Aberrant neuromuscular junctions and delayed terminal muscle fiber maturation in α-dystroglycanopathies

Mariko Taniguchi1, Hiroki Kurahashi3, Satoru Noguchi4, Takayasu Fukudome5, Takeshi Okinaga2, Toshifumi Tsukahara6, Youichi Tajima7, Keiichi Ozono2, Ichizo Nishino4, Ikuya Nonaka4 and Tatsushi Toda1,*

1Division of Clinical Genetics, Department of Medical Genetics and 2Department of Pediatrics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, 3Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan, 4National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan, 5Division of Clinical Research, Nagasaki Medical Center of Neurology, Kawatanamachi, Nagasaki 859-3615, Japan, 6Center for Nano Materials and Technology, Japan Advanced Institute of Science and Technology, Tatsunokuchi, Ishikawa 923-1292, Japan and 7Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan

Received October 15, 2005; Revised and Accepted February 28, 2006

Recent studies have revealed an association between post-translational modification of α-dystroglycan (α-DG) and certain congenital muscular dystrophies known as secondary α-dystroglycanopathies (α-DGopathies). Fukuyama-type congenital muscular dystrophy (FCMD) is classified as a secondary α-DGopathy because the responsible gene, fukutin, is a putative glycosyltransferase for α-DG. To investigate the pathophysiology of secondary α-DGopathies, we profiled gene expression in skeletal muscle from FCMD patients. cDNA microarray analysis and quantitative real-time polymerase chain reaction showed that expression of developmentally regulated genes, including myosin heavy chain (MYH) and myogenic transcription factors (MRF4, myogenin and MyoD), in FCMD muscle fibers is inconsistent with dystrophy and active muscle regeneration, instead more of implicating maturational arrest. FCMD skeletal muscle contained mainly immature type 2C fibers positive for immature-type MYH. These characteristics are distinct from Duchenne muscular dystrophy, suggesting that another mechanism in addition to dystrophy accounts for the FCMD skeletal muscle lesion. Immunohistochemical analysis revealed morphologically aberrant neuromuscular junctions (NMJs) lacking MRF4 co-localization. Hypoglycosylated α-DG indicated a lack of aggregation, and acetylcholine receptor (AChR) clustering was compromised in FCMD and the myodystrophy mouse, another model of secondary α-DGopathy. Electron microscopy showed aberrant NMJs and neural terminals, as well as myotubes with maturational defects. Functional analysis of NMJs of α-DGpathy showed decreased miniature endplate potential and higher sensitivities to d-Tubocurarine, suggesting aberrant or collapsed formation of NMJs. Because α-DG aggregation and subsequent clustering of AChR are crucial for NMJ formation, hypoglycosylation of α-DG results in aberrant NMJ formation and delayed muscle terminal maturation in secondary α-DGopathies. Although severe necrotic degeneration or wasting of skeletal muscle fibers is the main cause of congenital muscular dystrophies, maturational delay of muscle fibers also underlies the etiology of secondary α-DGpathies.

*To whom correspondence should be addressed. Tel: +81 668793380; Fax: +81 668793389; Email: toda@clgene.med.osaka-u.ac.jp

© The Author 2006. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
INTRODUCTION

Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800) is an autosomal recessive muscular dystrophy and the second most common childhood muscular dystrophy in Japan, following Duchenne muscular dystrophy (DMD) (1). Clinical manifestations of FCMD include severe congenital muscular dystrophy from early infancy, cobblestone lissencephaly and eye malformation. We previously isolated the responsible gene for FCMD, termed *fukutin* (2,3). Recently, it has been postulated that *fukutin* modulates the glycosylation of α-dystroglycan (α-DG), a major component of the dystrophin–glycoprotein complex (4,5). FCMD is classified as one of the congenital muscular dystrophies, such as laminin-α2-deficient congenital muscular dystrophy (MDC1A) (6). Recently, FCMD has also been classified as a secondary α-DGopathy, as mutations in genes encoding glycosyltransferases result in hypoglycosylated α-DG (7). α-Dystroglycan binds to extracellular matrix proteins such as laminin, agrin and perlecan, which are important in maintaining muscle cell integrity (8). Hypoglycosylated α-DG provokes the post-translational disruption of dystroglycan–ligand interactions in the skeletal muscle of patients, leading to the severe phenotypes of congenital muscular dystrophies (7). Other glycosyltransferases include POMGnT1 (protein O-mannose β-1, 2-N-acetylglucosaminyltransferase 1), POMT1 and POMT2 (protein O-mannosyltransferases 1 and 2), fukutin-related protein (FKRP) and LARGE; mutations in these genes induce human muscle–eye–brain disease, Walker–Warburg syndrome and congenital muscular dystrophy type 1C/1D, and mouse myodystrophy, respectively (9–14).

Primary characteristics of the so-called ‘muscular dystrophy’ such as DMD include necrotic change and active regeneration of muscle fibers. From infancy, DMD patients usually show dystrophic change in skeletal muscle, accompanied by elevation of serum creatine kinase (CK) levels. However, DMD patients usually maintain their gait until early adolescence. In contrast, FCMD patients show severe phenotypic characteristics from very early infancy, and few patients can acquire gait regardless of serum CK levels (1). Skeletal muscle fibers in FCMD are extremely small, irregular in cell size and architecturally disorganized, and extensive fibrosis prevails from the early infantile stage. However, only a small number of muscle fibers show severe necrotic change or active myofibril regeneration, and satellite cells are also fewer than those of DMD (1,15,16). These phenotypic differences promote the hypothesis that another mechanism may also account for the pathophysiology of secondary α-DGpathies.

