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α-dystroglycanのO-mannose型糖鎖と細胞外matrix結合に異常をきたす先天性筋ジストロフィーの病態解明と治療法の開発

平成１５－１７年度　総合研究報告書

主任研究者　清水　輝夫

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研究要旨

α-dystroglycan（αDG）の O-mannose 型糖鎖の先天的形成不全が推定される先天性筋ジストロフィー群、特に本邦独特の福山型先天性筋ジストロフィー Fukuyama type congenital muscular dystrophy FCMD、フィンランドにみられる Muscle-eye-brain 病 MEB、および Walker-Warburg 症候群 WWS の分子病態解明と治療法の開発を目的とした。その結果、筋基底層蛋白 laminin α2 に結合する成分であるαDG の O-mannosyl glycan(Sia α2-3Gal β1-4GlcNAc β1-2Man α1-O Ser/Thr)の合成過程の障害で当該３疾患が発症することが確立でき、この糖鎖の骨格筋発生における重要性が確認された。即ち、①当該３疾患筋は、抗αDG 糖鎖抗体の染色性不良、抗αDG コア蛋白質抗体の染色性良好、オーバーレイアッセイでのαDG・ラミニン結合不良の3特徴を共通してもっている、②第２段階 GlcNAc β1→2Man α1-O Ser/Thr の転移酵素 POMGnT1(1p33-34)はゴルジ体膜にあり、その遺伝子の点変異や小欠失により POMGnT1 活性が完全消失して MEB を発症させる、③第１段階糖転移酵素活性 POMT(Man α1→O Ser/Thr)は２つの成分 POMT1/2 の複合体として ER 膜にて機能し、POMT1（9q34.1）と POMT2(14q24.3)それぞれの点変異や小欠失により POMT 活性が完全消失して WWS（約１割）を発症させる、④FCMD(9q31)の責任蛋白質 fukutin には、POMGnT1 に類似して N 端に膜貫通ドメインと stem region、C 端に DxD motif をもつ糖転移酵素類似ドメインがあるが糖転移酵素活性はなく、N 端の膜貫通ドメインを介して POMGnT1 と結合するゴルジ体膜蛋白質であり、POMGnT1 活性の modulator と推量される、⑤αDG の O-mannosyl glycan 合成の第３段階(Gal β1→4GlcNAc β1-2Man α1-O Ser/Thr)、４段階(Sia α2→3Gal β1-4GlcNAc β1-2Man α1-O Ser/Thr)の酵素活性の異常をきたす疾患はみつかっていない。⑥αDG の O-mannosyl glycan 異常によるラミニン結合不全は当該３疾患の骨格筋以外に、末梢神経系（基底層と Schwann 細胞の髄鞘最外側膜の間）、脳表（基底層と脳表アストログリア限界膜の間）にもあてはまり、末梢神経（根）髄鞘形成異常と脳の第１層（カハール・レチウス細胞）および第５、６層細胞の走行異常（白質内滞留と過遊走）が存在する、⑦RNAi の実験から、この糖鎖形成を障害すると体壁骨格筋の形成障害がショウジョウバエでも再現され、この糖鎖は骨格筋発生に必須の成分である、⑧新しいαDG 糖鎖異常症として dystrophy chicken が存在する、⑨治療面で、βDG 分解系として matrix metalloproteinase type 2 & 9 が同定され、この分解系は Duchenne 型および肢帯型筋ジストロフィーsarcoglycanopathy にて特異的に亢進する。しかし FCMD では非亢進であり FCMD の治療には応用できない。FCMD など基底膜が損傷する疾患には、むしろ骨髄移植による幹細胞導入の方が有望である、などが結論された。類似疾患 FKRP 異常症、Large 異常症と dystrophy chicken、およびこれらの治療法は今後に残された課題である。

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A.　研究目的

α-Dystroglycan(αDG)のO-mannose型糖鎖と細胞外matrix結合に異常が疑われる先天性筋ジストロフィー群（α-dystroglycanopathy；福山型先天性筋ジストロフィーFCMD常劣、Muscle-eye-brain病MEB常劣、Walker-Warburg syndrome WWS常劣、FKRP異常症、Large異常症）の分子病態解明と治療法の開発のため、❶αDGのO-mannosyl glycan (Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-O Ser/Thr)の合成各段階の酵素の同定、❷福山型先天性筋ジストロフィーの原因蛋白質fukutinの生理機能の解明、❸脳・末梢神経病態の解明、❹治療法の開発を行う。

B.　研究方法

❶αDGのO-mannosyl glycan合成の各段階の酵素の遺伝子解析および酵素活性の生化学的測定法を確立する（H15-17；遠藤、千葉）。❷fukutinの生化学的糖転移酵素活性を検討し、その生理機能を解明しfukutin結合分子を解析する（H15-17；戸田）。❸フクチン欠損キメラマウスの脳・末梢神経病態を病理形態学的方法で検討する（H17；寺島、松村・清水）。❹治療法に関連して、１）薬物治療のため、ジストロフィー筋のDG分解系metalloproteinase活性の関与とその阻害薬効果を検討する（H15-17；松村・清水）。２）遺伝子・細胞治療のため基盤研究を行う（H15-17；砂田・戸田）。

（倫理面への配慮）

「ヒトゲノム・遺伝子解析研究に関する倫理指針（平成１３年３月２９日文科省・厚労省・経済省告示第１号）」、国立精神・神経センター倫理規定を遵守し、各研究施設の定める倫理規定にもとづいた倫理委員会の承認を得るものとする。

C.　研究結果及び考察

（１）α-Dystroglycan（αDG）は骨格筋を含むほとんどすべての細胞の形質膜に存在し基底層蛋白質lamininと結合する成分で、その分子の中１／３はproline、serine、threonineが多く、serine、threonineに結合するO型糖鎖が多数結合するmucin domainである。一分子あたり約14-15個のO-mannosyl glycan(Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-O Ser/Thr)が存在し、基底層接着分子laminin α2のG domainに結合する。このO-mannosyl glycan合成の第二段階GlcNAcβ1→2Manα1-O Ser/Thrの酵素POMGnT1遺伝子をクローニングした。同遺伝子は、1p33-34に局在、UDP-N-acetylglucosamine:protein O-linked mannose-β-1,2-N-acetylglucosamine transferaseをコードする。本遺伝子がMEBの原因遺伝子であることを解明し、α-dystroglycanopathy研究仮説に直接的根拠が得られた点でepoc-making rearchとなった。

