B. 研究方法

DMRV のモデルマウス(DMRV-/-hGNETg)は、GNE-KO マウスと変異GNE トランスジェニックマウスの掛け合わせにより作製した。

R-fluorbiprofen は 25mg／kg 体重の量を、毎日２回、経口ゾンデを用いて、胃内へ投与した。アミロイド蓄積が考えられる生後 40 週から投与を開始した。または、飼料に混ぜて投与した。この場合、10mg/kg 体重/日（マウス一匹あたり、体重 30g と、1 日 4g の飼料を食べるとして計算した）で、1 週間ごとに餌料は交換した。

GNE 発現アデノウイルスベクターは、ヒト GNEcDNA を Myc タグに繋いで、５型アデノウイルスベクターである pAxCAwtit2 にクローニングした。大腸菌でのコスミドコンストラクションの後、プラスミドに変換し、COS 細胞で発現を確認した。コスミドは、完全長ゲノム導入法により、HEK273 細胞へトランスフェクション、アデノウイルスに変換し、HEK273 細胞でクローン化とウイルスの増幅を行なった。このベクターは CAG プロモーターにより、普遍的な遺伝子を発現することが出来る。ウイルスの培養細胞への感染は、セミコンフルエントな時期を用いた。

（倫理面への配慮）

すべての動物実験は、国立精神・神経センター神経研究所動物実験に関する倫理指針に従い行い、同研究所小型実験動物倫理問題検討委員会にて審査・承認を得ている。すべての組み換え DNA 実験は、カルタヘナ議定書に基づく「遺伝子組み換え生物等の使用等の規制による生物の多様性の確保に関する法律」と関係省令を遵守し、国立精神・神経センター神経研究所組み換え DNA 実験安全委員会の審査・承認を得て行なっている

C. 研究結果

経口ゾンデによる *R*-fluorbiprofen 投与（mg/Kg 体重）において、投与マウスは、投与後３日目から、重篤な体重減少を示し、1－2 週間で、約 25%の個体が死に至った。他のマウスも体重減少が顕著に認められた。病理解剖の結果、消化管より多量の出血が認められた。また、一度に多量の *R*-fluorbiprofen を投与するには、その溶解度に問題があった。

そこで、*R*-fluorbiprofen 入りの飼料を用いた、自由摂取による投与を試みた。投与を開始した５個体のうちで、開始当初は顕著な体重の減少は認められなかったが、うち１個体で、投与２週後から体重の減少を認め、3 週目で死亡した。他のマウスには何ら目立った変化は無く、今も投与を続けている。

GNE 発現アデノウイルスベクターを定法に従い、完全長ゲノム導入法にて作製した。増幅・精製したアデノウイルスは、生物学タイター6×10^7 pfu/ml(物理化学タイター比：8000)と極めて純度が低いものであった。このアデノウイルスを用いて、CHO 細胞への感染実験を行なった。ウエスタンブロットの結果、75kDa の単一バンドを示した。これは予測分子量と完全に一致するものであった。抗 Myc 抗体を用いた、免疫細胞染色では、核を除く細胞質全体に渡って、ほぼ均一な染色が見られた。以前に報告されたような核やゴルジ体への局在は見られなかった。培養筋管細胞でもほぼ同様の局在が確かめられた。また、これらの GNE 過剰発現細胞では際立った形態変化は観察されなかった。レクチン染色においても、シアル酸反応性レクチンの染色に際立った変化は無かった。

