Primary collagen VI deficiency is the second most common congenital muscular dystrophy in Japan

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ABSTRACT

Objectives: To determine the frequency of primary collagen VI deficiency in congenital muscular dystrophy (CMD) in Japan and to establish the genotype-phenotype correlation.

Methods: We performed immunohistochemistry for collagen VI in muscles from 362 Japanese patients with CMD, and directly sequenced the three collagen VI genes, *COL6A1*, *COL6A2*, and *COL6A3*, in patients found to have collagen VI deficiency.

Results: In Japan, primary collagen VI deficiency accounts for 7.2% of congenital muscular deficiency. Among these patients, five had complete deficiency (CD) and 29 had sarcolemma-specific collagen VI deficiency (SSCD). We found two homozygous and three compound heterozygous mutations in *COL6A2* and *COL6A3* in all five patients with CD, and identified heterozygous missense mutations or in-frame small deletions in 21 patients with SSCD in the triple helical domain (THD) of *COL6A1*, *COL6A2*, and *COL6A3*. All mutations in SSCD were sporadic dominant. No genotype-phenotype correlation was seen.

Conclusion: Primary collagen VI deficiency is the second most common CMD after Fukuyama type CMD in Japan. Dominant mutations located in the N-terminal side from the cysteine residue in the THD of *COL6A1*, *COL6A2*, and *COL6A3* are closely associated with SSCD.

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Ullrich congenital muscular dystrophy (UCMD) is a form of congenital muscular dystrophy (CMD) clinically characterized by congenital muscular weakness, proximal joint contractures, distal joint hyperlaxity, and normal intelligence as originally described,[1] and now includes additional features such as protuberant calcanei, facial muscle involvement, and high-arched palate.[2,3]

Mutations in the three collagen VI genes, *COL6A1*, *COL6A2*, and *COL6A3*, are reported to cause two types of myopathies, Bethlem myopathy and UCMD. Bethlem myopathy is a relatively mild dominant inherited myopathy,[4] while UCMD was thought to be autosomal recessive, albeit recent reports of dominant mutations.[5-7]

In skeletal muscle, collagen VI is normally observed in the interstitium and strongly delineates the sarcolemma. In patients with UCMD, collagen VI is deficient either completely, which we refer to as complete deficiency (CD), or sarcolemma-specifically, which we consider as sarcolemma-specific collagen VI deficiency (SSCD).[8,9]

The spectrum of clinical features in reported patients has been shown to be widely variable, with the maximal functional ability ranging from inability to acquire ambulation to mild weakness and with the ability to run,[10-14] as opposed to the previously known phenotype of UCMD.

The true frequency of collagen VI deficiency has not been established; therefore, we

Supplemental data at www.neurology.org

See also page 1043

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ventured on this study to know the frequency of primary collagen VI deficiency among patients with CMD, and to study genotype-phenotype correlation among patients.

METHODS **Patients.** All clinical materials used in this study for diagnostic purposes were obtained with informed consent. A total of 362 Japanese patients with diagnosed CMD based on clinical and pathologic observations from 1978 to 2004 were included. The criteria of CMD are with onset in utero or during the first year of life, and showing dystrophic changes in muscle pathology.[15] Available blood samples from the patients' relatives were also included in the analysis. DNA samples from 50 subjects without any known muscle disease were also studied.

The clinical features of patients were analyzed by careful review of their medical records provided by their attending physicians.

Immunohistochemistry. Immunohistochemical staining was performed on 6 μm serial cryosections of muscle, as described previously.[5,9] Briefly, sections were incubated in mixtures of rabbit polyclonal antibody against collagen IV (Abcam, Cambridge, UK) diluted 1:500, and mouse monoclonal antibody against collagen VI (ICN Biomedicals, Aurora, OH) diluted 1:500 for 1 hour. After washes with phosphate buffered saline, mixtures of either antimouse IgG Alexa 488, antirabbit IgG Alexa 568 conjugates (Invitrogen, Carlsbad, CA) diluted 1:500 or appropriate peroxidase-conjugated secondary antibodies were applied for 30 minutes. These sections were observed under fluorescence microscopy. In addition, collagen VI was also detected by using Basic DAB Detection Kit (Ventana Medical Systems Inc., Tucson, AZ).

Sequence analysis of collagen VI genes. Direct sequencing was performed in all three collagen VI genes in patients found to have collagen VI deficiency by immunohistochemistry. Genomic DNA was extracted from either frozen muscle biopsy samples or peripheral blood lymphocytes using standard protocols.[16] PCR primers were designed to amplify all the exons of *COL6A1*, *COL6A2*, and *COL6A3* and their flanking intronic regions. Amplified fragments were directly sequenced using BigDye Terminator v3.1 Cycle Sequencing system on ABI3100 automated Genetic Analyzer (Applied Biosystems, Foster City, CA). When aberrant splicing was suspected, total RNA was extracted from fibroblasts or frozen muscles using Totally RNA Kit (Nippon Gene, Tokyo, Japan) and was reverse transcribed with oligo $(dT)_{15}$ primer using SuperScript III (Invitrogen). RT-PCR was performed using relevant exonic primers, and the amplified fragment was directly sequenced. Sequence data were analyzed with the SeqScape (Applied Biosystems) program and compared with the genomic or cDNA sequences of collagen VI genes in database (Genbank GeneID and mRNA Genebank): *COL6A1* 1291, NM_001848; *COL6A2* 1292, NM_001849; and *COL6A3* 1293, NM_004369. A hundred control chromosomes were examined for each novel mutation by restriction enzyme analysis and direct sequencing.

Genotype-phenotype correlations. The clinical characteristics collected from attending physicians were demographic data, floppiness, presence of joint contractures or hyperlaxity, congenital hip dislocation, mental retardation, high arched palate, and pertinent laboratory examinations including serum creatine kinase, electrocardiogram, and echocardiogram studies. Severity of the condition was reflected by the walking ability of patients, which is defined as the number of years from the age the patient acquired independent ambulation to the age when the patient becomes wheelchair-bound.

Mean (±) SD of the characteristics were computed and subjected to either a univariate analysis (Fisher exact test) or χ^2 test, whichever was appropriate, with significant p value set at < 0.05.

RESULTS Immunohistochemical analysis revealed that 34 of 362 (9.4%) patients had collagen VI deficiency. Five patients had CD, whereas 29 showed SSCD (figure 1).

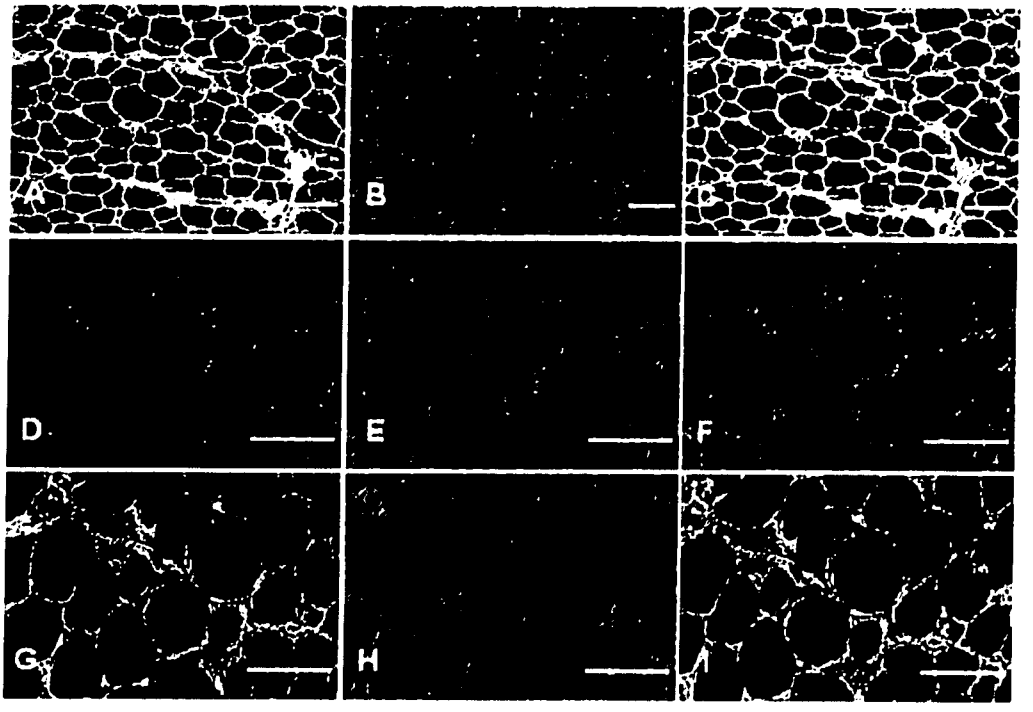
Direct sequencing of *COL6A1*, *COL6A2*, and *COL6A3* genes revealed mutations in a significant number of patients with collagen VI deficiency, apart from the several polymorphisms that we have found (table E-1 on the *Neurology* Web site at www.neurology.org). Mutations were found in all patients with CD. Among these, two mutations were previously reported,[8,9] while in the other three patients, novel mutations were seen in *COL6A2* and *COL6A3* homozygously or compound heterozygously (table 1, figure 2). Genomic analysis of DNA from their parents led us to identify that the heterozygous mutation identified in patients was carried by each parent.

In the 29 patients with SSCD, heterozygous mutations in the collagen VI genes were seen in 21 of them. Six different mutations were identified in *COL6A1*, five in *COL6A2*, and three in *COL6A3* (table 1). *COL6A1* c.868G>A and c.1056 + 1g>A mutations were previously reported in Bethlem myopathy,[17] while *COL6A1* c.850 g>A, c.868G>A and *COL6A3* c.6210 + 1G>A mutations were documented in UCMD.[5,7] The other 10 mutations were novel.

All these mutations were located in the triple helical domain (THD) in the three collagen VI genes (figure 2). Ten patients had missense glycine substitutions in *COL6A1* and *COL6A2*, and one had lysine-to-arginine mutation. All the mutations were predicted to cause single amino acid replacements or small in-frame deletions of 99 bp or shorter, however, the basic structure of G-X-Y was maintained in these deletions. All the mutations were located in the N-terminal side from the cysteine residue of the THD. We also analyzed genomic DNA from healthy parents and siblings of 11 patients and did not find any mutation, highly suggesting that the mutations were de novo.

