Nervous System & Sugar Chain

English

神経筋接合部におけるパールカンの役割

パールカンはすべての基底膜に存在するほか、軟骨などに存在するヘパラン硫酸プロテオグリカンである。５つの機能ドメインからなるコアタンパク質は400kDaに及び、N末端とC末端にヘパラン硫酸鎖の結合部位が存在する。パールカンは成長因子シグナルを修飾し、細胞の増殖、分化を制御するなどの種々の生物学的活性を持つ。これらの結果はほとんど*in vitro*の実験で確認されてきたが、近年、遺伝子改変マウスの解析や、ヒト遺伝性疾患の同定によりパールカンの発生や疾患における重要性が明らかになってきた。

我々は二つの予後の異なる疾患の原因遺伝子がともにパールカンであることを報告した。パールカンの機能完全欠損はノックアウトマウスの症状とよく似た周産期致死性のSilverman-Handmaker 型軟骨異形成症(DDSH)を、機能部分欠損では軟骨病変と筋持続収縮（ミオトニア）を併せ持つSchwartz-Jampel症候群 (SJS)を発症することを示した。さて、パールカンは筋組織においても基底膜の重要な構成成分であるが、発生が成熟するに従い神経筋接合部に多く存在してくる。神経筋接合部においては、アセチルコリン(ACh)とニコチン型ACh受容体の結合が後シナプス膜の脱分極を引き起こし筋が収縮する。筋の収縮と弛緩を速やかに制御するため、ここにはACh受容体やアセチルコリンエステラーゼ(AChE)をはじめとする多くの分子が集合して共同して働く必要がある。AChE はAChを加水分解することにより、この反応を終息させるとともに、AChのリサイクルに貢献する酵素である。神経筋接合部ではコラーゲン様の分子（collagen-like tail subunit; ColQ）ドメインをもつ基底膜特異的なAChEが存在するが、*in vitro*の実験結果から、この分子がパールカンと結合することが想定されていた。パールカンノックアウトマウスは周産期に死亡するが、筋の発生、分化、神経支配、神経筋接合部形成は少なくともこの時期まではほぼ正常に達成されていた。ところが、ACh受容体、アグリン、ラプシン、αジストログリカンといった神経筋接合部集合分子の集積に異常がないのに、AChEのみが特異的に欠損していることが解った。ノックアウトマウス筋全体の生化学的解析ではColQ フォームを含めたすべてのAChEが発現していることから、産生されたColQフォームが神経筋接合部に局在化

することの障害によると考えられる。この結果はAChEの神経筋接合部への局在にパールカンが必須の働きをすることを示唆している。このことがSJSにおける筋の持続収縮（ミオトニア）と関連すると考えられ、新しいモデルマウスを使った電気生理学的解明や治療実験が期待される。

