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Jan A.M. Smeitink, Rob C.A. Sengers
and J.M. Frans Trijbels

Oxidative Phosphorylation in Health and Disease

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To all patients suffering from oxidative phosphorylation disorders

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FOREWORD

The reason I accepted to write the foreword to this fine book —and, I suspect, the reason I was asked to write it— is that I had the good fortune to witness and to some extent share the first steps of "mitochondrial medicine". I remember when, as a medical student, I read with excitement the exemplary piece of clinical investigation in which Rolf Luft and coworkers documented that the culprit for the severe hypermetabolism that afflicted a young Swedish woman was not a dysfunction of the thyroid gland, but loose coupling of muscle mitochondria. Ten years later, as a postdoctoral fellow in neurology with Lewis P. Rowland and a guest in the laboratories of C. P. Lee and Britton Chance, I struggled to isolate intact muscle mitochondria from the second patient with Luft disease and could hardly believe my eyes when the pen of the oxygen electrode recorder plunged after the addition of substrates, unresponsive to ADP or oligomycin! In the intervening years, substantial morphological and biochemical evidence had been provided to support the mitochondrial nature of several human diseases, as initially suggested by Luft.

Much of these data came from the University of Nijmegen, where, under the leadership of Rob Sengers, collaboration between basic scientists and clinicians —"translational research" as it is now called— was fostered and continues to this day. It is, therefore, historically appropriate that both the editors and several contributors of this book belong to that institution. Far from being parochial, however, the book includes experts from many countries.

In the spirit of multidisciplinary collaboration that has characterized mitochondrial research from its inception —in the absence of animal models, patients were of equal interest to clinical and basic scientists— this book includes updates on the normal structure, function, and molecular biology of the mitochondrial respiratory chain, "practical" chapters on the usefulness and limitations of traditional diagnostic methodologies (morphology, biochemistry, molecular genetics), and an overview of the diagnostic promise of new technologies (chips, proteomics).

While the unchecked —and uncheckable— hypermetabolism of Luft disease has not yet been seen more than twice, clinical heterogeneity, indeed clinical "arlequinism", characterizes mitochondrial diseases, which is hardly surprising, considering that mitochondria are present in every tissue of the body and that mitochondrial genetics is more akin to population genetics than to the more "disciplined" mendelian genetics. The diagnostic challenge often leads either to underdiagnosing ("what is this confusing clinical presentation?") or to overdiagnosing ("this clinical presentation is so confusing that it must be a mitochondrial disease!"). To help clinicians avoid these extremes, the book contains critical reviews of symptoms and signs, alone or

variously associated into syndromes, as well as useful updates on the genetic defects of either the mitochondrial or the nuclear genome responsible for many disorders.

Until not too long ago, mitochondrial diseases appeared to be an inextricable clinical morass: now, as new molecular defects are being discovered at rapid pace, a rational classification is emerging, based on clinical and genetic criteria. A case in point is progressive external ophthalmoplegia (PEO), a very common manifestation of mitochondrial dysfunction. Sporadic patients commonly harbor large-scale rearrangements. Maternally inherited PEO is due to point mutations in mtDNA, most often the A3243G MELAS mutation, but also many other mutations in mtDNA-encoded tRNA genes. Patients with autosomal dominant PEO and multiple mtDNA deletions have mutations in at least three —but certainly more— genes, *ANT1*, *Twinkle*, and *POLG*. Finally, autosomal recessive PEO can be due to mutations in *TP*, causing both multiple deletions and depletion of mtDNA, to mutations in *POLG*, or to mutations in *TK2* or *dGK*, associated with mtDNA depletion.

We have been much less successful in unraveling the pathogenic mechanisms of mitochondrial diseases, but the multiple possible deleterious effects of mitochondrial dysfunction are reviewed in one chapter, including energy shortage, excessive production of radical oxygen species, altered calcium homeostasis, unchecked apoptosis, a mixture of the above, or all of the above.

As always in medicine, the goal is to heal. Here, again, we have been slow in meeting the challenge, but promising new approaches are being tested in vitro and the availability —at long last— of "mito-mice" will allow to test some therapies in vitro. The promise of experimental animals and the promise of novel therapeutic strategies are discussed in two chapters.

This book speaks to a large audience, but especially to clinicians from all subspecialties of medicine, residents and medical students, and to all those who want to understand mitochondria, these ancient guests, and how the fire that they have brought to our cells can be dimmed by mutations in their own genome or in "ours."

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CHAPTER 1

The Human OXPHOS System:
Structure, Function and Physiology

Immo E. Scheffler

Abstract

In this review the composition, structure and function of the complexes of the mammalian mitochondrial electron transport chain and the ATP synthase are highlighted in the context of the Chemiosmotic Hypothesis and our understanding of oxidative phosphorylation. Thus, a firm biochemical foundation is established for the diagnosis of human mitochondrial diseases. The significant insights achieved to date also lead to new questions about the biogenesis of these multisubunit complexes, their interactions, their regulation, and the integration of their activities with other mitochondrial and cellular functions.

Introduction

Bioenergetics began with experiments by Lavoisier in which he determined that the amount of heat given off per unit volume of carbon dioxide produced was the same in a guinea pig as from the combustion of charcoal. Although one can quibble with the precision of the measurements, the famous Frenchman raised the idea that living beings were "sustained by fire". One hundred and sixty years later mitochondria were identified as the "powerhouse of the cell". Living organisms, with all their highly evolved structures, their ability to process information, and their capacity for proliferation, are subject to the laws of thermodynamics. This multitude of activities is driven in heterotrophic organisms by the energy released from the metabolism ("combustion") of carbohydrate.

The growth of physiology and bioenergetics, initially with reference to muscle activity, is a fascinating story from the nineteenth and early twentieth century culminating in major breakthroughs after around 1950. Fatty acid oxidation and the Krebs cycle reactions were localized in mitochondria. Oxygen consumption (respiration) was shown to take place in mitochondria of almost all eukaryotic cells, and the combustion of carbohydrates provided sufficient energy. The major challenge, therefore, was to understand how this chemical free energy could be stored, transported within the cell, and utilized in the performance of diverse types of biological work (muscle contraction, the biosynthesis of macromolecules, the many ion pumps and transport systems, information storage).

One of the first major breakthroughs came from the discovery of ATP, and other high-energy phosphates. The universal function of ATP has led to its designation as the "energy currency" of the cell. By coupling its hydrolysis to other, endergonic (energy-requiring) reactions, these thermodynamically unfavorable reactions could be driven forward. The mechanism is well understood for the biosynthesis of macromolecules. The understanding of the role of ATP hydrolysis in the activity of various molecular motors (myosins, dyneins, kinesins) has also made great strides in the past decades. Hydrolysis of ATP is accompanied by conformational changes in the motor, and brownian movements are 'rectified' by the interaction of the motor

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with polar macromolecular assemblies.[1] The most difficult problem continues to be the elucidation of the molecular mechanism of various ATP-driven ion pumps.

It is estimated that a human being turns over about half of his/her body weight of ATP per day, even in the absence of strenuous exercise. The emphasis here has to be on turnover: there is a continuous production and consumption of ATP. How is the constant supply of ATP maintained? We now understand the operations of two significant mechanisms. Glycolysis produces ATP by a series of substrate level phosphorylations in the course of the conversion of glucose to two molecules of lactate. The overall reaction is inefficient, and not sustainable for long because of problems with the removal of lactate, but it has the great advantage of being a fast reaction that can be activated rapidly. The rate can be tuned efficiently by means of allosteric mechanisms to meet the requirements of the moment, of prime importance during muscle activity. The pathway of glycolysis is familiar, and it should be recognized that ATP synthesis during glycolysis is fundamentally different from oxidative phosphorylation.

ATP synthesis by oxidative phosphorylation constitutes the major mechanism in all organisms living under aerobic conditions. Glucose is oxidized completely to CO_2, and a much larger release of energy is obtained. It is relevant that none of the oxygen consumed appears in the CO_2 evolved in the overall process. The pathway from glucose to pyruvate, and via acetyl-CoA into the Krebs cycle, is well known. This cycle (the "hub of metabolism") is important not only in the breakdown of carbohydrates (catabolism). It also plays a critical role in the generation of numerous small intermediates that serve as starting materials for biosynthetic (anabolic) pathways. The oxidation of the carbohydrate to CO_2 is coupled to the reduction of NAD^+ to NADH (and some $FADH_2$). However, just as there is no large excess of ATP to be used up, there is also only a limited supply of [NAD^+ + NADH] in a cell. NADH must be reoxidized and recycled.

Most of the NADH is reoxidized in mitochondria, and even NADH produced by glycolysis in the cytosol under aerobic conditions is brought into the mitochondrial matrix by shuttle mechanisms. A complex series of inter-conversions and reactions maintains a redox balance in cells as reflected, for example, by the ratio of oxidized vs. reduced glutathione.

The oxidation of NADH can be described by the reaction

$$\mathbf{2\ NADH + 2\ H^+ + O_2 \rightarrow 2\ NAD^+ + 2\ H_2O\ \Delta G'^{o} = -\ 220\ kJ/mol\ NADH}$$

Early in the 20th century when the importance of enzymes became apparent, it was perhaps natural to assume that a 'single' enzyme would catalyze the above reaction, and the name 'Atmungsferment' was championed by one of the most prominent biochemists of that era, O Warburg. A role for cytochromes was suggested by the pioneering work of Keilin, causing a major controversy, because the oxidation of NADH could be inhibited by carbon monoxide, but a reaction of cytochromes with carbon monoxide could initially not be demonstrated. The issue was resolved by the discovery of cytochromes aa_3.

Complexes of the Electron Transport Chain

Composition and Structure

After mitochondria became identified as the site of respiration, biochemical analyses in the period of ~1955-1975 established the major features of the electron transport chain in the inner mitochondrial membrane. The idea evolved that the oxidation of NADH by oxygen did not take place in an active site of a single enzyme. The understanding of a sequential flow of electrons from NADH to oxygen through many proteins was achieved with the contributions from many individuals.[2,3] Kinetic analyses as well as the employment of a series of newly characterized inhibitors by Chance and coworkers established an order in which cytochromes served as intermediates in the transfer of electrons from NADH to oxygen. Cytochromes consist of an apo-protein associated with a heme group. Another heme protein with an illustrious history in biochemistry is hemoglobin, serving as the oxygen carrier in the blood. The heme

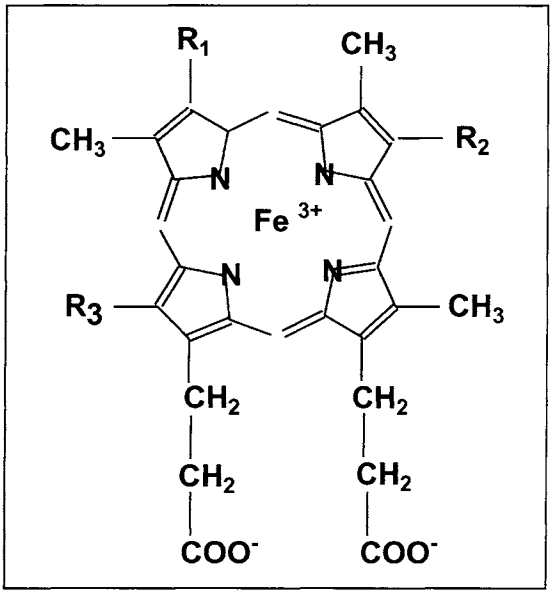

Figure 1. Structure of a heme prosthetic group. Cytochromes a, b, c differ in the nature of the groups R_1, R_2, and R_3 attached to the porphyrin ring. Cytochrome c is the only cytochrome in which the heme is covalently linked to the apo-protein via two cysteine side chains.

consists of a planar porphyrin ring structure with a Fe-ion in the center. The porphyrin ring can be substituted with a variety of side chains, and the nature of these side chains as well as the "microenvironment" of the heme group provided by the surrounding side chains of the protein determine the redox potential of the heme. Thus, although the oxidation-reduction reaction in every case involves an inter-conversion of Fe^{2+}/Fe^{3+}, the redox potential appropriate for the position of the cytochrome in the overall electron transport chain determines the direction of electron flow. The discovery and characterization of cytochromes also contributed to a solution of another significant fundamental problem: how is a protein converted into a conductor of electrons? Quantum-mechanical tunneling can account for a jump over a limited distance within a protein, but the heme groups provide relay-stations for electrons passing through proteins.

Three major cytochromes have been characterized in mitochondria (Fig. 1). Type a and b cytochromes have heme groups associated with the apo-protein by noncovalent interactions, while the heme group in cytochrome c is covalently linked to the protein through thioether bonds with two cysteine residues. The soluble cytochrome c was the first to be purified, in contrast to the other mitochondrial cytochromes. The apo-protein has a molecular mass of ~ 13 kDa; its amino acid sequence is highly conserved in evolution, and it has served as a model protein for the construction of phylogenetic trees.

A combination of spectroscopic and kinetic studies helped to establish the order of the electron flow from NADH (or succinate) as follows:

cytochrome b → cytochrome c1 → cytochrome c → cytochromes aa3 → oxygen

The cytochromes, except cytochrome c, were found embedded in the inner mitochondrial membrane, but a more complete understanding of their structure had to wait for a better understanding of biological membranes in general.

Two other basic discoveries were important for the understanding of the mitochondrial electron transport chain. First, coenzyme Q (ubiquinone) was shown to participate in mitochondrial electron transport via a redox cycle involving the oxidized form, ubiquinone, a relatively stable free radical intermediate (semiquinone), and finally the fully reduced ubiquinol (Fig. 2). Each step requires one electron and one proton. Experimental studies and theoretical

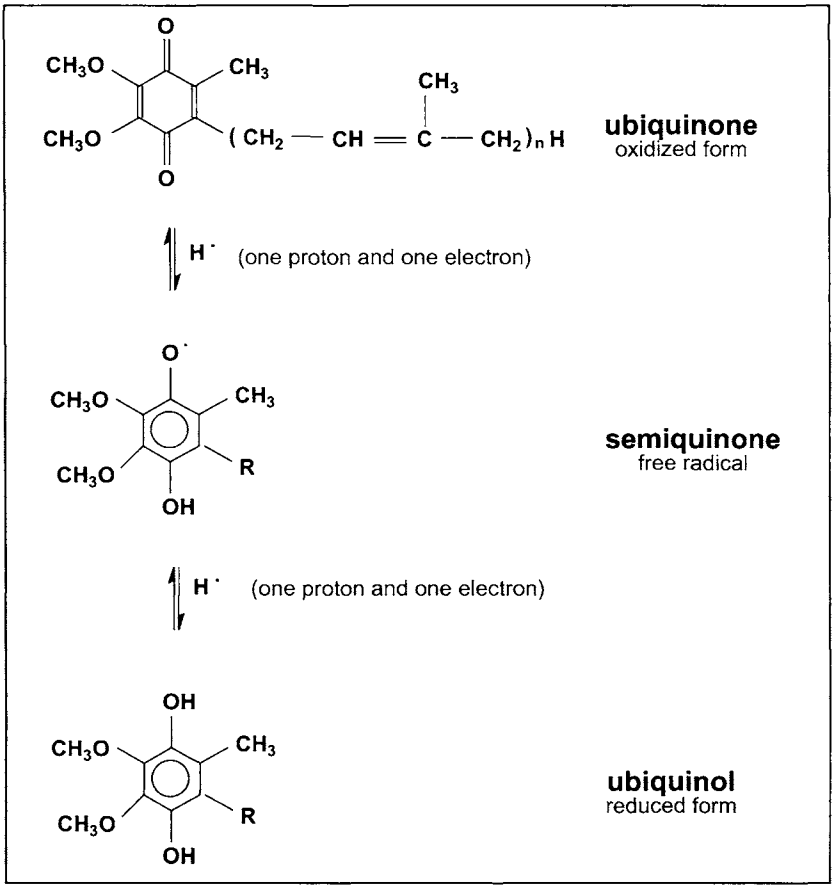

Figure 2. Structural formulae for the oxidized and reduced forms of ubiquinone. The aliphatic side chain, R, is a polyisoprene chain with ten monomeric units in animals.

considerations suggested that the ubiquinone participates 'upstream' of the cytochromes, and that it was an intermediate in the oxidation of both NADH and succinate as follows:

NADH + H^+ + Q → NAD^+ + QH_2

Succinate + Q → Fumarate + QH_2

Significantly, ubiquinone is insoluble in aqueous solution and 'soluble' in the lipid membrane because of its long polyisoprene chain. At the same time it was predicted to be mobile in the membrane. Our current understanding of biological membranes still leaves us with considerable uncertainty about the precise orientation of ubiquinone in the lipid bilayer. Fatty acid side chains of the typical phospholipid have 16 to 18 carbons, compared to 50 carbons in the polyisoprene chain of Q_{10}. What constraints, if any, limit not only the lateral mobility in the plane of the membrane, but also the mobility of the functional quinone/quinol group across the membrane, or in and out of the various binding sites on complexes I, II, and III? While the long polyisoprene chain is found naturally, experiments have shown that water-soluble quinones with only one isoprene unit can also serve as substrates in in vitro assays.

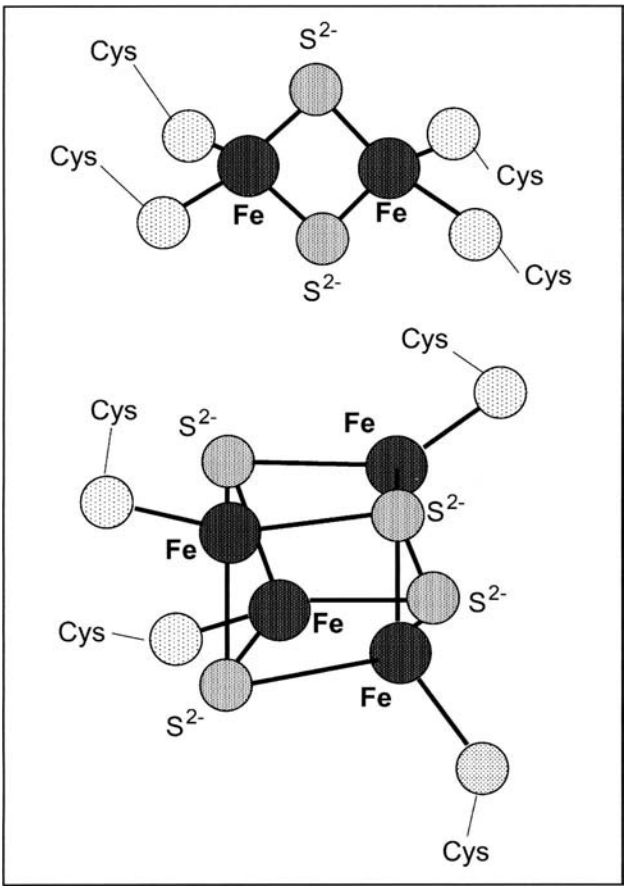

Figure 3. Structures of two iron-sulfur centers, [2Fe-2S] at the top, and [4Fe-4S] at the bottom. Cysteine side chains from the protein and sulfide groups are shown. The entire unit acts as an electron acceptor or donor, with the charges distributed over all the iron ions.

A second important discovery was the discovery of nonheme iron-sulfur centers.[4,5] These very interesting structures represent a second means of incorporating Fe-ions into proteins, and thus provide another redox couple for electron transport. They were missed originally because they have relatively low extinction coefficients in the visible and accessible uv range, but biochemical analyses revealed the presence of iron above the level accounted for by the hemes. Three types of clusters are found in mammalian mitochondria: [2Fe-2S], [3Fe-4S], and [4Fe-4S], represented by [xFe-yS] (Fig. 3). Two or more (x) ferric and ferrous ions are complexed with cysteine side chains, and in addition the cluster contains (y) sulfide ions (S^{2-}). The metal ions in the cluster form a conjugated system such that single electrons are gained or lost by the entire cluster, not an individual, identifiable Fe ion. The nature of the cluster and the environment of the surrounding protein side chain(s) determine the redox potential of the cluster. More than one cluster may be associated with a single protein subunit. A detailed study of these clusters became possible by their distinct electron paramagnetic resonance (EPR) spectra in their oxidized and reduced states.

The biogenesis of these clusters has been elucidated in part only relatively recently from a combination of molecular and genetic studies in yeast.[6-10] A protein complex with at least ten

subunits is responsible for the assembly of these clusters in the mitochondrial matrix. It should be added that iron-sulfur clusters are also found in cytosolic and nuclear proteins, raising novel questions about their export from mitochondria.

