表1　RNA結合蛋白質とリピートとの結合[9]

	CUG-BP	MBNL1	PKR(p20)
MS2-2	−	−	−
UG 24	++++++	−	−
CA 24	−	−	−
CUG 7	−	−	−
CUG 16	−	++	−
CUG 21	−	+++	−
CUG 37	−	++	−
CUG 41	+	++	−
CUG 70	−	++	+
CCUG 7	+	−	−
CCUG 22	−	++++	−
CCUG 50	−	+++	−
CAGG 22	−	−	−
CAGG 50	+	−	−

ていた)に注目した．これはショウジョウバエの筋肉発生分化や目の光受容体の分化を司る*muscleblind*のオーソログだからである．このMBNL1は，**表1**で明らかなように，CUGリピートとCCUGリピートの両方に結合することが初めて証明された．MBNL1は，CHHG(Hはグアニン以外のヌクレオチド)に最も強く結合することがわかった[9]．**図4**のように，MBNL1が結合するのは二重鎖を形成するRNAではなく，少しこぶ(ミスマッチを含むヘアピン)のある二重鎖RNAであることも推測された．

図2のCには，*ZNF9*遺伝子のイントロンにある$(TG)_n(TCTG)_n(CCTG)_n$から転写された$(UG)_n(UCUG)_n(CCUG)_n$とGST-MBNL1の結合様式を示す．このゲルリターデーション・アッセイで明らかなように，GST-MBNL1はこの配列にもきちんと結合し，抗GST抗体の添加によって移動度が変化することがわかる．

これらの実験により，MBNL1がDMの症状を決める最有力の候補にあがった．

7. MBNLファミリー

ヒトMBNLには3つのホモログが存在する．MBNL1，MBNL2(MBLL)，MBNL3(MBXL)である．興味深いことに，この3種類の蛋白質は，どれも核内に存在するリピートRNAと共局在することが示されている．

2003年の終わりに，MBNL1のノックアウトマウス(MBNL1-KO)が発表された[10]．これによれば，MBNL1-KOはミオトニアや白内障というDM症状を呈するとともに，トロポニンTのスプライシングパターンも変化していることがわかり，DMのモデルになることがわかった．すなわち，MBNL1もCUG-BP同様，スプライシングを調節している可能性がある．

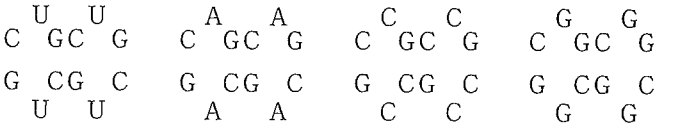
著者らは，まず，MBNL1-3の基質特異性を調べてみた．その結果，MBNL1だけではなく，MBNL2もMBNL3もCUGならびにCCUGリピートに結合することがわかった(未発表)．この結果は，MBNL群がリピート領域に結合する能力をもっているということである．**図5**に示すように，本来RNAスプライシングを調節しているMBNL1-3やCUG-BPが伸長リピートを含むmRNAにトラップされ，働くべきところで働くことができなくなったことがDMの症状に関係する，というのが現在のところまでの結論である．

8. CELFファミリー

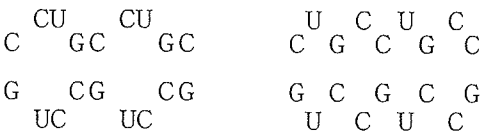
CUG-BPなどのRNA結合蛋白質ファミリーをCELFファミリーと呼ぶ．CUG-BPはCELF1とも呼ばれ，CUG-BP2/ETR-3はCELF2である．CELFはヒトでは全部で6つ存在しており，主にスプライシング調節因子であろうと考えられている．また，翻訳制御にもかかわっているという話もある．

問題は，MBNLファミリーとCELFファミリーとの関係である．双方合わせて10種類近くの因子がmRNAのスプライシングにかかわることから，これらの発現組織の違いによってスプライシングパターンが変化する可能性，相乗効果など，これらの機能分担によってもスプライシングが促進されたり抑制されたりすることなどがわかれば，今後のDM研究は飛躍的に進むだろう．

1. CNG



2. CCUG



3. CAG/CUG

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
GUCGUCGUCGUCGUCGUCGUCGUCGUCGUCGUC

図 4　MBNL1 が結合するリピート

MBNL1 は，この図の CNG リピートや CCUG リピートに結合するが，CAG/CUG の二重鎖には結合しない．

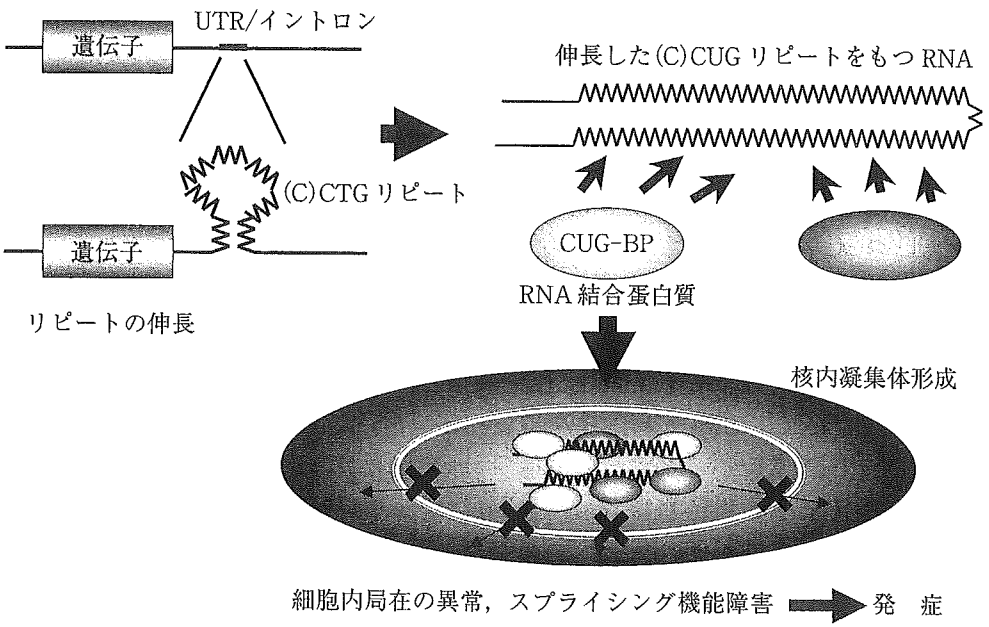


図 5　MBNL1 と ZNF9 の CCUG リピートとの結合

9. 酸化ストレス説

DM1 と DM2 の症状がすべてリピート RNA 結合蛋白質捕捉で説明できるわけではない．現在までに数々の細胞変化が指摘されているが，最もよく知られているのが DM1 に対する酸化ストレスである．

これは，長い CTG リピートを導入した DM cDNA を安定形質発現させた C2C12 細胞や者由来の線維芽細胞が酸化ストレスに弱いこから唱えられたものである[11]．人工的に酸化トレスを与えると，これらリピート伸長細胞

アポトーシスを起こして死んでいくことが明らかになった．また，これらの細胞死は，N-アセチルシステインや水溶性ビタミンE(Trolox)の添加で抑制されることがわかり，確かに酸化ストレスが細胞死に関与していることが示された[12]．

一方，著者らは160CTGリピートを含むDMPK cDNAを導入した細胞と5CTGリピートを導入した細胞の発現プロファイルをDNAチップを用いて解析し，リピートが長い細胞では酸化ストレス後，αBクリスタリン，endosulfine，MBNL1の発現低下を認めた[13]．これらが，リピート伸長細胞における酸化ストレス脆弱性にどうかかわっているか，そのメカニズムは不明だが，160CTG導入細胞でのMBNL1の発現の低下は，これらの間に何らかの関連があることを示唆するものである．

おわりに
—治療への展望—

DMの効果的な治療法はまだ見つかっていない．酸化ストレスを抑えるビタミンEが既に治療に用いられているが，細胞死阻害の実験結果からすると，効果を得るには多少，投与量が足りない可能性がある．

また，RNAに焦点が絞られている現在，MBNL1の発現量の調節などが新しい治療の標的になってきている．この場合，ホモログであるMBNL2やMBNL3の代償作用も考慮に入れなければならず，今少し基礎研究の充実が望まれる．とにかく，遺伝子ベースの治療法がようやく目の前に見えてきたという段階であり，これからの研究の発展が望まれる．

文　献

1) 紀　嘉浩ほか：病気を起こす反復配列．日経サイエンス　**395**(9)：58-65，2004.
2) Ranum LP, Day JW: Myotonic dystrophy: RNA pathogenesis comes into focus. Am J Hum Genet **74**: 793-804, 2004.
3) Day JW, et al: Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. Neurology **60**: 657-664, 2003.
4) Sasagawa N, Ishiura S: Myotonic dystrophy protein kinase. Wiley Encyclopedia Mol Med **5**: 2203-2205, 2002.
5) Takahashi N, et al: Coexpression of the CUG-binding protein reduces DM protein kinase expression in COS cells. J Biochem **130**: 581-587, 2001.
6) Mankodi A, et al: Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science **289**: 1769-1773, 2000.
7) Timchenko LT, et al: Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implication for myotonic dystrophy. Hum Mol Genet **5**: 1115-1121, 1996.
8) Takahashi N, et al: The CUG-binding protein(CUG-BP) binds specifically to UG dinucleotide repeats in a yeast three-hybrid system. Biochem Biophys Res Commun **277**: 518-523, 2000.
9) Kino Y, et al: Muscleblind protein, MBNL1/EXP, binds specifically to CHHG repeats. Hum Mol Genet **13**: 495-507, 2004.
10) Kanadia RN, et al: A muscleblind knockout model for myotonic dystrophy. Science **302**: 1978-1980, 2003.
11) Usuki F, et al: Differential signaling pathways following oxidative stress in mutant myotonin protein kinase cDNA transfected C2C12 cell lines. Biochem Biophys Res Commun **267**: 739-743, 2000.
12) Usuki F, et al: In vivo protection of a water-soluble derivative of vitamin E, Trolox, against methylmercury-intoxication in the rat. Neurosci Lett **304**: 199-203, 2001.
13) Takeshita Y, et al: Decreased expression of alpha-B-crystallin in C2C12 cells that express human DMPK/160CTG repeats. Basic Appl Myol **13**: 305-308, 2003.