D. 考察

DMRV骨格筋に蓄積しているベータアミロイドタンパク質の生成を抑える目的で、γセクレターゼの切断生成物の特異性をAβ1-42からAβ1-40に変換する薬剤である非ステロイド性抗炎症剤*R*-fluorbiprofenが用いられている。非ステロイド性抗炎症剤としては、fluorbiprofenのラセミ体が用いられてきたが、そのR体は細胞毒性が低く、γセクレターゼの特異性を変化させる特異的作用を持つことが知られている。ベータアミロイド高発現マウスにおいてなされた研究では、25mg/kg/日のdoseにて、*R*-fluorbiprofenを3日間経口投与したところ、Aβ1-42の産生を、特異的に60%程度抑えた。また、Aβ1-40の産生に関しては、ほとんど阻害されなかったと報告している。しかしながら、その後の報告では、25mg/kg/日のdoseでの長期投与では、2週間で85%のマウスが死に至ったとある。我々が、最初に用いた25mg/kg/日というdoseは、その報

告を再現したものであるかも知れない。また、10mg/kg/日のdoseでの14日間の投与では、なんら致死性は報告されていない。*R*- fluorbiprofenを食餌にて、10mg/kg体重/日のdoseでの投与では、1個体のみが、1週間で致死に至った。この個体の解剖所見では消化管からの少量の出血は認めたが、元々のDMRVマウスの致死率を反映するものと考えられた。
アデノウイルスベクターの作製では、純度は低いものの、精製することが出来た。培養細胞に導入し、発現タンパク質を確認した。以前の報告と同様の分子量をもつタンパク質の発現を確認した。細胞内分布では、生化学的に示されたように、細胞質への分布が確認された。しかしながら、一部の報告にあるような細胞核やゴルジ体への局在は確認されなかった。なぜ、細胞内局在にこのような相違を生じるかはわからない。

E. 結論
DMRV モデルマウスに対して、ベータアミロイドの蓄積を抑制する薬剤 *R*-fluorbiprofen の投与を行なった。遺伝子治療基礎実験を行なうため、ヒト GNE を高発現しうるアデノウイルスベクターを開発した。

F. 健康危険情報
特になし

G. 研究発表
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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   特になし

2. 実用新案登録
   特になし

3. その他
   特になし

Ⅲ.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名： 論文タイトル名. 発表誌名 巻号： ページ, 出版年
Malicdan MCV, Noguchi S, Nishino I: Autophagy in a Mouse Model of Distal Myopathy with Rimmed Vacuoles or Hereditary Inclusion Body Myopathy. ***Autophagy*** 3: 396-398, 2007
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Ⅳ.　研究成果の刊行物・別刷

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Addendum

Autophagy in a Mouse Model of Distal Myopathy with Rimmed Vacuoles or Hereditary Inclusion Body Myopathy

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KEY WORDS

DMRV, hIBM, sialic acid, amyloid, GNE, myopathy, autophagic vacuoles, model mouse, cellular stress

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Addendum to:

A Gne Knockout Mouse Expressing Human V572L Mutation Develops Features Similar to Distal Myopathy with Rimmed Vacuoles or Hereditary Inclusion Body Myopathy

M.C. Malicdan, S. Noguchi, I. Nonaka, Y.K. Hayashi and I. Nishino

Hum Mol Genet 2007; 16:115-28

ABSTRACT

Distal myopathy with rimmed vacuoles (DMRV) or hereditary inclusion body myopathy (hIBM) is an autosomal recessive disorder clinically characterized by weakness that initially involves the distal muscles, although other muscles can be affected as well. Pathological hallmarks include the presence of rimmed vacuoles (RVs) and intracellular Congo red-positive depositions in vacuolated or nonvacuolated fibers. Mutations in the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (*GNE*) gene, which encodes the rate-limiting enzyme in sialic acid biosynthesis, are causative of DMRV/hIBM. Recently, we have generated a mouse model (*Gne*$^{-/-}$hGNEV572L-Tg) for this disease, and have shown that these mice exhibit hyposialylation and intracellular amyloid deposition before the characteristic RVs are detected, indicating that autophagy is a downstream phenomenon to hyposialylation and amyloid deposition in DMRV/hIBM.