All 34 patients (17 boys and 17 girls) with col-

Figure 1 Double immunostaining for collagen VI (green) and collagen IV (red)



(A through C) Normal. (D through F) Complete collagen VI deficiency (CD). (G through I) Sarcolemma-specific collagen VI deficiency (SSCD). In normal control, collagens VI and IV are colocalized in the sarcolemma as demonstrated by yellow in the sarcolemma (C). As expected, in patients with complete collagen VI deficiency (CD), only collagen IV expression is seen (E and F). However, in patients with sarcolemma-specific collagen VI deficiency, collagen VI is expressed in the interstitium while only collagen IV is seen in the sarcolemma (I), demonstrating the sarcolemma-specific mode of collagen VI deficiency (SSCD). (D through F) Patient 5, (G through I) Patient 15 in table 2. Bar denotes 50 μm.

lagen VI deficiency were without apparent family history and thus were considered to be sporadic cases (table 2). Most of the patients, 27/30 (90%), were floppy infants. Torticollis was seen in 12/29 (41%) and congenital hip dislocation in 16/31 (52%). Motor development was delayed in 32/33 (97%) as they acquired head control at the mean age of 4.7 months, sat without support at 12 months, and walked without support at 23 months (data not shown). Intellectual development was normal except in one female patient. Proximal dominant muscle weakness was seen in all patients in various degrees. Distal joint hyperlaxity was seen in 27/29 (93%), while proximal joint contractures were noted in 20/30 (67%). Protuberant calcanei was observed in 23/26 (88%) and high arched palate in 20/28 (71%). Serum creatine kinase levels were either normal or mildly elevated. Independent ambulation was not achieved in six patients, and three were still able to run. The clinical course of the disease was seemingly progressive, especially in patients showing typical UCMD phenotype. In these patients, assisted ventilation or respiratory support was required from the first decade of life. No cardiac complication was found on electrocardiogram or echocardiography in our series, as previously reported by others.[10,14]

For comparison between the modes of collagen VI deficiency and the clinical features, the differences between CD (n = 5) and SSCD (n = 29) were statistically analyzed across the phenotypic characteristics shown in table 2. No statistical differences were found between CD and SSCD ($p > 0.1$). We also analyzed genotype-phenotype correlations in terms of mutated genes: *COL6A1* (n = 12), *COL6A2* (n = 5), and *COL6A3* (n = 4). However, no statistical differences were seen among them ($p > 0.1$). In addition, the comparison between the patients with and without mutations in terms of clinical features did not show statistical difference. We did not find any mutation in eight patients with SSCD. There was no phenotypic difference between patients with SSCD with and without mutations.

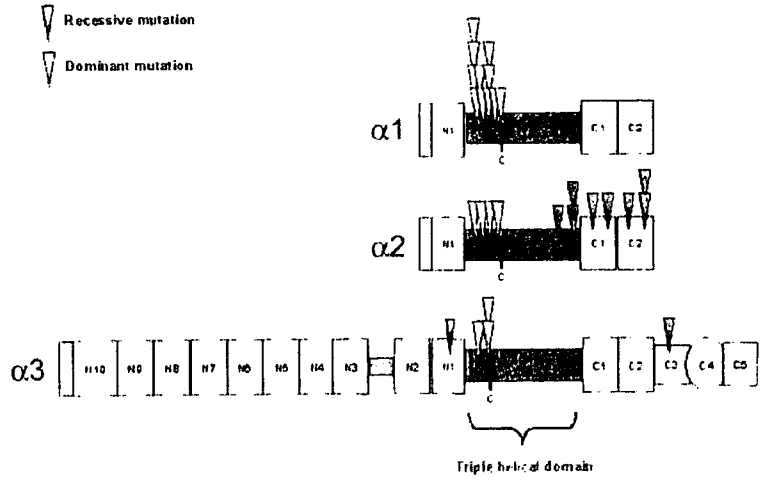
DISCUSSION Fukuyama type CMD (FCMD) is the most frequent CMD and is found almost exclusively in Japan, accounting for 49.2% in our series, although a patient with an extremely severe lethal disease who was clinically dissimilar to but genetically proven to have FCMD was re-

Table 1 Mutations in collagen VI genes in patients with collagen VI deficiency

Gene	Location	Domain	Nucleotide change	Predicted consequence	No. of patients
Mutations in patients with CD					
COL6A2	intron 19	THD	c.1572+1G>C	p.Gly508_Pro524del	1 (hom)
	exon 28	C2	c.2678_2700del23	p.Pro893fs	
	intron 23	THD/C1	c.1771-2A>T	p.Gly591fs	1 (c.het)
	exon 26	C1	c.2279_2280del2	p.Asp761fs	
	intron 14	THD THD/C1	c.1270-1G>C	p.Gly424_Lys444del	1 (c.het)
	intron 23		c.1771-3G>C	p.Gly591fs	
COL6A3	exon 12	N1	c.5692delG	p.Val898fs	1 (c.het)
COL6A3	exon 40	C3	c.8737delG	p.Ala2913fs	
Heterozygous mutations in patients with SSCD					
COL6A1	exon 9	THD	c.850G>A	p.Gly284Arg	4
	exon 10	THD	c.868G>A	p.Gly290Arg	2
	exon 12	THD	c.956G>T	p.Lys319Asn	1
	exon 13	THD	c.958_966del9	p.Gly320_Lys322del	3
	exon 13	THD	c.967_975del9	p.Gly323_Lys325del	1
	intron 14	THD	c.1056+1G>A	p.Gly335_Asp352	1
COL6A2	exon 6	THD	c.812G>A	p.Gly271Asp	1
	intron 6	THD	c.856-2A>G	p.Gly286_Lys309del	1
	exon 7	THD	c.875G>T	p.Gly292Val	1
	exon 8	THD	c.901G>T	p.Gly301Cys	1
	exon 8	THD	c.902G>A	p.Gly301Asp	1
COL6A3	intron 15	THD	c.6157-2A>G	p.Gly2053_Pro2070del	1
	intron 16	THD	c.6210+1G>A	p.Gly2053_Pro2070del	2
	intron 16	THD	c.6210+2T>A	p.Gly2053_Pro2070del	1

CD = complete deficiency; THD = triple helical domain; hom = homozygous change; c.het = compound heterozygous change; SSCD = sarcolemma-specific collagen VI deficiency.

Figure 2 Schematic diagram of the domain structure of the protein chains encoded by *COL6A1-3*



The identified putative recessive mutations in complete deficiency (CD) are indicated by blue arrows. Probable dominant mutations in sarcolemma-specific collagen VI deficiency (SSCD) are indicated by yellow arrows. Each triple helical domain (THD) contains a single cysteine residue (depicted as "C") which is important in dimer assembly. Mutations in SSCD are clustered in the N-terminal side from the cysteine residue of the THD

ported from Turkey.[18] Merosin-negative CMD accounts for 38 to 46% of patients with CMD in Western countries while it accounts for only 2.8% in our series.[19,20] The frequency of each form of CMD varies considerably in different ethnic groups.

We identified 34 patients with collagen VI deficiency among 362 Japanese patients with CMD (9.4%). Among all the CMD patients, 26 (7.2%) patients had mutations in either one of the collagen VI genes, indicating that primary collagen VI deficiency is the second most common CMD in Japan after FCMD.

Collagen VI is a ubiquitously expressed extracellular matrix protein composed of three chains, α1 (VI), α2 (VI), and α3 (VI), encoded by three genetically distinct genes: *COL6A1*, *COL6A2*, and *COL6A3*. The three collagen VI chains have a THD consisting of 335–336 amino acids G-X-Y repeats in central portion,[21-23] and are assembled into monomers through those domains. These monomers assemble intracellularly into dimers

Table 2 Clinical, pathologic, and genetic features of the patients with collagen VI deficiency

Patient	Age at last consultation, y	Sex	Collagen VI	Floppy infant	Torticollis	Congenital hip dislocation	Mental retardation	Proximal joint contracture	Distal joint hyperlaxity	Protuberant calcanei	High arched palate	Serum creatine kinase*	Independent ambulation, y†	Ability to run	Gene	Mutations
1	[illegible]	M	CD	No	Yes	No	No	No	Yes	No	No	937	ND	ND	[illegible]	c.[illegible]772+1G>C (homo. ygous)
2	[illegible]	[illegible]	CD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	367	0	No	COL6A2	c.2678-2_2680del23 (homozygous)
3	[illegible]	[illegible]	CD	Yes	No	Yes	No	No	Yes	Yes	Yes	343	2+	Yes	COL6A2	c.[illegible]>A c.[illegible]_2280del2
4	[illegible]	M	CD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	440	0	No	COL6A2	c.1270-1G>C c.[illegible]71-3C>C
5	[illegible]	M	CD	Yes	No	Yes	No	[illegible]	Yes	Yes	No	[illegible]3	ND	ND	COL6A3	c.5692delG c.8[illegible]97delG
6	10	M	SSCD	ND	ND	ND	No	Yes	Yes	Yes	Yes	Normal	0	No	COL6A1	c.850G>A
7	11	M	SSCD	Yes	No	No	No	Yes	Yes	Yes	Yes	242	10	No	COL6A1	c.850G>A
8	16	F	SSCD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	170	0	No	COL6A1	c.850G>A
9	8	F	SSCD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	195	0	ND	COL6A1	c.850G>A
10	[illegible]	[illegible]	SSCD	Yes	No	Yes	No	No	Yes	ND	ND	417	3+	No	COL6A1	c.868G>A
11	6	F	SSCD	Yes	No	Yes	No	Yes	Yes	Yes	Yes	138	[illegible]	ND	COL6A1	c.868G>A
12	[illegible]	M	SSCD	Yes	Yes	Yes	No	Yes	Yes	[illegible]	Yes	201	ND	ND	COL6A1	c.956G>[illegible]
13	14	F	SSCD	Yes	ND	Yes	No	ND	ND	Yes	ND	372	ND	ND	COL6A1	c.958_966del9
14	10	[illegible]	SSCD	Yes	ND	No	No	ND	Yes	Yes	Yes	343	[illegible]	No	COL6A1	c.958_966del9
15	7	F	SSCD	Yes	No	No	No	No	Yes	No	Yes	289	6+	No	COL6A1	c.958_966del9
16	4	[illegible]	SSCD	Yes	No	No	No	Yes	ND	ND	Yes	76	ND	No	COL6A1	c.967_975del9
17	10	M	SSCD	Yes	No	No	No	No	Yes	Yes	No	354	9+	Yes	COL6A1	c.1056+1G>A
18	[illegible]6	M	SSCD	No	No	No	No	No	Yes	ND	No	191	12	No	COL6A2	c.812C>A
19	18	M	SSCD	No	No	No	No	No	No	ND	ND	197	17+	No	COL6A2	c.856-2A>G
20	8	M	SSCD	Yes	No	Yes	No	Yes	Yes	Yes	Yes	329	5	No	COL6A2	c.875G>[illegible]
21	8	M	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	No	207	6+	No	COL6A2	c.901G>T
22	8	M	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	[illegible]1	6+	Yes	COL6A2	c.902G>A
23	3	F	SSCD	ND	No	Yes	No	No	ND	ND	No	148	ND	No	COL6A3	c.6157-2A>G
24	6	[illegible]	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	[illegible]4	4	No	COL6A3	c.6[illegible]G>A
25	9	F	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	539	8+	No	COL6A3	c.6210+1G>A
26	4	[illegible]	SSCD	Yes	No	No	No	No	Yes	Yes	No	136	2+	No	COL6A3	c.6210+1G>A
27	11	F	SSCD	Yes	No	Yes	No	Yes	Yes	ND	No	80	ND	ND	None	
28	9	M	SSCD	Yes	[illegible]	Yes	No	Yes	Yes	Yes	Yes	[illegible]	ND	No	None	
29	1	M	SSCD	ND	ND	ND	No	ND	ND	ND	ND	319	ND	ND	None	
30	1	[illegible]	SSCD	Yes	No	Yes	Yes	No	Yes	[illegible]	Yes	[illegible]5	6	No	None	
31	14	M	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	ND	113	5	No	None	
32	[illegible]	M	SSCD	ND	ND	ND	No	ND	ND	ND	ND	ND	ND	ND	None	
33	9	F	SSCD	Yes	No	Yes	No	Yes	No	No	No	380	8+	ND	None	
34	[illegible]	M	SSCD	Yes	No	Yes	No	Yes	Yes	[illegible]	Yes	99	[illegible]+	No	None	
		M:F = 1:1	CD:SSCD = 5:29	Yes 27/30 = 90%	Yes 12/29 = 41%	Yes 16/31 = 52%	No retardation 33/34 97%	Yes 20/30 = 67%	Yes 27/29 = 93%	Yes 23/26 = 88%	Yes 19/28 = 68%	High creatine kinase 24/33 = 72%				

