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d) Complex IV（ミトコンドリア呼吸鎖複合体 IV）欠損症
Complex IV deficiency

✓ 疾患の要点

- Complex IV が欠損ないし活性が低下することにより，エネルギー産生低下が起こり臓器障害を引き起こす
- ミトコンドリア DNA および核DNA の両方とも原因になりうる
- Complex IV 欠損症の頻度は，はっきりと記載されたものは少ない．筆者らの酵素診断の中では呼吸鎖異常症全体の約16%（300 例中49 例）である
- mtDNA の異常では Leigh 脳症，MELAS，（運動不耐性）ミオパチー，LHON，急性脳症，心筋症，前立腺癌など臨床病型は様々である．核DNA では SURF1 など Leigh 脳症を呈するものが多い．遺伝子ごとに特徴的な症状が報告されている
- 診断は complex IV の低下・欠損を酵素学的に証明することである（in vitro 酵素活性，BN-PAGE 解析，免疫染色など）

　　　　　　　　　　　　　　　　　　　　　　　　　　　　　

■欠損酵素：シトクローム c オキシダーゼ（cytochrom c oxidase），EC 1.9.3.1
■遺伝情報／遺伝形式／ OMIM：表 1 中に記載

ミトコンドリア呼吸鎖複合体 IV（complex IV）はシトクローム c オキシダーゼ（cytochrom c oxidase: CCO, あるいは COX）とも呼ばれ，電子伝達系の終末部にあり，還元型シトクローム c を酸化するとともに，酸素を還元し水 2 分子をつくる酵素である．

この complex IV の欠損ないし活性が低下によってエネルギー産生が低下して臓器障害を引き起こすものが，ミトコンドリア呼吸鎖複合体 IV 欠損症（complex IV 欠損症）である．臨床的に最も common な神経障害は Leigh 脳症であるが，新生児・乳児ミトコンドリア病，心筋症といった臨床型もよく見られる．
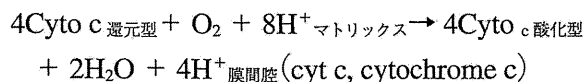
疫　学

筆者らの酵素診断の中では complex I 欠損症，複数の呼吸鎖欠損症の次に高い頻度であり，単独呼吸鎖欠損症の中では，complex I 欠損症に次いで多い．呼吸鎖異常症全体の約14%（232 例中 33 例）である．また，Leigh 脳症を起こす原因としても多いが，ミオパチー単独症状から，多臓器障害にわたるまで症状は多彩であり，発症年齢も新生児から成人までさまざまである．

選択的 complex IV 欠損症（Isolated complex IV deficiency）をきたす遺伝子は mtDNA，核 DNA と

も 1990 年代後半から見つかりはじめているが，現在は新規遺伝子は核 DNA の報告のみである．これらの遺伝子を表 1 にまとめた．さらに図 1 に complex IV のアセンブリー過程のモデル[1]を示すので，参考にしていただきたい．また，最近のわが国における Complex IV 欠損症については，最近 Tanigawa ら が，2 例の *Surf1* 異常 に 基づく Leigh 脳症の症例報告を報告している[2]．

病因・病態

Complex IV の酵素反応は次の通りである．

$$4\text{Cyto c}_{還元型} + O_2 + 8H^+{}_{マトリックス} \rightarrow 4\text{Cyto c}_{酸化型} + 2H_2O + 4H^+{}_{膜間腔} (\text{cyt c, cytochrome c})$$

この酵素は，還元型シトクローム c を酸化型にする．これに伴い酸素が還元されて水 2 分子を生じる．

MitoMap の HP（http://www.mitomap.org/ MITOMAP）を参照すると，mtDNA 由来のものとしては，シトクローム c の I〜Ⅲ をコードしている遺伝子（*MT-CO1-3*）内に 60 を超えるの点変異の報告がなされている[3]．Leigh 脳症，MELAS，（運動不耐性）ミオパチー，LHON，急性脳症，心筋症，前立腺がんなど臨床病型は様々であり，重症度は症例によって異なる．

次に核 DNA 由来の既知の変異について述べ

表 1 Complex IV 欠損症関連の核遺伝子のまとめ

遺伝子	正式名称	局在	OMIM	code している タンパク・働き	遺伝形式	報告されている疾患
サブユニット異常						
COX6B1	cytochrome c oxidase subunit VIb polypeptide 1	19q13.1	124089	サブユニット 6B	A.R.	乳児ミトコンドリア病
COX4I2	cytochrome c oxidase subunit IV isoform 2	20q11.21	607976	サブユニット 4 isoform 2	A.R.	膵外分泌不全, 赤血球異型性貧血 (Dyserythropoeitic Anemia), 頭頂部の頭蓋骨骨化過剰の合併症例
COX7B	cytochrome c oxidase subunit VIIb	Xq21.1	300887	サブユニット VIIb	A.R.	先天性皮膚形成不全症(APLCC)
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	7p21.3	603833	additional COX subunit	A.R.	Leigh 脳症
アセンブリータンパク・呼吸鎖生合成 (biogenesis) の異常						
SURF1	surfeit 1	19q13.12	185620	アセンブリー因子	A.R.	Leigh 脳症や重篤な神経疾患
SCO2	SCO cytochrome oxidase deficient homolog 2	22q13.33	604272	copper transport	A.R.	乳児ミトコンドリア病 (心筋症, 脳筋症)
SCO1	SCO cytochrome oxidase deficient homolog 1	17p13.1	603644	copper transport	A.R.	乳児ミトコンドリア病 (心筋症, 脳症, 肝症)
COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase	17p12	602125	Heme A farnesyltransferase	A.R.	尿細管障害を伴う脳症, Leigh 脳症, 乳児ミトコンドリア病 (難聴, 心筋症)
COX15	COX15 homolog, cytochrome c oxidase assembly protein	10q24	603646	Heme A synthesis	A.R.	Leigh 脳症 (長期生存例あり) や乳児ミトコンドリア病 (脳症, 心筋症, 小頭症)
COX20	COX20 cytochrome C oxidase assembly factor	1q44	614698	アセンブリー因子	A.R.	小児の成長障害, 筋緊張低下, 小脳失調
ETHE1	ethylmalonic encephalopathy 1	19q13.31	608451	sulfur dioxygenase ; Ethylmalonic acid metabolism	A.R.	エチルマロン酸脳症
FASTKD2	FAST kinase domains 2	2q33.3	612322	アポトーシスに関与・？	A.R.	乳児ミトコンドリア病(脳症)
COA (C2orf64)	cytochrome c oxidase assembly factor 5	2q11.2	613920	アセンブリー因子	A.R.	新生児期のミトコンドリア心筋症
ミトコンドリア DNA の発現に関わる異常						
LRPPRC	leucine-rich pentatricopeptide repeat containing	2p21	607544	アセンブリー因子	A.R.	Leigh脳症 (French-Canadian Leigh disease)

る. はじめにサブユニット異常についてである.

COX6B1 遺伝子異常については, 2008 年にこの遺伝子異常により重篤な乳児の脳筋症をきたした症例が報告された[4]. 数少ないサブユニットを code した核の遺伝子である.

COX4I2 遺伝子異常は, 脂肪吸収障害を伴う膵外分泌不全(cystic fibrosis や Shwachman-Diamond 症候群や Pearson 症候群などで見られる)や, 赤血球異型性貧血(dyserythropoeitic Anemia)や, 頭頂部の頭蓋骨骨化過剰きたす症例で見つかっている[5].

次はアセンブリータンパクの異常である.

Surf1 は病因遺伝子としては最も有名であり, complex IV 欠損によって生じる Leigh 脳症の主要因となる遺伝子である. また, その他の重篤な神経疾患や多臓器障害もきたす. 特に新生児期〜乳児期に発症することが多い[6].

SCO1 および SCO2 はミトコンドリア内の銅の成熟化とサブユニット II の合成に関わる. 前者は肝症＋脳症, 心筋症＋脳症を引き起こした報告がある[7][8].

COX10 は Heme A farnesyltransferase をコードしており complex IV の機能発現と Complex IV 内のヘム A に関わる成熟機構に関係する. この遺伝子の phenotype は, 表 1 に示すように Leigh 脳症を含めさまざまである. Leigh 脳症の症例や乳児ミトコンドリア病の症例は輸血が必要な重度の貧血をきたしている[9]. 初回報告例は尿細管障害を伴う脳症としての報告であるが, 本遺伝子異常に特異的ではないだろう.

COX15 は HemeA 合成に関わる遺伝子であり, complex IV の生合成に関わるタンパクである. Leigh 脳症の長期生存例の報告がある一方, 新生児早期の致死性心筋症でも報告がある[10].

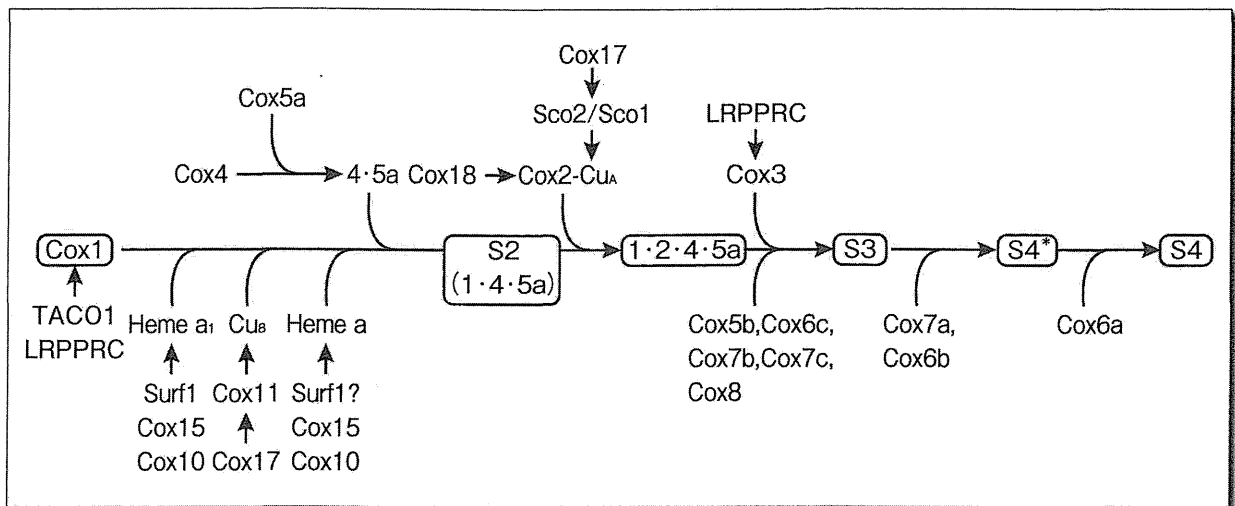ETHE1 はエチルマロン酸血症(脳症)として知られ, ミトコンドリアマトリックス内にある硫化