DMRV or hIBM is an early adult-onset autosomal recessive myopathy which usually presents as a gradually progressive myopathy predominantly affecting the distal muscles,[1,2] although proximal muscles can also be affected. On muscle biopsy, a variety of abnormalities is seen, including the characteristic finding of RVs,[3-5] intracellular deposition of Congo red-positive materials such as β-amyloid and α-synuclein,[6] abnormal phosphorylation of tau,[7,8] activation of the ubiquitin proteasome system,[9] and activation of the lysosomal system.[10] Until now, however, the precise mechanism that leads to rimmed vacuole formation and/or symptoms in DMRV has remained elusive. Several studies have alluded to the presence of RVs as an upstream event, which could be responsible for the muscle weakness seen in patients. DMRV is associated with mutations in the *GNE* gene,[11,12] which codes for a bifunctional enzyme that catalyzes the rate-limiting step in sialic acid biosynthesis.[13] Hyposialylation thus has been thought to contribute to the overall pathomechanism of this myopathy,[14] although its precise role has not been clarified. The causative nature of these mutations in the *GNE* gene was confirmed by the mouse model which we recently generated.[15] Further, we have shown that sialic acid is reduced in the serum, muscle, and other organs of these mice, underscoring the importance of hyposialylation in the DMRV/hIBM pathomechanism.

Rimmed vacuoles in DMRV/hIBM are actually clusters of autophagic vacuoles (AVs) and myeloid bodies.[16] The autophagic nature of these vacuoles was supported by the observations of acid-phosphatase-rich primary lysosomes, clathrin-positive granules, and the presence of cathepsins B and L in fibers with RVs.[17]

Autophagy is a major cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles in eukaryotic cells that is activated under various conditions, including trophic stress, a large number of intracellular/extracellular stimuli, invasion of microorganisms, and nutritional deprivation. Nevertheless, the autophagic vacuoles observed in these myopathies were thought to be mainly secondary to abnormal lysosomal function with regard to their effects on muscle fiber breakdown.[17,18] In DMRV/hIBM, it has been suggested that the presence of abnormal autolysosomes and/or autophagosomes is due to the following possibilities: (1) an excessive amount of substrate in otherwise normally functioning lysosomes, which can include misfolded glycoproteins that were not degraded in the ER;[19] (2) a response to oxidative stress, possibly due to hyposialylation, as sialic acid has been shown to be a scavenger of hydrogen peroxide;[20] (3) a secondary response to ER stress;[19] (4) defective lysosomal enzymes, which might be generated by muscle fiber endogenous cytotoxins, or by hyposialylation; (5) nondegradation of substrates because of certain conformational changes, including exposure of normally hidden hydrophobic residues; (6) slowing of the degradation rate of segregated cellular components within autolysosomes; and (7) nonprogression of the autophagic process.