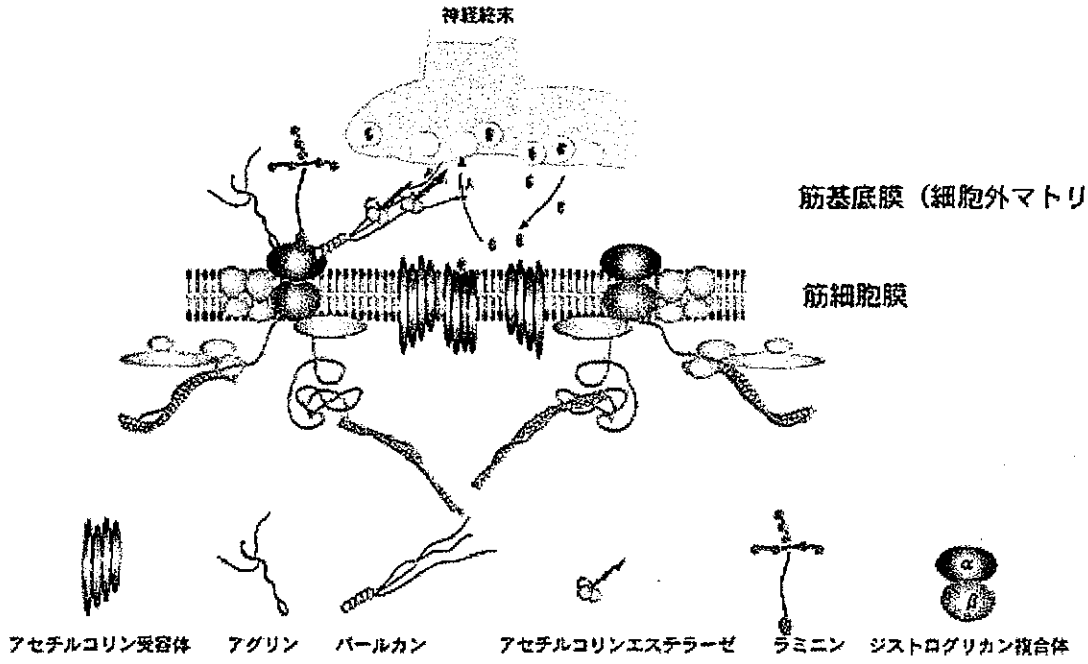

図1 神経筋接合部でのパールカンの機能

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Links PG-A09 基底膜のプロテオグリカン（朔 敬）

2004年8月6日

遺伝子改変マウスの解析から解明されるパールカンの神経筋機能への関与

平澤(有川)恵理

基底膜型ヘパラン硫酸プロテオグリカンであるパールカンは多くの生物学的活性が報告されてきた．発生や疾患におけるパールカンのはたらきを研究するため，ノックアウトマウスを作製した．解析の結果から軟骨の発生，分化にパールカンが必須であることが判明し，症状の類似性と遺伝子座の解析から，2つの予後の異なるヒト疾患を同定した．また，ノックアウトマウスの神経筋接合部においてアセチルコリンエステレースが特異的に欠損することと，パールカン機能部分欠損疾患であるSchwartz-Jampel症候群の筋異常収縮との関連性を考察する．

Key Words　パールカン　ヘパラン硫酸プロテオグリカン　神経筋接合部　ミオトニア

はじめに

パールカンは種々の生物学的活性をもつ多機能細胞外マトリックス分子として注目されるヘパラン硫酸プロテオグリカンである．すべての基底膜に存在するが，軟骨や脳などその他の組織でも発現している．パールカンの発見は，20年ほど前にさかのぼり，当初腺組織などにおける形態形成や糸球体基底膜におけるはたらきが注目された．その後，線維芽細胞増殖因子（FGF）をはじめとする成長因子や細胞膜受容体とも結合してさまざまな細胞内シグナリングを修飾制御する機能分子としてもはたらき，器官の形態形成過程などに重要なはたらきをすることなどがわかってきた[1,2]．また，血管新生，組織の修復，がん細胞の増殖，転移，浸潤などにも関与していると考えられ，さらにパールカンと疾患との関連に興味がもたれている[3]．

I. パールカンのドメイン構造と機能

パールカンのコア蛋白質の分子量は400K以上あり，5つの異なった機能ドメインをもち，*in vitro*の研究成果から，それらのドメインの構造と生物活性の関連がわかってきた（図1）．ドメインⅠはコア蛋白質のN末端にあり，3カ所のヘパラン硫酸鎖の結合部位が存在する．このヘパラン硫酸鎖はFGF2と結合して細胞分裂や血管新生を促進するとされる．この領域はもっとも相同性の低い特異的部位である．ドメインⅡは低分子量リポ蛋白質（LDL）受容体のLDL結合部位の配列と相同性があり，4つのシステインに富むモチーフが存在する．ドメインⅢは，ラミニン短鎖N末端領域と高い相同性をもち，3つの球状のサブドメインとシステインに富むEGF様の繰返し配列をもつ．ドメインⅣはNCAMの免疫グロブリン様構造部位と相同性の高い配列が繰り返し存在し，もっとも大きいドメインである．この領域の前半は他の基底膜蛋白質と結合性をもち，パールカンの細胞外マトリックスでの局在に強く関与していると思われる[4]．もっともC末端のドメインⅤはラミニンα鎖やアグリンのC末端球状Gドメインと相同性をもつ3つのサブドメインと，それらを分断するEGF様配列からなり，α-ジストログリカンやインテグリンβ1などの細胞表面受容体と結合することから，細胞接着への関与など特

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Roles of perlecan in neuromuscular function : Studies in knockout mice and human disorders