*Normal value (NV) = 51 to 197 U/L, except in Patient 23 (NV = 40 to 116 U/L) and Patient 27 (NV = 30 to 50 U/L).
†Number of years from the age when patients acquired independent ambulation to the age when they become wheelchair-bound; indicates that the patient is still able to walk independently until the time of examination.
CD = complete deficiency; SSCD = sarcolemma-specific collagen VI deficiency; ND = not determined; MR = mental retardation.

and tetramers, after which the tetramers are secreted to extracellular space and are associated with each other to form collagen VI microfibrils.[24] Collagen VI is thought to anchor the basement membrane in the skeletal muscle by interacting with collagen IV, a major component of the basal lamina.[25] The cysteine residues are located in the THD at position 89 in both the α1 (VI) and α2 (VI) chains, and are obviously involved in stabilizing collagen VI dimer. In the α3 (VI) chain, these are located at position 50, and are known to link to the scissor-like connections used in the formation of tetramers.[21]

The three collagen VI genes are composed of 107 exons, thus mutation screening is quite a challenge, aside from the known fact that it is highly polymorphic.[6] Mutation analysis of all the exons and exon-intron boundaries of the *COL6A1, COL6A2,* and *COL6A3* have led us to identify mutations in our patients with collagen VI deficiency. Among these 34 patients, 5 had CD and 29 showed SSCD.

All five patients with CD had homozygous or compound heterozygous mutations in collagen VI genes, a phenomenon which is compatible with autosomal recessive mode of inheritance.[3,6,8,9,11-13] Interestingly, in the two patients (Patients 1 and 2) who had homozygous mutations in *COL6A2*, no consanguinity among their parents was noted, similar to all other patients. Although we did not find these mutations in 100 control chromosomes, we still could not exclude the possibility that we are dealing with a common mutation because we were not able to perform haplotype analysis due to limited number of patients.

Among 29 patients with SSCD, 21 had heterozygous mutations in collagen VI genes (72.4%). None of the healthy family members examined, including parents, had the corresponding mutation, indicating that all these mutations were de novo dominant mutations.

Interestingly, all these mutations were present in the THD, and they were either missense or in-frame. Most of all missense mutations affected glycine residues in G-X-Y motif in the THD, emphasizing the importance of glycine residues in the THD. None of these in-frame mutations affected the triple-amino acid repeating frame, G-X-Y, which is thought to play an essential role in forming the triple helix among the three collagen VI chains. This notion is further supported by our observation that collagen VI microfibrils with the THD mutation have reduced capacity to facilitate adhesion of cells to the extracellular matrix.[26]

Furthermore, all the dominant mutations that we identified and those previously reported were located in the N-terminal side from the cysteine residue in the THD.[5,6,11,27] The cysteine residues are believed to be crucial in the formation of the triple helical structures in collagen VI.[28] Thus the substitutions or deletions in the N-terminal side from the cysteine residue are likely to affect the conformation around the cysteine residues and, subsequently, the formation of the functional higher structure of collagen VI complex.

All heterozygous mutations found in patients with SSCD were de novo, and none of the patients with SSCD had a family history suggesting autosomal dominant inheritance. This is most likely because these dominant mutations are associated with a rather severe phenotype not allowing patients to produce offspring. In contrast, in Bethlem myopathy, the phenotype is mild enough for patients to produce children. This probably explains why Bethlem myopathy shows autosomal dominant inheritance.

One patient with CD had a homozygous mutation in the THD (Patient 1, table 2). Although his parents had the mutation in heterozygous mode, they were reported to be healthy. Another patient (Patient 4) whose parents were also healthy had compound heterozygous in-frame deletions (51 and 48 bp) in the THD in *COL6A2*. These mutations were located in the C-terminal side from the cysteine residue in the THD, unlike mutations in SSCD. These results indicate that substitutions or deletions in the N-terminal side from the cysteine residue in the THD are associated with dominant mutations in SSCD,[29] while mutations in the C-terminal side from the cysteine residue in the THD are recessive mutations associated with CD.

We did not find any mutations in 8 of 29 patients with SSCD. Recently, CMD with joint hyperlaxity, clinically similar to UCMD, was mapped to chromosome 3p23-21, reinforcing the idea that mutations in genes other than *COL6A1, COL6A2,* or *COL6A3* can also cause a clinically similar disease condition.[30] For example, a protein interacting with collagen VI in the sarcolemma might be defective. However, there remains a possibility that mutations are present in the promoter regions or introns, or that we might have overlooked mutations.

From the clinical point of view, patients with collagen VI deficiency showed a wider clinical spectrum than previously thought, ranging from typical UCMD to a much milder condition.

Interestingly, three patients can still run at the time of examination, which is unusual for classic

UCMD. Of note is the observation that in four patients who had the same c.850G>A mutation in *COL6A1* (Patients 6, 7, 8, and 9), three of them never acquired independent ambulation, but one remained ambulant for 10 years. These data imply that the mutation is not predictive of the phenotype of patients, at least in the ability to walk; however, we could not make a direct conclusion regarding this because of the small population.

We did not find any genotype-clinical phenotype correlation, similarly to a previous report.[27] In addition, genotype-pathologic phenotype correlation was also absent, as exemplified by some patients showing much milder clinical phenotype than classic UCMD although they had collagen VI deficiency. Therefore, to call the condition collagen VI deficiency may be more appropriate. Our observation that there was no correlation between immunohistochemical patterns (SSCD and CD) and clinical phenotype in collagen VI deficiency suggests that the residual amount of collagen VI may not at all be correlated with the phenotype, and that the main pathomechanism of the disease can possibly be due to the disruption of collagen VI anchorage to the basal lamina.[8,9]

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Reduced cell anchorage may cause sarcolemma-specific collagen VI deficiency in Ullrich disease

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ABSTRACT

Background: *COL6* gene mutations are associated with Ullrich congenital muscular dystrophy (UCMD), which is clinically characterized by muscle weakness from early infancy, hyperlaxity of distal joints, and multiple proximal joint contractures. We previously reported that the majority of patients with UCMD have sarcolemma-specific collagen VI deficiency (SSCD). More recently, we found heterozygous *COL6A1* glycine substitutions in patients with UCMD with SSCD.

Objective: To elucidate how *COL6A1* glycine mutation leads to SSCD.

Methods: We evaluated the synthesis, formation, and binding of collagen VI to the extracellular matrix in fibroblasts with p.G284R mutation in *COL6A1*.

Results: Collagen VI was normally secreted into the cultured medium in fibroblasts harboring p.G284R mutation. When the medium with normal collagen VI was added to collagen VI-deficient fibroblast culture, collagen VI bound surrounding the cells, while collagen VI with p.G284R mutation did not. Cell adhesion of fibroblasts with p.G284R mutation was markedly reduced similarly to that of collagen VI-deficient cells. Interestingly, this reduction in adhesion of the cells with p.G284R mutation was recovered by the addition of the medium with normal collagen VI, which would suggest a therapeutic strategy for a replacement therapy.

Conclusion: Heterozygous glycine substitution in *COL6A1* may cause decreased binding of collagen VI microfibrils to the extracellular matrix resulting in sarcolemma-specific collagen VI deficiency. ***Neurology*® 2007;69:1043–1049**

Ullrich congenital muscular dystrophy (UCMD) is an inherited muscular disorder clinically characterized by muscle weakness, distal joint hyperlaxities, and proximal joint contractures.[1] Patients with UCMD show deficiency of collagen VI. We have previously demonstrated two modes of collagen VI deficiency: complete deficiency and sarcolemma-specific collagen VI deficiency (SSCD). In SSCD, collagen VI is present in the interstitium but is barely detectable in the sarcolemma.[2] The complete deficiency of collagen VI is associated with recessive mutations in collagen VI genes,[2-6] but the primary cause of SSCD has not yet been determined.[2]

Collagen VI is an extracellular matrix (ECM) consisting of three chains: α1, 2, and 3, which are encoded by *COL6A1*, *COL6A2*, and *COL6A3* genes.[7] Association of the three subunits to form monomers is by staggered assembly into dimers,[8] which subsequently align to form tetramers. After being secreted, these tetramers associate end-to-end to form the characteristic beaded microfibrils.[9-11]

Recently, heterozygous missense mutations that substitute the glycine in the Gly-*X*-Y amino acid repeat in the triple helical domain including p.G284R in *COL6A1* have been

Supplemental data at www.neurology.org

See also page 1035

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identified in patients with UCMD.[12-14] Previous reports alluded to the effects of single amino acid substitutions on collagen VI structure and function in causing UCMD[12,14,15] as well as in Bethlem myopathy,[15-17] a milder autosomal dominant muscle disease allelic to UCMD.[18-22] However, the molecular pathomechanism has not been clearly determined.