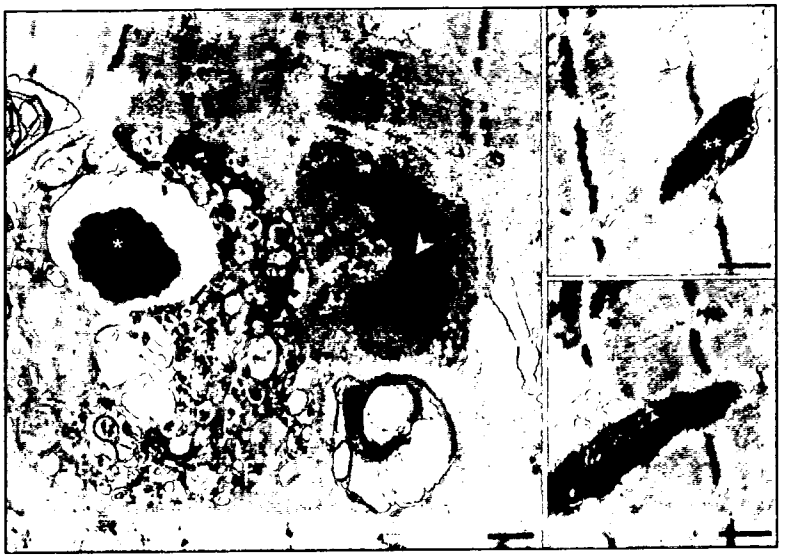

Figure 1. Electron microscopy findings in the *Gne*$^{-/-}$ *GNE*V572L-Tg mice (gastrocnemius, 52 wks ♀). Various cellular debris are enclosed by nascent AVs (arrows) and degradative AVs (double arrows). Large, osmiophilic deposits can likewise be seen (asterisk). Filamentous and granular amyloid deposits are seen (arrowhead). In areas where the myofibrillar structure is relatively preserved, dense ovoid bodies are seen (double asterisks). These are sometime associated with autophagic vacuoles (double arrowheads), suggesting that these deposits predate RV formation. Bars represent 1μm.

In the *Gne*$^{-/-}$h*GNE*V572L-Tg mice, we observed pathological changes that correlate with age. Before 30 weeks of age, the muscle pathology is mainly characterized by variation in fiber size. In addition, scattered angular fibers are seen from 30 weeks. At around 32–34 weeks, intracellular depositions of amyloid are noted in several fibers. After 40 weeks, the characteristic RVs are observed. Similar to humans, these RVs have high acid phosphatase activity and are highlighted by various lysosomal antibodies, aside from other proteins, implicating the lysosomal system as one of the processes whereby the proteins that contribute to the disease symptoms are degraded. Moreover, by ultrastructural studies (Fig. 1), these vacuoles contain multilamellar bodies, electron-dense bodies, and various heterogeneous materials and cytoplasmic debris that are limited by a double membrane, indicating that these are nascent AVs (arrow). To a lesser extent, some vacuoles have a single limiting membrane, assuming the characteristics of degradative vacuoles (double arrows). In other areas, however, some cellular debris are observed without any limiting membrane, suggesting that the limiting membrane may have ruptured. In general, these AVs are often associated with areas where filamentous/granular deposits, which are possibly amyloid, are observed (arrowhead). Occasionally, large aggregations of deposits are seen with these AVs, and they are surrounded by filamentous structures (asterisk).

Interestingly, similarly large, dense and ovoid bodies, which can sometimes be granular and could be possibly considered as amyloid deposits, are seen in areas where the myofibrillar structure is well preserved (double asterisks). Interestingly, we have observed that these probable amyloid deposits can also be seen in areas that appear normal, i.e., myofibrils are intact, strongly suggesting that the presence of such deposits precedes the accumulation of AVs. In nearby areas, these dense deposits are noted to have AVs within their substance (double arrowheads).

In the DMRV/hIBM mouse, the presence of amyloid deposits could be secondary to a spontaneous autophagic process. The alternative possibility is that autophagy is a reactive process, a response to an abnormal