Although expression profiling of skeletal muscle from patients with DMD, MDC1A and α-sarcoglycanopathy have been described (17–19), no similar analysis has been reported for FCMD and other secondary α-DGpathies. To investigate the molecular mechanism of FCMD and other secondary α-DGpathies, we profiled gene expression in FCMD skeletal muscle using cDNA microarray and subsequent quantitative real-time polymerase chain reaction (PCR). Here we demonstrate that aberrant neuromuscular junctions (NMJs) and maturational delay of muscle fibers are significant to the mechanism underlying secondary α-DGpathies.

RESULTS

Aberrant muscle regeneration is suggested by gene expression profiling of FCMD skeletal muscle

Gene expression profiling of FCMD skeletal muscle was performed using a custom cDNA microarray. Clustering analysis showed similar overall expression profiles of muscle from four FCMD patients, aged 20 days to 1 year, 6 months (Fig. 1A). This similarity is independent of age and histology of the muscle specimen in our samples.

We analyzed individual genes showing distinct expression patterns in FCMD skeletal muscle compared with normal children or DMD patients. Most genes encoding muscle components were down-regulated in FCMD. Among these, *myosin light chain 1*, *3* and *4* (*myl1*, *3* and *4*) were up-regulated in DMD skeletal muscle, in contrast with FCMD (Fig. 1B). Expression of the developmentally regulated myosin heavy chains (*MYHs*), *MYH1*, *MYH2* and *MYH7* (slow, adult-type), was down-regulated in FCMD but not in DMD, whereas expression of *MYH8* (fast-type) showed no significant change in FCMD compared with DMD or normal controls. Slow-type MYHs (*MYH1*, *MYH2* and *MYH7*) are present in mature muscle fibers and crucial for sarcomere assembly to maintain muscle integrity, whereas fast-type or developmental MYHs (*MYH3*, *MYH4* and *MYH8*) are seen in early immature myoblasts or in regenerating fibers. These observations suggest that expression of mature muscle components is suppressed in FCMD skeletal muscle at all ages examined.

With regard to muscle fiber differentiation, myogenic factors including *MyoD*, *myf5* and *myogenin* (*myf4*) showed insufficient signal for the analysis. It is noteworthy, however, that *MRF4* (*myf6*) was down-regulated in FCMD. Expression of the alpha-type cholinergic receptor (*CHRNA4*), which is known to be regulated by *MyoD* and *MRF4* (20,21), was much higher in FCMD patients than in normal controls.

We next performed real-time quantitative PCR to further investigate skeletal muscle differentiation. We compared mRNA expression in FCMD muscle with normal or DMD skeletal muscle, as DMD is a good example for active regeneration, in which expression of muscle component and myogenic factor mRNA expression is expected to be up-regulated. Although *CHNRA* was up-regulated in DMD, as predicted, its expression was even higher in FCMD (Fig. 2A and B). Among these cholinergic receptor subtypes, gamma-type cholinergic receptor (*CHNRG*), which is a component of fetal isoforms, was up-regulated, whereas epsilon-type cholinergic receptor (*CHNRE*), which only comprises adult isoforms (22), was down-regulated in FCMD (Fig. 2B). *MYH* slow-type (*MYH7*) was down-regulated in FCMD, consistent with the microarray analysis, whereas expression of fast-type MYH (*MYH1b*) was not altered in FCMD. Interestingly, although *MyoD* and *myogenin* were up-regulated in both DMD and FCMD, *MRF4* was down-regulated in FCMD muscle but up-regulated in DMD (Fig. 2A and B). *MRF4* expression is known to be up-regulated in the late phase of muscle regeneration or differentiation, followed by sequential expression of *MyoD*, *myf5* and *myogenin*, indicating significant roles in terminal differentiation (20,21). These results suggest that FCMD skeletal muscle undergoes an unbalanced differentiation process.
Final maturation step is retarded in FCMD skeletal muscle

To investigate how differentiation is impaired, we examined histological specimens of FCMD skeletal muscle. Marked interstitial tissues with numerous small, round-shaped immature fibers and some necrotic fibers increased with age were seen in FCMD skeletal muscle specimens. Interstitial tissue is prominent from early infancy and progresses with age (Fig. 3A–C), and skeletal muscle from an FCMD fetus also shows rich interstitial tissues (Fig. 3E). Although necrotic change in muscle fibers is not so marked as in DMD fibers, DMD muscle shows less marked fibrosis and more mature fibers, despite more active necrotic and regenerating processes (Fig. 3D). Overall, FCMD muscle is reminiscent of fetal muscle; skeletal muscle from a normal fetus appears rich in fibrous tissues and small, round-shaped immature myotubes (Fig. 3F).

Muscle fiber type is easily identified by ATPase staining. Normally, type 2C fibers are mainly seen in fetal muscle fibers or in regenerating fibers. However, in ATPase-stained cryospecimens, FCMD muscle showed a significantly higher percentage of undifferentiated type 2C muscle fiber contents relative to DMD or control samples ($P < 0.005$) (Fig. 3G and H, Table 1).

