一致と臨床症状の特徴からSJSをパールカン異常により起こる第二の疾患の有力な候補と考え，前述のアプローチを進め，3症例で5種類の遺伝子変異を同定した[18]．

SJS遺伝子座が染色体1番1p36.1から1p34に局在することを示したNicoleらは，最近さらにこの領域を狭めることにより，1p36.1からp35にマップされていたパールカン遺伝子がSJS遺伝子座に含まれることを見いだし，さらに2家系のSJSでエキソンスキッピング，ミスセンス，ノンセンス変異の3種類の変異を報告している[19]．筆者らは，SJSでの蛋白質レベルの解析も行い，DDSHと異なりSJSではパールカンが細胞外に分泌され，筋基底膜に局在しているということを確認した．これはSJSがDDSHと比べ，明らかに良好な経過をとる大きな理由と考えられる．

V. 先天性筋無力症候群とSJSにおけるAChE欠損と発症機構

ノックアウトマウスの神経筋接合部の解析からSJSにおけるパールカン分子の欠損が，AChEのNMJ基底膜への結合，集束に影響し，神経筋の興奮とその解除に異常をきたす可能性が示唆された．SJSにおいてパールカンが部分欠損すると生成されたAChEの基底膜への局在化が部分的に障害されることが予想される．ヒト遺伝性疾患の一つに終板AChEの完全欠損する先天性終板AChE欠損症がある．これは常染色体劣性のまれな疾患で，*ColQ*遺伝子の異常により終板におけるAChEの欠損をひき起こす．臨床的には乳児期または小児期に始まる易疲労性と脱力を主症状とする先天性筋無力症候群で，躯幹筋の脱力と易疲労性のため，起立歩行時に側彎，前彎などの姿勢異常を示す．AChEの欠損症ではシナプス間隙に過剰のAChが存在する．このため，アセチルコリン受容体（AChR）の開口時間の延長による脱分極性ブロックやAChRの脱感作をきたし，神経筋の興奮伝達が阻害され，筋力低下の原因となる．AChEの完全欠損であるこの先天性筋無力症候群とAChEの部分欠損が想定されるSJSにおけるミオトニアという臨床的に相反する症状をつなぐ発生機序は非常に興味深い．脱分極の程度が軽い場合はブロックではなく持続性の筋収縮をきたす可能性がある．今後，神経終板の微小電位や微細構造の解析による検討が必要となる．

VI. アルツハイマー病の老人斑へのパールカン関与の可能性：パールカン全長過剰発現マウスの解析

中枢神経系におけるパールカンの機能はまだ十分解明されていないが，アルツハイマー病におけるアミロイド斑，神経原線維への蓄積が報告されている[20]．これに対し，Hartらはパールカン全長を過剰発現させたトランスジェニックマウスの作製，解析によりアミロイド斑形成を誘発する試みを行った[21]．しかし，正常パールカンの過剰発現だけではアミロイド斑，神経原線維の形成は誘発されないことがわかった．また実験的に発現させたトランスジェニックマウスの糖鎖が，ヒト生体中の糖鎖と同一の性質をもつものかは不明であり，病的状態での糖鎖修飾の違いなど，今後の研究発展が望まれる．アミロイド斑，神経原線維に蓄積するヘパラン硫酸プロテオグリカンは主としてアグリンであるという報告もあり[22]，まだまだ不明の点が多い．

●おわりに

ノックアウトマウスの解析から，まったく予後の異なる2つのヒト遺伝性疾患が同定された．症例数の蓄積による遺伝子変異と臨床症状の解明が期待される．これまでの症例の解析では，重症度は遺伝子変異の部位に特異的ではなく，パールカン蛋白質が細胞外に存在できるかどうかによっている．変異パールカン蛋白質の細胞内での認識，排除，分解機構，細胞外への分泌機構はまだ不明であるが，コンホメーションの変化や糖鎖の変化が大きく関与していると思われる．パールカンはヘパラン硫酸プロテオグリカンとして分類されているが，組織によってコンドロイチン硫酸鎖をつける可能性もあり，またグルコサミノグリカン（CAG）鎖も組織により，あるいは発生段階により異なる修飾をうけていることが想定される．さらに興味深いことに，神経筋接合部に特異的なヘパラン硫酸鎖を認識する抗体の報告があり[23,24]，神経系のシナプスにもその機能により異なるヘパラン硫酸鎖が付加されるとすると，神経機能の複雑さを修飾する機構として糖鎖の多様性と有用性が示されていくことになる．

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ONLINE MUTATION REPORT

Very low penetrance in 85 Japanese families with facioscapulohumeral muscular dystrophy 1A

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J Med Genet 2004;41:e12 (http://www.jmedgenet.com/cgi/content/full/41/1/e12)

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common form of muscular disorder with an autosomal dominant trait, and its frequency is about one in 20 000. It is characterised by weakness and atrophy of the facial, shoulder girdle, and upper limb muscles. The pelvic girdle and lower limbs subsequently also become involved, and, eventually, 20% of patients have to use wheelchairs by the age of 40 years.[1] Most patients develop clinical symptoms in late childhood or adolescence, although the onset of the disease and its clinical severity are heterogeneous.

The FSHD locus was mapped to the subtelomeric region of the long arm of chromosome 4 by genetic linkage analysis.[2–4] More than 95% of patients with FSHD had a small (<35 kb) *Eco*RI fragment on chromosome 4q35 on southern blotting analysis with the probe p13E-11 (FSHD1A; MIM 158900).[5–8] This *Eco*RI fragment contains tandem repeats of the 3.3 kb *Kpn*I unit (D4Z4). The number of D4Z4 repeats varies from 11 to 150 in healthy people, although the number is fewer than 11 in patients with FSHD1A.[6 8] Although no responsible gene has been isolated within the FSHD region, the number of D4Z4 repeats is a critical determinant of the age of onset and clinical severity of the disease. In general, 1–3 D4Z4 repeats are associated with a severe form of the disease that presents in childhood, 4–7 repeats with the most common form of FSHD, and 8–10 repeats with a milder disease and reduced penetrance.[8–12]

Probe p13E-11 crosshybridises with chromosome 10q26, which contains highly homologous 3.3 kb *Kpn*I repeated units. As the *Bln*I restriction enzyme site exists exclusively within each unit derived from 10q26, but not in D4Z4 (a unit from 4q35), double enzyme digestion with *Eco*RI and *Bln*I can discriminate between the 4q35 (*Bln*I resistant) fragments and 10q26 (*Bln*I sensitive) fragments.[13] The highly homologous structure means that the subtelomeric interchromosomal translocation between chromosomes 4 and 10 occurs often (in about 20–30% of people) and has been suggested to contribute to deletion of *Kpn*I repeats on chromosome 4q35.[14–16] The frequency of translocation, however, was not significantly different between healthy people and those with FSHD.[17]

More complicatedly, some people have five *Eco*RI fragments in total, including one additional *Bln*I resistant fragment. These people were suggested to have somatic mosaicism of the 4q35 region and two cell populations with different fragment sizes.[10 18–21]

To clarify the frequency of the *de novo* mutation, the penetrance, and influence of the shortened repeats on clinical symptoms, we performed clinical and genetic analyses on patients with FSHD1A and both parents of each patient.

PARTICIPANTS AND METHODS

We extracted genomic DNA from peripheral blood lymphocytes with a standard technique after informed consent was obtained. We analysed 255 DNA samples, including samples from 85 Japanese patients with FSHD1A and both parents of each patient.

We used pulsed field gel electrophoresis (PFGE) and conventional gel electrophoresis to determine the size and chromosomal origin (chromosome 4 or 10) of each of the fragments. The DNA was double digested with *Eco*RI/*Hind*III and *Eco*RI/*Bln*I for PFGE and with *Eco*RI and *Eco*RI/*Bln*I for the conventional study. After we transferred the DNA to Hybond N^+ (Amersham Biosciences, Tokyo, Japan), we performed overnight hybridisation at 65°C with the probe p13E-11, as described previously.[8] We also used probe pMA13 to identify people with a deletion of the p13E-11 recognition site and with hybrid repeats that consisted of clusters of type 4 and type 10 *Kpn*I units. pMA13 is a 1.3 kb *Stu*I digested fragment within the *Kpn*I unit. We scanned the hybridised membranes and stored the image data for densitometric analysis. We estimated the intensity of each fragment with densitometry and BAS2500 (Fuji Photo Film, Tokyo, Japan). For people with somatic mosaicism, we estimated the proportion of cells with a small *Eco*RI fragment by comparing the labelled intensity with the expected intensity.

Key points

- Facioscapulohumeral muscular dystrophy (FSHD) is a common autosomal dominant muscular dystrophy.
- Most patients with FSHD have fewer numbers of tandem repeated 3.3 kb *Kpn*I units on chromosome 4q35 (FSHD1A), and southern blotting analysis with the probe p13E-11 shows a small *Eco*RI fragment (<35 kb).
- To clarify the deletion mechanism and influence of shortened repeats on clinical symptoms, we examined 85 Japanese unrelated patients with FSHD1A and both parents of each patient.
- In 35 (41%) families, only the proband had a small *Eco*RI fragment and these were suggested to be *de novo* mutations. In the remaining familial cases, somatic mosaicism of the 4q35 region was seen in 17/50 (34%) parents with a small *Eco*RI fragment. This suggests that deletion of the 4q35 region of the chromosome was generated often during mitosis and transmitted to the next generation.
- Although almost complete penetrance of FSHD is known, no clinical symptoms were seen in 26 (52%) parents who carried a small *Eco*RI fragment (including people with mosaicism) in this study.
- The high frequency of parents without the disease but with deletion of the 4q35 region implies the role of additional factors in the development of the clinical symptoms of FSHD.

We used the *BglII/BlnI* dosage test with the probe p13E-11, as reported previously, to detect the interchromosomal translocation.[22] This method characterises the first *KpnI* repeat as a *BlnI* resistant 4.0 kb (chromosome type 4q) fragment or a *BlnI* sensitive 1.8 kb (chromosome type 10q) fragment. We calculated the intensity ratio of the two bands and classified all people in accordance with the number of chromosomes with *BlnI resistant* (type 4q) *KpnI* units: nullsomy (N: two type 10q repeats on chromosome 4), monosomy (M: one type 10q repeat on chromosome 4), disomy (D: standard), trisomy (T: one type 4q repeat on chromosome 10), or quatrosomy (Q: two type 4q repeats on chromosome 10).

RESULTS

All 85 unrelated patients had clinical symptoms consistent with FSHD and a small *EcoRI fragment* of the 4q35 region <35 kb. The fragment sizes were 10–27 (mean 17.5) kb.

In 35/85 (41%) families, only the proband had a small *BlnI* resistant *Eco*RI fragment; these cases were suggested to be the result of *de novo* mutations (fig 1). Nineteen patients were men and 16 women. The size of the small *Eco*RI fragment varied from 10 to 27 (mean 15.2) kb. All but one proband had four *Eco*RI fragments (derived from both chromosomes 4 and 10). One patient had five *Eco*RI fragments that included a faint 10 kb fragment, and this was suggested to be somatic mosaicism. The *BglII/BlnI* dosage test showed that 5/29 (17%) of the *de novo* probands, including one patient with mosaicism, had one type 4 repeat on chromosome 10 (trisomy), and others had a standard disomic pattern (data not shown).