POMGnT1は660アミノ酸からなるII型の膜タンパク質で、N端に膜貫通部位と幹領域が、C端側に酵素活性部位がある。脳神経細胞、末梢神経、筋に特異的に発現し、筋細胞のゴルジ体膜に局在する。N-型糖鎖の生合成（GlcNAc→Man-Asn）に関わる糖転移酵素α-3-D-mannoside β-1,2-N-acetylglucosaminyl transferase I(GnT-I)とは23.2%の相同性を有していた。POMGnT1をHEK293T細胞に発現させるとPOMGnT活性は約100倍増大し、一方GnT-I活性は検出されなかった。

本遺伝子は1p33-34にマップされ、MEB原因遺伝子の存在領域1cMの中に存在した。そこで6症例のMEB患者において本遺伝子を解析したところそれぞれ異なる6種の変異を見出した。3例のsplicing donor site変異のホモ(2例: IVS17+1G>T, 1例: IVS17+1G>A)、1例のミスセンス変異のホモ(1743G>A, Ser550Asn)、1例の1塩基欠失によるフレームシフト変異のホモ(1813delC)、1例のミスセンス変異(1572C>G, Pro493Arg)と1塩基欠失(1970delG)によるフレームシフト変異の複合ヘテロであった。その後の検索でフィンランド人以外にも、イタリア人、ベルギー人、アメリカ人、韓国人、日本人のMEB患者にも見つかり、現在までに１３種の変異が見つかっている。従って、本疾患は従来考えられたフィンランド人に多いわけではなく人種を超えて存在することがわかった。

いずれも変異遺伝子産物の酵素活性は著しく低下していた。また、MEB 骨格筋膜の抗α-dystroglycan 糖鎖抗体の染色性が不良、抗α-dystroglycan コア蛋白質抗体の染色性は良好、オーバーレイアッセイでのαDG のラミニン結合は不良であったことから、本酵素の target として、αDG の O-mannosyl glycan の形成が選択的に欠損していると考えられた。即ちMEB では Man-αDG のままのため、ラミニン結合が不良であり、基底膜・αDG・dystrophin・細胞内骨格の架橋構造が破綻していると考えられる。

以上よりPOMGnT1 糖転移酵素遺伝子は MEB の原因遺伝子であり、哺乳類の O-mannose 型糖鎖が筋および脳で重要な働きを果たしていることが考えられる。筋ジストロフィーの原因遺伝子として糖鎖転移酵素が同定され、実際に患者で変異が確認されたのは初めてのこととなった。Loss of POMGnT1 function（αDG の O-mannosyl glycan の形成障害→laminin との結合不全）により Muscle-eye-brain 病 MEB が発症する。

（2）αDG の O-mannosyl glycan 合成の第一段階酵素活性 protein-O-mannosyl transferase POMT は Man α1-O Ser/Thr を触媒し、ヒト・ラットを含む哺乳類、ショウジョウバエともに酵母や C. elegans 同様に POMT1/POMT2 複合体として活性を有し、ER 膜で機能する。いずれか一方では酵素活性はない。その活性は、合成ヒトαDG peptide の serine/threonine OH と dolichol-phosphate、mannose 存在下で測定できる。

Walker-Warburg 症候群 WWS の約 10 %の患者で 7 種の POMT1 変異（9q34.1）が報告された。いづれも点変異と小欠失である。検討したところ変異 POMT1 と野生型 POMT2 の発現系ではどの組み合わせでも失活しており POMT1 遺伝子異常で POMT 活性が完全に失われていることが証明された。その後、POMT2 の遺伝子異常(14q24.3)が 4 種報告されたが、POMT1 異常症と同様の機序であった。これで、WWS の約 1 割が説明できる。

ショウジョウバエの POMT1/POMT2 のいずれの RNAi でも、αDG の O-mannose 転移酵素活性が失活し、ショウジョウバエ体壁骨格筋の形成に重篤な障害をきたし、体軸がねじれる。

また、MEB 骨格筋膜と同様に、抗αDG 糖鎖抗体の染色性が不良、抗αDG コア蛋白質抗体の染色性は良好、オーバーレイアッセイでのαDG・ラミニン結合は不良であったことから、本酵素の target として、αDG の O-mannosyl glycan の形成が選択的に欠損していると考えられた。即ち WWS では無糖鎖-αDG となっているため、ラミニン結合が不良であり、基底膜・αDG・dystrophin・細胞内骨格の架橋構造が破綻していると考えられる。

以上のことから、ヒトを含む哺乳類、ショウジョウバエなど高等動物では、αDG 糖鎖の形成が最も初期段階(ER 膜)で欠損しているため、ラミニン結合が不良となり、基底膜・αDG・dystrophin・細胞内骨格の架橋構造が破綻していると考えられる。

（3）FCMD の責任蛋白質 fukutin は、N 型糖転移酵素 GnT I や POMGnT1 と類似して、N に膜貫通ドメインと幹領域をもち、C 端に DxD motif を持つ酵素活性ドメインを有する。しかし fukutin には糖転移酵素活性はなく、N 端にある膜貫通ドメインを介して POMGnT1 と結合する Golgi 体膜蛋白質である。第 92 番アミノ酸で N-glycan1個が付加している。Fukutin は POMGnT1と複合体を形成し、αDG の O-mannosyl glycan 合成の第二段階（GlcNAcβ1→2Man α1-O Ser/Thr）に関与する modulator と推定される。この考えは、FCMD 筋や次に述べる fukutin 欠損キメラマウス筋での、抗αDG 糖鎖抗体の染色性不良、抗αDG コア蛋白質抗体の染色性良好、オーバーレイアッセイでのαDG・ラミニン結合不良の結果と良く一致し、αDG の O-mannosyl glycan の形成が選択的に欠損していると考えられた。即ち FCMD では MEB 同様に Man-αDG となっているため、ラミニン結合が不良であり、基底膜・αDG・dystrophin・細胞内骨格の架橋構造が破綻していると考えられる。

FCMD および myd mouse（Large 異常症）の骨格筋では、筋分化の後期に発現する転写因子 myf 6 と myosin heavy chain 2 & 7 の発現が低下し、筋細胞核や筋線維の成熟異常がみられる。また、神経筋接