Using immunohistochemical analysis, we examined MYH subtypes to confirm the differentiation impairment in FCMD and in myodystrophy mouse ($myd$), which is another model of secondary α-DGpathies. In normal muscle from age-matched controls, no staining of developmental or neonatal MYH (Fig. 3I and J) was seen. In contrast, FCMD and $myd$ muscle fibers stained positively for developmental and neonatal MYHs (Fig. 3M and N). These positive fibers corresponded with those staining positive for fast-type MYHs in a serial section (Fig. 3M–O, arrows). Similar staining patterns were observed in skeletal muscle from an FCMD fetus. It is unlikely that all fibers showing developmental MYH expression are derived from regenerating fibers, as few active regenerating or necrotic fibers are seen in the hematoxylin and eosin (HE) specimen at any ages (Fig. 3A–C). Similar staining patterns were observed in skeletal muscles from an FCMD fetus and adult $myd$ (data not shown). It is unlikely that all fibers showing developmental MYH expression are derived from regenerating fibers, as few active regenerating or necrotic fibers are seen in the HE specimen (Fig. 3A–C).

These results induce the possibility that maturation might be slowed or arrested in FCMD and $myd$ skeletal muscles, and possibly this is common in secondary α-DGpathies. It also
implies that secondary α-DGpathies have more complex etiology than the so-called ‘muscular dystrophy’, and that may be partly explained by a maturational defect.

NMJ abnormalities induce maturational delay in secondary α-DGpathies

Microarray analysis showed a reduction in MRF4 expression in FCMD. Using immunohistochemistry, we further investigated MRF4 expression in FCMD and in myd. Immunoreactivity against MRF4 antibody yielded strong signals, which co-localized with the nucleus and NMJs (Fig. 4A). In normal skeletal muscles, anti-MRF4 antibody yielded weak signals which were not merged with NMJ (Fig. 4A, lower columns). Similar results were obtained in myd (data not shown). Regarding the fact that MRF4 is required at the time and place of NMJ development during skeletal muscle differentiation (23), these results prompt the hypothesis that the differentiation process of muscle fibers arrests at this point in secondary α-DGpathies.

We next examined the morphology of NMJs in both FCMD and myd by staining acetylcholine receptor (AChR) in NMJs with anti-α-bungarotoxin (Fig. 4B). Almost all the NMJs of FCMD and myd showed sparse, weak staining (Fig. 4B, lower columns), in contrast with the dense pattern in normal skeletal muscle (Fig. 4B, upper columns). In normal skeletal muscles, the borders of positive signals were characteristically flared because of multiple layers of synaptic folds, whereas borders in FCMD and myd appear smooth and simple, and synaptic folds—particularly secondary folds—were seldom observed. This signal pattern reflects deteriorated or non-deteriorated cluster of AChR on NMJs in secondary α-DGpathies.

Electron microscopic examination of these secondary α-DGpathies revealed aberrant NMJ lesions with abnormal neural endings. NMJs with fewer synaptic folds and secondary clefts were seen in all NMJs of FCMD and myd (Fig. 5A–F). In addition, the muscle fibers showed characteristics of immaturity, consistent with our hypothesis that the myotubes are maturationally arrested (Fig. 5G and H). These fibers are distinct from the active regenerating

Figure 2. Differential expression of muscle components and myogenic factors in skeletal muscles from FCMD, DMD and normal children. (A) PCR products show that MyoD and myogenin, which are sequentially expressed in the early phase of muscle regeneration, are up-regulated in DMD and FCMD; however MYH and MRF4 are down-regulated in FCMD but not DMD. (B) Quantitative real-time PCR analysis of mRNA expression. Each bar represents the mean value and 95% confidence interval of duplicate experiments in two patients for each disease and normal control. White bar, normal children; black bar, DMD; gray bar, FCMD. Expression levels are plotted as values normalized to gapdh. *P < 0.005 (Student’s t-test).
myotubes seen in DMD muscle fibers, in that ribosome particles appear quite poor.

We performed functional analysis of the morphologically aberrant NMJs in secondary α-DGpathy by measuring miniature endplate potential (MEPP) and endplate potential (EPP) of myd mice (Table 2). The amplitudes of MEPP were markedly lower in myd mice than in normal littermates (P < 0.005). In contrast, quantal content of EPP was increased in myd (P < 0.005). The reduction of MEPP amplitude could be compensated by the increased quantal content, and the safety margin of neuromuscular transmission is considered to be maintained in myd mice. The number of endplates recorded in myd mice was much fewer than in normal littermates. However, the amount of d-Tubocurarine that can inhibit the muscle contraction induced by the EPP was distinctively low for myd muscle relative to that of normal littermate (Table 2). These findings implicate, combined with the morphological observation, that most of the endplates in myd are not adequately innervated, but a small number of NMJs functionally compensate the low MEPP amplitude to maintain the safety margin of neuromuscular transmission.