トリプレットリピート病と蛋白質の進化

Triplet-repeat disorders and evolution of proteins

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◎翻訳領域に存在するトリプレットが伸長することで発病する一群の疾患の発症メカニズムが明らかになりつつある．また，特殊なポリアミノ酸(とくに，疎水性アミノ酸)の性質によって細胞死が引き起こされることがわかってきた．同時に，ヒトゲノムの解析から蛋白質の進化的な観点からも興味深い知見が得られている．

Key word：トリプレットリピート病，ポリアミノ酸，細胞死，蛋白質の進化

トリプレットリピート病は奇妙な遺伝様式で知られる一群の疾患で，そのきわだった特徴は表現促進現象(anticipation)である[1]．幾世代にも発症が認められる家系では親よりも子，子よりも孫の発症が早く，しかも重篤な症状を呈する．最初にみつかった脆弱X症候群や筋強直性ジストロフィーでは非翻訳部位に存在する三塩基の伸長が原因であるのに対し，翻訳領域に存在するCAG三塩基繰り返しの伸長は蛋白質内のグルタミン残基の伸長をもたらし，種々の神経症状を発現させる．後者の代表例がHuntington病や脊髄小脳失調症1，2，3型で，これらでは異常蛋白質の蓄積が神経細胞死を引き起こすという説が有力になっている．非翻訳領域のトリプレットの伸長はDNAのメチル化異常やRNA結合蛋白質の機能異常を引き起こす[2,3]が，翻訳領域での伸長はアミノ酸ポリマーが挿入されたことによる蛋白質の機能獲得が原因らしい．また，最近になってグルタミンだけではなくアラニンやロイシンの伸長が疾患を引き起こすこともわかりポリアミノ酸病としての一群の発症メカニズムに注目が集まっている(表1)．興味深い点はこれらの多くが行動異常を引き起こすということである[4]．

本稿ではこのポリアミノ酸病に焦点を絞り，ポリアミノ酸がなぜ細胞死を誘導するのかについて，また，ポリアミノ酸の進化的機能についても議論してみたい．

ポリグルタミン病

図1はCAG三塩基の伸長を簡単な図にまとめたものである．この伸長は発生の早い段階で起こることが知られており，Huntington病の場合には男性の精子ですでにCAGが伸長していたり，また成人脳ではリピート長にモザイク性が認められることから，減数分裂時または体細胞分裂の初期に伸長したと考えられている．現在までに知られているトリプレットリピート病ではCAGリピートの伸長がきわめて多く，このトリプレットだけがなぜ伸びやすいのかについては確固たる証明はされていない．図2にはHuntington病の責任遺伝子Huntingtinのアミノ酸配列を示す．HuntingtinのN末端から18番目からグルタミン(一文字記号Q)が続くが，この長さが34個以下であれば発病しないが，36個を超えるとHuntington病になることが知られている．

表 1　ポリアミノ酸領域の伸長による疾患

ポリグルタミン	Huntington 舞踏病(HD) 歯状核赤核淡蒼球ルイ体萎縮症(DRPLA) 球脊髄性筋萎縮症(SBMA) 脊髄小脳失調症(SCA)1, 2, 6, 7, 17 型 Machado-Joseph 病(MJD)
ポリアラニン	眼咽頭型筋ジストロフィー(OPMD) X 染色体連鎖性精神遅滞・てんかん X 染色体連鎖性精神遅滞・成長ホルモン欠損 先天性中心性低換気症候群(CCHS) 鎖骨頭蓋形成異常症(CCD) 合多指症(SPD) 手足性器症候群(HFGS) 瞼裂縮小・下垂・内眼角贅皮 2 型(type 2 BPES) 全前脳症(HPE)
ポリアラニン or ポリロイシン	Huntington 病様 2(HDL2)
ポリアスパラギン酸	仮性軟骨発育不全症候群(PSACH) 多発性骨端形成不全症(MED)

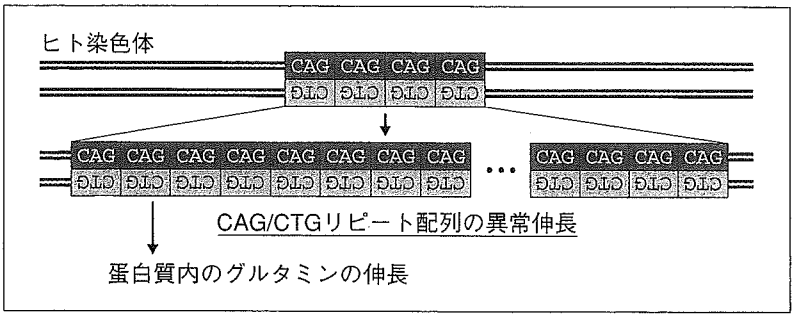

図 1　トリプレットリピート病

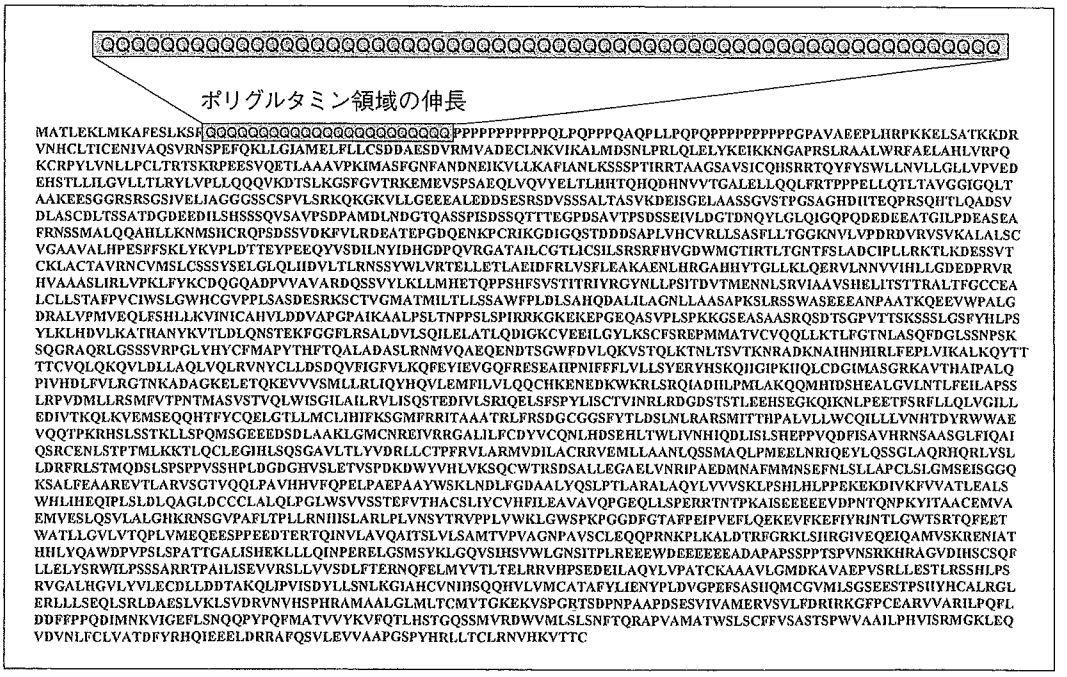

図 2　Huntingtinにおけるポリアミノ酸領域の伸長

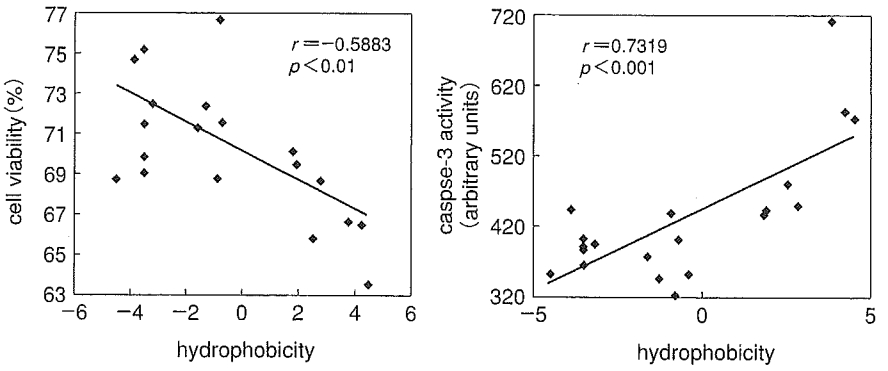

図 3　ポリアミノ酸による細胞毒性と疎水性(Kyte, J. and Doolittle, R. F. : *J. Mol. Biol.*, **157** : 105-132, 1982 より)
点は個々のアミノ酸を示す.

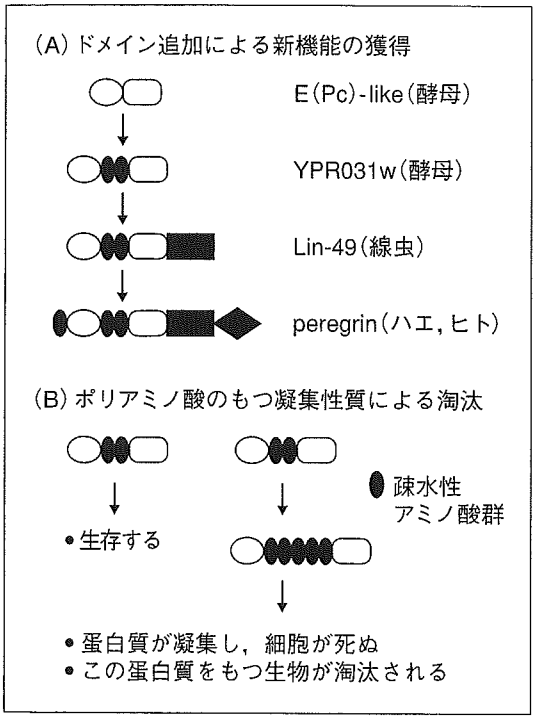

図 4　蛋白質の進化
A : ドメイン追加による新機能の獲得, B : ポリアミノ酸のもつ凝集性質による淘汰.

モデル細胞の構築

そこで著者らはポリグルタミンを含むポリアミノ酸がなぜ病気を引き起こすのかを知るために, 黄色蛍光蛋白質 YFP の C 末端側に 30 個の同一アミノ酸をつなげた人工遺伝子を作製し, これを細胞に発現させてみた[5]. その結果, ロイシンを 30 個つなげるとその細胞は死にやすくなることが明らかになった. 細胞死を起こすときにはカスパーゼ 3 の活性が高くなることがわかりアポトーシスが起こっていることもわかった. ロイシンの後, 以下, イソロイシン, バリンとその傾向は続いた. ところが, 予想に反してグルタミンを 30 個つないでもアラニンを 30 個つないでも細胞は死ぬことがなかった. 検討の結果, 図 3 に明らかなように, アミノ酸の疎水度と細胞毒性に相関関係があることが明らかになったのである[6]. また, 疎水度に比例してカスパーゼ 3 の活性も上昇していることがわかり細胞死はアポトーシス様であることがわかった. また, 疎水性ポリアミノ酸は細胞内では核周囲に凝集体をつくることも明らかになった.

以上の結果をまとめると図 4 のようになる. 一般に蛋白質は原核生物から真核生物に, そして脊椎動物へと進化するときに, 図 4-A のように機能を複雑化させてきたと考えられている. これはゲノムに再編(重複など)が起こって, 1 つの蛋白質にドメインが追加され, だんだん蛋白質が複数の機能を備えるようになってきたらしい. そのとき, たまたま図 4-B のようにポリアミノ酸が伸長した場合, 一定以上に伸びた疎水性ポリアミノ酸は, 細胞に毒性を与え, 細胞が死に, 最終的にその生物が死滅するのではないか, と著者らは考えた. 現在のところ, 核周囲への蛋白質の凝集が細胞死の引き金になるのか, 凝集自体に毒性があるのかについては結論は出ていない. 今後, 各種シグナル(たとえば, 核・Golgi 等局在シグナル)を付与したコンストラクトを用いた結果を比較し, どちらが正しいか検証したい.