図1　哺乳類の complex IV アセンブリーモデル

（Fornuskova D, et al: Novel insights into the assembly and function of human nuclear-encoded cytochrome c oxidase subunits 4, 5a, 6a, 7a and 7b. Biochem J **428**: 363-374.2010 より引用）

物の異化に関わる sulfur dioxygenase をコードしている遺伝子である．硫化物は強力な complex IV 阻害物質であるため，何らかの硫黄物による complex IV 活性の間接的な低下が機序として示唆されている．エチルマロン酸血症は，タンデムマス（アシルカルニチン分析，C4-6 上昇）および尿中有機酸分析にて化学診断が可能である．

FASTKD2 はこれまで新生児のミトコンドリア病（脳症）の報告がある[11]．*FASTKD2* はミトコンドリア内部分画のタンパクを code しており，アポトーシスへの関与が考えられている．

C2orf6 は Complex IV のアセンブリー過程の早期に関与する因子と考えられており，新生児の肥大型心筋症として報告されている[12]．

LRPPRC はケベック州（Saguenay-Lac-Saint-Jean 地域）のフランス系カナダ人に好発する Leigh 脳症を起こす病因遺伝子として知られている[13]．この遺伝子はサブユニットやアセンブリータンパクではなく，mtDNA の発現に関わることがわかってきた．

NDUFA4 は complex I のサブユニットをコードしている遺伝子と思われたが，実際には Leigh 脳症の患者で complex IV のサブユニットであることが示唆された[14]．

診断と鑑別診断

本症の診断は，臨床症状や CT，MRI などの画像所見，生化学所見などから呼吸鎖異常症を疑い，皮膚線維芽細胞，または障害臓器（肝臓，筋肉，心筋）などを用いて呼吸鎖酵素活性を測定することである．Complex III のアッセイと同様にこの酵素アッセイは非常に速いため，手際よく測定しないと偽性 complex IV 欠損症をつくってしまう．また，COX 染色は病理検査としても行われており，合わせて評価することは有意義である．

治療と予後

本症の根治的治療法はなく，一般的に高脂肪食およびミトコンドリアカクテル等を使用していくことになる．

Complex IV 欠損症に関して，ベザフィブレート（Bezafibrate: BZF）を使用することにより，complex IV の活性が上昇し ATP 産生が増すことが *COX10* や他のアセンブリー因子欠損マウスで示されており[15]，さらに complex IV 欠損症症例の培養細胞でも complex I, III, IV 活性の上昇効果が示されている．

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e) Complex V (ミトコンドリア呼吸鎖複合体 V) 欠損症
Complex V deficiency

☑ 疾患の要点

- Complex V が欠損ないし活性が低下することにより，エネルギー産生低下が起こり臓器障害を引き起こす
- ミトコンドリア DNA および核DNA の両方とも原因になりうる
- Complex V 欠損症の頻度は，酵素診断されているうち約4% が complex V 欠損症である mtDNA の異常は酵素診断されることは少なく，それを含めると相当数になると思われる
- mtDNA の異常では，Leigh 脳症，MELAS，(運動不耐性)ミオパチー，LHON，急性脳症，心筋症，前立腺癌など臨床病型は様々であり，重症度は症例によって異なる．核DNA では新生児ミトコンドリア病(先天性高乳酸血症)が多く，生化学的に 3-メチルグルタコン酸尿症を呈することが多い
- 診断は Complex V の低下・欠損を酵素学的に証明することである(*in vitro* 酵素活性，BN-PAGE 解析など)

···

■欠損酵素：ATP 合成酵素(ATP sythetase)，EC 3.6.1.14
■遺伝情報／遺伝形式／OMIM：表 1 中に記載

ミトコンドリア呼吸鎖複合体 V(complex V)は ATP 合成酵素(ATP sythetase)であり，呼吸鎖の最終段階に位置する．電子伝達によって complex I, III, IV は H^+低濃度のマトリックスから H^+高濃度の膜間部(サイトゾルにつながっている)にプロトンを汲み出す．こうして生じる電気化学勾配(プロトン駆動力)に従ってプロトンが膜間腔からマトリックス内に流入するときに，内膜に結合した ATP 合成酵素が ADP と無機リン酸から ATP を合成する(図 1)[1]．

この complex V = ATP 合成酵素の欠損ないし活性が低下によってエネルギー産生が低下して臓器障害を引き起こすものが，ミトコンドリア呼吸鎖複合体 V 欠損症(complex V 欠損症)である．Complex V 欠損症の臨床診断としては Leigh 脳症，新生児・乳児ミトコンドリア病，心筋症などさまざまである．

疾　学

Complex V 欠損症の頻度は，はっきりと記載されたものは少ない．Honzik らはオーストリアとチェコでの 20 年間で新生児に発症した 129 人の MRCD 患者のうち，31 人(24%)が complex V 欠損であると報告しており，欧州(特に東欧地域)に

おける新生児発症 MRCD においては大きな割合を占めている[2]．その多くは *TMEM70* 遺伝子異常である．また，Rodenberg の論文を読み解くと，酵素診断されているうち約 4% が complex V 欠損症(ちなみに complex I 欠損が 31%，複数の呼吸鎖欠損が 27%，complex IV 欠損が 19%，complex III 欠損が 12%，complex II 欠損が 7%)である[1]．しかし complex V 欠損症の多くの症例は，新生児のミトコンドリア病であり，高アンモニア血症を認めたり，尿中有機酸分析にて 3-メチルグルタコン酸が検出されたりすることが多く，診断の十分な手がかりとなる．筆者らは全国の新生児高乳酸血症や高アンモニア血症の症例に対して，尿中有機酸分析を含む代謝スクリーニング検査を行う機会が多いが，3-メチルグルタコン酸はほとんど検出されておらず，わが国での発症は欧州ほど多くことが予想される．

mtDNA 由来では a，A6L サブユニットをコードしている，*MT-ATP6*，*MT-ATP8* 遺伝子が病因遺伝子として挙げられる．特に *MT-ATP6* 遺伝子異常による Leigh 脳症をはじめとしたミトコンドリア病の報告は多い．
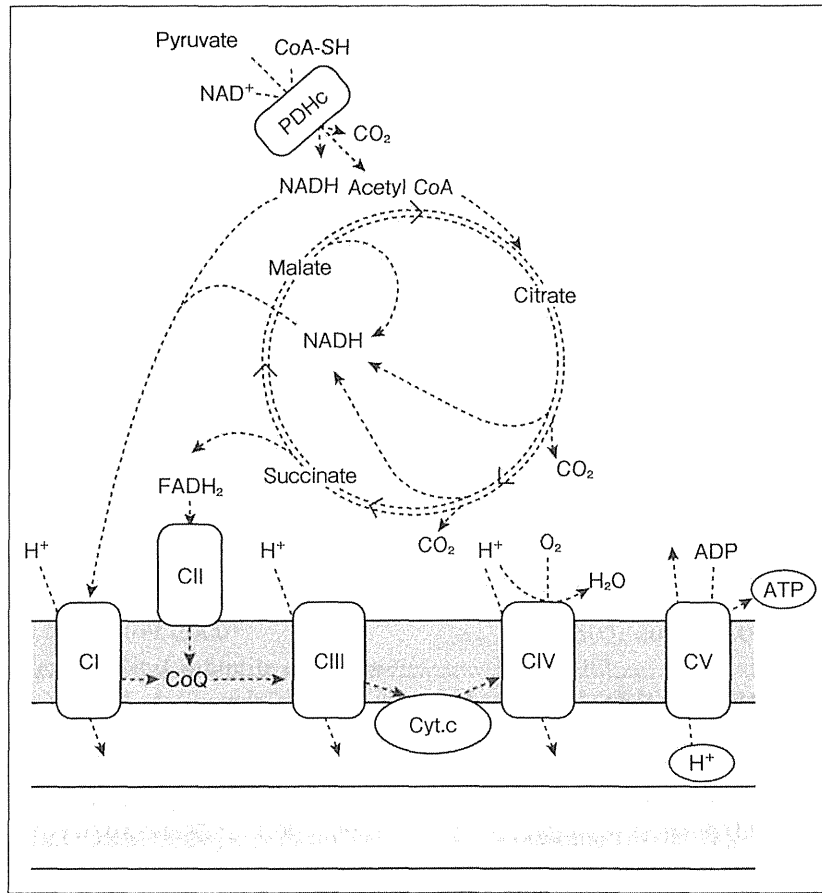
図1　TCA回路およびミトコンドリア呼吸鎖の模式図
(Rodenburg RJ: Biochemical diagnosis of mitochondrial disorders. J Inherit Metab Dis 34: 283-292. 2011 より抜粋，改変)

病因・病態

　現在病因遺伝子として報告されているものは，本酵素のサブユニット異常およびアセンブリー因子を含む呼吸鎖生合成(biogenesis)の異常である．これらの異常に伴い complex V の酵素活性が低下し，症状が出現する．本症は，新生児期に症状を呈することが多い．

　以下，既知の病因遺伝子について述べる．

　mtDNA 由来の病因遺伝子としては，サブユニット a を code している MT-ATP6 とサブユニット A6L を code している MT-ATP8 である．Complex V 欠損症で最初に発見された遺伝子異常は，MT-ATP6 である[3]．これまで MT-ATP6 遺伝子内に 20 を超えるの点変異の報告がなされている (http://www.mitomap.org/MITOMAP)．特に m.8993T>G/C および m.9176T>G/C 変異は，孤発性運動失調，NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa)，両側線条体壊死，Leigh 脳症