In this article, using fibroblasts with the p.G284R mutation, the synthesis, formation, and functions of collagen VI microfibrils were analyzed.

METHODS Clinical materials. All clinical materials used in this study were acquired with informed consent. Biceps brachii muscle was biopsied for diagnostic purpose. Based on our immunohistochemical and genetic screening of Japanese patients who were diagnosed with UCMD based on typical clinical features, i.e., muscle weakness, hyperextensibility of distal joints, and contractures of proximal joints, we found five patients with mutations in *COL6* gene: four with SSCD had a heterozygous c.850G>A (p.G284R) mutation in *COL6A1* gene, and one with complete deficiency harbored compound heterozygous mutations in *COL6A3*. In this study, we analyzed fibroblasts from foreskin biopsy which were available in two of the four patients with SSCD, and in the patient with complete deficiency of collagen VI.

Immunohistochemical and histologic staining. Immunohistochemical and histologic staining used in this study have been described previously.[3] Briefly, sections were incubated in mixtures of mouse monoclonal antibody against collagen VI (1:500 dilution) (ICN Pharmaceuticals) and rabbit polyclonal antibody against collagen IV (1:500 dilution) (Abcam Ltd.) for 1 hour. After phosphate buffered saline (PBS) washes, mixtures of anti mouse IgG Alexa 488, anti-rabbit IgG Alexa 568 conjugates (1:500 dilution) (Molecular probes) were applied for 30 minutes.

Cell culture. Fibroblasts from two patients with UCMD with p.G284R mutation, one with collagen VI complete deficiency, and two controls were cultured to reach confluence on 100 mm collagen I coated dishes in 10% fetal bovine serum/Dulbecco's modified Eagle's medium under humidified 5% CO_2 at 37 °C.

For enhancement of expression, cells were cultured in the presence of 0.25 mM L-ascorbic acid (Sigma) for 3 days. For cell detachment test, cultured fibroblasts were treated with 10 mM EDTA-PBS (pH. 8.0) at 37 °C for 1 hour. After washing with PBS, cells that remained on the dish were counted in a constant area (0.25 mm × 0.25 mm, total of 8 areas). Student *t* test was used for statistical analysis.

The cultured medium in each patient's cells was changed with the medium similar to which control or patient cells had been cultured for 3 days in the presence of 0.25 mM L-ascorbic acid. The cultured media for control or patient cells for exchanging were prepared by centrifugation at 2,000 rpm for 10 minutes. The amount of collagen VI in the clarified media was measured by Western blot using Quantity One software (PDI, Inc.). Cells were further cultured for 3 days in the exchanged medium containing relatively the same amount of collagen VI. They were subjected to cell detachment test and immunocytochemical staining.

For evaluating the substrate-retained collagen VI molecules, cells were mechanically removed by pipetting and the remaining proteins were extracted for Western blot analysis.

Immunocytochemical staining. The fibroblasts cultured on collagen I-coated cover slips were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.25% Triton X-100-PBS on ice for 10 minutes, followed by incubation with 1% bovine serum albumin in PBS for 1 hour. Cells were incubated for 1 hour in mixtures of mouse monoclonal antibody against collagen VI (1:500 dilution) (ICN Pharmaceuticals). After PBS washes, these were incubated with mixtures of antimouse IgG Alexa 488 and TOTO-3 to stain nuclei (1:600 dilution) (Molecular probes) for 30 minutes.

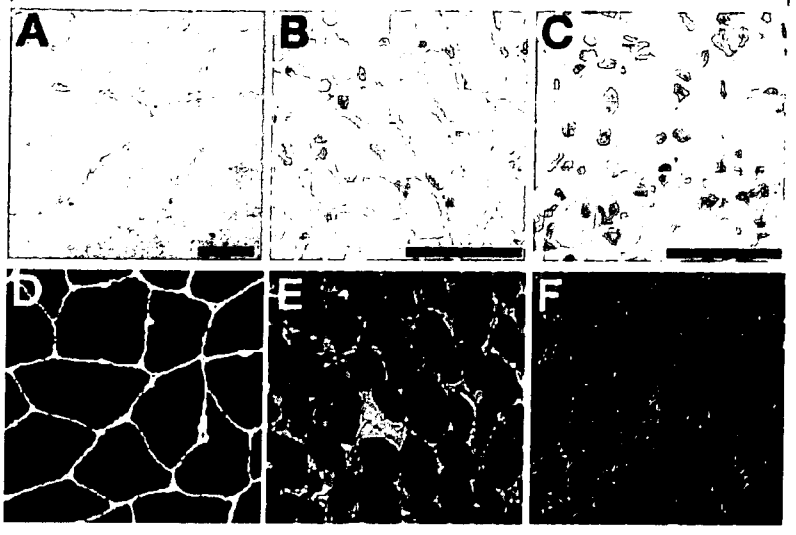
Western blot analysis. Proteins in the cultured medium and cell extract, as well as the proteins that remained on the dish after pipetting, were electrophoresed on 5–17.5% polyacrylamide gel under reduced condition and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). The remaining proteins on dish with or without EDTA treatment were extracted with a solution containing 2 M thiourea, 7 M urea, 4% CHAPS, 10 mM Tris-HCl (pH 8.5). After blocking, the proteins were allowed to react with rabbit polyclonal antibody against the subunits of collagen VI (1:50 dilution) (Abcam) or rabbit polyclonal antibody against fibronectin (1:3,000 dilution) (Chemicon) and incubated with peroxidase-conjugated, antirabbit IgG (1:3,000 dilution) (TAGO Inc.). Visualization of proteins was done using ECL Western blotting detection reagents and analysis system (Amersham Biosciences).

Negative staining electron microscopy. Culture media from control and patient cells were purified by centrifugation and adsorbed onto thin bar grids covered with a thin layer of carbon for 5 minutes. The grids were washed with PBS, and stained with 2% phosphotungstic acid. The dried sample was observed in a HITACHI H-600 transmission electron microscope (Hitachi) operated at 120 kV accelerating voltage.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis. The remaining protein (50 μg) from control and UCMD cells after EDTA treatment were labeled with Cy3 and Cy5 minimal dyes (Amersham Biosciences), following manufacturer's instructions. Mixture of both labeled samples was subjected to isoelectric focusing for separation in the first dimension by IPG gels (covering the range pH 3 to 10) using the Ettan IPGphor isoelectric focusing system (Amersham Biosciences); SDS-PAGE was then performed on a 10% polyacrylamide gel in second dimension. The Cy3/Cy5 signals were separately detected using Typhoon 9400 (Amersham Biosciences). DeCyder software (Amersham Biosciences) was used for quantitation and comparison of Cy3 and Cy5 intensities of all spots. Protein mass fingerprint analysis based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on spots was performed for the identification of proteins (Hitachi Science Systems, Ltd.).

RESULTS Brief clinical summary of the patients with p.G284R mutation. The patients with p.G284R mutation in *COL6A1* showed typical

Figure 1 Hematoxylin and eosin staining and immunohistochemistry of muscle sections



Muscle sections from normal control (A, D), patient with Ullrich congenital muscular dystrophy (UCMD) with p.G284R mutation (B, E), and patient with UCMD collagen VI complete deficiency (C, F) were stained with hematoxylin and eosin, and immunostained by anti-collagen VI antibody (D through F). Both collagen VI (green) and collagen IV (red) are present in sarcolemma in control muscle, as indicated in merged images (yellow, D). In contrast, collagen VI is only seen in the interstitium but not in the sarcolemma in the patients with UCMD with p.G284R mutation in *COL6A1* (E). In the case of complete deficiency of collagen VI, collagen VI is absent in the muscle section (F). Bars denote 50 μm.

clinical phenotypes of UCMD, including muscle weakness, hyperextensibility of distal joints, and contractures of proximal joints. All were sporadic cases. No genotype-phenotype correlation between SSCD and complete deficiency of collagen VI was shown.

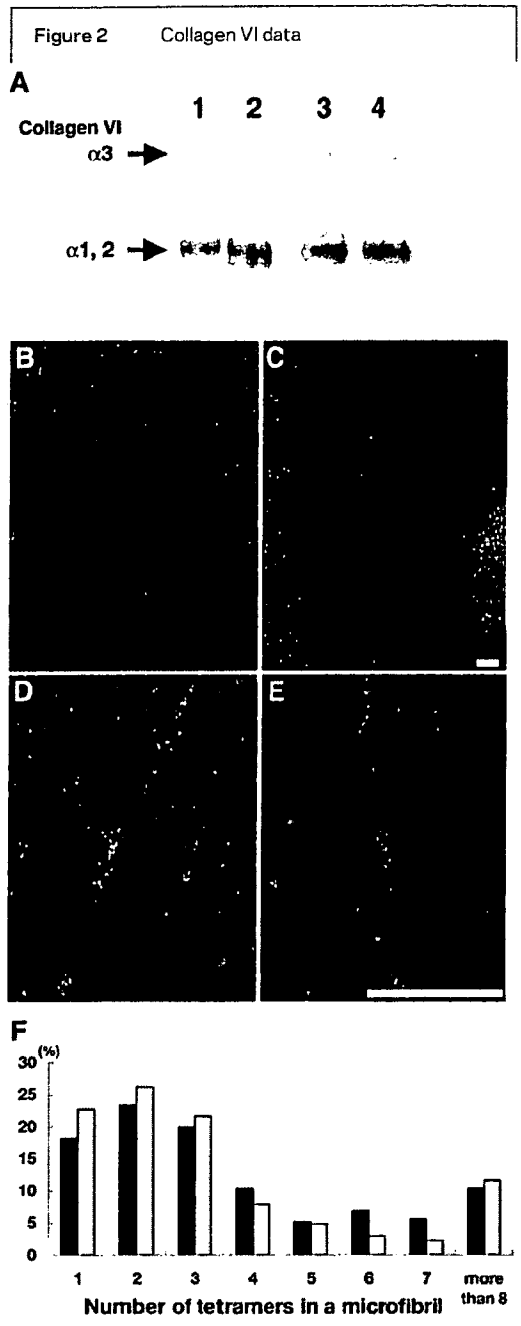
Localization of collagen VI in the skeletal muscle with p.G284R mutation. In the biopsied muscles from patients with UCMD with p.G284R mutation, double immunostaining of collagen VI and collagen IV, which is the major component of the basal lamina, revealed SSCD (figure 1, B and E). We also examined collagen VI microfibrils on electron microscopy; microfibrils were present in the interstitium, but they did not bind to the basement membrane (data not shown), similar to the previous report.[2] In the muscles from a patient with compound heterozygous mutations, collagen VI was completely deficient in the muscle (figure 1, C and F).