The remaining 50 patients were familial cases; one parent had a small *Eco*RI fragment of the same size as that of the proband. Twenty-three patients inherited a small *Eco*RI fragment from their father and 27 from their mother.

Surprisingly, 26 (52%) of the parents (11 fathers and 15 mothers) with a small *EcoRI* fragment had no clinical symptoms (fig 1). Overall, 17/85 (20%) of families had a parent with somatic mosaicism. The age range of parents with a small *EcoRI fragment* who were unaffected by the disease was 40–75 (mean 57.4) years at the time of examination, and the size of the short *Eco*RI fragment varied from 10 to 26 (mean 19.3) kb. The other parents who had a small *Eco*RI fragment (12 fathers and 12 mothers) had

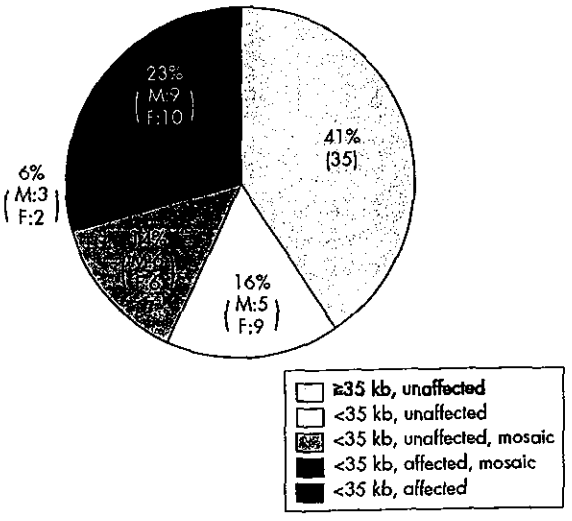

Figure 1 Results of southern blotting analysis and clinical symptoms of 85 families of patients with FSHD1A. M, male; F, female.

clinical symptoms consistent with FSHD. The age range of parents with symptoms of FSHD was 38–67 (mean 52.8) years, and the small fragment sizes ranged from 12 to 25 (mean 18.7) kb. Interestingly, no correlation was seen between the development of clinical symptoms and the size of the small *EcoRI fragment* (fig 2). One mother who had a 25 kb *Eco*RI fragment had weakness in the shoulders, but no muscle weakness was observed in one father with a 14 kb fragment.

Southern blotting analysis showed that 17/50 carrier parents with a deleted allele had five *EcoRI* fragments that included a fainter, small *Eco*RI fragment (fig 3). Double digestion with *EcoRI and BlnI showed one more BlnI* resistant fragment than was expected from the *BglII/BlnI* dosage test (data not shown). Densitometrical analysis confirmed that the radiolabelling intensity of these small *Eco*RI fragments was fainter than expected and varied from 15% to 90%. These results suggest that 17/50 (34%) of parents with a small *EcoRI* fragment had somatic mosaicism of the 4q35 region and two cell populations with different combinations of *Eco*RI fragments. Five parents with mosaicism (three fathers and two mothers) had clinical symptoms consistent with FSHD, but 12 parents with mosaicism (six fathers and six mothers) were unaffected (fig 1). The size and intensity of a small *Eco*RI fragment were variable and did not correlate with the clinical features of the disease. A man with mosaicism with a 20 kb fragment was affected, while a man with a 10 kb fragment with mosaicism was asymptomatic (fig 2). In addition, a man with a 17 kb fragment in 90% of cells was unaffected, but women with the same sized fragment in 46% and 52% of cells had clinical symptoms consistent with FSHD (data not shown).

The interchromosomal translocation between 4q35 and 10q26 was seen in 4/13 (31%) people with somatic mosaicism. Three parents (one father and two mothers) had three type 4 repeats (trisomy), and one father had monosomy of a type 4 repeat (data not shown).

DISCUSSION

Facioscapulohumeral muscular dystrophy is a dominantly inherited common muscular dystrophy with a high occurrence of new mutations.[1 10 11 23 24] Most patients with FSHD had a deletion of tandem repeated 3.3 kb *KpnI units* on chromosome 4q35, but the deletion mechanism of the repeat units is not known. Genetic analysis of 85 unrelated Japanese patients with FSHD1A and both parents of each patient by southern blotting analysis found that 41% of cases were the result of *de novo* mutations. A high proportion of *de novo* mutations may be caused by the specific structure of the region associated with FSHD on chromosome 4.

A possible consideration with respect to the shortened repeats is interchromosomal translocation between the subtelomeric region of chromosomes 4 and 10, which is observed in 20–30% of the healthy population.[14 17] Frequently observed recombination implies a role for deletion of the 4q35 region; however, the ratio of translocation is similar between healthy people and patients with the disease.[17] The translocation ratio in the 35 patients with *de novo* mutations in our study was not significantly different from that reported previously in healthy people.[17] Further studies are needed to elucidate the exact role of interchromosomal translocation for the deletion of repeated units on 4q35.

Another possible cause is somatic mosaicism. Somatic mosaicism of the 4q35 region was seen in 15–20% of the healthy parents of patients with FSHD.[1 20 23–25] Van der Maarel *et al* reported that the patient or an asymptomatic parent had somatic mosaicism in 40% of families with *de novo* cases of FSHD.[23] On the other hand, only 3% of random blood donors have somatic mosaicism.[16] In our study, 17/50 (34%) parents

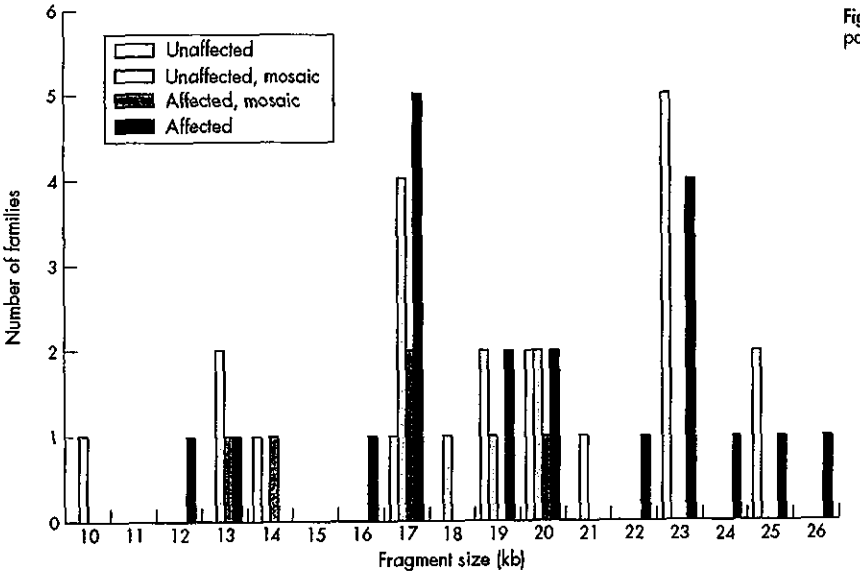

Figure 2 *EcoRI* fragment size of parents with a deleted allele.

with a deleted allele had somatic mosaicism of the 4q35 region, and 17/85 (20%) of patients with FSHD1A inherited a small *EcoRI* fragment from parents with mosaicism. These results suggest that somatic mosaicism is one of the major factors in the development of FSHD1A. A previous study showed that 46% of people with mosaicism had one or more *BlnI* resistant units on chromosome 10; that is nearly fivefold more frequent than in healthy people.[21] In the present study, however, 3/13 (23%) of parents with mosaicism had type 4q *BlnI* resistant units on chromosome 10; this proportion was similar to that in healthy people.[17]

In the people with mosaicism, the size and intensity of a small *EcoRI* fragment were variable and did not correlate with the clinical features of the disease. This result may not be surprising, however, because somatic mosaicism occurs in the early embryonic stage, and the percentage of cells with

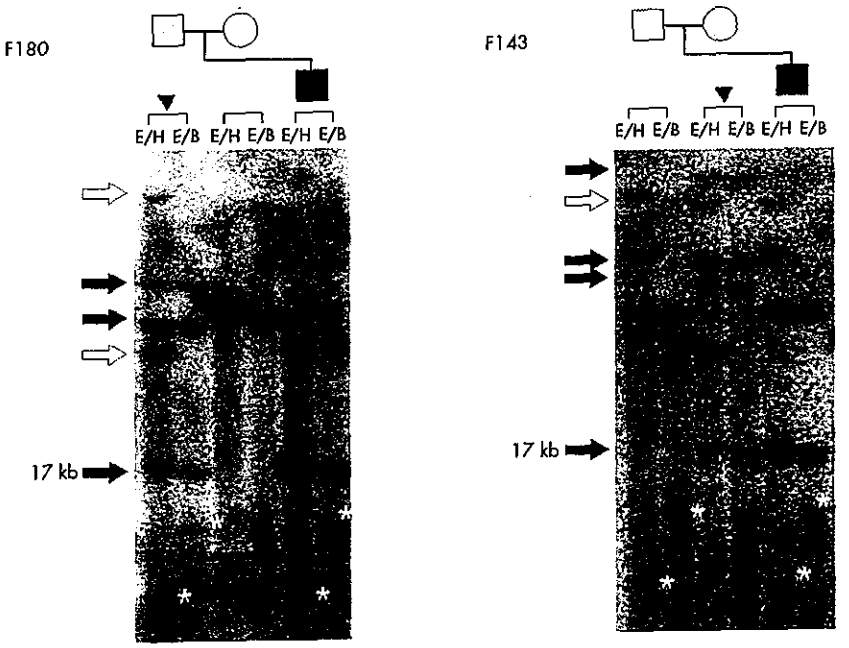

Figure 3 Southern blotting analysis of two families with a proband with FSHD1A and a parent with somatic mosaicism. DNA was digested by *EcoRI/HindIII* (E/H) and *EcoRI/BlnI* (E/B) and separated by PFGE. The father in F180 (arrowhead) had three *BlnI* resistant *EcoRI* fragments (black arrows) that included a faint small (17 kb) *EcoRI* fragment. The affected son inherited the small fragment from his father. Fragments derived from chromosome 10 are shown with white arrows. The mother in F143 (arrowhead) had four *BlnI* resistant *EcoRI* fragments (black arrows) that included a faint 17 kb fragment and one *BlnI* sensitive *EcoRI* fragment (white arrow). This mother was confirmed to have one type 4 repeat on chromosome 10 (trisomy) by the *BglII/BlnI* dosage test. The affected son carried this 17 kb fragment. Alleles from chromosome Y are marked with asterisks.

deleted alleles will vary between tissues. In our study, no significant difference was seen between the sexes in the clinical symptoms in parents with mosaicism, although asymptomatic female carriers of mosaicism have been reported as predominant.[10 21 25]