ヒトゲノムの検索

この結果を受けて著者らはヒトゲノムの網羅的

表 2　ヒトゲノム中のポリアミノ酸含有蛋白質

	11～20	21～30	31～40	41～
Ala	37	0	0	0
Arg	0	0	0	0
Asn	0	0	0	0
Asp	3	0	0	0
Cys	0	0	0	0
Gln	28	15	3	0
Glu	26	3	1	0
Gly	18	2	0	0
His	12	0	0	0
➡Ile	0	0	0	0
➡Leu	2	0	0	0
Lys	0	0	0	0
Met	0	0	0	0
➡Phe	0	0	0	0
Pro	16	1	0	0
Ser	8	4	0	2
Thr	2	0	0	0
Trp	0	0	0	0
Tyr	0	0	0	0
➡Val	0	0	0	0

検索を行うことにした[7]．ヒトのゲノムは約 30 億塩基対から構成され，24,000 個の遺伝子をコードしている．この蛋白質産物をすべてサーチし，ポリアミノ酸をもっている蛋白質がいくつ存在するかを調べたところ，驚くべき結果が得られた(表 2)．表には，蛋白質中にポリアミノ酸を 11～20 個，21～30 個，31～40 個，41 個以上もつものがヒトゲノム中にいくつあるかを調べたものである．

まず注目すべきは矢印のところで，ポリイソロイシン，ポリフェニルアラニン，ポリバリンをもつ蛋白質がない，という点である．また，ポリロイシンをもつものが 2 つしかない，という点である．これに加えてポリアミノ酸病として注目を集めているポリグルタミン含有蛋白質が何と 44 個，ポリアラニン含有蛋白質が 37 個も存在するということも予想外であった．

これはなにを意味しているのであろうか．著者らの仮説“疎水性ポリアミノ酸が細胞死を引き起こすために，生命体は進化の過程でポリアミノ酸含有蛋白質を捨て去った”の一部はたしかに正しいものと思われる．なぜなら，ポリ(疎水性)アミノ酸含有蛋白質は，ヒトだけではなくすべての生命体で保存されていないからである．それではなぜヒトは，疾患を引き起こすと考えられるポリグルタミン含有蛋白質を，こんなにも多くもってるのであろうか．逆に，細胞に害はないと思わるポリアルギニンやポリリシンをもつ蛋白質が在しないことも不思議な現象である．これらの答は得られていないが，著者はポリグルタミン生命体にとって何らかの必須の生理作用をもつめに，細胞死を引き起こすぎりぎりの長さのもを保持せざるをえなかった，という仮説をとりい．転写因子のいくつかのなかにポリグルタミが保存されていることも，それを支持するものある．またこれ以外にもアミノ酸固有の化学的質も無視することはできず，システインやヒスジンなどは細胞内の酸化還元状態や重金属イオ動態を変化させ細胞を死に至らしめる可能性が，り，塩基性アミノ酸は核内の恒常性を異常な状に変えるのかもしれない．

おわりに

トリプレットリピート病のなかで翻訳領域中三塩基の伸長によって起こるポリアミノ酸性疾は比較的原因の解明が進んでいる．しかし，疾で一番多いポリグルタミン病ですら，発症機構異論が投げかけられている[8]．ポリグルタミン含蛋白質がアポトーシスを誘導するという人もいば，逆に細胞死を防ぐように働くという説も提されている．グルタミンの数を 30 個から 150 個上げると細胞が死ぬという著者らの結果は前者近いが，ヒトゲノムにこれほど多くのポリグルミン含有蛋白質が存在することの説明はまだでていない．また，Huntington 病のようにグルタンが 34 個から数個増えただけで急激に発病す理由も明らかになっていない．この非常に厳密決まっているグルタミン数は，たぶん，特徴的高次構造に依存する可能性が強いが，今後のさなる蛋白質の性質と細胞内凝集メカニズムの解が待たれている．

文献

1) 石浦章一・他：トリプレットリピート病の発症メカニズム．現代化学，**403**：46-51，2004.
2) Ranum, L. P. W. and Day, J. W.：Myotonic dystrophy：RNA pathogenesis comes into focus. *Am. J. Hum. Genet.*, **74**：793-804, 2004.

3) Kino, Y. et al. : Muscleblind protein, MBNL1/EXP, binds specifically to CHHG repeats. *Hum. Mol. Genet.*, **13** : 495-507, 2004.
4) McGuffin, P. et al. : Toward behavioral genomics. *Science*, **291** : 1232-1233, 2001.
5) Oma, Y. et al. : Intracellular localization of homopolymeric amino acid-containing proteins expressed in mammalian cells. *J. Biol. Chem.*, **279** : 21217-21222, 2004.
6) Oma, Y. et al. : Comparative analysis of the cytotoxicity of homopolymeric amino acids. *Biochim. Biophys. Acta.*, **1748** : 174-179, 2005.
7) 石浦章一：タンパク質の進化に関する一考察．遺伝，**58**(4)：88-92，2004.
8) Bates, G. : Huntingtin aggregation and toxicity in Huntington's disease. *Lancet*, **361** : 1642-1644, 2003.

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◆Drug-Eluting Stent——最新情報

（企画：田辺健吾／三井記念病院循環器内科）

＊＊

冠動脈インターベンション(PCI)後の再狭窄を予防する強力なデバイスとして，薬剤溶出性ステント(DES)が登場した．わが国でもシロリムス溶出性ステント(Cypher™)が，2004年8月に保険償還され，日常臨床において使用可能となり普及率は急速に上昇している．本特集ではCypher™およびTAXUS™の各トライアルの最新エビデンスから次世代DESの展望，さらにはDES時代のIVUSの役割，DESのdark side，DESを用いたPCI vs. CABGのトライアル，抗血小板療法や適応に関する日本特有の問題など，いまDESの臨床使用にあたり最も必要かつ注目の最新情報を，光と影の両面から第一線の識者により解説していただく．

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ORIGINAL ARTICLE

Autophagic Vacuoles with Sarcolemmal Features Delineate Danon Disease and Related Myopathies

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Abstract

Among the autophagic vacuolar myopathies (AVMs), a subgroup is characterized pathologically by unusual autophagic vacuoles with sarcolemmal features (AVSF) and includes Danon disease and X-linked myopathy with excessive autophagy. The diagnostic importance and detailed morphologic features of AVSF in different AVMs have not been well established, and the mechanism of AVSF formation is not known. To address these issues, we have performed detailed histologic studies of myopathies with AVSF and other AVMs. In Danon disease and related AVMs, at the light microscopic level, autophagic vacuoles appeared to be accumulations of lysosomes, which, by electron microscopy consisted of clusters of autophagic vacuoles, indicative of autolysosomes. Some autolysosomes were surrounded by membranes with sarcolemmal proteins, acetylcholinesterase activity, and basal lamina. In Danon disease, the number of fibers with AVSF increased linearly with age while the number with autolysosomal accumulations decreased slightly, suggesting that AVSF are produced secondarily in response to autolysosomes. Most of the AVSF form enclosed spaces, indicating that the vacuolar membranes may be formed in situ rather than through sarcolemmal indentation. This unique intracytoplasmic membrane structure was not found in other AVMs. In conclusion, AVSF with acetylcholinesterase activity are autolysosomes surrounded by secondarily generated intracytoplasmic sarcolemma-like structure and delineates a subgroup of AVMs.

Key Words: Autophagic vacuole, Autophagy, Danon disease, LAMP-2, Lysosome.

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INTRODUCTION

Danon disease, an X-linked vacuolar cardiomyopathy and myopathy, is caused by primary deficiency of lysosome-associated membrane protein-2 (LAMP-2), a major lysosomal membrane protein (1–4). Muscle biopsies contain small autophagic vacuoles with cytoplasmic debris. The membranes of these vacuoles have structural features of sarcolemma and biochemical activities of acetylcholinesterase (AChE) and nonspecific esterase (NSE) (5). Although some sarcolemmal proteins, including dystrophin, have been detected in vacuolar membranes (3), the presence of other sarcolemmal proteins has not been studied. In addition, the pathomechanism by which LAMP-2 deficiency leads to the formation of these peculiar autophagic vacuoles with sarcolemmal features (AVSFs) is still unknown.

AVSFs are also seen in X-linked myopathy with excessive autophagy (XMEA) (6), infantile autophagic vacuolar myopathy (AVM) (7), and adult-onset AVM with multiorgan involvement (8). XMEA is clinically characterized by a mild pure skeletal myopathy. In contrast, infantile AVM involves both cardiac and skeletal muscles and patients die within several months after birth, whereas adult-onset AVM affects multiple organs including liver, kidney, and skeletal muscles. All of these diseases show multilayered basal lamina and the deposition of C5b-9 over the surface of the muscle fiber; these features are not seen in Danon disease. Nevertheless, these diseases are likely to share a common pathomechanism since they also have AVSF similar to those seen in Danon disease (9).

To delineate subtypes of AVMs and to gain insights into their pathomechanisms, we have performed detailed histologic evaluations of muscle from patients with Danon disease, XMEA, infantile AVM, and adult-onset AVM, and from LAMP-2 deficient mice (10, 11). Moreover, to evaluate the specificity of the AVSF we have also characterized autophagic vacuoles in other lysosomal myopathies, including acid maltase deficiency (AMD), sporadic inclusion body myositis (SIBM), and distal myopathy with rimmed vacuoles (DMRV), which has recently been shown to be the same disease as hereditary inclusion body myopathy (HIBM).

MATERIALS AND METHODS

Patients

We examined skeletal muscles of ten affected men from 8 families with genetically confirmed Danon disease. We also

confirmed this diagnosis by immunohistochemistry to demonstrate absence of LAMP-2 in skeletal muscle. Age at muscle biopsy varied from one year to 29 years, average 15 years ± 9. One patient underwent 2 biopsies from his left biceps brachii muscle at ages one year and from his right quadriceps femoris muscle at age 16 years (12). We also studied muscle from a 2-month-old boy with infantile AVM (7), a 41-year-old man with adult-onset AVM with multiorgan involvement (8), and an 18-year-old man with probable XMEA who showed typical clinicopathologic features of the disease but without a family history of myopathy.

Control specimens were obtained from 10 individuals with morphologically normal muscle. In addition, we also studied muscle from 21 patients with AMD (9 infants, 6 children, and 6 adults), 18 patients with DMRV/HIBM, and 20 patients with SIBM. We confirmed that all DMRV/HIBM patients had mutations in the gene encoding UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (13).

Histochemistry

All biopsy specimens were taken from either the biceps brachii or quadriceps femoris muscle. These tissue samples were frozen in liquid nitrogen-cooled isopentane for histochemistry and immunohistochemistry. Transverse serial frozen sections of 8-μm thickness were stained with hematoxylin and eosin (H&E), modified Gomori trichrome, and a battery of histochemical methods, including AChE and NSE stains.