(MILS; Maternally Inherited Leigh Syndrome ともいう)といった症状を呈することが多い[4]．その他多くの変異が報告されているが，Leigh 脳症，MELAS，(運動不耐性)ミオパチー，LHON，急性脳症，心筋症，前立腺癌など臨床病型はさまざまであり，重症度は症例によって異なる．MT-ATP8 遺伝子異常による MRCD の報告は 2008 年から，脳症，心筋症などが報告されている[5)6)]．

　次に核 DNA 由来の既知の変異について述べる (表1)．サブユニット異常に関して，現在報告されている遺伝子は ATP5E (サブユニット ε)と ATP5A1 (サブユニット a)でありかつ，それぞれ1例ずつの報告である．ATP5E 遺伝子は22歳女性の報告で，新生児期に発症し，乳酸アシドーシスと 3-メチルグルタコン酸高値を認め，軽度の精神遅滞，末梢神経障害を呈しているという報告である[7]．ATP5A1 遺伝子は新生児の進行性脳症の原因として報告されている[8]．

　最も多い核由来の異常は TMEM70 (Transmem-

brane protein 70)である．*TMEM70* はミトコンドリア内膜に関連しているタンパクで，complex V の生合成に重要である（ancillary factor）ことがわかっている[5]．臨床的には新生児ミトコンドリア病を呈する．すなわち高乳酸血症，脳症，心筋症，奇形，新生児白内障，消化管の機能不全，先天性の筋緊張亢進など多彩な症状を呈しうる[9][10]．Honzik は 25 症例の *TMEM70* 異常症例をまとめている[10]．それによると早産や IUGR も 7 割近く認めている．無呼吸発作，筋緊張低下がともに92%，肥大型心筋症が 76%，停留精巣が 67%，尿道下裂が 54%，高乳酸血症（5 〜 36mmol/L）を92%，高アンモニア血症（100 〜 520 μmol/L）を86% に認めている．40% が 6 週以内に死亡しており，予後はよくない．この遺伝子異常はロマニー族（Romanies）に集積していることが明らかになっている．本症は尿中有機酸分析で 3-メチルグルタコン酸高値が必発である．*ATPAF2* 遺伝子は*ATP12* 遺伝子とも言われ，complex V のアセンブリータンパクである．*TMEM70* と同様に重症な新生児ミトコンドリア病を呈し，脳症，奇形等を呈した報告がある[11]．

診断と鑑別診断

本症は大きな特徴は，新生児期に発症することが多いことである．したがって新生児高乳酸血症や新生児心筋症などの中に含まれていることにな

る．Complex V の酵素活性での診断は困難なことが多い．一部の新生児ミトコンドリア病は尿中 3-メチルグルタコン酸の有無である程度鑑別できる．また，組織や培養細胞を用いた BN-PAGE 解析は，他の呼吸鎖欠損と同様に診断の一助になる．

治療と予後

本症の根治的治療法はなく，一般的に高脂肪食およびミトコンドリアカクテルなどを使用していくことになる．Complex V 欠損症に特化した治療法は今のところ出てきていない．また，新生児ミトコンドリア病は多くは致死性の経過をとることが多く，予後は非常に厳しい．エクソーム解析やiPS 細胞の活用など新しい技術によって，新たな治療展開が生まれることを期待したい．

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表2 ATP 合成酵素の組成

バクテリア	哺乳類	酵母菌
α (55.2 kDa)	α (55.1 kDa)	α (55.3)
β (50.1)	β (51.6)	β (52.5)
γ (32.4)	γ (30.2)	γ (30.6)
δ (19.3)	OSCP	OSCP (20.9)
ε (14.9)	δ (15.1)	δ (14.5)
−	ε (5.7)	ε (6.6)
−	Inhibitor protein	
a	a	Subunit 8 (5.87)
b	b	Subunit 6 (27.9)
c	c	Subunit 9 (7.79)
−	d	d(19.66)
−	e	
−	f	b(P25)
−	g	
−	A6L	
−	F6	

表1　Complex V 欠損症関連の核遺伝子のまとめ

遺伝子	正式名称	局在	OMIM	コードしているタンパク・働き	遺伝形式	報告されている疾患
サブユニット異常						
ATP5E	ATP synthase, H$^+$ transporting, mitochondrial F1 complex, epsilon subunit	20q13.32	606153	subunit ε	A.R.	新生児ミトコンドリア病(22歳時に報告)
ATP5A1	ATP synthase, H$^+$ transporting, mitochondrial F1 complex, alpha subunit 1	18q21	164360, 615228	subunit α	A.R.	新生児ミトコンドリア病(進行性脳症)
呼吸鎖生合成 (biogenesis) の異常						
ATPAF2 (*ATP12*)	ATP synthase mitochondrial F1 complex assembly factor 2	17p11.2	608918	F_1 のアセンブリー因子	A.R.	新生児ミトコンドリア病(脳筋症)
TMEM70	transmembrane protein 70	8q21.11	612418	Complex V の生合成に関わる,アセンブリー因子?	A.R.	新生児ミトコンドリア病(心筋症,脳筋症),3-メチルグルタコン酸尿症(ほぼ必発)

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Original article

Beneficial effect of pyruvate therapy on Leigh syndrome due to a novel mutation in PDH E1α gene

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Abstract

Leigh syndrome (LS) is a progressive untreatable degenerating mitochondrial disorder caused by either mitochondrial or nuclear DNA mutations. A patient was a second child of unconsanguineous parents. On the third day of birth, he was transferred to neonatal intensive care units because of severe lactic acidosis. Since he was showing continuous lactic acidosis, the oral supplementation of dichloroacetate (DCA) was introduced on 31st day of birth at initial dose of 50 mg/kg, followed by maintenance dose of 25 mg/kg/every 12 h. The patient was diagnosed with LS due to a point mutation of an A–C at nucleotide 599 in exon 6 in the pyruvate dehydrogenase E1α gene, resulting in the substitution of aspartate for threonine at position 200 (N200T). Although the concentrations of lactate and pyruvate in blood were slightly decreased, his clinical conditions were deteriorating progressively. In order to overcome the mitochondrial or cytosolic energy crisis indicated by lactic acidosis as well as clinical symptoms, we terminated the DCA and administered 0.5 g/kg/day TID of sodium pyruvate orally. We analyzed the therapeutic effects of DCA or sodium pyruvate in the patient, and found that pyruvate therapy significantly decreased lactate, pyruvate and alanine levels, showed no adverse effects such as severe neuropathy seen in DCA, and had better clinical response on development and epilepsy. Though the efficacy of pyruvate on LS will be evaluated by randomized double-blind placebo-controlled study design in future, pyruvate therapy is a possible candidate for therapeutic choice for currently incurable mitochondrial disorders such as LS.
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Keywords: Leigh syndrome; PDH E1α mutation; Pyruvate; Lactic acidosis; Therapy

1. Introduction

LS, originally reported as subacute necrotizing encephalomyelopathy by Dr. Denis Leigh in 1951 [1], is an early-onset progressive neurodegenerative disorder characterized by developmental delay or regression, lactic acidosis, and bilateral symmetrical lesion in the basal ganglia, thalamus, and brainstem [2]. The clinical presentations of the disease are heterogeneous, due to the severity of biochemical defects caused by mutations in both nuclear and mitochondrial genes involved in energy metabolism. Though many molecular defects are reported to be associated with LS [3], the underlying gene defects remain unidentified in nearly half of the patients [4,5]. Since LS is associated mainly with the respiratory chain deficiency, there is no established treatment except for a limited number of patients such as those with thiamine-responsive pyruvate dehydrogenase deficiency [6], or those with defects in the biosynthetic pathway of coenzyme Q [7]. We have proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the

glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm, (b) pyruvate can activate PDHC by inhibiting pyruvate dehydrogenase kinase, and (c) pyruvate can scavenge hydrogen peroxide by non-enzymatic reaction [8]. Recently, we reported that pyruvate produced a slightly favorable change in the plasma lactate and pyruvate levels in LS with cytochrome c oxidase deficiency [9]. In the present report, we describe a clinical experience of pyruvate therapy in a child with LS having PDH deficiency caused by a novel mutation in PDH E1α gene.

2. Patient and methods

2.1. Patient

The 5-years-old boy, presented as severe psychomotor retardation with severe lactic acidosis, was born weighing 1797 g at full term gestational age as the second child of unconsanguineous parents. He was transferred to neonatal intensive care units because of fatal distress with the severe lactic acidosis. The concentrations of lactate and pyruvate in blood were 6–10 times higher than normal range, with normal lactate/pyruvate ratio (Table 1). He was under respiratory care with medication of severe metabolic acidosis. Aminogram of his plasma showed an elevated alanine concentration of 1.82 mM (normal range, 0.21–0.52). Since he was showing continuous lactic acidosis, the oral supplementation of DCA was introduced on 31st day of birth at initial dose of 50 mg/kg, followed by maintenance dose of 25 mg/kg/every 12 h. Though he showed severe floppy infant, his mechanical ventilation has been terminated at the 45th day of birth, and starting oral administration of ingredient nutrient. Although the concentrations of lactate and pyruvate in blood were

Table 1
Biochemical parameters during therapy with none, DCA, or pyruvate.

	None ($n = 8$)	DCA therapy ($n = 12$)	Pyruvate therapy ($n = 10$)
Lactate (mM) (normal: 0.03–0.17)	9.6 ± 0.54	8.6 ± 2.63	5.28 ± 1.73[a,b]
(Range: minimum–maximum)	(8.70–10.10)	(3.56–12.70)	(2.73–7.75)
Pyruvate (mM) (normal: 0.003–0.10)	0.69 ± 0.13	0.61 ± 0.19	0.42 ± 0.13[a,b]
(Range: minimum–maximum)	(0.49–0.82)	(0.31–0.93)	(0.26–0.68)
L/P ratio (normal: 10–15)	14.5 ± 3.10	14.2 ± 2.12	12.6 ± 1.52
(Range: minimum–maximum)	(10.6–18.7)	(11.5–17.9)	(10.5–15.1)
Alanine (mM) (normal: 0.21–0.52)	1.7 ± 0.28	1.13 ± 0.27[a]	0.77 ± 0.38[a]
(Range: minimum–maximum)	(1.11–1.82)	(0.76–1.51)	(0.39–1.42)

All date are presented as mean ± SD during each treatments.
Lactate, pyruvate L/P ratio, and alanine were analyzed the significance between periods of none, DCA and pyruvate therapy using the two-tailed Mann–Whitney U-test. P value less than 0.05 showed significant.
[a] It showed significance between none and DCA or pyruvate therapy.
[b] It showed significance between DCA and pyruvate therapy. n: number of measurements.