Analysis of collagen VI secreted in the cultured medium from patients with UCMD with p.G284R mutation. In fibroblasts from two patients with UCMD with the p.G284R mutation, collagen VI was present in the extracellular areas, as in control. On Western blot analysis, collagen VI subunits (α1, 2, and 3) were detected in the whole extract from fibroblasts and in the cultured medium, with similar size and amounts as in control cells (figure 2A). Ultrastructural analysis of collagen VI microfibrils with p.G284R mutation showed that tetramers were secreted from the cells; moreover, microfibrils were assembled in comparable length with control, including long microfibrils which consisted of more than 8 tetramers (figure 2, B through E). The shape and the length of each tetramer unit in microfibrils were also normal (figure 2, D and E).

Binding capacity of collagen VI with or without p.G284R mutation to ECM. In normal cells, collagen VI was localized in the ECM surrounding cells (figure 3A). On the other hand, collagen VI was absent in the ECM of cells from the patient with compound heterozygous mutations (figure 3E), consistent with the complete deficiency of collagen VI in muscle (figure 1F). When cultured medium of control cells containing normal collagen VI was added to these collagen VI-deficient cells, we proceeded to evaluate the binding of collagen VI to ECM on days 1, 2, and 3. Collagen VI was detected in the ECM of fibroblasts from patients with complete deficiency of collagen VI on days 2 and 3 (figure 3, B, C, and D). However, when cultured medium containing collagen VI with p.G284R mutation was added to collagen VI-deficient cells, collagen VI was only detected on day 3, in extremely reduced amount than that of normal cells after the incubation for 3 days (figure 3, F, G, and H).

Recovery of the adhesion ability of patients with UCMD's cells by treatment with the cultured medium containing normal collagen VI. In order to examine the effect of *COL6* mutation on the attachment of fibroblasts, we assessed cell adhesion of patients with UCMD's cells on a dish after treatment with EDTA. There was no difference in cell adhesion on dish in medium after washing with PBS among normal cells, p.G284R, and collagen VI-deficiency cells (data not shown). However, after EDTA treatment, in the cells with p.G284R mutation and collagen VI-deficient cells, the number of cells retained on the dish was reduced to approximately 30% of that of control ($p < 0.001$) (bars no. 1 in figure 4A).

Interestingly, by the addition of medium containing normal collagen VI to p.G284R mutated cells and collagen VI-deficient cells, the number of the retained cells on dish was restored to the level of control cells (p.G284R and collagen VI complete deficiency shown by bars no. 3, 5 in figure 4A). However, using the medium from p.G284R culture which contained the same amount of collagen VI did not restore the number



(A) Collagen VI in the fibroblasts or the cultured medium. Collagen VI is present in the extract of cells on dish (lane 1, control cells; lane 2, p.G284R mutation) and in the cultured medium of cells (lane 3, control cells; lane 4, p.G284R mutation). Collagen VI with p.G284R is produced and secreted to cultured medium in the same mass and amount compared to control cells. (B through E) Negative staining electron microscopy of secreted collagen VI. Collagen VI microfibrils in the cultured medium secreted from control cells (B, D) or cells with this p.G284R mutation (C, E) were visualized by negative staining electron microscopy. Long microfibrils are present in both control and patient (B, C). The magnified images show that the structure of tetramer is normal in length and shape. Bars denote 100 nm (D, E). (F) Quantitative analysis of association of collagen VI tetramers. The distribution of the numbers of tetramer in one microfibril is shown as a percentage of the total number of microfibrils. Black bars show the results of 231 microfibrils from control cells. White bars show 163 microfibrils from Ullrich congenital muscular dystrophy patient cells.

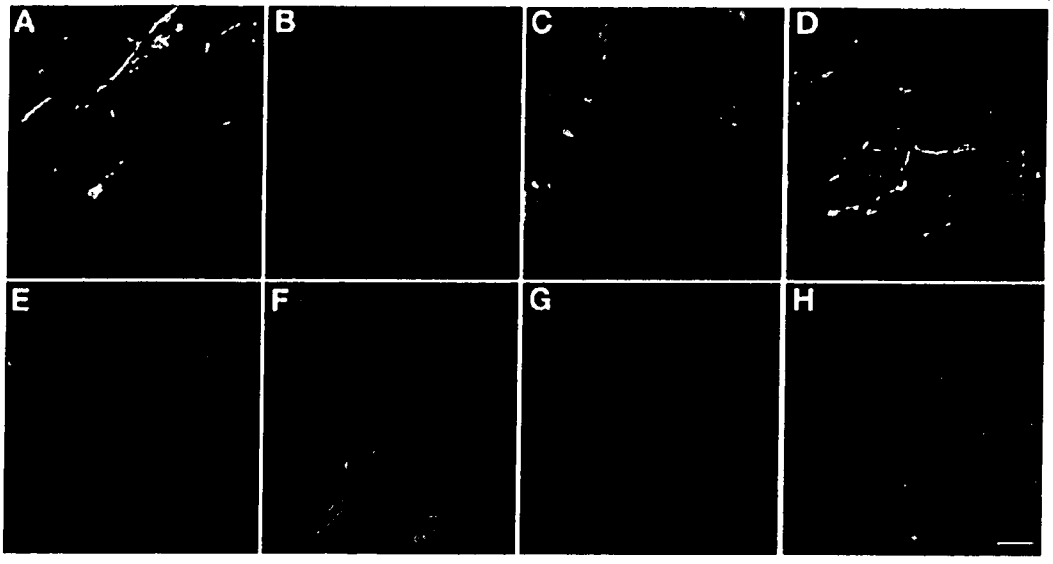
of collagen VI deficient cells retained on dish (bar no. 6 in figure 4A). Our analysis of substrate-retained collagen VI supported these findings. Only after treatment with the cultured medium containing normal collagen VI, we observed the recovery of substrate-retained collagen VI in the patient's cells with p.G284R mutation and collagen VI deficiency (figure 4B, lanes 3, 5). However, the addition of the cultured medium containing collagen VI with p.G284R mutation did not recover the reduction of the amount of collagen VI in the ECM of p.G284R mutated and collagen VI deficient cells (figure 4B, lanes 4, 6). In contrast, the amount of fibronectin bound to the substrate showed no difference between control and the mutant cells (figure 4B).

We further analyzed the remaining proteins bound to the substrate in order to examine influences of the alteration of collagen VI molecule to other proteins. In the 2D-PAGE analysis, only two groups of spots from 937 spots detected showed marked reduction (>fivefold) in the patient with p.G284R as compared to control. MALDI-TOF-mass spectrometry analysis revealed that these proteins were collagen VI α 1 and α 2. In contrast, the amount of the other substrate-bound proteins on dish was not greatly altered (see supplemental data at www.neurology.org).

DISCUSSION In this study, we used fibroblasts from patients with UCMD with heterozygous c.850G>A (p.G284R) mutation in *COL6A1* gene in the *N*-terminal region of the triple helical domain. Mutations in this region have been reported to be associated with UCMD and Bethlem myopathy.[12-16] Muscles from all four patients with this mutation showed the characteristic features of SSCD, just as in previously reported patients.[2,12,14,22] In our series, we were not able to establish genotype-phenotype correlation between SSCD and complete deficiency of collagen VI.

On electron microscopy, the shape and the length of collagen VI microfibrils secreted from cultured fibroblasts from the patients seemed to be normal, indicating that the mutation of p.G284R does not affect the production and formation of the microfibrils. Our data contradict previous reports showing that mutations in the triple helical domain of *COL6A1* are associated with abnormal tetramer formation and unnaturally long microfibrils.[12,17] These results suggest that glycine substitution in the triple helical do-

Figure 3 Immunocytochemical staining of collagen VI binding to the cells with the complete deficiency of collagen VI



Collagen VI (green) is clearly present in extracellular area of the control fibroblasts (A). In the cells of complete deficiency, collagen VI is absent (E). Culturing with control medium (B, 1 day; C, 2 days; D, 3 days) or in p.G284R medium (F, 1 day; G, 2 days; H, 3 days). In culture with control medium, collagen VI is present on dish on days 2 and 3; in p.G248R medium (F through H), collagen VI is detected on dish on day 3 in extremely reduced amount. Bar denotes 20 μm. Nuclei of cells are stained with TOTO-3 (blue).

main may cause a variety of influences on the formation of collagen VI.

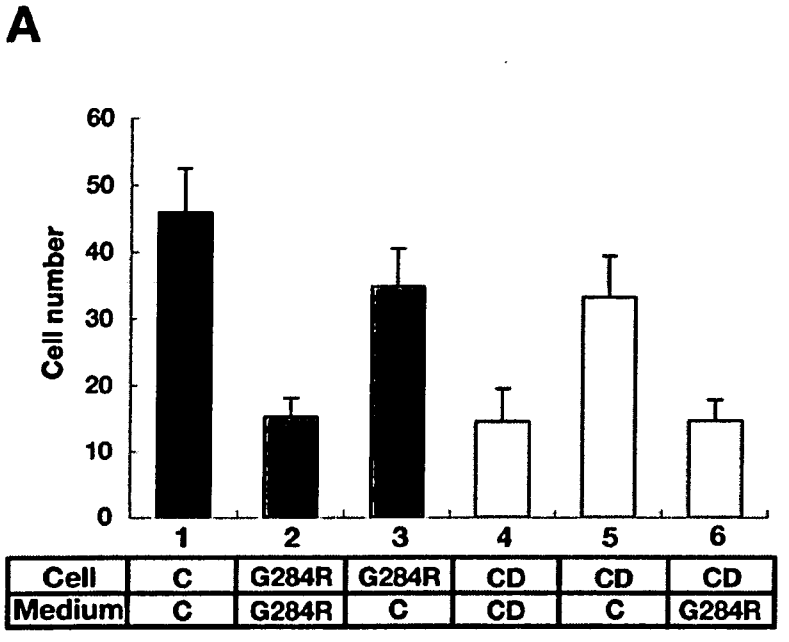
We evaluated the function of mutated collagen VI in p.G284R using patients with UCMD's fibroblasts. The immunocytochemical staining of the collagen VI deficient cells after incubation with cultured medium clearly show that collagen VI in the cultured medium of UCMD cells with p.G284R mutation had less binding capacity than that of control cells. These results suggest that the microfibrils with the mutated collagen VI α1 chain alter the affinity of collagen VI to its binding partners. Previous studies showed the presence of aggregated filaments sometimes assuming dot/spot-like appearance in the fibroblasts with the same mutation p.G284R,[14] which may support our contention that the alteration in the collagen VI microfibrils by this mutation can influence the interaction of collagen VI microfibrils in ECM.