A major advance in the field came from the successful fractionation of the electron transport chain into four complexes (I - IV) by Hatefi.[11-13] Mitochondrial membranes were solubilized and further fractionated by a judicious combination of detergents and buffers. A fifth complex (V) was also purified and identified as the ATP synthase, an essential part of the oxidative phosphorylation mechanism (Fig. 4). The properties of each complex were defined by assays with specific substrates. Hatefi exploited the observations that cytchrome c and ubiquinone were mobile carriers in the electron transport chain that could be removed, or added from an exogenous source. The reactions catalyzed by each complex were the following:

Complex I: $NADH + Q + H^+ \rightarrow NAD^+ + QH_2$

Complex II: $Succinate + Q \rightarrow Fumarate + QH_2$

Complex III: $QH_2 + 2\ cyt\ c(Fe^{3+}) \rightarrow Q + 2\ cyt\ c(Fe^{2+}) + 2\ H^+$

Complex IV: $4\ cyt\ c(Fe^{2+}) + 4\ H^+ + O_2 \rightarrow 4\ cyt\ c(Fe^{3+}) + 2\ H_2O$

Complex V could be assayed as an ATPase, the reverse of the synthase reaction that can occur without any input of energy.

Complex I

The mammalian NADH-ubiquinone oxidoreductase (complex I) is made up of (at least) 46 distinct subunits.[14,15] The cDNAs for each of these subunits have been cloned from several organisms. With the completion of the human genome project the locations of all genes are also known. They are scattered widely over many autosomes, and two of the genes are on the X chromosome. Seven of the 46 subunits are encoded by mitochondrial DNA (ND1, 2, 3, 4, 4L, 5, and 6), and these seven, together with seven nuclear genes, are homologous to genes found in prokaryotes.[16-18] The functionally equivalent complex in prokaryotes is therefore much simpler in structure, and a major challenge is to identify the function of the 32 additional proteins in the mammalian complex.

A crystal structure for complex I is not yet available. Low resolution structures have been obtained by electron cryomicroscopy.[19-22] The overall shape is that of a 'boot' or 'L', and one can distinguish two or three subdomains (Fig. 4). An integral membrane subcomplex contains all the subunits encoded by mtDNA (ND1, 2, 3, 4, 4L, 5, 6) and more than ten subunits encoded by nuclear genes. A prominent peripheral membrane subcomplex extending into the matrix contains 21 subunits including the 75 kDa, 51 kDa, 49 kDa, 30 kDa and 24 kDa subunits. The low-resolution structure defines a neck region, but it is not yet possible to unambiguously designate specific subunits making up the neck. A fractionation procedure by Walker's lab has defined three subcomplexes: a peripheral membrane subcomplex, Iα, and two integral membrane subcomplexes, Iβ and Iγ, where Iγ is believed to include the neck region.[23,24] Subcomplex Iα is made up of a flavoprotein (51kDa protein with FMN), and at least 7 subunits with iron sulfur centers.[25] Clearly, the binding site for the substrate NADH is close to the flavin mononucleotide, and the Fe-S clusters must constitute a path for electrons from the flavin to ubiquinone. There are at least two quinone binding sites, but their precise location is still unclear. The Iα subcomplex can be functionally defined as an NADH dehydrogenase, while the neck region has been proposed to be related to multisubunit hydrogenases.[26] The integral membrane subcomplex is still the most mysterious with regard to structure and function, but it must be responsible for proton pumping from the matrix to the intermembrane space. The oxidation of one molecule of NADH is accompanied by the net transfer of four protons from the matrix to the IMS. How electron transport through complex I drives proton

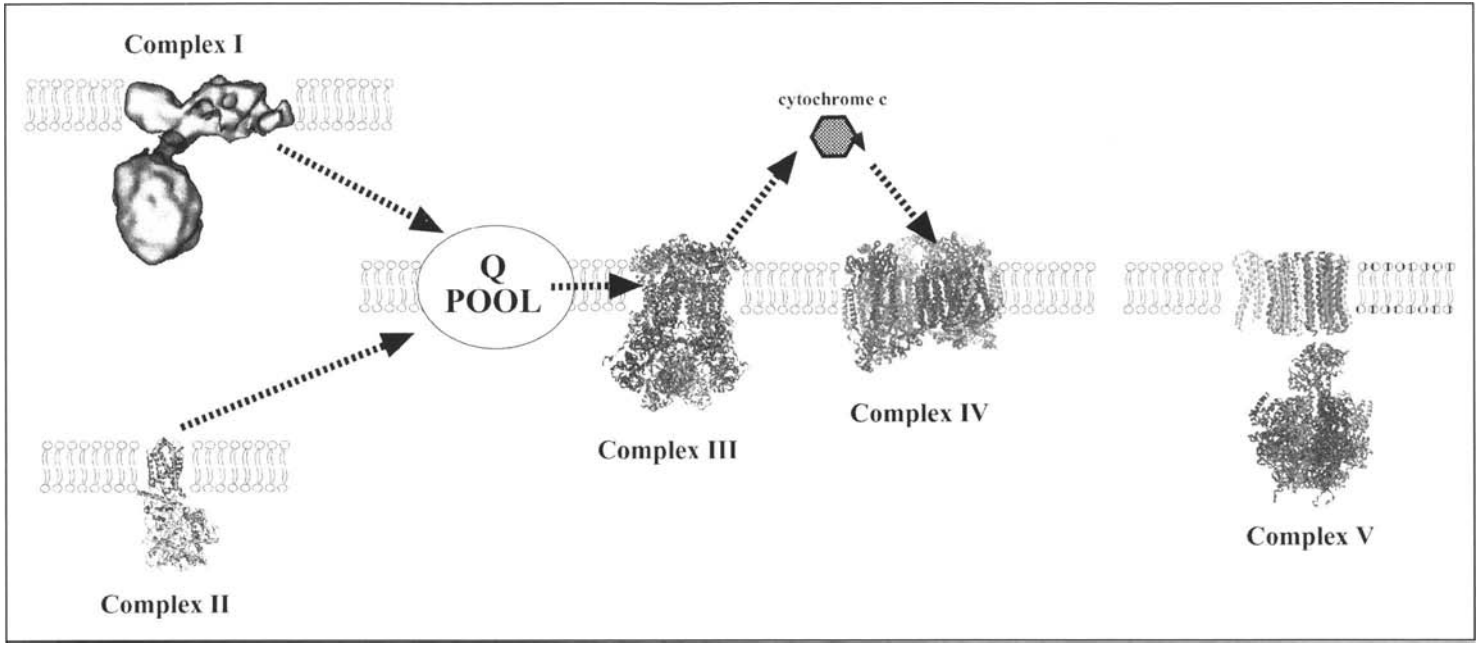

Figure 4. Schematic view of the electron transport chain (complexes I – IV), and ATP synthase (complex V). The structures for the individual complexes were provided by Dr. A. Matsuno-Yagi at the Scripps Research Institute.

pumping is a significant and challenging future problem. Two distinct mechanisms have been speculated about; one is associated with the redox cycle of ubiquinone (another Q cycle?) (Fig. 5), and another is proton translocation driven by conformational changes in the complex. It has been hypothesized that both mechanisms may operate side by side, each contributing to the total proton transport. In this context an interesting recent study with the *E. coli* complex has revealed two conformations of the complex.[27] The discovery that the complex I from *Klebsiella* pumps sodium ions instead of protons appears to eliminate a Q cycle-dependent mechanism.[28,29]

The role of many of the subunits is still obscure. They could play a role in assembly, stabilization or regulation of the complex. Since an equivalent complex does not exist in the common yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, genetic studies have been limited. Genetic experiments with other lower eukaryotes (*Neurospora crassa*, *Chlamydomonas*, the yeast *Yarrowia lipolytica*)[30-33] are promising. The presence of a number of very small subunits of less than 10 kDa size is particularly puzzling.[34-37] One of these, the MWFE subunit encoded by the X-linked *NDUFA1* gene has been shown to be absolutely essential for the assembly of a functional complex I.[38,39] Two other proteins essential for assembly as shown by genetic experiments in *Neurospora* are the acyl carrier protein,[40] and a factor referred to as CIA30.[41] The mitochondrial acyl carrier protein is required for lipoic acid synthesis.[42,43] Finally, a relatively recent addition to the number of complex I subunits is the bovine homologue of GRIM-19, a gene product induced by interferon-β and retinoic acid. Its implication in programmed cell death suggests a new connection between the mitochondrial electron-transport chain and apoptosis.[44]

There have been several recent reports that specific subunits of complex I are phosphorylated by a cAMP-dependent protein kinase.[45,46] The 18-kDa IP subunit (*NDUFS4* gene) has been definitely identified, while a small phosphorylated subunit of ~6kDa (tentatively identified as the MWFE subunit) remains to be verified.[47] Such post-translational modifications may play a regulatory role, but a physiological basis for such a regulation is not yet clear. Are these subunits phosphorylated constitutively, or is this phosphorylation a mechanism of fine-tuning the activity of this complex? What are the physiological signals controlling the relevant kinases and phosphatases? A strong indication of the potential importance of phosphorylation comes from the finding of a child with a fatal neurological syndrome in which a mutation in the *NDUFS4* gene prevented cAMP-dependent activation of the complex.[48]

Complex II

Complex II (succinate-ubiquinone oxidoreductase) is the simplest of the complexes of the electron transport chain (Fig. 4).[49-51] Its four subunits are encoded by nuclear genes (*SDHA-D*) in most organisms including mammals. Two subunits form a peripheral membrane subcomplex. The 70kDa flavoprotein (FP; gene *SDHA*) binds FAD as cofactor near the active site for succinate binding. The flavin is covalently attached to a highly conserved histidine. A 28 kDa iron protein (IP; gene *SDHB*) contains three iron-sulfur clusters ([2Fe-2S], [3Fe-4S], and [4Fe-4S]) for electron transport between the flavin moiety and the final acceptor ubiquinone. The peripheral complex is anchored to the membrane by interactions with two integral membrane proteins (C_{II-3} and C_{II-4}; genes *SDHC* and *SDHD*). Both of these subunits have three transmembrane helices, and each subunit contributes histidine sidechains for binding a b-type cytochrome. Replacement of either H128 or H135 with arginine in C_{II-3} by site-directed mutagenesis leads to an inactive complex, or causes failure of assembly (Scheffler lab, unpublished observations). Two ubiquinone-binding sites must also be provided by the integral membrane subunits. The amino acid sequences for the FP and IP subunits are highly conserved from prokaryotes to eukaryotes, while the integral membrane proteins show less homology or similarity. However, four highly conserved histidine residues are present in the C_{II-3} protein of many diverse organisms.

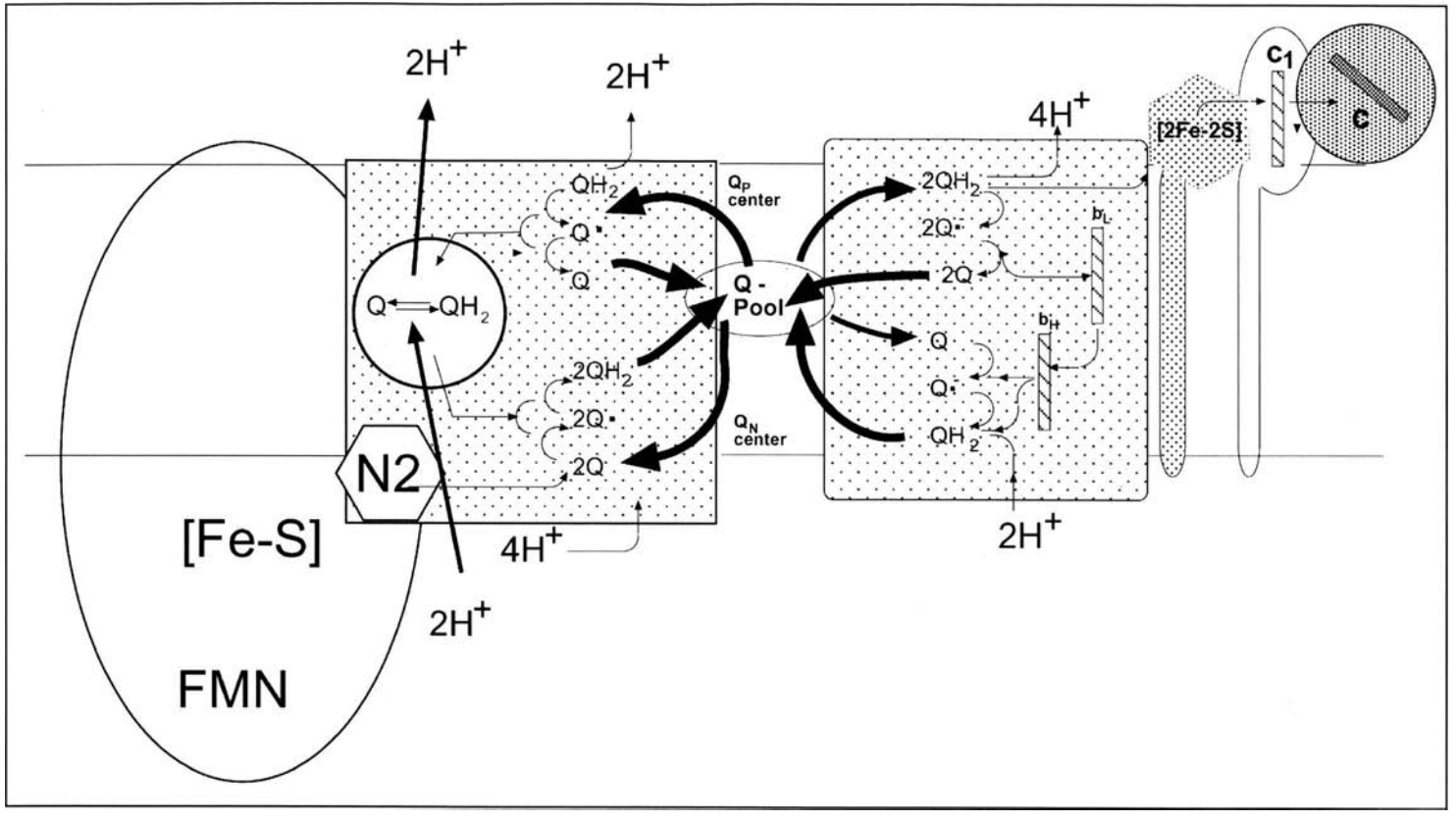

Figure 5. Composite drawing of the Q cycle as proposed by Trumpower for complex III, and adapted to complex I by the author, based on proposals by Ohnishi and coworkers. The presence and proposed function of the third ubiquinone in complex I is still somewhat hypothetical.

A crystal structure for the mitochondrial complex II is not yet available; however, the structures for the closely related fumarate reductase (QFR) and succinate dehydrogenase (SQR) from *E. coli* can contribute very important clues.[52,53] The prokaryotic SQR complex has in turn been shown to have great similarities to the mammalian complex.[49-51] In the absence of proton pumping the challenge of defining a path for protons does not arise. Electron transport can be predicted with considerable precision. It is noteworthy, that QFR does not have a b-type cytochrome. The cytochrome b present in SQR and the mammalian complex II has not been implicated in electron transport by spectroscopic measurements, but it seems to play a role in the assembly and/or stabilization of the integral membrane subcomplex. This problem requires further investigation.

The complex II from *Saccharomyces cerevisae* probably resembles the mammalian complex closely, and therefore molecular-genetic studies in yeast have contributed significantly to its understanding.[51] From a structural perspective, two quinone binding sites have been defined and partially characterized by site-directed mutagenesis.[54] They can be modeled into the complex using the *E. coli* SQR as a guide. Additional genetic studies in yeast have provided answers to questions related to the biogenesis of the complex.[55-57]

Complex III

Complex III, ubiquinone-cytochrome c reductase, often abbreviated to cytochrome c reductase, is also referred to as the bc_1 complex after the two cytochromes associated with it. The mammalian complex III contains 11 subunits, of which one, cytochrome b, is encoded by the mitochondrial genome. A functionally equivalent complex in bacteria contains only three homologous subunits: cytochrome b, cytochrome c_1, and the Rieske iron-sulfur protein. Just as in the case of complex I, eukaryotes have a significantly larger number of subunits for which a function is not immediately apparent. Electron microscopic studies as well as biochemical isolation procedures suggest that the complex is a dimer in vivo (Fig. 4). However, the significance of dimerization for function has not been explicitly demonstrated.

Biochemical as well as immunochemical studies had predicted the topology and major features of the subunits. Cytochrome b with eight transmembrane helices is associated with two heme groups (b_L and b_H) distinguishable by spectroscopic techniques, and there is a third heme in cytochrome c_1. Another functionally important group is the iron-sulfur cluster [2Fe-2S] in the Rieske protein. Decades of research culminated in 1997 with the publication of the complete crystal structure of the bovine complex at 2.9 A resolution. The structure has been further refined since then.[58-60] A curious feature of the structure is the large domain (about half the molecular mass) on the matrix side of the membrane, made up of two so-called 'core proteins'. These appear to have no functional role in electron transport or proton pumping. They may contribute significantly to the stabilization of the dimer, and both have homology to the α and β subunits of the mitochondrial matrix protein processing peptidase (MPP). The closely related mammalian MPP is soluble in the matrix, but in plants the MPP has actually been found to be associated with complex III.

The two b-type hemes are embedded in the membrane, but the heme-containing domain of cytochrome c_1 and the protein domain surrounding the [2Fe-2S] cluster of the Rieske protein are found on the side of the membrane (P side) facing the intermembrane space. Electrons flowing through these two metal ion centers are passed to the soluble cytochrome c in the intermembrane space.

The crystal structures for the first time clearly defined the two ubiquinone binding sites (Q_N and Q_P)* and the sites where several well-known inhibitors (antimycin A, myxothiazol) bind to complex III. The function of the complex is to oxidize QH_2 and transfer the electrons to cytochrome c. Cytochrome c can accept only one electron at a time, and QH_2 can give up

* The two quinone binding sites are also referred to as the Q_i site (quinol reducing) and Q_o site (quinol oxidizing), respectively.

one electron (and one proton) to form the relatively stable semiquinone, followed by a second electron/proton transfer to yield the fully oxidized quinone. This simple sequence is not followed, however. Instead, a more complicated scheme, the Q cycle, has been proposed to explain two aspects of the mechanism of complex III (Fig. 5). First, the flow of electrons through the four metal centers must be consistent with the redox potentials of the various centers. Second, a long-standing challenge has been to explain how electron transport through the complex causes the complex to function as a proton pump.

P. Mitchell was the first to propose that the mobility of the ubiquinone in the membrane might play a role in proton translocation by discharging the protons vectorially on one side of the membrane.[61] The modified Q cycle by Trumpower and colleagues is best described with reference to a schematic diagram (Fig. 5).[62-64] In the first, bi-furcated step one molecule of QH_2 (in the Q_P site) is partially reduced with one electron being transferred to the [2Fe-2S] center in the Rieske protein, and from there to cyt c_1, and finally to cyt c. Two protons are released into the IMS. A second electron is then transferred via cyt b_L and cyt b_H to a Q bound at the Q_N site, forming the semiquinone. This sequence is repeated: a second QH_2 is oxidized at the Q_P site and the semiquinone at the Q_N site is fully reduced to QH_2. Thus, a total of four protons are released into the IMS, and two protons are picked up from the matix ending up in the quinone pool.

The scheme is consistent with observations made with several distinct inhibitors binding at or near the Q-binding sites: myxothiazol binds at or near Q_P and inhibits electron flow from that site to the b_L cytochrome. Stigmatellin also binds at or near the QP site, but its binding domain is distinct from that of myxothiazol. Both inhibitors can be used in combination. Antimycin A binds at or near the Q_N site and prevents electron transfer from cyt b_H to the quinone. Thus, reversed electron transport can be observed from cyt c, and the cytochrome b can be readily reduced in the presence of antimycin, while myxothiazol or stigmatellin slow down this reverse electron flow considerably.

Controversy about some of the mechanistic aspects of the proposed bifurcation of the electron flow from reduced QH_2 are countered by the observation that the domain of the Rieske protein with the [2Fe-2S] cluster can swing back and forth between two conformations. In the oxidized form it is close to the quinol-binding site to accept an electron, but once reduced it dissociates to move closer to the cyt c_1; the electron from the remaining semiquinone now is shuttled to the cyt b, while the [2Fe-2S] cluster is reoxidized by electron transfer to cyt c_1. A detailed aspect of this mechanism includes the protonation and deprotonation of the Rieske protein at a critical and conserved tyrosine residue close to the iron-sulfur cluster. Experiments with *Rhodobacter spheroides,*[65,66] yeast[67] and *Paracoccus denitrificans*[68] have confirmed the importance of this residue in determining the redox mid-potential and the pH dependence of the rate of quinol oxidation.