合部にも著しい異常像があり、αDGのクラスタリングも不良である。従って、FCMD筋は筋発生後期の段階に留まり、その段階で筋細胞死機序が発症していると結論できる。

fukutin 欠損キメラマウスの末梢神経、特に神経根の部分で太い有髄線維中心に著しい異常があり、末梢神経最外層での抗αDG糖鎖抗体の染色性が不良、抗αDGコア蛋白質抗体の染色性は良好で、オーバーレイアッセイでのαDG・ラミニン結合能は著減している。従って、FCMDでは著明な末梢神経髄鞘形成不全をともなっており、骨格筋同様の機序がある。

fukutin 欠損キメラマウスの大脳皮質構築異常について第1層のカハール・レチウス細胞、第5層の錐体路ニューロンが脳表のグリア境界膜を越えて過遊走している。また、第5層細胞群と第6層細胞群は大脳白質内に異所性分布をする。この結果、従来FCMD脳では脳表アストログリアとその基底膜がつくるグリア限界膜に存在するαDGのO-mannosyl glycanの形成不良のため限界膜が脆弱となり、脳室壁から脳表へと移動する神経細胞が一部限界膜を超えて過遊走となる、との説明であったが、この機序は第5、6層細胞にあてはまるが2-4層細胞にはあてはまらないことが判明した。

新たにヒトfukutin創始者変異ノックインマウスを作成し、ノックインホモとノックイン／ヌル複合ヘテロの両者でPOMGnT活性の約80％低下を認めた。しかし、両者とも筋ジストロフィーの発症はなく、複合ヘテロで軽微な大脳皮質層構造異常がみられたのみである。治療へのヒントの可能性がある。

以上から、fukutinはゴルジ体膜でPOMGnT1酵素と結合して、何らかの機序でGlcNAcβ1→2Manα1-O Ser/Thr of αDGに関与し、その欠損にてManα1-O Ser/Thr of αDGしかできないためラミニン結合が不良となり、基底膜・αDG・dystrophin・細胞内骨格の架橋構造が破綻すると考えられる。この結果、FCMDとMEB、WWSはα-dystroglycanopathyであると結論できる。この説は、骨格筋、末梢神経(基底層とSchwann細胞の髄鞘最外層)、脳表(基底層とアストログリア限界膜)に当てはまる。

(4) dystrophic chickenの骨格筋膜のαDGの各種レクチンとの結合実験から、dystrophic chickenのαDGにはGalβ1-3GalNAc、terminal GalNAcが多く、Siaα2-3Galと総シアール酸が減少していることが骨格筋および心筋で明らかになり、抗αDG糖鎖抗体の染色性が不良、抗αDGコア蛋白質抗体の染色性は良好で、オーバーレイアッセイでのαDG・ラミニン結合能が著減している。POMT活性、POMGnT活性、Gal転移活性、シアール酸転移活性には異常なく、O-mannosyl glycan以外のαDG糖鎖異常症の可能性がある。FKRP異常症(19q、先天性MDC1Cと肢帯型LGMD2I)、Large異常症(マウス筋ジストヒト肢帯型)についで新たなα-dystroglycanopathyと考えられる。その実態解明は今後に残された。

(5) FCMDの治療法開発の観点から、①βDGの分解機転の抑制と②遺伝子治療、③細胞治療を試みた。①Dystroglycanにはlaminin結合成分で形質膜外表に存在するα-dystroglycan αDGとdystrophinと結合し細胞内シグナル伝達を担う膜貫通成分β-dystroglycan βDGがあり、そのノックアウトマウスは胎生致死であること、dystroglycanに結合または機能連関する蛋白質(ラミニンなど)により多数の筋ジストロフィーが存在することはすでに周知のことである。これらの筋ジストロフィーに共通する機序として、基底層laminin・dystroglycan・dystrophin・細胞内actin骨格の架橋構造(dystroglycan架橋構造)の破綻がある。その破綻に際し生じるdystroglycan複合体の分解機構としてβDG分解がある。βDGは膜貫通蛋白(43 kDa)であり、metalloproteinase MMPにより13 kDa細胞膜外ドメインが細胞外に放出され、30 kDa成分が残存する。その結果αDGは、膜から遊離、基底層成分からも遊離する。この機序は、筋以外ではがん細胞の転移に関係していると考えられる。この機序は、当初想定していた膜結合型MMPではなく分泌型MMP types 2と9が活性をもち、特異的阻害剤で抑制できる。この分解機序はDMD、sarcoglycanopathy、cardiomyopathic hamsterの骨格筋・心筋で特異的に見られるが、FCMD、fukutin欠損キメラマウス、

MEB、WWS のα-dystroglycanopathy には存在しなかった。従って、FCMD を MMP 阻害薬によって薬物治療できる可能性はない。

②myc-tag をつけたマウス fukutin 遺伝子を電気穿孔法で fukutin 欠損キメラマウス前脛骨筋へ導入すると、fukutin 発現、抗αDG 糖鎖抗体染色・laminin 結合能の回復をみとめる線維が少数みられたが、効率は悪く治療法としては不向きである。

③正常マウスの骨髄由来 ES 細胞を中胚葉へ分化・誘導すると、沿軸中胚葉細胞群の中に筋原細胞が存在する。骨髄移植法にて筋基底膜が障害されるラミニン欠損症マウスや mdx マウスに経静脈的に導入すると骨格筋に生着分化する幹細胞が豊富に存在することが判明し、FCMD モデルマウスで試みる価値がある。

D.　結論

αDG の O-mannosyl glycan 合成の各段階酵素が決定され、fukutin は酵素そのものではなく、POMGnT1 に結合して第２段階酵素活性に関与する Golgi 体膜成分であった。同時に福山型、MEB、WWS の先天性筋ジストロフィーの基本的分子病態も解明され、それらの責任遺伝子・蛋白質の解析、RNAi 実験から、αDG の O-mannosyl glycan は脊椎・無脊椎動物界に広く存在し、基底膜と細胞膜の接着構造をもつすべての臓器に存在して、その発生に基本的役割をもっていること、筋と神経系（中枢および末梢）の形成に決定的影響を持つことを世界に先駆けて解明でき、臨床医学的に先天性筋ジストロフィーの基本病態に関与する essential factor であることを確立した。特に、日本独特で、Duchenne/Becker 型についで多い筋ジストロフィーFCMD の病態が解明できたことは、国際的にも評価されている。未だ、残された先天性筋ジストロフィー（FKRP 異常症、Large 異常症）や dystrophy chicken の解明、これらの治療法開発が残った点が心残りである。

今後の問題点

先天性筋ジストロフィーは欧米に多く脳奇形をともなわないラミニン欠損症と脳奇形を伴う非ラミニン欠損症とに大別でき、後者の大半がα-dystroglycanopathy として説明できるようになり、病態解明と診断法（遺伝子診断法、蛋白生化学的酵素診断法）が大幅に進歩した。今後の問題として、①α-dystroglycanopathy の中枢神経系には脳奇形を伴うのが一般的であるが、中には障害を出さない症例や病系が存在する。このので病態解明は治療法の開発に直接的に関係すると思われる。特に O-mannose glycan αDG が laminin 以外に結合する蛋白が存在する点は重要なことである。②Large 異常症 (myd)、FKRP 異常症、dystrophic chicken の解明が待たれる。③WWS の約 10 %が POMT 異常症として説明可能であるが、残りの 90 %は未解明である。④骨髄移植法など治療の開発が残された、などである。