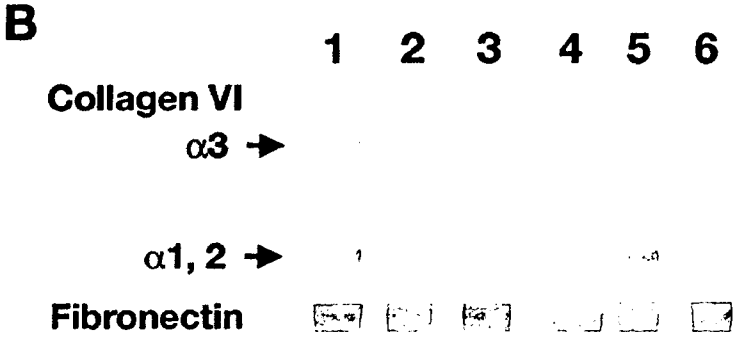
The results in the cell attachment test demonstrated that the adhesion ability of cells with the p.G284R and complete deficiency of collagen VI was less compared to that of control cells. The amount of the retained collagen VI on dish after the washing was reduced in the patients with UCMD (p.G284R and collagen VI complete deficiency) compared to control. These findings suggested that the reduction of the binding capacity by p.G284R mutation may cause an abnormality in the adhesion of fibroblasts from patients with UCMD. Since collagen VI is thought to anchor cells to the ECM, loss of the binding capacity should result in reduced cell adhesion. Our findings indicate that the interaction of collagen VI is related to cell adhesion between fibroblasts and ECM.

The specific loss of collagen VI in sarcolemma of these patients, which is the pathologic finding in muscle sections, suggests a possibility that this feature may be caused by the reduction of binding capacity of collagen VI microfibrils to other molecules as a results of the heterozygous mutation in the *COL6A1* triple helical domain. Collagen VI microfibrils have been shown to bind to cell-surface receptors, integrins,[23] and NG2,[24,25] and interact with several ECM components including fibrillar collagens,[26,27] collagen IV,[28] fibronectin,[29,30] biglycan, decorin,[31-33] and microfibril-associated glycoprotein-1 (MAGP-1).[34] Further studies on the binding partners of collagen VI in the ECM or on the cellular membrane may provide clues in understanding the function of collagen VI and the pathomechanism behind UCMD.

Our results on the mutant cells cultured in normal medium are of note. Interestingly, the adhesion ability of the cells with *COL6A1* p.G284R mutation and complete deficiency was restored by switching the medium to the one containing normal collagen VI microfibrils, indicating that

Figure 4 Recovery of binding ability of patients with Ullrich congenital muscular dystrophy (UCMD)'s cells by changing cultured medium from normal or p.G284R fibroblasts





(A) Number of remaining cells on the dish after detachment with EDTA solution. After changing into control medium (containing collagen VI), the number of cells became comparable to control (no. 1). On the other hand, UCMD cells, p.G284R (gray bar, no. 2), or collagen VI complete deficiency (open bar, no. 4), whose medium have not been changed, do not show recovery of cell attachment ($p < 0.001$, the number of counted area = 8). The cultured medium containing collagen VI with p.G284R likewise can not restore adhesion capacity of collagen VI complete deficiency cells (open bar, no. 6). Error bars indicate standard deviations. (B) The cells are cultured in three types of medium: original medium from cells (lanes 1, 2, 4); medium from control cells containing normal collagen VI (lanes 3, 5); or medium from cells with p.G284R mutation (lane 6). After the detachment of the cells by 10 mM EDTA, collagen VI is observed in control cells (lane 1). Cells with p.G284R mutation cultured in their own medium showed no detectable collagen VI (lane 2); however, after culture in the normal medium, collagen VI is observed in similar amounts to the control cells (lane 3). In collagen VI deficient cells, collagen VI is deficient in their own medium, but is detected when normal medium is used (lane 5). Interestingly, medium from p.G284R cells contains similar amounts of collagen VI protein to control cells (figure 2, lane 4), but collagen VI deficient cells cultured in this medium showed no detectable collagen VI (lane 6).

the function of mutant fibroblasts can be rescued by adding normal collagen VI. Moreover, recovering only by the addition of cultured medium containing normal collagen VI indicates that other functions and molecules of cells from patients with UCMD are normal. Results of recovery test using collagen VI-deficient fibroblasts suggested that collagen VI microfibrils with p.G284R mutation are less likely to interact with collagen VI binding partners, and thus, affecting the function of cells in attaching to other ECM proteins. These results demonstrated that the addition of normal collagen VI microfibrils possessing intact affinity to its binding partners rescues abnormalities in these cells from UCMD. This denotes that therapeutic use of normal collagen VI in patients with UCMD with SSCD may be plausible.

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Defective peripheral nerve myelination and neuromuscular junction formation in fukutin-deficient chimeric mice

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Abstract

Dystroglycan is a central component of the dystrophin–glycoprotein complex that links the extracellular matrix with cytoskeleton. Recently, mutations of the genes encoding putative glycosyltransferases were identified in several forms of congenital muscular dystrophies accompanied by brain anomalies and eye abnormalities, and aberrant glycosylation of α-dystroglycan has been implicated in their pathogeneses. These diseases are now collectively called α-dystroglycanopathy. In this study, we demonstrate that peripheral nerve myelination is defective in the fukutin-deficient chimeric mice, a mouse model of Fukuyama-type congenital muscular dystrophy, which is the most common α-dystroglycanopathy in Japan. In the peripheral nerve of these mice, the density of myelinated nerve fibers was significantly decreased and clusters of abnormally large non-myelinated axons were ensheathed by a single Schwann cell, indicating a defect of the radial sorting mechanism. The sugar chain moiety and laminin-binding activity of α-dystroglycan were severely reduced, while the expression of β1-integrin was not altered in the peripheral nerve of the chimeric mice. We also show that the clustering of acetylcholine receptor is defective and neuromuscular junctions are fragmented in appearance in these mice. Expression of agrin and laminin as well as the binding activity of α-dystroglycan to these ligands was severely reduced at the neuromuscular junction. These results demonstrate that fukutin plays crucial roles in the myelination of peripheral nerve and formation of neuromuscular junction. They also suggest that defective glycosylation of α-dystroglycan may play a role in the impairment of these processes in the deficiency of fukutin.

Keywords: dystroglycan, fukutin, glycosylation, myelination, neuromuscular junction.

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Dystroglycan (DG) is encoded by a single gene and cleaved into two proteins, α- and β-DG, by post-translational processing (Ibraghimov-Beskrovnaya *et al.* 1992). In skeletal muscle, DG is a key component of the dystrophin–glycoprotein complex (DGC). α-DG is a highly glycosylated extracellular peripheral membrane protein that binds to several extracellular matrix (ECM) proteins, such as laminin, agrin, and perlecan (Ervasti and Campbell 1993; Bowe *et al.* 1994; Peng *et al.* 1998). The mucin-like domain of α-DG binds to laminin G like domains of these ligands (Kanagawa *et al.* 2004) and certain sugar chain structures of α-DG, including Siaα2–3Galβ1–4GlcNAcβ1–2Man–Ser/Thr, are involved in this binding (Chiba *et al.* 1997). On the other hand, the transmembrane protein β-DG anchors α-DG to the cell membrane, and the cytoplasmic domain of β-DG interacts with dystrophin, which binds to F-actin (Jung *et al.* 1995).

Dystroglycan is also expressed in various non-muscle tissues including peripheral nerve. DG is expressed in Schwann cells, where it localizes to the outer membrane apposing the basal lamina (Saito *et al.* 1999) and the microvilli at the nodal axoglial interface (Saito *et al.* 2003). The Schwann cell DG interacts with laminin and agrin (Yamada *et al.* 1996; Previtali *et al.* 2003) and forms a

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Abbreviations used: AChE, acetylcholinesterase; AChR, acetylcholine receptor; DG, dystroglycan; DGC, dystrophin-glycoprotein complex; ECM, extracellular matrix; FCMD, Fukuyama-type congenital muscular dystrophy; NMJ, neuromuscular junction; α-BTX, α-bungarotoxin.

DGC-like complex with Dp116, utrophin, sarcoglycans, dystrophin-related protein 2 (DRP2) and L-periaxin (Saito *et al.* 1999; Imamura *et al.* 2000; Sherman *et al.* 2001). We have previously demonstrated that Schwann cell DG is necessary for myelination of peripheral nerve (Saito *et al.* 2003).

Recently, primary mutations of the genes encoding known or putative glycosyltransferases have been identified in several forms of congenital muscular dystrophies, which are characterized by muscular dystrophy with onset during the neonatal period accompanied by variable brain and ocular anomalies (Muntoni *et al.* 2004). In these diseases, laminin-binding activity of α-DG has been shown to be greatly reduced in skeletal muscle because of aberrant glycosylation of α-DG (Michele *et al.* 2002), and they are now correctively called α-dystroglycanopathy (Toda *et al.* 2003). Among them, Fukuyama-type congenital muscular dystrophy (FCMD), which is caused by the mutation of the *fukutin* gene, is one of the most common autosomal recessive disorders in Japan (Kobayashi *et al.* 1998). Fukutin is a ubiquitously expressed Goldi-resident protein shearing homology with fringe-like glycosyltransferases (Aravind and Koonin 1999). Because the constitutive fukutin knockout is embryonic lethal in mice, we generated fukutin-deficient chimeric mice and demonstrated that these mice developed severe muscular dystrophy, brain anomaly and eye abnormality (Takeda *et al.* 2003; Chiyonobu *et al.* 2005; Kurahashi *et al.* 2005).