Immunohistochemistry

We performed indirect immunofluorescence staining on 5-μm serial cryosections of muscle according to previously described methods (14). These sections were incubated at 37°C for 2 hours with primary mouse monoclonal IgG antibodies against AChE, lysosomal membranous proteins: LAMP-1, lysosomal integral membrane protein-1 (LIMP-1), LIMP-2, and 19 primary monoclonal or polyclonal antibodies against various sarcolemmal proteins and extracellular matrix proteins (Tables 1 and 2). We also used antibodies against an intralysosomal protein, cathepsin L, and endosomal proteins, VAMP-7, Rab5, transferrin receptor (TfR), and low-density lipoprotein receptor (LDL-R). These were subsequently incubated at room temperature for 1 hour with a secondary antibody, fluorescein isothiocyanate (FITC)-labeled goat F(ab')2 anti-mouse IgG (Leinco Technology, St. Louis, MO) or anti-rabbit IgG (H&L) (Leinco). For double immunolabeling using mouse monoclonal anti-LIMP-1 and rabbit polyclonal anti-dystrophin antibodies (a generous gift from Dr. Imamura), we used two secondary antibodies: FITC-labeled anti-mouse IgG (Leinco) and rhodamine-labeled anti-rabbit IgG (Leinco). We also have stained serial sections with Alexa 488 conjugated α-bungarotoxin (Molecular Probe, Eugene, OR) and were examined by fluorescence microscopy. Furthermore, in other sections, after incubation with primary antibodies we stained with the avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA) using another secondary antibody: biotinylated goat anti-mouse IgG (Vector). The reaction was visualized with 3,3′-diaminobenzidine (DAB) as the substrate, yielding a brown reaction product. Normal mouse IgG, diluted to the same concentration as the primary antibodies, was used as a negative control.

To estimate presence of the sarcolemmal proteins in vacuolar membrane, we scored the signal of the antibodies from negative (−) to strong (+++) relative to their immunoreactivity in the sarcolemma. The strong score (+++) indicates that the reactivity level in vacuoles equals that in the sarcolemma. Moreover, we counted the numbers of 1) muscle fibers with intracytoplasmic vacuoles highlighted with dystrophin, and 2) muscle fibers with intracytoplasmic overexpression of LIMP-1, in randomly selected fields of all the patients, and calculated the average percentages of both types of muscle fibers in each patient. Statistical analysis of the correlation between the age of the patients and the numbers of muscle fibers immunoreacting dystrophin or LIMP-1 was performed using linear regression.

Electron Microscopy

For electron microscopy, biopsy specimens were fixed in buffered 2% isotonic glutaraldehyde at pH 7.4, postfixed in osmium tetroxide, and embedded in Epoxy resin. Ultrathin sections were stained with uranyl acetate and lead nitrate, and examined with an H-7000 electron microscope (Hitachi, Tokyo, Japan).

Immunoelectron Microscopy

We performed immunoelectron microscopy by preembedding labeling methods. We used muscle biopsy specimens frozen in liquid nitrogen-cooled isopentane without paraformaldehyde prefixation. The specimens were cut in a cryostat into 10-μm transverse sections without thawing and fixed in chilled 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4) for 10 minutes. The fixed sections were washed 5 times in phosphate-buffered saline (PBS). To eliminate nonspecific reactions, sections were incubated for 30 minutes at room temperature in PBS containing 10% normal goat serum and 1% bovine serum albumin (BSA) with PBS. The sections were then incubated at 4°C overnight with one of the following primary mouse monoclonal IgG antibodies: LIMP-1 and the C-terminus of dystrophin. After washing for 30 minutes in PBS, the sections were incubated at 4°C overnight with a secondary antibody: 10-nm-gold-labeled rat anti-mouse antibody (British Biocell International, Cardiff, UK). Subsequently, the sections were fixed in 0.5% glutaraldehyde and postfixed in osmium, and embedded in Epoxy resin. Ultrathin sections were counterstained with uranyl acetate and lead nitrate.

LAMP-2-Deficient Mice and Pathological Methods

We analyzed tibialis anterior muscle from 2 LAMP-2-deficient mice (10, 11) at ages 4 months and 16 months and age-matched normal mice. Muscle specimens were frozen in liquid nitrogen-cooled isopentane for histochemistry and immunohistochemistry or fixed with glutaraldehyde for electron microscopy. Transverse serial frozen sections of 10-μm thickness were stained with H&E, modified Gomori trichrome,

TABLE 1. Summary of Histochemistry and Immunohistochemistry in Various Myopathies with Autophagic Vacuoles

			Expression on Vacuolar Membrane		
	Manufacturer of Antibody	Dilution	Danon Disease and Related AVMs	Rimmed Vacuolar Myopathies	AMD
Histochemistry					
NSE	–	–	+++	–	–
AChE	–	–	+++	–	–
PAS	–	–	+	–	+++
Acid P	–	–	± to ++	++	++
Immunohistochemistry					
AChE	Chemicon, Temecula, CA	1:2000	+++	–	–
AChR	Molecular Probe, Eugene, OR	1:300	–	–	–
C-terminus of Dystrophin	Novocastra, Newcastle Upon Tyne, UK	1:100	+++	– to +	– to +
Rod domain of Dystrophin	Novocastra	1:50	+++	– to +	– to +
N-terminus of Dystrophin	Novocastra	1:20	+++	– to +	– to +
α-Sarcoglycan	Novocastra	1:100	+++	– to +	– to +
β-Sarcoglycan	Novocastra	1:100	+++	– to +	– to +
γ-Sarcoglycan	Novocastra	1:200	++	– to +	– to +
δ-Sarcoglycan	Novocastra	1:50	+++	– to +	– to +
α-Dystroglycan	Upstate, Lake Placid, NY	1:100	++	– to +	– to +
β-Dystroglycan	Novocastra	1:200	+++	– to +	– to +
Dystrobrevin	RDI, Flanders, NJ	1:100	++	– to +	– to +
Dysferlin	Novocastra	1:50	++	– to +	– to ±
Utrophin	Novocastra	1:50	+	– to ±	–
Caveolin-3	Transduction Labs, Lexington, KY	1:100	++	– to +	– to +
β-Spectrin	Novocastra	1:100	++	– to +	– to +
Laminin α2	Chemicon,	1:5000	++	– to +	– to +
Integrin β1	Genex, Helsinki, Finland	1:100	+++	– to +	– to +
Perlecan	Chemicon	1:100	++	– to +	– to +
Agrin	A generous gift from Dr. Sugiyama (32)	1:100	++	– to +	– to +
Fibronectin	Biomedical Tech., Stoughton, MA	1:1000	++	– to ±	– to ±
Collagen IV	Novocastra	1:1000	– to +	– to ±	– to ±
Collagen VI	ICN, Aurora, OH	1:500	– to +	–	– to ±

Both antibodies against fibronectin and agrin were rabbit polyclonal antibodies. All the other antibodies were mouse monoclonal antibodies. AChR was evaluated by binding to α-bungarotoxin. AMD, acid maltase deficiency; NSE, non-specific esterase; AChE, acetylcholinesterase; PAS, periodic acid Schiff; Acid P, acid phosphatase; AchR, acetylcholine receptor.

and a battery of histochemical methods, and the same immunohistochemical methods described above.

RESULTS

Histochemistry and Immunohistochemistry

By routine histologic studies, the vacuolar membranes in Danon disease, probable XMEA, infantile AVM and adult-onset AVM were essentially identical (Table 1). All muscle samples showed mild to moderate variation in fiber size. There were no necrotic fibers except in muscle from adult-onset AVM, which revealed a few necrotic and regenerating fibers. There were scattered small basophilic granules rather than vacuoles in the muscle fibers in H&E-stained sections (Fig. 1). Histochemistry revealed AChE and NSE activities in the vacuolar membranes and the vacuolar structures of the granules. Immunohistochemistry also confirmed presence of AChE in those vacuoles. However, they did not bind to α-bungarotoxin, indicating the absence of acetylcholine receptors (AChRs) in the vacuolar membranes.

By immunohistochemistry, the AVSF reacted for all the tested sarcolemmal and extracellular matrix proteins in the vacuolar membranes in muscle from patients with Danon disease and related AVMs, although reactivity levels of the proteins were variable (Table 1; Fig. 1). However, only collagens IV and VI showed less intense reactivity in the vacuolar membranes than that in the sarcolemma. Most of the AVSFs were scattered throughout the cytoplasm rather than clustered in the subsarcolemmal region. On serial transverse 5-μm sections, most of the AVSFs formed a closed space and the vacuolar membranes were not connected to the sarcolemma with only a few exceptions (Fig. 1Y). Longitudinal sections demonstrated the oval shape of the AVSF, confirming the closed structure of the vacuoles (Fig. 1Z). Vacuolar membranes connected to the sarcolemma were seen in only 2 patients; both were more than 20 years old.

In muscle from patients with Danon disease, LIMP-1, a lysosomal membrane protein, showed accumulations scattered

TABLE 2. Summary of Lysosomal and Endosomal Proteins for Immunohistochemistry in Danon Disease and Related AVMs

Antigen	Manufacturer	Dilution	Expression in the Muscle Fibers
Lysosomal protein			
LAMP-1	Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA	1:100	++
LAMP-2	DSHB	1:100	−
LIMP-1	DSHB	1:100	+++
LIMP-2	A generous gift from Dr. Tanaka (10)	1:200	+
Cathepsin L	Abcam, Cambridge, UK	1:100	+
Endosomal protein			
Rab5	BD Bioscience, Franklin Lakes, NJ	1:50	+
LDL-R	Progen Biotechnik, Heidelberg, Germany	1:100	+
VAMP-7	A generous gift from Dr. Galli (29)	1:200	++
Transferrin R	Lab Vision, Fremont, CA	1:100	+

Antibody against LIMP-2 was rabbit polyclonal and antibody against LDL-R was chicken polyclonal. All the other antibodies were mouse monoclonal.

throughout the fibers in a distribution identical to that of the small basophilic granules on H&E-stained sections (Fig. 2; Table 2), indicating that most autophagic vacuoles in Danon disease are autolysosomes. These autolysosomal accumulations were surrounded by dystrophin-positive membranes in some fibers but not in others (Fig. 2). LAMP-1 and LIMP-2 showed slightly increased expression in fibers with LIMP-1-positive granules (data not shown). Muscle fibers with dystrophin-positive vacuoles accounted for 0.5% to 14.3%, increasing in proportion with age ($y = 0.016 + 0.40x$, $r = 0.94$; Fig. 3). Muscle fibers with autolysosomal accumulations, both with and without dystrophin-positive vacuolar membranes, accounted for 23.7% to 28.7%, showing a slight tendency to decrease with age ($y = 28.6 - 0.15x$, $r = 0.71$; Fig. 3).

LDL-R, TfR, and Rab5 showed mild upregulation mainly in fibers with autolysosomal accumulations in Danon disease and related AVMs (Table 2). Cathepsin L was expressed weakly, mainly in fibers with autolysosomal accumulations. Only VAMP-7 was strongly expressed, mainly in the non-vacuolated fibers without autolysosomal accumulations.

There were occasional intracytoplasmic vacuoles with sarcolemmal proteins in muscles from patients with other AVMs (i.e. DMRV/HIBM, SIBM, and AMD) but their presence was less consistent than in Danon disease and related AVMs. In addition, they never showed AChE or NSE activity. In DMRV/HIBM and SIBM, fibers with sarcolemmal protein-associated vacuoles accounted for approximately 5% to 15% of fibers with rimmed vacuoles (Fig. 4; Table 1). In AMD, sarcolemmal and extracellular matrix proteins were present in some vacuolar membranes. The frequency of fibers with sarcolemmal proteins-associated vacuoles was less than 5% of vacuolated fibers in infantile AMD, and 10% to 15% of vacuolated fibers in childhood and adult-onset AMD.