Hypoglycosylation of α-DG as the etiology of non-clustering AChR in NMJs

We performed immunostaining to examine core α-DG in muscle fibers. In normal skeletal muscles, α-DG localized to the NMJ and sarcoplasmic membrane (Fig. 6A). In contrast, FCMD and myd showed substantial α-DG on the sarcoplasmic membrane, but only weak signals were observed in thin NMJs, indicating a failure of α-DG aggregation (Fig. 6A, normal NMJs, arrows; FCMD and myd, arrowheads). We also examined staining of glycosylated α-DG (IIH6). As expected, we saw no signal on NMJs or on the sarcoplasmic membrane in FCMD and myd (data not shown), implying that glycosylation

Table 1. Muscle contents and type 2C fibers in biopsied specimen of FCMD, DMD and normal children

| Disease | Age       | Muscle (%) | Type 2C (%) | Fibrosis (%) |
|---------|-----------|------------|-------------|--------------|
| FCMD (F1) | 20 days  | 71         | 26          | 27           |
| FCMD (F2) | 7 months | 53         | 26          | 41           |
| FCMD (F3) | 1-year 4 months | 60      | 25         | 42           |
| FCMD (F4) | 1-year 6 months | 52     | 19         | 43           |
| DMD*     | 3–9 years | 61         | 9          | 30           |
| Normal*  | 1 year    | >95        | <5         | <5           |

*Average, n = 10.

Figure 3. HE and ATPase stains of biopsied FCMD skeletal muscle, used for microarray analysis. Each specimen shows marked fibrosis with numerous small immature muscle fibers, which is seen from early infancy (A, 20 days; B, 7 months; C, 1-year 6 months), and progresses with age. DMD muscle (D, 5 years) shows less marked fibrosis and less frequent immature fibers despite more active necrotic and regenerating processes. Note that the pathological findings of FCMD skeletal muscles are similar to those of fetal skeletal muscles (E, FCMD fetus, 19 weeks; F, normal fetus, 21 weeks). Also note many undifferentiated immature type 2C fibers stained darkly for ATPase under both alkaline (pH 10.4) (G) and acid (pH 4.6) (H) pre-incubations. Immunostaining for MYH subtypes shows positive staining of developmental and neonatal MYH and decreased staining of slow-type MYH, which are distinct from normal muscles (normal child muscles, 1 year, I–L; FCMD, 1 year, M–P in sequential cryosections). Scale bars = 100 µm.
is crucial for α-DG aggregation and also for the subsequent clustering of AChR in NMJs. α-DG is expressed on both the muscle peripheral membrane and the peripheral nerve terminal at NMJs (24). Thus, we examined whether a pre-synaptic or post-synaptic lesion contributes to aberrant NMJ formation.

Staining for synaptophysin at the pre-synaptic region or for fasciculin at the synaptic gap showed abnormal patterns similar to that of α-bungarotoxin (Fig. 6B). These observations indicate that NMJ abnormalities in secondary α-DGpathies may arise not only at the post-synaptic muscle peripheral membrane, but also by pre-synaptic hypoglycosylated α-DG.

Utrophin and dystrophin are expressed abundantly in pre- and post-synaptic regions of mature NMJs and suggested to play an important role for synaptic maturation and the maintenance of NMJs (25). To analyze aberrations of the distribution of utrophin and dystrophin, we performed immunostaining for utrophin and dystrophin in NMJs. Examination under confocal microscopy allowed a precise view of both proteins on the sarcolemmal membrane. In NMJs from a normal sample, utrophin strongly stains exclusively at fine primary and secondary synaptic folds, tangled with dystrophin staining just beneath the muscle peripheral membrane (Fig. 6C, left column; Fig. 6D, upper columns). In contrast, NMJs from secondary α-DGpathies show thinner, fold-less and weak signals for both utrophin and dystrophin (Fig. 6C, right column; Fig. 6D, middle and lower columns).

Figure 4. Immunohistochemistry of MRF4 in secondary α-DGpathy and aberrant NMJs in secondary α-DGpathy. (A) Fluorescence image of MRF4 (green), α-bungarotoxin staining of AChR on NMJ (red) and DAPI-stained nuclei (blue) in normal and FCMD skeletal muscles. In normal muscle, NMJs stain strongly, merging with MRF4 staining and DAPI (arrows, upper columns). In FCMD, the staining pattern of MRF4 in the nucleus of muscle fibers is markedly decreased and no merging stain with NMJ is seen (arrowhead). (B) Compared with normal AChR on NMJs (red) stained by α-BTX (upper columns), scattered, fold-less staining pattern is present in both FCMD and myd (lower columns). Scale bars = 5 μm.

Figure 5. Electron microscopic examinations of NMJs and skeletal muscle from secondary α-DGpathies. Aberrant NMJs and myotubes with maturational arrest are seen in secondary α-DGpathies. Compared with normal (A, human; B, wt mouse), NMJs in FCMD (C and D) and myd (E and F) show simpler secondary clefts and wider synaptic clefts with occasional multilayered basal lamina (D and F, white arrows). Moreover, maturationally arrested myotubes are seen in secondary α-DGpathy. Three cells (1–3) share a common basement membrane (G), and at higher magnification, these myotubes contain poorly organized myofibrils (black arrows) (H). In contrast with early regenerating fibers in normal regenerating myotubes, ribosome particles are not abundant in FCMD, indicating maturational arrest of myotubes in secondary α-DGpathy. Abbreviation: PC, primary cleft; NT, nerve terminal; BM, basal lamina. Scale bars = 1.0 μm.


**DISCUSSION**

FCMD has long been classified as ‘muscular dystrophy’, although the clinical characteristics differ from those of DMD. Muscular dystrophy is defined generally by necrotic change and active regeneration of muscle fibers. However, FCMD muscle in infantile stage seems more likely to have additional features, implying a more complex pathogenesis for FCMD. Indeed, our microarray analysis showed that general expression profiling clusters FCMD and DMD distinctively. Expression of mature muscle components was surprisingly low in FCMD, indicating less active regeneration of muscle fibers. We also saw similar expression profiles among all FCMD patients in our samples, indicating that FCMD is a chronic rather than progressive disorder, at least at the infantile period.