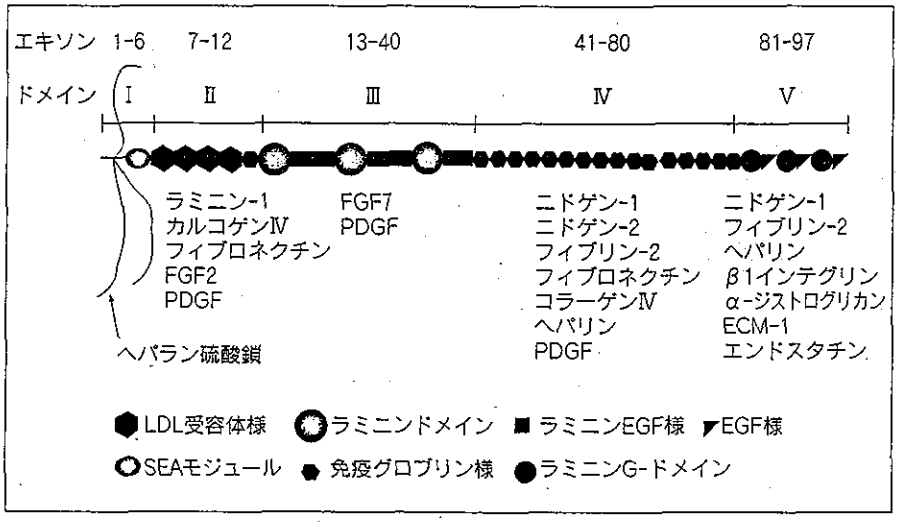

図1　パールカン分子の機能ドメインとその結合蛋白質

殊な機能が想定されている[5]．

II. パールカンノックアウトマウスの作製と解析によるパールカンの機能解明

筆者らはこの多様な機能をもつと予想されるパールカンの *in vivo* での役割を明らかにし，その欠損が発生にどのような障害をもたらすか，また，どのような疾患に結びつくのかを調べるために，パールカン遺伝子のノックアウトマウスを作製した[6]．このマウスの一部は頭部形成異常のため胎生10.5日ころに早期胎生期致死を呈するが，大部分のものは胎生14.5日ころから徐々に軟骨形成異常を発症し，出生ころには明らかな四肢短縮を呈し，呼吸障害を伴って死亡することがわかった（図2）．

この四肢短縮は内軟骨性骨化の障害によるが，膜性骨化は逆に亢進し，四肢の長管骨は横径が増し，太く短く発達する．脊椎の椎体部分の発生も内軟骨性骨化によるので，長管骨と同様の変化をとる．Costellらの作製したノックアウトマウスも同様の表現型を呈した[7]．パールカンのノックアウトマウスにおける内軟骨性骨化の障害の原因としては，パールカン欠損により軟骨分化に深くかかわるシグナル機構が変化した可能性と，軟骨マトリックス中のパールカンの欠損が軟骨マトリックス分子の形成に直接影響を与え，これを脆弱化した可能性が考えられるが，おそらくその両方の影響があると思われる．成長板での軟骨最終分化におけるシグナル機構において重要な分子として，FGF受容体3（FGFR3）やインディアンヘッジホッグ（Ihh）などが挙げられるが，これらはヘパラン硫酸鎖との結合が知られる．しかし，パールカンにおける主要なヘパラン硫酸鎖結合部位を有するドメインI欠損マウスが大きな異常を呈さなかったという報告があり，単純には説明できない．軟骨において糖鎖の結合部位や糖鎖合成に違いがある可能性も残される．軟骨細胞の最終分化帯である肥大軟骨細胞周囲でのパールカンの局在を考えると，細胞表面でのFGFシグナルの調整やマトリックスと細胞の接着などをつかさどる役割などが想定される．パールカンのFGF低親和性受容体としての生物学的機能やインテグリン，ファイブロネクチンとの結合能を考えると非常に興味深い点である．ノックアウトマウス解析の結果，ほとんどの器官は一応の発生，分化を示し，出生まで至った．また，基底膜も組織学的，電子顕微鏡学的に，多くの組織でほぼ正常な構造をとった．しかし，その一方で，基底膜をもたない軟骨では致命的な異常を呈した．