When we consider the penetrance of FSHD, our findings are important. Despite the clinical heterogeneity, even in the same family, patients with FSHD usually become symptomatic in the second decade of life. Penetrance increases in an age dependent manner and has been estimated to be <5% for children aged 0–4 years, 21% for those aged 5–9 years, 58% for those aged 10–14 years, 86% for those aged 15–19 years, and 95% for those aged ≥20 years.[26] Another study showed that non-penetrance at the age of 60 years has been estimated as 2–5%.[27] From these studies, FSHD was suggested to be a highly penetrant disease. Our retrospective study, however, showed that penetrance was low, being estimated at 59% (excluding somatic mosaicism) and 48% (including somatic mosaicism). The number of mothers without mosaicism who were unaffected by the disease was higher than the number of fathers; however, no significant differences between sexes were seen in the numbers of affected parents and of parents with mosaicism. The size of small *Eco*RI fragments of unaffected parents was variable; the smallest fragment was 14 kb. This fragment was estimated to contain only two *Kpn*I repeated units (D4Z4), which generally causes severe phenotypes from childhood. Further clinical follow up studies of parents with a small *Eco*RI fragment who are unaffected by FSHD are needed. Penetrance, however, seems to be lower than previously reported. Even in random blood donors, 3–6% of people have FSHD sized type 4 repeat arrays.[8 16 28] The existence of asymptomatic people with a small *Eco*RI fragment strongly suggests the involvement of additional unknown factors in the development of clinical symptoms. Position effect variegation, which induces allele specific transcriptional repression of genes located centromerically, has been proposed as the molecular mechanism of FSHD. Recently, unexpected gene expression that was related inversely to the number of repeat units was reported in the muscles of patients with FSHD.[29] In addition, one of the two variants of the 4q subtelomere was reported to be associated uniquely with patients with FSHD.[30] Additional studies are needed to clarify the molecular pathomechanism of this complicated disease.

ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid for Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labour and Welfare and Ichiro Kanehara Memorial Foundation, Japan.

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Received 29 March 2003
Accepted 16 June 2003

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Sarcolemma-specific collagen VI deficiency in Ullrich disease

Mutations in collagen VI are a cause of the severe congenital muscular dystrophy, Ullrich disease. Ishikawa et al. report eight patients with Ullrich disease with a different abnormality: collagen was present in the interstitium but absent from the sarcolemma. They suggest that the failure of collagen VI to anchor the basal lamina to the interstitium can cause Ullrich disease.

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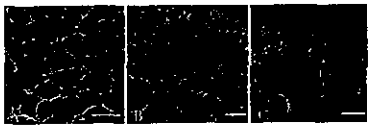
Collagen VI expression and muscle weakness

Commentary by Carolyn Sewry, MD

There is growing interest in the role of the extracellular matrix in neuromuscular disorders. Protein complexes associated with dystrophin connect the cytoskeleton of the muscle fiber to the extracellular matrix via transmembrane proteins. The precise function(s) of the complexes have not been fully elucidated but current hypotheses favor membrane stability and signaling as the most probable. What is clear, however, is that defects in several of the components can lead to malfunctioning of the muscle fiber, and can result in primary and secondary alterations in protein expression, giving rise to specific neuromuscular phenotypes. Collagen VI is unusual in that recessive mutations usually give rise to Ullrich disease, a form of congenital muscular dystrophy (UCMD), but dominant mutations lead to a milder disorder, Bethlem myopathy. UCMD



Immunostaining for collagen VI (A: normal, B: Fukuyama-type congenital muscular dystrophy, and C: Ullrich disease). Collagen VI is present in the interstitium but is markedly reduced or absent in the sarcolemma in an Ullrich disease patient.

is a severe disorder with onset at birth or in early infancy and characterized by hypotonia, contractures of proximal joints, and distal joint laxity. Affected UCMD patients often are unable to walk and develop respiratory failure. Muscle weakness in Bethlem myopathy, however, is milder and only slowly progressive. The article by Ishikawa et al. highlights the general role of immunohistochemistry in identifying alterations in protein expression in recessively inherited conditions, especially subtle ones, and emphasizes the importance of assessing the basal lamina. It also illustrates the fact that ultrastructural studies can provide new information when combined with the molecular genetic characterization of disease. In addition, their results lend further support to molecular heterogeneity associated with the Ullrich phenotype. It is, however, surprising that mutations in collagen VI were found in only one of their eight patients, suggesting either that a molecular deficit was overlooked or that other genes are more commonly mutated. However, two potential candidates, biglycan and decorin, were excluded.

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Ullrich disease due to deficiency of collagen VI in the sarcolemma

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Abstract—The authors identified eight patients with Ullrich disease in whom collagen VI was present in the interstitium but was absent from the sarcolemma. By electron microscopy, collagen VI in the interstitium was never linked to the basal lamina. These findings suggest that in these patients it is not the total absence of collagen VI from the muscle but the failure of collagen VI to anchor the basal lamina to the interstitium that is the cause of Ullrich disease. Only one of the patients had a mutation in the collagen VI gene, suggesting that the primary abnormality in most of the patients involved some other molecules.

NEUROLOGY 2004;62:620–623

Ullrich disease is an autosomal recessive disorder characterized clinically from birth or early infancy by congenital muscular dystrophy, with contractures of the proximal joints and hyperextensibility of the distal joints, high-arched palate, and protuberant calcanei with normal intelligence.[1] Recently, complete loss or reduction of collagen VI due to collagen VI gene mutations has been associated with Ullrich disease.[2-6] Collagen VI is thought to play a role in connecting the basal lamina to the interstitium and a defect in this function is implicated in Ullrich disease.[5,7] We report eight patients with Ullrich disease in whom collagen VI was present in the interstitium but was specifically absent in the sarcolemma, bolstering the hypothesis that Ullrich disease is due to the loss of mechanical anchoring of the basal lamina to the interstitium.

Materials and methods. *Patients.* We studied eight Japanese patients with the diagnosis of Ullrich disease based on typical clinical features, i.e., delayed motor milestones, hyperextensibility of distal joints, and contractures of proximal joints. All were sporadic cases. Serum creatine kinase (CK) was mildly elevated in two-thirds of the patients (table). Biceps brachii muscle was biopsied in all patients.

Histochemical and immunohistochemical analysis. Muscle biopsy samples were frozen in liquid nitrogen-cooled isopentane for histochemistry and immunohistochemistry. Eight-micrometer-thick transverse serial sections were stained with hematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical techniques. We also immunostained biopsy sections with monoclonal antibodies against collagen VI (1:500) (ICN Biomedicals, Aurora, OH), fibronectin (1:200) (CHEMICON, Temecula, CA), integrin α7,[8] and α-dystroglycan (1:100) (Upstate, Lake Placid, NY), and the polyclonal antibody against collagen IV (1:2000) (Advance, Tokyo, Japan). We visualized the monoclonal antibodies by avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA) using biotinylated goat anti-mouse IgG (Vector) and 3,3'-diaminobenzidine except for double immunostaining for collagen IV and VI, for which we used two secondary antibodies—FITC-labeled anti-mouse immunoglobulin G (IgG) (Leinco Technology, St. Louis, MO) and rhodamine-labeled anti-rabbit IgG (Leinco)—and the sections were examined by fluorescence microscopy.

Sequence analyses. Total RNA was extracted from frozen muscle using Totally RNA Kit (Nippon gene, Tokyo, Japan) and was reverse transcribed into cDNA with oligo $(dT)_{20}$ primer using the ThermoScript RT-PCR System (Life Technologies, Carlsbad, CA).

In COL6A1 and *COL6A2*, we amplified two overlapping fragments, encompassing nt 35 through 1299 and nt 1280 through 3133 (NM001848) (all nucleotide numbers are based on the open reading frame [ORF] in the cDNA sequence indicated by each accession number), and nt 21 through 1390 and nt 1292 through 3136 (NM001849), respectively, which cover the entire ORF. In *COL6A3*, we amplified three overlapping fragments, encompassing nt 64 through 3406, nt 3323 through 6347, and nt 6257 through 9610 (NM400369). We directly sequenced the amplified fragments with the PCR primers[9] and relevant internal primers using BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster, CA), and electrophoresed the samples using ABI PRISM 377 and 3100 DNA sequencer (PE Biosystems). We also sequenced the amplified *COL6A2* genomic fragments in lymphocyte DNA

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Received May 19, 2003. Accepted in final form November 3, 2003.

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Table *Clinical summary of the patients*

Characteristics	Patient							
	1	2	3	4	5*	6	7	8
Sex	M	F	F	M	M	F	M	M
Age at muscle biopsy	11 y	2 y 3 m	2 y	3 y 3 m	3 y	1 y	4 y	10 y
Consanguinity	–	–	–	–	–	–	–	–
Condition at birth								
Hypotonia	+	+	+	NA	–	+	+	+
Contracture of proximal joints	+	+	+	NA	–	+	+	+
Development								
Head control, m	6	5	4	3	4	3	4	6
Sitting alone	2 y	1 y	1 y 6 m	NA	9–10 m	1 y	NA	3 y
Ambulation	NW	2 y 2 m	NW	1 y 4 m	1 y 6 m	NW	1 y 10 m	2 y 10 m
Present status								
Contracture of proximal joints	+	+	+	+	+	+	+	+
Loose distal joints	+	+	+	+	+	+	+	+
Absent tendon jerks	+	+	+	+	+	+		+
High-arched palate	+	+	+	NA	+	–	–	+
Mental retardation	–	–	–	–	–	–	–	–
Scoliosis	++	++	++	NA	+	+	++	+
Torticollis	–	+	–	–	+	–	+	+
Protrusion of calcaneus bone	++	+	+	NA	NA	NA	+	–
EMG	Myogenic	Myogenic	NE	Myogenic	NE	NE	N	N
CK (IU/L)	NA	164–342	290	242	113	232	270–400	129

* The patient who had a compound heterozygous mutation in *COL6A2* gene.

NA = detailed information not available; NW = never walked; NE = not examined; N = normal; CK = creatine kinase.

from Patient 5 encompassing intron 22 through 23 and intron 26 through exon 27.

With biglycan and decorin, we amplified each exon and flanking sequences by PCR in DNA from the patients and directly sequenced the amplified fragments (primer information available upon request).

Electron microscopy. For electron microscopy, a portion of the muscle biopsy was fixed in 2% glutaraldehyde and postfixed in osmium tetroxide, dehydrated in graded alcohol series, and then embedded in Epon (Taab Laboratories Equipment Ltd., Aldermaston, UK). Ultrathin sections were stained with uranyl acetate and lead citrate.

Results. *Histochemical and immunohistochemical analyses.* All the biopsies showed variation in muscle fiber size, increased endomysial connective tissue, and regenerating fibers. There were necrotic fibers in six biopsies (figure).