Complex IV

Complex IV is the site where molecular oxygen is converted to water. The electron donor is cytochrome c, the soluble, small cytochrome in the intermembrane space. The name cytochrome oxidase is the common name for this complex. The mammalian enzyme is composed of thirteen subunits, and as found for complexes I, III, and V, the cytochrome oxidases in lower eukaryotes have fewer subunits (e.g., 9 in yeast). It is likely that the extra subunits have a role in assembly, stabilization or regulation rather than in the basic mechanism of electron transfer from cyt c (Fe^{2+}) to oxygen. The three largest subunits found in all eukaryotic organisms are encoded by mitochondrial DNA, and not surprisingly, these subunits are also found in the prokaryotic complex where only three or four subunits are found.

The study of this complex can be said to have begun with the discovery and spectroscopic characterization of the cytochromes a and a_3 by Keilin in 1925, and they culminated with the publication of the crystal structure of the bovine complex,[69-71] shortly after the simpler bacterial complex from *Paracoccus denitrificans* had been solved in 1995.[72] In the intervening years many basic properties of the complex had been determined, but the crystal structure provided

final confirmation and a considerable refinement and additional detail. The x-ray structure revealed two additional metal centers containing Mg^{2+} and Zn^{2+}. These are not implicated in electron transport, but serve in the stabilization of the tertiary structure of the protein(s). Heme a and heme a_3 are prosthetic groups associated with subunit I which also includes the Cu_B redox center. Subunit II binds the second copper ion (Cu_A), and subunit III is likely to be involved in proton pumping/transport. These three subunits constitute the functional core of the complex, similar to that found in prokaryotes. Genetic studies in yeast have demonstrated that subunits IV, V, VI, VII or VIIa are required for assembly of an active complex, and it is likely that the same is true in mammalian mitochondria. The four additional subunits found in vertebrates could also be needed for assembly, or they could play a role as allosteric regulatory proteins sensitive to adenine nucleotide levels.[73-77] It is noteworthy that complex IV is the only complex in which tissue-specific isozyme subunits have been found so far.[78-80] Knock-out mice missing the gene for CoxVIaH suffer from cardiac dysfunction.[81]

The path of the electron from cytochrome c to oxygen through the various metal centers was worked out from spectroscopic and kinetic studies and supported by the crystal structure. The first bi-nuclear copper center (Cu_A) is localized in a domain that extends into the IMS for interaction with cyt c. The electron is then transferred via heme a to heme a_3, where heme a_3 forms a second binuclear center with the copper ion Cu_B. In the binuclear centers the electrons are de-localized. Oxygen as the terminal electron acceptor binds close to the Cu_B center. A total of four electrons have to be transferred successively from four molecules of reduced cyt c to convert one O_2 molecule to two water molecules. An oxygen-oxygen bond has to be split in the process. Intermediates of partially reduced oxygen must be held in place until the reaction is complete and two innocuous water molecules are released. Premature release of partially reactive oxygen (ROS) species would be deleterious, causing apoptosis and aging. However, there is a general consensus that the active site for oxygen in complex IV is designed to prevent such a potentially deleterious reaction, and the more commonly accepted mechanism for ROS production is leakage of electrons from high potential sites on complexes I, II, and III. Detailed schemes and structures of intermediates have been proposed, e.g.,[82] but there are continuing arguments about the rate limiting steps, and particularly about the most challenging problem of how this redox reaction is coupled to the net transfer of protons across the membrane. A total of eight protons are taken up from the matrix side; four of those end up in water molecules, and four are expelled into the intermembrane space.[71,83-86] In this case no mobile, lipid-soluble carrier such as ubiquinone can be invoked, and the translocation of protons must be driven entirely by allosteric changes occurring within the protein complex, similar to the mechanism established for bacteriorhodopsin.[87,88] Which step in the successive electron transfers is a key step? And, what is the precise path of the protons through the complex and across the membrane? In general, protons are passed from strategically located amino acid side chains whose pK is dependent on the changing charges in the various metal centers. From the crystal structure certain candidates can be identified, and experiments with mutated subunits (in simpler genetic systems) can provide important clues. The discussion at some level has included notions such as a "proton wire" and quantum mechanical tunneling of protons.[85,89-91] There is also a distinct possibility that the number of protons pumped is not an integral number, and slippage may occur.

The Ratio of Complexes I - IV: Supercomplexes

A problem that was addressed some time ago and revisited recently was to ask how many complexes of each type were present in the electron transport chain, in other words, what is the stoichiometry for the different complexes? For bovine heart mitochondria the relative number of complexes I, II, III, IV, and V was reported as [1.1+/-0.2] : [1.3+/-0.1] : [3] : [6.7+/-0.8] : [3.5+/-0.2],[92] not very different from the earlier estimate.[13] Technical problems make such measurements difficult to perform with other tissues.

The inner mitochondrial membrane has one of the highest protein to lipid ratios of any biological membrane, and questions were raised whether this membrane could indeed be viewed as a fluid mosaic model, that is, as a lipid bilayer in which protein complexes "float like icebergs". Fluidity in this membrane was established, and lateral diffusion of protein complexes appeared to be possible.[93,94] Thus, the complexes in the inner membrane were considered to float about randomly and independently, but most significantly, the mobile carriers ubiquinone and cytochrome c were thought to shuttle electrons between these complexes. This view has been challenged in recent years.[92,95,96] The development of novel conditions for solubilization of membrane complexes, and fractionations on sucrose gradients, or by Blue Native Gel electrophoresis, has revealed that complexes I – IV may be aggregated into supercomplexes. The implications of these findings are not yet completely clear. The assembly and the stability of these complexes could be influenced by such interactions, and the "mobile" carriers ubiquinone and cytochrome c could be trapped within such supercomplexes, speeding up the reactions (analogous to a mechanism known as substrate channeling). Distinct complexes between complex I, III, and IV have been observed in mammalian mitochondria, and between complex III and complex IV in yeast.[95,96] There may be more than one cytochrome oxidase per supercomplex, and in yeast the stoichiometry may vary with the growth conditions.

Reactive Oxygen Species

The oxidation of NADH by molecular oxygen does not occur in the active site of a single enzyme ("Atmungsferment"), but instead takes place with these two substrates separated over a considerable distance by an 'electron transport chain'. A problem recognized early in these investigations was that there is a potential leakage of electrons from a high-potential site to a suitable acceptor; its small size and solubility make the oxygen molecule a prominent candidate. Estimates of the amount of oxygen diverted to reactive oxygen species (ROS) vary widely. The first reaction is the addition of an electron to an oxygen molecule to form the superoxide radical $O_2^{\cdot-}$ Several reactions follow, with or without the participation of metal ions:

$$2\ O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (1)$$

$$O_2^{\cdot-} + Fe(III) \rightarrow O_2 + Fe(II) \qquad (2)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^{\cdot} \qquad (3)$$

The sum of reactions (2) and (3) yields

$$O_2^{\cdot-} + H_2O_2 + H^+ \rightarrow O_2 + H_2O + OH^{\cdot} \qquad (4)$$

The three species, $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$, are considered the reactive oxygen species (ROS), although it is the hydroxyl radical ($OH^{\cdot}$) that is probably the most reactive and damaging. As discussed above, the formation of the superoxide is thought to occur from leakage from high-potential, upstream sites of the electron transport chain. The nature of these sites has by no means been settled. One school of thought places the emphasis on the semiquinone species, i.e., the intermediate in the interconversion of ubiquinone (Q) and ubiquinol (QH_2). Depending on diffusion and exchange in the quinone pool in the membrane, oxygen and the semiquinone, $QH^{\cdot}$, could potentially interact quite readily:

$$OH + O_2 \rightarrow O + H^+ + O_2^{\cdot-}$$

In the literature it is generally stated that the superoxide is produced by complexes I and III. Complex II, which also includes ubiquinones in its redox reaction, has only recently been mentioned as a potential source of ROS.[97,98] Also, arguments have been advanced that one or more iron-sulfur clusters in complex I may be the source of the electron leak.[99] Several spectrophotometric methods for measuring ROS are available; they typically measure the oxidation of a chromophore by the superoxide or hydrogen peroxide, and not the formation of the highly

reactive and short-lived hydroxyl radical. In whole cells and mitochondria the precise source of the ROS cannot be determined by such measurements, but must exploit the use of specific substrates, inhibitors and mutants.

While there is an inherent low rate of leakage from the electron transport chain, it is apparent that various perturbations caused by mutations, drugs and inhibitors, and hyperoxia can lead to significant increases in ROS production. For example, mutations in the ce*SDHC* gene in *Caenorhabditis elegans* have been shown to increase ROS production,[100] decrease life-span, and cause other age-dependent alterations.

The topic of free radicals in health and disease is a vast one.[101] ROS can have necessary and beneficial functions (in cellular defense systems and signal transduction), but they are primarily harmful in excess. Prominent theories on aging place much of the blame on reactive oxygen species. Superoxide dismutases (SOD), catalase, and a variety of peroxidases are scavenging enzymes constituting our primary defence against ROS. Thus, a low level of leakage may be constitutive, unavoidable, and relatively harmless over a short period of time because of adequate scavenging mechanisms. Over a long period of time there is likely to be an accumulation of damage in mitochondrial DNA; damaged lipids and proteins may turn over rather than accumulate.

Other chapters in this volume will deal with mitochondrial diseases caused by mutations in nuclear and mitochondrial DNA. In trying to understand the pathology of such diseases, an initial focus is clearly on an energy deficiency and its consequences for a particular tissue. It is conceivable, however, that specific mutations may be deleterious not so much because of a reduction in the capacity for oxidative phosphorylation, but because they can elevate ROS production, and therefore accelerate certain age-dependent processes, or cause cell death by apoptosis.

Inhibitors of Electron Transport

Historically the elucidation of the electron transport chain has benefited from the use of a limited number of specific and potent drugs. Rotenone can inhibit complex I and therefore respiration driven by NADH producing reactions. In isolated mitochondria succinate can still act as an effective substrate. Antimycin A can inhibit both succinate and NADH-dependent respiration, and therefore it must act downstream from the point where electron transport from succinate and NADH converge. Cyanide and azide were found to be very effective inhibitors at the end of the chain, i.e., they could occupy the site in cytochrome oxidase where oxygen normally acts as the terminal acceptor. Thus, in the presence of rotenone and oxygen, electrons can be 'drained' from the chain and all the cytochromes can be found in the oxidized state for spectroscopic characterization. Alternatively, in the presence of cyanide and succinate, all cytochromes can be reduced. By reducing all cytochromes in the absence of oxygen and then admitting oxygen, kinetic studies of the oxidation of cytochromes could establish the order $b \rightarrow c_1 \rightarrow c \rightarrow a \rightarrow a_3$.[102]

In recent years the emphasis has changed. There are practical applications, because some of these drugs may have applications as insecticides or in the control of other pests (rodents). More often specific drugs are used in structurefunction studies and in detailed mechanistic studies.[103] The use of antimycin A, myxothiazol and stigmatellin in the formulation of the Q cycle and the study of electron flow in the bc_1 complex (III) has already been mentioned. Another view of the function of this complex and its interaction with these inhibitors has been presented by Matsuno-Yagi and Hatefi.[104]

There are numerous inhibitors of complex I. They all appear to inhibit electron transfer from the high potential iron-sulfur cluster (N-2) to ubiquinone, but they are distinguishable by being either noncompetitive inhibitors (rotenone, phenoxan, aurethin) or partially competitive inhibitors (piericidin A, aurachins A and B, thiangazole, phenalamid A2). It emerges that there are at least two quinone binding sites, and there has been a strong incentive to find inhibitors that can differentiate between these two sites.[105,106] The designer drug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was discovered to cause symptoms of parkin-

sonism[107,108] because it is metabolized to the 1-methyl-4-phenylpyridinium ion (MPP^+) which has been shown to be an inhibitor of complex I.[109,110] Consequently, a defect in complex I was hypothesized to be a primary cause of Parkinson's disease. However, evidence for any mutations in complex I genes (nuclear or mitochondrial) as a primary genetic basis for Parkinson's disease is unconvincing.

The most commonly used inhibitor of complex II is the substrate analogue, malonate, binding at the active site for succinate. A series of 2-alkyl-4,6-dinitrophenols have been described as noncompetitive inhibitors of the mammalian complex II.[111] It is likely that they bind near the quinone binding site(s).

In addition to cyanide and azide, nitric oxide at nanomolar concentrations has been shown to be a competitive inhibitor of cytochrome oxidase.[112-115] It is significant that this gas is produced endogenously by the mitochondrial nitric oxide synthase (NOS), and thus the physiological implications for mammals have to be explored. In addition, nitric oxide can be oxidized or reduced yielding intermediates that can react further to form various reactive nitrogen species (RNS) (e.g., peroxynitrate, $ONOO^-$).

The ATP Synthase

A Molecular Motor

The oxidation of NADH (and succinate) via the electron transport chain constitutes the energy source for the phosphorylation of ADP to ATP, but how this exergonic reaction is coupled to the endergonic phosphorylation reaction was a contentious topic for almost two decades after the identification of mitochondria as the "powerhouse of the cell".[3] One school of thought was strongly influenced by insights from reactions now referred to as 'substrate level phosphorylations', in which inorganic phosphate is activated on an intermediate, forming a high energy phosphate bond (e.g., a mixed anhydride, X~P). The phosphate is then transferred to ADP. After many fruitless attempts to identify the X~P intermediate (or a protein side chain to which inorganic phosphate was linked), the solution came from an ingenious hypothesis first proposed by P. Mitchell in 1961.[116-120] The hypothesis was so unorthodox that it was very slow to be understood and accepted,[121,122] but eventually it revolutionized thinking about bioenergetics and mechanisms of energy interconversion in mitochondria, chloroplasts and photosynthetic bacteria. The Chemiosmotic Hypothesis is now universally accepted in its generalized form. Electron transport through a series of integral membrane complexes serves to translocate protons across a membrane. The result is an electrochemical gradient storing the chemical free energy released from the redox reactions (Fig. 6).

The protonmotive force, $\Delta\mu_H$, has two components: a chemical concentration gradient representing a difference in proton concentrations across the membrane (ΔpH), and a membrane potential ($\Delta\Psi$) created due to the charge transfer associated with proton translocation. Thus,

$$\Delta\mu_H = 2.3\,RT\,\Delta pH + Z\,F\,\Delta\Psi$$

where Z is the charge of the proton, F is the Faraday constant, and $\Delta\Psi$ is the membrane potential. The genius of the Mitchell Hypothesis is the recognition that electron transport and ATP synthesis can be physically separated and executed by distinct protein complexes, and yet coupled by means of the creation of this electrochemical gradient across a common membrane. Electron transport drives a proton circuit, and proton flux through complex V drives phosphodiester bond formation to make ATP.

Ideas about the mechanism involved evolved in parallel with ideas and insights about ATP-dependent ion pumps. The molecular mechanisms by which such pumps operate were not understood then, and today there are still many missing details. Nevertheless, the rationale for the existence of such pumps first became apparent from the understanding of membrane potentials and action potentials in neurons and axons. ATP hydrolysis is the source of free energy for pumping ions across a membrane. Experimentally a large ion gradient could make

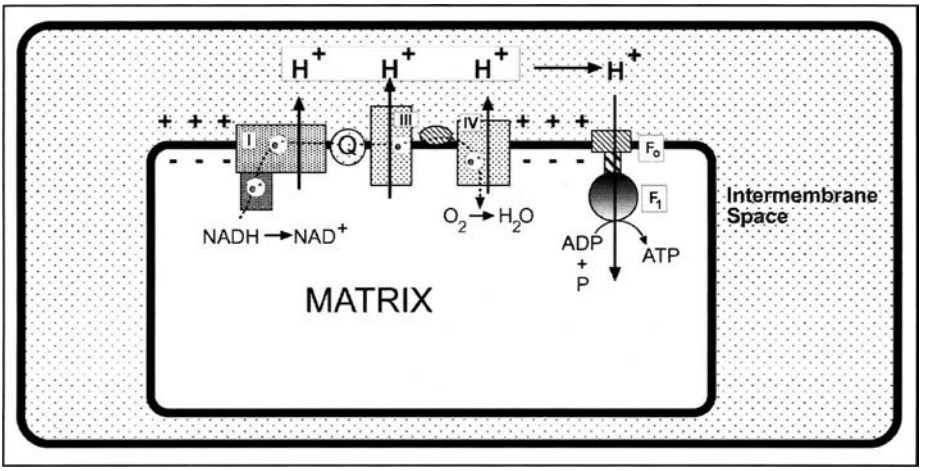

Figure 6. Schematic drawing showing the electron transport chain and ATP synthase in the inner mitochondrial membrane and the relevant activities in the chemi-osmotic hypothesis for oxidative phosphorylation.

such pumps run in reverse. Therefore, if a sufficiently large ion gradient could be established by some mechanism, it could serve to drive ATP synthesis by ions flowing from high to low chemical potential. One of the more impressive and convincing experiments of this type was to combine bacteriorhodopsin from a prokaryote (*Halobacter halobium*) with the ATP synthase (complex V) from a vertebrate (bovine heart) in synthetic lipid vesicles. Illumination activated the bacteriorhodopsin as a proton pump and set up a protonmotive force, $\Delta\mu_H$, capable of driving ATP synthesis in the presence of ADP and inorganic phosphate.

Biochemical and genetic experiments over three decades set the stage for the detailed description of one of the most remarkable structures in biology: a molecular rotary engine. Complex V was clearly not required or associated with electron transport. It could be assayed as an ATPase, and thus was an obvious candidate for carrying out the reverse reaction, ATP synthesis. The complex was first detected by electron microscopy as lollipop-like structures attached to the inner membrane and extending into the matrix of mitochonria.[123] The heads could be dissociated from the stalk as soluble complexes and reassociated with the membrane with the help of an oligomycin-sensitivity-conferring-protein (OSCP).[124-126] Sensitivity to oligomycin made complex V easily distinguishable from other cellular ATPases.

The biochemical characterization of the purified complex led to the identification of at least 15 subunits in mammals, 13 subunits in yeast, and an even smaller number of related subunits in the corresponding bacterial complex. The soluble head complex contained the peptides α, β, γ, δ, ε in the ratio 3:3:1:1:1, and it became known as the F_1-ATPase, in contrast to the F_o subcomplex with the major subunits a, b, and c (stoichiometry: ab_2c_n, where n=9-12). The F_o-subcomplex was an insoluble integral membrane complex.

It is difficult to do justice to the many ingenious and diverse studies over a period of two decades (for authoritative reviews see refs. 128-132);[127-131] they culminated in the x-ray structure, first of the F_1-ATPase, and subsequently of the combined F_1- F_o complex from yeast.[132,133] In the crystallized yeast complex only the α, β, γ, δ, ε, and c subunits were detectable, leaving the interaction of the ab_2 stator with the c_n rotor and with the F_1 hexamer (via the δ subunit)*

*There is some potential confusion, because the δ subunit in animal mitochondria is the ε subunit in bacteria. Compare the schematic diagrams of the Walker group describing the yeast complex in a recent issue of Science (286: 1700 (1999)) with the diagram in the commentary by Fillingame depicting the *E. Coli* complex.

as a problem for the future, although cross-linking studies and others have yielded a complete lower resolution picture (Fig. 7). Each c subunit has two transmembrane helices connected by a loop on the matrix side; together they form a larger barrel-shaped structure with its center connected to the γ subunit via the δ subunit. In the crystal structure from the yeast complex the surprising finding was that the number of c subunits was 10, not 9 or 12 as expected (see below).

