表1 複合体II欠損症の分類

1. 遺伝子異常による分類
   a. flavoprotein(Fp: SDHA): 遺伝性Leigh脳症
   b. iron-sulfur protein(IP: SDHB): 遺伝性傍神経節腫，遺伝性褐色細胞腫
   c. integral membrane protein(SDHC): 遺伝性傍神経節腫
   d. integral membrane protein(SDHD): 遺伝性傍神経節腫

2. 生化学的分類
   a. 複合体II単独欠損症
      1) SDH欠損のあるもの
      2) SDH欠損のないもの
      3) SDH欠損が不明のもの
      4) 酸素消費のみで異常が証明されたもの
   b. 他の酵素複合体欠損を伴う複合体II欠損症
      1) 複合体I欠損を伴うもの
      2) 複合体I，III欠損を伴うもの
      3) 複合体I，III，IV欠損を伴うもの

3. 臨床的分類
   a. 乳児重症型(致死型)脳筋症
   b. 幼児-若年発症型脳筋症(Leigh脳症)
   c. 筋症(ミオグロビン尿，易疲労性)
   d. 遺伝性傍神経節腫
   e. 他の疾患に続発するもの
      1) fumarase欠損
      2) Zellweger症候群
      3) Friedreich失調症
      4) 家族性ジストニア
      5) Huntington病
      6) Leber's hereditary optic neuropathy(LHON)

下がみられる場合と欠損線維がモザイクに染色される場合[2]があり，その遺伝子異常との相関(genotype-phenotype correlation)は不明である．

構成遺伝子が単離されて以来，種々の病態で遺伝子異常が同定され報告された．1995年，Bourgeronらは，Leigh脳症を示す姉弟で，初めてflavoprotein(Fp: SDHA)遺伝子のArg554 Trpの変異を報告した[3]．患者の両親はいとこ婚であり，患者ではホモプラスミーを呈していた．その後Astutiらも，遺伝性の褐色細胞腫と傍神経節腫を来している患者でiron-sulfur protein subunit(IP: SDHB)の変異を初めて報告した[4]．Niemannらは，常染色体優性遺伝を示す傍神経節腫3型の患者で，integral membrane protein subunits(SDHC)の変異を初めて報告した[5]．Baysalらは，同じく遺伝性傍神経節腫を来す患者で，integral membrane protein subunits(SDHD)の遺伝子変異を初めて報告した[6]．

しかしながら，複合体IIの欠損と遺伝性傍神経節腫の因果関係はいまだ解明されていない．

3. 病因論的事項

複合体IIが核の支配であることより，本症の遺伝形式としてメンデル遺伝(常染色体性劣性遺伝)を考えねばならない．しかし，病期が進行すると，他の電子伝達系酵素欠損同様に複合的な酵素活性低下が起こってくる．

4. 病態(症候論と検査成績)

臨床病型として，乳児期早期に発症し1歳前に致死性の経過をとる乳児重症型(致死型)脳筋

症，幼児期から学童期に発症し，緩徐進行性の脳筋症の型をとる病型(Leigh脳症)，ミオグロビン尿や易疲労性を特徴とする筋型，遺伝性傍神経節腫を来す病型，および他の疾患に続発するものが考えられる．

Riggsらは，1984年，知的退行，ミオクローヌスてんかん，低身長を伴う進行性脳筋症を示す姉弟例で，複合体IIの生化学的酵素欠損症を初めて報告した[7]．筋生検では，ミトコンドリアの増生と脂肪滴の沈着を認めた．筋組織でのSDH，および電子伝達系複合体I，複合体IV酵素活性がすべて正常であったことから，SDHとコエンザイムQの間に異常があると推測された．

Behbehaniらは，同年，重度の筋力低下と脱水時の反復性嘔吐，代謝性アシドーシスを来し5カ月で死亡した患児で，筋内の複合体II活性欠損症を報告した[8]．電顕的に異常ミトコンドリアの筋鞘膜下の集積を示すragged-red fibersを多数認め，脂肪滴も蓄積していた．

1988年，Martinらは，精神発達遅滞の3歳になる男児で，コエンザイムQの欠損を報告した[9]．患児は，近位筋優位の筋力低下と不全麻痺を伴う動揺性歩行および大脳基底核の両側性壊死を示していた．血中および髄液中の乳酸は持続的に高値であった．筋生検では脂肪滴の軽度増加以外は正常所見であった．

1992年，Robinsonらは，子宮内発育遅延があり生後6日で呼吸不全により死亡した女児および生後3カ月で死亡した兄の兄妹で，培養皮膚線維芽細胞，および分離筋ミトコンドリアのピルビン酸脱水素酵素，複合体II，IVの活性低下を報告した[10]．各複合体のポリペプチドは，培養皮膚線維芽細胞や筋肉では分子集合できないが，肝では分子集合可能であることを見いだした．これは，酵素複合体の複数の欠損がサブユニットレベルで証明された初めての報告である．

1993年，Rotigらは，糖尿病，視神経萎縮，難聴を伴うWolfram症候群で7.6kbのミトコンドリアDNAの欠失により，複合体IIを含む電子伝達系酵素複合体の欠損を報告した[11]．

1994年，Smithらは Leber's hereditary optic neuropathy(LHON)で，11778変異，3460変異をもつ患者間で重症度と喫煙状態との関係を比較検討し，電子伝達系酵素複合体IIの欠損を報告した[12]．

1994年，Arpaらは，血族結婚のある両親から生まれた22歳の女性で，全身の筋力低下と易疲労性，高CK血症がある複合体II欠損症を報告した[13]．筋生検では，筋線維径の大小不同，タイプI線維優位，筋鞘膜下のDNPH陽性顆粒および中性脂肪滴の増加がみられ，電顕では結晶様封入体を有する異常ミトコンドリアがみられた．

その後，ミトコンドリアtRNAの点変異，家族性ジストニア，Zellweger症候群，Huntington病，Friedreich失調症[14]などでも本酵素欠損が報告されている．

5. 診断と鑑別診断

最も信頼できる複合体II活性の測定は，新鮮な生検骨格筋検体からミトコンドリア分画を分離精製し，コハク酸などを基質とした酸素消費能の測定(oxograph)，SDH，コハク酸からcytochrome Cまで鉄硫黄蛋白画分を含めた酵素活性測定を同時に行うことである．この場合，細胞質の夾雑蛋白の混入の指標として，citrate synthaseなどに対する比活性で補正する方法もとられている．その評価には，様々な因子が関与するため，経験ある施設で行うことが望ましい．また，筋組織化学的にSDH染色を行い染色性を検討する必要がある．

6. 治療と予後

今までにコエンザイムQ10，イデベノン，メナジオン，リボフラビン，コルチコステロイド，カルニチン，クレアチン，ビタミンKおよびC，ジクロロ酢酸，カルジオクロームなどが投与されてきた．本症に対する特異的な治療薬はなく，以上に述べた対症療法が重要である．

[補　記]

最近，複合体II酵素に臓器特異性を示すsub-

unitが存在することが報告された(東京大学大学院生物医化学北潔博士による私信). 今後の研究の展開が注目される.

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臨床編 II

ミトコンドリア病(狭義)　欠損複合体別分類と臨床

複合体 III

Complex III

古賀靖敏　　柊山　了

Key words: 複合体III，チトクロームb，コエンザイムQ，ミオグロビン尿症，ragged-red fiber

1. 概念・定義

複合体III(ubiquinol-cytochrome c oxidoreductase，EC: 1.10.2.2)は，ミトコンドリア呼吸鎖の電子伝達系複合体の2番目の酵素である．複合体IもしくはIIからubiquinonolによって伝えられた電子をチトクロームcに渡し，プロトンをミトコンドリア内膜側から外膜へ転換する役割を担う．この電子の授受により，複合体IIIはubiquinonolを酸化し，チトクロームcを還元する．

複合体IIIは，少なくとも11個のサブユニットからなる酵素複合体である．そのうち1個は，ミトコンドリアDNAにコードされたチトクロームb(subunit III)で，残りはすべて核DNAにコードされる．核のサブユニットは，core protein I，core protein II，チトクローム c1(subunit IV)，Rieske iron-sulfur protein(subunit V)および少なくとも6つのポリペプチドからなり，分子集合により活性を獲得する．核DNA由来のサブユニットの多くは，他の酵素複合体と同様N末端にプレ配列(ミトコンドリアターゲティングシグナル)をもつ前駆体蛋白として細胞質で合成される．その後，細胞質シャペロン系(Hsp70シャペロン)により，細胞内小器官であるミトコンドリア外膜のインポート受容体に輸送される．

この複合体IIIの活性が低下するものを複合体III欠損症という．多くのミトコンドリア病，その他の症候群で本酵素の活性低下が報告されている．

2. 分　　類

現在考えられる複合体III欠損症の分類を，表1に示す．本症の臨床型としては，Leber hereditary optic neuropathy(LHON)，運動不耐性ミオパチー，ミオグロビン尿と高乳酸血症を示すもの，乳児致死性多臓器不全症，心筋症，Parkinson病，Huntington病，Fanconi症候群など多様性に富む病像を示す．

遺伝子異常が判明したものでは，ミトコンドリアにコードされたチトクロームb遺伝子の異常として，LHON症[1]，運動不耐性ミオパチー[2]，ミトコンドリア脳筋症[3]，大腸直腸癌[4]で多くの報告がある．一方，核の遺伝子異常による複合体III欠損症は，2001年，de Lonlayらにより初めて報告された．新生児期に近位尿細管障害，肝障害，脳症で発症した児で，複合体IIIの分子集合に必須の核DNAにコードされたBCS1L遺伝子の変異を初めて見いだした[5]．この変異は，複合体III欠損症患者の1/3にみられる変異のホットスポットであることが判明した．複