Table 2
Entire clinical course and symptoms.

	Clinical course		
	None	DCA	Pyruvate
Study periods	1 month (1 m)	17 months (2–18 m)	58 months (1 year 6 months–6 years 4 months)
Hospitalization (day)	31	124	3
Emergency visit (time)	0	14	4
Diagnosis by EEG	Infantile epilepsy	West syndrome or Lennox–Gastaut syndrome	Lennox–Gastaut syndrome
Convulsion			
Frequency	15 or more/days	18 or more/days	2–3/months
Duration	5–15 s/Epilepsy	5–20 s/Epilepsy	5–10 s/Epilepsy
Series formation	None	Series formation	No series formation
Anticonvulsants	Phenobarbital 20 mg/kg/day	Carbamazepine 10 mg/kg/day Valproate 10–15 mg/kg/day Clobazam 1.0 mg/kg/day Zonisamide 2–4 mg/kg/day	Carbamazepine 10 mg/kg/day Valproate 15 mg/kg/day Clobazam 1.5 mg/kg/day Zonisamide 2–4 mg/kg/day
JMDRS	58	58	57
Developments	Severe floppy infant Respiratory care	Cannot head control Cannot sit alone Cannot rolling over Floppy infant Eating mainly by S-tube	Floppy infant Head control (21 months) Rolling over (42 months) Sit alone (56 months) Eating mainly by mouth

slightly decreased by DCA, his clinical conditions were deteriorating progressively. He could not fix the head control, and roll over at 6 months of age. He was diagnosed with West syndrome at 6 months-old because of his intractable generalized convulsions. Though he received two types of anti-convulsants as shown in Table 2, his convulsion did not stop and showed several seizures a day with series formation. Brain MRI on 7-months-old showed a premature myelination and atrophy in frontal lobe with callosal hypoplasia, and brainstem abnormality. He showed severe floppiness, loose head control, inability to sit alone and roll over, feeding difficulty, and no significant words at the age of 18 months-old. His EEG pattern changed to Lennox–Gastaut syndrome at that time (Fig. 1A). Nerve conduction velocity in both motor and sensory nerve showed low amplitude with delayed velocity indicating
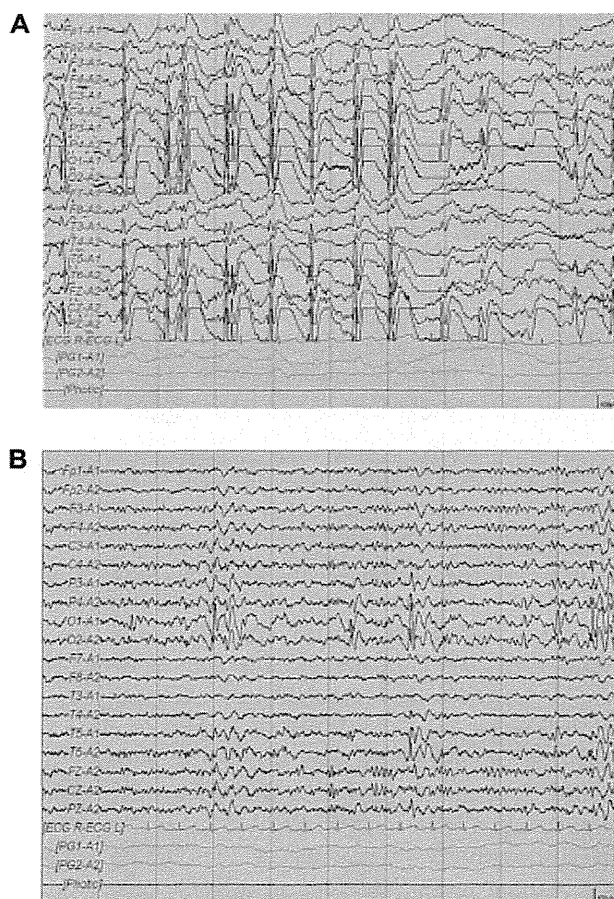

Fig. 1. (A) EEG taken at 18 months old. A grossly abnormal inter-ictal EEG showed continuous, high-amplitude, sharp-slow-waves or spike-slow-waves indicating a multifocal and generalizing sharp-slow-wave-discharges at 1.5–2.5 Hz. Patient showed intractable epilepsy with 15–20 times a day of grandmal, and/or myoclonic type seizure. (B) EEG taken at 36 months old. An abnormal inter-ictal EEG pattern showed with continuous, sharp-slow-waves or spike-slow-waves. However it showed low-amplitude and less multi-focality. Patient showed no grandmal or myoclonic type seizure by daily base frequency.

severe neuropathy. At this point, we thought that severe neuropathy seen in the patient may caused by the severe adverse effects of DCA, since he received the DCA supplementation for more than 17 months period. Because of the severe neuropathy, we decided to terminate the DCA at his age of 18 months-old, and after received written informed consent, we started the oral supplementation of sodium pyruvate at 0.5 g/kg/day TID. Three months later, he started to roll over and showed the facial expression of happiness and sadness. He could start to chatter and swallow the liquid food. Six months after starting pyruvate supplementation, he had almost no epileptic seizure and was demonstrated the significant improvement by EEG (Fig. 1B). The entire clinical course is summarized in Fig. 2 and Table 2.

The lactate and pyruvate concentrations in cerebral spinal fluid were 8.23 mM, and 1.26 mM under the period of DCA therapy, and 4.61 mM and 0.68 mM under the period of pyruvate therapy (Fig. 2).

2.2. Lactate, pyruvate, L/P ratio and alanine determination

In order to investigate the energy state of patient in each time period of therapy, we measured the plasma level of lactate, pyruvate and aminogram including alanine, 8 times in the periods of 31 days with free of DCA and pyruvate, 12 times in 17 months during DCA therapy, and 10 times in 58 months during pyruvate therapy. Analysis of amino acids was performed on protein-free extracts of fresh plasma using described methods.

2.3. Enzyme assays

The PDHC activity in cultured skin fibroblasts was assayed using two different concentrations of TPP (0.4 and 1104 mM) after the activation of PDHC using DCA as previously described [10].

2.4. Genetic analysis

Mutation analysis of the E1α gene, a major cause of PDHC deficiency, was performed using genomic DNA from cultured skin fibroblasts. For the genetic analysis of the 11 exons of the E1α gene, the individual exons were amplified using primer pairs and conditions as described previously [11].

2.5. Statistical analysis

Statistical analysis of the biochemical data including lactate, pyruvate, L/P ratio, and alanine was performed using two-tailed Mann–Whitney U-test or Student's t-test. A value of $P < 0.05$ was considered as statistically significant.
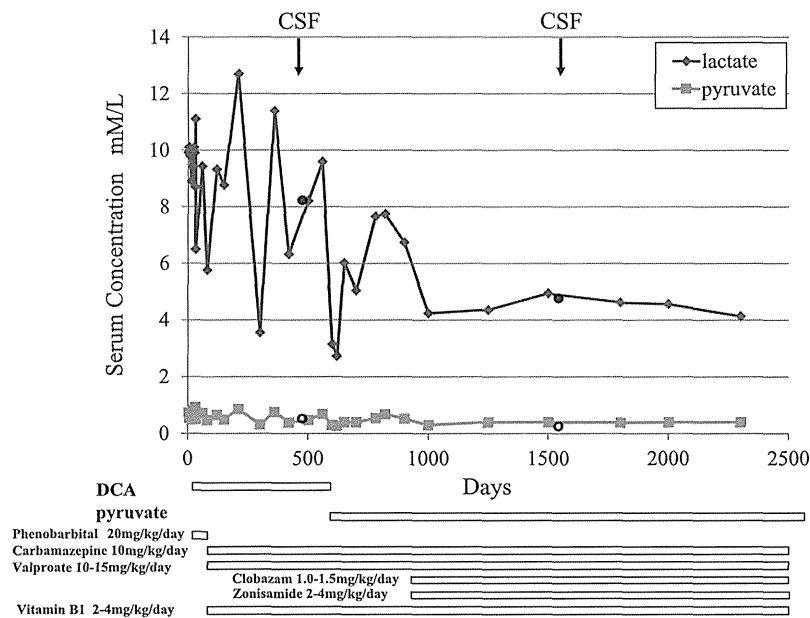
Fig. 2. Entire clinical course.

3. Results

Since patient showed lactic acidosis with normal lactate/pyruvate ratio, we measure the PDHC activity in cultured skin fibroblasts cells. The PDHC activity was 0.94 in the presence of DCA and 0.4 mM TPP (normal: 4.07 ± 0.68 nmol/min/mg protein). Mutation analysis of PDH E1α subunits revealed a point mutation of an A–C at nucleotide 599 in exon 6, resulting in the substitution of aspartate for threonine at position 200 (N200T). Though this mutation has not been reported before, we considered it as the responsible gene defect in this patient because; (1) no other mutations were found in entire PDH E1α gene, (2) conserved amino acid in different species, (3) mother has the mutation in hemizygous condition, and (4) no same mutation found in 50 normal females.

The laboratory data before, and after the treatment by DCA, and after pyruvate treatment are shown in Table 1 and Fig. 2. The concentration of lactate and pyruvate in blood before the treatment was 51–58 times higher than normal range, with normal lactate/pyruvate ratio (Table 1). The concentration of alanine was also increased 2.1–3.5 times higher than normal range. After the treatment by DCA, though the concentration of lactate and pyruvate showed no significance, the concentration of alanine was significantly decreased. The patient showed intractable seizures, and decreased the activity of daily living. After the treatment by pyruvate, the concentration of lactate and pyruvate were significantly decreased in comparison with those without therapy, and with DCA treatment, with significantly decreased level of alanine (Table 1 and Fig. 2). The concentrations

of lactate and pyruvate in the CSF were also significantly decreased with significantly decreased plasma level of alanine (Fig. 2).