A summary of the mechanism as it is understood today can be made as follows. The $\alpha_3\beta_3$ hexamer forms a barrel-shaped structure with alternating α and β subunits and with a three-fold rotational symmetry; there are three active sites for nucleotide binding. However, the insertion of the highly asymmetrical γ subunit through the interior of the barrel introduces an asymmetry in the interactions with each αβ dimer such that its conformation and hence the conformation of its substrate binding site is different for each dimer. The rotation of the γ subunit induces a series of alterations that constitute the enzyme cycle. In other words, the binding of the substrates, ADP and P_i, the formation of the triphosphate, ATP, and the release of the product constitute a sequence of basic steps that are determined by the affinity of the active site for substrates, intermediates and products. A rotating three-site model had first been proposed by Boyer in 1982,[128] and refined over the years.[127,129] Detailed considerations of the occupation of the three sites at any given moment in vivo or in vitro are still being debated.[134] A central argument, however, the generation of three distinct sites in the $\alpha_3\beta_3$ hexamer, was spectacularly confirmed by the crystal structure. A second important aspect was that the catalytic cycle was brought about by the actual rotation of the γ subunit. While the crystal structure was highly suggestive, a convincing experimental proof was provided by the experiments of Noji and colleagues.[135]

The next challenge was to elucidate a mechanism that can provide the driving force for the rotation generated by the flow of protons through the F_o subcomplex. Structural and experimental evidence strongly suggest that the γ subunit is rigidly attached with the help of the ε subunit to the cylindrical structure formed by 9-12 c subunits; the cylindrical structure formed by these integral membrane subunits therefore must also rotate within the membrane around an axis perpendicular to the membrane. Such a model assigns the ab integral membrane proteins the role of a stator that remains at a fixed position in the membrane and also holds the $(\alpha\beta)_3$ in place by interactions between peripheral membrane domains of the two b subunits and the outside of the $(\alpha\beta)_3$ barrel (via the δ subunit?) (Fig. 7; bottom).

A still hypothetical model proposes that protons enter a site on the a subunit from the IMS. They are then transferred to a c subunit where they remain trapped until the c subunit has been rotated to a position where the proton can be released on the matrix side. This F_o motor has also been described as a "pure brownian ratchet that uses the binding and release of protons flowing through it to rectify its rotational diffusion".[1] A complete x-ray structure of the F_o subcomplex with both the ab_2 and c subunits will be required to allow detailed speculations about the path of the proton. However, elegant genetic studies with the *E. coli* ATP synthase have implicated several specific side chains of the a and c subunits in this proton translocation.[136-139]

There remains a problem arising from the experimentally observed ratio of ~three protons passing through the F_o subcomplex per ATP produced. It seemed obvious to assume that three protons cause a 120 degree turn, and therefore one would also have expected to have a three-fold rotational symmetry associated with the assembly of c-subunits, matching that of the $(\alpha\beta)_3$ complex. Again, the interaction with the ab_2 integral membrane proteins would introduce asymmetry in the overall structure. Some elegant experimental studies by Fillingame and coworkers with the *E. coli* F_o subcomplex had supported a model with 12 c subunits.[138,140] The structural studies with the yeast complex with 10 c subunits raise the possibility that the number need not be a multiple of three, and may vary from organism to organism. Another conclusion may be that the H^+/ATP ratio is not an integer, allowing slippage in proton translocation and rotation. A theoretical discussion of the mechano-elastic properties of this "electrochemical transducer with rotary mechanics" has been presented.[141]

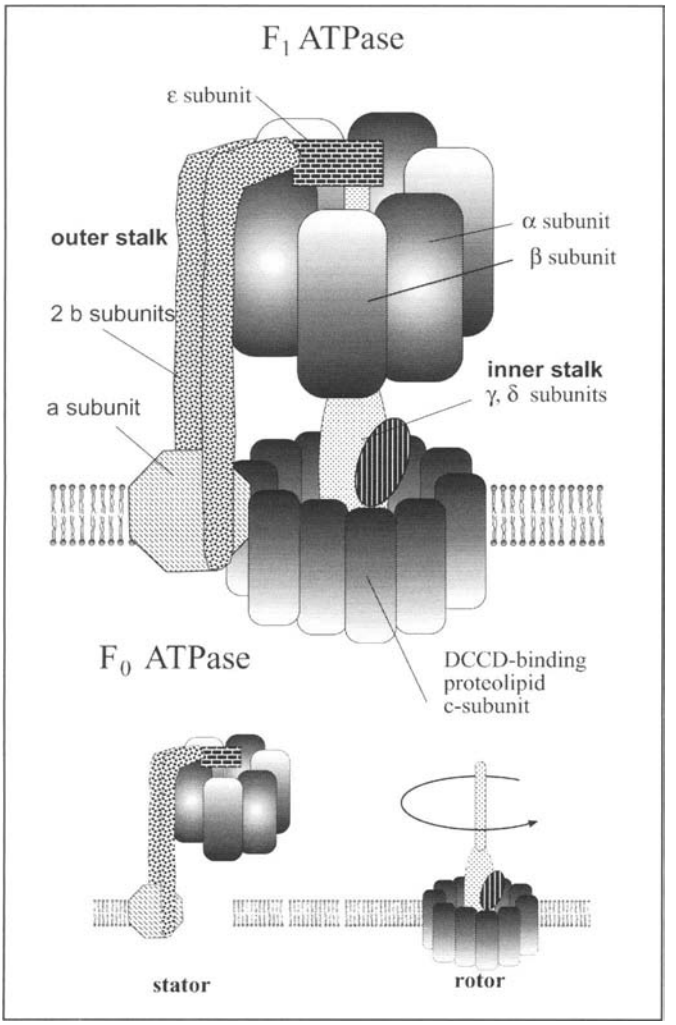

Figure 7. Schematic drawing of complex V (ATP synthase) at the top, and the individual components (stator and rotor) of the molecular rotary engine.

The most penetrating insights were obtained from a combination of biochemical, genetic and structural techniques applied to the bacterial and yeast complexes, but the basic principles for the operation of this molecular machine apply equally well to the mammalian complex. The structures and function of the extra subunits found in the higher eukaryotes still need to be integrated into the picture.

A small peptide of 84 amino acids has been characterized from bovine heart that can act as an F_1 ATPase inhibitor, IF_1. Homologues have been described in other organisms. Its structure and interaction with the ATP synthase is quite well understood.[142] Highly conserved histidine residues at the C-terminus are thought to make the C-terminus a pH- sensitive switch. Its function is to prevent the complex to act as an ATPase under anaerobic conditions when the membrane potential/proton gradient is collapsed. It thus prevents the loss of ATP from the pump running in reverse of its normal direction.

Regulation of Oxidative Phosphorylation

In studies with isolated mitochondria a well-known distinction is made between tightly coupled and uncoupled mitochondria. In coupled mitochondria the respiration rate will be very low even in the presence of abundant substrates (such as succinate) when no ADP and inorganic phosphate are present. The chemiosmotic hypothesis provides an explanation: in the absence of a mechanism to dissipate the electrochemical proton gradient electron transport comes to a halt. Electron transport through a given complex will not be able to overcome the "resistance" from a very high membrane potential and proton gradient. An alternative is to uncouple mitochondria by synthetic or natural uncouplers. Dinitrophenol and carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) have found applications in this context as small molecule uncouplers that can shuttle protons across the inner membrane. Subsequently, a family of uncoupling proteins were discovered. The first, UCP1, is clearly responsible for thermogenesis in brown adipose tissue of newborns and hibernating animals.[143] Others (UCP2 - UCP5) can act as uncouplers in certain experimental conditions, but their physiological and biochemical properties have not been completely elucidated, and controversies continue about their role in thermogenesis or fatty acid metabolism.[144]

The overall regulation of oxidative phosphorylation can be discussed with a focus on three aspects (Fig. 8): (1) the availability, production and turnover of oxidizable substrates such as NADH, succinate and fatty acids; (2) the rate of electron transport from complex I (or II) to oxygen; and (3) the availability and concentration of ADP (and P_i) from the turnover of ATP by all the energy consuming processes of the cell. This makes it a formidable task to discuss regulation of oxidative phosphorylation, because undoubtedly there are many cell and tissue-specific conditions and parameters to consider. A discussion of abnormal, pathological metabolic states are beyond the scope of this review. The concentration of ADP in the mitochondrial matrix may also be controlled by the activity of the ADP/ATP translocator that exchanges adenine nucleotides across the inner membrane.

Another question is whether the electron transport chain, and specific complexes within it, can play a regulatory role under certain conditions or in specific tissues. This issue becomes particularly pertinent for the understanding of mitochondrial diseases and the pathological consequences of mutations in either mitochondrial DNA or the relevant nuclear genes. If one thinks of electron transport as a series of reactions, as suggested by the existence of distinct complexes and the mobile carriers between them, one can ask whether a particular complex is rate limiting, i.e., a bottleneck, in the conventional sense of viewing the problem, or with a particularly high control coefficient in the more up-to-date formalism of flux control theory?[145]

Applications of flux control theory to mitochondria have been published by several authors.[146-151] Different respiratory complexes have different control coefficients, but in general these coefficients are all relatively low (< 0.2), and for a given complex the control coefficient has been found to be variable between different tissues. For example, the control coefficient for cytochrome oxidase is 0.2 in muscle and 0.01 in brain or liver.[151] The value of the control coefficient is also related to the "threshold value". If respiration is measured in the presence of increasing concentrations of a specific inhibitor, and compared with the remaining activity of the targeted complex, then it is typically observed that there is no significant inhibition of respiration until the activity of the complex is inhibited 80-90%, the threshold value. The threshold value is inversely related to the control coefficient. Other chapters in this volume will again address threshold values in relation to partially active complexes and mitochondrial diseases.

A number of papers by Kadenbach and colleagues have argued that ATP and/or ADP can exert direct control over the activity of cytochrome oxidase (complex IV) by an allosteric mechanism.[76,77,152] Such observations made in vitro with a purified complex remain to be fully integrated into a physiological framework. Another intriguing aspect of complex IV is that it is the only complex known for which tissue specific isozymes/subunits have been found.[153] The structural consequences of such substitutions also remain to be fully explored.

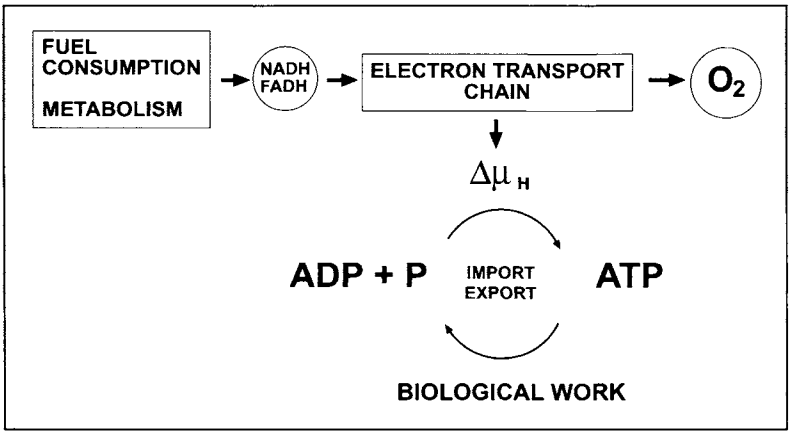

Figure 8. Schematic representation and interrelationship of the major activities: metabolism to produce $NADH/FADH_2$, respiration to recycle $NADH/FADH_2$, coupling to ATP synthesis (oxidative phosphorylation) and recycling of ATP to ADP by biological work. All of these functions are coordinated by regulatory and feedback mechanisms.

Finally, there are several publications with evidence for the phosphorylation of subunits of electron transport complexes by cyclic AMP-dependent or other protein kinases.[45,46,48,154,155] What remains to be elucidated fully is whether phosphorylation is constitutive and necessary to activate the complex, or whether the control of phosphorylation is a means of modulating the activity.[156]

Many of the mechanisms introduced above lend themselves to the relatively rapid control of the rate of oxidative phosphorylation in response to external stimuli, and the mechanism is readily reversible. A major topic of its own is to consider how oxidative phosphorylation (total capacity) is regulated in different tissues by a variety of mechanisms that potentially include the following: gene expression (coordinate (?) transcription of multiple genes, and post-transcriptional activities), biogenesis of mitochondria and mitochondrial morphology (density of cristae), subcellular localization of mitochondria and more.[3] The role of iron and its incorporation in hemes and iron-sulfur centers has been emphasized.[157]

Assembly of Electron Transport Complexes

A major challenge for the future is to understand how these multi-subunit complexes are assembled in and on the inner mitochondrial membrane. Complex I will serve as an example to illustrate the different aspects of biogenesis that remain to be further elucidated.

The expression of at least 39 nuclear and 7 mitochondrial genes contributes to the formation of a functional complex I. Thirty nine proteins made in the cytosol must be imported into mitochondria: integral membrane proteins with multiple transmembrane regions have to pass though the outer membrane (TOM complex) and then be inserted into the inner membrane with the help of TIM22 and several proteins in the IMS (Tim9-Tim10,...).[158] Proteins made in the mitochondrial matrix must be inserted into the membrane, with the help of the Oxa1 protein[159] and others. Are these two processes interdependent? One possibility is that these proteins are inserted independently, and subsequently they cluster and associate in the plane of the membrane to serve as a docking site for the attachment of the peripheral membrane subcomplex. The attachment of the peripheral proteins may also stabilize the integral membrane subcomplex. Alternatively, insertion of one or more integral membrane proteins may be dependent on the presence of other integral membrane subunits. The proteins of the peripheral membrane subcomplex are imported into the matrix by the TOM-TIM23 route. Folding,

association with cofactor (FMN), and formation of the 7 iron-sulfur centers may occur before, during or after assembly of the subcomplex. Preliminary evidence suggests that this subcomplex (or portions of it) is assembled in the matrix and subsequently attached to the integral membrane subcomplex. Genetic experiments with lower eukaryotes that have a complex I (*Neurospora crassa*, *Chlamydomonas*, the yeast *Yarrowia lipolytica*[30-33]) are very promising and will accelerate as the genomic databases expand.

The use of small interferring RNAs will expand the range of experimental approaches for mammalian mitochondria.

Experiments addressing the assembly of complex IV (cytochrome oxidase) in yeast (*Saccharomyces cerevisiae*) have introduced the need for "assembly factors". Assembly factors have been proposed to participate transiently in the formation of the final complex. Yeast mutants exist that fail to assemble a functional cytochrome oxidase, even though all the structural genes for the known subunits are normal.[160-164] Similarly, among the patients with reduced cytochrome oxidase activity some have been characterized with novel defective genes (e.g., SURF-1) homologous to those for assembly factors in yeast.[165-172]

Conclusions

Many decades have brought us from the simple notion of an "Atmungsferment" to our present understanding of the electron transport chain made up of four major complexes and a total of more than 70 proteins, multiple heme groups and iron-sulfur centers. The crystal structures of all but complex I have been solved, shedding much light on structure as related to function. The complexity of the whole chain is designed to capture a large amount of free energy released from the oxidation of NADH, and to convert this energy into the more useful "currency" for biological work, ATP. A truly revolutionary idea was that this free energy could be stored in the form of an electrochemical gradient across the inner mitochondrial membrane, composed of a proton gradient and a membrane potential. Oxidative phosphorylation thus became decomposed into two distinct and physically separable processes: electron transport coupled to proton pumping out of the matrix (carried out by the electron transport complexes I-IV), and ATP synthesis carried out by a molecular rotary engine (complex V) driven by proton flow back into the mitochondrial matrix.

The big picture is fairly complete. Major problems for the future are to understand assembly, and what role is played by additional proteins found in (higher eukaryotes), but not in the comparable prokaryotic complexes. What regulatory or modulatory role do they play, if any? How is the activity of the whole ensemble regulated over the long term (differentiated cells) and over the short term (adaptation to metabolism and stress)? What can go wrong, and what are the physiological consequences for a complex organism such as humans?

Finally, the past few years have made it abundantly clear that mitochondria are not just the powerhouse of the cell, but are very intimately integrated into the life, function and death of a cell. In particular, their role in regulating intracellular calcium levels is becoming very apparent, and it should be recognized that all of the transport processes (ions, metabolites) in and out of mitochondria are ultimately also driven by electrochemical and ion gradients established and controlled by the electron transport chain.

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CHAPTER 2

Molecular Biology of the OXPHOS System

Richard C. Scarpulla

Abstract

The mitochondrion contains a circular DNA genome (mtDNA) that serves as the basis for its own genetic system. This system is semiautonomous because the coding capacity of mtDNA is limited to 13 subunits of the respiratory chain apparatus and the rRNAs and tRNAs necessary for their translation. The inheritance of mtDNA differs from that of nuclear DNA in that it segregates randomly during mitosis and meiosis and is transmitted exclusively through the female germ line. Nucleus-encoded enzymes and factors direct the transcription and replication of mtDNA within the mitochondrial matrix. Mitochondrial translation also relies upon nucleus-encoded ribosomal proteins, synthetases and translation factors. In recent years, molecular mechanisms for the bi-genomic control of mitochondrial biogenesis and function have been elucidated.

Introduction

A variety of mutations in mitochondrial DNA (mtDNA) have been associated with human pathology.[1] Pathogenic mutations in mtDNA all affect the OXPHOS system because the mitochondrial genome is essential to the biogenesis of the respiratory chain apparatus. These observations have sparked considerable interest in the molecular mechanisms of mtDNA expression, inheritance, maintenance and replication. Much progress has been made in understanding the molecular biology of the OXPHOS system and major inroads have been made in elucidating nucleo-mitochondrial interactions (see refs. 2, 3). This chapter will present a general treatment of the basic molecular genetics of OXPHOS with the major focus on mammalian systems, although analogies to the yeast system will occasionally be incorporated. Recent findings on the regulation of nuclear genes governing the OXPHOS system will also be presented.

mtDNA

Observations made in the late 1950s and early 1960s pointed to the existence of a mitochondrial genetic system that was separate from that of the nucleus (see ref. 4). Early work demonstrated that isolated mitochondria had a protein synthetic machinery that could synthesize a small number of proteins. Further studies established the existence of mitochondrial ribosomes, rRNA and tRNA. Surprisingly, the system displayed antibiotic sensitivities that were more akin to prokaryotic translation than to eukaryotes. A major advance was the discovery of mitochondrial DNA in yeast and in other organisms.[5] Since these early discoveries, much has been done to define the structure and gene organization of mtDNA. The first complete mtDNA sequence was obtained from humans and sequences of mitochondrial genomes from many organisms have now been catalogued (http://megasun.bch.umontreal.ca/). A striking result from this work is that a similar complement of genes is conserved in mtDNAs from

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all multicellular organisms. In vertebrates these include genes for 13 protein subunits of respiratory chain complexes I, III, IV and V, two rRNAs and 22 tRNAs (Fig. 1).

In stark contrast to the nuclear genome, mtDNA in mammals and other vertebrates exhibits extreme economy of sequence organization. Human mtDNA exists as a covalently closed circle of about 16.6 kb. Its genes are in a head to tail arrangement with little or no intergenic regions and are completely devoid of introns. Certain respiratory chain protein genes overlap and the adenine nucleotides of UAA termination codons are supplied by polyadenylation.[6] Protein coding and rRNA genes are interspersed with tRNA genes which punctuate the sites of RNA processing (Fig. 1). The only substantial noncoding region is the D-loop, which gets its name from the triple stranded structure or displacement loop that is formed by association of the nascent H-strand in this region. The D-loop is the site of transcription initiation from bi-directional promoters and also contains the origin of H-strand DNA replication.[4] It should be noted that the structural economy found in vertebrates does not exist in plants and fungi where the mitochondrial genomes are much larger and contain intergenic regions, introns and multiple promoters and transcriptional units.[7,8] Another interesting anomaly is that the mito-

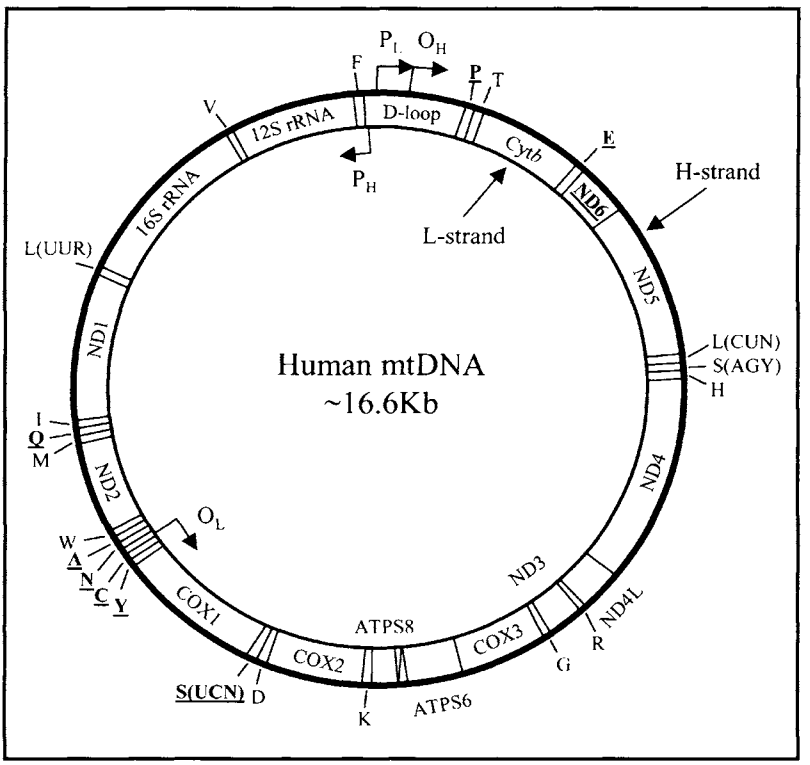

Figure 1. Human mitochondrial DNA (mtDNA). Genomic organization and structural features of human mtDNA are depicted in a circular genomic map. Protein coding and rRNA genes are interspersed with 22 tRNA genes (denoted by the single letter amino acid code). The D-loop regulatory region contains the L- and H-strand promoters (P_L and P_H, respectively) along with the origin of H-strand replication (O_H). The origin of L-strand replication (O_L) is displaced by approximately two-thirds of the genome within a cluster of five tRNA genes. Protein coding genes include: Cytochrome oxidase (COX) subunits 1, 2 and 3; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5 and 6; ATP synthase (ATPS) subunits 6 and 8; cytochrome *b* (Cyt*b*). ND6 and the 8 tRNA genes encoded on the L-strand are in bold type and underlined, all other genes are encoded on the H-strand.

chondrial genetic system utilizes genetic codes that differ slightly from the universal nuclear code and that these differences are species specific. For example, in humans the universal AUA (isoleucine) and UGA (stop) codons specify methionine and tryptophan, respectively, in mitochondria (see ref. 9).