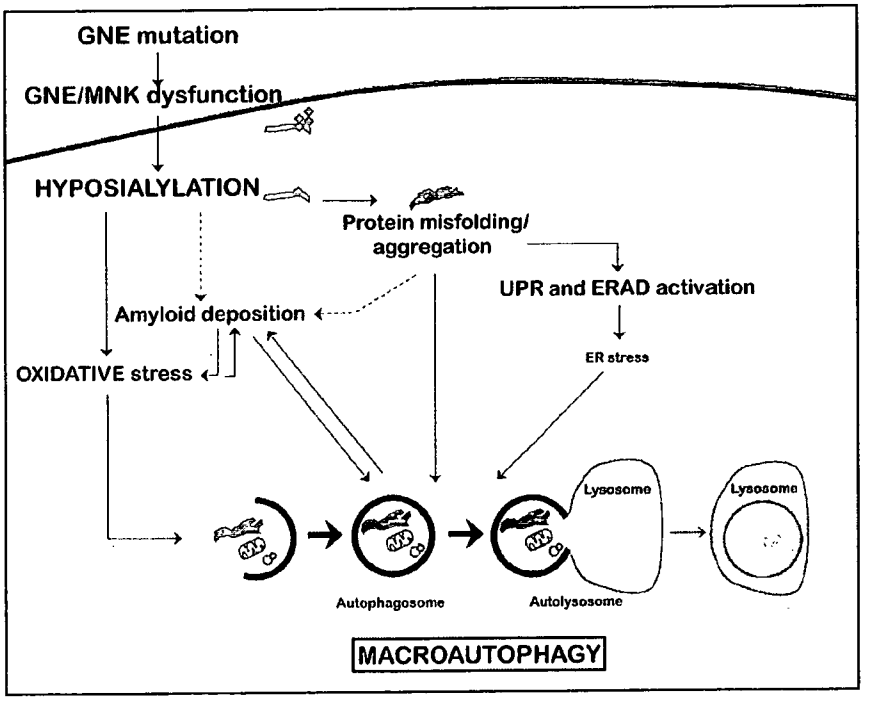

Figure 2. Mechanism of autophagy in DMRV/hIBM. Hyposialylation, secondary to *GNE* mutations, can result in three possible outcomes. First, it can lead to abnormal protein configuration or misfolding. Exposure of hydrophobic domains makes these proteins prone to aggregation; UPR and ERAD pathways are subsequently activated, but an increase in the amount of substrate protein could lead to ER stress. Second, hyposialylation can affect normal processing of other proteins, such as amyloid, which could bring about abnormal amyloid depositions. In addition, amyloid deposition can also trigger oxidative stress. Third, as sialic acid has been shown to scavenge hydrogen peroxide, hyposialylation can contribute to oxidative stress. Thus, in DMRV/hIBM, the autophagic process can commence from the following: protein misfolding/aggregation, amyloid deposition, ER stress, and oxidative stress. Alternatively, AVs can be a site of amyloidogenic processing of the amyloid precursor protein. Further, the presence of accumulating AVs and their association with lysosomal markers indicate that autophagy does not progress normally.

conformation (exposure of hydrophopic surfaces) of a certain protein which was not successfully cleared by cytosolic proteases. Protein aggregation then can ensue, shielding the hydrophobic patches; however, aggregation makes these proteins ostensibly resistant to attack by cytosolic proteases, leaving macroautophagy as the only viable possibility for their removal. We think that the lysosomal system is activated because of the presence of these deposits, which are most likely amyloid.

The presence of intracellular amyloid deposits in these mice and the association of these deposits with an autophagic process have widened the perspective in understanding the pathomechanism of DMRV/hIBM. In a previous report, these AVs have been implicated to be a site of amyloidogenic amyloid precursor protein, APP, processing;[21] in addition, intralysosomal Aβ accumulation has been found to be induced by oxidative stress, implying that oxidative stress could be an upstream event to amyloid deposition and/or RV formation. Moreover, a recent report demonstrated that reactive oxygen species may contribute to the formation of autophagosomes.[22] Alternatively, because amyloid is itself a glycoprotein, hyposialylation may lead to protein misfolding and eventually could lead to the activation of autophagy and/or oxidative stress. The conundrum remains, thus far unanswered, of how hyposialylation could lead to either protein misfolding or oxidative stress. Nonetheless, Figure 2 is a schema of how *GNE* mutations could trigger autophagy. Clearly, addressing these issues in future studies will help us have a better understanding of the pathomechanism of DMRV/hIBM.

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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

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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.[5,8] 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).

