage.

As pointed out above, the two strains show phenotypic divergences. A/J mice display a later onset and a slower progression of muscular disease compared with SJL mice[47,48], and the two strains show differences in their gene expression profiles. Similarly, dysferlinopathies in humans are a clinically heterogeneous group of disorders[78]. It has been considered that through probing of the causes of interstrain differences, A/J and SJL strains of mice might prove useful in providing clues regarding the causes of clinical heterogeneity in humans and in identifying targets for stopping or slowing the progression of the disease. At present, however, we have little knowledge of the causes of interstrain differences.

Development of Therapies for Muscular Dystrophies, Including Dysferlinopathy

Transfection of cDNA by plasmid vectors

Plasmid vectors can transfer large cDNA fragments (for example, full-length dystrophin cDNA) and they are noninfectious and nonimmunogenic[8]. Although these characteristics are major advantages compared with viral vectors, plasmid vectors provide less-efficient transfection than do viral vectors.

Transfection of cDNA by viral vectors

The AAV vector is currently one of the most promising viral vectors for transfection of cDNA. AAV can package and protect recombinant DNA strands as large as 6.0 kb, but virions carrying larger DNA strands are preferentially degraded by the proteasome[79]. Furthermore, AAV vectors can induce immune reactions[80,81].

In gene therapy for LGMD2B, the size of the dysferlin cDNA prevents its direct incorporation into an AAV vector for therapeutic gene transfer into muscle. To bypass this limitation, Lostal *et al.* have split dysferlin cDNA at the exon 28/29 junction and have cloned it into two independent AAV vectors, each carrying the appropriate splicing sequences[82]. Intramuscular injection of the corresponding vectors into a dysferlin-deficient mouse led to the expression of full-length dysferlin for at least 1 year.

Exon skipping by antisense oligonucleotides

Exon skipping by antisense oligonucleotides is among the most advanced therapies for muscular dystrophy (especially DMD). GSK-2402968 is currently advancing to Phase III clinical trials (http://clinicaltrials.gov/ct2/results?term=GSK-2402968), and a Phase II study of AVI-4658 is ongoing (http://clinicaltrials.gov/ct2/results?term=AVI-4658). However, exon skipping by antisense oligonucleotides has some disadvantages[8] in that the therapy needs regular repeated administration because the method modifies only the process of mRNA splicing, and different antisense oligonucleotides are needed for different types of dystrophin gene deletion.

Stem or progenitor cell transplantation

Myoblasts/satellite cells, hematopoietic stem cells, mesenchymal stem cells, bone-marrow side population, muscle side population, muscle-derived stem cells, adipose-derived stem cells, endothelial progenitor cells and vessel-associated stem cells have all been studied for their possible use in cell-based therapies[11,83]. Several clinical trials involving injection of myoblasts or pericytes from HLA-matched donors have begun or are planned, and the number of such trials will increase in the near future[11]; however, little information is currently available regarding the safety and efficacy of these techniques in clinical use.

In a study of a cell-based therapy for LGMD2B, human and mouse dysferlin proteins were detected one month after transplantation in all SCID mice transplanted with normal human myoblasts and in SJL mice transplanted with allogeneic primary mouse muscle cell cultures[84]. The number of dysferlin-positive fibers was 40–50% and 20–30% in SCID and SJL muscle sections, respectively. In another study, it was shown immunohistochemically that a small number of human cells from human umbilical cord blood became grafted into recipient SJL mice muscle and expressed both dysferlin and human-specific dystrophin 12 weeks after transplantation[85].

Read-through by small-molecule drugs

Phase IIb trials of ataluren (PTC124), which causes read-through for the nonsense mutation DMD/BMD, has been suspended or terminated because the primary end-point did not reach statistical significance within the 48-week duration of the study (http://clinicaltrials.gov/ct2/results?term=PTC124).