We confirmed maturational delay and aberrant NMJs in FCMD skeletal muscle fibers by expression profiling, morphological and histochemical examination. These findings are common to secondary α-DGpathies but are not seen in DMD. In this study, we demonstrate that the etiology of secondary α-DGpathy skeletal muscle abnormalities may stem from maturational arrest caused by aberrant NMJs in addition to dystrophy. MDC1A, clinical characteristics of which are similar to FCMD, is described with an initial phase of necrosis and regeneration in the early steps of the disease (6). Although aberrant NMJ is also seen in MDC1A (27), the muscle fragility due to defect in the component of basement membrane mainly affects the phenotype and causes necrosis and regeneration.

A considerable body of evidence indicates that muscle differentiation ceases at NMJ formation in FCMD. First, immature type 2C fibers are predominant in FCMD (Table 1). At the initiation of muscle differentiation, satellite cells proliferate to become myoblasts, fuse to organize myotubes. These early myotubes contain type 2C fibers. Following NMJ formation, immature fibers are induced to differentiate further into mature muscle fibers such as type 1, 2A and 2B. Second, down-regulated expression of matured MYHs in FCMD also suggests arrest at this stage. Following completion of NMJ formation, embryonic, neonatal-type MYHs in immature myofibers are replaced by adult-type, slow MYHs, which are induced by extracellular matrix (ECM) components, growth factors or programmed cell differentiation (28).

Third, MRF4, which is postulated to be induced by AChR clustering in NMJ formation, is down-regulated in FCMD. Normally, during the early phase of muscle development, a series of myogenic regulatory genes such as myf5, MyoD and myogenin are sequentially expressed, followed by MRF4 up-regulation just after NMJ formation. AChRs bind to myotubes, associate with specific factors and trigger a signal to induce expression of myogenic factors including MRF4, which is suggested to play a crucial role in muscle terminal maturation and maintenance (20,21,29). Therefore, MRF4 is distinct from MyoD, myogenin and myf5 in that it is expressed mainly in matured myotubes and myofibers. MRF4 has been reported to co-localize with AChR in NMJs and to function in terminal muscle maturation and fiber maintenance (30). It is reasonable to assume that MRF4 function is required at the time and place of NMJ development during skeletal muscle differentiation (23). These results prompt the hypothesis that the differentiation process of muscle fibers arrests at this point in FCMD and myd.

Fourth, the fact that high CHRNA and low CHRNA expression in FCMD muscle relative to that in age-matched normal control or DMD muscle is striking, although the age of the DMD patient was slightly higher than that of the FCMD patients. It clearly indicates that most of the AChR in FCMD muscle is fetal type. It also supports our hypothesis that skeletal muscle in secondary α-DGpathy is immature and that delay of differentiation might be involved with maturation defect of NMJs.

What causes aberrant NMJs in secondary α-DGpathies? Normally, NMJs are built by a dense tangle of sarcoplasmic membrane and neural endings through a layer of basement membrane. It is possible that connections between the neural terminal and glycosylated α-DG, made through a layer of basement membrane and mediated by molecules such as laminin or agrin, are important to normal NMJ formation and subsequent muscle differentiation (31,32). MDC1A, clinical characteristics of which are similar to FCMD (6), also shows aberrant NMJs with fewer synaptic folds (27). In contrast, Musk or rapsyn deficiency does not resemble severe muscular dystrophy in spite of the abnormal NMJ formation. The interaction of laminin and α-DG is thought to play an essential role in the transition of AChR microaggregates into macroaggregates at the developing NMJ, followed by the concentration of α-DG on NMJs (32,33). These facts lend support to our hypothesis that the laminin/α-DG interaction fulfills a pivotal function in normal NMJ formation.

Alternatively, it is also possible that attachment of neural terminals to NMJs is affected pre-synaptically in secondary α-DGpathies. Abnormalities in the pre-synaptic peripheral nerve would affect the neural endings of NMJs, leading to

---

**Table 2. Functional analysis of NMJs in myd mice**

|        | MEPP amplitude (mV) | Quantal content (m) | d-Tubocurarine concentration (μg/ml) |
|--------|---------------------|--------------------|-------------------------------------|
| Control 1 | 0.52 ± 0.07 (n = 16) | 49.1 ± 5.5 (n = 10) | 0.38 |
| Control 2 | 0.64 ± 0.10 (n = 18) | 52.8 ± 3.8 (n = 14) | 0.45 |
| myd 1   | 0.32 ± 0.04 (n = 11) | 65.6 ± 6.5 (n = 5)  | 0.30 |
| myd 2   | 0.36 ± 0.06 (n = 7)  | 75.4 ± 38.7 (n = 5) | 0.30 |

*aValues given are mean ± SEM and number of endplates (in parentheses).
*bThe amount of d-Tubocurarine that can inhibit the muscle contraction induced by the EPP."
deficient differentiation signal transduction to FCMD muscle fibers and arrested post-synaptic muscle differentiation. It has been suggested that α-DG in the central or peripheral nervous system is hypoglycosylated in secondary α-DG pathies (34). O-Mannose-type glycoprotein is suggested to contribute to the stability and maintenance of muscle cell membrane, synaptic formation and myelination of peripheral nerves, although the precise mechanism of α-DG activity in peripheral nerve tissue is unclear. The dystrophin–glycoprotein complex on Schwann cells is also thought to be important in peripheral myelogenesis, regeneration, differentiation, apoptosis and polarity of skeletal muscle cells (35). Although Ishii et al. (36) reported that electron microscopy of Schwann cells on FCMD muscle revealed no pathologic findings, neural transmission may be developmentally impaired and collapsed as a result of hypoglycosylated α-DG.