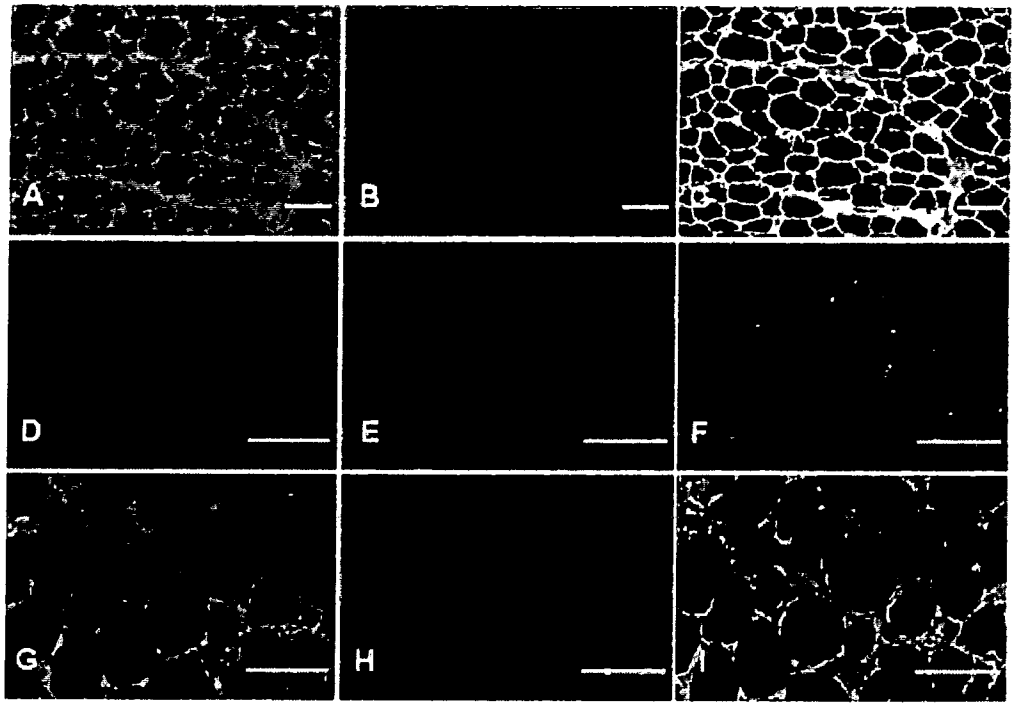
Direct sequencing of *COL6A1, COL6A2,* and *COL6A3* genes revealed mutations in a significant number of patients with collagen VI deficiency, apart from the several polymorphisms that we have found (table E-1 on the *Neurology* Web site at www.neurology.org). Mutations were found in all patients with 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.

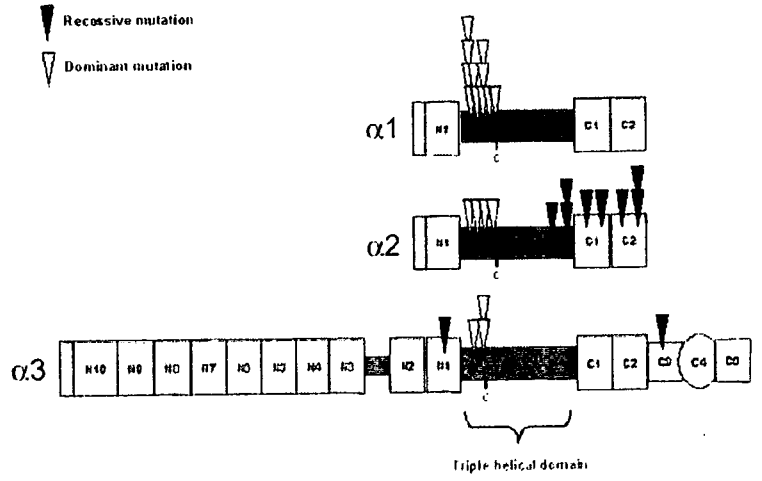
DISCUSSION Fukuyama type CMD (FCMD) is the most frequent CMD and is found almost exclusively in Japan, accounting for 49.2% in our series, although a patient with an extremely severe lethal disease who was clinically dissimilar to but genetically proven to have FCMD was re-