Yasutoshi Koga, Ryo Fukiyama: Department of Pediatrics and Child Health, Kurume University School of Medicine　久留米大学医学部小児科

表1 複合体III欠損症の分類

1. 遺伝子異常による分類
   a. 複合体IIIサブユニットの核遺伝子異常
      1) core protein I
      2) core protein II
      3) cytochrome c1 (subunit IV)
      4) Rieske iron-sulfur protein (subunit V)
      5) BCS1L
   b. 複合体IIIサブユニットのミトコンドリア遺伝子異常
      1) cytochrome b (subunit III)
   c. その他のミトコンドリア遺伝子異常
      1) A5874G mutation at mitochondrial tRNA (Tyr) gene
   d. 他の核遺伝子異常に伴う複合体III欠損症
      1) 複合体IのNDUFS4変異
2. 成因不明の生化学的異常による複合体III欠損症
   a. coenzyme Q deficiency
3. 臨床的分類
   a. 乳児致死型多臓器疾患
   b. 運動不耐性ミオパチー
   c. 類組織球性心筋症(ミトコンドリア心筋症)
   d. Leber hereditary optic neuropathy (LHON)
   e. Parkinson病
   f. Huntington病
   g. De Toni-Debre-Fanconi症候群
   h. colorectal cancer
   i. 顔面肩甲上腕型ジストロフィー
   j. 小脳失調，ミオクローヌス，性腺発育不全
   k. West症候群

合体IIIの核遺伝子の解明により，核遺伝子の変異も今後多く見いだされると考えられる．

3. 病因論的事項

複合体IIIが核とミトコンドリアDNAの両支配であることより，本症の遺伝形式としてメンデル遺伝(常染色体性劣性遺伝)，母性遺伝の両者を考えねばならない．しかし，病期が進行すると，他の電子伝達系酵素欠損同様に複合的な酵素活性低下が起こってくるため，生化学的手法のみでは一次的異常がわかりにくい．また，ミトコンドリア遺伝子のtRNA遺伝子異常もしくは大欠失症例で，複合体IIIの異常の報告が散見されるが，特定の複合体異常に起因するものではないため，この稿では割愛する．

4. 病態(症候論と検査成績)

複合体III欠損症にみられる症候としては，乳児致死性多臓器障害，運動不耐性ミオパチー，ミトコンドリア心筋症，その他種々の症候群に随伴する酵素欠損症の4病型に大別できる[6]．

a. 乳児致死性多臓器障害

高乳酸血症，低血糖，尿細管障害，けいれんで3生日で死亡した男児で，骨格筋および肝の複合体III酵素活性特異的に低下していた[7]．骨格筋および心筋のチトクロームb含量は正常の75%まで低下しており，ウエスタンブロットでの筋，肝，心筋のnon-heme iron-sulfur proteinのみ著明に低下していた．新生児期早期に著明なアシドーシス，腎尿細管障害，けいれんを来す病像は，核の遺伝子異常が判明したBCS1L遺伝子変異症[5]の病像と類似するものである．

b. 運動不耐性ミオパチー

多くの軽症型はこの臨床病型をとる[8]．運動負荷により，高乳酸血症を来す．筋病理では，多くの例にragged-red fiberと脂肪滴の沈着がみられる．酵素生化学的に，複合体IIIの活性の低下は認められるものの，酵素蛋白レベルの解析結果は特異的なポリペプチドの低下とはならず，遺伝的に均一とは考えられない．

Andreuらは，進行性の運動不耐症と近位筋の筋力低下を示す患者でチトクロームb遺伝子の種々の変異を報告した．Keightleyらは，労作時の易疲労性と高乳酸血症を示す患者で複合体III欠損症として1983年に報告されていたものを再検索し，チトクロームb遺伝子のG15242Aのストップコドン変異を同定した．患者では，この変異が筋肉で87%，末梢血白血球で0.7%であったが，正常コントロールでは見つからなかった．免疫組織化学的検索では，Rieske iron-sulfur proteinの染色性と変異の含量は逆相関し，33%以上の変異筋線維ではすべて免疫性が消失した．一方，ragged-red fiberでの変異含量は平均89%であり，両者に正の相関がみられた．

現在までに報告されたチトクロームb変異は，

ミスセンス変異，ナンセンス変異，小欠失があるが[2]，臨床的重症度と遺伝型の相関解明(genotype-phenotype correlation)は今後の課題である．

c. ミトコンドリア心筋症

Papadimitriouらは，生後4週で心不全で死亡した女児で，巨大ミトコンドリアの集積を伴う心筋肥大を認め，分離骨格筋ミトコンドリアの検索でチトクロームbの低下を伴う複合体III欠損症を報告した[9]．Marin-Garciaは，虚血性心筋症を呈した患者でチトクロームbのC15452A変異を見いだした．複合体IIIの酵素活性はコントロールの50%以下に低下し，病因と考えられた[10]．

d. 他の症候群に随伴する複合体III酵素欠損

Musumeciらは，2001年，反復性ミオグロビン尿，けいれん，小脳失調，精神遅滞を来し，筋生検でragged-red fiberと脂肪滴の沈着がみられた患児で，筋コエンザイムQの欠損を報告した[11]．すべての患者は，コエンザイムQの補充療法に反応し，筋力の増強，小脳失調の改善，けいれんの軽症化がみられたという．

Ranaらは，Parkinson病の患者でチトクロームb遺伝子の4塩基欠失が複合体IIIの分子集合を阻害することを報告した[12]．この変異を高頻度に有する患者由来の細胞では，core protein Iは保たれていたがRieske iron-sulfur proteinは著明に減少し，過酸化水素の産生が亢進していた．このことから，Parkinson病でみられる酸化的リン酸化の様々な程度の違いがミトコンドリアDNAの変異と関連づけられた．

5. 診断と鑑別診断

最も信頼できる複合体III活性の測定は，新鮮な生検骨格筋検体からミトコンドリア分画を分離精製し，αケトグルタール酸，コハク酸などを基質とした酸素消費能の測定(oxograph)，αケトグルタール酸，コハク酸からチトクロームcまで鉄硫黄蛋白画分を含めた酵素活性測定を同時に行い，かつ分光学的にチトクローム含量を測定することである．この場合，細胞質の夾雑蛋白の混入の指標として，citrate synthaseなどに対する比活性で補正する方法もとられている．その評価には，様々な因子が関与するため，経験ある施設で行うことが望ましい．

6. 治療と予後

今までにコエンザイムQ_{10}，イデベノン，メナジオン，リボフラビン，コルチコステロイド，カルニチン，クレアチン，ビタミンKおよびC，ジクロロ酢酸，カルジオクロームなどが投与されてきた．本症に対する特異的な治療薬としてユビキノン誘導体は使用すべきであり，以上に述べた対症療法が重要である．

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REGULAR PAPER

Koji Ikezoe · Masahiro Nakagawa · Chuanzhu Yan
Jun-ichi Kira · Yu-ichi Goto · Ikuya Nonaka

Apoptosis is suspended in muscle of mitochondrial encephalomyopathies

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Abstract Over the past few years, many studies have been done on the apoptotic involvement in muscle fiber degeneration in various myopathies, but the occurrence of apoptosis in muscles of mitochondrial encephalomyopathies is still controversial. To confirm whether apoptotic processes are truly related to muscle fiber degeneration in mitochondrial encephalomyopathies, we performed the TUNEL method not only at the light microscopic (LM) but also at the electron microscopic (EM) level for muscles of five MELAS, five CPEO and five MERRF patients and five control muscles. Immunohistochemical studies of Bcl-2, Bax, cytochrome *c*, Apaf-1, activated caspase-3 and human inhibitor of apoptosis protein XIAP, and immunoblotting of Apaf-1 and XIAP were also carried out. In LM-TUNEL, MELAS, CPEO and MERRF patients had only very small numbers of TUNEL-positive myonuclei: 0.13±0.10%, 0.15±0.14% and 0.04±0.09%, respectively. Almost all of them were seen in ragged-red fibers (RRFs). EM-TUNEL showed no significant increase of DNA fragmentation in RRFs despite mild peripheral chromatin condensation. However, Bax and Apaf-1 expression and cytochrome *c* release from mitochondria were seen in RRFs. Caspase-3 activation was confirmed in 9.0±3.7%, 12.0±4.4% and 12.4±3.8% of RRFs in MELAS, CPEO and MERRF, respectively, but not in control muscles. Almost all RRFs showed sarcoplasmic expression of XIAP. Thus, there is a possibility that, although apoptotic reactions started in muscles of mitochondrial encephalomyopathies, their execution is rarely completed. Sarcoplasmic expression of XIAP probably leads to the suspension of the apoptotic process in mitochondrial encephalomyopathies.