Naturally, our data do not rule out the other possibilities for immaturity of the skeletal muscles of α-DGPathies. Although the expression profile and histochemical data indicate that skeletal muscles are in a persistent undifferentiated state, it is possible that the regeneration process fulfills a crucial function in the phenomenon observed in skeletal muscles of α-DGPathies. It is also possible that most of the muscle fibers are under denervation status, because motor neurons are unable to maintain strong attachments to myofibers, leading to a constant stimulation of denervation signal pathways. Although we demonstrated substantial evidences for the aberrant NMJ, the pathogenesis of skeletal muscles in α-DGPathies is likely to be a combination of these problems and of multifactorial origin.

Taken together, these findings show that muscle fibers in secondary α-DGPathies are developmentally arrested more to ‘dystrophic’, perhaps because hypoglycosylated α-DG precludes proper aggregation on NMJs, preventing AChR clustering. These defects may disrupt terminal muscle maturation, which is induced after innervation of neurons on the muscle peripheral membrane via a basement membrane layer. Dystrophic changes traditionally thought to underlie ‘muscular dystrophy’ are caused by attenuated physical connections between α-DG and the muscle basement membrane. We propose that the muscle lesion in secondary α-DGPathies is caused by complex pathogenesis, not only by dystrophic change but more importantly, maturational arrest resulting from chronically delayed terminal muscle fiber maturation and NMJ deficiency. To date, no clinical approaches to secondary α-DGPathies exist. These findings open a possible

![Figure 6](https://academic.oup.com/hmg/article-abstract/15/8/1279/644547)

**Figure 6.** Aberrant NMJs and lack of α-dystroglycan aggregation in NMJs affect pre- and post-synaptic formation of NMJs in secondary α-DGpathy. (A) α-DG (green) strongly co-localizes with α-BTX in NMJs of normal skeletal muscle, merged yellow (arrows). However in secondary α-DGpathies, the lack of α-dystroglycan aggregation on aberrant formed NMJs is seen despite positive staining on muscle peripheral membrane, as evidenced by the lack of co-localization signal in merged columns (arrowheads). (B) Green-stained pre-synaptic markers (fasciculin, AChE marker; synaptophysin, pre-synaptic vesicle marker) also show the aberrant staining pattern of NMJs, shown in merged staining with α-BTX (red) in myd. (C) Utrophin remains on sarcoplasmic membrane of FCMD muscle fibers. (D) Confocal imaging of utrophin and dystrophin on NMJs of secondary α-DGpathy. Merged column in normal NMJs (arrow) shows that fine primary and secondary folds lined with utrophin are tangled with dystrophin staining on NMJs, which also line the peripheral membrane of muscle fibers. Note that in secondary α-DGpathies, secondary folds are severely lacking (arrowheads). Thin, scattered utrophin staining and markedly decreased staining of dystrophin on NMJs are seen in both FCMD and myd. Scale bars = 5 μm.
avenue for treating muscle tissues in these congenital muscular dystrophies, via targeted induction of NMJ maturation in muscle fibers.

MATERIALS AND METHODS

Samples
All clinical materials were collected for diagnostic purposes. Four muscle specimens (biceps bracket) from FCMD patients (ages: 20 days, 7 months, 1-year 4 months, and 1-year 6 months) were used in the analysis. Genetic screening identified a homozygous retrotranspositional insertion into the 3' untranslated region of fukutin in all FCMD patients (3). For the non-dystrophic muscle controls, muscle RNAs from two children (ages: 1 year) was used. These patients were selected based on normal laboratory findings, normal plasma CK levels and no histopathological evidence for muscular dystrophy. We also obtained myodystrophy (Large myd) mice and control littermates (Large myd or Large +/−), aged 3 and 6 months, by mating heterozygous pairs provided by Jackson Laboratories.

RNA isolation and expression profiling
Generation of cDNA microarrays containing skeletal muscle transcripts has been reported previously (19). Using similar methods, we constructed a new cDNA chip containing 5600 genes expressed in skeletal muscle. RNA isolation, hybridization and detection methods also have been reported previously. Microarray experiments were carried out using a competitive hybridization method with two labeled targets: one for muscle RNAs from FCMD patients or normal children, and another for pooled muscle RNAs (Origene), which served as a template control for per-chip normalization. Each analysis was conducted at least twice. The hybridization intensities of each spot and the background intensities were calculated using a ScanArray 5000 microarray scanner with Quant Array software (Perkin-Elmer Life Science).

Microarray data analysis
Analysis of microarray data was performed using Genespring version 6.1 (Silicon Genetics) software. Data used for further analysis were calculated using a previously reported method (19). To avoid ‘false-positive’ signals, we excluded genes from the analysis for which average normal expression level constraints are under 500. We sorted 1790 genes from a total of 5600 for further analysis.