4. Discussion

LS, the most dominant sub-type of mitochondrial disorders in children, are clinically more severe and patients usually die before the first decade of the life. In another words, LS showed the most severe cytopathy among subtypes of mitochondrial disorders. Therapeutic target of mitochondrial angiopathy is now on-going of L-arginine as an investigator-mediated clinical trial on MELAS [12]. However there are no clinical trial of therapeutic approach for mitochondrial cytopathy especially LS. Since the severe adverse events of DCA reported in 2006 [13], the new therapeutic drugs to prevent or improve the mitochondrial cytopathy or lactic acidosis have to be developed as a substitute for DCA.

In the present study, we reported a patient with LS caused by a novel PDH E1α mutation who responded to pyruvate administration for 3 years period. Pyruvate therapy significantly decreased the lactate, pyruvate and alanine levels, showed no adverse effects such as severe neuropathy seen in this patient under the DCA therapy, and had better clinical response on development and epilepsy. It was reported that pyruvate percolates through the blood brain barrier via monocarboxylate transporters and provides an excellent energy state for neurons and astroglia [14]. As shown in our patient (Fig. 2), pyruvates decreased lactate and alanine levels not only in blood but in CSF, and improved the electroencephalogram in our patient, suggested that pyruvate

may pass through blood-brain barrier and improve the metabolic condition in the brain in our patient. We have proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm [8], (b) pyruvate can activate the pyruvate dehydrogenase complex (PDHC) by inhibiting the pyruvate dehydrogenase kinase [8,9], and (c) pyruvate can scavenge the hydrogen peroxide by a non-enzymatic reaction [15]. Pyruvate improved the hemodynamic condition by intracoronary infusion in patients with congestive heart failure [16,17], or the neurological recovery following cardiopulmonary arrest and resuscitation [18]. In our patient, we determined the daily supplement of pyruvate by the presence of diarrhea as adverse effects or by the capacity of amount of oral administration. In our patient, daily administration of sodium pyruvate resulted in 0.5d/kg/day TID. The exact pharmacological mechanisms why serum pyruvate is also decreased after the pyruvate therapy, have to be clarified in future study, by using proteome analysis or comprehensive multiple analysis of total cell metabolism.

Considering the progressive nature of LS, pyruvate may prevent the neurodegeneration and lactic acidosis in our patient. Though the efficacy of pyruvate on LS will be evaluated by randomized double-blind placebo-controlled study design in future, pyruvate therapy is a possible candidata for therapeutic choice for currently incurable mitochondrial disorders such as LS.

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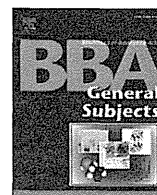
Review

Molecular pathology of MELAS and L-arginine effects☆,☆☆

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ABSTRACT

Background: The pathogenic mechanism of stroke-like episodes seen in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) has not been clarified yet. About 80% of MELAS patients have an A3243G mutation in the mitochondrial tRNA$^{Leu(UUR)}$ gene, which is the base change at position 14 in the consensus structure of tRNA$^{Leu(UUR)}$ gene.

Scope of review: This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes at the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

Major conclusions: Molecular pathogenesis is mainly demonstrated using ρ^0 cybrid system. The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of tRNA$^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-tRNA$^{Leu(UUR)}$ versus uncharged tRNA$^{Leu(UUR)}$ molecules; 3) the accumulation of aminoacylation with leucine without any misacylation; 4) accumulation of processing intermediates such as RNA 19, 5) wobble modification defects. All of these loss of function abnormalities are created by the threshold effects of cell or organ to the mitochondrial energy requirement when they establish the phenotype. Mitochondrial angiopathy demonstrated by muscle or brain pathology, as SSV (SDH strongly stained vessels), and by vascular physiology using FMD (flow mediated dilation). MELAS patients show decreased capacity of NO dependent vasodilation because of the low plasma levels of L-arginine and/or of respiratory chain dysfunction. Although the underlying mechanisms are not completely understood in stroke-like episodes in MELAS, L-arginine therapy improved endothelial dysfunction.

General significance: Though the molecular pathogenesis of an A3243G or T3271C mutation of mitochondrial tRNA$^{Leu(UUR)}$ gene has been clarified as a mitochondrial cytopathy, the underlying mechanisms of stroke-like episodes in MELAS are not completely understood. At this point, L-arginine therapy showed promise in treating of the stroke-like episodes in MELAS. This article is part of a Special Issue entitled Biochemistry of Mitochondria.

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1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (OMIM 540000), characterized by an

early onset of stroke-like episodes, was first described by Pavlakis and colleagues in 1984 [1]. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [2], about 80% of MELAS patients have an A3243G mutation in the mitochondrial tRNA$^{Leu(UUR)}$ gene (OMIM 590050) [3–5]. Although more than 25 years have passed since MELAS was first defined clinically and pathologically, the pathogenesis of the stroke-like episodes is still uncertain. Mitochondrial angiopathy with degenerative changes in small arteries and arterioles, which has been reported in many MELAS patients [6,7], is suggested by the observation of strong succinate dehydrogenase activity in the wall of blood vessels (SSVs) [8]. In spite of the fact that many therapeutic trials have been conducted to cure mitochondrial disorders, no trial has been successful, though several clinical trials are still on-going. Based on the hypothesis that stroke-like episodes in MELAS are caused by segmental impairment of vasodilatation in intracerebral arteries, we use L-arginine in MELAS patients during the acute phase to cure the symptoms or to

decrease the frequency and/or the severity of the stroke-like episodes [9,10,11]. This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes in the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

2. Molecular pathophysiology of mitochondrial cytopathy in MELAS

2.1. Characteristics of tRNA$^{Leu(UUR)}$ gene and structure stabilization of mutant

A point mutation in the structural gene for a tRNA may be expected to result in a deficiency in translation. However, inhibition of translation due to a mutated tRNA gene may occur at several levels. The base change at position 14 in the consensus structure of tRNA$^{Leu(UUR)}$ is an invariant A in bacterial and cytosolic eukaryotic tRNAs and is typically involved in the tertiary folding of classical tRNAs (Fig. 1A) [12]. Because of above reason, the A3243G mutation is primarily thought to disrupt the tertiary interaction between the highly conserved np A14 (>90% for adenine) and U8, a binding that stabilizes the L-shaped tertiary fold [13,14], which results in partially folded tRNA transcripts into the
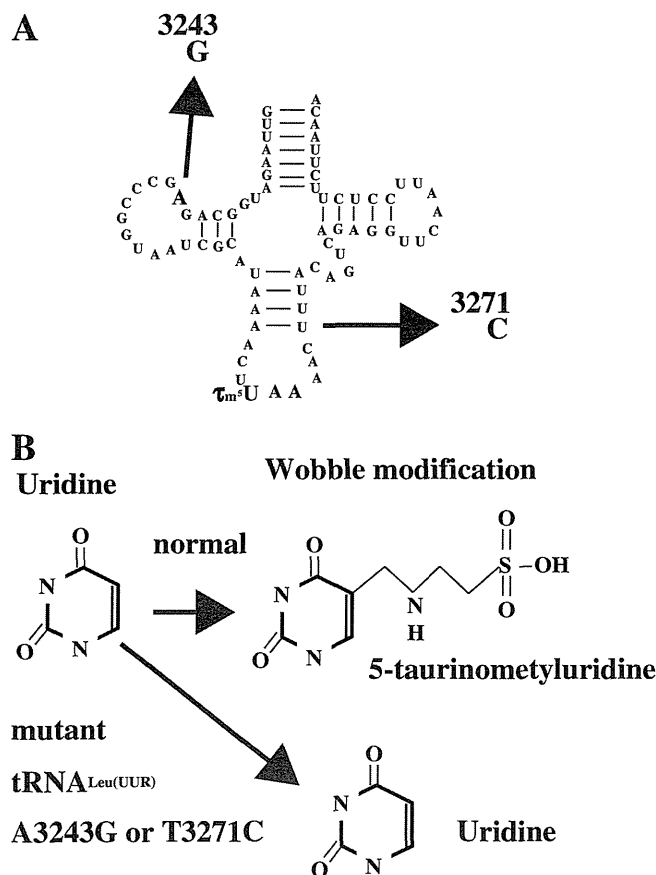

Fig. 1. tRNA$^{Leu(UUR)}$ structure and wobble modification. tRNA$^{Leu(UUR)}$ structure. An A to G change at position 14 in the consensus structure of tRNA$^{Leu(UUR)}$, which is thought to disrupt the tertiary folding of classical tRNAs [12], results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [14,15]. B. Wobble modification The wild-type tRNA$^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base [35], however its modification is absent in the tRNA$^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. The wobble modified uridine in the wild-type tRNA$^{Leu(UUR)}$ is 5-taurinomethyluridine (sm5U). The U on the bold indicates the unmodified uridine present in the mutant tRNAs.

L-shaped structure with an acceptor branch but with a floppy anticodon branch [15]. The mutant tRNA is able to adapt to the synthetase, but results in incorrect tRNA processing and enzyme maturation and accordingly defects in a variety of biochemical pathways. The mutation may directly affect the mitochondrial tRNA function in translation, such as structure stabilization, methylation, amino-acylation, and codon recognition, or alternatively, may affect recognition of the tRNA by an enzyme not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA.

2.2. ρ⁰ cybrid system in MELAS

King et al. developed the technologies whereby the mitochondria from cells derived from patients are transferred to a cell line lacking mtDNA (so called ρ^0 cybrid system), which allowed to conduct the study of the genotype-phenotype relationships in mitochondrial function [16,17]. In this manner, it is possible to create trans-mitochondrial cell lines containing different proportions of mutated mtDNA from 0% to 100%, and to study the effects of a given mutant load on the activity of respiratory chain complexes, mitochondrial respiration and cell growth, as well as mitochondrial tRNA stability, methylation, aminoacylation, codon recognition and threshold effects. First application of this technique to an A3243G mutation related to molecular basis of MELAS, has been reported by Chomyn et al. [18], and King et al. [19] independently. Mutant transformants showed protein synthesis defects clearly, and demonstrated that there was the direct evidence between single nucleotide change at 14th position of an A to G transition in the mitochondrial tRNA$^{Leu(UUR)}$ gene and mitochondrial dysfunction. However, the reduction in labeling of the various mitochondrial translation products in mutant was not correlated with their UUR-encoded leucine content. King also reported the similar effects in transformants having a T3271C mutation [19]. This ρ^0 cybrid system becomes the orthodox and powerful tool when one evaluates the pathogenicity of any nucleotide changes in the mitochondrial DNA.