By immunohistochemistry, collagen VI was present in the interstitium and sarcolemma in normal controls and in muscle samples from patients with other forms of congenital muscular dystrophy (CMD) (see the figure, A and B). In our patients, however, collagen VI was markedly reduced or absent in the sarcolemma while it was present in the interstitium (see the figure, C). We confirmed the specific absence of collagen VI from the sarcolemma by double immunostaining for collagen VI (see the figure, A through C) and collagen IV (see the figure, D through F), a major component of basal lamina. In Fukuyama-type CMD (FCMD) and non-Fukuyama-type CMD (nonFCMD) muscles, collagen VI and collagen IV were colocalized in the sarcolemma (see the figure, G and H). By contrast, in patients with Ullrich disease, only collagen IV, but not collagen VI, was present in the sarcolemma although collagen VI was present in the interstitium (see the figure, I).

On immunostaining for proteins interacting with collagen VI, integrin α7, α-dystroglycan, and fibronectin, all were present in the sarcolemma, as in controls (data not shown).

Sequence analyses. None of our patients had mutations in genes encoding collagen VI subunits, biglycan, and decorin, except that Patient 5 had a compound heterozygous mutation in *COL6A2* gene. On direct sequencing of the RT-PCR products from Patient 5, there were skipping of entire exon 23 and a six-bp deletion in exon 26, both in a heterozygous manner. In the genomic DNA, we found a heterozygous G-to-A substitution at position +5 in intron 23 and the corresponding heterozygous six-bp deletion in exon 26. The latter mutation deleted one of the two tandem repeats of the sequence CATCGG in nt 2268–2273 and 2274–2279 in *COL6A2* ORF, which is predicted to delete isoleucine and glycine, at residues 759 and 760 (or 757 and 758) (data not shown). We also sequenced DNA from the parents of Patient 5 and found the former mutation in the mother and the latter in the father, both in heterozygous mode. Both mutations were absent in 100 normal chromosomes.

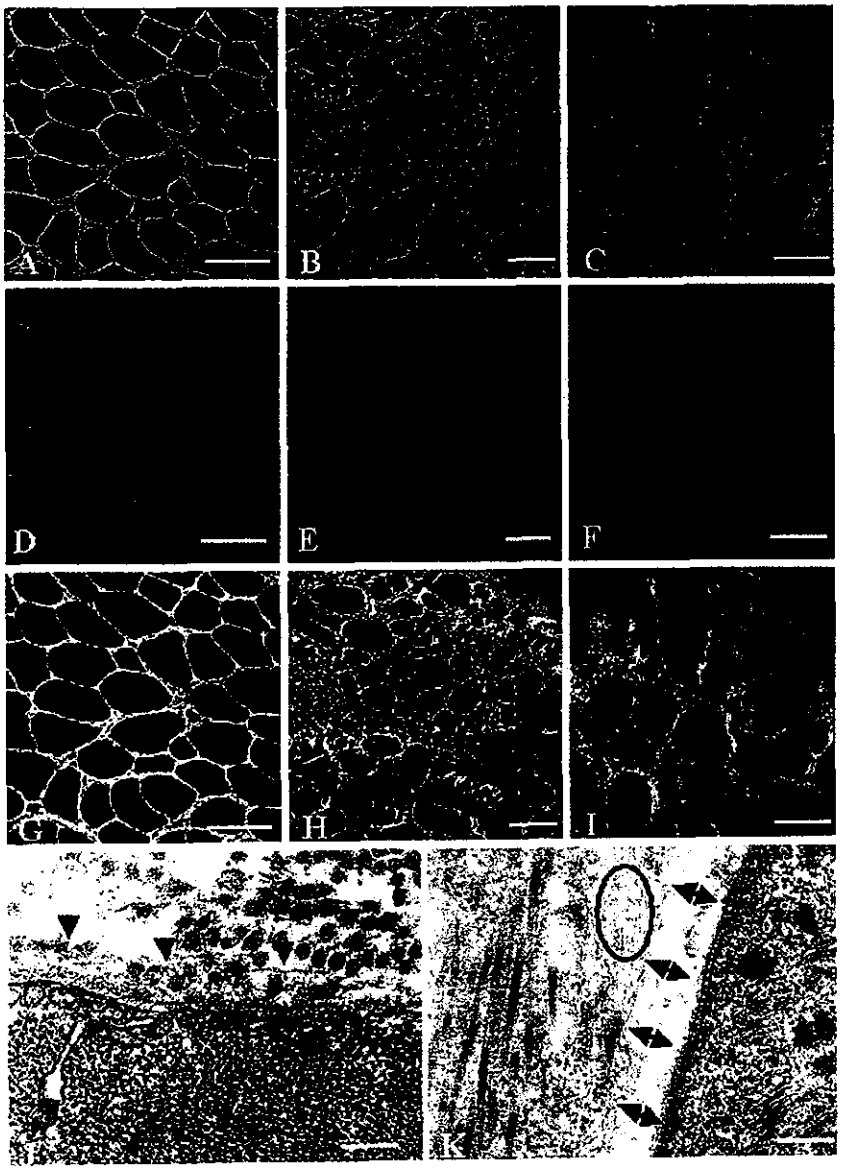

Figure. Pathologic features of the disease. (A–C) Immunostaining for collagen VI (A: normal, B: Fukuyama-type CMD [FCMD], and C: Ullrich disease). Collagen VI is present in the interstitium but is markedly reduced or absent in the sarcolemma in an Ullrich disease patient. (D–F) Immunostaining for collagen IV (D: normal, E: FCMD, and F: Ullrich disease). Collagen IV is present in the sarcolemma. (G–I) Superimposed images (G: nonFCMD, H: FCMD, and I: Ullrich disease). Both collagen VI and collagen IV are present in sarcolemma in other congenital muscular dystrophies, as indicated by yellow (G and H). In contrast, only red is seen in the sarcolemma in Ullrich disease although interstitium is stained green (I). (J and K) Electron micrographs (J: FCMD and K: Ullrich disease). Microfibrils usually link to the basal lamina, as exemplified in FCMD muscle (arrowheads) (J). In contrast, in Ullrich disease, the basal lamina is intact and microfibrils are present in the interstitium (encircled), but is never associated with the basal lamina (arrowheads) (K). (Bar = 50 μm in A-I; 1 μm in J and K.)

Electron microscopy. The basal lamina was intact even in degenerating muscle fibers (see the figure, J). Collagen fibrils in the interstitium appeared normal with a periodic pattern of about 65 nm intervals. Microfibrils, which are known to be collagen VI, were present in the interstitium, but they were never linked to the basal lamina by electron microscopy (see the figure, K).

Discussion. Three types of collagen VI immunostaining pattern have been reported in Ullrich disease: normal, complete absence, and generalized reduction (partial deficiency).[3-6] The eight patients in this study had a new mode of collagen VI involvement, its almost complete absence specifically from the sarcolemma, but not from the interstitium.

Among our eight patients, only one had a compound heterozygous mutation in the *COL6A2* gene, but even these mutations may not be pathogenic because they are in-frame, although they were absent in 100 normal chromosomes in our study. Alternatively, mutations may exist in the noncoding regions, which we did not sequence. Collagen VI is thought to anchor the basement membrane in skeletal muscle by interacting with collagen IV, a major component of the basal lamina.[7] However, by electron microscopy, there was no connection between collagen VI microfibrils in the interstitium with the basal lamina even though both the basal lamina and collagen fibrils were morphologically intact. These findings suggest that not only the absence of collagen VI from skeletal muscle but also the absence of collagen VI from the sarcolemma alone, both of which result in the loss of anchoring between the basal lamina and the interstitium, can cause Ullrich disease. Thus, our findings indicate genetic heterogeneity in Ullrich disease with collagen VI abnormality.

Proteins interacting with collagen VI are natural

candidates to be the molecule primarily responsible in our patients. Indeed, mice deficient in the sarcolemmal protein, biglycan, which is also thought to bind to collagen VI, were reported to show a reduction in collagen VI, especially in the sarcolemma.[10] We, therefore, investigated the proteins that potentially bind to collagen VI, including biglycan, decorin, integrin α7, α-dystroglycan, and fibronectin. However, integrin α7, α-dystroglycan, and fibronectin were all present by immunohistochemistry. Furthermore, no mutations were found in biglycan and decorin genes, suggesting that these proteins are unlikely to be involved. Nevertheless, there still remains the possibility that other proteins that interact in a similar fashion with collagen VI could be responsible for the disease in our patients and further studies are necessary to identify the primary cause.

Acknowledgment

The authors thank the patients and their families for their cooperation and support of this research and F. Uematsu, K. Tatezawa, and K. Murayama for technical assistance.

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POMT1 mutation results in defective glycosylation and loss of laminin-binding activity in α-DG

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Abstract—Walker–Warburg syndrome (WWS) is a congenital muscular dystrophy associated with neuronal migration disorder and structural eye abnormalities. The mutations in the *O*-mannosyltransferase 1 gene (*POMT1*) were identified recently in 20% of patients with WWS. The authors report on a patient with WWS and a novel *POMT1* mutation. Their patient expressed α-dystroglycan (α-DG) core protein, but fully glycosylated α-DG antibody epitopes were absent, associated with the loss of laminin-binding activity.

NEUROLOGY 2004;62:1009–1011

Walker–Warburg syndrome (WWS; MIM 236670), Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800), and muscle-eye-brain disease (MEB; MIM 253280) are closely related congenital muscular dystrophies (CMDs) with cobblestone lissencephaly and eye abnormalities. Although they are known to be caused by the mutations of different genes encoding putative glycosyltransferases,[1] it now is clear that the mutations of each gene produce overlapping clinical phenotypes.[2,3] In addition, they share a similar pattern of selective loss of α-dystroglycan (α-DG) on immunohistochemical study.[1] A recent study showed hypoglycosylation of α-DG and loss of binding activity of α-DG to laminin, neurexin, and agrin in FCMD, MEB, and the mutant myodystrophy (Large^{myd}) mouse, suggesting a defect in the same post-translational modification pathway of glycosylation in α-DG.[4]

Mutations in the *O*-mannosyltransferase 1 gene (*POMT1*) were implicated recently in 20% of patients with WWS.[5] The laminin-binding site in α-DG is thought to reside in *O*-mannosyl-linked carbohydrate side chains, which may require POMT1 for synthesis.[6]

We report our experience with a Japanese boy with WWS and a novel *POMT1* mutation, who showed reduced glycosylation and loss of laminin-binding activity of α-DG in skeletal muscle.

Additional material related to this article can be found on the Neurology Web site. Go to www.neurology.org and scroll down the Table of Contents for the March 23 issue to find the title link for this article.