Electron Microscopy and Immunoelectron Microscopy

In Danon disease and related AVMs, electron microscopy revealed scattered clusters of autophagic vacuoles containing cytoplasmic debris, electron dense materials, and myeloid bodies. Some of these autophagic vacuoles had basal lamina on the luminal side, while other clusters were not limited by a membrane (Fig. 5).

Immunoelectron microscopy showed many autophagic vacuoles; however technical limitations posed by preparing samples from frozen tissue without prefixation prevented us from clearly defining vacuolar membranes. At higher magnification, dystrophin signals were detected on the cytoplasmic side of the vacuolar membrane and along the periphery of the vacuoles (Fig. 5). In contrast, the LIMP-1 antibody signals were associated with autophagic materials including glycogen particles and cytoplasmic debris within the vacuoles, suggesting that the vacuoles are limited by membranes with sarcolemmal features and contain multiple small autophagic vacuoles derived from autolysosomes.

Muscle Pathology in Mice

Muscles from LAMP-2-deficient mice at both 4 and 18 months of age showed features of AVSFs at both light and electron microscopic levels. There were slight variations in fiber size and small vacuoles with basophilic granules by H&E. The granules contained acid phosphatase-positive material. These AVSFs had AChE and NSE activities similarly to those in Danon disease. The frequency of muscle fibers with the AVSFs decorated by NSE and AChE activities was 0.4% at 4 months and 8% at 16 months (data not shown). On immunohistochemistry, the vacuolar membranes were stained with antibodies against dystrophin and other sarcolemmal proteins as well as extracellular matrix proteins, whereas LAMP-2 was completely absent in the muscle. On electron microscopy, there were scattered intracytoplasmic autophagic vacuoles with glycogen particles and cytoplasmic debris (data not shown).

DISCUSSION

In muscle from patients with Danon disease and related AVMs, the membranes of AVSF showed immunoreactivity for all of the sarcolemmal and extracellular matrix proteins tested. Dystrophin and dystrobrevin are cytoskeletal proteins localized along the cytoplasmic side of the sarcolemma (15). Sarcoglycans and β-dystroglycan are transmembranous proteins and are components of "dystrophin bolts" (16). Utrophin

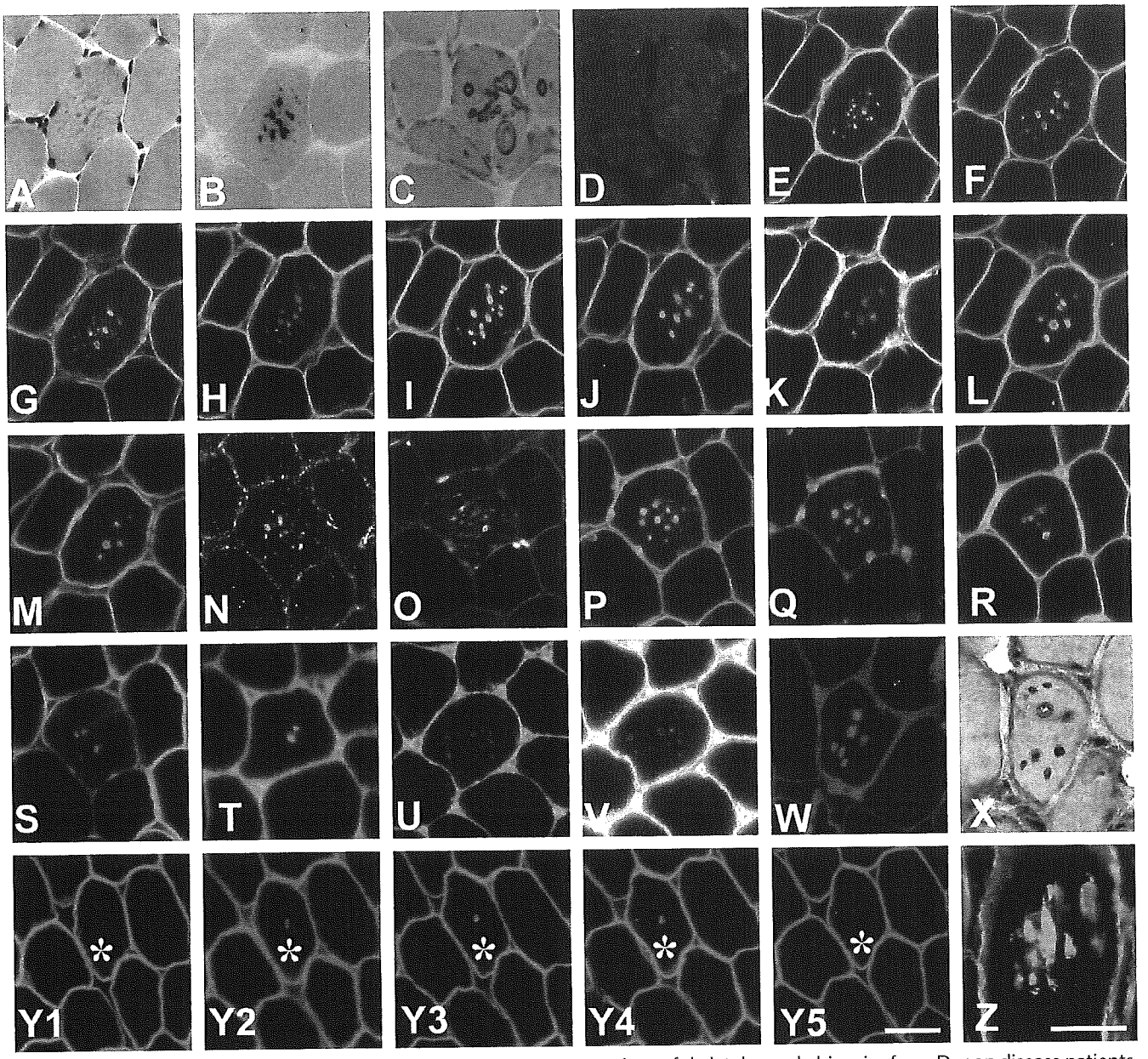

FIGURE 1. Histochemistry and immunohistochemistry. Transverse sections of skeletal muscle biopsies from Danon disease patients. Several fibers contain scattered tiny basophilic intracytoplasmic vacuoles **(A)**: H&E. The vacuolar membrane has high nonspecific esterase **(B)** and acetylcholinesterase **(C)** activities. None of the vacuoles bind to α-bungarotoxin **(D)**. Sections were stained with antibody against the C-terminus of dystrophin **(E)**, the rod domain of dystrophin **(F)**, the N-terminus of dystrophin **(G)**, laminin α2 **(H)**, α-sarcoglycan **(I)**, β-sarcoglycan **(J)**, γ-sarcoglycan **(K)**, δ-sarcoglycan **(L)**, dystrobrevin **(M)**, α-dystroglycan **(N)**, utrophin **(O)**, dysferlin **(P)**, β-dystroglycan **(Q)**, perlecan **(R)**, caveolin-3 **(S)**, collagen IV **(T)**, fibronectin **(U)**, collagen VI **(V)**, integrin β1 **(W)**, and agrin **(X)**. The vacuolar membranes were immuno-positive with most of the primary antibodies, although reactivity of these proteins was variable. The results are summarized in Table 1. Transverse 5-μm serial sections **(Y1–Y5)** and longitudinal section **(Z)** of muscle from Danon disease patient showing immunoreaction for dystrophin. Vacuolar membrane in muscle fiber (*) is not connected to the sarcolemma but is closed. Longitudinal section shows that the vacuoles are spherical or oval. **(D–W, Y1–Y5, Z)**: FITC-labeled staining; **(X)**: DAB staining. **(C–S, U, V, Y1–Y5)**: serial sections. Scale bars: **(A–W, Y1–Y5)** = 20 μm; **(Z)** = 30 μm.

is a submembranous protein structurally similar to dystrophin and is widely expressed, albeit at low levels, in the sarcolemma (17). Integrin β1 and α7 are transmembranous proteins and form a complex with each other in the sarcolemma (18). Dysferlin and caveolin-3 are also sarcolemmal proteins and are responsible for limb-girdle muscular dystrophy (LGMD) 2B and LGMD 1C, respectively (19, 20). Extracellular proteins, collagen IV, perlecan, fibronectin, agrin, and laminin, are the

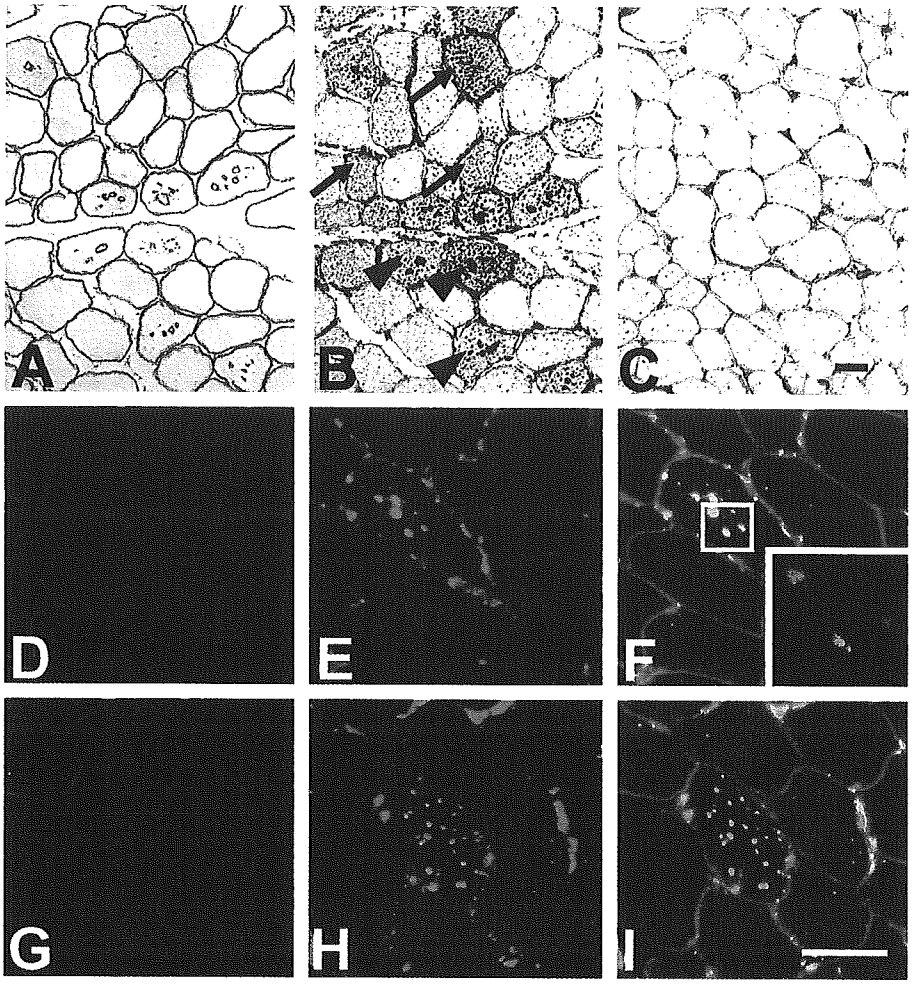

FIGURE 2. Indirect immunohistochemistry. Transverse sections of skeletal muscle stained with DAB for dystrophin **(A)** and LIMP-1 **(B, C)**. **(A, B)** Danon disease patient; **(C)**: Normal control. In Danon disease some muscle fibers express both LIMP-1 and dystrophin (arrowheads), whereas some muscle fibers show overexpression of LIMP-1 with absence of dystrophin (arrows). Normal control showed almost no expression of LIMP-1 **(C)** in muscle fibers. Scale bar = 40 μm. Double immunohistochemistry. Transverse sections of skeletal muscle from Danon disease patient, stained for dystrophin and LIMP-1. LIMP-1 is strongly accumulated inside the muscle fibers **(D, G)**. In some muscle fibers, LIMP-1-positive accumulations are clearly surrounded by dystrophin immunopositive membrane **(D–F)**. These vacuoles are the AVSF. In other muscle fibers, LIMP-1-positive accumulations are not surrounded by dystrophin **(G–I)**. **(D, G)**: dystrophin; **(E, H)**: LIMP-1; **(F, I)**: merged. Scale bar = 30 μm.

main components of the basal lamina. Collagen VI is present in the interstitium but is associated directly with collagen IV (21). We observed very little staining of only collagens IV and VI in vacuolar membranes, indicating that the membranes hardly contain these collagens. Based on our findings, we deduce that the vacuolar membrane of AVSFs in Danon disease and related AVMs have most of the sarcolemmal proteins ranging from cytoplasmic dystrophin to the extracellular laminin.