2.3. Transcription termination of mitochondrial RNAs in MELAS

The mammalian mitochondrial tRNAs are transcribed as part of larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences (Fig. 2). The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. In these polycistronic molecules, the tRNA structures are believed to act as recognition signals for the processing enzymes which make precise endonucleolytic cleavages at the 5' and 3' ends of the tRNA sequences in the primary transcripts, yielding the mature rRNAs, mRNAs, and tRNAs [21]. The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, terminates at the 3'-end of the 16S rRNA gene just before the tRNA$^{Leu(UUR)}$ gene. This transcript, corresponding to the ribosomal genes, is processed to yield the mature rRNAs and, due to its very high rate of synthesis, is responsible for the bulk of the rRNA formation [22]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the tRNA$^{Leu(UUR)}$ gene, and which promotes termination of transcription (Fig. 3A) [22,23]. Since this mutation is located exactly in the middle of termination protein binding domain, the A3243G mutation in the tRNA$^{Leu(UUR)}$ gene has been shown in vitro to impair the binding of this protein factor and to affect the efficiency of transcription termination at the end of the 16S rRNA gene [23]. However, in vivo analysis using ρ^0 cybrid system provided no evidence to support above data. There were no alterations of size of the tRNA$^{Leu(UUR)}$ or of the immediately downstream-encoded ND1 mRNA or of the 16S rRNA, as detectable by changes in their electrophoretic mobility [18]. The steady-state amounts of mitochondrial rRNAs, mRNAs, and tRNA$^{Leu(UUR)}$ are not significantly affected by the MELAS mutation in ρ^0 cybrid system. The discrepancy of the data described above may be explained by the possibility that the
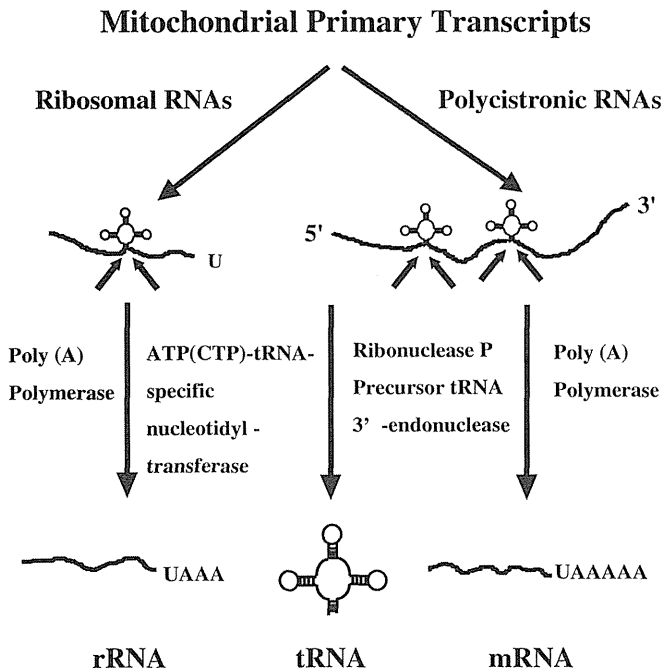
Fig. 2. Mammalian mitochondrial transcription system. The human mitochondrial RNAs are transcribed as a larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences.
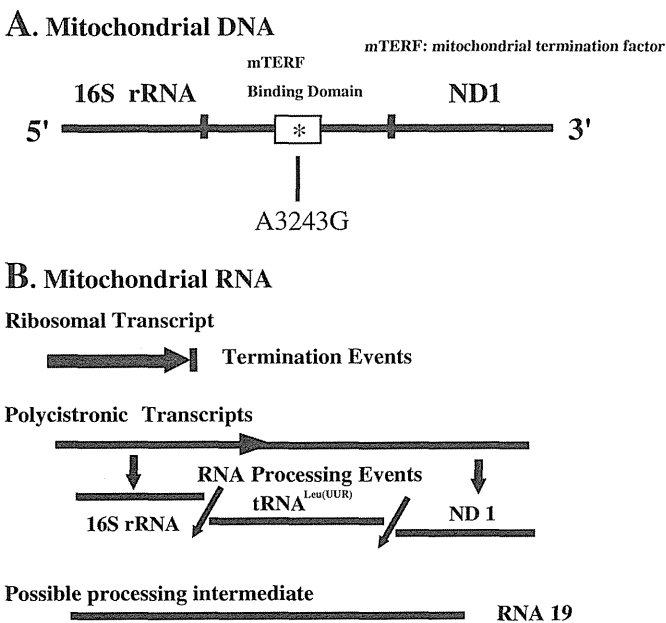

Fig. 3. Post-transcriptional modification. A. Transcription termination. The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the tRNA$^{Leu(UUR)}$ gene, and which promotes termination of ribosomal transcription. B. Post-transcriptional modification and RNA 19 The increase of RNA 19, corresponding to the 16S rRNA + tRNA$^{Leu(UUR)}$ + ND 1 genes, found in mutant tRNA$^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids as efficiently as in wild-type cybrids [19]. RNA 19 is also accumulated in muscle specimens from 8 MELAS patients [26]. The proportion of mutated RNA in RNA 19 fraction is always higher than those in the percentage of mutation in mitochondrial DNA, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30].

reduction in affinity of mTERF for the mutated target sequence is compensated by hyper-expression of the protein. Anyway, using genetic, biochemical, and morphological techniques, it was found that the mutant, but not wild-type cybrids, displayed quantitative deficiencies in cell growth, protein synthesis, and respiratory chain activity [19].

2.4. Processing of polycistronic transcripts in MELAS

It was found that there was an accumulation of a previously unidentified RNA transcript in mutant cybrids (A3243G or T3271C), designated as RNA 19, corresponds to the 16S rRNA + tRNA$^{Leu(UUR)}$ + ND1 genes, which are contiguous in the mtDNA (Fig. 3B) [19]. The ratios of mtDNA-encoded rRNAs to mRNAs were not found to be altered in these in vitro experiments. In order to analyze whether the MELAS mutation is associated with errors in transcription termination and processing of the polycistronic transcripts in the region of the mutation, it was performed fine mapping of the mature transcripts derived from the 16S rRNA, tRNA$^{Leu(UUR)}$, and ND 1 genes in both wild-type and mutant cybrids. It was also analyzed the steady-state levels of tRNA$^{Leu(UUR)}$ by high-resolution RNA transfer hybridizations. It was found that mutation has no effect in vivo on the accuracy of transcription termination at the end of the ribosomal RNA genes, on the precise endonucleolytic cleavage of the polycistronic RNA at tRNA$^{Leu(UUR)}$, or on the post transcriptional addition of -CCA at the 3′ end of tRNA$^{Leu(UUR)}$ [24]. On the other hand, the experiments using plasmids carrying tRNA$^{Leu(UUR)}$ inserts (wild type, as well as A3243G) which designated to evaluate the endonucleolytic 3′-end processing and CCA addition at the tRNA 3′ terminus, showed that A3243G mutation reduced 2.2 fold of the efficiency of 3′-end cleavage, and almost has no abnormal effects on CCA addition [25].

2.5. Accumulation of RNA 19 in MELAS cybrids and organs from patients

The increased amounts of the transcript corresponding to the 16S rRNA + tRNA$^{Leu(UUR)}$ + ND 1 genes, designated as RNA 19, found in mutant tRNA$^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids (A3243G or T3271C) as efficiently as in wild-type cybrids [19]. It was demonstrated that RNA 19 is accumulated in muscle specimens from 8 MELAS patients who have a heterogeneous percentage of mutation (58% to 99%) in the A3243G of tRNA$^{Leu(UUR)}$ gene [26]. An increase in the levels of RNA 19 was observed in nearly all tissues examined from these patients, which do not provide evidence for tissue-specific differences in mitochondrial RNA processing. The elevation of steady-state levels of RNA 19 have also reported in skeletal muscle and fibroblasts of a patient with mitochondrial myopathy and a complex I deficiency who harbored an A to G transition in tRNA$^{Leu(UUR)}$ gene at position 3302 [27]. Thus, altered RNA processing may be associated with other point mutations in tRNA$^{Leu(UUR)}$ gene associated with MELAS. It also analyzed a mutated proportion of RNA 19 in an RNA fraction obtained from sampled skeletal muscles from 6 unrelated patients with MELAS. The proportion of mutated RNA in RNA 19 fraction exceeded 95% in all patients, although the percentage of mutation in mitochondrial DNA ranged from 54 to 92, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30]. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243G, T3271C and A3302G mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the tRNA$^{Leu(UUR)}$ (Fig. 4) [31]. Though the reason why RNA 19 was elevated in patients who have the point mutation of tRNA$^{Leu(UUR)}$ gene is unknown, we believe that elevated levels of RNA 19 may play an important role in the pathogenesis of this disorder.
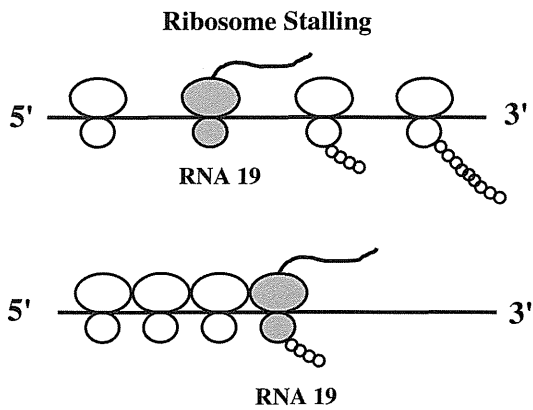
Ribosome Stalling

Fig. 4. xRibosomal stalling. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243 mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the tRNA$^{Leu(UUR)}$ [31].