Methods. *Patient.* The patient was a Japanese boy aged 3.5 years from apparently nonconsanguineous parents. No other family member was affected. Prenatal ultrasonography showed that the patient had a meningoencephalocele. He was born at gestational week 38 by Cesarean section with a body weight of 2,042 g. He was floppy with an enlarged head. He underwent surgery to remove a meningoencephalocele, and a ventriculoperitoneal shunt was added 21 days after birth. Mild microphthalmia and corneal clouding also were observed. Serum creatine kinase levels were markedly elevated to 600 to 31,000 IU/L (upper normal limit, 70 IU/L). He exhibited markedly delayed milestones. He could not control his head, roll over, or sit. He showed lack of facial expression with an inability to smile and never developed the ability to speak. Brain MRI revealed agyric frontal and temporo-occipital lobes mixed with pachygyric parietal cortex. Hypoplasia of brain stem and cerebellum also was observed (figure 1). EEG showed multifocal spikes, and the muscle biopsy showed marked increase in fatty tissue with evidence of necrosis and regeneration. The mutational analysis for fukutin and protein *O*-mannose β-1,2-N-acetylglucosaminyl-transferase gene (*POMGnT1*) did not show any abnormalities.

Immunohistochemistry and immunoblotting studies. The following antibodies were used: monoclonal anti-α-DG (VIA4-1, Upstate Biotechnology, Lake Placid, NY), polyclonal goat anti-α-DG (GT20ADG),[4] monoclonal anti-β-DG (43DAG1/8D5, Novocastra Laboratories, Newcastle upon Tyne, UK), monoclonal anti-laminin-α2 chain (5H2, Chemicon, Temecula, CA), monoclonal antidystrophin C-terminal (Dy8/6C5, Novocastra Laboratories), and monoclonal antisarcoglycan antibodies (Novocastra Laboratories). The detailed techniques of the immunohistochemistry, immunoblotting, and laminin overlay assays have been described previously.[4,7]

Mutation analysis. Genomic DNA was extracted from frozen muscle tissue using standard method with informed consent. Primer

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Supported by Grants-in-Aid for Research on Psychiatric and Neurologic Diseases and Mental Health from the Ministry of Health, Labor, and Welfare and the Ichiro Kanehara Memorial Foundation, Japan. K.P.C. is an investigator for the Howard Hughes Medical Institute.

Received May 27, 2003. Accepted in final form November 10, 2003.

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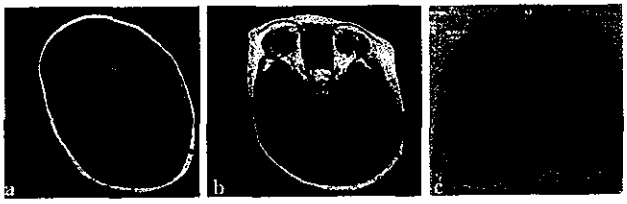

Figure 1. Brain MRI of patient at age 3 years shows agyric frontal and temporo-occipital lobes mixed with pachygyric parietal cortex, hypoplasia of brain stem and cerebellum, and defect of septum pellucidum. The periventricular white matter change (A and B, TR540/TE15; C, TR5400/TE90) also is seen.

pairs were designed to amplify all coding exons and flanking intronic sequences of *POMT1*. The amplified products were sequenced using an ABI PRISM 3100 (Applied Biosystems, Foster City, CA). For the detection and screening of L421del (1260 to 1262 delCCT) in exon 13 of *POMT1*, primers F-CAGTAGCAGCAACTCATGGG, R-ACGGTTGTGGCTGCTATAGC, and restriction enzyme *AvaI* were used. One hundred healthy Japanese individuals served as control subjects.

Results. *Immunohistochemical and immunoblotting analyses.* The immunohistochemical analysis revealed an almost complete loss of immunoreactivity with VIA4-1 anti-α-DG antibody in the patient, whereas anti-α-DG core protein GT20ADG showed membrane staining in each muscle fiber (figure 2). Immunoreaction against the laminin-α2 chain was reduced slightly, but β-DG (see figure 2), dystrophin, and sarcoglycans (not shown) were well preserved.

Immunoblotting analysis using GT20ADG showed a band with a reduced molecular mass, whereas VIA4-1 showed no detectable band for α-DG in the patient (figure 3, A and B). The molecular weight shift observed in our patient (>60 kDa) was almost identical to those reported in FCMD and MEB.[4] On laminin overlay assay, the patient's muscle showed an almost complete loss of laminin-binding activity of α-DG (figure 3C).

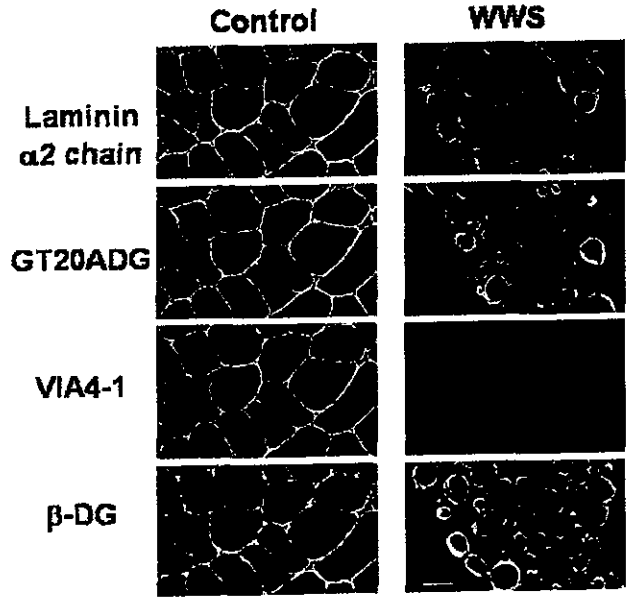

Figure 2. In the patient (with Walker–Warburg syndrome [WWS]), a complete loss of immunoreactivity is observed with the monoclonal antibody VIA4-1 against α-dystroglycan (α-DG), whereas it appears normal around muscle fibers when the polyclonal antibody GT20ADG against α-DG was used. β-DG is well preserved, but the laminin-α2 chain shows mild reduction; bar = 20 μm.

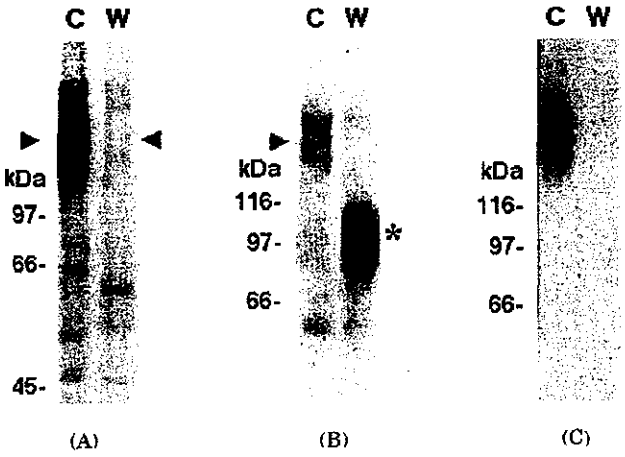

Figure 3. (A) The immunoblotting study using the antibody VIA4-1 showed a broad band around 156 kDa (arrowheads) in control skeletal muscle (C) that is undetectable in the patient (W). (B) The immunoblotting study using the antibody GT20ADG showed a band with a reduced molecular mass (≅90 kDa, asterisk) in the patient, whereas the normal band of α-dystroglycan (α-DG) at 156 kDa was detected in the control. (C) The laminin overlay assay showed loss of band in the patient, suggesting there is an almost complete loss of laminin-binding activity in α-DG from the patient's muscle. M = molecular mass.

Mutation analysis. We found a homozygous deletion of three base pairs (1260 to 1262 delCCT) in *POMT1*, which is expected to delete single amino acid leucine at position 421 (see figure E-1A on the *Neurology* Web site). No identical mutation was present in 100 normal Japanese control subjects (see figure E-1B on the *Neurology* Web site). The amino acid sequence alignment showed that the deleted amino acid leucine and surrounding primary sequence are highly conserved among different species (see figure E-1C on the *Neurology* Web site).

Discussion. In this study, we identified a deletion of the single amino acid leucine at position 421 of *POMT1* from the patient's DNA. This is considered to be a causative mutation for several reasons. First, the same change was not found among 100 Japanese control subjects. Next, the deleted amino acid leucine is located within a highly conserved region of the gene and is conserved among different species. A previously reported V428D mutation also is only seven amino acids downstream to ours.[5] These findings suggest that this conserved region plays an important role in the proper function of the protein.

Our patient showed exceptionally long survival for WWS because most patients with WWS die during infancy and rarely survive beyond age 3

years. Because complete agyria is common in patients with WWS, the pattern of the cortical dysplasia in our patient—agyria mixed with parietal pachygyria in MRI—could be considered milder than typical WWS. Thus, our patient showed intermediate phenotype between WWS and MEB in terms of clinical severity and MRI finding. However, the diagnosis of WWS seems more accurate than MEB or FCMD in our patient because he had a meningoencephalocele, which is almost exclusively seen in WWS.[8] There are some recent reports documenting the remarkable clinical variability originating from the mutation of the same genes causing CMDs, and thus, it is possible for *POMT1* mutations to produce a more benign WWS phenotype like that seen in our patient.[2,3,9]

Although the immunoreactivity against the antibody VIA4-1 was lost completely in our patient, the reaction against the antibody GT20ADG was well preserved. Because the antibody GT20ADG recognizes the core protein of α-DG, our results indicate that α-DG localizes at the surface membrane of skeletal muscle but that the epitope for VIA4-1 antibody was specifically disrupted or masked.[4] Because the antibody VIA4-1 is thought to recognize, at least in part, the carbohydrate epitope of α-DG, the glycosylation status of α-DG is likely to be altered in our patient.[4] The results of immunoblotting and laminin overlay assays further support this speculation. The α-DG from normal skeletal muscle is a heavily glycosylated protein with a molecular weight of 156 kDa. Thus, the reduction of molecular weight, seen only by GT20ADG, is likely to be related to the loss of glycoconjugates from α-DG. Accordingly, the loss of laminin-binding activity shown in the laminin overlay assay most likely is caused by the loss of glycoconjugate, which is thought to be a laminin-binding ligand of α-DG.[6] A brain-selective deletion of dystroglycan in mice was shown recently to cause CMD-like brain malformations and defective laminin binding, giving strong evidence that abnormalities of dystroglycan underlie the neuronal migration disorder seen in this group of disorders.[10] Because similar pattern of glycosylation-deficient disruption of dystroglycan function has been observed in FCMD, MEB, and Large^{myd} mice,[4] it is likely that WWS shares a similar pathomechanism with them. In addition, the complete loss of the laminin-binding activity of α-DG in our patient with WWS is almost identical to that observed in FCMD.[4]

Our study proves that WWS caused by the mutation of *POMT1* coexists with other types of CMDs in the Japanese population. We also demonstrated that WWS is a member of the group of CMDs associated with defective glycosylation of α-DG that results in the loss of function of α-DG as a matrix receptor.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 279, No. 12, Issue of March 19, pp. 11402–11407, 2004
Printed in U.S.A.