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Disruption of dystroglycan axis by β-dystroglycan processing in cardiomyopathic hamster muscle

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Abstract

α-Dystroglycan is a cell surface peripheral membrane protein which binds to the extracellular matrix, while β-dystroglycan is a type I integral membrane protein which anchors α-dystroglycan to the cell membrane via the N-terminal extracellular domain. The complex composed of α- and β-dystroglycan is called the dystroglycan complex. Although defects of the dystroglycan gene have not been identified as the primary causes of hereditary diseases in humans, secondary but significant abnormalities of the dystroglycan complex have been revealed in severe muscular dystrophies, including sarcoglycanopathy (LGMD2C, D, E and F). In this study, we investigated proteolytic processing of β-dystroglycan and its effect on the extracellular matrix–cell membrane linkage in cardiomyopathic hamsters, the model animals of LGMD2F. Compared to normal controls, proteolytic processing of β-dystroglycan was activated in the skeletal, cardiac and smooth muscles of cardiomyopathic hamsters and this resulted in the partial disruption of the dystroglycan complex in these tissues. These phenomena were observed from the early phase of muscle degeneration process. Our results suggest that proteolytic processing of β-dystroglycan disrupts the extracellular matrix–cell membrane linkage via the dystroglycan complex and this may play a role in the molecular pathogenesis of muscle degeneration in cardiomyopathic hamsters.
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Keywords: Dystroglycan; Extracellular matrix; Cardiomyopathic hamster; Sarcoglycanopathy

1. Introduction

The dystroglycan complex is a cell membrane-spanning complex composed of α- and β-dystroglycan, which are encoded by a single gene *Dag1* and cleaved into two proteins by posttranslational processing [1]. α-Dystroglycan is a cell surface peripheral membrane protein which binds to laminin in the basement membrane, while β-dystroglycan is a type I integral membrane protein which anchors α-dystroglycan to the cell membrane via the N-terminus of the extracellular domain and binds to the cytoskeletal protein dystrophin via the C-terminal cytoplasmic domain [1–7]. Thus, the dystroglycan complex provides a tight link between the extracellular matrix (ECM) and intracellular cytoskeleton. While the role of the dystroglycan complex in the assembly and maintenance of the basement membrane remains controversial [8–11], these facts indicate that the dystroglycan complex needs to be disrupted efficiently when tissue remodeling takes place under various circumstances and suggest that a specific device may exist for this purpose. As such a candidate, we have reported a matrix metalloproteinase (MMP) activity that cleaves the extracellular domain of β-dystroglycan [12]. The finding that the C-terminal proteolytic fragment of β-dystroglycan loses the ability to bind to α-dystroglycan indicates that the dystroglycan complex is disrupted by this process [12].

The intriguing question would be if proteolytic processing of the dystroglycan complex plays a role in the molecular pathogenesis of muscle degeneration in muscular dystrophies. One condition suitable for testing this possibility is cardiomyopathic hamsters, muscle degeneration of which is caused by the defect of the δ-sarcoglycan gene [13–15]. In muscle, the dystroglycan complex interacts with the sarcoglycan complex,

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comprised of α-, β-, γ-, δ-sarcoglycan and sarcospan, to form the dystrophin–glycoprotein complex (for a review see Refs. [16–20]). While defects of the *Dag1* gene have not been identified as the causes of hereditary diseases in humans, defects of the α-, β-, γ- and δ-sarcoglycan genes have been identified as the causes of severe forms of limb-girdle muscular dystrophies, LGMD2D, E, C and F, respectively [16–20]. Cardiomyopathic hamsters are thus the model animals of LGMD2F. At present, the molecular mechanism by which the deficiency of δ-sarcoglycan results in muscle degeneration in cardiomyopathic hamsters remains unclear. However, several lines of evidence indicate that the dystroglycan complex is severely disrupted and this plays a role in the muscle degeneration process of these animals as well as other types of sarcoglycanopathy and their model animals [19,21–27]. In the present study, we investigated proteolytic processing of β-dystroglycan and its effect on the ECM–cell membrane linkage via the dystroglycan complex in cardiomyopathic hamsters.

2. Materials and methods

2.1. Miscellaneous

Three to fifteen percent SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously [12,25,28,29]. The monoclonal antibody 43DAG/8D5 against the C-terminal 15 amino acids of β-dystroglycan, IIH6 against the carbohydrate residues of α-dystroglycan and 2D9 against the proximal portion of the G domain of laminin α2 chain are kind gifts from Drs L.V.B. Anderson (Newcastle General Hospital), K.P. Campbell (University of Iowa) and H. Hori (Tokyo Medical and Dental College), respectively [1,5,28,30]. *N*-Biphenyl-sulfonyl-phenylalanine hydroxamic acid (BPHA) is a hydroxamate derivative and a specific MMP inhibitor that has narrow ranges of target specificity and low levels of cytotoxicity [12,31].

2.2. Biochemical analysis of the dystroglycan complex in hamster tissues

F1B hamsters and BIO 14.6 and TO-2 cardiomyopathic hamsters were purchased from Bio Breeders Inc. (MA, USA) and handled according to the animal welfare regulations of Teikyo University School of Medicine. After sacrifice of animals by decapitation under anesthesia with diethylether, skeletal (quadriceps femoris) muscle, cardiac muscle, smooth muscle (small intestine) and brain were dissected out quickly. For immunoblot analysis, the tissues were extracted quickly by homogenizing and boiling in a buffer containing 80 mM Tris–HCl, (pH 6.8), 10% SDS, 1% β-mercaptoethanol, 115 mM sucrose, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA, unless stated otherwise [12,25]. The dystroglycan complex was isolated from the skeletal muscle crude membranes by wheat germ agglutinin (WGA) affinity chromatography as described previously [12]. Briefly, the crude membranes were extracted, at 4 °C for 2 h, with 50 mM Tris–HCl (pH 7.4), 0.5 M NaCl, 0.75 mM benzamidine and 0.1 mM PMSF (buffer A) containing 1% Triton X-100. After centrifugation, the supernatant was incubated with WGA-Sepharose 6MB (Amersham Pharmacia Biotech, Little Chalfont, UK) overnight at 4 °C. After extensive washing with buffer A containing 0.1% Triton X-100, the bound fractions were eluted by the same buffer containing 0.35 M *N*-acetyl-D-glucosamine.