Antibody treatment

A safety trial of a neutralizing antibody to myostatin, MYO-029, has been conducted in adult muscular dystrophies (Becker muscular dystrophy, facioscapulohumeral dystrophy and LGMD)[86]. Although MYO-029 has good safety and high tolerability, no improvements were noted in the exploratory end points of muscle strength and function. This probably occurred because the study was not designed to seek efficacy.

In a study of an antibody therapy for LGMD2B, administration of an antibody for tumor necrosis factor (TNF) resulted in dose-dependent reductions in inflammatory change, necrosis and fatty/fibrous change. These findings suggest that TNF does indeed play a role in damage to muscle in SJL mice[87].

Because these therapeutic methods for dysferlinopathy are still not under evaluation in clinical studies, immediate development of a therapy for dysferlinopathy is a desirable goal.
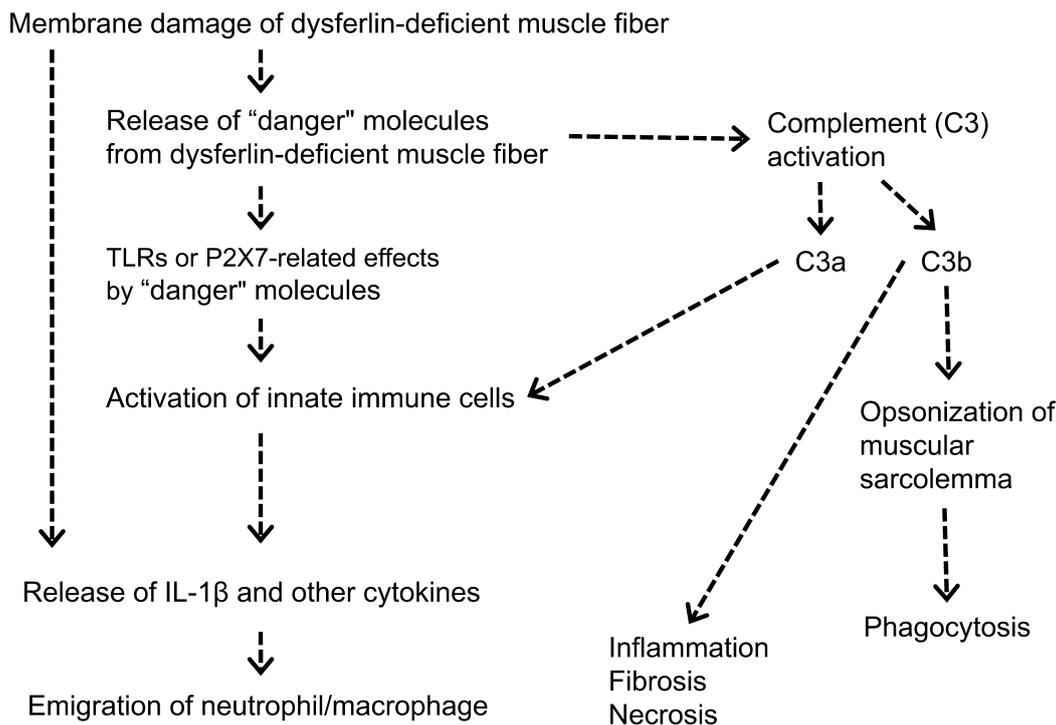
Fig. 6. Estimated inflammatory process in dysferlin-deficient skeletal muscle. Plasma membrane damage to dysferlin-deficient muscle fibers causes a release of "danger" molecules (heat-shock proteins, high mobility group box-1, ATP, etc.). These "danger" molecules are recognized by receptors on innate immune cells and muscle cells and they stimulate the production of proinflammatory cytokines. Moreover, the released "danger" molecules activate the complement system and stimulate C3a or opsonizing C3b. The proinflammatory mediator C3a can trigger the production of proinflammatory cytokines from cells and render the local vascular endothelium "leaky"; this is followed by attraction of the migration of neutrophils and macrophages. C3b binds to the negatively charged sarcolemma, stimulating phagocytosis. These processes cause more-severe muscular degeneration/necrosis.