Reduction of UDP-*N*-acetylglucosamine 2-Epimerase/ *N*-Acetylmannosamine Kinase Activity and Sialylation in Distal Myopathy with Rimmed Vacuoles*

Received for publication, December 3, 2003, and in revised form, December 30, 2003
Published, JBC Papers in Press, January 5, 2004, DOI 10.1074/jbc.M313171200

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Distal myopathy with rimmed vacuoles is an autosomal recessive muscle disease with preferential involvement of the tibialis anterior that spares the quadriceps muscles in young adulthood. In a Japanese patient with distal myopathy with rimmed vacuoles, we identified pathogenic mutations in the gene encoding the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase, which catalyzes the initial two steps in the biosynthesis of sialic acid. In this study, we demonstrated the relationship between the genetic mutations and enzymatic activities using an *in vitro* expression assay system. Furthermore, we also showed that the levels of sialic acid in muscle and primary cultured cells from DMRV patients were reduced to 60–75% of control. The reactivities to lectins were also variable in some myofibers, suggesting that hyposialylation and abnormal glycosylation in muscles may contribute to the focal accumulations of autophagic vacuoles, amyloid deposits, or both in patient muscle tissue. The addition of ManNAc and NeuAc to primary cultured cells normalized sialylation levels, thus demonstrating the therapeutic potential of these compounds for this disease.

Distal myopathy with rimmed vacuoles (DMRV)[1] is an autosomal recessive disorder characterized clinically by the preferential involvement of the tibialis anterior muscle, sparing the quadriceps muscles as originally described in 1981 (1, 2). The age at onset is relatively late with a mean of 26 years. Muscle biopsy of this disorder is characterized by many rimmed vacuoles, which are particularly abundant in atrophic fibers. Necrotic and regenerating fibers are rarely seen (3). The nucleus occasionally contains tubulofilamentous inclusions of 15–20 nm in diameter.

Hereditary inclusion body myopathy (HIBM) is an autosomal recessive disorder that presents with adult-onset slowly progressive distal and proximal weakness and has characteristic pathological features in muscle tissue, including rimmed vacuoles and filamentous inclusions, that are similar to those seen in DMRV (4). Gene loci of both diseases have been mapped to chromosome 9 (5, 6). HIBM is caused by mutations in the UDP-GlcNAc 2-epimerase/ManNAc kinase gene (*GNE* gene) (7). Previously we identified homozygous and compound heterozygous mutations in the *GNE* gene in 27 DMRV patients (8), demonstrating that the two diseases are allelic.

UDP-GlcNAc 2-epimerase/ManNAc kinase is a dual functional enzyme catalyzing two initial steps in the biosynthesis of sialic acid (9). This enzyme catalyzes the conversions of UDP-GlcNAc to ManNAc and ManNAc to ManNAc 6-phosphate. Despite the identification of the *GNE* gene mutations, we still do not fully understand how these mutations contribute to the pathophysiology in DMRV/HIBM. Several questions remain unanswered. 1) What is the status of sialylation activity in the patients with *GNE* mutations? One would expect sialylation to be impaired but not completely absent in DMRV/HIBM patients, because sialic acid is essential for embryonic development (10). In fact, homozygous null mutations have never been identified in patients (8, 11). 2) Why are symptoms restricted to the skeletal muscles? *GNE* transcripts are expressed in various tissues and are especially predominant in the liver (12). 3) Why do mutant proteins not complement each other in patients who have heterozygous mutations in each of the two domains? The two domains in GNE protein have been reported to catalyze the enzymatic reactions separately and independently (13). To address these questions, we studied the relationships between mutations and enzymatic activity using *in vitro* expression and enzymatic assay systems. We also determined the levels of sialylation in sera, muscles, and primary cultured cells from DMRV patients and normal individuals.

EXPERIMENTAL PROCEDURES

Mutation and Sialic Acid Analyses of Patients—All of the patients were Japanese. The patients were diagnosed as having DMRV based on both clinical features and muscle pathology. Numbering of the patients followed the protocol presented in our previous report (8). Gene analyses of DMRV patients were performed as described previously (8). Primary fibroblasts were obtained from patient 18 and patient 19, whose mutations were reported previously. Primary skeletal myocytes were obtained from patient 5 as reported previously. Sialic acid contents in sera were measured with a SIALIZYME-550 kit (Fujirevio, Tokyo, Japan), and those in muscle and cells were determined by the

* This study was supported by a grant for research on psychiatric and neurological diseases and mental health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: DMRV, distal myopathy with rimmed vacuoles; HIBM, hereditary inclusion body myopathy; MAM, *Maackia amurensis* lectin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimido ester; SSA, *Sambucus sieboldiana* agglutinin; SBA, soybean agglutinin; GNE, UDP-GlcNAc 2-epimerase/ManNAc kinase; WGA, wheat germ agglutinin.

thiobarbituric acid method. Informed consents were obtained from all subjects using a form approved by the Ethical Review Board at the National Center of Neurology and Psychiatry (Tokyo, Japan).

Expression of Recombinant GNE Proteins—The cDNA for wild-type *GNE* was obtained by reverse transcribed-PCR from normal muscle RNA and cloned into pCR-blunt vector (Invitrogen). The cDNAs for *GNE* mutants were obtained by reverse transcribed-PCR from skeletal muscle RNA of DMRV patients or by site-directed mutagenesis from wild-type cDNA. All cloned muscle cDNAs were sequenced by ABI cycle-sequencing procedures using an ABI 3100 (Applied Biosystems, Foster City, CA). The sequenced and inserted cDNAs were cut out with EcoRI and blunted, and the purified cDNA fragments were inserted in-frame into the expression vector, pCMV-Myc (Invitrogen). The expression constructs were transiently transfected into COS-7 cells using LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol. After 24 h, the Myc-tagged wild-type GNE and the mutant proteins were extracted from transfected cells. UDP-GlcNAc 2-epimerase activity was measured as described previously. The ManNAc kinase assay was performed with slight modification according the previous report (13).

Cross-linking of GNE Mutant Proteins—To analyze the oligomer structure of wild-type and mutant GNE, cell lysates were subjected to a reaction with 10 mM MBS for 30 min at room temperature for cross-linking. The Myc-tagged cross-linked products were purified with anti-Myc-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and eluted by boiling in 2% SDS solution. The products were subjected to SDS-PAGE and Western blot using anti-Myc 9E10 antibody (Santa Cruz Biotechnology).

Lectin Staining and Protein Analysis of Skeletal Muscles from Patients—Biotin-labeled *Maackia amurensis* (MAM), soybean agglutinin (SBA), and *Sambucus sieboldiana* agglutinin (SSA) (Seikagaku Kogyo, Tokyo, Japan) and fluorescein isothiocyanate-labeled streptavidin (Vector Laboratories, Burlingame, CA) were used for the staining of muscle sections. Unfixed 10-μm-thick muscle sections were blocked in 2% casein/phosphate-buffered saline and stained with lectin solution for 2 h at room temperature. The proteins were extracted from the skeletal muscle sections using 8.5 M urea, 0.5% Nonidet P-40 and analyzed by two-dimensional PAGE. Monoclonal antibodies, H4A3 (for LAMP-1), 43DAG1/8D5 (for β-dystroglycan), and VIA4-I (for α-dystroglycan) were used for Western blotting. Laminin binding to α-dystroglycan was examined as described previously (14).

Cell Cultures—COS-7 cells were cultured in 10% fetal bovine serum/Dulbecco's modified Eagle's medium in 5% CO_2. Primary fibroblasts and myoblasts from DMRV patients and normal individuals were cultured in 10% fetal bovine serum, Dulbecco's modified Eagle's medium, and Ham's F-12 medium in 5% CO_2. The myoblasts were induced to myogenic differentiation by switching the medium to 5% horse serum, Dulbecco's modified Eagle's medium, and Ham's F-12 medium. At 24 h before lectin staining or sialic acid determination, the medium was replaced with serum-free Dulbecco's modified Eagle's medium and Ham's F-12 medium with or without 5 mM GlcNAc, ManNAc, or NeuAc. Cells were fixed and permeabilized as described previously (15). Biotin-labeled SBA, wheat germ agglutinin (WGA) (Seikagaku Kogyo), an antibody against desmin (ICN Pharmaceuticals, Costa Mesa, CA), and Alexa Fluo 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) were used for staining the cells.

RESULTS

Novel Mutations in GNE Gene in DMRV Patients—In the previous study, we identified 12 different *GNE* mutations in either homozygous or compound heterozygous states in 27 DMRV patients (8). From these results, we concluded that DMRV is allelic to HIBM. Subsequently, we identified six patients harboring five different mutations, of which three were novel mutations: 1622C→T, 89G→C, and 2173G→A (Table I). These novel mutations were absent in 100 control chromosomes from normal Japanese individuals.

Enzymatic Activities of GNE Mutants in DMRV Patients—Site-directed mutagenesis of the GNE protein has shown that the two enzymatic activities are separately and independently catalyzed by two domains, an N-terminal epimerase domain and a C-terminal kinase domain (13). Previously, we reported reductions in UDP-GlcNAc 2-epimerase activities in leukocytes from the patients with mutations in the *GNE* gene (8). However, the enzyme activity was too weak in leukocytes to clearly demonstrate correlations between gene mutations and the enzymatic activities. To clarify this relationship, we generated recombinant proteins with each of the 13 species of mutations that we identified. All but one of the mutant and wild-type recombinant GNE proteins were expressed in COS cells and migrated at 75 kDa in SDS-PAGE. The single exception was a recombinant protein with a deletion of amino acids 206–256 (Δ206–256) caused by exon 4 skipping. The abnormal protein was degraded in COS cells (Fig. 1*A*). We determined the specific activities of UDP-GlcNAc 2-epimerase and ManNAc kinase of the mutant proteins relative to wild type (Fig. 1*B*). The endogenous activities in mock transfected COS cells were determined to correct for the background enzyme activity. UDP-GlcNAc 2-epimerase activities in mutants C13S, H132Q, D176V, D177C, V331A, and D378Y were reduced to less than 20% of the control. In contrast, the I472T and G708S mutants each revealed an ~50% reduction, and V572L, A630T, and A631V each showed only a 20–30% reduction in activity as compared with wild-type cells. ManNAc kinase activity was retained in the N-terminal mutants C13S, H132Q, D176V, D177C, V331A, and D378Y, whereas the C-terminal mutants I472T, V572L, A630T, A631V, and G703S showed dramatic reductions in activities. These data were essentially compatible with a prior report (13). Interestingly, the A524V mutant preferentially affected UDP-GlcNAc 2-epimerase activity, although the mutation is in the kinase domain. None of the DMRV mutants showed complete loss of UDP-GlcNAc 2-epimerase or ManNAc kinase activities.

TABLE I
Identified mutations

The mutations were identified in patients diagnosed as having DMRV based on both clinical features and muscle pathology. Numbering of patients followed that in our previous report (8). Exon indicates the exon number where mutation was found. Protein domain is predicted ones from the sequence homology as previously (13).