Quantitative real-time PCR
Two patients with FCMD (ages: 10 months and 1 year), two patients with DMD (ages: 1 and 7 years) and two normal children (ages: 1 and 2 years) were used for quantitative RT–PCR using skeletal muscle RNAs. Single-strand cDNA was produced with random primers, and quantitative real-time RT–PCR using SYBR-green was performed using the ABI Prism 7900 sequence detection system (Applied Biosynthesis). Data analysis was performed in duplicate experiments. Statistical significance was evaluated using Student’s t-test, and P < 0.05 was considered significant. The primers used for the experiments are shown in Supplementary Material, Table S1. Gapdh was used as an internal control.

Imaging analysis
HE staining and ATPase staining were performed on cryosections. ATPase staining was performed at pH 9.4–10.6 and 4.2–4.6. For ATPase staining, average data from 10 DMD patients (ages: 3–9) and 10 normal control cases (ages: around 1 year) were selected. Scion Image Beta 3b (Scion Corporation) was used for estimating the content of type 2C fibers, muscle fibers, adipose tissues and interstitial tissues (Table 1). Statistical analysis was performed using Student’s t-test.

Immunohistochemistry
All muscle specimens were processed for cryosectioning (8-μm thick) and fixed in 50% ethanol and 50% acetic acid for 1 min. Anti-core α-dystroglycan [GT20ADG, gift from Dr Kevin Campbell (5)], rhodamine-conjugated α-bungarotoxin (BTX, Molecular Probes), anti-synaptophysin (NCL-SYNApP, Novocastra), Alexa-fluor488 labeled anti-fasciculin2 (F4293, Sigma), anti-MRF4 (C-19, Cruz Biotechnology), anti-utrophin (UT2, gift from Dr Michihiko Imamura), anti-human dystrophin (NCL-DYS2, Novocastra), anti-human dystrophin (MANDRA-1, Sigma) and anti-myosin heavy chain for developmental, neonatal, fast and slow types (NCL-MHCd, MHCn, MHCf and MHCs, respectively, Novocastra) were used to stain NMJs.

Electron microscopy
Muscle specimens were obtained from four patients diagnosed as FCMD (ages: 6 months to 4 years old) and from two normal children (ages: 1 year). Five NMJs were found in three FCMD patients’ skeletal muscle and four normal NMJs were examined in two normal children. Intercostal muscles and soleus muscles were dissected from myodystrophy and control mice, and cut into 1-mm thick cubes. Four NMJs were examined in myd and normal controls, respectively. Samples were fixed in 2% glutaraldehyde and embedded in epoxy resin as described previously (36). Ultrathin (50–90-nm thick) sections were cut on an Ultracut S ultramicrotome (Reichert).

Electrophysiologic examination
Diaphragms with its motor nerve were dissected and used for the conventional intracellular microelectrode study (37). MEPPs, EPPs and resting membrane potentials (RMPs) were recorded. For EPP recording, the phrenic nerve was stimulated using a suction electrode at 0.5 Hz. d-Tubocurarine chloride (CuraRen, Sigma) was used at a concentration sufficient to inhibit muscle contraction. The potentials were corrected for non-linear summation and the last 64 responses in a train of 114 were saved for later analysis. The quantal content m was calculated by the variance method. MEPP and EPP amplitudes were corrected to a standard RMP of −80 mV. To correct MEPP amplitude by the fiber diameter, the geometric...
mean of the shortest and longest diameters of muscle fibers was determined in 30 randomly selected muscle fibers in cryostat sections.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
We are grateful to Drs Fumiaki Saito and Shin‘ichi Takeda for their critical comments, Fumie Uematsu and Eiji Oiki for technical support and Dr Jennifer Logan for editing the manuscript. This work was supported by a Health Science Research Grant, Research on Psychiatric and Neurological Diseases and Mental Health, from the Ministry of Health, Labor, and Welfare of Japan; and by the 21st Century COE program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflict of Interest statement. None declared.