Keywords Apoptosis · Mitochondria · X-linked inhibitor of apoptosis protein · Ragged-red fiber · Mitochondrial encephalomyopathy

K. Ikezoe · M. Nakagawa · C. Yan · I. Nonaka
Departments of Ultrastructural Research,
National Institute of Neuroscience,
National Center of Neurology and Psychiatry (NCNP),
Kodaira, Tokyo, 187-8502, Japan

Y. Goto (✉)
Department of Mental Retardation and Birth Defect Research,
National Institute of Neuroscience, NCNP,
Kodaira, Tokyo, 187-8502, Japan
Tel.: +81-42-3461713, Fax: +81-42-3461743

K. Ikezoe
Department of Neurology, Kyushu Koseinenkin Hospital,
Kitakyushu, Fukuoka, 806–8501, Japan

J. Kira
Department of Neurology, Neurological Institute,
Graduate School of Medical Sciences, Kyushu University,
Maidashi, Fukuoka, 812-8502, Japan

M. Nakagawa
Department of Plastic Surgery,
Graduate School of Medical Sciences, Tokyo University,
Hongo, Tokyo, 113-8655, Japan

Introduction

Mitochondrial encephalomyopathy is a group of heterogeneous disorders caused by mitochondrial abnormalities leading to a decrease in ATP synthesis [31]. There are three major forms of mitochondrial encephalomyopathy: mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode (MELAS) [34], myoclonus epilepsy associated with ragged-red fibers (MERRF) [12] and chronic progressive external ophthalmoplegia (CPEO) [21]. These three diseases are caused by mitochondrial DNA (mtDNA) mutations [13, 14, 38, 44]. In muscle pathology, myopathic changes of varying degrees are seen, and the presence of ragged-red fibers (RRFs) is the morphological hallmark [33].

Apoptosis is a process of active cell death under the strict control of genes. It is involved in the pathogenesis of many diseases: cancer, autoimmune diseases and neurodegenerative diseases [17]. Many signaling pathways leading to apoptosis have been identified so far. Two major pathways, through Fas stimulation [1] and through cytochrome *c* release from mitochondria [5, 40], have been

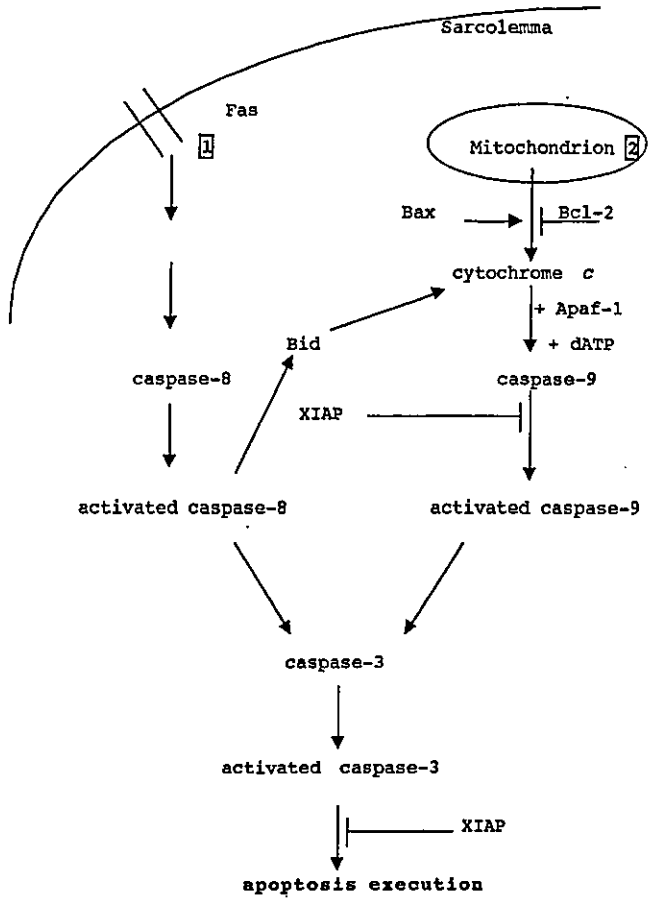


Fig. 1 Apoptosis signaling pathways. Two major apoptotic signaling pathways have been extensively studied so far: the pathway through Fas stimulation (*1*) and that through cytochrome *c* release from mitochondria (*2*). XIAP can inhibit the activation of caspase-9 in the latter pathway and the proteolytic activity of activated caspase-3 in both pathways (*XIAP* X-linked inhibitor of apoptosis protein)

extensively studied (Fig. 1). There is a great possibility that apoptosis, as well as abnormal energy metabolism, is related to the pathomechanism of mitochondrial encephalomyopathies [19].

During the past few years, several studies have been made of apoptotic involvement in mitochondrial encephalomyopathies [29, 37]. However, the occurrence of apoptotic muscle fiber degeneration in them is still controversial. Moreover, there are no reports dealing with the apoptotic signaling pathways in muscles of mitochondrial encephalomyopathies. There are some reports that skeletal muscle cells are refractory to apoptotic processes, especially those through cytochrome *c* release: in normal human skeletal muscles, Apaf-1 (apoptotic protease-activating factor-1), which activates caspase-9 together with cytochrome *c* from mitochondria is not expressed [4]. In muscle biopsy specimens of polymyositis and inclusion body myositis, X-linked inhibitor of apoptosis protein (XIAP) was reported to inhibit apoptotic cell death [24].

To determine whether apoptotic processes actually take place in muscles of mitochondrial encephalomyopathies, and if so, which signaling pathways are utilized, we performed the terminal deoxynucleotidyl transferase mediated dUTP mediated nick end-labeling (TUNEL) method not only at the light microscopic (LM) but also at the electron microscopic (EM) level. Immunohistochemical studies of the apoptosis-related proteins Bcl-2, Bax, cytochrome *c*, Apaf-1, activated caspase-3 and XIAP were also carried out.

The results suggest that the apoptotic reaction had started but rarely completed, probably due to strong sarcoplasmic expression of XIAP in RRFs in the muscle biopsy specimens of mitochondrial encephalomyopathies. We discuss what the findings mean in the pathogenesis of the diseases.

Materials and methods

Patients

We selected five cases of MELAS, five of CPEO and five of MERRF whose muscle biopsy specimens contained more than 5% RRFs (Table 1). All MELAS patients had the most common A-to-G point mutation at nucleoside position (np) 3,243; all MERRF had an A-to-G mutation at np 8,344. In CPEO, all patients had mtDNA deletions that had confirmed the diagnosis. All patients examined in this study showed muscle weakness and atrophy. Controls consisted of five muscle biopsy samples with nondiagnostic findings.

TUNEL methods

For the TUNEL method at the light microscopic level (LM-TUNEL), we used an in situ apoptosis detection kit (Takara Shuzo, Japan) according to the manufacturer's instructions on frozen muscle biopsies. We examined at least 2,000 muscle fibers in each muscle biopsy sample.

For the TUNEL method at the electron microscopic level (EM-TUNEL), we used an Apop Tag kit (Intergen, N.Y.). Ultrathin sections taken from the same epoxy resin-embedded blocks used for conventional EM observation were mounted on nickel grids, and the surface was etched lightly with saturated sodium metaperiodate for 3 min. Subsequently, these ultrathin sections on the grids were incubated with working buffer containing terminal deoxynucleotidyl transferase (TdT), digoxigenin-dideoxyuridine triphosphate (dig-ddUTP) and dideoxyadenosine triphosphate (ddATP) at 37°C for 1 h, and then incubated with a blocking buffer (300 mM sodium chloride·30 mM sodium citrate) at 37°C for 30 min. Subsequently, these sections were incubated with sheep anti-digoxigenin antibody conjugated to 10-nm colloidal gold (British BioCell, UK) at room temperature for 1 h [16, 28]. Finally, these sections were lightly stained with uranyl acetate and then examined with an H-7000 transmission EM (Hitachi, Japan). We examined at least 20 myonuclei in each muscle of mitochondrial encephalomyopathy patients and control muscles.

We used prednisolone-treated rat thymuses as positive controls for apoptotic cells: a Wistar rat at 4 weeks of age was injected with prednisolone (5 mg/kg) intraperitoneally and killed 4 h later [10].

Immunohistochemistry

For immunohistochemical analyses, we utilized the immunoperoxidase technique: an avidin-biotin complex method using antibodies against Bcl-2 (1:40, monoclonal, Dako, Denmark), Bax (1:30, polyclonal, Calbiochem, Cambridge, UK), Apaf-1 (1:800, polyclonal, Imgenex, San Diego, Calif.), activated caspase-3 (1:50, polyclonal, PharMingen, Tokyo, Japan) and XIAP (1:250, Trans-

Table 1 Summary of patients (*RRFs* ragged-red fibers, *CC* cytochrome *c*, *Co* control)

Patient		Age/sex	Total fibers[a]	RRFs[b] (%)	TUNEL-positive myonuclei[c] (%)	Bax[d] (%)	CC[e] (%)	Apaf-1[f] (%)	Activated caspase-3[g] (%)	XIAP[h] (%)
MELAS	1	4/F	2,600	20	0.2(1.0)	>90	0.8	30	13	>90
	2	9/M	2,700	12	0.25(2.1)	>90	2.2	25	11	>90
	3	41/F	2,500	10	0(0)	>90	0.8	25	5	>90
	4	26/F	3,200	8	0.1(1.3)	>90	2.5	20	11	>90
	5	18/M	2,200	12	0(0)	>90	0	20	5	>90
Mean ± SD[i]					0.13±0.10 (0.88±1.00)		1.26±1.05	25.0±3.6	9.0±3.7	
CPEO	1	41/M	3,200	15	0.25(1.7)	>90	1.0	30	12	>90
	2	54/F	3,000	20	0.3(1.5)	>90	0.5	30	10	>90
	3	39/M	2,600	15	0(0)	>90	0	20	7	>90
	4	4/F	2,100	10	0.2(1.9)	>90	2.3	25	12	>90
	5	22/F	3,800	12	0(0)	>90	1.0	25	19	>90
Mean ± SD[i]					0.15±0.14 (1.02±0.94)		0.96±0.77	26.0±8.0	12.0±4.4	
MERRF	1	6/F	2,400	5	0(0)	>90	1.7	25	14	>90
	2	13/F	3,700	5	0(0)	>90	1.1	30	18	>90
	3	12/F	2,800	10	0(0)	>90	0	20	10	>90
	4	25/M	3,200	8	0.2(1.2)	>90	0.4	15	8	>90
	5	22/F	2,900	10	0(0)	>90	0	20	12	>90
Mean ± SD[i]					0.04±0.09 (0.24±0.54)		0.64±0.67	22.0±5.7	12.4±3.8	
Co	1	6/F	2,000	0	0(0)					
	2	15/M	2,100	0	0(0)					
	3	35/M	3,200	0	0(0)					
	4	50/F	2,500	0	0(0)					
	5	64/M	2,400	0	0(0)					
Mean ± SD[i]					0±0 (0±0)					