Patient	Mutation	Exon	Predicted amino acid alteration	Protein domain
28	578A→T	E3	D176V	Epimerase
	1765G→C	E10	V572L	Kinase
29	578A→T	E3	D176V	Epimerase
	578A→T	E3	D176V	Epimerase
30	578A→T	E3	D176V	Epimerase
	1622C→T	E9	A524V	Kinase
31	89G→C	E2	C13S	Epimerase
	89G→C	E2	C13S	Epimerase
32	578A→T	E3	D176V	Epimerase
	2173G→A	E12	G708S	Kinase
33	1765G→C	E10	V572L	Kinase
	1765G→C	E10	V572L	Kinase

Oligomerization of GNE Mutants—GNE protein forms a homohexamer by oligomerization (13). We examined whether the mutations affect the oligomerization of GNE molecules, because homohexamer structure of GNE protein was reported to be essential for UDP-GlcNAc 2-epimerase activity (13). The recombinant mutant proteins were subjected to the cross-linking with MBS. Fig. 1*C* shows the electrophoretic tracing patterns of cross-linked products of wild-type and mutant GNE proteins. The product of wild-type GNE predominantly migrated at >400 kDa, which corresponds to homohexamer. C13S, V572L, A630T, and A631V gave cross-linked products similar to that of wild-type GNE, whereas the mutants H132Q and D176V mainly generated a 200-kDa product, and D177C, V331A, D378Y, and A524V produced 98-kDa proteins. These data indicate that the hexameric oligomerization is necessary for UDP-GlcNAc 2-epimerase activity and that N-terminal mutants generally fail to form large oligomers although the molecular region responsible for the oligomerization is not clearly

TABLE II

The list of their mutations, GNE activities, and the results of lectin staining of patients used in this study

The muscle specimens from patients 5 and 8, the fibroblasts from patients 18 and 19, and the myotubes from patient 5 were used in this study. ND, not determined.

Patient	Mutation	Predicted amino acid alteration	GNE activity[a]	MNK activity[b]	SSA staining in skeletal muscle	SBA staining in skeletal muscle	WGA staining in cultured cell	SBA staining in cultured cell
			%	%				
5	IVS4+4A→G	Exon 4 skipping	ND	ND	Variable	Positive in atrophic fibers	Negative in plasma membrane	Positive
	1765G→C	V572L	68.2	8.3				
8	578A→T	D176V	18.2	86.5	Variable	Positive in atrophic fibers	ND	ND
	1043T→C	V331A	16.1	114				
18	578A→T	D176V	18.2	86.5	ND	ND	Weak	Positive
	1466T→C	I472T	47.5	4.7				
19	578A→T	D176V	18.2	86.5	ND	ND	Weak	Positive
	578A→T	D176V	18.2	86.5				

[a] *GNE activity represents* UDP-GlcNAc 2-epimerase activity.
[b] MNK activity represents ManNAc kinase activity as percentage relative to that of wild type GNE.

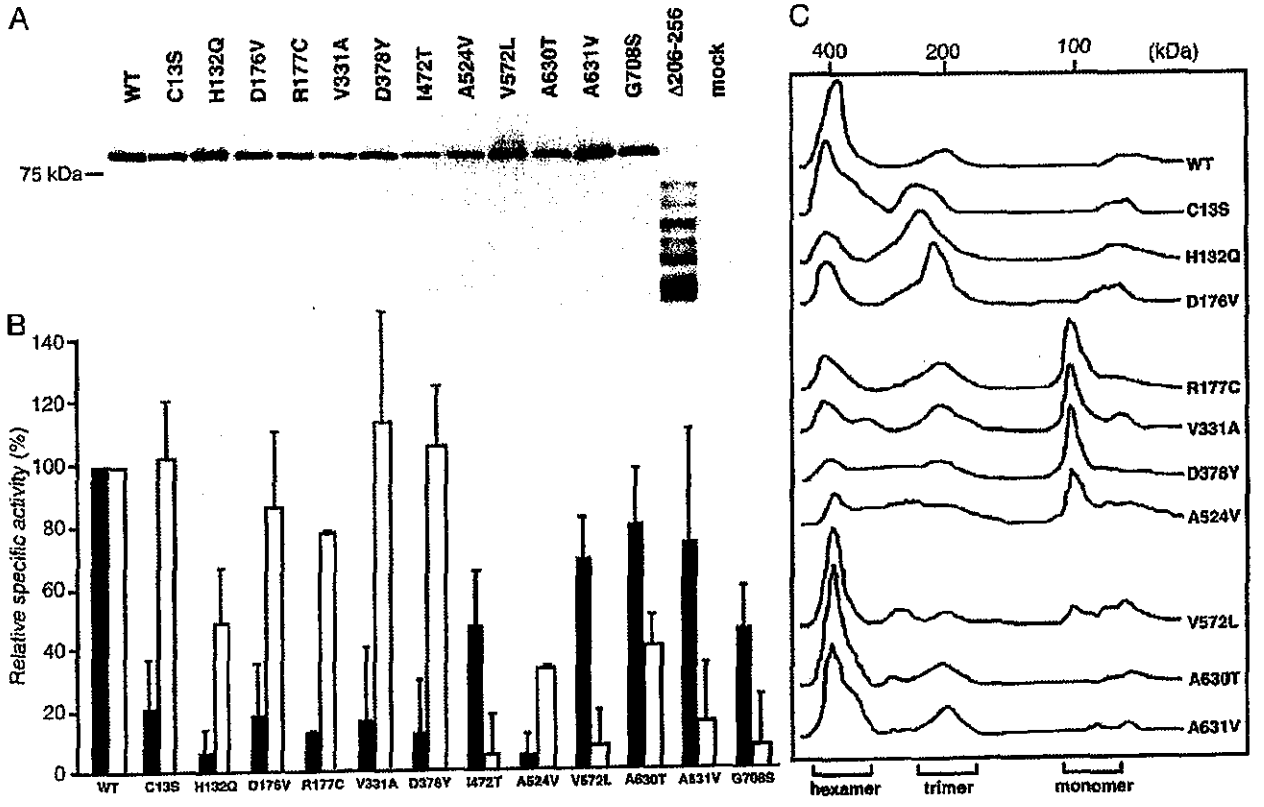

FIG. 1. **Enzymatic activities and oligomerization of mutant GNE proteins.** *A*, Myc-tagged wild-type (*WT*) and mutant GNE proteins expressed in COS cells were separated in SDS-PAGE and Western blotted with anti-Myc antibody. Only the mutant protein with the deletion of amino acids 206–256 was degraded in the cells. *B*, UDP-GlcNAc 2-epimerase (*black*) and ManNAc kinase (*white*) activities were measured *in vitro*. The *bar graphs* display relative specific activities presented as percentage of wild-type activities. *C*, wild-type and mutant GNE proteins were cross-linked with MBS and analyzed by immunoblotting. The immunoreactive patterns of cross-linked products were shown. The predicted migration positions of hexamer, trimer, and monomer are represented at the *bottom*.

related to the predicted oligomerization domains based on the primary structure.

Sialic Acid Contents in Skeletal Muscles and Primary Cells from DMRV Patients—*GNE* gene mutations reduced the enzymatic activity of GNE protein. These results led us to hypothesize that sialylation should be affected in the tissues of DMRV patients. We measured the sialic acid content in sera and muscles from DMRV patients (Fig. 2*A*). In sera, no difference was detected between patients and normal controls, whereas in skeletal muscle, a 25% reduction of sialic acid was observed in DMRV muscles. We also assessed the status of sialylation in DMRV muscles by lectin staining. We used three lectins: SSA for detection of Siaα2–6Gal/GalNAc, MAM for Siaα2–3Gal, and SBA for GalNAcα1–3Gal (16–18) (Fig. 2*B*). The results of lectin staining are summarized in Table II. SSA uniformly stained sarcolemma in control muscle, whereas it faintly stained sarcolemma and strongly stained interstitial tissues in DMRV muscle (Fig. 2*B*, *panels d–f*). MAM strongly stained sarcolemma and interstitial tissues in both the control and patient muscles. We did not observe any reduction in MAM staining in

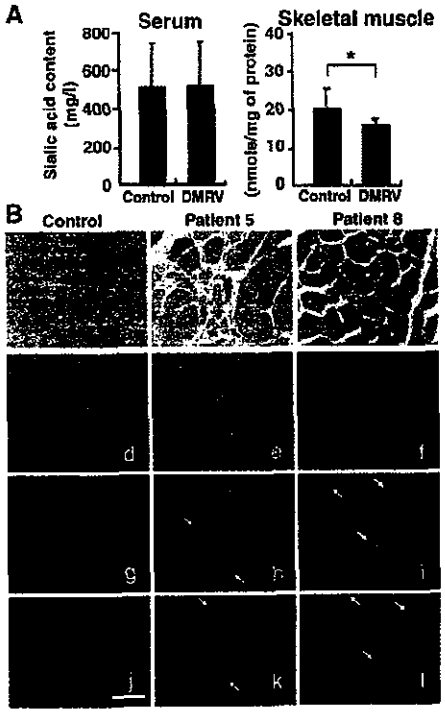

FIG. 2. **Sialylation of sera and muscles from DMRV patients and controls.** *A*, sialic acid contents of the serum from control ($n = 7$) and DMRV patients ($n = 9$, patients 1, 5, 6, 7, 12, 13, 17, 26, and 28) were measured using a SIALIZYME-550 kit. The sialic acid contents of muscles from the control ($n = 7$) and DMRV patients ($n = 4$, patients 5, 8, 9, and 13) were measured by the thiobarbituric acid method. *, $p < 0.05$. *B*, lectin staining and immunohistochemical staining of α-dystroglycan in skeletal muscles from control (*a*, *d*, *g*, and *j*) and DMRV patients 5 (*b*, *e*, *h*, and *k*) and patient 8 (*c*, *f*, *i*, and *l*). *a–c*, stained with hematoxylin and eosin; *d–f*, stained with SSA lectin; *g–i*, stained with SBA lectin; *j–l*, stained with an antibody for α-dystroglycan (VIA4-1). *Arrows* indicate rimmed vacuoles. SBA lectin strongly stained sarcolemma and cytoplasmic areas in the cluster of atrophic or rimmed vacuoles containing myofibers.