2.6. Aminoacylation

The decrease in level of total tRNA$^{Leu(UUR)}$ observed in the mutant cell lines (46–62% of the control values) could arise either from decreased rate of formation from the corresponding primary heavy strand transcript or from a decreased metabolic stability [32]. The increased amount of RNA 19, which may be a precursor of tRNA$^{Leu(UUR)}$, was demonstrated in ρ^0 cybrid system as well as somatic tissues in MELAS patients. RNA 19 may suggest the former possibility. On the other hand, the A3243G mutation could perfectly destabilize the tertiary structure of the molecule, and mutant tRNA$^{Leu(UUR)}$ becomes more susceptible to nucleolytic attack [13,33]. It is proposed that mutant tRNA induces the misincorporation of amino acids in mitochondrial DNA encoded polypeptides. However, the demonstration of aminoacylation by mutant tRNA has been little pursued because a chemical amount of the mutant tRNA has not been purified, probably due to technical difficulties. In 2000, Yasukawa succeeded in purifying the mutant tRNA$^{Leu(UUR)}$ molecules in a chemical amount by taking advantage of the solid phase probing method [34], and clearly demonstrated that the mutant tRNA$^{Leu(UUR)}$ is aminoacylated with leucine only. However the extent of aminoacylation of the mutant tRNAs was relatively low. The total amounts of leucyl-tRNA$^{Leu(UUR)}$ with the mutations were estimated to be less than 30% that of the wild-type counterpart [35]. To determine if the decreased fraction of aminoacylated tRNA$^{Leu(UUR)}$ in mutant cells was due to a defect in the ability of mutant tRNA to be aminoacylated by the human mitochondrial leucyl-tRNA synthetase, Park et al. examined the aminoacylation kinetics of wild-type and mutant tRNA$^{Leu(UUR)}$, using both native and in vitro transcribed tRNA$^{Leu(UUR)}$ [36]. An A3243G mutant tRNA$^{Leu(UUR)}$ was 25-fold less efficiently aminoacylated in vitro, compared to the wild-type tRNA$^{Leu(UUR)}$. There are many evidences that aminoacylation capacities in tRNA $^{Leu(UUR)}$ gene mutations are reduced [37]. The reduced amount of aminoacyl-tRNA$^{Leu(UUR)}$ with the A3243G mutation could explain the reduction in protein synthesis.

2.7. Modified defects at wobble position in mitochondrial tRNA gene

A number of reports suggest that a decrease of protein synthesis cannot explain the decline in respiratory enzyme activity or in oxygen consumption [38,39]. Even when the mitochondrial protein synthesis rate was normal, the enzymatic activity of complex I was observed to be significantly affected in cybrid clones containing 60% to 95% mutant mtDNA. The muscle form of Complex I deficiency turned out to be MELAS clinically and was confirmed to have an A3243G mutation in all patients [40]. Thus, the decrease in protein synthesis may not itself contribute directly to the pathogenesis caused by mitochondrial

dysfunction. Some unusual mobilities of proteins in SDS-polyacrylamide gel electrophoresis have been reported [18,38], which strongly suggest that amino acids were misincorporated into the proteins synthesized in the mitochondria with the mutant mtDNA. The steady-state amounts of tRNA$^{Leu(UUR)}$ with the A3243G or the T3271C mutation in the respective cybrid clones were about 30% that of the wild-type in the control cybrid clones with wild type mtDNA [35]. In contrast, the steady-state amounts of tRNA^{Phe} and tRNA^{Ile} (encoded upstream and downstream of the tRNA$^{Leu(UUR)}$gene) remained unchanged in both the mutant and control cybrid cells. The life span of the mutant tRNA$^{Leu(UUR)}$ is significantly shortened. The half-life of the wild-type tRNA$^{Leu(UUR)}$ was estimated to be about 56 h, whereas those of the A3243G and T3271C mutants were only about 6 and 12 h. Therefore the reduced steady-state levels were due to the shortened life spans of the mutant tRNAs. Yasukawa found that the wild-type tRNA$^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base (Fig. 1B). In contrast, this uridine modification is absent in the tRNA$^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. It is interesting to note that both of the mutant tRNA$^{Leu(UUR)}$ are deficient in the modification at the wobble position despite having mutations at different positions. Modified defects at wobble position in mitochondrial tRNA gene are also demonstrated by primer extension methods [41]. The deficiency in uridine modification at the wobble position in the mutant tRNA$^{Leu(UUR)}$ strongly suggests mistranslation by these mutant tRNAs according to the mitochondrial wobble rule, which is also demonstrated in other tRNA mutation in MERRF (myoclonus epilepsy with ragged-red fibers) [42–45]. Although mutant tRNA$^{Leu(UUR)}$ does not follow the wobble rule, the mutant tRNA$^{Leu(UUR)}$ is aminoacylated with only leucine, not with other aminoacids. The stability and aminoacylation of the mutant tRNA$^{Leu(UUR)}$ were found to be decreased, suggesting that the molecular pathogenesis of MELAS could be a combination of a lowered availability of aminoacyl tRNA$^{Leu(UUR)}$ and defective translation. This is the first observation of a common modification defect affected by different point mutations within a single tRNA gene.

2.8. Threshold effects in various steps in the cell or in the organs

The phenotypic threshold effect observed at the single-cell level could arise when the products of the wild-type mtDNA can no longer "complement" the effects of the mutated ones [46,47]. For instance, a heteroplasmic mutation in mtDNA will result in the co-existence of mutated mRNAs, mutated tRNAs and defective respiratory chain subunits along with their wild-type homologues. These wild-type molecules may be sufficient to support normal function of the organelle until their levels fall below a critical value (threshold), at which point they can no longer compensate for the effect of the mutation, leading to impairment of mitochondrial function. The phenotypic threshold effect is based on this reserve of different macromolecules (mRNAs, tRNAs, subunits), and can then be considered as a protective mechanism providing a safety margin against the effects of deleterious mutations. Above complementation can occur at different levels of mitochondrial gene expression, such as 1) gene transcription, 2) structural stability of the tRNAs, 3) maturation process of the tRNAs, ribosomal RNA, and mRNAs, 4) wobble modification of tRNAs, 5) aminoacylation, 6) translation, 7) molecular assembly of the active form of enzyme complexes in harmony with mitochondrial and nuclear-encoded polypeptides, 8) locate to the mitochondrial inner membrane, 9) biochemical overall function of mitochondria in the cell, 10) biochemical overall function of mitochondria in the organ, 11) original threshold of organ to the mitochondrial energy requirement. The cells which require high energy states, such as neurons, muscles, heart, and kidneys, may be more severely affected by the threshold level of mutation than cell that require low energy levels. The phenotypes in the severity of the disease may influence various factors listed above and are more complicated to elucidate.

2.8.1. Summary of molecular mechanisms of mitochondrial cytopathy

The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of tRNA$^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-tRNA$^{Leu(UUR)}$ versus uncharged tRNA$^{Leu(UUR)}$ molecules; 3) accumulation of processing intermediates such as RNA 19, 4) wobble modification defects leading to translation defect. The A3243G mutation shows dominant negative effects in the processing system of mitochondrial transcription seen in both trans-mitochondrial cell and muscles in MELAS patients. Molecular mechanisms described above may contribute to respiratory chain enzyme defects, especially complex I, and lead to the mitochondrial cytopathy seen in the MELAS patients. Moreover the A3243G mutation affects the nuclear background [46,47], resulting in a high glycolytic rate, increased lactate production, reduced glucose oxidation, impaired NADH-response, reduced mitochondrial membrane potential, markedly reduced ATP production, deranged cell calcium handling with an increased cytosolic calcium handling with an increased cytosolic calcium load, an increased amount of reactive oxygen species in cybrid cells, reduced insulin secretion, premature aging, and deregulation of genes involved in the metabolism of amino groups and urea genesis. The above mechanism may lead to the cytotoxic edema seen in stroke-like episodes in MELAS.

3. Pathophysiology of mitochondrial angiopathy in MELAS

3.1. Hypotheses of stroke-like episodes in MELAS

The primary cause for stroke-like episodes in young MELAS patients— whether 1) mitochondrial cytopathy, 2) mitochondrial angiopathy, 3) non-ischemic neurovascular cellular mechanism, or combined—remains controversial. Mitochondrial cytopathy is caused by an oxidative phosphorylation defect in neurons, glia, or both as supported by evidence of an oxidative phosphorylation defect described by molecular pathogenesis section. Mitochondrial angiopathy is caused by the endothelial dysfunction evidenced by pathological, vascular physiological [11], or therapeutic findings [9,10]. Finally, the non-ischemic neurovascular cellular mechanism has been recently proposed by the clinical and neuroimaging data by Iizuka et al. [3].

3.2. Mitochondrial angiopathy in MELAS

Mitochondrial angiopathy with degenerative changes in small arteries and arterioles in the brain has been reported in autopsy cases of MELAS patients [6,7]. The mitochondria in the endothelium and smooth muscle cells of cerebral arterioles and capillaries also proliferate in a similar fashion as an area of ragged-red fibers (RRFs). Abnormal accumulation of mitochondria in vascular endothelial cells and smooth muscle cells is responsible for the infarct-like lesions [48]. These blood vessels have been designated as strongly succinate dehydrogenase-reactive vessels (SSVs), since they are rich in abnormal mitochondria [8]. Unlike RRFs and SSVs seen in MERRF and Kearns–Sayre syndrome (KSS), RRFs and SSVs seen in MELAS are typically cytochrome c oxidase (COX) positive, while those seen in MERRF or KSS are mostly COX negative, what is known as the "MELAS paradox"[49]. Since nitric oxide (NO) can bind to the active site of COX and displace heme-bound oxygen, hyperactive COX may decrease the regional NO concentration and lead to the segmental vasodilatation defect in SSV regions. Although infarct-like lesions histopathologically and stroke-like episodes clinically may not be caused simply by occlusion or obliteration of small vessels, this mitochondrial angiopathy, which can be severe in pial arterioles and small arteries, seems to explain the distribution of multiple areas of necrosis [50]. Since MELAS was associated with respiratory dysfunction, accumulated superoxide radical anion may react with nitric oxide to create the powerful oxidant hydroxypernitrite which may induce the neuronal apoptosis or cell damage [51]. All findings, described here,

suggest that mitochondrial angiopathy is a unique and common change in all MELAS brains examined. This pathological abnormality, called mitochondrial angiopathy, may lead to the vasogenic edema seen in stroke-like episodes in MELAS.