[a]Total number of muscle fibers examined
[b]% of RRFs among all muscle fibers
[c]% of muscle fibers with light microscopic TUNEL-positive myonuclei among all muscle fibers (among all RRFs)
[d]% of RRFs with Bax expression among all RRFs
[e]% of RRFs showing diffuse staining of cytochrome *c* among all RRFs
[f]% of RRFs with Apaf-1 expression among all RRFs
[g]% of RRFs with activated caspase-3 expression among all RRFs
[h]% of RRFs showing cytoplasmic expression of XIAP among all RRFs
[i]These data were analyzed by Student's *t*-test.

duction Laboratories, Lexington, Ky.) and the immunofluorescence technique using an antibody against cytochrome *c* (1:250, monoclonal, PharMingen), on serial 6-μm cryostat sections of muscle biopsy specimens. In addition, we used nonimmune mouse IgG or rabbit serum in place of these primary antibodies as negative controls.

Immunoblotting

Muscle cryosections were boiled for 5 min in a buffer containing 10% sodium dodecyl sulfate (SDS), 70 mM TRIS-HCl (pH 6.7), 10 mM EDTA and 5% 2-mercaptoethanol. After centrifugation, the protein content of the supernatants was estimated using the Bio-Rad protein assay system. The muscle extracts were then separated by SDS-polyacrylamide gel electrophoresis (5–10% gradient gel) and electrotransferred to a nitrocellulose membrane. Anti-Apaf-1 (1:1,000) and XIAP (1:500) antibodies were applied. These antibodies were revealed by horseradish peroxidase-linked sheep anti-rabbit immunoglobulin and enhanced by chemiluminescence (Amersham, Ill.). Whole cell lysate of human embryonic kidney 293 cells and HeLa cells, positive controls for Apaf-1 and XIAP, respectively [24, 25], were also examined in the same manner.

Results

TUNEL methods

At the LM level, two MELAS, two CPEO and four MERRF cases plus all control muscles showed no TUNEL-positive myonucleus. The remaining three MELAS, three CPEO and one MERRF cases had TUNEL-positive myonuclei. The average incidences were 0.13±0.10% in MELAS, 0.15±0.14% in CPEO and 0.04±0.09% in MERRF (Table 1). Almost all of them were seen in RRFs except for MERRF patient 4 (Fig. 2). At the EM level, control muscle showed a small number of immunogold particles linked to DNA fragments, as previously reported (Fig. 3C, D) [22]. On the other hand, many DNA fragments were demonstrated in the lymphocytes with condensed chromatin and shrunken cytoplasm in the thymuses treated with prednisolone (Fig. 3A, B). About half of the myonu-

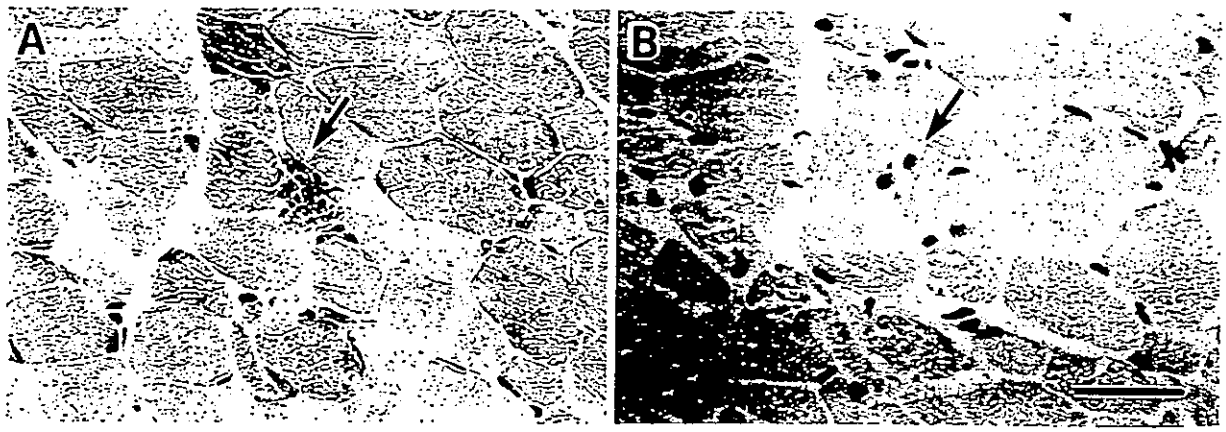


Fig. 2A, B LM-TUNEL method. A TUNEL-positive myonucleus (*arrow*) in an RRF of CPEO patient 1. All fibers with TUNEL-positive myonuclei are RRFs except those of MERRF patient 4 (not shown here). **A** Modified Gomori trichrome; **B** lightly counterstained with hematoxylin (*LM* light mocroscopic, *RRF* ragged-red fiber, *CPEO* chronic progressive external ophthalmoplegia, *MERRF* myoclonus epilepsy associated with ragged-red fibers). **A, B** Serial sections, *bar* **B** (also for **A**) 50 μm

clei in RRFs showed mild peripheral condensation of chromatin and hypertrophied nucleoli compared to those in control muscles and non-RRFs in mitochondrial encephalomyopathies. However, the number of DNA fragments even in myonuclei of RRFs was not significantly increased in comparison with control muscles (Fig. 3E, F). In addition, we found no DNA fragments in mitochondria of any case.

Immunohistochemistry

Bcl-2

In the mitochondrial encephalomyopathies, there was weak expression of Bcl-2 in the sarcoplasm of all type 2 non-RRFs and all RRFs regardless of fiber type. In control muscles, all type 2 fibers showed weak expression.

Bax

In contrast to Bcl-2, Bax was strongly expressed in the sarcoplasm of almost all RRFs but not in control muscles at all (Fig. 4B).

Cytochrome c

Cytochrome *c* stain showed a granular appearance in type 1 fibers of control muscles (Fig. 5A, C). This appeared to reflect its localization in mitochondria. On the other hand, there were several RRFs with diffuse cytochrome *c* staining in all patients, showing probable cytochrome *c* leakage from mitochondria to sarcoplasm (Fig. 5B, D) [3]. These fibers also showed positive Apaf-1 expression.

Apaf-1

In mitochondrial encephalomyopathies, 25.0±3.6%, 26.0±8.0% and 22.0±5.7% of RRFs in MELAS, CPEO and MERRF muscles, respectively, were positively stained (Fig. 4C). Non-RRFs were occasionally stained. In control muscles, no fiber was stained.