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	5	M	CD	No	Yes	No	No	No	Yes	No	No	337	ND	ND	*COL6A2*	c.1572+1G>C (homozygous)
2	4	F	CD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	361	0	No	*COL6A2*	c.2678_2700del23 (homozygous)
3	4	F	CD	Yes	No	Yes	No	No	Yes	Yes	Yes	343	2+	Yes	*COL6A2*	c.1771-2A>T c.2279_2280del2
4	4	M	CD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	440	0	No	*COL6A2*	c.1270-1G>C c.1771-3G>C
5	2	M	CD	Yes	No	Yes	No	Yes	Yes	Yes	No	413	ND	ND	*COL6A3*	c.5692delG c.8737delG
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	270	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	5	F	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	3+	ND	*COL6A1*	c.868G>A
12	13	M	SSCD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	201	ND	ND	*COL6A1*	c.956G>T
13	14	F	SSCD	Yes	ND	Yes	No	ND	ND	Yes	ND	372	ND	ND	*COL6A1*	c.958_966del9
14	10	F	SSCD	Yes	ND	No	No	ND	Yes	Yes	Yes	343	5	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	F	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	16	M	SSCD	No	No	No	No	No	Yes	ND	No	191	12	No	*COL6A2*	c.812G>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>T
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	257	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	16	F	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	164	4	No	*COL6A3*	c.6210+1G>A
25	9	F	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	Yes	539	8+	No	*COL6A3*	c.6210+1G>A
26	4	F	SSCD	Yes	No	No	No	No	Yes	Yes	No	336	2+	No	*COL6A3*	c.6210+2T>A
27	11	F	SSCD	Yes	No	Yes	No	Yes	Yes	ND	No	80	ND	ND	None	
28	9	M	SSCD	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	47	ND	No	None	
29	1	M	SSCD	ND	ND	ND	No	ND	ND	ND	ND	319	ND	ND	None	
30	17	F	SSCD	Yes	No	Yes	Yes	No	Yes	Yes	Yes	215	6	No	None	
31	14	M	SSCD	Yes	Yes	No	No	Yes	Yes	Yes	ND	113	5	No	None	
32	4	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	4	M	SSCD	Yes	No	Yes	No	Yes	Yes	Yes	Yes	299	2+	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

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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.[7] 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, antirabbit 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.

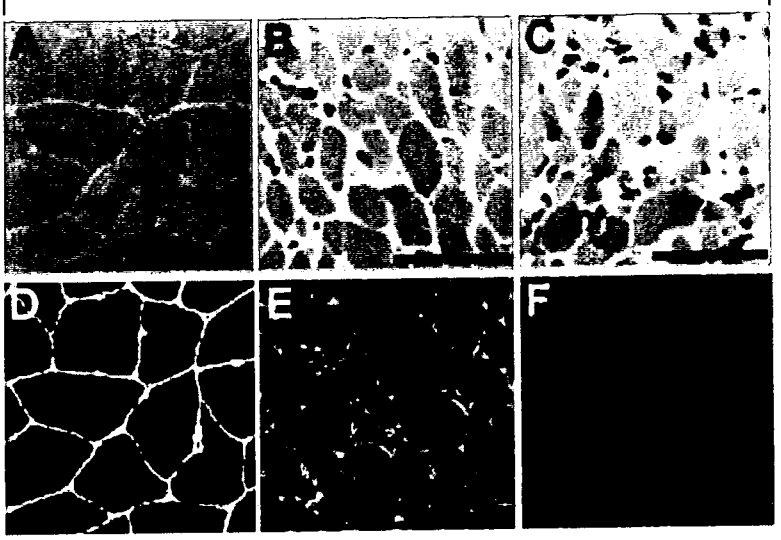
Western blot analysis. Proteins in the cultured medium and cell extract, as well as the proteins that remained on the dish after pipetting, were electrophoresed on 5–17.5% polyacrylamide gel under reduced condition and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). 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