The vacuolar membranes of AVSF have abundant activities of AChE similar to neuromuscular junctions. Nevertheless, they are distinct from motor endplates because the membranes lacked AChRs. In the early stages of formation of the neuromuscular junction, AChE and AChRs are localized diffusely throughout the sarcolemma. When axon terminals make contact with muscle cells, postjunctional folds are quickly formed. In this process, AChE and AChRs accumulate at junctions and disappear from the extra-junctional sarcolemma (22, 23). These facts support our hypothesis that the vacuoles are intracellular enclosed spaces, because, if AVSF were derived from sarcolemma, then AChE-expressing vacuoles should be located near neuromuscular junctions rather than scattered in the cytoplasm. Furthermore, the presence of AChE without AChRs clearly indicates that the vacuolar membranes are distinct from either junctional or extra-junctional sarcolemma and suggests that they are formed through a unique process.

In the intracellular degradative process called autophagy, "isolation membranes" initially sequesters portions of cytoplasm to be degraded and forms "autophagosomes," which then fuse with lysosomes and become "autolysosomes." The cytoplasm sequestered in autolysosomes is then digested by proteolytic enzymes provided by the lysosomes. Most autophagic vacuoles in Danon disease are autolysosomes rather than autophagosomes, which lack enzymatic activity. These are indicated by the demonstration of many LIMP-1-positive accumulations scattered throughout the fibers (24, 25) and the autophagic nature of the vacuoles on electron microscopy. Actually, small basophilic granules on hematoxylin and eosin are most likely these autolysosomal accumulations as suggested by their pattern of distribution and the fact that lysosomes are basophilic on H&E. Moreover, some clusters of autolysosomes are surrounded by membranes with sarcolemmal features but others are not. In support of this notion, ultrastructural studies identified 2 types of autophagic vacuoles: 1) clusters of autophagic vacuoles not surrounded by membranes or basal lamina, and 2) vacuoles containing various

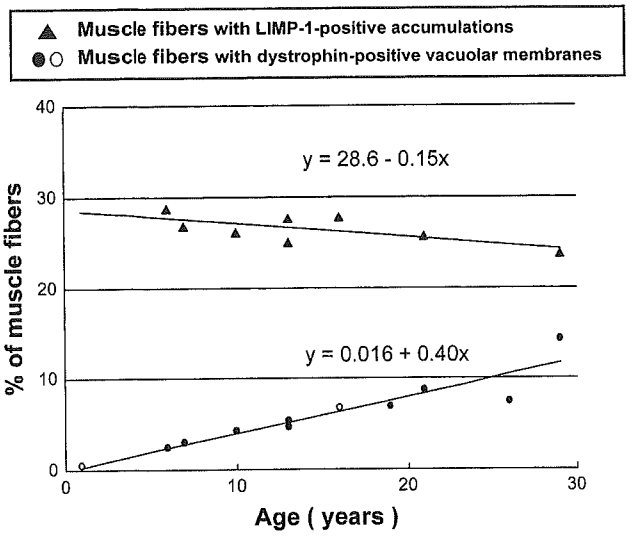

FIGURE 3. Relationship between age of the patients with Danon disease and number of muscle fibers with vacuoles highlighted with dystrophin or LIMP-1 on immunohistochemistry. The open circles show the only patient who had 2 muscle biopsies. The muscle fibers (circles) with intracytoplasmic vacuoles surrounded by dystrophin immuno-positive membrane (AVSFs) increased with age (r = 0.936). The muscle fibers (triangles) with overexpression of LIMP-1 showed a slight decrease with age (r = 0.353). r, Pearson's linear correlation coefficient.

autophagic materials encircled by membranes with basal lamina along the luminal side.

The proportion of muscle fibers with AVSFs increased with age in Danon disease and LAMP-2-deficient mice. In contrast, muscle fibers with LIMP-1-positive autolysosomal accumulations existed even in young patients and decreased slightly with age. It is most likely that the formation of these autolysosomal accumulations, which are clusters of autophagic vacuoles seen on electron microscopy, is a primary change in muscle fibers of Danon disease and related AVMs. The formation of peculiar membranes with sarcolemmal features around the autophagic vacuoles is hence a secondary phenomenon. Since muscle symptoms progress slowly in Danon disease, the development of muscle symptoms might be associated more closely with the formation of the unusual autophagic vacuoles rather than directly with the deficiency of LAMP-2.

LAMP-1 is the autosomal paralogous counterpart of LAMP-2 and both are thought to protect lysosomal membrane and cytoplasm from proteolytic enzymes within the lysosomes. LAMP-2 is tissue-specific but unlike LAMP 1, which is ubiquitously expressed, its expression is likely to be specifically regulated (26). Inhibition of LAMP-1 function results in failure of fusion of lysosomal and plasma membranes and therefore impaired exocytosis (27), a process usually by which cytoplasmic debris in the autophagosomes are extruded out from the cell through the sarcolemma (28). We therefore assume that LAMP-2 deficiency might likewise be related to dysregulation of exocytosis, leading to the development of the

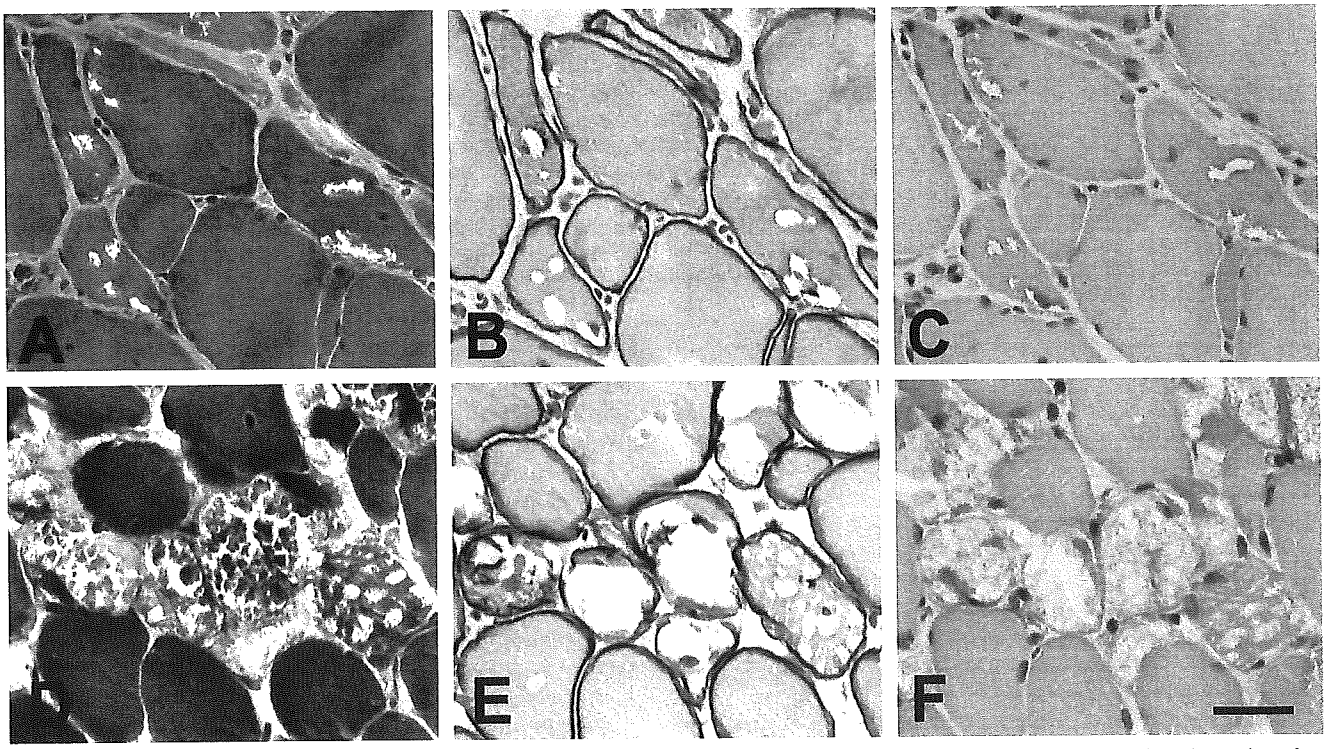

FIGURE 4. Transverse serial sections of muscle of patient with DMRV/HIBM **(A–C)** and with childhood AMD **(D–F)**. Only a few rimmed vacuoles in DMRV/HIBM showed presence of dystrophin. In AMD, dystrophin is present on some vacuolar materials. However, no vacuolar membranes have AChE activity in DMRV/HIBM and AMD. **(A, D)**: Gomori-trichrome stain; **(B, E)**: AChE stain; **(C, F)**: immunohistochemistry against dystrophin with DAB. Scale bar = 30 μm.