Although involvement of the peripheral nervous system has not been characterized in FCMD so far, it is intriguing to hypothesize that the deficiency of fukutin may cause abnormal myelination of peripheral nerve because of aberrant glycosylation of Schwann cell α-DG. In addition, α-DG is a receptor for agrin, a potent regulator of acetylcholine receptor (AChR) clustering at the post-synaptic membrane of neuromuscular junction (NMJ) (Bowe *et al.* 1994). Several lines of evidence have implicated DG in the formation and maintenance of NMJ (Cote *et al.* 1999; Peng *et al.* 1999; Grady *et al.* 2000; Jacobson *et al.* 2001). These observations prompted us to hypothesize that the clustering of AChR at NMJ may be altered in α-dystroglycanopathy. To test this possibility, we investigated the status of myelination of peripheral nerve and clustering of AChR at NMJ in the fukutin-deficient chimeric mice.

Materials and methods

Generation of fukutin-deficient chimeric mice

The design of the fukutin targeting construct and generation of fukutin-deficient chimeric mice were reported previously (Takeda *et al.* 2003). We used mice with more than 80% contribution of fukutin$^{-/-}$ cells judged by chimerism of coat color as fukutin-deficient chimeric mice and those with 0% contribution as their control.

Antibodies

Rabbit polyclonal antibody against 34 amino acids in the C-terminal domain of human α-DG (DKGGLSAVDAFEIHVHRRPQGDRA-PARFKAKFVG) was generated and affinity purified (AP1530). Mouse monoclonal antibody IIH6 against sugar chain of α-DG and 8D5 against C-terminal domain of β-DG were kindly gifted by Dr K. P. Campbell (University of Iowa) and the late Dr L. V. B. Anderson (Newcastle General Hospital), respectively. Mouse monoclonal antibody 2D9 against laminin α2 chain was kindly provided by Dr H. Hori (Tokyo Medical and Dental University). Mouse monoclonal antibody against C-terminal end of dystrophin (MANDRA 1) and affinity isolated rabbit anti-laminin were obtained from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibody against synaptophysin and mouse monoclonal anti-dystrobrevin was purchased from Novocastra and BD Biosciences (San Jose, CA, USA), respectively. Mouse monoclonal antibodies against agrin, laminin β1 and β1-integrin (clone MB1.2) were from Chemicon (Temecula, CA, USA). Mouse monoclonal anti-laminin γ1 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). FITC- or Cy3-conjugated secondary antibodies were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and horseradish peroxidase-labeled secondary antibodies were obtained from Roche (Basel, Switzerland).

Histopathological, immunohistochemical and electron microscopic analyses

Histopathological analysis was performed on the fukutin-deficient chimeric mice and age matched control mice between the ages of post-natal day 15 and 24 months using standard frozen section, epon section, and electron microscopic techniques. Immunofluorescent microscopic analysis of peripheral nerve and skeletal muscle was performed as described previously (Michele *et al.* 2002). To obtain longitudinal images of NMJs, sternocleidomastoid muscle was fixed in 1% paraformaldehyde in PBS for 20 min and incubated with 30% sucrose overnight. Then, the specimens were cryosectioned at 40 μm thickness, immunostained and observed using LSM310 confocal microscope (Zeiss, Carl Zeiss, Göttingen, Germany). For the detection of NMJs, tetramethylrhodamine-labeled α-bungarotoxin (BTX), Alexa fluor 488-labeled α-BTX (Invitrogen-Molecular Probes, Carlsbad, CA, USA) or FITC-labeled fasciculin 2 was employed. Fasciculin 2 (Sigma) was labeled with FITC using Fluoreporter FITC protein labeling kit (Molecular Probe) according to the protocol provided by the manufacturer. The density of myelinated nerve fibers was calculated by counting the number of myelinated fibers in a spinal root and dividing the number by the area, using ImageJ software (NIH, Bethesda, MD, USA). The density was calculated in 10 roots and the statistical difference was evaluated by *t*-test.

In situ ligand overlay assay

Recombinant rat C-terminal agrin (R & D Systems, Minneapolis, MN, USA) or mouse EHS laminin (Biomedical Technologies Inc., Stoughton, MA, USA) was labeled with FITC using Fluoreporter FITC protein labeling kit (Invitrogen-Molecular Probe). Frozen quadriceps muscle of fukutin-deficient chimeric mice and control mice was cryosectioned at 8 μm thickness. After blocking with 3% BSA in buffer A (10 mmol/L triethanolamine, pH 7.6, 140 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$), the cross sections were

overlaid with FITC-labeled agrin or laminin in buffer A for 3 h. Later the specimen were washed with buffer A and observed using immunofluorescent microscope (Carl Zeiss). In some experiments, they were double stained with tetramethylrhodamine-labeled α-BTX.

Immunoblotting, blot overlay and solid-phase binding assays

For biochemical analysis, tissues were isolated and disrupted using Polytron (Kinematica, Littau-Lucerne, Switzerland) followed by Dounce homogenization in 50 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 0.6 μg/mL pepstatin A, 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin, 0.75 mmol/L benzamidine, and 0.1 mmol/L PMSF. After briefly spinning down debris, the homogenate was applied to 3–12% SDS-PAGE and resolved under reducing condition. Immunoblot analysis was performed as described previously (Michele *et al.* 2002). Blot overlay assay was performed as described previously (Michele *et al.* 2002), using EHS laminin, human merosin (laminin-2) (Chemicon) and recombinant rat C-terminal agrin as probes. Anti-agrin antibody (Chemicon) was used to detect the bound agrin. Solid-phase binding assay was performed as previously described (Michele *et al.* 2002).

Results

Myelination of peripheral nerve is defective in fukutin-deficient chimeric mice

To see if the deficiency of fukutin affects peripheral nerve architecture, we evaluated cross sections from various nerves of fukutin-deficient chimeric mice using light microscope. In the chimeric spinal roots, sciatic nerves and tibial nerves from post-natal day 15 (P15) to 23 months of age, the density of myelinated nerve fibers was decreased compared with control (Figs 1a and b). Morphometric quantification confirmed significant reduction of myelinated fiber density in the chimeric mice (Fig. 1c). Remarkably, in the spinal roots and sciatic nerves of P30 chimeric mice, there were numerous abnormally pale-staining areas, which consisted of clusters of densely packed non-myelinated axons (Figs 1d and e). Moreover, in the spinal roots of the old chimeric mice (>20 months old), nerve bundles in which myelinated fibers were strikingly decreased or lost were occasionally observed (Figs 1f and g).

We performed electron microscopic analysis of peripheral nerve of the chimeric mice. Because the aforementioned morphological abnormalities were most prominent in the spinal roots of old chimeric mice, we analyzed the chimeric mice of 20–23 months of age. In the spinal roots of the chimeric mice, non-myelinated axons with abnormally large caliber were frequently observed (Fig. 2a). Typically, these non-myelinated axons were larger than 1 μm in diameter and surrounded by much smaller non-myelinated axons. In many cases, ensheathment of these abnormally large non-myelinated axons was incomplete and their cytoskeletal structures were obscure, indicating they were degenerated (Fig. 2a). Occasionally, Schwann cells that appeared to ensheath multiple myelinated axons were observed (Fig. 2b). In the nerve bundles where severe decrement of myelinated fibers was observed under light microscope (Figs 1f and g), Schwann cells surrounding numerous degenerated axons with or without myelin or myelin ovoids were found (Fig. 2c). Many non-myelinated axons were very large in diameter and their cytoskeletal structures were obscure (Fig. 2c). In the Schwann cell cytoplasm, numerous degradated mitochondria and other cell organelles were accumulated (Fig. 2c). On the other hand, the outer membrane of Schwann cells in these degenerated nerve bundles were normally surrounded by typical mature basal lamina (Fig. 2d).

Aberrant glycosylation and reduced laminin-binding activity of α-DG in the peripheral nerve of fukutin-deficient chimeric mice

To see if the deficiency of fukutin affects the expression of the components of the DGC in peripheral nerve, we examined the sciatic nerve of fukutin-deficient chimeric mice by immunofluorescent microscope. The immunoreactivity of α-DG revealed by antibody against sugar chain moiety of α-DG (IIH6) was localized to the outermost layer of myelin sheath in the control mice, whereas the signal was severely decreased in the chimeric mice (Fig. 3). In sharp contrast, the immunoreactivity of α-DG revealed by antibody against core protein of α-DG (AP1530) was localized to the outermost layer of myelin sheath in both control and chimeric mice, and was indistinguishable between them (Fig. 3). Other components of the DGC, including β-DG, utrophin, dystrobrevin, and ECM proteins, such as laminin α2, β1 and γ1 chains were all localized to the outer membrane of myelin sheath or endoneurial basal lamina in both control and chimeric mice, and their expression was indistinguishable between them (Fig. 3).