Mitochondrial Inheritance

The fact that mtDNA is a compartmentalized extrachromosomal element contributes to a mode of inheritance that differs from that of nuclear genes. Early work in yeast led to the observation that certain respiratory chain mutations displayed a cytoplasmic inheritance pattern that resulted from the random segregation of mtDNA molecules during mitosis. This provided genetic evidence that mitochondria had their own DNA before its existence was demonstrated physically. Somatic mammalian cells generally have 10^3-10^4 copies of mtDNA with approximately 2-10 genomes per organelle.[10] These genomes replicate in a relaxed fashion that is independent of the cell cycle that is defined by nuclear DNA replication (see refs. 2, 4, 9) Some mtDNA molecules undergo multiple rounds of replication while others do not replicate. This, along with random sampling during cell division, allows the segregation of sequence variants during mitosis.[11]

In mammals, mtDNA is strictly maternally inherited (see refs. 11, 12). The paternal lineage does not contribute mtDNA to the offspring nor is there known to be recombination between maternal and paternal sequence variants. The paternal mtDNA is lost during the first few embryonic cell divisions. In addition, because mtDNA is a multicopy genome, an individual may harbor more than a single sequence, a condition referred to as heteroplasmy. A sequence variant that is detrimental may be tolerated in low copy because the defective gene product(s) it encodes do not reach the threshold for disrupting cellular function. However, sequence variants are known to segregate rapidly from heteroplasmy to homoplasmy in passing from one generation to the next.[13] This can result in offspring in which the detrimental variant predominates, leading to a defective mitochondrial phenotype. The molecular basis for this rapid meiotic segregation has been explored and has been ascribed to a bottleneck or sampling error in the female germ line.

A massive amplification of mtDNA occurs during oogenesis from about 10^3 copies in the primary oocyte to approximately 10^5 copies in the mature oocyte (see ref. 11). Replication of mtDNA is halted in the mature oocyte and the existing population of mtDNA molecules is partitioned to the daughter cells during early cell divisions until the copy number is diluted to approximately that found in somatic cells (Fig. 2). Embryonic replication does not resume until the blastocyst stage of development.[14] For unknown reasons, the mitochondrial number is reduced to about 50 in primordial germ cells and increases to about 200 in the oogonia. Assuming the normal somatic cell number of genomes per organelle, the mtDNA copy number in germ cell progenitors is extremely small, on the order of 50 to 100 copies. This small number is consistent with a genetic bottleneck and, in fact, heteroplasmic mice constructed from two domestic strains were used to establish that nearly all of the mtDNA segregation occurs between the primary germ cells and the oogonia. Thus, the genetic bottleneck occurs early in the pathway of oogenesis and segregation of sequence variants is complete before the formation of primary oocytes. It is interesting to note that significant mitotic segregation does not appear to occur during embryogenesis. In addition, defective variants are not selectively eliminated or reduced during oogenesis or early embryonic development indicating that optimal mitochondrial respiratory chain function is not required for these processes.[11]

Replication, Transcription, RNA Processing

MtDNA Replication

Replication and transcription of mtDNA is completely dependent on nucleus-encoded gene products. In mammalian cells, replication is most intense near the nucleus and newly repli-

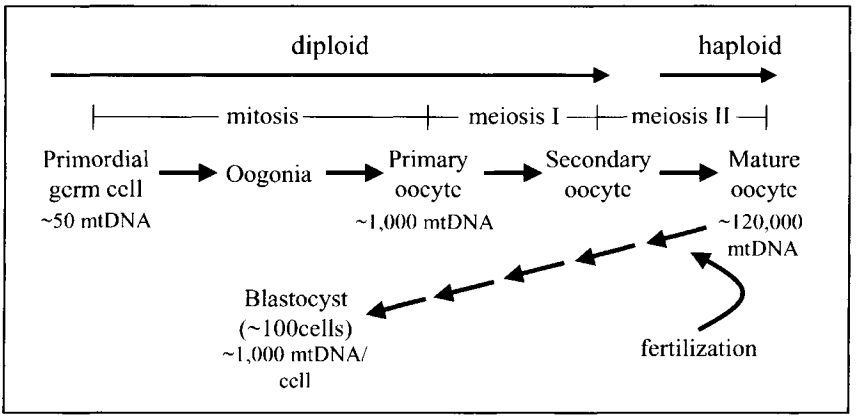

Figure 2. MtDNA copy number during oogenesis and early embryogenesis. Diagram depicts the amplification of maternal mtDNA from low copy in primordial germ cells to high copy in the mature oocyte and its dilution to approximately somatic cell levels during formation of the blastocyst.

cated molecules distribute outwardly through the mitochondrial network.[15] The overwhelming majority of evidence points to a mechanism of bi-directional replication where the replication origins for the two strands, termed heavy (H) and light (L) based on their buoyant densities, are displaced by about two-thirds of the genome.[16,17] This results in temporal as well as spatial separation of initiation events (Fig. 1). The D-loop regulatory region contains bi-directional promoters, (P_H) and (P_L), for transcribing H and L strands as well as the H-strand replication origin (O_H). As depicted in Figure 3, the RNA transcript initiated at P_L is cleaved in the vicinity of three evolutionarily conserved sequence blocks (CSB I, II, and III), and H-strand replication is initiated at the sites of these cleavages.[18] Thus, transcription is coupled to replication and the sites of RNA cleavage are transition sites between RNA and DNA synthesis. A decision must be made to continue transcription through the CSBs or to truncate the nascent RNA to initiate DNA replication. Although nothing is known of how the transition is regulated, a stable RNA-DNA hybrid that requires CSBs I and III has been demonstrated.[19] Even after DNA synthesis begins, the nascent strand is often terminated downstream from a conserved element referred to as a termination associated sequence (TAS).[20] This event may be important in controlling mtDNA levels and accounts for the triple-stranded D-loop structure.

Once the nascent H strand traverses two-thirds of the genome, L strand replication is initiated at O_L which is a short noncoding region within a cluster of tRNA genes.[16,17,21] Upon displacement of the parental H strand, O_L is thought to form a stem-loop structure.[22] This serves as the recognition site for a mitochondrial primase that produces a short RNA primer for the initiation of L strand replication. Initiation of DNA synthesis occurs near a G+C rich region at the base of the stem. The primase has been only partially purified and is thought to require RNA for catalytic activity.[23]

It should be noted that replication intermediates that are consistent with coupled leading and lagging strand replication from a single origin have also been detected.[24] This mode of replication was initially observed under conditions where cells were recovering from transient mtDNA depletion[24] but is now thought by the authors to represent the predominant mechanism of replication in dividing cells.[25] This conclusion has been challenged by the proponents of the classical strand-displacement model on the basis that the new model is supported mainly by the detection of replication intermediates using two-dimensional gel electrophoresis.[26] The precise structure of these intermediates has not been confirmed by other means and they may be the products of transcriptional events. By contrast, multiple lines of experimental evidence,

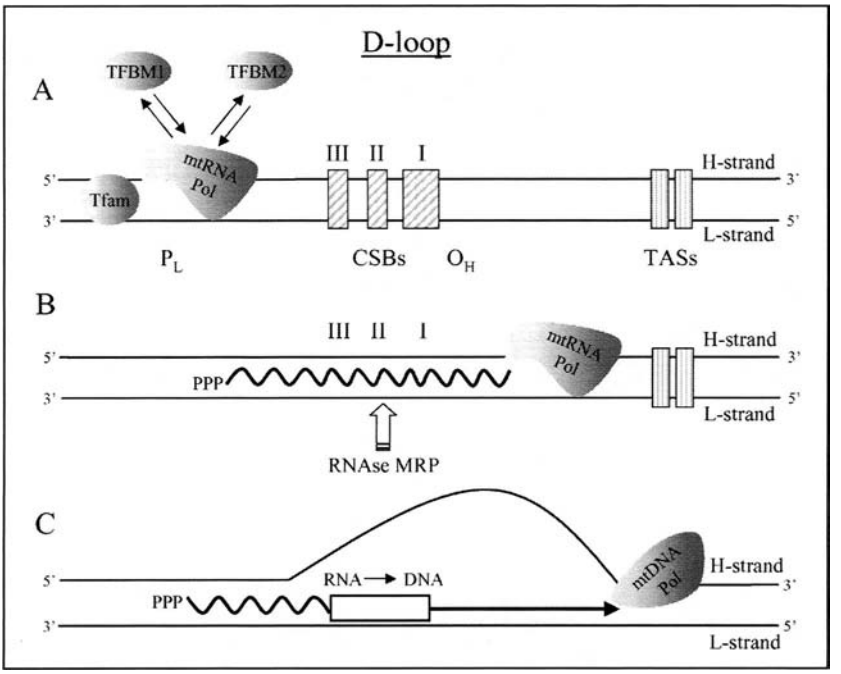

Figure 3. Schematic representation of the initiation of mtDNA transcription and replication within the D-loop regulatory region. A) Transcription is initiated at P_L by mtRNA polymerase (mtRNA Pol) in the presence of Tfam, a stimulatory factor that unwinds DNA, and one of the two isoforms of mtTFB (TFBM1 or TFBM2) which presumably act as dissociable specificity factors. The latter resemble rRNA dimethyltransferases and thus may also function in RNA modification or processing. B) The nascent RNA transcript is extended around the genome but with some frequency is cleaved at specific sites in the vicinity of the conserved sequence blocks (CSBs) by RNAse MRP. These truncated RNAs serve as primers for mtDNA replication initiated at O_H. C) The RNAse MRP cleavage sites correspond to the heterogeneous 5' ends of the newly synthesized H-strand and represent the transition sites between RNA and DNA synthesis. Nascent H-strands may terminate at termination associated sequences (TASs) giving rise to the D-loop structure.

including the precise mapping of replication origins and the detection of the predicted replication intermediates by electron microscopy, support the strand-displacement model.[16,26]

Many of the key players in mtDNA transcription and replication have been characterized in recent years and all of these are products of nuclear genes. DNA polymerase γ, the only known mitochondrial DNA polymerase, is a heterodimer of large (125-140kD) and small (35-54kD) subunits and is highly conserved from yeast to man.[27] A mutation of the large subunit in yeast that eliminates catalytic activity results in a loss of mitochondrial DNA without affecting cell viability.[28] Polymerase γ has both a 5' → 3' polymerase as well as a 3' → 5' exonuclease that eliminates mis-incorporated bases and facilitates the fidelity of mtDNA replication.[29] Both activities are associated with the large subunit. The function of the small subunit remains unknown, although it likely contributes to primer recognition and processivity.[30,31] The primer RNA for H-strand replication is generated by cleavage of the L-strand transcript by mitochondrial RNA processing (MRP) endonuclease (Fig. 3). This ribonucleoprotein contains a nucleus-encoded RNA that is essential for catalysis (MRP RNA) and is most abundant in the nucleolus where it participates in the processing of 5.8S rRNA precursors.[17] Although its association with mitochondria has been questioned,[32] both its cleavage specificity and in situ hybridization profile argue strongly for its function in mtDNA replication.[33-35] RNAse MRP

cleaves an R-loop containing the H-strand origin of replication at specific sites that match the in vivo priming sites.

During replication, exposed single-stranded regions are bound by mitochondrial single-stranded binding protein (mtSSB). In yeast, mtSSB is required for mtDNA maintenance, consistent with its role in mtDNA replication.[36] Genes for mammalian homologues have been characterized[37] and the crystal structure of human mtSSB has been solved. The mtSSB is structurally distinct from nuclear SSB but bears a strong structural similarity to the *E. coli* protein.[38] Topoisomerases and helicases have also been associated with mitochondria. Of particular note is a newly discovered mitochondrial protein designated as 'twinkle' because of its punctate cytoplasmic staining pattern.[39] Twinkle has a helicase domain resembling that of bacteriophage T7 gene 4 and mutations in the human nuclear gene encoding twinkle are associated with the autosomal dominant form of progressive external ophthalmoplegia. The inherited form of this disease is characterized by multiple deletions in mtDNA. Although a mechanism has yet to be elucidated, the genetic evidence suggests that twinkle function is essential for maintaining the integrity of the mitochondrial genome.

Transcription

Transcription initiation has also been well characterized. In yeast, transcription is initiated at approximately twenty transcriptional units throughout the genome (see ref. 7). In contrast, vertebrate transcription is initiated at two promoters, P_H and P_L for heavy and light strands respectively. These are spaced only 150 nucleotides apart within the D-loop regulatory region.[40,41] The H- and L-strand transcriptional units differ from most nuclear genes in that they are polygenic, specifying more than one RNA gene or mRNA. In addition to the RNA primer for H-strand replication, P_L also directs the synthesis of a transcript that is processed to one mRNA and eight of the 22 tRNAs (Fig. 1). The polygenic transcript directed by P_H is processed to 14 tRNAs, 12 mRNAs and the two rRNAs. The activities of both promoters require a 15 nucleotide conserved sequence motif that defines the core promoter. In addition, both promoters share an upstream enhancer that serves as the recognition site for Tfam (previously mtTF-1 and mtTFA) an HMG box protein that stimulates transcription through specific binding to the upstream enhancers (Fig. 3). Tfam also binds nonspecifically to apparently random sites on mtDNA.[42-44]

Enzymes and factors involved in mtDNA transcription have been identified and characterized. In yeast, transcription is directed by a 145kD core polymerase encoded by *RPO41* and a 43kD specificity factor, also known as sc-mtTFB, encoded by *MTF1* (see refs. 2, 4, 7). The polymerase shares sequence similarities with the T7 and T3 bacteriophage polymerases, which are also comprised of a single subunit. The primary structure of the sc-mtTFB specificity factor bears some resemblance to prokaryotic sigma factors[45] but a recent crystal structure reveals significant homology to rRNA methyltransferase.[46] The polymerase and specificity factor transiently interact and both are required for specific transcription initiation in vitro.[47] Genetic evidence supports a functional interaction between the two factors in vivo as well.

A vertebrate polymerase and a specificity factor that is required for specific initiation has been characterized biochemically in *Xenopus laevis*.[48] Although purification of the human polymerase has been elusive, a human cDNA that encodes a protein with sequence similarity to yeast mitochondrial and phage polymerases has been identified in database screenings.[49] The encoded protein localizes to mitochondria suggesting that it is a bona fide mitochondrial polymerase. A human mtTFB cDNA has also been isolated and the encoded protein has properties consistent with it being a functional homologue of sc-mtTFB.[50] The protein is localized to mitochondria, can bind DNA and stimulates transcription from an L- strand promoter in vitro. More recently, two isoforms of h-mtTFB, termed TFBM1 and 2, have been identified.[51] TFBM1 is identical to the original isolate but has about one-tenth the transcriptional activity of TFBM2. Both proteins work together with Tfam and mtRNA polymerase to direct proper initiation from H- and L-strand promoters (Fig. 3) and, like the yeast factor, both are related to

rRNA methyltransferases. It has yet to be determined whether the proteins are bi-functional or whether they evolved a single function from an ancestral methyltransferase. The availability of these human cDNAs should open the way for mechanistic studies on mammalian mitochondrial transcription.

Tfam is thus far the most well-characterized vertebrate factor involved in mitochondrial transcription initiation. As mentioned above, it was first identified as an HMG-box protein that recognizes enhancer elements in P_L and P_H.[17,47,52] Like other HMG proteins, Tfam can bend and unwind DNA, properties potentially linked to its ability to stimulate transcription upon binding DNA immediately upstream from the sites of transcription initiation.[43,53] In addition to specific promoter recognition, Tfam binds nonspecific DNA with high affinity. This property along with its abundance in mitochondria suggests that it plays a role in the stabilization and maintenance of the mitochondrial chromosome through its phased binding to nonpromoter sites. ABF2, a related HMG box factor from Yeast, resembles Tfam and is required for mtDNA maintenance and respiratory competence.[54] Expression of Tfam in ABF2-deficient Yeast cells can rescue both phenotypes suggesting that the two proteins are functionally homologous. Despite this functional complementation, ABF2 lacks an activation domain present in Tfam and does not stimulate transcription. A Tfam knockout mouse displays embryonic lethality and a depletion of mtDNA confirming an essential role for the protein in mtDNA maintenance in mammals.[55] In addition, Tfam levels correlate well with increased mtDNA in ragged-red muscle fibers and decreased mtDNA levels in mtDNA-depleted cells.[56] The correlation with mtDNA content is also observed for mtSSB in contrast to polymerase γ, which is expressed constitutively.[57] Despite these intriguing correlations, it is unclear which, if any of these, is the key limiting factor whose expression is regulated in controlling mtDNA copy number.[58]

Termination and RNA Processing

Transcription termination and RNA processing also play an important role in governing the steady-state levels of mitochondrial transcripts. Transcripts from P_H initiate either upstream from the $tRNA^{Phe}$ gene that precedes the 12S rRNA or near the 3'-end of $tRNA^{Phe}$ near its border with 12S rRNA. Initiation is more frequent at the upstream site and these transcripts terminate at a strong bi-directional terminator that is downstream from the 16S rRNA gene while a minority of transcripts traverse the entire H-strand.[59] Termination also occurs in the opposite direction thus attenuating L-strand transcription before a region where no L-strand genes are present. These termination events are thought to control the ratio of rRNA to mRNA in the mitochondrial matrix. The terminator consists of 28 nucleotides within the $tRNA^{Leu}$ gene immediately downstream of 16S rRNA. This sequence binds a trans-acting factor called mTERF, a 34kD protein that specifies site-specific transcription termination in vitro.[60] A structural prediction based on a mTERF cDNA revealed three leucine zippers that are involved in intramolecular interactions and facilitate binding of the protein to the target DNA. However, although the expressed recombinant protein displayed the expected binding specificity, it was not sufficient to direct termination in vitro suggesting that an additional component(s) may be required.[61]

Little is known about the regulation of RNA processing in mitochondria although the enzymatic machinery is at least partially characterized. One unique feature of mitochondrial genomic organization is that tRNA genes are dispersed around the mtDNA and flank the rRNA genes and nearly all of the protein coding genes (Fig. 1). Thus, most RNA processing sites occur at the junctions between tRNAs and other transcripts. This suggests that tRNA secondary structure may serve as the signal for enzymatic cleavage and release of individual RNA species.[6] In those cases where tRNA genes are not at the junctions, the adjacent RNAs may form structures that resemble the tRNA cleavage sites. Several enzymatic activities have been implicated in RNA maturation in human cells using an in vitro system, although there is some controversy as to the precise identity of the enzymes involved.[62] It is generally believed

that the 5'-end is processed by an RNAse P activity and the 3'-end by an unidentified endonuclease. Whether the human mitochondrial RNAse P has the same H1 RNA present in nuclear RNAse P and also the same substrate specificity remains controversial. In yeast, the mitochondrial RNAse P is comprised of a nucleus-encoded protein and a mitochondrially-encoded RNA that is necessary for catalysis.[63] Once the tRNAs are excised, CCA is added to their 3'-ends by an ATP(CTP)-tRNA-specific nucleotidyltransferase. The processed mRNAs are polyadenylated by a mitochondrial poly(A) polymerase but are lacking 5'-untranslated regions or a 7-methylguanylate cap structure. The rRNAs are modified by a short 3'-addition of adenyl residues.