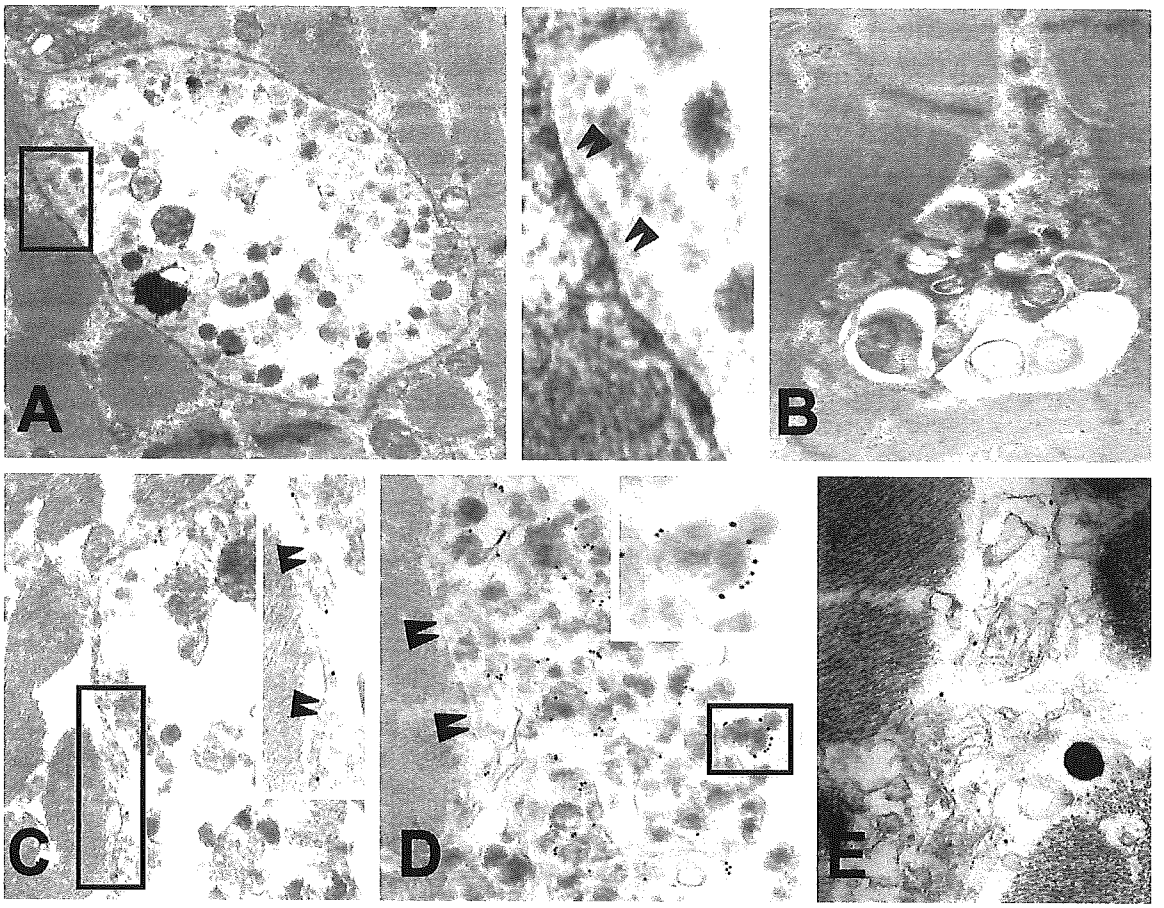

FIGURE 5. Electron micrograph in muscle from Danon disease patient. Scattered in the muscle fibers were clusters of autophagic vacuoles **(A)** containing cytoplasmic debris, electron dense material, and myeloid bodies. Some of these clusters were encircled by a membrane with basal lamina (paired arrowheads) on its luminal side, while other clusters are not limited by a membrane **(B)**. Electron immunohistochemistry after single labeling with dystrophin or LIMP-1 antibody shows localization of the proteins in autophagic vacuoles **(C–E)**. In the clusters with membranes, that is, the AVSF **(A)**, the immunogold particles show dystrophin **(C)** along the vacuolar membrane (paired arrowheads), and the immunogold particles show LIMP-1 **(D)** around autophagic material inside autophagic vacuoles. In contrast, in the clusters not surrounded by membranes, immunogold particles show LIMP-1 around autophagic materials with absence of dystrophin **(E)**. Original magnifications: **(A)** 15,000×; **(B)** 18,000×; **(C)** 20,000×; **(D)** 18,000×; **(E)** 30,000×.

unusual autophagic vacuoles with unique sarcolemmal features.

TfR and LDL-R are found in the membranes of recycling endosomes. In contrast, Rab5 and VAMP-7 are present in the membranes of early and late endosomes (29). We revealed the presence of all of these proteins in the fibers with autophagic vacuoles, indicating that in addition to the lysosomal system, the endosomal system is activated in Danon disease and related AVMs. Interestingly, VAMP-7 was increased in nonvacuolated fibers without autolysosomal accumulations, suggesting that maturation to late endosomes could prevent the formation of the unique vacuolar membranes.

Most of the vacuolar membranes were closed and were not connected to the sarcolemma in Danon disease. The autolysosomes containing cytoplasmic debris are therefore seen to be entrapped within the lumen of the vacuoles, and as such can be possibly considered to be extracellular space. Together with the observations that most AVSF did not accumulate in the subsarcolemmal region but were scattered in the cytoplasm, our findings suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism other than indentation of sarcolemma. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes (Fig. 6). The membranes surrounding the autophagic vacuoles might have originated from the lysosomal membrane or the isolation membrane that elongates and develops into the membrane of autophagosome (30), or is formed in situ and entirely de novo. If the vacuolar membranes are formed within the muscle fibers, it is compatible with the observation that the vacuolar membranes lack collagens IV and VI because collagens are thought to be produced mainly in the interstitium. Further studies are still necessary to understand the mechanism of the formation of these peculiar vacuolar membranes.

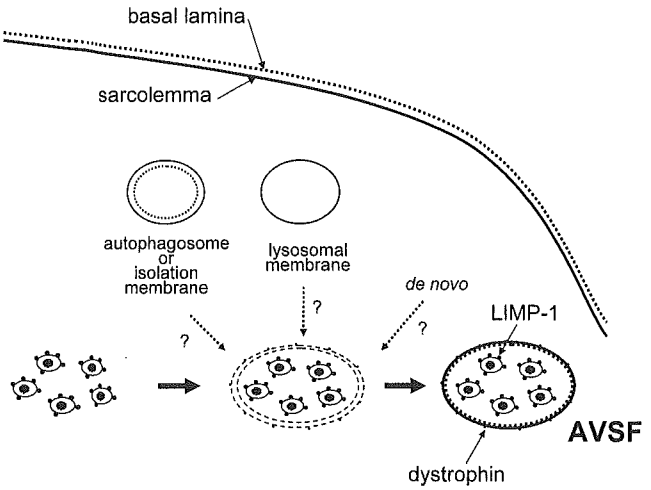

FIGURE 6. Schematic diagram of autophagic vacuoles in muscle fiber of patients with Danon disease. The membranes of the AVSFs were closed and were not connected to the sarcolemma. We suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes, as illustrated.

In XMEA, vacuolar membranes have not been reported to have AChE activity (6). However, the strong similarity of the pathologic characteristics to Danon disease naturally raised the question of whether AChE activity is present in the vacuolar membranes in XMEA. Indeed, our Japanese patient with probable XMEA showed AChE activity in the vacuolar membranes. Therefore, Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement share a common pathologic feature, namely, AVSF with AChE activity in the vacuolar membranes. Nevertheless, XMEA, infantile AVM, and adult-onset AVM are genetically different from Danon disease, as indicated by the presence of LAMP-2, which is absent in Danon disease. The observation that some features are not seen in Danon disease, like the presence of multilayered basal lamina and the deposition of C5b-9 over the surface of the muscle fiber, raise a possibility that some of these diseases might be allelic to XMEA, albeit different clinical phenotypes.

The autophagic vacuoles in AMD and rimmed vacuoles were reported to occasionally show presence of dystrophin. Nevertheless, these vacuoles are distinct from the AVSF seen in Danon disease and related AVMs, because the frequency of the vacuoles with sarcolemmal features is much less and most of sarcolemmal proteins are not consistently present in the vacuolar membranes of AMD and the rimmed vacuolar myopathies. Moreover, the activities of AChE and NSE were never found in the vacuolar membranes of these myopathies. In addition, on electron microscopy, the vacuolar membranes with basal lamina, such as those seen in Danon disease and related AVMs, were not found in AMD, DMRV/HIBM, or SIBM. According to the classification of De Bleecker et al, the AVSF may belong to type 1 vacuoles as the boundaries of type 1 vacuoles reacted for laminin, β-spectrin, and dystrophin (31). However, type 1 vacuoles were thought to be open to extracellular space and arise from invagination of the sarcolemma. Moreover, the membranes of AVSF have not only many sarcolemmal and extracellular proteins but also AChE activity, and may be formed in situ in cytoplasm as described above. Therefore, we think that the AVSF are a new, highly specific subtype of type 1 vacuoles.

Although the mechanism of their production still remains a mystery, overall, AVSF with AChE activity delineate Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement from other AVMs. Most likely, this unique pathologic finding will probably be found in more diseases and therefore the list of AVMs in this group is likely to expand.

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REFERENCES

1. Nishino I, Fu J, Tanji K, et al. Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). Nature 2000;406:906–10
2. Danon MJ, Oh SJ, DiMauro S, et al. Lysosomal glycogen storage disease with normal acid maltase. Neurology 1981;31:51–57
3. Sugie K, Yamamoto A, Murayama K, et al. The clinicopathological features of genetically confirmed Danon disease. Neurology 2002;58: 1773–78
4. Nishino I, Yamamoto A, Sugie K, Nonaka I, Hirano M. Danon disease and related disorders. Acta Myologica 2001;20:120–24
5. Murakami N, Goto Y, Itoh M, et al. Sarcolemmal indentation in cardiomyopathy with mental retardation and vacuolar myopathy. Neuromuscul Disord 1995;5:149–55
6. Kalimo H, Savontaus ML, Lang H, et al. X-linked myopathy with excessive autophagy: A new hereditary muscle disease. Ann Neurol 1988;23: 258–65
7. Yamamoto A, Morisawa Y, Verloes A, et al. Infantile autophagic vacuolar myopathy is distinct from Danon disease. Neurology 2001;57:903–5
8. Kaneda D, Sugie K, Yamamoto A, et al. A novel form of autophagic vacuolar myopathy with late-onset and multiorgan involvement. Neurology 2003;61:128–31
9. Nishino I. Autophagic vacuolar myopathies. Curr Neurol Neurosci Rep 2003;3:64–69
10. Tanaka Y, Guhde G, Suter A, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000;406: 902–6
11. Saftig P, Tanaka Y, Lullmann-Rauch R, von Figura K. Disease model: LAMP-2 enlightens Danon disease. Trends Mol Med 2001;7:37–39
12. Sugie K, Koori T, Yamamoto A, et al. Characterization of Danon disease in a male patient and his affected mother. Neuromuscul Disord 2003;13: 708–11
13. Nishino I, Noguchi S, Murayama K, et al. Distal myopathy with rimmed vacuoles is allelic to hereditary inclusion body myopathy. Neurology 2002;59:1689–93