2.3. Biochemical analysis of the dystroglycan complex of RT4 cells

The rat schwannoma cell line RT4 was kindly provided by Drs A. Asai (University of Tokyo) and Y. Kuchino (National Cancer Center, Tokyo, Japan) [12,29]. RT4 cells were grown in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum, 16.7 mM glucose, 2 mM glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin. Culture medium was changed every 3 days. When cells grew to near confluence, culture medium was discarded by decanting and living cells were harvested by scraping the culture dishes with rubber policemen. The crude membranes were prepared as described previously [12,28].

3. Results

3.1. Proteolytic processing of β-dystroglycan in the muscle of cardiomyopathic hamsters

We have reported previously that, due to proteolytic processing of the 43 kDa full-size β-dystroglycan (β-DG_{full}), a 30 kDa fragment of β-dystroglycan (β-DG_{30}) is created in several bovine tissues [12]. BIO 14.6 and TO-2 cardiomyopathic hamsters are the descendants from the same ancestral strain and have the identical mutation of the δ-sarcoglycan gene, a genomic deletion including the first exon [13–15]. We performed immunoblot analysis of β-dystroglycan processing in the tissue homogenates of BIO 14.6 and TO-2 cardiomyopathic hamsters, using F1B hamsters as normal controls. First, we analyzed 12-week-old animals showing severe degeneration of skeletal and cardiac muscles (Fig. 1). We have shown previously that β-dystroglycan processing occurs physiologically in the majority of normal bovine tissues, except skeletal muscle, cardiac muscle and brain [12]. This was also the case in hamsters and β-DG_{30} was not detectable in the skeletal and cardiac muscles of F1B hamsters (Fig. 2a). In BIO 14.6 and TO-2 cardiomyopathic hamsters, on the other hand, β-DG_{30} was abundantly present in the skeletal and cardiac muscles

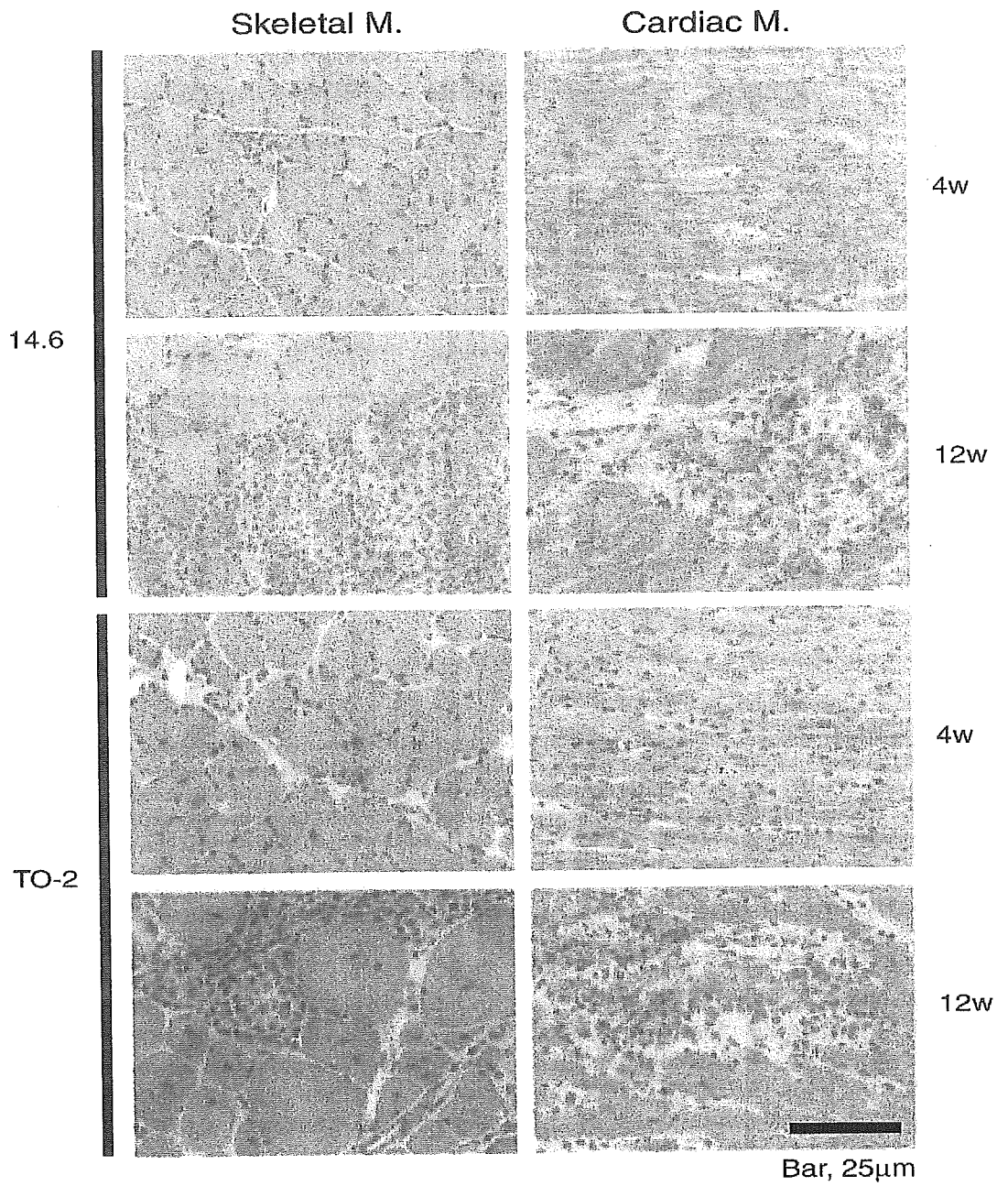

Fig. 1. Dystrophic changes in the skeletal and cardiac muscles of cardiomyopathic hamsters. Shown are the H&E stained cryosections of skeletal (quadriceps femoris) and cardiac muscles of BIO 14.6 and TO-2 cardiomyopathic hamsters. Dystrophic changes, including muscle fiber necrosis, variation of muscle fiber size, internal nucleation, proliferation of interstitial connective tissue and inflammatory cell infiltration, were present in the skeletal and cardiac muscles of both strains of hamsters. These changes were more severe in 12-week- old animals than 4-week- old animals. Bar, 25 μm.

(Fig. 2a), indicating excessive β-dystroglycan processing in the striated muscle of these two strains.