III. 神経筋接合部におけるヘパラン硫酸プロテオグリカンの重要性とパールカンのユニークな機能

神経筋接合部は高度に発達した基底膜構造であり，運動神経からの電気的興奮をアセチルコリン（ACh）による化学的興奮に置き換え，筋収縮・弛緩を制御している．筋の収縮・弛緩をすみやかに行うため，アセチルコリン受容体，アセチルコリンを分解するアセチルコリンエステレース（AChE）のほか，MuSK（muscle specific kinase），ニューレグリン，ラプシン，ジストログリカン，ラミニンなどの分子が神経筋接合部構成分子として集束してい

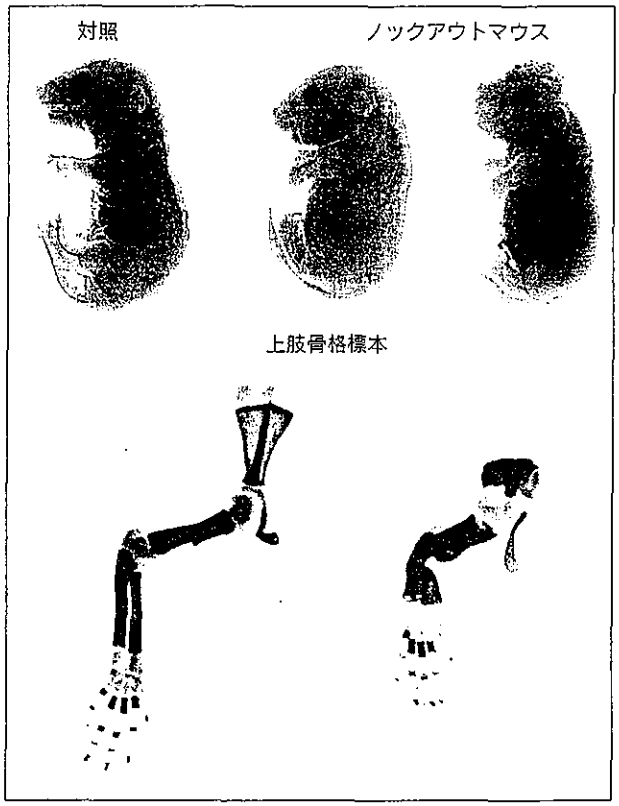

図2 胎生18.5日パールカンノックアウトマウスの外観と上肢の骨格標本
内軟骨性骨化の障害により短縮を認める．ノックアウトマウス右は脳ヘルニア合併例．骨格標本は鎖骨，肩甲骨を含む上肢骨格標本．軟骨部分がアルシャンブルーによって青く，骨部分がアリザリンレッドによって赤く染色されている．ノックアウトでは軟骨マトリックスの染色性が悪く．長管骨が太く短い．

る（図3）．神経筋接合部においてはいくつかのヘパラン硫酸プロテオグリカンが特異的に発現し，重要なはたらきをすることが推定されている[8]．たとえばアグリンは神経筋接合部形成のための必須分子であり，その遺伝的欠損により神経筋接合部が形成されなくなることが知られている．パールカンは筋基底膜に存在するが，発生が進むにつれ神経筋接合部に多く発現するようになり，神経筋接合部における重要性が示唆された[9]．筆者らはパールカンノックアウトマウスにおける神経筋接合部の解析を行った．機能完全欠損であるノックアウトマウスは出生時までに死亡するが，神経筋接合部発生および形成を観察することができる．新生ノックアウトマウスの横隔膜，肋間筋，骨格筋などにおいて，末梢神経の支配，神経筋接合部へのアセチルコリン受容体，アグリン，ラプシンなどの分子の集合などがみられ，ほぼ正常な神経筋接合部が形成されているにもかかわらず，AChEの局在のみが欠損していることを示した[10]．筋全体の生化学的検討ではAChEの各アイソフォームは確認されたことから，生成されたAChEの基底膜への局在化の障害と考えられた．