Recombination and Repair

The mitochondrial genome acquires mutations at a rate 10 to 20 times faster than the nuclear genome resulting in a higher rate of molecular evolution and to the accumulation of disease mutations.[64] Several contributing factors to this phenomenon include the absence of mitochondrial histones, the existence of replicative intermediates with extensive regions of single-stranded structure and the lack of nucleotide excision repair pathways. In addition, although a high frequency of genetic recombination between mitochondrial genomes occurs in yeast, no recombination between paternal and maternal genomes or between heteroplasmic sequence variants has been observed in vertebrates. However, while deficient in nucleotide excision and mismatch repair, mitochondria are capable of base excision repair in response to oxidation and alkylation.[65] Damaged bases may be generated spontaneously or removed enzymatically by DNA glycosylases. For example, differential splicing of the transcript for uracil DNA glycosylase dictates whether the enzyme is localized to the nucleus or mitochondria and other glycosylases may be targeted to mitochondria as well. In addition to glycosylases, mitochondria possess other necessary activities for a base excision repair pathway including an endonuclease, ligase and polymerase and the reaction has been reconstituted in vitro using mitochondrial enzymes.[66] This mode of repair is significant considering that mtDNA is located near the respiratory chain which produces the bulk of reactive oxygen species that can oxidize DNA and other macromolecules. It has been postulated that oxidative damage to mtDNA may contribute to degenerative diseases and aging.

Mitochondrial Translation System

The mtDNA contribution to the mitochondrial translation system is restricted to the production of 2 ribosomal RNAs and 22 tRNAs. This number of tRNAs is smaller than the 32 required by the wobble hypothesis. One explanation for this difference is that for those amino acids with four possible codons, a U is present in the wobble position of a mitochondrial tRNA that allows recognition of all four codons (see refs. 2, 9). The protein components necessary for translation, including ribosomal proteins, tRNA synthetases, and the initiation and elongation factors, are all encoded by nuclear genes. Mitochondrial translation is bacteria-like both in its sensitivity to antibiotics that act on the ribosome, and in the use of N-formylmethionyl-tRNA for initiation. In addition, it was recognized soon after their discovery that mitochondrial ribosomes were smaller than those found in the cytosol with mammalian mitoribosomes having a sedimentation coefficient of 55S.[67] However, mitoribosomes are larger and more massive than bacterial ribosomes and they also have more protein subunits, shorter RNAs (16S and 12S) and are lacking 5S rRNA. Additionally, mitoribosomal proteins bear no close sequence similarity to either bacterial or eukaryotic ribosomal subunits suggesting that they are subject to different selective constraints.[68]

Many of the mechanistic details of mitochondrial translation in vertebrate systems have yet to be uncovered. In contrast to their cytosolic counterparts, mitochondrial mRNAs are lacking both a 5'-untranslated region and a 7-methylguanylate cap structure which facilitate ribosome binding and scanning to localize the initiation codon. The absence of these features may account for the production of larger amounts of mRNA with reduced translational efficiency.

Binding of the ribosome to mitochondrial mRNA is not sequence specific and apparently occurs in the absence of initiator tRNA. Initiation factors may be involved in initiator codon recognition but the only such factor identified in mammalian mitochondria is mtIF-2. Human and bovine homologues have been cloned and display sequence similarity with GTPases and IF-2 from *E. coli*.[69,70] The mitochondrial protein binds the small ribosomal subunit and facilitates binding of fMet-tRNA. MtIF-2 is subsequently released from the complex upon GTP hydrolysis followed by association of the large subunit to complete the initiation complex. In addition, mitochondrial elongation factors (mtEF-Tu, mtEF-Ts and mtEF-G) have been isolated and cDNAs obtained.[71,72] The structural and functional characteristics of these factors bear a strong resemblance to prokaryotic elongation factors.

Bi-Genomic Expression of the Respiratory Chain

Nuclear Activators and Coactivators

The limited coding capacity of mtDNA necessitates that the nuclear genome contributes the majority of gene products that are essential for mitochondrial function. Of the 100 or so respiratory chain proteins required for electron transport and oxidative phosphorylation, most are the products of nuclear genes. The nucleus also controls the biogenesis of the respiratory apparatus and the maintenance of mtDNA by providing all of the structural and enzymatic machinery required for mitochondrial transcription, translation and DNA replication. However, the nuclear contribution is not limited to the expression of the respiratory chain. Essential pathways for the oxidation of pyruvate and fatty acids, the biosynthesis of heme and certain amino acids are at least partially associated with the mitochondria.[73,74]

It has been well established in yeast that the regulated expression of nuclear genes in response to environmental signals is a key mechanism for mediating changes in respiratory metabolism. The availability of oxygen and nonfermentable carbon sources regulates the expression of many nuclear genes encoding respiratory chain proteins.[75] This occurs via the activation or induction of specific transcriptional activators and repressors that are targets of metabolic signaling pathways. Respiratory gene expression is also subject to regulation in mammals in response to diverse signals. These include hormonal stimulation, cyclic nucleotides,[76] oncogenic transformation, contractile activity,[77] temperature, and unidentified stimuli during pre[78] and post-natal[79] development. In many of these cases, respiratory chain subunits are induced at the mRNA or protein level but the specific regulatory mechanisms are not understood.

In recent years, a number of transcriptional regulators have been implicated in the expression of nucleus-encoded respiratory genes in mammals. Two such factors, NRF-1 and NRF-2, were identified as part of the characterization of cytochrome *c* and cytochrome oxidase promoters (see refs. 74, 80). It is now clear that one or both of these factors act on the majority of nuclear genes encoding subunits of the respiratory chain complexes. They are also involved in the expression of mitochondrial transcription and replication factors (Tfam, mtTFB and MRP RNA), heme biosynthetic enzymes and other proteins required for respiratory function. Thus, as summarized in Figure 4, these factors have the potential to integrate the expression of nuclear and mitochondrial genetic systems in response to cellular energy demands. NRF-1 is a transcriptional activator that binds a G + C rich pallindromic recognition site as a homodimer.[81] It exists as a phosphoprotein in vivo and phosphorylation enhances its DNA binding and transcriptional activities. NRF-1 phosphorylation also contributes to the growth-regulated induction of cytochrome *c*.[82] Targeted disruption of the NRF-1 gene in mice results in lethality at the blastocyst stage of development and depletion of mitochondrial DNA.[83] NRF-2 (originally defined in mice as GABP) has multiple subunits that either bind DNA or contribute activation or cooperative binding functions to a heterotetrameric complex.[84,85] This complex binds to tandemly arranged sites in many respiratory promoters (see refs. 3, 74, 80, 84, 86).

Although NRFs are key players, the nuclear genetic control of mitochondrial function cannot be explained by these factors alone. Several respiratory genes do not contain recognition

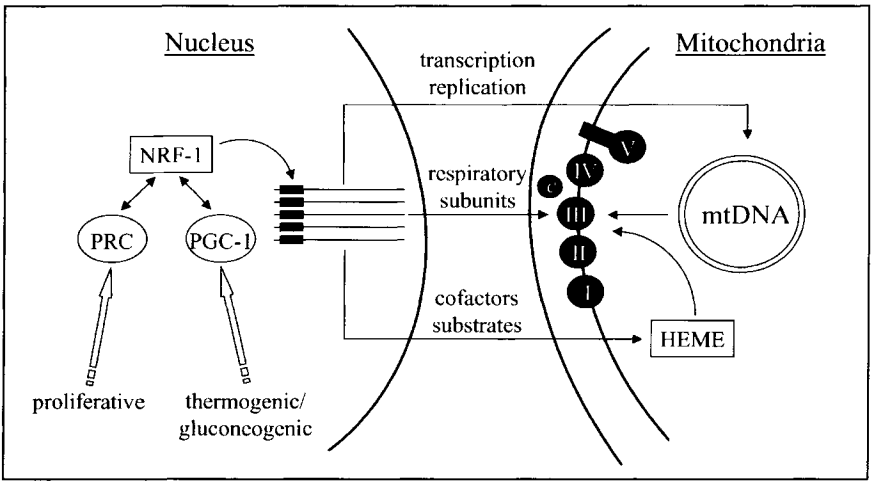

Figure 4. Activator-coactivator interactions in mitochondrial biogenesis. Nuclear respiratory factor 1 (NRF-1) is representative of transcription factors that act on nuclear genes whose products are required for expression and function of the mitochondrial respiratory apparatus. NRF-1 is a target for a small family of transcriptional coactivators whose members include PRC and PGC-1. The expression of these coactivators is regulated by proliferative, thermogenic and gluconeogenic signals thus placing NRF-1 and its target genes under the control of key signaling pathways. Activator-coactivator interactions may serve an integrative function in regulating cellular energetics.

sites for NRF-1 or NRF-2 and a number of other transcription factors have been implicated in respiratory gene expression. These include Sp1, CREB, YY1 and muscle-specific factors among others (see refs. 3, 86). In addition, nuclear genes for other mitochondrial functions (e.g., fatty acid oxidation) are controlled by transcription factors (e.g., PPARα) that do not act on respiratory promoters.[87] These observations beg the question of how multiple transcription factors can be integrated into a program of mitochondrial biogenesis. Part of the explanation came with the discovery of PGC-1, a transcriptional coactivator that induces mitochondrial biogenesis by interacting with NRF-1, PPARα and possibly other nuclear factors.[88,89] PGC-1 is markedly induced in brown fat during adaptive thermogenesis and has the remarkable property of being able to induce mitochondrial biogenesis when expressed ectopically in cultured cells or in transgenic mice.[3,89] In the presence of NRF-1, PGC-1 can trans-activate NRF-1 target genes that are necessary for the biogenesis of mitochondria and the expression of a functional respiratory chain (Fig. 4). PGC-1 interacts with NRF-1 in vitro and in vivo and a dominant negative allele of NRF-1 interferes with the ability of PGC-1 to induce mitochondrial proliferation.[88] PGC-1 also interacts with PPARα to induce the enzymes of fatty acid oxidation.[89] PPARα is a major activator of this pathway and is enriched in tissues with high oxidative energy demands. PGC-1 can bind PPARα and trans-activate PPARα-dependent promoters. Thus, the functional interplay between PGC-1 and certain nuclear transcription factors appears to define a major regulatory pathway for the biogenesis of mitochondria.

Recently, transcriptional coactivators related to PGC-1 have been identified. PGC-1 related coactivator (PRC) has several structural features in common with PGC-1 including an activation domain, an LXXLL coactivator signature, and an RNA recognition motif.[90] PRC is indistinguishable from PGC-1 in its ability to interact with NRF-1 and to activate NRF-1 target genes. However, it differs from PGC-1 in its mode of regulation. PRC is not significantly induced during adaptive thermogenesis but is induced when cells are stimulated to proliferate by serum growth factors (Fig. 4). PRC is down regulated when cells exit the cell cycle upon contact inhibition or withdrawal of serum. The results suggest that PRC may control mito-

chondrial biogenesis in response to proliferative signals. PGC-1β is a second PGC-1 family member that is closely related to PGC-1 and shares a similar tissue distribution.[91] Although it is not induced in brown fat upon cold exposure, it is induced in liver in response to fasting. This latter property is shared with PGC-1 which has recently been implicated in the induction of gluconeogenesis.[92] Thus, the differential regulation of members of this family of coactivators may help coordinate the biogenesis of mitochondria with pathways of cellular energy metabolism.

Retrograde Regulation

A second mode of bi-genomic regulation concerns the response of nuclear genes to changes in mitochondrial activity. This phenomenon is well-studied in yeast and has been termed retrograde regulation (see ref. 2). In yeast cells lacking mtDNA (ρ^0 cells) the nuclear *CIT2* gene, encoding peroxisomal citrate synthase, is markedly induced. This enzyme is part of the glyoxylate cycle and its induction in response to a defect in mitochondrial respiration allows cells to convert two carbon compounds such as acetate to carbohydrate.[93] The peroxisomal citrate synthase and other peroxisomal proteins are regulated by the basic helix-loop-helix transcription factors Rtg1p and Rtg3p.[94] A third protein Rtg2p facilitates the translocation of Rtg1p and Rtg3p to the nucleus in response to a mitochondrial deficiency. This retrograde pathway allows cells to adapt to defects in respiratory energy production.

Although the Rtg pathway has not been identified in vertebrates, there are a number of examples where nuclear gene expression appears to be altered by mitochondrial deficiency. In certain mitochondrial diseases, defective mitochondria proliferate in diseased muscle fibers giving rise to ragged red fibers.[95] Specific nuclear genes involved in ATP production also display elevated expression in cells with mtDNA mutations.[96] A change in the pattern of nuclear gene expression, involving proteins of the mitochondrial inner membrane as well as intermediate filaments and ribosomes, is observed in human cells upon depletion of mtDNA.[97] Chicken cells depleted of mtDNA or treated with the mitochondrial protein synthesis inhibitor chloramphenicol have increased levels of mRNAs for elongation factor 1α, β-actin, v-*myc* and GAPDH.[98] Presumably, these examples represent nuclear responses to deficiencies in ATP production. Although no unifying mechanisms have been advanced to explain these phenomena, recent studies suggest that retrograde signaling may be mediated by calcium.[99] Mitochondrial impairment, either by depletion of mtDNA or by metabolic inhibitors, mediates a stress response that coincides with elevated cytosolic calcium levels. The response includes increased expression of calcium-responsive transcription factors and cytochrome oxidase subunit Vb. Calcium has also recently been linked to a PGC-1-dependent pathway of mitochondrial biogenesis in skeletal muscle.[100] Transgenic mice, expressing a constitutively active calcium/calmodulin-dependent protein kinase in skeletal muscle, display increased mtDNA copy number and respiratory cahin enzymes as well as elevated PGC-1 levels. Thus, calcium may be an important link between the relay of extracellular signals to the nucleus and the bi-directional communication between nucleus and mitochondria. Much needs to be done before it is clear whether the various changes in gene expression that coincide with mitochondrial impairment represent a physiologically meaningful pathway of retrograde regulation in vertebrate cells.

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CHAPTER 3

Clinical Diagnosis of Oxidative Phosphorylation Disorders

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Abstract

Oxidative phosphorylation defects are a common group of inborn errors of metabolism. Patients may present to a variety of different physicians and at any age. Whilst some patients present with a characteristic phenotype that allows early diagnosis, in many the clinical features are suggestive rather than diagnostic. Neurological features are often prominent in all age groups, but the involvement may be diffuse or remarkable specific (for example optic atrophy alone). In other patients, involvement of other systems is more prominent and in some there is evidence of multiorgan failure. Clinical investigations such as imaging, cardiac studies, and endocrine investigations are often supportive of a diagnosis and form an important part of the clinical investigation. The clinical diagnosis of defects of oxidative phosphorylation is likely to remain a challenge with only the alert clinician identifying the difficult cases.

Introduction

The process of mitochondrial oxidative phosphorylation ultimately results in the condensation of inorganic phosphate and adenosine diphosphate (ADP) to produce adenosine triphosphate (ATP), a readily utilisable energy source. It is dependent on five multi-subunit polypeptide complexes [I→V] located within the inner mitochondrial membrane, only one of which (complex II), is wholly encoded by the nuclear genome, the others comprising of subunits encoded by both the nuclear and mitochondrial genomes. These polypeptide complexes utilize flavins, nicotinamides, cytochromes, iron-sulphur centres, and in the case of complex IV, copper ions to transfer electrons by a series of concomitant oxidation and reduction steps. Through this succession of oxido-reduction reactions electrons pass along the 'mitochondrial respiratory chain' of complexes (I→IV) and in doing so, generate an electrochemical gradient by fuelling the extrusion of protons from the matrix across the inner membrane at complexes I, III, and IV. ATP is then generated by the dissipation of this proton gradient through complex V (ATP synthase).

Disorders of this process of mitochondrial oxidative phosphorylation present at any age and the ubiquitous tissue requirement for ATP often results in many different systems or organs being affected. Consequently the clinical features are extremely variable and patients present to an assortment of distinct clinical specialities. The diverse clinical referral pattern observed in these diseases, results in most centres having relatively little collective clinical experience, despite disorders of oxidative phosphorylation proving to be quite common as a group. The exceptionally variable nature of mitochondrial disease warrants its entry into the differential

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diagnosis of many different conditions and it is often only due to the astute clinician that the diagnosis is given initial consideration.

In approaching problems of clinical diagnosis it is essential to have a clear perspective on the relative prevalence of various diseases which may enter the differential. We therefore provide an overview of current evidence and difficulties encountered in establishing the epidemiology of oxidative phosphorylation diseases before going on to describe the many different clinical features observed in patients with these disorders. However before doing so, it is important to highlight that patients may have features which do not conform to the recognised clinical syndromes, that clinical diagnosis must be intimately linked to first rate laboratory facilities and finally, that without an initial clinical awareness, no diagnosis will be established. Having confirmed a diagnosis of an oxidative phosphorylation disorder, the clinical management of these patients then relies on a multidisciplinary team approach that includes paediatric experts in inborn errors of metabolism, neurologist / paediatric neurologist, nurse specialist, physical, occupational and speech therapists.

Epidemiology of Defects of Mitochondrial Oxidation

Establishing the prevalence of defects of oxidative phosphorylation presents many difficult problems. The clinical presentation varies considerably and patients may present with features which are common and nonspecific such as isolated deafness or diabetes mellitus. In addition, the accurate diagnosis of mitochondrial disease may be difficult and in some patients requires a biopsy of affected tissue such as muscle. These problems compound the ethical issues surrounding presymptomatic genetic testing in adults and children. Thus until recently, it has generally been accepted that defects of mitochondrial oxidation are exceedingly rare and patients were predominantly investigated in laboratories with a special interest in these disorders. This has led to complex and patchy referral patterns that do not lend themselves to epidemiological investigations. It is because of these difficulties that most epidemiological studies were originally designed to determine the frequency of a particular mitochondrial DNA (mtDNA) mutation in patients with a specific disease phenotype, but recently there have been a number of studies designed to determine the true prevalence of mitochondrial disorders at a population level.

The Frequency of Mitochondrial Disease in Patients with a Specific Disease Phenotype

Leber's hereditary optic neuropathy (LHON) is the most common mtDNA disorder.[1] In the premolecular era, LHON was estimated to affect 1 in 50,000 of the general population, based upon large family studies with a characteristic phenotype and a maternal inheritance pattern. It is now well recognized that the vast majority of cases of LHON are due to one of three point mutations of mtDNA affecting complex I, or *ND*, genes (see below). In the Australian population, 2% of individuals who have invalid blind pensions harbour one of these mtDNA mutations.[2] Recently, we have determined the incidence of mtDNA mutations in the North East of England.[3] The minimum point prevalence of visual failure due to LHON within our population was 3.22 per 100,000 (95% CI: 2.47-3.97 per 100,000) and the minimum point prevalence for mtDNA LHON mutations was 11.82 per 100,000 (95% CI: 10.38-13.27 per 100,000). These results indicate that LHON is not rare, but has a population prevalence similar to autosomally-inherited neurological disorders.