To see if this phenomenon is specific to striated muscle, we analyzed brain and smooth muscle of 12-week- old BIO 14.6 and TO-2 cardiomyopathic hamsters. Similar to bovine brain, β-DG_{30} was not detectable in the brain of F1B hamsters (Fig. 2a). Interestingly, β-DG_{30} was also undetectable in the brain of BIO 14.6 and TO-2 cardiomyopathic hamsters (Fig. 2a), indicating that β-dystroglycan processing was not activated in the brain of these two strains. We have shown previously that β-DG_{30} is barely detectable in normal bovine smooth muscle [12]. Similarly, β-DG_{30} was barely detectable in the smooth muscle of F1B hamsters (Fig. 2a). However, β-DG_{30} was more abundant in the smooth muscle of BIO 14.6 and TO-2 cardiomyopathic hamsters than F1B hamsters, indicating excessive β-dystroglycan processing beyond the physiological level in the smooth muscle of these strains (Fig. 2a).

These results indicated muscle-specific activation of β-dystroglycan processing in the advanced stages of BIO

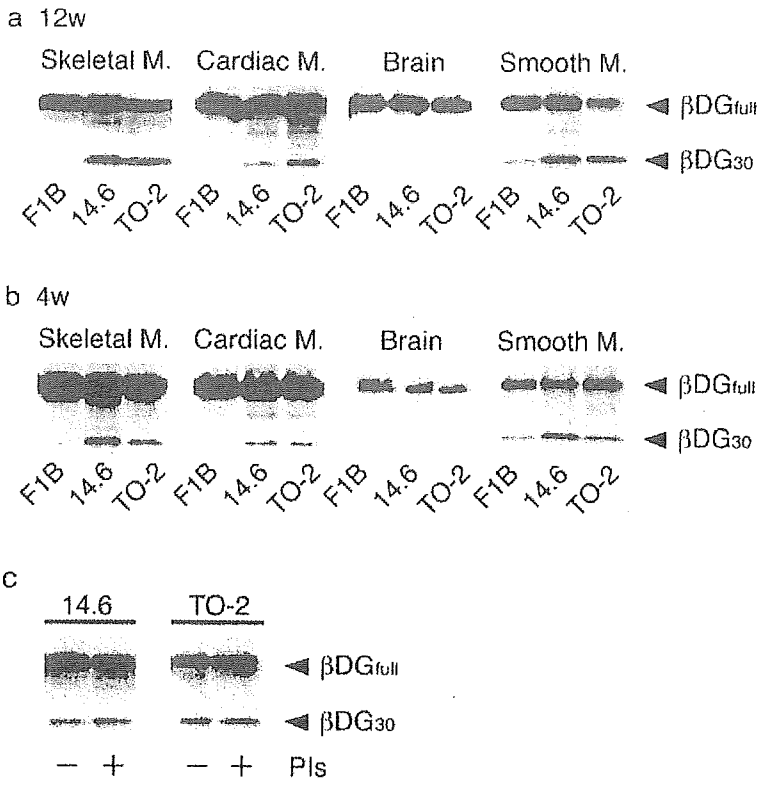

Fig. 2. β-Dystroglycan processing by MMP in cardiomyopathic hamsters. Tissue homogenates of normal (F1B), BIO 14.6 and TO-2 cardiomyopathic hamsters were analyzed by immunoblotting using 43DAG/8D5. (a) Twelve-week- old hamsters. While only β-DG_{full} was detectable in the skeletal and cardiac muscles of F1B hamsters, β-DG_{30}, in addition to β-DG_{full}, was detected in the skeletal and cardiac muscles of BIO 14.6 and TO-2 cardiomyopathic hamsters. β-DG_{30} was undetectable in the brain of either F1B or BIO 14.6 and TO-2 cardiomyopathic hamsters. While β-DG_{30} was barely detectable in the smooth muscle of F1B hamsters, it was more abundant in the smooth muscle of BIO 14.6 and TO-2 cardiomyopathic hamsters. (b) Four-week- old hamsters. Changes similar to those of 12-week- old hamsters were observed. (c) The skeletal muscles of 12-week- old BIO 14.6 and TO-2 cardiomyopathic hamsters were extracted by the SDS buffer with (+) or without (−) various protease inhibitors (PIs), including 20 mg/ml BPHA, 0.6 mg/ml pepstatin A, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM benzamidine, 1 mM PMSF, 1 mM EDTA and 1 mM EGTA. The proteolysis of β-dystroglycan was not significantly inhibited by the presence of protease inhibitors in the SDS buffer.

14.6 and TO-2 cardiomyopathic hamsters. To see if this is the consequence of severe muscle degeneration in the advanced stages, we next analyzed 4-week- old animals showing only mild muscle degeneration (Fig. 1). The results were similar to those of 12-week- old animals and β-DG_{30} was detectable in the skeletal and cardiac muscles of BIO 14.6 and TO-2 cardiomyopathic hamsters, but not F1B hamsters (Fig. 2b). In addition, β-DG_{30} was more abundant in the smooth muscle of BIO 14.6 and TO-2 cardiomyopathic hamsters than F1B hamsters, and β-DG_{30} was undetectable in the brain of F1B, BIO 14.6 or TO-2 cardiomyopathic hamsters (Fig. 2b). These results indicated that β-dystroglycan processing started in the early phase of muscle degeneration in BIO 14.6 and TO-2 cardiomyopathic hamsters.

We presume that β-dystroglycan processing in the muscles of BIO 14.6 and TO-2 cardiomyopathic hamsters is not due to degradation during the preparation process in vitro, because the muscles were extracted quickly by homogenizing and boiling in a buffer containing 10% SDS. To further confirm this, we extracted the muscles by the SDS buffer with or without various protease inhibitors, including BPHA, a hydroxamate derivative which inhibits the proteolysis of β-dystroglycan effectively [12]. If the proteolysis of β-dystroglycan occurs during the preparation process, it will be significantly inhibited by the presence of these protease inhibitors in the SDS buffer. However, the proteolysis of β-dystroglycan was not significantly inhibited by the protease inhibitors (Fig. 2c). These results indicated that the proteolysis of β-dystroglycan in the muscles of BIO 14.6 and TO-2 cardiomyopathic hamsters occurred before the preparation process, most likely in vivo. This is also consistent with our previous finding that the proteolysis of β-dystroglycan occurs in vivo in certain cultured cells [12].

The dystroglycan complex can be isolated from the detergent extracts of the membranes by WGA affinity chromatography, because it binds to WGA lectin with high affinity [5,12]. To compare the integrity of the dystroglycan complex between normal and cardiomyopathic hamsters, we solubilized the skeletal muscle crude membranes with Triton X-100 and applied the solubilates to WGA affinity chromatography. In normal hamsters, the core components of the dystroglycan complex, α-dystroglycan, β-DG_{full} and laminin α2 chain, all bound to WGA-Sepharose (Fig. 3).