Ⅳ．パールカン欠損による2種類のヒト遺伝性疾患の同定

1．パールカン機能完全欠損による遺伝性疾患の同定

ノックアウトマウスの解析結果から，軟骨分化におけるパールカンの重要性が示され，その後ヒトでのパールカン欠損病を見いだす大きな鍵を与えた．筆者らは，パールカン欠損マウスの表現型から，ヒトでのパールカン欠損疾患が存在するものと考え，その探索を開始した．候補となる疾患の同定に際しては，ヒトでのパールカン欠損病でもノックアウトマウスと同じような表現型を呈する可能性から，ノックアウトマウスでの特徴的な骨X線所見，骨成長板の病理所見と合致する常染色体劣性遺伝形式軟骨異形成症を探した．その結果，周産期致死性の軟骨異形成症（Silverman-Handmaker型dyssegmental dysplasia；DDSH）の表現型[11]がパールカンノックアウトマウスのそれときわめて類似していることがわかった．この疾患では患児は体幹，四肢とも著しく短縮し，四肢長管骨の彎曲，椎体骨の大小不同など，ノックアウトマウスと非常によく似た骨格異常を示すが，さらに一部の例で脳ヘルニアを合併するとされる点もノックアウトマウスと共通し，非常に有力な候補と考えられた．この遺伝子は100 kb以上の巨大遺伝子であるため，その欠損病を探すアプローチとして，各ドメインに対する抗体を用いた免疫染色によりスクリーニングを行い，cDNAを用いたRT-PCRでmRNAレベルの異常を検出し，染色体DNAレベルでの変異を同定するという方法を用いてパールカン遺伝子変異を探索した．その結果，3症例2種類の遺伝子変異を同定した[12,13]．これらの変異は，それぞれフレームシフトを起こし，変異の直後で転写を終了するアウトオブフレーム変異であった．筆者らはさらに，蛋白質レベルの解析を行い，遺伝子変異によりこの疾患ではパールカンが細胞外に分泌されず，細胞外マトリックスとして機能しないことがわかった．すなわち，パールカン機能完全欠損に起因する症状と考えられた．

2. パールカン機能部分欠損による遺伝性疾患の同定

パールカン分子は前述したように複数のドメインからなる巨大な多機能分子と考えられ，ヒト疾患を考慮するにあたっては，部分欠損などによる他の表現型も考慮する必要がある．そこで，パールカン遺伝子がマップされている染色体1p36に連鎖する疾患を探したところ，Schwartz-Jampel症候群（SJS）が候補として挙げられた．詳細なマッピングの結果，SJSの遺伝子座がパールカン遺伝子と450 kb以内の距離にあることがわかった．SJSは別名，軟骨異栄養性筋強直症（chondrodystrophic myotonia）とよばれ，ミオトニア症状と軟骨異常を伴う遺伝性疾患で，DDSHと異なり患者は生存する．患児は一般に出生時には明らかな症状を認めず，乳児期以降，低身長や特徴的な顔貌に気づかれ，3歳くらいまでに診断される．顔面筋の緊張のため眼裂は狭小となり，口を尖らせた仮面のような顔貌を呈する．その他の合併症としては，小眼症，白内障，斜視，眼振などの眼症状がある．高口蓋，低位耳介などの小奇形もしばしば合併する．骨格異常としては，低身長，大関節の屈曲拘縮，脊椎の後彎が認められる．X線所見にて，扁平椎，骨端，骨幹端異形成がみられるが，骨端，骨幹端異形成は大関節に限られる．大腿骨頭の変化は比較的強く，内反股を認めることがある．この疾患では，骨格異常とミオトニアという臨床症状のユニークな組合せから，その病因解明に興味がもたれていた．また，ミオトニア症状に関しても，他のミオトニア疾患で観察されるミオトニアと異なった特徴をもっており，病因遺伝子の分子機構解明が待たれていた．パールカンは筋細胞をとりまく基底膜にも存在し，神経筋結合部（NMJ）にはとくに多く局在する．Cエレガンスにおけるパールカン相同体と考えられる*unc-52*の欠損では，筋線維の横紋構造（myofilament lattice）が破壊され胎生致死を呈することが知られており[14]，パールカンと筋疾患の関連の可能性が示唆されていた．また，パールカン分子の生物学的機能の一つに，NMJ特異的な非対称型AChEのコラーゲン様ドメイン（collagen-like tail；ColQ）と結合することが知られている．ColQはAChEの構造のうち神経筋接合部基底膜に接着する尾部構造である[15,16]．それゆえ，パールカンはNMJの基底膜にAChEを結合，集束させることにより，AChによる神経筋の興奮とその解除を速やかに調整する機能をもつと予測される[17]．筆者らは，遺伝子座の