3.3. Non-ischemic neurovascular cellular mechanism

Iizuka et al. proposed that the stroke-like episodes in MELAS may reflect neuronal hyperexcitability (epileptic activity), which increases energy demand and creates an imbalance between energy requirements and the adequate availability of ATP due to an oxidative phosphorylation defect, particularly in the susceptible neuronal population [3,52]. The generalized cytopathic mechanism and non-ischemic neurovascular cellular mechanism reflect the so-called mitochondrial cytopathy theory.

3.4. Neuro-imaging analysis in stroke-like episodes

Unlike thrombotic or embolic stroke usually seen in adult patients, the stroke-like episodes in MELAS are atypical because they affect young people and are often triggered by febrile illnesses, migraine-like headaches, seizure, psychological stress, and dehydration. Many neuro-imaging studies have been reported at different phases of onset from stroke-like episodes in MELAS through the use of computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), single emission computed tomography (SPECT), and positron emission tomography (PET). Calcification of the basal ganglia is frequently observed in MELAS by CT even before starting the stroke-like episodes. MRI scans of acute stroke-like events show an increased signal on T2-weighted or on fluid attenuation inversion recovery (FLAIR). The regions do not conform to the territories of large cerebral arteries but rather affect the cortex and subjacent white matter with sparing of deeper white matter. Acute changes in these regions may fluctuate, migrate, or even disappear during the time course. Cerebral angiograms in MELAS patients have confirmed absence of large-vessel pathology by demonstrating normal results, increased size of caliber arteries, veins, or capillary blush with early venous filling, with the exception of several case reports [53,54]. MRS studied revealed that the decrease in N-acetylaspartate (NAA), which is thought to be an amino acid specific to neurons, and an increase of lactate, which is reflected of anaerobic metabolism by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS), were in evidence in the affected areas at acute stroke-like episodes. Kubota et al. reported that L-arginine infusion protect the accumulation of lactate by MRS analysis in stroke-like episodes in MELAS [55]. The increased level of lactate on ^{1}H-MRS is also recognized even in normal appearing regions [56]. Phosphorus MRS studies have shown decreased levels of high-energy phosphate compounds in the brains of MELAS patients [57], showing that mitochondrial cytopathy constantly exists in the MELAS patient. SPECT studies have generally revealed that the increased tracer accumulation was reported in acute (several days) and subacute stage (month) from the onset of stroke-like episodes and lasted for several months. In the chronic stage (several months or years later), the decreased tracer accumulation was reported. However, in the hyperacute stage (3 h after the onset of stroke-like episodes), we observed hypoperfusion by SPM-SPECT analysis [58]. Moreover, the hypoperfusion and the hyperperfusion areas are both demonstrated in the MELAS patients not only at an acute phase but at an interictal phase, showing that MELAS has inappropriate cerebral circulation [54]. Moreover, MELAS showed hypoperfusion in the posterior cingulated cortex by SPM-SPECT, which is the common finding in Alzheimer disease, and may be related to the dementia state usually seen in the progressive stage of MELAS. There are several PET studies using (rCMRO$_2$), [^{62}Cu]-diacetyl bis (N4-methylthiosemicarbazone) (^{62}Cu-ATSM), and [^{18}F]-fluorodeoxy-glucose (^{18}FDG) in stroke-like regions [59,60]. All of the PET studies of

patients have revealed decreased oxygen consumption relative to glucose utilization, further confirming the impairment of oxidative phosphorylation [61]. The dissociation in PET findings between cerebral glucose and oxygen metabolism may be the characteristic feature of MELAS, suggesting the mitochondrial cytopathy theory or non-ischemic neurovascular cellular mechanism. Diffusion-weighted (DWI) imaging is a new MRI technique for detecting diffusion of water molecules. Using DWI, local water mobility can be assayed as the absolute value of tissue water and expressed as the apparent diffusion coefficient (ADC). It has been shown using a stroke model in rats that ADC (a marker for cytotoxic brain edema) significantly declined within the first 5–10 min after stroke onset, while T2-relaxation time (a marker for vasogenic brain edema) increased as early as at the first T2-imaging time-point (20–35 min after embolization) [62]. The acute phase of stroke-like lesions in MELAS appear as a high signal on DWI with normal or increased ADC values, suggesting vasogenic edema which support the mitochondrial angiopathy theory [63,64]. On the contrary, many case reports found a decrease in ADC, which suggests mitochondrial cytopathy theory [65]. Recently, it was reported that increased and decreased ADC portions are mixed in stroke-like lesions, in which the increased ADC portion showed disappearance of the lesions thereafter, and the decreased portion showed persistent lesions. They suggested that there might be different levels of mitochondrial energetic transport impairment, correlated with cellular dysfunction. Specifically, this would be a mild energy failure resulting in moderate cellular dysfunction, responsible for vasogenic edema (high ADCs) and a severe energy failure resulting in irreversible cellular failure with cytotoxic edema (low ADCs) [66].

3.5. Endothelial dysfunction in MELAS

Physiologically, MELAS patients have a decreased vasodilation capacity in small arteries examined by flow mediated vasodilatation (FMD) methods, sized from 3 to 5 mm in their diameter [11]. MELAS patients have significantly decreased levels of L-arginine at acute phase of stroke-like episodes, which plays an important role in endothelial-dependent vascular relaxation [67], vasodilatation may be more severely affected in MELAS. Since MELAS patients have defective respiratory chain enzyme activities, a high NADH/NAD+ratio inhibits the NO synthetase reaction to cause a decreased production of NO at the endothelial cells or smooth muscle cells in the artery. In addition, ADMA (asymmetrical dimetyl-arginine), a risk factor of ischemic heart disorders, was relatively increased in MELAS patients [10], which may lead to a negative effect on the endothelial NO synthetase activity. If hyperactive COX may decrease the regional NO concentration as described in "MELAS paradox" [49], all of the above scenarios lead to the segmental vasodilatation defect especially in the segment of SSV regions in the cerebral artery or arterioles. The investigator-mediated clinical trial of L-arginine on MELAS (Dr. Koga as a principle investigator) to cure the symptoms of stroke-like episodes at acute phase, and to prevent or decrease the severity of stroke-like episodes at interictal phase of MELAS are on-going at 15 institutions of university hospital in Japan.

3.5.1. Summary of mitochondrial angiopathy and L-arginine effects

Pathophysiological mechanisms of mitochondrial angiopathy and the effects of L-arginine are summarized in Fig. 5.

4. Conclusion and future direction

The possible pathogenic mechanism of stroke-like episodes in MELAS may not be simple but complicated as described by the mechanisms in mitochondrial cytopathy and in mitochondrial angiopathy. Mitochondrial cytopathy has been demonstrated clearly as molecular and cellular defects by trans-mitochondrial cellular models. Mitochondrial angiopathy also has been demonstrated in brain and muscle pathology and vascular physiology. Although the results of
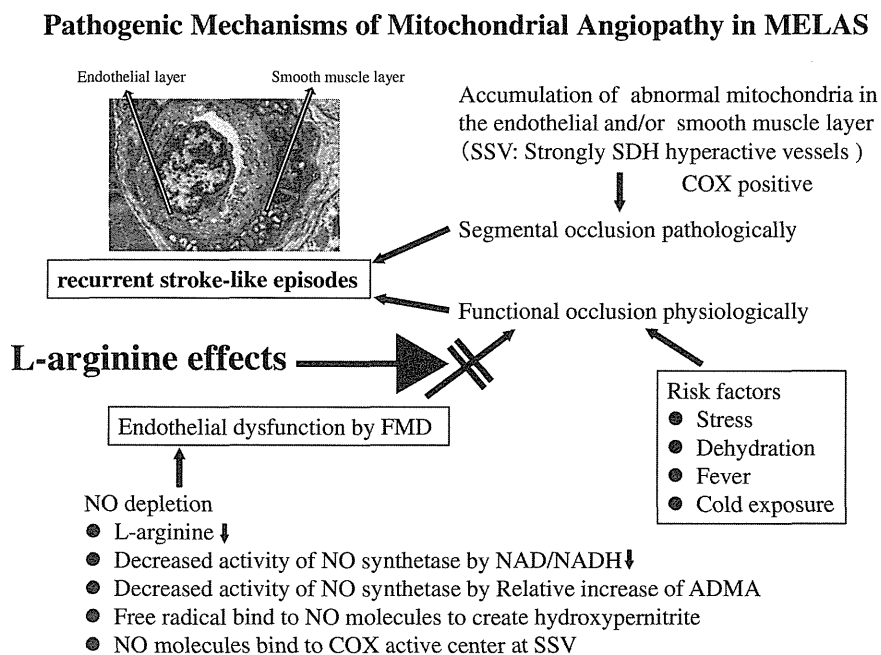
Pathogenic Mechanisms of Mitochondrial Angiopathy in MELAS

Fig. 5. Pathogenic mechanisms of mitochondrial angiopathy in MELAS. Segmental occlusions of small artery or arteriolen are evident in brain as well as in muscle pathology, in which abnormal accumulations of mitochondria have seen in endothelial and smooth muscle layers [6,7]. This phenomenon is recognized as SSVs in muscle and brain in MELAS [8], whereby mitochondrial function is more profoundly defective than the rest of the vessels, and demonstrated as endothelial dysfunction by FMD physiologically [11]. In MELAS patients, decreased levels of L-arginine is reported at acute phase of stroke-like episodes, a potent donor of NO, is also responsible for NO-dependent vascular dilatation defect. The decreased NAD/NADH ratio and accumulation of superoxide come from respiratory chain deficiency results in the inhibition of NO synthetase at generation process and decrease NO molecules by binding to create hydroxypernitrite, also contribute to the NO-dependent vasodilation abnormality. Since SSVs has usually high COX-positive feature histochemically, high COX activity decrease the residual NO molecules by binding to COX reactive center. The mental stress, dehydration, fever and cold exposure are also very important factors to increase the risk of the stroke-like episodes in MELAS.