Consistent with these results, immunoblotting with antibody against sugar chain moiety of α-DG (IIH6) revealed severe reduction of immunoreactivity of 120 kD α-DG in the chimeric sciatic nerve compared with control (Fig. 4a). In contrast, antibody against core protein of α-DG (AP1530) demonstrated only slight reduction of 120 kD α-DG in the chimeric sciatic nerve (Fig. 4a). Interestingly, this antibody detected an additional band of 80 kD, suggesting that considerable portion of α-DG might be aberrantly hypoglycosylated and migrate faster in the chimeric sciatic nerve (Fig. 4a, arrowhead). On the other hand, the expression of β-DG, laminin β1, γ1 chain and Dp116, a Schwann cell specific isoform of dystrophin, was indistinguishable between the control and chimeric mice (Fig. 4a). As sugar chain structure of α-DG plays an essential role in the binding of laminin, we assessed the laminin-binding activity of α-DG in sciatic nerve. Blot overlay assay demonstrated that the binding of laminin-2 to α-DG was greatly reduced in the chimeric sciatic nerve (Fig. 4b). To further confirm the reduction of laminin-binding activity in the chimeric sciatic

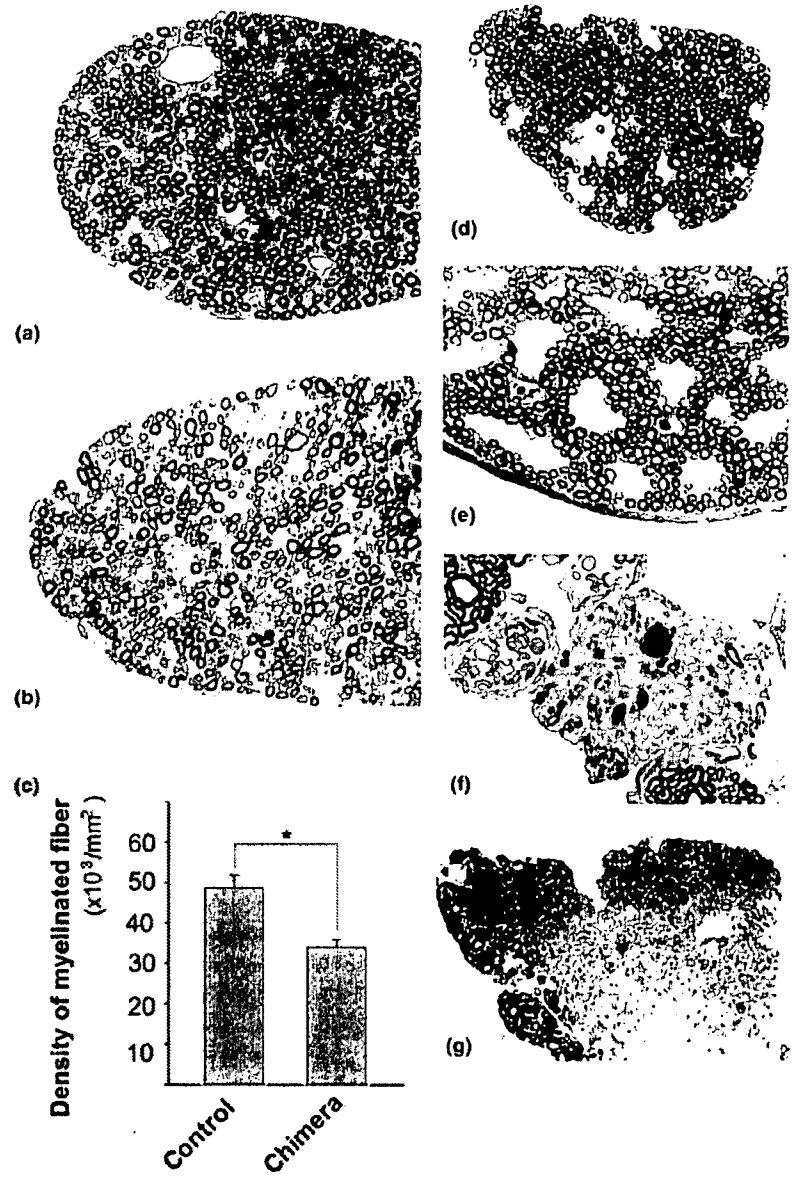

Fig. 1 Defects of myelin formation and radial sorting of axons in the peripheral nerve of fukutin-deficient chimeric mice. (a and b) Toluidine blue-stained epon sections of spinal roots from the post-natal day 15 (P15) control (a) and chimeric mice (b). In the chimeric nerves, the density of myelinated nerve fibers was decreased compared with control. (c) Morphometric quantification revealed significant reduction of myelinated fiber density in the spinal roots of P15 chimeric mice. (d and e) In the roots and sciatic nerves of P30 chimeric mice, there were numerous abnormally pale-staining areas, which were clusters of densely packed non-myelinated axons. (f and g) In the spinal roots of old chimeric mice, nerve bundles in which myelinated fibers were strikingly decreased or lost, were occasionally observed. (f) 20-month old mouse; (g) 23-month old mouse.

nerve, we performed quantitative solid-phase assay. The total laminin-binding activity of chimeric sciatic nerve was decreased to 20% of control (Fig. 4b).

Preservation of β1-integrin in the peripheral nerve of fukutin-deficient chimeric mice

We also analyzed the expression of β1-integrin, which has been shown to play a role in peripheral myelination (Feltri *et al.* 2002). Immunohistochemical analysis revealed that β1-integrin was equally localized to the outermost layer of myelin sheath in both control and chimeric mice (Fig. 5a). Immunoblot analysis also confirmed that the expression of β1-integrin was indistinguishable between the control and chimeric mice (Fig. 5b).

Architecture of neuromuscular junction is defective in fukutin-deficient chimeric mice

To evaluate AChR clustering, quadriceps muscle was stained with Alexa fluor 488-labeled α-bungarotoxin (α-BTX), a specific marker for AChR. Interestingly, NMJs of the chimeric mice were much smaller than control (Fig. 6a). We also analyzed the *en face* topology of NMJ labeled with α-BTX in longitudinal sections of sternocleidomastoid muscle. In the control mice, the pattern of AChR staining was smooth and continuous in appearance (Fig. 6b). On the other hand, NMJs of the chimeric mice showed a fragmented and discontinuous pattern of AChR staining defined by some discrete cups (Fig. 6b).

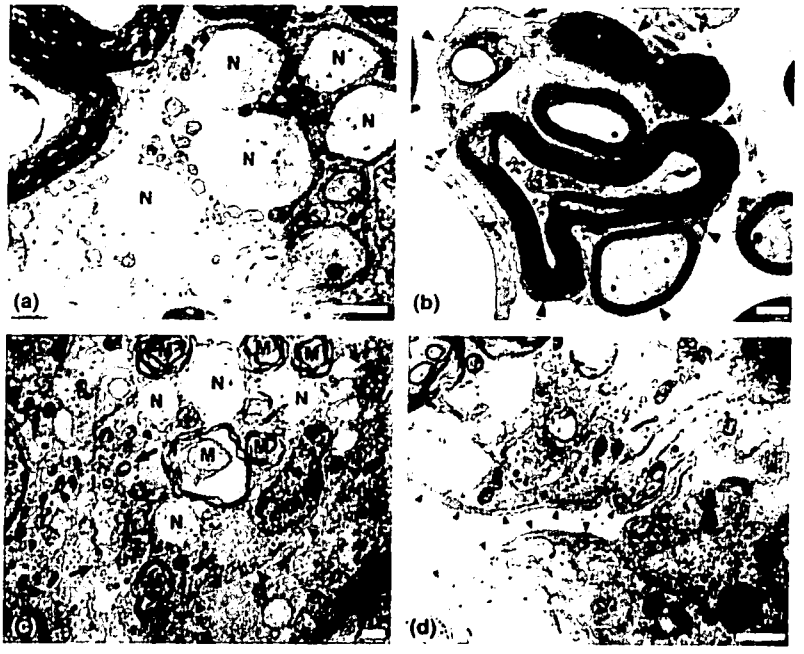

Fig. 2 Ultrastructural abnormalities of peripheral nerve in fukutin-deficient chimeric mice. Electron microscopic analysis of the spinal roots was performed using chimeric mice of 20 (a, c, d) and 23 (b) months of age. (a) Non-myelinated axons with abnormally large caliber were frequently observed. They were surrounded by much smaller non-myelinated axons. Ensheathment of these abnormally large non-myelinated axons was incomplete and their cytoskeletal structures were obscure, indicating that they were degenerated. N: degenerated large non-myelinated axons. (b) A single Schwann cell appeared to ensheath multiple myelinated axons and a non-myelinated axon (arrow). Arrowheads indicate the outer membrane of a Schwann cell. (c) Schwann cells surrounded many degenerated axons with or without myelin or myelin ovoids. Many of the non-myelinated axons were very large in diameter and cytoskeletal structures inside of the axons were obscure. In the Schwann cell cytoplasm, numerous degradated mitochondria and other cell organelles were accumulated (arrow). M: degenerated myelinated axons or myelin ovoid, N: degenerated abnormally large non-myelinated axons, SN: Schwann cell nuclei. Arrowheads indicate the outer membrane of a Schwann cell. (d) The Schwann cell outer membrane of degenerated nerve bundles was normally surrounded by mature basal lamina (arrowhead). Scale bar indicates 1 μm.

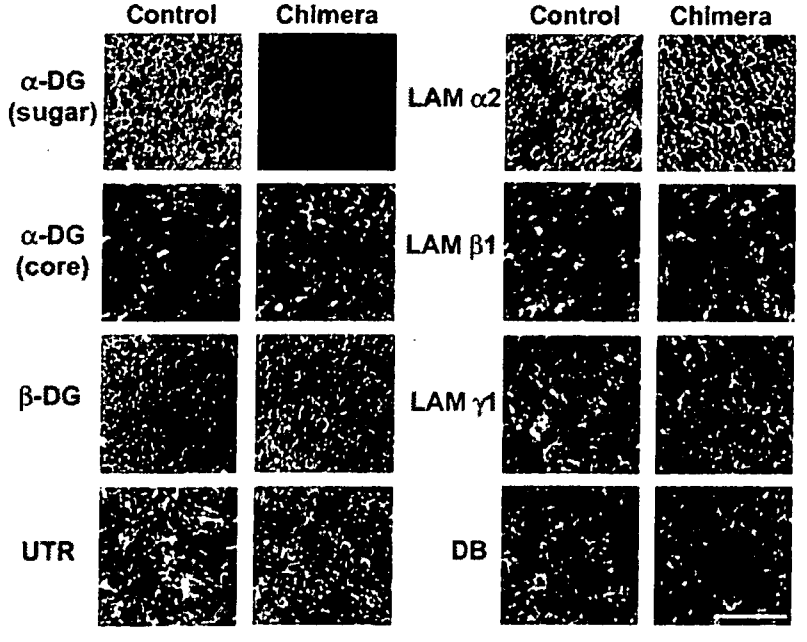

Fig. 3 Abnormal expression of α-DG in the peripheral nerve of fukutin-deficient chimeric mice. Immunohistochemical analysis revealed that the immunoreactivity for sugar chain moiety of α-DG recognized by monoclonal antibody IIH6 was localized to the outermost layer of myelin sheath in the control sciatic nerve, whereas the signal was severely decreased in the chimeric sciatic nerve. In contrast, the immunoreactivity of α-DG revealed by antibody against its core protein (AP1530) was localized to the outermost layer of myelin sheath in both control and chimeric mice, and was indistinguishable between them. β-DG, utrophin, dystrobrevin, laminin α2, β1 or γ1 were normally expressed at the outer membrane of myelin sheath or endoneurial basal lamina in both control and chimeric sciatic nerves. UTR, utrophin; LAM, laminin; DB, dystrobrevin. Scale bar indicates 50 μm.