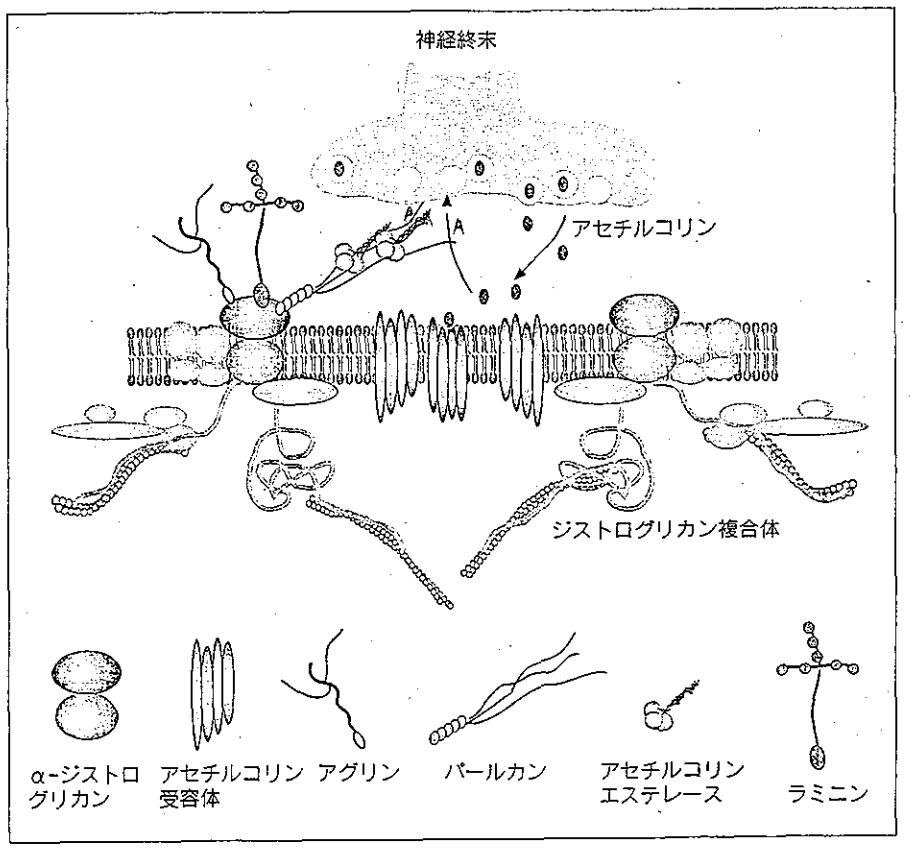

図3　神経筋結合部の模式図
パールカン，ラミニン，アグリンはいずれもその球状ドメインでα-ジストログリカンに結合し，神経筋接合部に集合するとされる．神経終末から放出されるアセチルコリンは受容体に作用した後，速やかにアセチルコリンエステレースにより分解され，筋収縮のシグナルは終了する．パールカンはこのアセチルコリンエステレースの局在に必須と考えられる．

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，軀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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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 *EcoRI* 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 *EcoRI* fragment varied from 10 to 27 (mean 15.2) kb. All but one proband had four *EcoRI* fragments (derived from both chromosomes 4 and 10). One patient had five *EcoRI* 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 *EcoRI* fragment of the same size as that of the proband. Twenty-three patients inherited a small *EcoRI* 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 *EcoRI* fragment varied from 10 to 26 (mean 19.3) kb. The other parents who had a small *EcoRI* fragment (12 fathers and 12 mothers) had 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 *EcoRI* fragment had weakness in the shoulders, but no muscle weakness was observed in one father with a 14 kb fragment.

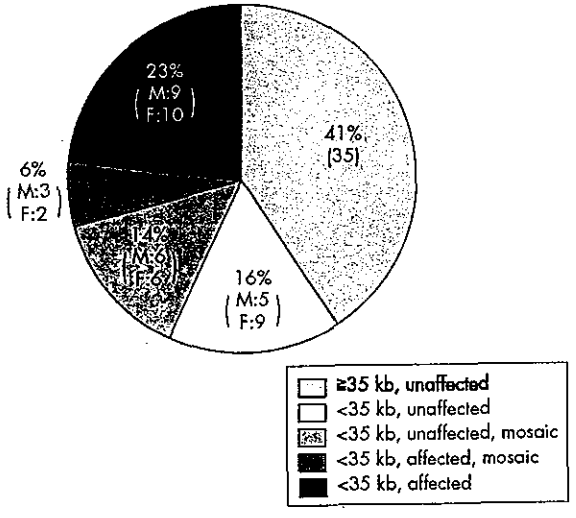

Figure 1 Results of southern blotting analysis and clinical symptoms of 85 families of patients with FSHD1A. M, male; F, female.