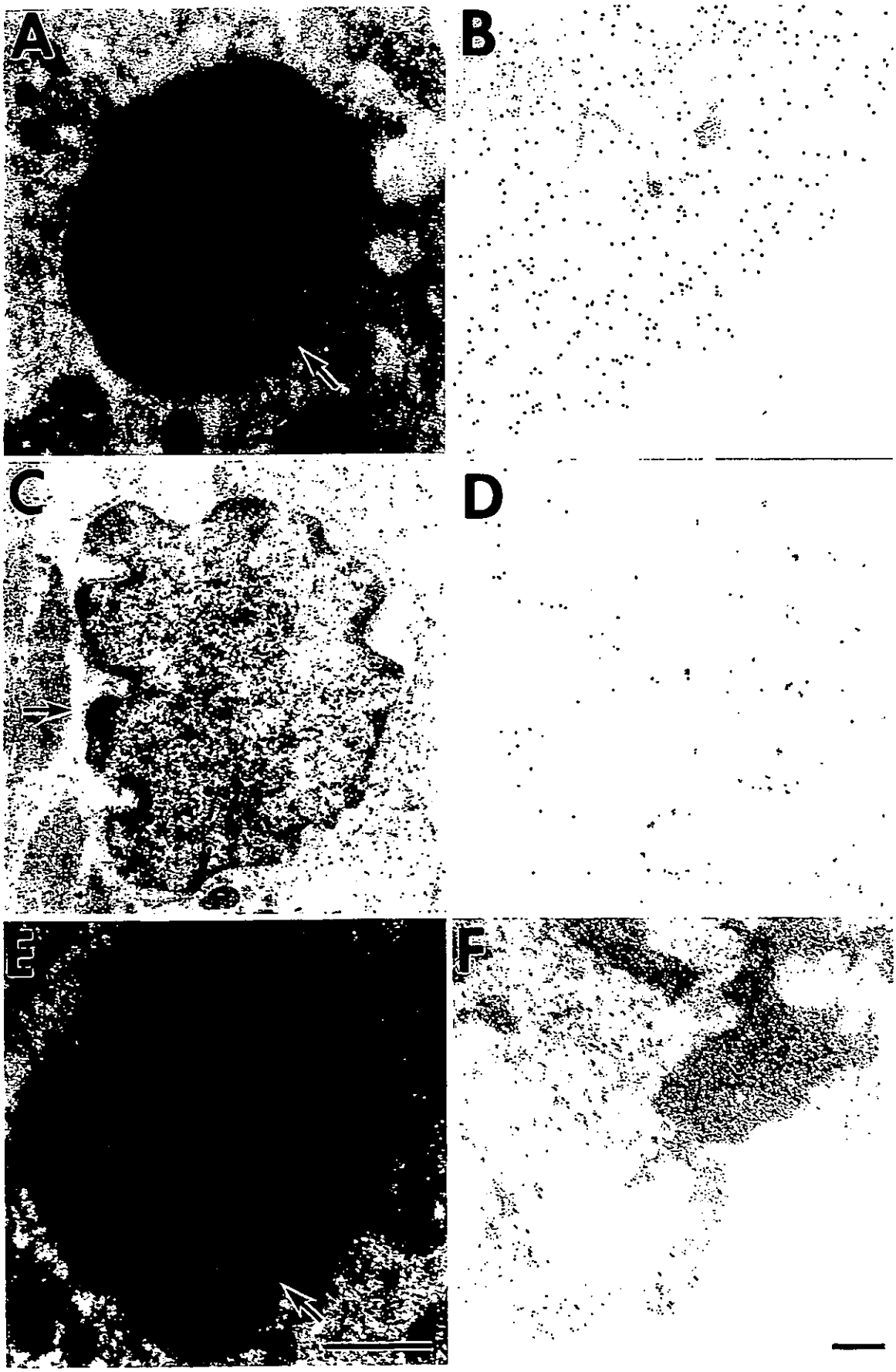
Activated caspase-3

In mitochondrial encephalomyopathies, 9.0±3.7%, 12.0±4.4% and 12.4±3.8% of RRFs in MELAS, CPEO and MERRF, respectively, showed caspase-3 activation in their sarcoplasm (Fig. 4D). Non-RRFs did not show activation of caspase-3, except for MERRF patient 4, who had a few non-RRFs with TUNEL-positive myonuclei and showed caspase-3 activation in several non-RRFs. In control muscles, caspase-3 activation was not seen. Almost all RRFs with activated caspase-3 showed Apaf-1 expression.

XIAP

Almost all RRFs showed sarcoplasmic expression of XIAP (Fig. 6). Non-RRFs in mitochondrial encephalomyopathies and fibers in control muscles showed sarcolemmal and occasional sarcoplasmic expression, regardless of the fiber type.

Fig. 3A–F EM-TUNEL method. **A** A typical apoptotic thymic ▶ lymphocyte of a rat treated with prednisolone. **B** Higher magnification of the portion indicated by the *arrow* in **A**. Gold particles linked to fragmented DNA are accumulated in condensed chromatin. **C** A normal myonucleus in a control muscle. **D** At higher magnification only a small number of gold particles are seen mainly in the area of heterochromatin. **E** A representative myonucleus in an RRF showing peripheral condensation of chromatin and enlarged nucleolus. MELAS patient 1. **F** Gold particles are seen in peripherally condensed chromatin area. However, the number of particles is not significantly increased as compared to **D**. Photographs **B, D, F** are less exposed than **A, C, E**, respectively, to make gold particles clearer (*EM* electron microscopic, *MELAS* mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episode). *Bars* **E** (also for **A, C**) 1 μm, **F** (also for **B, D**) 200 nm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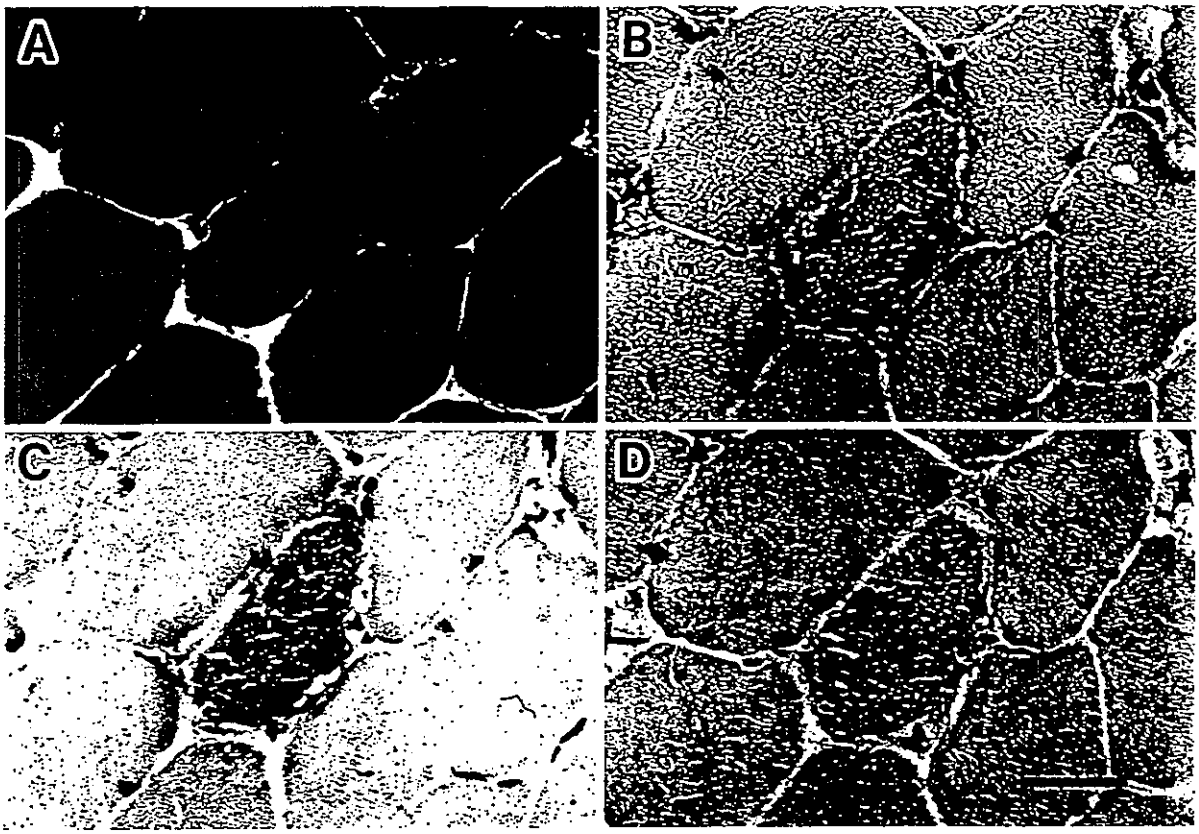

Fig. 4 Immunohistochemical detection of apoptosis-related proteins Bax, Apaf-1 and activated caspase-3 in an RRF of CPEO patient 2. **A** A typical RRF. **B, C** Bax (**B**) and Apaf-1 (**C**) are expressed in the sarcoplasm of this RRF. **D** Activation of caspase-3 is also seen in the sarcoplasm of this RRF. **A** Modified Gomori trichrome; **B–D** diaminobenzidine, lightly counterstained with hematoxylin. **A–D** Serial sections; *bar* **D** (also for **A–C**) 30 μm

Immunoblotting

Apaf-1

Apaf-1 was positively expressed in all mitochondrial encephalomyopathy muscles examined in this study, although its expression was weak compared to that in human embryonic kidney 293 cells used as a positive control of Apaf-1. In control muscles, no expression was seen (Fig. 7). The results confirmed increased Apaf-1 expression in RRFs as found immunohistochemically.

XIAP

XIAP was strongly expressed in both mitochondrial encephalomyopathy and control muscles (Fig. 8). No significant differences of the area and density of bands were found among them.

Discussion

In this study, we demonstrated that Bax and Apaf-1 are expressed and cytochrome *c* is released from mitochondria, resulting in caspase-3 activation, especially in RRFs. However, the ratios of TUNEL-positive myonuclei are much lower than that of the fibers showing caspase-3 activation. In other words, it is possible that apoptosis in muscles biopsy specimens of mitochondrial encephalomyopathies is suspended or rarely completed at least in some fibers. In addition, sarcoplasmic expression of XIAP, an inhibitor of caspases, is found in RRFs in mitochondrial encephalomyopathies.