The second most common mtDNA defect is the A3243G point mutation in the leucine (UUR) tRNA gene.[1] There has been great interest in the role of mtDNA mutations as a cause of diabetes mellitus. Different values for the frequency of the A3243G point mutation in patients with diabetes mellitus depend upon the subgroup of diabetics under study and ethnic background. In the general diabetic population, the A3243G mutation is found in 0.1-1% of Caucasian and Japanese populations. A maternal relative with diabetes increases the likelihood

that the disorder is due to a mtDNA defect, with values ranging between 1.6%[4] and 5.5%,[5] and deafness and diabetes increase the frequency of the A3243G mutation still further.[5] Overall, the prevalence of diabetes in Western Europe is between 3% and 6% of the general population, and thus the prevalence of mitochondrial diabetes due to the A3243G mutation is conservatively estimated at 3-6/100,000 of the general population.[6]

Population-Based Studies of Mitochondrial Disease

In the first of these studies, Majamaa and colleagues[4] studied 245,201 adults in Northern Finland. They identified individuals with clinical features and family history suggestive of mitochondrial disease, and determined the frequency of the A3243G mutation. Of the 615 patients identified on clinical grounds, 480 were screened for the A3243G mutation and they detected 11 independent maternal pedigrees transmitting the A3243G mutation, giving an overall point prevalence of 16.3/100,000 of the adult population (95% C.I. 11.3-21.4/100,000). Subgroup analysis revealed a high prevalence of the A3243G mutation in certain subgroups of the Finnish population. Deafness is a common feature of mitochondrial disease,[7] and 7.4% of adults in Northern Finland with deafness and a family history of deafness had the A3243G mutation. Patients with the A3243G mutation often present with recurrent occipital infarction[8] and, in Northern Finland, 6.9% of individuals with occipital stroke had the A3243G mutation.[9]

We recently determined the point prevalence for adult mitochondrial DNA (mtDNA) disease for the mid-year period of 1997 in the Northeast of England.[1] Patients were ascertained through the hospital referral system over a ten-year period. Each individual underwent a series of investigations including clinical studies, a muscle biopsy, muscle histochemistry, mitochondrial respiratory chain studies, and mtDNA analysis on muscle DNA. We identified 104 cases (> 16 yrs of age to < 65 years of age for males, and to < 60 years of age for females) with mtDNA disease in an adult population of 1,582,584. After exploring the family history in each affected individual, we identified 161 maternal relatives at risk of inheriting the mtDNA defect, giving an estimated minimum prevalence of mtDNA defects of 12.48/100,000 for the population below retirement age. Overall, it appears that mtDNA disease is at least as common as many other neurological disorders, including sporadic diseases such as amyotrophic lateral sclerosis,[10] inherited disorders such as Huntington's disease,[11] and common forms of muscular dystrophy.[12]

Recent evidence from the Finnish population confirms the generally accepted view that mtDNA defects are a rare cause of mitochondrial oxidation disorders in children.[13] Over a seven-year period, Uusimaa and colleagues identified defects of oxidative phosphorylation in 26 children from a paediatric population of 146,482. Despite the high prevalence of the A3243G mutation within the study region,[4] only one child had an mtDNA defect (A3243G). Based upon their published data,[13] it is not possible to calculate an accurate value for the prevalence and incidence of childhood respiratory chain defects, but the data suggest that the prevalence of respiratory chain disease in children is in the same order of magnitude as mtDNA disease in the adult population. This conclusion is supported by a population based study from Victoria, Australia.[14] Between 1987 and 1996, the minimum birth prevalence of childhood respiratory chain disease was 4.7/100,000 births (95% C.I. = 3.2-5.0) from a largely Caucasian study group. It is possible to combine these data with the adult prevalence figures to calculate an approximate prevalence for all mitochondrial disease, because the point prevalence in adults of all ages corresponds to the birth incidence of individuals who will go on to develop mitochondrial oxidative phosphorylation disease in later life. Based upon the available evidence, a conservative estimate of the total number of individuals with defects of mitochondrial oxidation is 11.5/100,000, or 1 in 8500 of the general population. This figure corresponds to the point prevalence at any one time, or the birth incidence of individuals who will subsequently develop mitochondrial disease.

Clinical Features of Patients with Defects of Mitochondrial Oxidation

Neurological Features

In our experience neurological disease is the most consistently reported feature of mitochondrial dysfunction, but this may be biased because of the referral pattern to our centre. A number of syndromes have been characterized, many of them associated with, but not exclusive to, a specific type of mtDNA or nuclear defect. There are however, almost inevitably, a large proportion of patients with mitochondrial disease who either do not conform to the clinical criteria for a particular syndrome or present with only a few of the features. Neurological presentations such as seizures, migraine, stroke-like episodes, neuropathy or movement disorder are not uncommon, but clinicians often only consider mitochondrial disease when these features occur in conjunction with other symptoms such as deafness, diabetes or visual impairment. Isolated nonspecific symptoms such as fatigue and myalgia probably result in many patients not being referred at all, yet represent some of the most debilitating aspects of mitochondrial disease. In stark contrast, some patients with cardinal signs of respiratory chain dysfunction are fully investigated without any firm conclusions being reached.

Mitochondrial Myopathy

Myopathy is one of the hallmarks of mitochondrial disease and a prominent clinical feature of the syndromes described below. A significant number of patients with mitochondrial disease present with nonspecific disorders, but proximal weakness and exercise intolerance are the principle clinical features. Some of these presentations progress to involve other organ systems and become more obviously mitochondrial in origin, others remain confined to muscle with gradual deterioration in power and involvement of muscle groups outside the shoulder and hip girdles, including the diaphragm. However, fatigue and weakness are familiar symptoms to the neurologist and selecting those patients with myopathy for whom further mitochondrial investigations would be appropriate can be difficult. The finding of a proximal myopathy, in conjunction with other clinical features such as diabetes, sensory organ impairment or progressive multi-system disease, should prompt further investigation for mitochondrial disorders.

Chronic Progressive External Ophthalmoplegia (CPEO)

One of the commonest presentations in adults, this condition is defined by a slowly progressive paresis of eye musculature, bilateral ptosis and occasionally cardiac conduction defects.[15,16] Many of our patients first notice ptosis in their third or fourth decade, but age of onset is variable. As the disease progresses there is often associated proximal muscle weakness and fatigue though rarely to a debilitating extent. A sporadic, single deletion of mtDNA (4977 bp) is a common cause of CPEO, but other single deletions of variable length and some mitochondrial tRNA mutations result in an identical phenotype

Either recessive or dominant families with CPEO are well recognized and are associated with multiple species of deleted mtDNA.[17,18] In these patients the ophthalmoparesis and ptosis demonstrate a slowly progressive course with a wide range in the age of onset. Other features can include fatigue, optic atrophy, cataracts, ataxia, peripheral neuropathy, deafness, cardiomyopathy and depression. A number of causative nuclear gene mutations including *ANT-1*,[19] *Twinkle*[20] and *POLG*[21] have now been described in these families.

Kearns-Sayre Syndrome (KSS)

The onset of ophthalmoparesis and pigmentary retinopathy before the age of 20 years is characteristic of Kearns-Sayre syndrome. This sporadic condition is usually the result of either a large scale single deletion or complex rearrangements of mtDNA. Other clinical features include cerebellar ataxia, proximal myopathy, complete heart block, cardiomyopathy, endocrinopathies, short stature, deafness and an elevated CSF protein. As might be predicted from the early onset of this multisystem disorder life expectancy is considerably reduced.

Pearson (Bone Marrow Pancreas) Syndrome

Pearson syndrome is a rare disorder similar to KSS in that it results from large-scale rearrangements of mtDNA. In this instance however, clinical features of sideroblastic anaemia with pancytopenia and exocrine pancreatic dysfunction predominate in early life and frequently result in death during infancy. Survival through childhood leads to an improvement in anaemia but patients then develop the characteristic features of KSS. For Pearson's syndrome, KSS and Chronic Progressive External Ophthalmoplegia (CPEO) the clinical severity appears to correlate with the tissue localization of mutated mtDNA. In Pearson syndrome (and to a lesser extent KSS), mutated mtDNA can be demonstrated in a wide variety of tissues, whereas in CPEO the defective mtDNA is confined to muscle.

Mitochondrial Neurogastrointestinal Encephalopathy Syndrome (MNGIE)

This multisystem disorder is characterized by onset of chronic progressive external ophthalmoplegia, ptosis, gastrointestinal dysmotility (pseudo-obstruction), diffuse leukoencephalopathy, peripheral neuropathy, and myopathy. Histological and biochemical studies of MNGIE patients have confirmed the involvement of mitochondria in this disorder. The inheritance is autosomal recessive and it is due to mutations in the thymidine phosphorylase (*TP*) gene.[22,23]

Mitochondrial DNA Depletion Syndrome (MDS)

There are two distinct forms of this autosomal recessive disorder, (hepatocerebral and myopathic), with liver, muscle and kidney (Fanconi syndrome) being the organs most commonly affected. Both forms are often fatal in early childhood with encephalopathy or respiratory failure being the two principal causes of death.

These quantitative disorders of mtDNA probably occur because of a reduction in the pool of mitochondrial deoxyribnucleotides (dNTPs) necessary to synthesize mtDNA. The recent discovery in families with mtDNA depletion syndrome, of mutations in two independent enzymes that regulate this pool of dNTPs, deoxyguanosine kinase (*dGK*)[24] and thymidine kinase (*TK2*)[25] would seem to confirm such a mechanism. Interestingly from a clinical perspective, although both mutations result in mtDNA depletion, it appears that *dGK* mutations are specific for the hepatocerebral form while *TK2* mutations result only in the myopathic type.[26]

Mitochondrial Encephalopathy Lactic Acidosis and Stroke-Like Episodes (MELAS)

This clinical syndrome is characterized by parieto-occipital stroke-like episodes, which frequently do not conform to a single recognised vascular territory. Other features of MELAS include intermittent encephalopathic episodes associated with elevated plasma and CSF lactate, vomiting, migraine, dementia and focal or generalized epilepsy. The A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene was the first and most frequently described mtDNA mutation associated with this clinical phenotype.[27] However, other mutations have been described in association with this presentation and the A3243G mutation also causes other distinct clinical phenotypes such as diabetes and deafness.[28] Extraordinarily, such variations in phenotype can occur between individuals in the same family harbouring identical mutations.[29]

Myoclonus Epilepsy with Ragged Red Fibres (MERRF)

MERRF was initially reported in association with an A→G mutation in the gene encoding mitochondrial $tRNA^{Lys}$ at position 8344 and although other mutations in the same tRNA have now been reported, this remains the most common cause of this disease.[30,31] This severe neuromuscular disorder causes progressive myoclonus, focal and generalized epilepsy, muscle weakness and wasting, hypertrophic cardiomyopathy, dementia, deafness and cerebellar ataxia. Indeed the A8344G mutation has been identified as the cause of the original hereditary syndrome described by Ekbom of cerebellar ataxia, photomyoclonus, skeletal deformities and lipomata.[32,33]

Leigh Syndrome

Leigh syndrome is a progressive neuro-degenerative condition of infancy and childhood. The characteristic symmetric necrotic lesions distributed along the brainstem, diencephalon and basal ganglia were first described on post-mortem tissue,[34] but are readily visible on MRI or CT scan. The clinical presentation and course vary considerably but common features include signs of brainstem or basal ganglia dysfunction such as respiratory abnormalities, nystagmus, ataxia, dystonia, hypotonia and optic atrophy. Developmental delay and more particularly regression are prominent clinical features of this disorder and the latter may only be evident after a period of slow developmental progress. The clinical course can follow a stepwise deterioration with moderate recovery of developmental skills between episodes of regression or alternatively a slowly progressive decline. A severe failure of oxidative metabolism within the mitochondria of the developing brain due to a variety of biochemical and molecular defects, including nuclear and mtDNA mutations have been described in Leigh syndrome.[35,36] Inheritance can therefore be X-linked recessive, autosomal recessive, or maternal depending on the defect responsible.

Neuropathy, Ataxia and Retinitis Pigmentosa (NARP)

First described as a variable combination of developmental delay, retinitis pigmentosa, dementia, seizures, ataxia, proximal neurogenic muscle weakness, and sensory neuropathy in four members of a single family,[37] the phenotype has now been expanded to include cardiomyopathy and Leigh's syndrome. The original family had a heteroplasmic T→G transversion at nucleotide pair 8993 in subunit 6 of mitochondrial ATPase (ATP6), probably impairing ATP synthesis. A T→C mutation at 8993 has also subsequently been described with a generally milder clinical phenotype, but higher frequency of ataxia. Mutations in the mitochondrial ATPase subunits do not affect cytochrome oxidase activity and therefore NARP patients will have no evidence of mitochondrial myopathy on routine histochemical analysis.

Leber Hereditary Optic Neuropathy (LHON)

The first mitochondrial disease to be ascribed to a point mutation in mtDNA, LHON is an acute or subacute, bilateral, painless, central visual loss and the commonest cause of blindness in young men. The clinical condition was described as a familial neuro-ophthalmologic disease in 1871 but, it was not until over a century later that Wallace,[38] demonstrated the majority of LHON families harbour the same mtDNA mutation (G11778A). A number of mtDNA mutations have subsequently been described in association with LHON, but three mutations (G11778A, G3460A, and T14484C) are present in at least 90% of families. These mutations in the complex I (NADH: ubiquinone-oxidoreductase) encoding genes *ND4*, *ND1* and *ND6* respectively, are considered on the basis of their frequency, penetrance and clinical severity, to be primary LHON mutations. In this disorder there is an excess of affected males, but no X-linked visual-loss susceptibility locus has been found. Clinical examination reveals peripapillary telangiectasia (which gradually disappears with disease progression), microangiopathy, disk pseudo-oedema, and tortuous retinal vessels in over half of patients harbouring the G11778A mutation, with some apparently unaffected relatives demonstrating the same pathology. Onset of disease is commonest in the third or fourth decade with initial unilateral involvement being typical. Sequential involvement of the other eye commonly occurs within a 2-month period and subsequent decline in visual acuity of both eyes may be very sudden or slowly progressive over a period of several years. Both the disease penetrance and the clinical course appear to be determined by the mutation responsible, with measurements of final visual acuity ranging from 20/60 to no light perception at all. Similarly, the extent of visual recovery also varies in relation to the mutation, with only 4% of G11778A patients showing recovery an average of 36 months after onset; while 37% of T14484C patients recover after 16 months.[39,40]

Occasionally extra-ocular clinical features such as cardiac conduction defects (Wolff-Parkinson-White and Lown-Ganong-Levine (direct atrionodal pathway associated with

a short PR interval and no delta wave)) are evident and minor neurological problems are also not uncommon. Some mutations though, are associated with severe neurological problems including early onset dystonia accompanied by bilateral basal ganglial degeneration (G14459A).[41,42]

Cardiac Features of Mitochondrial Disease

As highlighted above many patients who present with neurological features may have cardiac involvement. This may include cardiomyopathy and conduction defects, both of which can be life threatening. In some patients however, cardiomyopathy may be the presenting or only feature of mitochondrial disease. Familial cardiomyopathies have been described in which the predominant cardiac features are of hypertrophic cardiomyopathy often with bi-ventricular involvement. Patients with this pattern of cardiac involvement should be further investigated for the possibility of mitochondrial disease.

Diabetes

Diabetes is commonly present in patients with mtDNA mutations and may be the presenting feature. The diabetes has a characteristic pattern in some patients and is due to failure of insulin secretion by the β-islet cells of the pancreas. These patients often present in their 20-40s with relatively mild diabetes which then rapidly progress to requiring insulin. In patients with mitochondrial diabetes, deafness is a frequently associated clinical feature.[28,43] Other endocrinopathies such as hypoparathyroidism are less common, but are well recognised in patients with Kearns-Sayre syndrome[44] and some point mutations.[45]

Childhood Presentations of Mitochondrial Disease

In general, childhood presentations of mitochondrial disease tend to be more severe than those with their onset in adult life and frequently involve many different organ systems. Hepatic dysfunction (MDS) and haemopoeitic stem cell failure (Pearson's syndrome) are uncommon features of mitochondrial disease but are seen more often in children than adults. Renal disease also appears to be a more prominent clinical feature of pediatric mitochondrial disorders, evident in both mitochondrial DNA depletion syndrome and complex III deficiencies (*BCS1L* mutations).[46] Developmental delay is a prominent but nonspecific finding that is much more discriminating as a diagnostic feature when found in conjunction with lactic acidaemia. Stepwise developmental regression, the loss of acquired skills, is however a much more specific indicator of a mitochondrial disorder (Leigh's syndrome). The child with mitochondrial disease, unlike the adult patient, is more likely to present to a limited variety of hospital specialists, (neonatologists or general paediatricians) and may be seen either with a crisis in the neonatal period or with nonspecific features such as failure to thrive, acquired infection (precipitating lactic acidaemia) or seizures.

Investigation of Suspected Mitochondrial Disease

The differential diagnosis for patients with defects of oxidative phosphorylation is extraordinarily diverse. Furthermore, the investigation of presumed mitochondrial disease is made more difficult because not only may the same genetic or biochemical defect present in a variety of different ways, but also the same clinical syndrome may be due to a variety of different biochemical or molecular defects. The detailed history and examination of a patient with suspected mitochondrial disease is therefore crucial to both initiation and interpretation of the relevant investigations. Common presenting symptoms include fatigue, myalgia and weakness but it is important to specifically address issues such as maternal health and obstetric history, family history of neonatal or childhood deaths, deafness, diabetes, cardiac symptoms, visual impairment and developmental delay. Identification of optic atrophy, ophthalmoparesis, hearing impairment, cardiac enlargement and the neurological signs associated with muscle, cerebellar, brainstem and basal ganglia involvement are essential elements of the examination.

Simple blood tests may provide some supporting evidence for the clinical diagnosis and initial investigations and should include creatine kinase, resting blood lactate, full blood count, thyroid and liver function, bone chemistry, random blood glucose and glycated haemoglobin (HbA1c). If the history and examination are suggestive of a specific mitochondrial syndrome e.g., MELAS, MERRF or NARP, then investigation for the common point mutations should be undertaken in blood. Results of such screening for mutations in blood should however be interpreted with caution as negative results do not exclude mitochondrial disease in a patient where there is a high index of clinical suspicion

All patients should have an ECG to investigate possible conduction defects or cardiac hypertrophy. Chest radiography and echocardiography are then appropriate investigations in those individuals with clinical or ECG evidence of cardiorespiratory involvement. Lumbar puncture is a valuable investigation as raised CSF lactate and mild protein elevation are consistent with mitochondrial dysfunction.[47] However, an increased CSF lactate following seizure activity or stroke, two common features of mitochondrial disease, should always be interpreted with caution. Electromyography may be normal, even in the presence of clinical myopathy and nerve conduction studies can demonstrate either an axonal or a mixed axonal-demyelinating peripheral sensorimotor neuropathy. A pattern of generalized slow waves, indicative of a subacute encephalopathy, or subclinical seizure activity may be evident on electroencephalogram (EEG). Cognitive impairment, central neurological signs, movement disorder or abnormal EEG all warrant some form of cerebral imaging. A variety of changes can be seen on computed tomography (CT) and magnetic resonance imaging (MRI), some of which are characteristic for specific mitochondrial disorders, e.g., the symmetrical hypodensities of brainstem, thalamus and basal ganglia seen in Leigh syndrome.

An increasing number of individuals are now alerted to the fact that they harbour oxidative phosphorylation disorders through the diagnosis of an affected family member. Individuals identified in this way often have little or no clinical evidence of disease when first diagnosed but should be made aware of both the possible risks to their health and that of their offspring. Indeed, there is an argument for routine follow-up of these cases with neurological / ophthalmolgical examination, measurement of HbA1c and ECG at regular intervals. Early recognition of diabetes or cardiac conduction defects may be life-saving.

Conclusions

Mitochondrial oxidation defects are important causes of disease with a minimal incidence of 1 in 8500 of the population. We believe this is likely to be an underestimation because of the difficulties in both recognising and diagnosing respiratory chain disease. The clinical diagnosis of defects of oxidative phosphorylation is likely to remain a challenge with only the alert clinician identifying the difficult cases. The clinician must be supported by access to laboratory facilities, preferably those which specialise in the investigation of these disorders.

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CHAPTER 4

Contribution of Histopathological Examination to the Diagnosis of OXPHOS Disorders

Martin Lammens and Henk ter Laak

Abstract

In muscle histopathological hallmarks for OXPHOS disorders are the so-called ragged-red fiber, the COX-negative or COX-deficient fiber, and the paracrystalline inclusions in mitochondria. Ragged-red fibers may be found in cases with mitochondrial DNA mutations. Up to now no morphological hallmarks were found for nuclear DNA mutations in genes coding for OXPHOS proteins. However, mutations in (nuclear) assembly genes for Complex IV may give rise to severe COX deficiency.

In the central nervous system, the peripheral nervous system, and in other organs, histopathological changes may be severe and specific for a particular OXPHOS disorder. However, direct indications to OXPHOS disorders are generally not present.

Introduction

The pathological diagnosis of OXPHOS disorders remains important, as it is often a precise starting point in the diagnostic process after the clinical suspicion has been raised. In most instances, the muscle biopsy is the most informative, even in cases in which strict muscle symptoms are not on the foreground. So, most of this chapter will be devoted to the findings in muscle biopsy. Morphological findings in other organs are often less specific, but still may be informative, be it at biopsy or at autopsy.