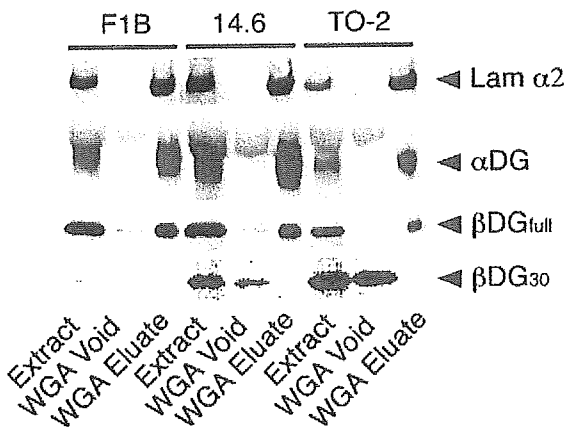

Fig. 3. Disintegration of the dystroglycan complex due to β-dystroglycan processing by MMP in the muscle of cardiomyopathic hamsters. The dystroglycan complex was isolated from the Triton X-100 extracts of hamster skeletal muscle membranes using WGA affinity chromatography. Shown are the Triton X-100 extracts, the voids and eluates of WGA affinity chromatography analyzed by immunoblotting using 43DAG/8D5, IIH6 and 2D9. In normal hamsters, the core components of the dystroglycan complex, α-dystroglycan, β-DG_{full} and laminin α2 chain, all bound to WGA-Sepharose. In BIO 14.6 and TO-2 cardiomyopathic hamsters, α-dystroglycan, β-DG_{full} and laminin α2 chain, but not β-DG_{30}, bound to WGA-Sepharose.

Likewise, α-dystroglycan, β-DG_{full} and laminin α2 chain all bound to WGA-Sepharose in BIO 14.6 and TO-2 cardiomyopathic hamsters (Fig. 3). However, β-DG_{30} did not bind to WGA-Sepharose and remained in the voids (Fig. 3), indicating that β-DG_{30} was not linked to laminin α2 chain via α-dystroglycan. Similar results were obtained using the cardiac and smooth muscle membranes (not shown). These results indicate that, due to processing of β-dystroglycan, a fraction of the dystroglycan complex was disrupted in the muscle of cardiomyopathic hamsters.

3.2. Dissociation of α-dystroglycan from the cell membrane due to processing of β-dystroglycan in RT4 cells

RT4 cells are convenient tools to investigate the proteolytic processing of β-dystroglycan in vitro, because they express both β-DG_{full} and β-DG_{30} constantly [12]. We speculated that this processing in RT4 cells was due to MMP, because (1) the cleavage site was localized to the extracellular domain of β-dystroglycan and (2) the processing was inhibited by specific inhibitors of MMP [12]. In this study, we further characterized this proteolytic activity in RT4 cells. We have shown previously that the proteolytic activity was associated with cells and not secreted into the culture medium, because the activity was retained in the total homogenates of harvested RT4 cells (Fig. 4). In this study, we fractionated the total homogenates of harvested RT4 cells into the membrane fraction. The proteolytic activity was not only retained but also augmented considerably in the membrane fraction (Fig. 4),

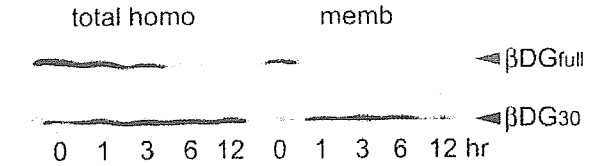

Fig. 4. Enrichment of the MMP activity that processes β-dystroglycan in the membrane fraction. The total homogenates or crude membranes of harvested RT4 cells were incubated at 37 °C for various time periods and analyzed by immunoblotting using 43DAG/8D5. When the total homogenates were further fractionated into the membrane fraction, the proteolytic activity was not only retained but also augmented considerably.

indicating that the activity was associated with the cell membrane.

To see the effects of β-dystroglycan processing on the stability of ECM–cell membrane linkage, we cultured RT4 cells in the presence or absence of BPHA [12]. We separated the total homogenates of harvested RT4 cells into the soluble fraction and crude membrane fraction by centrifugation. In the presence of BPHA, the processing of β-DG_{full} was inhibited and β-DG_{30} was not detectable in the total homogenates (Fig. 5a). After centrifugation, both β-DG_{full} and α-dystroglycan were completely recovered in the membrane fraction (Fig. 5a). In the absence of BPHA, processing of β-DG_{full} was active and β-DG_{30} was detected in the total homogenates (Fig. 5a). After centrifugation, β-DG_{30}, as well as β-DG_{full}, were recovered in the membrane fraction (Fig. 5a), consistent with the previous finding that β-DG_{30} retains the transmembrane domain [12]. On the other hand, only a small fraction of α-dystroglycan was recovered in the membrane fraction and a substantial amount was released to the soluble fraction (Fig. 5a), indicating the dissociation of α-dystroglycan from the cell membrane.

To further confirm this, we cultured and harvested RT4 cells in the presence or absence of BPHA. We then incubated the total homogenates of harvested cells in the presence or absence of BPHA at 37 °C for varying time periods and separated them into the soluble fraction and crude membrane fraction by centrifugation. In the absence of BPHA, β-DG_{full} decreased and β-DG_{30} increased with the progression of time (Fig. 5b). While both β-DG_{full} and β-DG_{30} were recovered in the membrane fraction after centrifugation, the amount of α-dystroglycan recovered in the membrane fraction decreased and instead that released to the soluble fraction increased with the progression of time (Fig. 5b). These events were suppressed considerably by BPHA (Fig. 5b). Similar results were obtained using another hydroxamate derivative L-*N*-(*N*-hydroxy-2-isobutylsuccinamoyl)-leucyl isobutyl amido (a kind gift of A. Okuyama, Banyu Tsukuba Research Institute) (not shown), which also inhibits β-dystroglycan processing [12]. Altogether, the results indicated that, in RT4 cells, the membrane-associated MMP activity dissociated α-dystroglycan from the cell membrane by processing