Many studies have been undertaken to clarify whether the apoptotic process is involved in the pathophysiology of various myopathies [3, 4, 15, 20, 24, 27, 29, 30, 32, 36, 37, 43]. Among these, it was reported that Apaf-1 was not expressed in human skeletal muscle cells at either the protein and mRNA levels and that therefore the occurrence of mitochondria-mediated apoptosis would be unlikely [4]. In the present study, we demonstrated Apaf-1 expression in muscles of mitochondrial encephalomyopathies by immunohistochemistry and immunoblotting. Moreover, caspase-9 activation, which is activated by Apaf-1 and cytochrome *c* from mitochondria, was demonstrated in muscle fibers of laminin α2-deficient mice [32] and of a patient with an unusual congenital myopathy [20]. The apoptotic process through cytochrome *c* release from mitochondria, which is required for Apaf-1 expression, was also reported in muscles of limb-girdle muscular dystrophy type

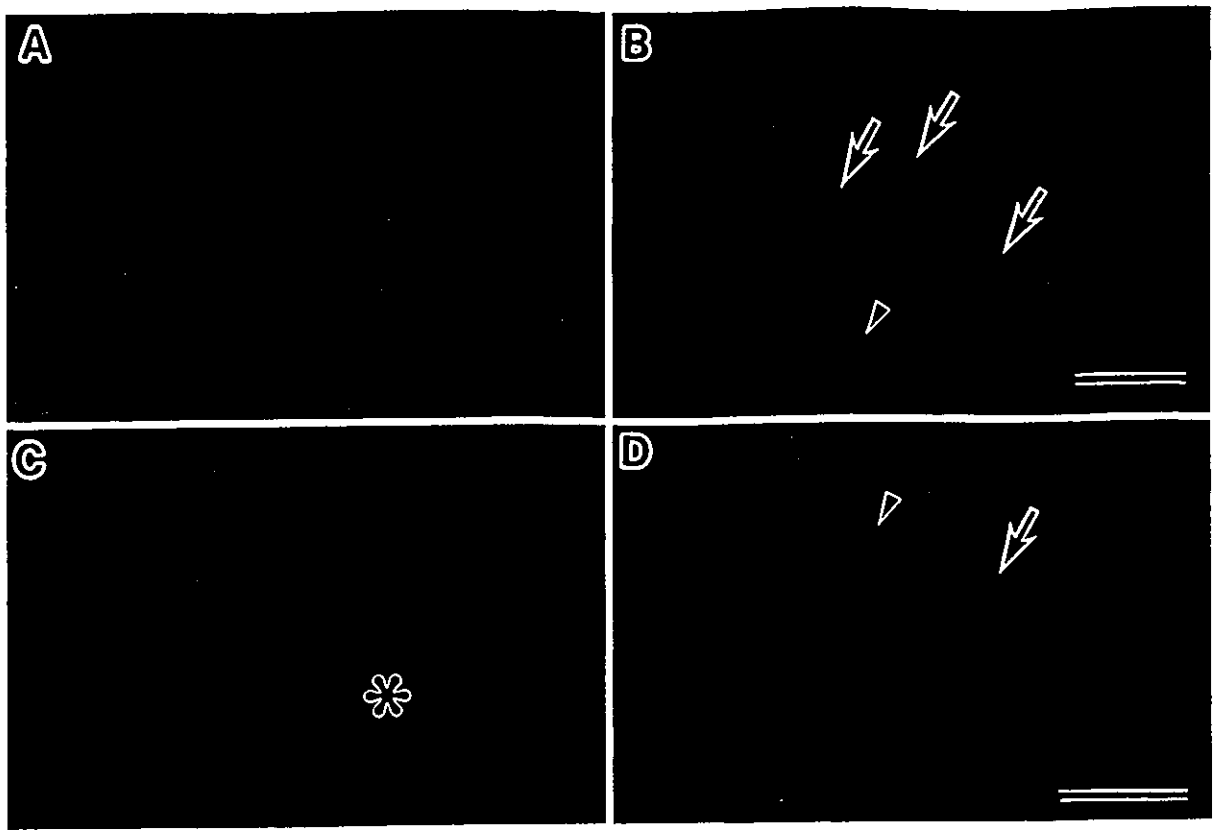

Fig. 5A–D Immunostaining of cytochrome *c*. **A** Type 1 fibers are weakly stained in all control muscles at this lower magnification. **B** RRFs in mitochondrial encephalomyopathy muscles show coarse granular appearance (*arrowhead*) to diffuse staining (*arrows*). **C** In control muscles, type 1 fibers showed more granular staining than type 2 fibers (*asterisk*). This seems to reflect the mitochondrial location of cytochrome *c*. **D** In mitochondrial encephalomyopathy muscles, RRFs show coarser granular appearance (*arrowhead*) than non-RRFs. The cytochrome *c* release from mitochondria is inferred from this diffusely stained RRF (*arrow*). **A–D** Immunofluorescence with FITC, **B**, **D** MELAS patient 2; *bars* **B** (also for **A**) 50 μm, **D** (also for **C**) 30 μm

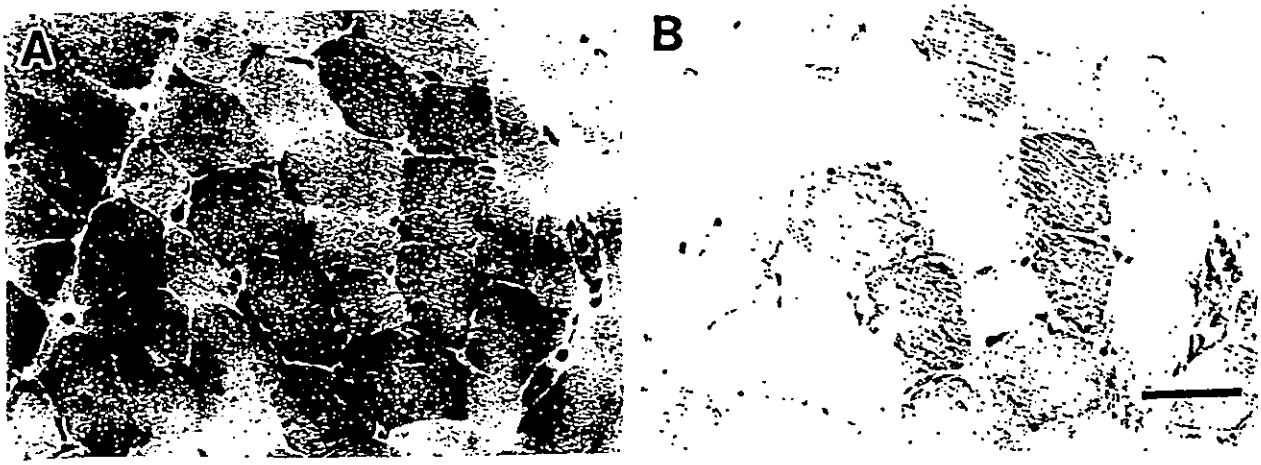

Fig. 6A,B Immunostaining of XIAP. **A** RRFs in a mitochondrial encephalomyopathy case, MELAS patient 1. **B** XIAP is expressed in the sarcoplasm of almost all RRFs of mitochondrial encephalomyopathies. A, B Serial sections; *bar* **B** (also for **A**) 50 μm

2A (LGMD2A) [3]. Taken together, there is little doubt that the apoptotic process due to cytochrome *c* release from mitochondria and including Apaf-1 expression and subsequent caspase-9 and -3 activation takes place in certain myopathies including mitochondrial encephalomyopathies.

Mirabella et al. reported apoptotic involvement in muscles of various mitochondrial encephalomyopathies [29]. They described 22 mitochondrial encephalomyopathy patients including 1 MERRF, 2 MELAS and 5 CPEO who showed more than 25% TUNEL-positive myonuclei. How-

Fig. 7 Immunoblotting using an antibody against Apaf-1. Extraction lysates of muscles from control muscles and mitochondrial encephalomyopathy muscles were loaded with 5 μg protein/lane. Whole cell lysate of human embryonic kidney 293 cells was also loaded as a positive control. All mitochondrial encephalomyopathy muscles show weak expression of Apaf-1, while all control muscles show no expression. *Lane 1* 293 cells; *lane 2* MELAS patient 1; *lane 3* CPEO patient 2; *lanes 4, 5* control muscles; *lanes 6, 7* MERRF patients 3, 4, respectively

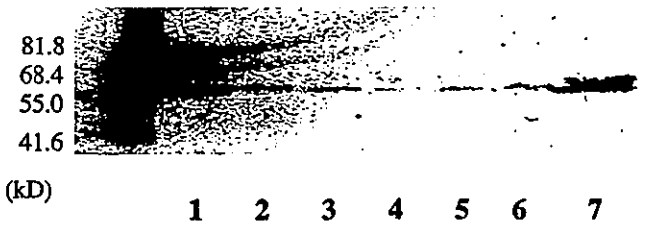

Fig. 8 Immunoblotting using an antibody against XIAP. Immunoblotting of XIAP was performed by a method similar to Apaf-1, but we used HeLa cell lysates as a positive control. All control and mitochondrial encephalomyopathy muscles examined show XIAP expression. *Lane 1* HeLa cells; *lanes 2–4* control muscles; *lane 5* MELAS patient 1; *lane 6* CPEO patient 2; *lane 7* MERRF patient 4

ever, in our study, the ratios of TUNEL-positive myonuclei were much lower (0.13±0.10% in MELAS, 0.15± 0.14% in CPEO and 0.04±0.09% in MERRF) than those they reported. Since mitochondrial encephalomyopathies progress slowly over many years, the ratios of TUNEL-positive myonuclei at the time of biopsy shown by Mirabella et al. seem to be too high. In fact, the ratio of TUNEL-positive myonuclei was 0.40±0.16% in muscles of LGMD2A [3] and that of apoptotic neurons was 0.02–0.09% in Alzheimer's disease brain [39]. LGMD2A and Alzheimer's disease are both long-standing degenerative diseases similar to mitochondrial encephalomyopathies. Further, Mirabella et al. did not reveal caspase-3 activation in muscle fibers but its expression. Because it was reported that caspase-3 is expressed even in normal muscle fibers [23], the finding of caspase-3 expression was hardly supportive of the high ratio of TUNEL-positive myonuclei.

Recently, Sciacco et al. claimed an absence of apoptosis in muscles of mitochondrial encephalomyopathies [37]. They reported that neither TUNEL-positive myonuclei nor Bcl-2 and Fas expression occurred in them. However, other molecules related to apoptotic signaling pathways, for example caspase-3, Apaf-1 and Bax, were not examined.