14. Takemitsu M, Nonaka I, Sugita H. Dystrophin-related protein in skeletal muscles in neuromuscular disorders: Immunohistochemical study. Acta Neuropathol 1993;85:256–59
15. Metzinger L, Blake DJ, Squier MV, et al. Dystrobrevin deficiency at the sarcolemma of patients with muscular dystrophy. Hum Mol Genet 1997;6:1185–91
16. Ozawa E, Nishino I, Nonaka I. Sarcolemmopathy: Muscular dystrophies with cell membrane defects. Brain Pathol 2001;11:218–30
17. Tinsley JM, Blake DJ, Roche A, et al. Primary structure of dystrophin-related protein. Nature 1992;360:591–93
18. Collo G, Starr L, Quaranta V. A new isoform of the laminin receptor integrin α7β1 is developmentally regulated in skeletal muscle. J Biol Chem 1993;268:19019–24
19. Minetti C, Sotgia F, Bruno C, et al. Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy. Nat Genet 1998;18: 365–68
20. Matsuda C, Aoki M, Hayashi YK, Ho MF, Arahata K, Brown RH Jr. Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy. Neurology 1999;53:1119–22
21. Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. J Biol Chem 1997;272:26522–29
22. Matthews-Bellinger JA, Salpeter MM. Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. J Neurosci 1983;3:644–57
23. Ishikawa Y, Shimada Y. Acetylcholine receptors and cholinesterase in developing chick skeletal muscle fibers. Brain Res 1982;281:187–97
24. Fukuda M. Biogenesis of the lysosomal membrane. Subcell Biochem 1994;22:199–230
25. Winchester BG. Lysosomal membrane proteins. Eur J Paediat Neurol 2001;5:11–19
26. Kannan K, Divers SG, Lurie AA, Chervenak R, Fukuda M, Holcombe RF. Cell surface expression of lysosome-associated membrane protein-2 (lamp2) and CD63 as markers of in vivo platelet activation in malignancy. Eur J Haematol 1995;55:145–51
27. Reddy A, Caler EV, Andrews NW. Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 2001;106:157–69
28. Engel AG. Acid maltase deficiency. In: Engel AG, Banqer BQ, eds. *Myology*, Vol. 2. New York, NY: McGraw-Hill, 1993:1533–53
29. Advani RJ, Yang B, Prekeris R, Lee KC, Klumperman J, Scheller RH. VAMP-7 mediates vesicular transport from endosomes to lysosomes. J Cell Biol 1999;146:765–76
30. Mizushima N, Yamamoto A, Hatano M, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001;152:657–67
31. De Bleecker JL, Engel AG, Winkelmann JC. Localization of dystrophin and β-spectrin in vacuolar myopathies. Am J Pathol 1993;143:1200–1208
32. Sugiyama JE, Glass DJ, Yancopoulos GD, Hall ZW. Laminin-induced acetylcholine receptor clustering: An alternative pathway. J Cell Biol 1997;139:181–91

Centronuclear myopathy in mice lacking a novel muscle-specific protein kinase transcriptionally regulated by MEF2

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Myocyte enhancer factor 2 (MEF2) plays essential roles in transcriptional control of muscle development. However, signaling pathways acting downstream of MEF2 are largely unknown. Here, we performed a microarray analysis using *Mef2c*-null mouse embryos and identified a novel MEF2-regulated gene encoding a muscle-specific protein kinase, Srpk3, belonging to the serine arginine protein kinase (SRPK) family, which phosphorylates serine/arginine repeat-containing proteins. The *Srpk3* gene is specifically expressed in the heart and skeletal muscle from embryogenesis to adulthood and is controlled by a muscle-specific enhancer directly regulated by MEF2. *Srpk3*-null mice display a new entity of type 2 fiber-specific myopathy with a marked increase in centrally placed nuclei; while transgenic mice overexpressing *Srpk3* in skeletal muscle show severe myofiber degeneration and early lethality. We conclude that normal muscle growth and homeostasis require MEF2-dependent signaling by Srpk3.

[*Keywords*: Myocyte enhancer factor 2; transcriptional regulation; serine arginine protein kinase (SRPK); Stk23/Srpk3; centronuclear myopathy]

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Skeletal muscle differentiation is cooperatively controlled by two families of transcription factors, the myogenic basic helix–loop–helix (bHLH) proteins and the myocyte enhancer factor 2 (MEF2) family of MADS domain proteins (Black and Olson 1998; Bailey et al. 2001; Pownall et al. 2002; Buckingham et al. 2003; Parker et al. 2003). Myogenic bHLH proteins, such as MyoD and myogenin, recognize a DNA sequence called an E box (CANNTG). Myogenic bHLH proteins associate and synergistically activate transcription with MEF2 factors, which bind to the A/T-rich DNA consensus (CTA-(A/T)$_4$-TA-G/A). Additionally, myogenic bHLH proteins activate their own expression and the expression of MEF2, while MEF2 stimulates expression of myogenic bHLH protein genes and the *Mef2c* gene (Cserjesi and Olson 1991; Lassar et al. 1991; Edmondson et al. 1992; Cheng et al. 1993; Yee and Rigby 1993; Wang et al. 2001; Teboul et al. 2002; Dodou et al. 2003). Such auto- and cross-regulatory interactions establish a mutually reinforcing circuit to achieve myogenesis.

The essential role of MEF2 in muscle development was first shown in *Drosophila* in which a loss-of-function mutation in the single MEF2 ortholog *D-mef2* results in a complete block to differentiation of all muscle lineages: somatic, cardiac, and visceral (Bour et al. 1995; Lilly et al. 1995). In mice, the existence of four *Mef2* genes—*Mef2a*, *Mef2b*, *Mef2c*, and *Mef2d*—with overlapping expression patterns makes it more difficult to assess the roles of these factors individually (Black and Olson 1998). Mice homozygous for a *Mef2c*-null allele show embryonic lethality around embryonic day 9.5 (E9.5) caused by improper development of the heart (Lin et al. 1997). The mutant hearts do not undergo looping morphogenesis, the future right ventricle does not form, and

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a subset of cardiac muscle genes is not expressed. MEF2 has also been implicated in maintenance of the slow fiber phenotype of skeletal muscle, in the control of striated muscle energy metabolism, and in pathological remodeling of the adult heart in response to stress signaling (Black and Olson 1998; McKinsey et al. 2002).

In principle, MEF2 may regulate muscle-specific target genes directly, or it may act indirectly by controlling the expression of subordinate transcription factors or signaling molecules that act as intermediaries to connect MEF2 to downstream targets that themselves are not dependent on MEF2-binding sites in their *cis*-regulatory regions. Indeed, vast arrays of direct and indirect targets of MEF2 in skeletal muscle cells in culture were recently described (Blais et al. 2005).

In an effort to identify MEF2 target genes that serve as "downstream" effectors of MEF2 action during muscle development, we performed a microarray analysis using *Mef2c*-null embryos and identified a novel muscle-specific protein kinase Stk23/Srpk3, which is encoded by a direct MEF2 target gene. *Srpk3*-null mice display a new entity of centronuclear myopathy, while transgenic mice overexpressing *Srpk3* in skeletal muscle show severe myofiber degeneration. These findings reveal an essential role for serine arginine protein kinase (SRPK)-mediated signaling in muscle growth and homeostasis downstream of MEF2 transcription factors.

Results

Srpk3: *a novel muscle-specific protein kinase gene*

In an attempt to identify novel MEF2-regulated genes, we compared the gene expression profiles of hearts from wild-type and *Mef2c*-null mouse embryos by an RNA microarray analysis. Because *Mef2c*-null embryos die around E9.5 (Lin et al. 1997), we used hearts from wild-type and null embryos at E9.0 prior to overt cardiac demise. Among the genes that were dysregulated in the *Mef2c* mutants, we found the expression of *Stk23* to be significantly decreased in the *Mef2c*-null hearts. The down-regulation of *Stk23* expression in the *Mef2c*-null hearts was confirmed by RT–PCR (Fig. 1a). Residual expression of *Stk23* in the mutants may reflect the presence of other MEF2 factors that partially compensate for MEF2C.

Stk23 was described in an analysis of human chromosomal DNA methylation as a potential protein-kinase-encoding gene (Grunau et al. 2000), but its expression profile and function have not been described. Structural comparison with various protein kinases clearly indicated that Stk23 possesses a bipartite kinase domain with high sequence similarity to the SRPK family kinases, Srpk1 and Srpk2 (Fig. 1b,c; Gui et al. 1994; Bedford et al. 1997; Kuroyanagi et al. 1998; Wang et al. 1998). As expected, Stk23 efficiently phosphorylated known SRPK

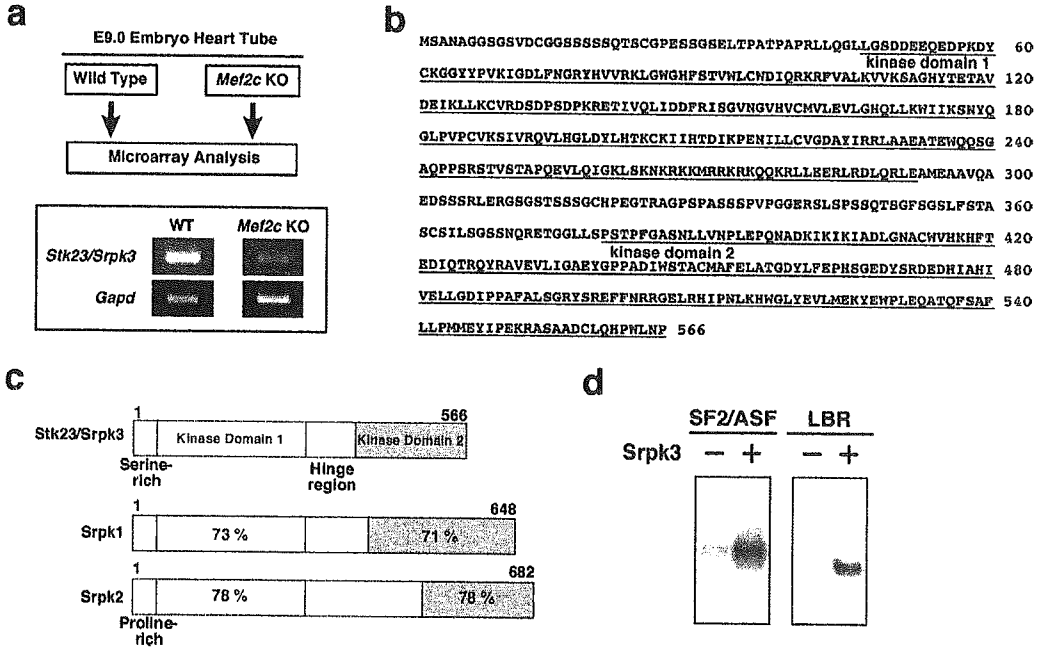

Figure 1. Identification of Stk23/Srpk3 as a novel SRPK. (*a*) Microarray analysis was performed using the hearts of E9.0 *Mef2c*-null and wild-type embryos. Down-regulation of the *Stk23/Srpk3* expression in the *Mef2c*-null hearts was confirmed by RT–PCR. (*Gapd*) Glyceraldehyde-3-phosphate dehydrogenase expression as a control. (*b*) Amino acid structure of mouse Stk23/Srpk3. Srpk3 is a 566-amino-acid protein that contains a bipartite kinase domain (underlined), N terminus, and a hinge region. (*c*) Schematic representation of mouse SRPK family kinases. Srpk3 shows high sequence similarity to Srpk1 and Srpk2 in the kinase domain, but not in the N terminus and hinge region. The percentages in the boxes are identities of Srpk1 and Srpk2 to Srpk3 at an amino acid level. Srpk3 and Srpk2 have serine- and proline-rich sequences in the N terminus, respectively. Amino acid numbers are also shown. (*d*) Phosphorylation assays using SR domain proteins. Srpk3 phosphorylated SF2/ASF and the N-terminal region of Lamin B Receptor (LBR) in vitro.