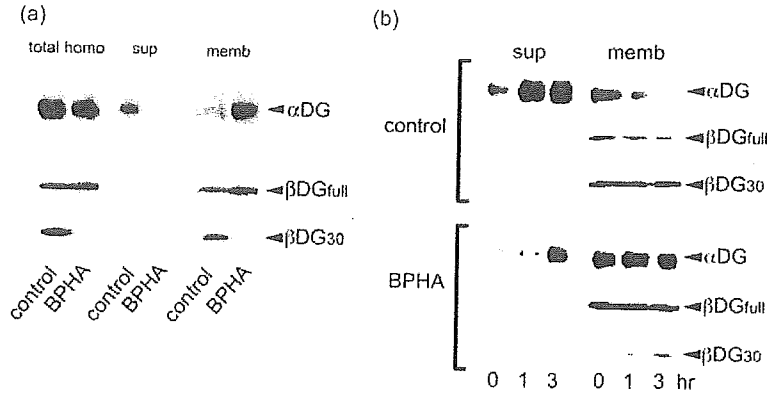

Fig. 5. Dissociation of α-dystroglycan from the cell membrane due to β-dystroglycan processing by MMP. (a) RT4 cells were cultured in the presence or absence of 20 μg/ml of BPHA for 3 days. The homogenates of harvested cells were separated into the soluble fraction and crude membrane fraction by centrifugation, and analyzed by immunoblotting using 43DAG/8D5. In the presence of BPHA, the processing of β-DG_{full} was inhibited and β-DG_{30} was not detectable in the total homogenates. After centrifugation, β-DG_{full} and α-dystroglycan were recovered in the membrane fraction. In the absence of BPHA, β-DG_{30}, in addition to β-DG_{full}, was detected in the total homogenates. After centrifugation, both β-DG_{full} and β-DG_{30} were recovered in the membrane fraction. While a small fraction of α-dystroglycan was recovered in the membrane fraction, a substantial amount was released to the soluble fraction. (b) After 3 days of culture in the presence or absence of 20 μg/ml of BPHA, RT4 cells were harvested, homogenized and incubated in the presence or absence of 20 μg/ml of BPHA at 37 °C for various time periods. Then the homogenates were separated into the soluble fraction and crude membrane fraction by centrifugation, and analyzed by immunoblotting using 43DAG/8D5. In the absence of BPHA, β-DG_{full} decreased and β-DG_{30} increased with the progression of time. While both β-DG_{full} and β-DG_{30} were recovered in the membrane fraction after centrifugation, the amount of α-dystroglycan recovered in the membrane fraction decreased and that released to the soluble fraction increased with the progression of time. These events were suppressed considerably by BPHA.

the extracellular domain of β-dystroglycan, thus disrupting the link between the ECM and cell membrane.

4. Discussion

Although defects of the *Dag1* gene have not been identified as the primary causes of hereditary diseases in humans, intriguing abnormalities of the dystroglycan complex have been revealed in severe muscular dystrophies. For instance, defective glycosylation of α-dystroglycan has been demonstrated in several forms of congenital muscular dystrophies, including Fukuyama-type congenital muscular dystrophy, MDC1C/LGMD2I, muscle–eye–brain disease, Walker–Warburg syndrome and $Large^{myd}$ mice, which are the model animals of congenital muscular dystrophy [32–41]. These diseases are all caused by the primary defects of the genes encoding glycosyltransferases, which are presumed to disturb glycosylation of α-dystroglycan. Because glycosylation of α-dystroglycan is crucial for the binding of laminin [5,42,43], its defect is expected to perturb this binding and result in the disruption of the ECM–cell membrane linkage via the dystroglycan complex. This scenario is supported by the finding that the antibody against the carbohydrate residues of α-dystroglycan involved in the binding of laminin induced a dystrophic phenotype in cultured muscle cells [44].

In the first half of the present study, we investigated proteolytic processing of β-dystroglycan and its effect on the ECM–cell membrane linkage via the dystroglycan complex in the muscle of cardiomyopathic hamsters, where α-dystroglycan has been reported to be dissociated almost completely from β-dystroglycan and the other components of the dystrophin–glycoprotein complex by several workers [21–24]. Compared to normal hamsters, proteolytic processing of β-dystroglycan was activated in the skeletal, cardiac and smooth muscles of cardiomyopathic hamsters in vivo. As a result, a fraction of the dystroglycan complex was disintegrated, causing a partial disruption of the ECM–cell membrane linkage in these tissues. These phenomena were observed from the early phase of muscle degeneration, indicating that it was not simply the consequence of severe muscle degeneration in the advanced stages. Altogether, these findings indicate that proteolytic processing of β-dystroglycan is involved in the muscle degeneration process of cardiomyopathic hamsters. It should also be noted, however, that only a small fraction of β-dystroglycan was proteolyzed and a substantial fraction remained intact in both 4- and 12-week- old animals (Fig. 2). This suggests that additional mechanisms should work to cause the severe disruption of the dystroglycan complex reported by other workers in these animals [21–24]. Alternatively, it could explain the apparent discrepancy that the deficiency of α-dystroglycan in these animals appears less severe in our study (Fig. 3) than in those by other workers [21–24].

While this study is the first to demonstrate the 30 kDa fragment of β-dystroglycan in animal models of neuromuscular diseases, Anderson et al. reported a similar fragment of β-dystroglycan in the biopsied skeletal muscle of sarcoglycanopathy patients [30]. Importantly, their finding was specific to sarcoglycanopathy and not observed in other diseases [30]. Aside from neuromuscular diseases, the 30 kDa fragment of β-dystroglycan has been detected in

rabbit cardiac muscle concomitant with the onset of irreversible ischemic injury [45] and in human breast and colon cancers together with the reduction of α-dystroglycan expression which correlated with higher tumor grade and stage [46]. Altogether, these findings raise an intriguing possibility that a mechanism similar to cardiomyopathic hamsters may work in a variety of pathological conditions, including sarcoglycanopathy, ischemic heart disease and cancer.

In the second half of the study, we characterized the proteolytic activity creating the 30 kDa fragment of β-dystroglycan in cultured RT4 cells, hoping that this would give us further insight into this mechanism. The results indicated that, in the case of RT4 cells, the protease was MMP enriched in the cell membrane fraction, raising the possibility that it might be a membrane-type MMP. The results also revealed that β-dystroglycan processing resulted in the dissociation of α-dystroglycan from the RT4 cell membrane, demonstrating for the first time the physical disruption of the ECM–cell membrane linkage. At present, we do not know if the protease creating the 30 kDa fragment of β-dystroglycan in cardiomyopathic hamsters is identical with that in RT4 cells. For this purpose, it is necessary to determine the N-terminal amino acid sequences of these fragments and to identify the proteases themselves by future studies. If they turn out to be identical, we can envision that, due to processing of the extracellular domain of β-dystroglycan by MMP, the link between the ECM and cell membrane via the dystroglycan complex is disrupted in the muscle of cardiomyopathic hamsters. This will have a profoundly deleterious effect on the muscle cell viability, because the disruption of the ECM–cell membrane linkage is expected to result in sarcolemmal instability, which will further augment muscle cell damage in a vicious cycle.

Acknowledgements

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