We found that XIAP expression in sarcoplasm of RRFs is dissimilar to that in control muscles [24]. It is possible that this difference in XIAP distribution contributes to the much lower ratio of TUNEL-positive myonuclei compared to that of caspase-3 activation in mitochondrial encephalomyopathies. XIAP inhibits caspase-9 activation (Figure 1) [6]. Nevertheless, we detected activated caspase-3, which is activated by activated caspase-9 in the apoptotic process through cytochrome *c* release from mitochondria. Activated caspase-3 activates caspase-activated DNase (CAD) [9] and apoptotic chromatin condensation inducer in the nucleus (Acinus) [35]. XIAP also inhibits the effect of activated caspase-3 [6]. Accordingly, DNA fragmentation and chromatin condensation hardly take place in muscles of mitochondrial encephalomyopathies.

There are three possible explanations for the "leaky" caspase-3 activation shown in this study. First, a different apoptotic signaling pathway other than mitochondria may be utilized. Caspase-3 is also activated by cleavage by activated caspase-8 without involving caspase-9 (Fig. 1). Caspase-8 is activated when the death receptors, one of which is Fas, are stimulated [1]. Therefore, the caspase-3 activation shown in this study may be due to the apoptotic pathway through Fas stimulation. In fact, Fas expression has been confirmed in muscles of mitochondrial encephalomyopathies [29]. Moreover, Asoh et al. [2] reported that mitochondrial dysfunction leads to up-regulation of Fas expression. Second, inhibitors of XIAP may play a role. Recently, two XIAP inhibitors, XIAP-associated factor 1 (XAF1) [26] and second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO) [7, 42] have been identified. These proteins can inhibit XIAP action and may lead to caspase-9 activation and the subsequent caspase-3 activation in muscles of mitochondrial encephalomyopathies. Third, XIAP itself may be gradually cleaved by other non-inhibited caspases or proteases [6]. The ability of XIAP to inhibit the activation of caspase-9 may then be reduced, and finally caspase-3 activation can take place.

Bax and Apaf-1 expression and caspase-3 activation are seen mostly in RRFs. This is probably due to a higher proportion of mutant mtDNA in RRFs than that in non-RRFs. The increased ratio of mutant mtDNA inhibits the respiratory chain and decreases ATP production [41]. Moreover, reactive oxygen species (ROS) generation in mitochondria is reported to increase under the conditions in which the respiratory chain is inhibited [11]. In fact, the immunostaining of 8-hydroxy-2'-deoxyguanosine, a marker of ROS generation was positive in RRFs examined in this study (data not shown). ROS in appropriate concentration can induce apoptosis [18]. In addition, it is reported that mtDNA abnormalities resulting in respiratory chain dysfunction increase Fas expression [2].

The reason the ratio of TUNEL-positive myonuclei in MERRF patients was smaller than those in MELAS and CPEO, in spite of similar expression of XIAP, is not clear. One explanation is that it was due to the limited numbers of muscles examined. Another is that the amount of ATP generated in RRFs in MERRF muscle is too low to accomplish the apoptotic execution, which is ATP dependent [8]. Respiratory chain complex IV (cytochrome *c* oxidase) deficiency is the most prominent in MERRF pa-

tients among the three diseases. Complex IV is the last electron acceptor in the mitochondrial respiratory chain, and its dysfunction leads to severe reduction in ATP generation.

In conclusion, it is possible that, although apoptotic reactions started in muscle biopsy specimens from mitochondrial encephalomyopathies, they are rarely completed at least in some fibers. It is likely that XIAP is a candidate for this delayed or suspended apoptotic process in muscles of mitochondrial encephalomyopathies.

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A Novel D104G Mutation in the Adenine Nucleotide Translocator 1 Gene in Autosomal Dominant Progressive External Ophthalmoplegia Patients with Mitochondrial DNA with Multiple Deletions

Hirofumi Komaki, MD,[1] Toshiyuki Fukazawa, MD,[3] Hideki Houzen, MD,[3] Kazuto Yoshida, MD,[4] Ikuya Nonaka, MD,[2] and Yu-ichi Goto, MD[1]

Autosomal dominant progressive external ophthalmoplegia is a mitochondrial disorder characterized by multiple large deletions of mitochondrial DNA. A recent study showed pathogenic heterozygous missense mutations in the heart/skeletal muscle isoform of the adenine nucleotide translocator 1 gene in autosomal dominant progressive external ophthalmoplegia patients. In one Japanese autosomal dominant progressive external ophthalmoplegia family, we found a novel A-to-G heterozygous mutation at nucleotide 311 of the adenine nucleotide translocator 1 gene, which segregated with affected individuals and could not be detected in the genomic DNA sequence of 120 normal controls. This mutation converted a highly conserved aspartic acid into a glycine at codon 104. Polymerase chain reaction analysis of single muscle fibers showed the presence of one type of deletion in each fiber, suggesting clonal expansion of mitochondrial DNA with deletions. These findings support the pathogenesis of the adenine nucleotide translocator 1 gene mutation in human disease.

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From the [1]Department of Mental Retardation and Birth Defect Research and [2]Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; [3]Department of Neurology, Hokuyukai Hospital, Hokkaido, Japan; and [4]Department of Neurology, Asahikawa Red Cross Hospital, Hokkaido, Japan.

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Address correspondence to Dr Goto, Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan.
E-mail: goto@ncnp.go.jp

Mitochondrial diseases comprise a wide spectrum of clinical phenotypes as a result of diverse mutations. Many pathogenic point mutations and rearrangements (deletions and duplications) in mitochondrial DNA (mtDNA) have been identified as the causes of the diseases. The representative pattern of the inheritance of mtDNA mutation is maternal transmission, although another is Mendelian inheritance, which is suggestive of a defect in nuclear DNA affecting mtDNA.[1]

Autosomal dominant progressive external ophthalmoplegia (adPEO) is a mitochondrial disorder characterized clinically by ptosis and progressive muscle weakness, especially of the external eye muscles. The disease onset is usually in early adulthood. The typical histological findings are ragged-red fibers (RRFs) with focal cytochrome *c* oxidase deficiency in patients' skeletal muscle. The patients have multiple large-scale deletions of mtDNA in the affected tissue. The patterns of inheritance indicate a nuclear gene defect predisposing them to secondary mtDNA deletions,[2,3] and there are three distinct autosomal loci for this disorder on chromosomes 4q34-35,[4,5] 10q24,[6,7] and 15q22-26.[8] Within these loci, mutations of adenine nucleotide translocator 1 (ANT1), C10orf2 (encoding Twinkle), and mtDNA polymerase γ genes have been associated with adPEO, respectively.

Human adenine nucleotide translocator exists as three isoforms that exhibit tissue-specific gene expression.[9] Because of its predominant expression in heart and skeletal muscle, ANT1 is designated as a heart/muscle isoform.[10] Its main function is a translocation of adenosine diphosphate and adenosine triphosphate across the inner mitochondrial membrane. Adenine nucleotide translocator also plays a central role in apoptosis as a central structural element of the permeability transition pore.[11]

Recently, two heterozygous missense mutations of ANT1 gene were identified in 5 4q-adPEO families and 1 sporadic patient.[5] We herein report a novel heterozygous mutation of the ANT1 gene in a Japanese adPEO family.

Patients and Methods

Autosomal Dominant Progressive External Ophthalmoplegia Pedigree

All affected family members had progressive ptosis and external ophthalmoplegia (Fig 1a). Patient II-7 first noticed mild bilateral progressive ptosis at age 48, and at age 55 she began to develop generalized muscle weakness. A physical examination at age 70 showed a very thin woman (weight, 28kg) with severe bilateral ptosis, ophthalmoplegia, generalized muscle weakness, and atrophy. Routine laboratory analyses, creatine kinase, lactate, pyruvate in both blood and spinal fluid, and electrocardiograms were normal. Brain magnetic resonance imaging showed diffuse abnormal signals in periventricular white matter. She developed heart failure at age 70 and died at age 71.

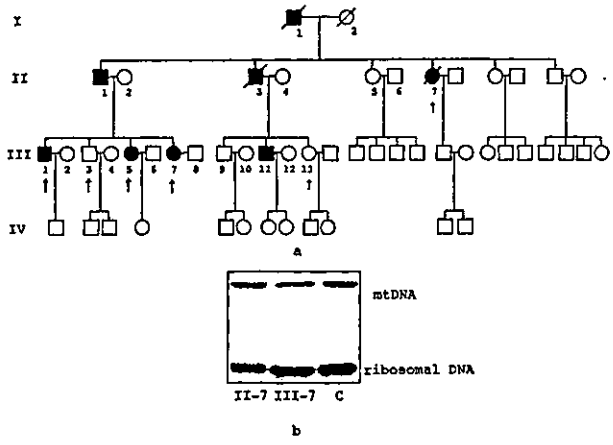

Fig 1. (a) Pedigree of the family with autosomal dominant progressive external ophthalmoplegia. Solid symbols represents affected individuals, and open symbols represent unaffected individuals. Arrows indicate the individuals subjected to mutation analysis. (b) Multiple mitochondrial DNA (mtDNA) deletions in muscle DNA with Southern blot analysis. Total DNA from a control subject (C) and Patients II-7 and III-7 were digested by restriction enzyme PvuII *to linealize the mtDNA, and Southern blot analysis was performed with mtDNA and 18S ribosomal DNA-specific probes.*

Patient III-7 was healthy until she started to show bilateral ptosis at age 33. A physical examination at age 41 showed bilateral ptosis and ophthalmoplegia. She had no sign of muscle weakness except for ophthalmoplegia. Routine laboratory analyses, creatine kinase, lactate, pyruvate in blood and spinal fluid, electrocardiograms, and brain computed tomography were normal. Histological studies on skeletal muscle biopsies for both II-7 and III-7 exhibited RRFs and cytochrome *c* oxidase deficiency in a few percent of the fibers and moderate type 2 fiber atrophy. Other affected members did not have definite symptoms associated with muscular and neurological diseases, including psychiatric manifestations, pigmentary retinopathy, hypogonadism, and respiratory insufficiency, except for ptosis and external ophthalmoplegia.