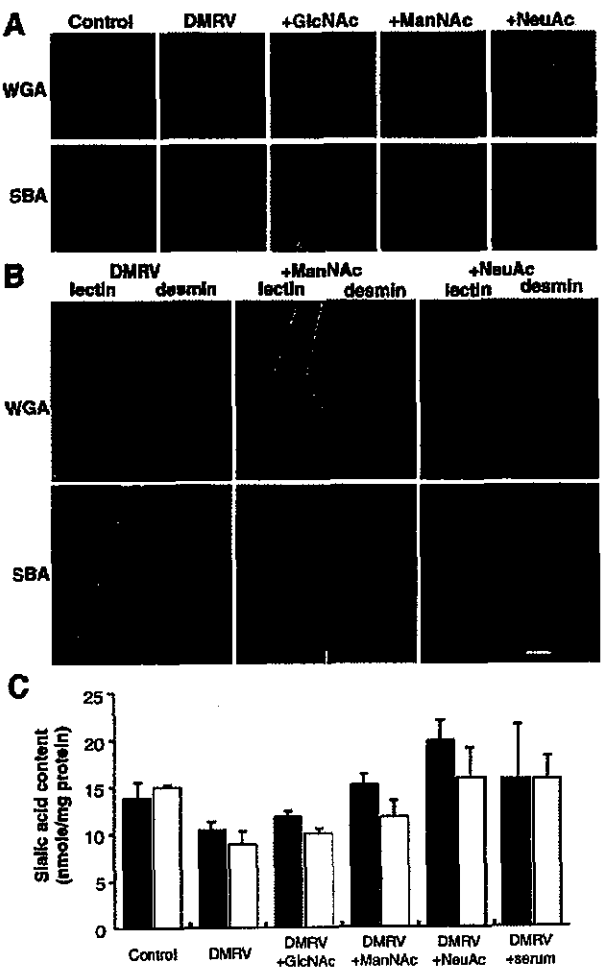

FIG. 3. **Recovery of the sialylation in DMRV cells by treatment with ManNAc and NeuAc.** *A*, the fibroblasts from patient 18 (DMRV) and control individuals were stained with WGA and SBA lectins. The patient 18 fibroblasts cultured in the presence of GlcNAc (*+GlcNAc*), ManNAc (*+ManNAc*) and NeuAc (*+NeuAc*) were also stained with both lectins. *B*, differentiated myotubes from patient 5 cultured without (*DMRV*) and with ManNAc (*+ManNAc*) and NeuAc (*+NeuAc*) were stained by WGA and SBA lectins and desmin antibody. Desmin-positive cells are myotubes. *C*, sialic acids in myotubes ($n = 4$ from patient 5, *black*) and fibroblasts ($n = 4$ from patients 18 and 19, *white*) were measured by the thiobarbituric acid method.

patients, which may be attributed to the strong intensity in our staining condition (data not shown). In contrast, SBA strongly highlighted the rimmed vacuoles containing fibers and the surrounding atrophic fibers in the patients both in sarcolemma and cytoplasm (Fig. 2*B*, *panels h* and *i*; see *arrows*), whereas it did not stain myofibers in the control (Fig. 2*B*, *panel g*). These data suggest that sialylation, other glycosylation, or both are at least partly disturbed in some myofibers in DMRV. Furthermore, we examined the expression of glycosylated α-dystroglycan in DMRV muscles using an antibody (VIA4-1) that recognizes a carbohydrate epitope. The α-dystroglycan staining was negative in rimmed vacuoles containing fibers and the surrounding atrophic fibers in one DMRV patient (Fig. 2*B*, *panel l*). However, positive staining in another patient demonstrated that α-dystroglycan expression varies among patients (Fig. 2*B*, *panel k*). Therefore, we concluded that the loss of α-dystroglycan staining is an extreme down-stream phenomenon in DMRV muscles. We also analyzed muscle sialylated glycoproteins (LAMP-1, and α- and β-dystroglycans) by one- or two-dimensional polyacrylamide gel electrophoresis, but when they were extracted in whole amounts, there was no significant change in the electrophoretic patterns of these proteins between control and patients (data not shown). Furthermore, we analyzed the laminin-binding property of α-dystroglycan from DMRV patients, and the α-dystroglycan showed a strong binding as the control (data not shown).

Restoration of Sialylation in DMRV Cells by Feeding with ManNAc and NeuAc—Previous studies reported that hyposialylation in *GNE−/−* embryonic stem cells and *GNE*-defective cells can be repaired by feeding with the natural sialic acid precursor ManNAc (10, 19). We examined the recovery of sialylation by the addition of NeuAc or ManNAc using primary cultured cells from patients and evaluated the sialylation status by lectin staining (Fig. 3*A*). We used two lectins, WGA, which specifically recognizes a cluster structure of sialic acids (20), and SBA. WGA strongly stained the perinuclear region in control fibroblasts. Fibroblasts from patient 18, who harbors compound heterozygous missense mutations D176V and I472T, showed weaker staining with WGA compared with normal controls (Fig. 3*A*; *WGA*, *DMRV*, and *Control*). SBA did not stain the fibroblasts from normal controls but strongly stained cells from DMRV patients. These results were also confirmed in myotubes from another DMRV patient (patient 5) who has compound heterozygous mutations causing exon 4 skipping and V572L (Fig. 3*B*) and fibroblasts from patient 19 with a homozygous D176V mutation (data not shown). The sialic acid

levels in skin fibroblasts and myotubes from DMRV patients were significantly decreased at ~60–74% of control cells when cells were cultured in serum-free medium (Fig. 3*C*). By adding ManNAc or NeuAc into the culture medium, sialic acid levels in the fibroblasts and myotubes were restored to normal levels (Fig. 3*C*, *+ManNAc and +NeuAc*). Furthermore, WGA staining of cells from patients also increased to normal levels, and particularly strong WGA staining was observed in the plasma membrane of myotubes. In contrast, the SBA staining in DMRV cells disappeared by the addition of either sugar (Fig. 3, *A* and *B*; *+ManNAc* and *+NeuAc*). The addition of GlcNAc into the medium had no effect on the staining pattern with either lectin (Fig. 3, *A* and *C*, *+GlcNAc*). These results suggest the potential therapeutic use of ManNAc and NeuAc.

DISCUSSION

In this study, we identified six additional DMRV patients with *GNE* mutations. Combined with prior results, the 1765G→C mutation accounts for 55% (36 of 66) of the abnormal alleles confirming the high frequency of this mutation in Japan. Haplotype analysis suggests that this common mutation is due to a founder effect (8). All of the mutations identified in DMRV patients caused reduction (but not total loss) of enzymatic activities of either *UDP-GlcNAc 2-epimerase* or ManNAc kinase. These results strongly suggest that DMRV is caused by partial loss of function of the gene product. Interestingly, we previously identified the compound heterozygous mutations D378Y and A631V in a North American DMRV patient of German and Irish origin; these mutations have also been identified in an Irish HIBM patient (13). D378Y reduced UDP-GlcNAc 2-epimerase activity, and A631V decreased ManNAc kinase activity. Together with clinical and pathological similarities, these biochemical and molecular genetic results suggest that DMRV and HIBM are actually the same disease.

Through our study, we obtained information about novel molecular aspects in GNE. The two catalytic domains of the GNE molecule do not always work separately or independently in contrast to a published report (13). For example, the A524V mutation is within the predicted ManNAc kinase domain; however, it strongly inhibited UDP-GlcNAc 2-epimerase. Interestingly, this mutant did not form an oligomeric structure similar to the other N-terminal mutants. The failure of oligomerization in this A524V mutant is probably responsible for the reduced UDP-GlcNAc 2-epimerase activity as suggested previously (13).

Sialylation was decreased in muscle and in cultured cells from patients but was not completely lost, because all of the mutant proteins with missense mutations partially retained both enzymatic activities. Sialic acid levels in sera from DMRV patients were normal. Sialic acids are predominantly produced in the liver and transferred to synthesized glycoproteins. The sialylated proteins are released into the blood plasma, and free sialic acid in the plasma is derived from desialylation of these glycoproteins. GNE is expressed in the liver in large amounts; therefore, the reduction in enzymatic activities by mutations may not significantly affect the synthesis of sialic acid in the livers of DMRV patients, and sialic acids are present at concentrations comparable with normal blood levels. In contrast, in DMRV skeletal muscles, the sialic acid contents are reduced. The reduced enzymatic activities along with weak expression of GNE protein are probably responsible for the more serious reduction in sialic acid synthesis in muscle tissue compared with plasma. Lectin staining showed abnormal staining only in some fibers, indicating that a restricted number of myofibers has glycosylation abnormalities. This selective involvement may be due to muscle uptake of sialic acid, which can compensate for the defect of sialic acid synthesis in most fibers and explains why patients are normal at birth and develop late onset myopathies.

By feeding DMRV myotubes and fibroblasts with NeuAc as well as ManNAc, sialic acid concentrations in the cells increased to normal levels. As reported previously (21), treatment with NeuAc resulted in more rapid and potent effects on the restoration of sialylation than treatment with ManNAc. This strongly suggests that pharmacological therapy may be effective against DMRV/HIBM. Interestingly, even in myotubes harboring mutations that severely decrease ManNAc kinase activity, sialylation was restored by treatment with ManNAc. Schwarzkopf *et al.* (10) also reported similar observations in the embryonic stem cell culture in which the *GNE* gene was disrupted. They suggested that another sugar kinase may convert ManNAc to ManNAc-6-phosphate in those cells; therefore, ManNAc kinase activity of GNE may not be essential for sialic acid synthesis. If so, then why do the *GNE* mutations retaining *UDP-GlcNAc 2-epimerase* activity cause loss of sialylation and disease? One possible explanation is that these mutations may destabilize the GNE molecule resulting in decreased amounts of mutated proteins. However, we did not detect any reductions in the expressed amounts or defects in oligomerization of ManNAc-mutated recombinant proteins. Further analysis is necessary to clarify the mechanisms for the rescue resulting from the addition of ManNAc.

Enhanced staining with SBA lectin was observed in the sarcolemma and within the cytoplasmic area of some myofibers. These fibers were clustered and tended to be atrophic or have rimmed vacuoles. There is a report describing negative staining with SBA in Duchenne and Becker muscular dystrophies (22), suggesting that it is probably not because of dystrophic changes of myofibers but rather because of the lack of sialic acids. This abnormal glycosylation apparently preceded the formation of rimmed vacuoles, which is a pathological hallmark of DMRV. These rimmed vacuoles were electron-microscopically recognized as focal accumulations of autophagic vacuoles, which sometimes surround degenerated myofibrils and amyloid deposits. However, it is unknown whether the focal accumulations of autophagic vacuoles are the cause or result of the degeneration of myofibrils and amyloid deposits. Hypo-sialylation and abnormal glycosylation could cause the misfolding of some glycoproteins, and thus these misfolded glycoproteins may be targets of autophagic degradation and also behave as cores for formation of amyloid deposits. In our study, dystroglycan and SSA lectin staining was variable among patients. One possible explanation is that sialylation may not be the direct cause of the disease. For example, the loss of GNE enzymatic activity may induce the accumulation of the substrate, UDP-GlcNAc, leading to the abnormal *O*-GlcNAc modification of various proteins in the cells (23). Nevertheless, this possibility may also be unlikely because the overexpression of *O*-GlcNAc transferase did not cause any morphological abnormality in skeletal muscles in mice (24). In the next step, further analyses using animal models, as well as further testing of therapy with ManNAc or NeuAc, will be necessary to clarify the pathomechanism of DMRV and HIBM and the pathway from hyposialylation to rimmed vacuole formation and muscle atrophy.

Acknowledgments—We thank Dr. M. Hirano (Columbia University) for reviewing the manuscript, C. Yoshioka (National Center of Neurology and Psychiatry) for technical assistance in drawing figures. The monoclonal antibody H4A3, developed by Drs. J. T. August and J. E. K. Hildreth, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.