REFERENCES
1. Fukuyama, Y., Osawa, M. and Suzuki, H. (1981) Congenital progressive muscular dystrophy of the Fukuyama type—clinical, genetic and pathological considerations. Brain Dev., 3, 1–29.
2. Toda, T., Segawa, M., Nomura, Y., Nonaka, I., Masuda, K., Ishihara, T., Sakai, M., Tomita, I., Orighi, Y., Suzuki, M. et al. (1993) Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31–33. Nat. Genet., 5, 283–286.
3. Kobayashi, K., Nakahori, Y., Miyake, M., Matsunuma, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M. et al. (1998) An ancient retrotransposon insertion causes Fukuyama-type congenital muscular dystrophy. Nature, 394, 388–392.
4. Hayashi, Y.K., Ogawa, M., Tagawa, K., Noguchi, S., Ishihara, T., Nonaka, I. and Arahata, K. (2001) Selective deficiency of alpha-dystroglycan in Fukuyama-type congenital muscular dystrophy. Nature, 418, 417–422.
5. Tome, F.M., Evangelista, T., Leclerc, A., Sunada, Y., Manole, E., Estournet, B., Barois, A., Campbell, K.P. and Fardeau, M. (1994) Congenital muscular dystrophy with merosin deficiency. CR Acad. Sci. III, 317, 381–385.
6. Muntoni, F., Brockington, M., Blake, D.J., Torelli, S. and Brown, S.C. (2002) Defective glycosylation in muscular dystrophy. Lancet, 360, 1419–1421.
7. Hohenester, E., Tisi, D., Talts, J.F. and Timpl, R. (1999) The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin. Mol. Cell., 4, 783–792.
8. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsushashi, H., Takahashi, S., Takeuchi, M. et al. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev. Cell., 1, 717–724.
9. Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayselari, H., Merlini, L., Chitayat, D., Dobyns, W.B. et al. (2002) Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker–Warburg syndrome. Am. J. Hum. Genet., 71, 1033–1043.
10. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabe, D., Sabatelli, P., Merli, I., Boon, M., Scheffer, H., Brockington, M., Muntoni, F. et al. (2005) POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker–Warburg syndrome. J. Med. Genet., 12, 907–912.
11. Grewal, P.K., Holzfeind, P.J., Bittner, R.E. and Hewitt, J.E. (2001) Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. Nat. Genet., 28, 151–154.
12. Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R.K., Voit, T., Merlini, L. et al. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. Hum. Mol. Genet., 12, 2853–2861.
13. Nonaka, I., Sugita, H., Takada, K. and Kumagai, K. (1982) Muscle histochernistry in congenital muscular dystrophy with central nervous system involvement. Muscle Nerve, 5, 102–106.
14. Terasawa, K. (1986) Muscle regeneration and satellite cells in Fukuyama type congenital muscular dystrophy. Muscle Nerve, 5, 465–470.
15. Chen, Y.W., Zhao, P., Borup, R. and Hoffman, E.P. (2000) Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. J. Cell Biol., 151, 1321–1336.
16. Haslett, J.N., Sanoudou, D., Kho, A.T., Bennett, R.R., Greenberg, S.A., Kohane, I.S., Beggs, A.H. and Kunkel, L.M. (2002) Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. Proc. Natl Acad. Sci. USA, 99, 15000–15005.
17. Noguchi, S., Tsukahara, T., Fujita, M., Kurokawa, R., Tachikawa, M., Toda, T., Tsujimoto, A., Arakata, K. and Nishino, I. (2003) cDNA microarray analysis of individual Duchenne muscular dystrophy patients. Hum. Mol. Genet., 12, 595–600.
18. Prody, C.A. and Merlie, J.P. (1991) A developmental and tissue-specific enhancer in the mouse skeletal muscle acetylcholine receptor alpha-subunit gene regulated by myogenic factors. J. Biol. Chem., 266, 22588–22596.
19. Fujisawa-Sehara, A., Nabeshima, Y., Komiya, T., Uetsuki, T., Asakura, A. and Nabeshima, Y. (1992) Differential trans-activation of muscle-specific regulatory elements including the myosin light chain box by chicken MyoD, myogenin, and MRF4. J. Biol. Chem., 267, 10031–10038.
20. Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature, 321, 406–411.
21. Sunyer, T. and Merlie, J.P. (1993) Cell type- and differentiation-dependent expression from the muscle acetylcholine receptor epsilon-subsitent promoter. J. Neurosci. Res., 36, 224–234.
22. Jacinthe, G. and Michael, F. (2001) Expression and localization of agrin during sympathetic synapse formation in vitro. J. Neurobiol., 48, 228–241.
23. Grady, R.M., Zhou, H., Cunningham, J.M., Heny, M.D., Campbell, K.P. and Sanes, J.R. (2000) Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin–glycoprotein complex. Neuron, 25, 279–293.
24. Helliwell, T.R., Man, N.T., Morris, G.E. and Davies, K.E. (1992) The dystrophin-related protein, utrophin, is expressed on the sarcolemma of limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Muscle Nerve, 15, 1288–1300.
29. Weis, J., Kaussen, M., Calvo, S. and Buonanno, A. (2000) Denervation induces a rapid nuclear accumulation of MRF4 in mature myofibers. *Dev. Dyn.*, 218, 438–451.

30. Zhou, Z. and Bornemann, A. (2001) MRF4 protein expression in regenerating rat muscle. *J. Muscle Res. Cel. Motil.*, 22, 311–316.

31. Campanelli, J.T., Roberds, S.L., Campbell, K.P. and Scheller, R.H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell*, 77, 663–674.

32. Kahl, J. and Campanelli, J.T. (2003) A role for the juxtamembrane domain of beta-dystroglycan in agrin-induced acetylcholine receptor clustering. *J. Neurosci.*, 23, 392–402.

33. Jacobson, C., Cote, P.D., Rossi, S.G., Rotundo, R.L. and Carbonetto, S. (2001) The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. *J. Cell Biol.*, 152, 435–450.

34. Saito, F., Moore, S.A., Barresi, R., Henry, M.D., Messing, A., Ross-Barta, S.E., Cohn, R.D., Williamson, R.A., Sluka, K.A., Sherman, D.L. et al. (2003) Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron*, 38, 747–758.

35. Leschziner, A., Moukhles, H., Lindenbaum, M., Gee, S.H., Butterworth, J., Campbell, K.P. and Carbonetto, S. (2000) Neural regulation of alpha-dystroglycan biosynthesis and glycosylation in skeletal muscle. *J. Nervechem.*, 74, 70–80.

36. Ishii, H., Hayashi, Y.K., Nonaka, I. and Arahata, K. (1997) Electron microscopic examination of basal lamina in Fukuyama congenital muscular dystrophy. *Neuromuscul. Disord.*, 7, 191–197.

37. Nagel, A., Lehmann-Horn, F. and Engel, A.G. (1990) Neuromuscular transmission in the mdx mouse. *Muscle Nerve*, 13, 742–749.