Southern blotting analysis showed that 17/50 carrier parents with a deleted allele had five *EcoRI* fragments that included a fainter, small *EcoRI* 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 *EcoRI* 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 *EcoRI* 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 *EcoRI* 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.[21] On the other hand, only 3% of random blood donors have somatic mosaicism.[16] In our study, 17/50 (34%) parents

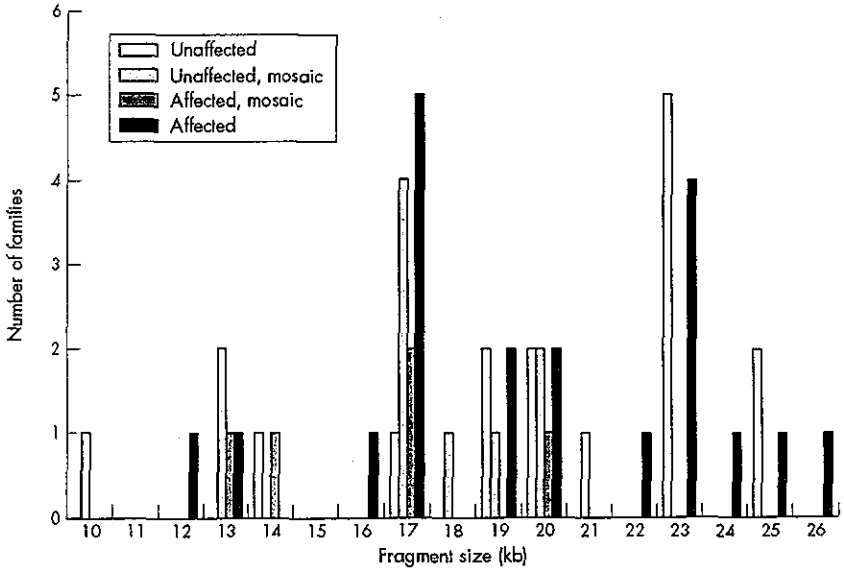

Figure 2 *EcoRI* fragment size of parents with a deleted allele.

with a deleted allele had somatic mosaicism of the 4q35 region, and 17/85 (20%) of patients with FSHD1A inherited a small *Eco*RI 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 *Eco*RI 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

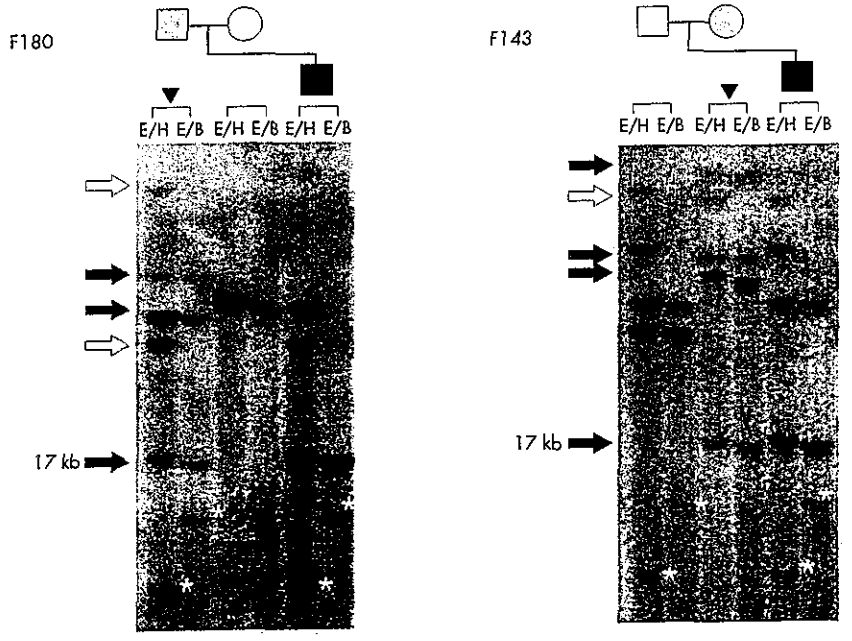

Figure 3 Southern blotting analysis of two families with a proband with FSHD1A and a parent with somatic mosaicism. DNA was digested by *EcoRI/HindIII* (E/H) and *EcoRI/BlnI* (E/B) and separated by PFGE. The father in F180 (arrowhead) had three *BlnI* resistant *EcoRI* fragments (black arrows) that included 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.