Identification of New Therapeutic Targets for Dysferlinopathy by Using Animal Models

Complement-associated factors

Complement factors are upregulated or activated in DYSF-deficient muscles from mice and humans[45,68]. This upregulation of complement factors was observed in DYSF-deficient mice before the onset of the obvious pathological markers and was normalized by muscle-specific expression of the DYSF transgene[45]. To confirm whether or not an activated complement system causes skeletal muscle damage, a gene for complement factor C3 or C5 was disrupted in DYSF-deficient mice[45]. Whereas genetic ablation of C5 had a minimal effect on muscle lesions in DYSF-deficient mice, a deficiency in C3 ameliorated histopathological changes in the skeletal muscles. In addition, deposition of C3 on the sarcolemma of quadriceps muscles in DYSF-deficient mice was confirmed by means of immunofluorescence analysis, whereas this staining pattern was not observed in DYSF/C3 double-deficient or wild-type mice. These results suggest that activated C3 is responsible for muscle damage in dysferlinopathy. Han[88] has proposed a mechanism for muscle damage caused by active C3, in which C3 is cleaved into C3a and C3b on activation of the complement system. C3a

is an anaphylotoxin that produces a local inflammatory response, whereas C3b serves as an opsonizing agent by coating the sarcolemma of dysferlin-deficient muscle. Opsonization of the sarcolemma, either with or without C5, enhances the phagocytosis of the target cell by macrophages, which are the predominant infiltrating cells in dysferlin-deficient muscles[48,89]. Figure 6 shows an estimated inflammatory process in dysferlin-deficient skeletal muscle.

Downregulation of decay-accelerating factor 1 (Daf1; also known as CD55), which acts as the complement inhibitor, induces increased susceptibility to complement attack in DYSF-deficient muscle cells[68]. However, in our study, SJL mice of all ages showed a marked downregulation of *Daf1/CD55* gene expression in skeletal muscles lacking obvious histopathological lesions[57]. Additionally, A/J mice showed no abnormality in *Daf1/CD55* gene expression levels in their skeletal muscles with histopathological lesions[57]. For these reasons, downregulation of *Daf1/CD55* alone cannot explain muscle damage in DYSF-deficient mice.

Endogenous danger/alarm factors

As previously mentioned, it has been proposed that DYSF deficiency in skeletal muscles results in release of endogenous danger/alarm signals, including HSPs, HMGB1

and ATP. These factors bind to the toll-like receptor P2X7, activating inflammasome[41], nuclear factor kappa-B and the complement pathways[45]. It is known that HSPs are antibody-dependently and antibody-independently activated in the complement system[90]. It has been reported that HMGB1 causes an irreversible decrease in the release of Ca^{2+} from the sarcoplasmic reticulum *in vitro*[91]. If the role of these factors in dysferlinopathy can be elucidated, the resulting knowledge may be helpful in preventing the progression of muscular lesions.

Conclusion

DYSF is involved in the membrane-repair process, intracellular vesicle system and development of T-tubules in skeletal muscles, and it interacts with MG53, annexins, caveolin-3, AHNAK, affixin, S100A10, calpain-3, tubulin and DHPR. In humans, a deficiency in DYSF induces dystrophic changes in the skeletal muscles of the limb girdle, the so-called LGMD2B and MM (which are collectively known as dysferlinopathy). LGMD2B occurs at a relatively high frequency in Japan, to the extent that the National Center of Neurology and Psychiatry stated in 2010 that dysferlinopathy has become the most common type of LGMD among the Japanese population. Because no therapeutic methods for dysferlinopathy are currently under evaluation in clinical studies, the immediate development of a therapy for dysferlinopathy is desirable. SJL and A/J mice are known to be naturally occurring animal models for dysferlinopathy. These models are therefore useful in verification experiments for new therapies and are valuable tools for identifying factors that accelerate dystrophic changes in skeletal muscle. However, it should be borne in mind that the genetic or immunological background of SJL or A/J mice could result in modifications of muscle damage in experiments involving these animal models.

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