We also performed sequence analysis of the ANT1 gene in 32 sporadic progressive external ophthalmoplegia patients with multiple mtDNA deletions.

Molecular Studies

Total DNA was extracted by the standard phenol/chloroform method from muscle specimens (II-7 and III-7) and blood (II-7, III-1, III-3, III-5, III-7, and III-13). Southern blot analysis was performed to detect the mtDNA rearrangements in muscle DNA. Total DNA was digested by restriction enzyme *PvuII* to linealize the circular mtDNA, and genomic blots of restriction digests of total DNA were hybridized with mtDNA and nuclear-encoded 18S ribosomal DNA gene probes.[12,13]

The four exons of the ANT1 gene were amplified by polymerase chain reaction (PCR) with the following ANT1-specific intronic primers (forward/reverse, 5′ to 3′): exon1, TAAGGGGGAGCTGCGGGCCA and ATATAGACACCCGAACGCCG; exon 2, AAATCTAGGAAGTGCAAACC and AGTTTATTTCAGTAGAGGAC; exon 3, CAGTGGCCTCTCTCCCTCCA and TAGCTTTTGCACCCAGGCTC; and exon 4, AGTCTCTTTCCTCCAGCGTT and AAGCGTGCATTAAGTGGTCT. Amplification products were directly sequenced as described elsewhere.[14]

The A-to-G mutation at nucleotide 311 was detected with the restriction enzyme *Alw I* (loss of a restriction site; Fig 2b). The *Alw I* restriction fragments were separated through 4% low melting agarose gel, stained with ethidium bromide, and photographed.

Single-muscle-fiber PCR was performed to detect mtDNA with large-scale deletion in each fiber with the previously described method.[15] The muscle fibers were isolated from 30μm-thick cryostat cross sections stained for succinate dehydrogenase activity to identify RRFs. DNA was extracted from the dissected normal and RRFs with alkaline extraction. Aliquots of the resulting 10μl solutions were subdivided into 10 PCR tubes, and PCR was carried out with 10 sets of primers to detect mtDNA with deletions with the previously described method (Fig 3).[16] The breakpoints of the deletions were detected by direct sequence as described previously.

Results

Southern blot analysis demonstrated full length, and the additional multiple fragments shorter than expected indicated that both II-7 and III-7 had mtDNA with

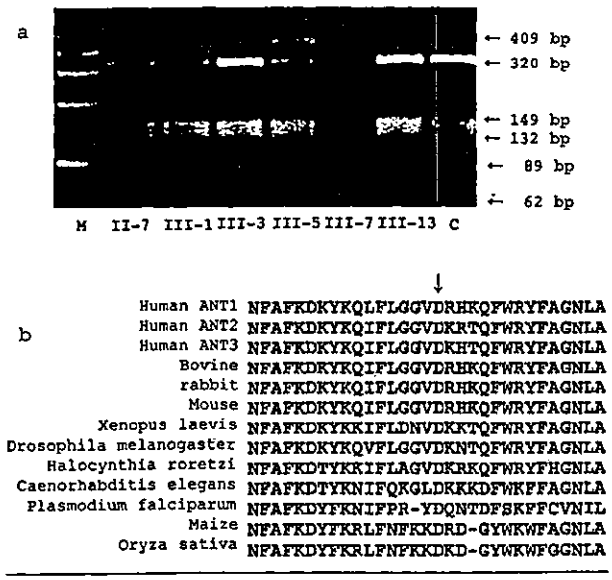

Fig 2. (a) Segregation of the mutation in the family. Genomic DNA were amplified by polymerase chain reaction (PCR) with a set of specific intronic primers for exon 2, and the products were digested restriction enzyme Alw I. *When the 775bp of the exon 2 PCR products were digested by* Alw I, *the following product sizes were generated—320, 149, 132, 89, 62, 13, and 10bp in the wild genome and 409, 149, 132, 62, 13, and 10bp in the mutant genome—and the restriction site was lost. (b) Sequence conservation of ANT1. D104 (arrows) is strictly conserved among species.*

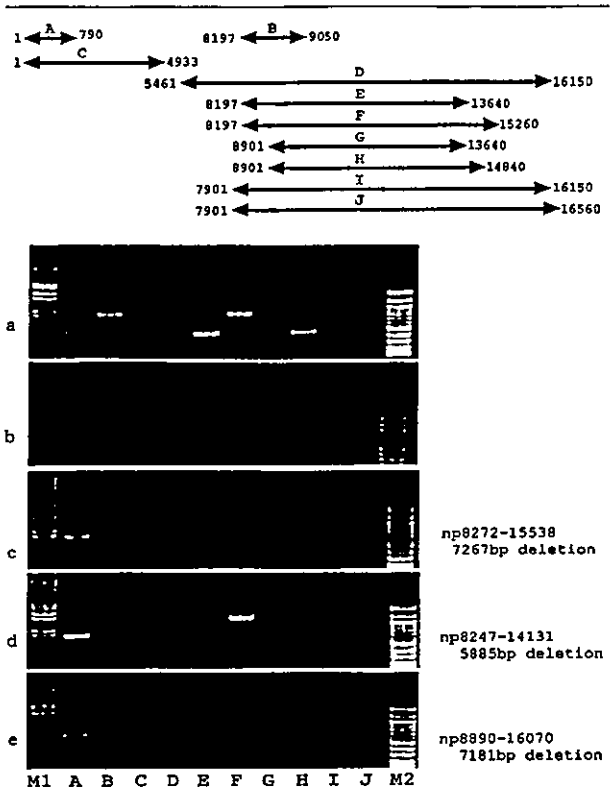

Fig 3. Single-muscle-fiber polymerase chain reaction (PCR) analysis of mitochondrial DNA (mtDNA). In the upper part of the figure, the lines flanked by arrows illustrate the primer pairs for PCR analysis as previously described.[15] The sequences of the primers are numbered according to the Cambridge mtDNA sequence. The lanes at the right and left show DNA size markers (M1, λ/Hind III, EcoRI double digest marker; M2, 100bp ladder marker). Primer pairs A and B were used to amplify wild-type mtDNA. Primer pairs C to M were used to amplify mtDNA with deletion. (a) PCR analysis from about 100 muscle fibers shows many amplified fragments indicating multiple mtDNA deletions. (b) Single-muscle-fiber PCR from nonragged-red fibers (nonRRFs) shows only expected normal fragments in lanes A and B. (c–e) Single-muscle-fiber PCR from three different ragged-red fibers (RRFs) shows some fragments shorter than expected. The identification of the breakpoints of the deletions by direct sequence of the fragments confirmed only one deletion in each RRF (right, breakpoints and deletion sizes).

deletions of different sizes showing multiple mtDNA deletions (see Fig 1b). To quantify the relative amount of mtDNA to that of nuclear DNA, we used the 18S ribosomal DNA genes as a probe.[13] With the signal as an internal control, mtDNA depletion was not detected in muscles of the 2 patients.

By the sequence analysis of the ANT1 gene, we found a novel mutation, A-to-G heterozygote mutation at nucleotide 311, which converted an aspartic acid into a glycine at codon 104 in exon 2 (data not shown). Other sequences were quite identical to normal ones. With a PCR-restriction fragment length polymorphism method, this mutation was detected in 4 affected family members (II-7, III-1, III-5, and III-7) but not in 2 unaffected family members (III-3 and III-13) or 120 normal individuals (see Fig 2a). No mutations were detected in 32 sporadic progressive external ophthalmoplegia patients.

Single-muscle-fiber PCR from nonRRFs showed only expected fragments (see Fig 3). Single-muscle-fiber PCR from three different RRFs showed some fragments shorter than expected. The identification of the breakpoints of the deletions by the direct sequence of the fragments confirmed that there was only one different type of deletion in each RRF. Fragments c and e had direct repeats flanking the breakpoints.

Discussion

We identified a novel A-to-G heterozygous mutation in exon 2 of the ANT1 gene in one Japanese adPEO family. We concluded that this mutation was potentially pathogenic for the following reasons. First, affected and unaffected family members demonstrated segregation with the mutation, which was not detected in normal individuals. Second, this mutation converted a strictly evolutionary conserved aspartic acid into a glycine, that is, an acidic into a nonpolar in a side chain (see Fig 2b). As mentioned previously, Kaukonen and colleagues[5] identified an ANT1 mutation, A114P, in 5 of 41 families of adPEO patients and V289M in 1 of 13 sporadic patients. The former mutation was located near the mutation we found, and the surrounding amino acids were relatively conserved, indicating that the region is functionally important.

Single-muscle-fiber analysis showed there was one type of deletion, and the type of deletion varied with each RRF. These findings suggest each mtDNA with deletion originated in a mitochondrion and expanded in a postmiotic cell, as discussed elsewhere.[16,17] Therefore, it is conceivable that a deletion is a relatively rare event. Secondary accumulations of multiple mtDNA deletions by nuclear gene defects have been observed in some mitochondrial diseases, including adPEO caused by mutated ANT1,[5] Twinkle (probably functioning as a mtDNA helicase),[7] and polymerase γ[8] genes and mitochondrial neurogastrointestinal encephalomyopathy caused by a mutated thymidine phosphorylase gene.[18] The pathomechanism of multiple mtDNA deletions is far from understood because the known functions of each defective gene vary.

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