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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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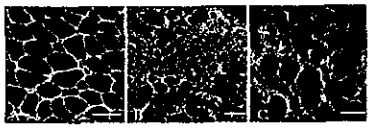
Collagen VI expression and muscle weakness

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.

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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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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.

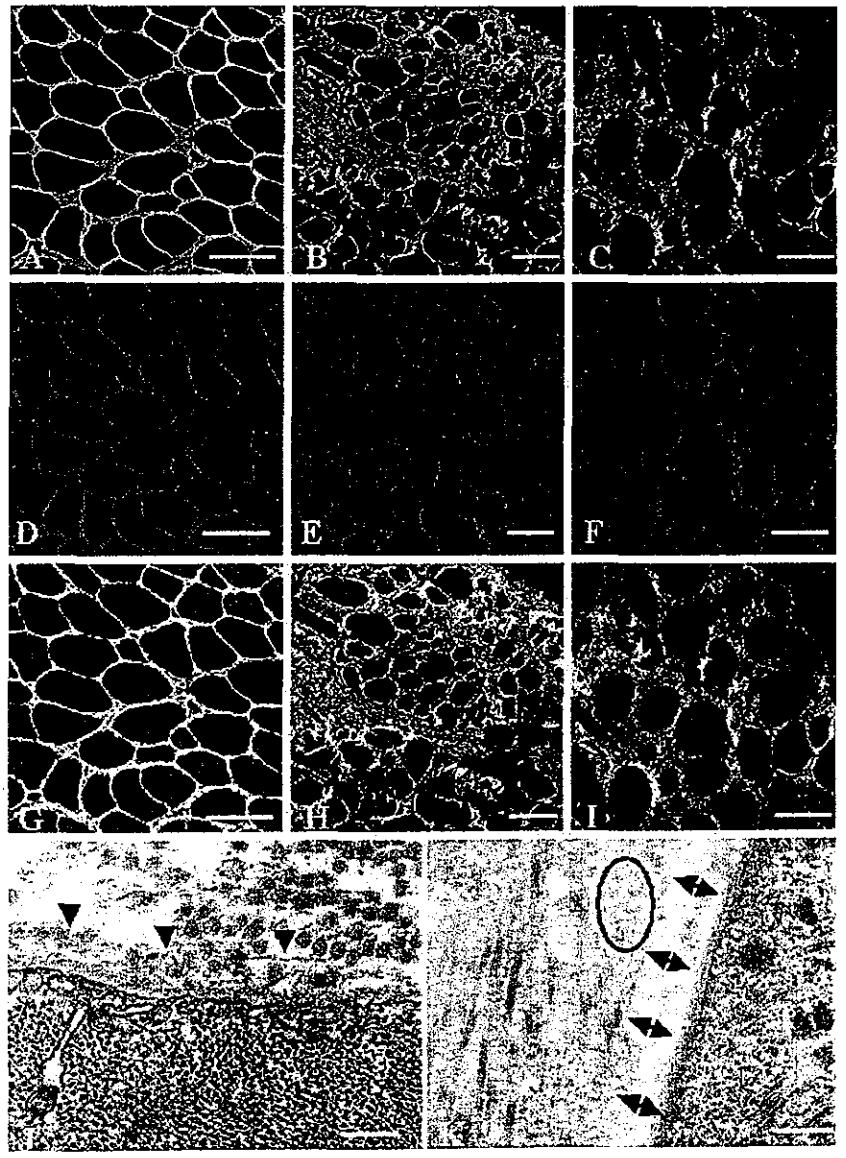

Figure. Pathologic features of the disease. (A–C) Immunostaining for collagen VI (A: normal, B: Fukuyama-type CMD [FCMD], and C: Ullrich disease). Collagen VI is present in the interstitium but is markedly reduced or absent in the sarcolemma in an Ulrich disease patient. (D–F) Immunostaining for collagen 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.

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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.

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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 anti-dystrophin 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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