Muscle Biopsy Diagnosis

Goals of Muscle Biopsy

A muscle biopsy is an important diagnostic tool when an OXPHOS disease is suspected. The muscle tissue is of course important to isolate mitochondria for biochemical examinations and may be the source of DNA and RNA for further molecular biological analysis. The primary goal of a muscle biopsy, however, is in most cases, to make a histopathological diagnosis. It is especially important to exclude other disease than OXPHOS diseases to explain symptoms such as muscle weakness, excessive fatigability, muscle pain, and other. On the other hand, some histopathological alterations may support the diagnosis of an OXPHOS disease. The presence of ragged-red fibers, the absence of oxidative stains in some fibers and the electron microscopic changes of mitochondria, are the most important signs of OXPHOS disease in the biopsy, as will be explained in the following paragraphs. Finally, pathological findings such as

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extensive fibrosis in the muscle biopsy may be important to explain certain secondary biochemical findings.

Methods

Biopsy

In our hospital, a needle biopsy under local anesthesia is preferred in adults. In children a surgical biopsy under general anesthesia is often performed. The advantage of a needle biopsy is obvious: there is no general or locoregional anesthesia necessary, it can be very quickly organized, and it may be repeated more than once if necessary. The prudent use of local anesthesia is important. As lidocaine and other anesthetics influence the oxidative phosphorylation, care must be taken not to immerse the muscle in the anesthetic. Therefore, a local anesthesia of the skin and subcutaneous tissue, without transgressing the muscular fascia is sufficient. Through one subcutaneous insertion of the needle, several pieces of muscle from different directions must be taken. The anesthesia is an important problem in children. In some centers, precautions are taken to prevent malignant hyperthermia.

As it is obvious that only fresh or freshly frozen tissue is sufficient for biochemical examination, the same holds true for expert pathological examination of the muscle biopsy. Special care is necessary to circumvent freeze artifacts. Therefore, the muscle tissue is shock frozen, immersed in cooled isopentane. For electron microscopic examination fixation of small pieces of tissue in buffered glutaraldehyde is preferred. In most cases the examination of paraffin embedded, formalin fixed muscle tissue is obsolete. Therefore, it is important to take the same precautions when taking postmortem muscle tissue.

In the cases, however, in which only paraffin material is available, there is some possibility to demonstrate the excessive presence of mitochondrial ribosomal DNA by in situ hybridization, and so reflecting the "ragged-red" fibers.[1] Even molecular biological examination of the tissue is still possible, but mostly remains second choice.

Histopathology

Since the process of oxidative phosphorylation takes place in the mitochondria, these organelles deserve special attention in the examination of muscle biopsies from patients suffering from oxidative phosphorylation disorders. In histology, mitochondria can be made visible by applying chemical methods onto sections cut from a biopsy specimen. In fact, although the term "histochemistry" includes all chemical methods to detect tissue constituents, the detection of enzymes (enzyme histochemistry), the detection of ultrastructure (electron microscopy), and the detection of proteins or other substances by antibodies (immune histochemistry), will be dealt with separately.

Histochemistry

In this section the HE stain, the modified Gomori trichrome stain, the Sudan Black B stain, and the PAS stain will be discussed especially in the context of revealing mitochondria.

HE (Hematoxylin-Eosin) Stain

This is the most frequently used stain in histology and histopathology. The method is simple, fast, and reliable and preparations can be stored for decades without any loss of quality. Oxidized hematoxylin (hematein) especially detects the frequently present phosphate groups in DNA an RNA, and the phosphate groups in the polar heads of phospoholipids, which are the basic elements of membranes. In this method, aluminum (Al^{3+}) cations are used as a mordant and in fact, these ions serve as a bridge between the hematein and the phosphate groups by coordinate bindings. This results in a blue color of cell nuclei and of all biological membranes including the membranes of mitochondria. Care must be taken not to loose these lipid-rich structures by ethanol before staining since ethanol dissolves all membranes and thus the mitochondria. This is the reason why in sections cut from paraffin-embedded specimens

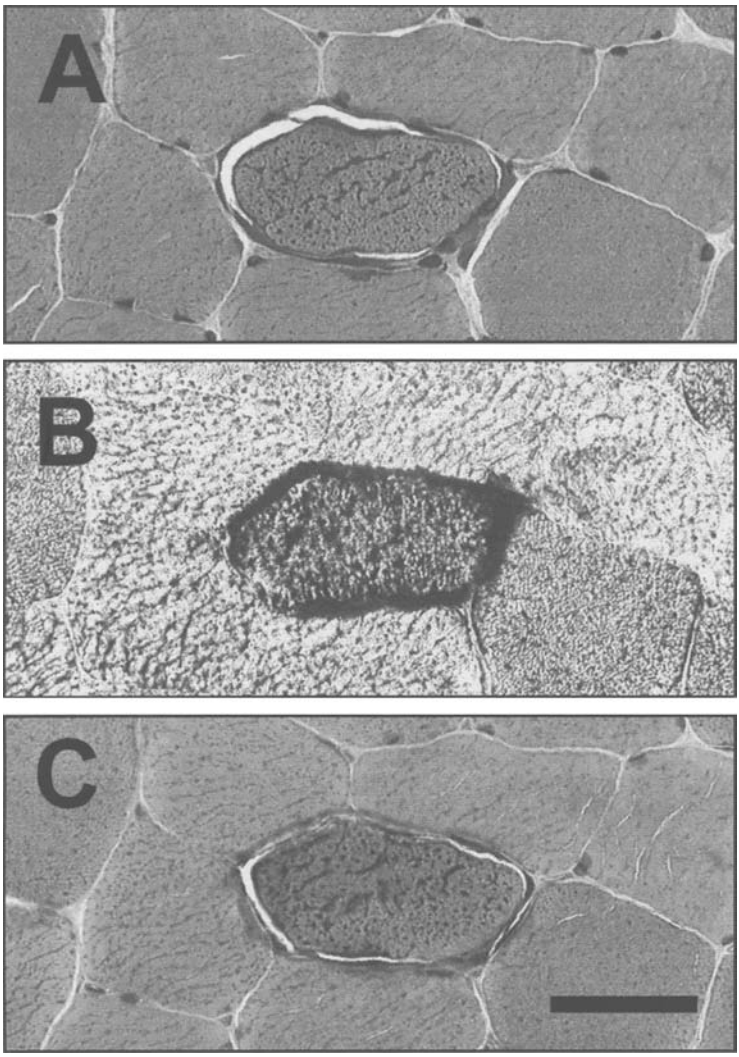


Figure 1. Serial sections of a ragged-red fiber. A) Hematoxylin-Phloxine (H-Phl) stain, B) Succinic dehydrogenase (SDH) stain, and C) Trichrome stain. Mitochondria and mitochondrial aggregates are clearly stained, especially in the center and at the border of the ragged-red fiber. Quadriceps biopsy from a 21-year-old patient with MELAS (3243A>G point mutation with 81% heteroplasmy in muscle and 30% in blood). Bar= 50 μm.

almost no mitochondria can be observed. If treated in a correct way, mitochondria or aggregates of mitochondria can be easily observed as blue-stained small dots in normal muscle fibers (Fig. 1A). In fact, the normal situation is slightly more complicated since there is a gradual increase of the mitochondrial density from the center to the edge of the muscle fiber. This may even give rise to the occurrence of so-called mitochondrial aggregates in some type I fibers directly beneath the sarcolemma, especially at locations where capillaries border the muscle fibers. Furthermore, type I fibers (the most oxidative ones) contain more mitochondria than type IIA and far more mitochondria than the type IIB fibers (the least oxidative fibers). How-

ever, since the blue color (also called "basophilia") may point to the presence of other organelle configurations (sarcoplasmic reticulum, lysosomes, and rimmed vacuoles) further analysis is necessary to confirm its mitochondrial nature.

After having applied hematein stain, the sections are normally counterstained with the pink-reddishly-staining eosin. However, in our laboratory we prefer to use phloxine instead of eosin since blue-stained basophilic structures contrast better against the brightly red background caused by phloxine.

Modified Gomori's Trichrome Stain

In this method[2] hematoxylin, chromotrope 2R and fast green are used. The signaling color is red (from chromotrope 2R) and fast green is used for background staining. The hematoxylin step in this method is rather used to mordant the sections with Al^{3+} ions to enable chromotrope 2R binding than staining it with hematoxylin. The positive structures are the same as in the HE stain (compare Figs. 1A and 1B). Furthermore, some pathological structures occurring in the field of neuromuscular disorders, such as nemaline rods and cytoplasmic bodies are also stained. The Gomori trichrome stain clearly demonstrates the hallmark for mitochondrial myopathies, the so-called ragged-red fiber (Fig. 1C). The ragged zones consisting of mitochondrial aggregates are redly stained.

Sudan Black B Stain

This is an important method because not only the normally occurring fat droplets in muscle fibers can be observed, but also phospholipids[3] and thus mitochondria (Fig. 2). Since fat droplets may be increased by number or size in mitochondrial disease, this method may contribute to the diagnosis of mitochondrial myopathy. Pretreatment with acetone dissolves the triglycerides in the fat droplets of fibers and in the fat cells without affecting the mitochondrial picture. In our laboratory we prefer this stain above other fat staining solutions (Sudan III, Sudan IV, and Oil red O) because of its high contrast and its affinity to phospholipids.

PAS (Periodic Acid-Schiff) Stain

Stored glycogen is the compound of interest in this method. As far as glycogen is concerned, this method is based on the formation of dialdehydes by oxidation of 1,2-glycol groups of glucose by periodic acid. Further reaction with Schiff reagent causes an intense red color. Since oxidation to aldehydes also takes place after substituting the -OH groups by amino or alkylamino groups and since sugars are abundantly present in glycoproteins and glycolipids, the unspecificity of this staining for glycogen is apparent. Pretreatment of a serial tissue section with amylase or simply saliva which degrades glycogen considerably increases specificity of the PAS stain. It is difficult to fix glycogen sufficiently in frozen sections as it easily moves away from its original positions. Therefore we recommend to perform the PAS procedure in semi-thin sections (1-micron-thick) obtained from specimens fixed for electron microscopy (glutaraldehyde). Since mitochondrial diseases may be accompanied by increased glycogen storage,[4] this method may be helpful. It is also indispensable in the diagnosis of glycogenoses, which are other diseases of energy metabolism.

Enzyme Histochemistry

Mitochondrial detection may be based on the structural properties and constituents of the mitochondrial membrane, but also on the activities of the various enzymes they contain. Appropriate substrates must be used to let the enzymes do their work optimally, so that insoluble stained compounds are formed. Generally, previous chemical fixation inactivates enzymes. By freezing the tissue and cutting it in a cryostat in frozen condition, enzyme activities can be shown after incubating them with the adapted substrates. Enzyme histochemistry started in the 1950s and nowadays more than a hundred techniques are available.[5] In this section three methods will be described which enable the detection of mitochondrial enzymes. Finally, the myofibrillar ATPase method will be discussed; this is a method that does not show mitochondrial activities but is generally used to discern type I, type IIA, type IIB, and type IIC fibers.

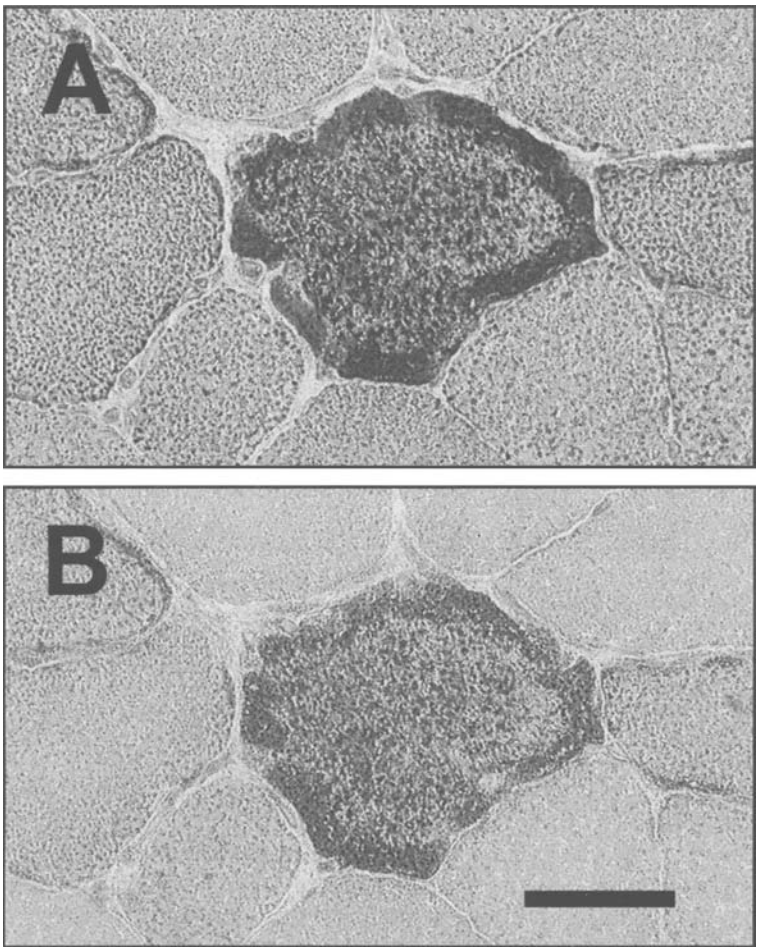


Figure 2. Serial section of a ragged-red fiber stained with Sudan Black B. In Figure A both fat droplets and phospholipids (especially the mitochondrial membranes) are stained. After pretreatment with acetone, fat droplets are solved and a mitochondrial staining remains (Fig. B). Quadriceps muscle biopsy from a 14-year-old boy with Kearns-Sayre syndrome (case 2). Bar= 50 μm.

SDH Stain

Succinic dehydrogenase (SDH) or complex II activity is shown by incubating the sections in a solution with Na-succinate (which is oxidized to fumarate) and a tetrazolium salt (NBT or nitro blue tetrazolium which is reduced to an insoluble blue-colored formazan). Succinic dehydrogenase is an enzyme of the Kreb's cycle or citric acid cycle; it is located in the inner mitochondrial membrane and is totally encoded by nuclear DNA. In vivo it delivers two electrons for the electron transport chain.

In normal tissue type I fibers show the most intense blue staining and type IIB fibers the least; these mitochondrial activities correlate well with the mitochondrial presence in HE- and trichrome-stained sections (Fig. 1). However, the detection of mitochondria with the SDH method is superior with respect to the HE stain and even better than the trichrome stain. Furthermore, the activities visible with the SDH stain are specific for succinic dehydrogenase and thus this staining method may be used as a mitochondrial marker since totally unstained muscle fibers are seldom observed.

NADH-TR Stain

Reduced nicotinamide adenine dinucleotide (NADH) is the substrate in this stain that may be oxidized by various NADH-dependant enzymes. The reaction results in the deposition of an insoluble formazan if a tetrazolium salt (NBT) was added at the same time. TR stands for tetrazolium reductase. Thus, in a muscle biopsy section the added NADH may be oxidized by complex I (an NADH-dehydrogenase with 7 subunits encoded by mitochondrial DNA) of the electron transport chain but also by at least one other NADH-dehydrogenase in the mitochondria.[6] Furthermore, the endoplasmic reticulum (usually called sarcoplasmic reticulum in muscle) contains NADH-oxidizing enzymes.[7] Unfortunately, no inhibitors are known that inactivate the latter enzymes. Although the staining results of this method are of excellent quality, the value of this stain for disorders of oxidative phosphorylation is limited.

COX Stain

Cytochrome oxidase or cytochrome c oxidase (COX) or complex IV is the last enzyme of the electron transport chain and is just as complex I and II located in the inner mitochondrial membrane. Cytochrome c oxidase is partly encoded by the nuclear genome (10 subunits) and partly by mitochondrial DNA (3 subunits). In vivo it transfers electrons from cytochrome c to oxygen so that together with protons water is formed. Cytochrome c must continuously be re-oxidized to keep the transport of electrons going.

In the enzyme histochemical method according to Novikoff and Goldfischer[8] benzidine is used as the electron donor. Although the method is in principle unspecific for cytochrome c oxidase, a high specific result is obtained in muscle.

In normal tissue, type I fibers show the most activity (see normal fibers in Fig. 4D) and type IIB fibers the least, just as the SDH and the NADH-TR stain. Contrarily to the SDH and NADH-TR stain, which rather reflect the mass of mitochondria as seen in the HE or trichrome stain, the COX activity of muscle fibers may be seriously decreased or even absent. Since type I and type IIA fibers normally contain the most mitochondria, COX activity decrease or absence is best observed in both these fiber types.

COX stain may be combined with SDH stain[9] in the same muscle section in order to discriminate between COX-positive ragged-red fibers (brown), COX-negative ragged-red fibers (blue), and COX-negative non-ragged-red fibers (blue); the extra subsarcolemmal staining makes the difference between the latter two fiber types.

ATPase (Myofibrillar) Stain

In fact two slices with sections are needed for typing type I, type IIA and IIB, and type IIC muscle fibers. Essentially the enzyme histochemical reaction takes place at pH 9.6 and concerns the enzyme ATPase located at the myosin filaments of the myofibrils. By preincubation at pH 4.2, type I, IIC, and other type II fibers can be observed; by preincubation at pH 4.6, type IIA and type IIB can be discerned.

Electron Microscopy

Although the global presence of mitochondria can be observed by the light microscopical methods already discussed, the fine structure of the organelles can be studied by transmission electron microscopy (Fig. 3). The spatial resolution for biological specimens in EM may be a 100 times better as compared to light microscopic observations. Preparations of excellent quality are obtained by chemically fixing the muscle tissue with glutaraldehyde followed by postfixing with osmium tetroxide. The fixed specimens are then dehydrated in ethanol or acetone and embedded in a resin. In our lab we use epon as a resin. Thin 70-nm-thick sections are cut and successively stained with uranyl acetate and lead citrate.

Immune Histochemistry

As has been mentioned above, it is possible to demonstrate a pathological excess of mitochondria by enzyme histochemical methods. Cytochrome c oxidase (COX) deficiency is genetically heterogeneous. Patients with COX deficiency secondary to mtDNA mutations have a specific pattern of subunit loss, which can be demonstrated with immune histochemistry with antibodies against different COX subunits.[10]

Molecular Diagnostic Methods

Molecular diagnostic methods such as polymerase chain reaction (PCR) and in situ hybridization (ISH) can be applied on tissue sections. However these techniques have not yet found their way in the all-day diagnostic work-up of a muscle biopsy. In situ PCR may be used to detect localized mtDNA deletions in individual fibers. It may be especially important to use in situ PCR to detect localized mtDNA depletion in cases where conventional methods cannot establish depletion uniformly in muscle biopsy.[11]

Morphological Hallmarks for Diagnosis of OXPHOS Disorders

Histopathological evidence for OXPHOS disorders can be obtained from HE-, SDH-, COX-, trichrome-, and antibody stained sections in light microscopy. In muscle the so-called ragged-red fiber (Fig. 1) and the COX-negative or COX-deficient fiber (Fig. 4D) are the most important markers for a mitochondrial disorder. A third important marker can only be observed by electron microscopy and concerns the presence of paracrystalline inclusions in mitochondria (Figs. 3B and 3E). In electron microscopy minor impressive changes such as increased size of mitochondria, the frequent presence of mitochondrial aggregates, mitochondria without cristae or with concentric cristae (Fig. 3C) or with peripheral cristae only (Fig. 3D), mitochondria with rounded dense and mitochondria with degenerating cristae (Fig. 3G) are of additive importance.

Ragged-Red Fiber

Ragged-red fibers represent the most important hallmark for mitochondrial proliferation in muscle fibers and can be observed with several histochemical and enzyme histochemical stains under the light microscope (see above). There is an increased presence of enlarged fat vacuoles. Generally, an increase of glycogen is present in these fibers, especially in the ragged region. Ragged-red fibers may also be recognized in HE stained sections as a basophilic ragged region (Fig. 1, 2, 4, and 5), on myofibrillar ATPase slides as an unstained region, in SDH- and NADH-TR sections as a strongly positive ragged region, and in Sudan Black B sections as a weakly gray ragged region.

It is difficult to give exact criteria for a ragged-red fiber. The ragged aspect is one criterion, no matter whether it concerns the total surface of the fiber or only a part of it. In some cases the total transverse area of a fiber is occupied by mitochondria without a discernable rim. These fibers must be classified as ragged-red fibers since they represent a more advanced stage of mitochondrial proliferation. The mitochondrial rim may be narrow and smooth; such fibers might be seen under various pathological or under special physiological conditions. In these cases often a small part of the circumference is concerned. Therefore is seems to be practical to use the term "ragged-red" only in cases with a more than 50% occupation of the circumference.

Analyzing muscle biopsy specimens may reveal that some ragged-red fibers, observed in the first serial sections of a series with different stains, have "disappeared" in the last ones. Thus, the ragged-red aspect is not necessarily continuously present along the length of a muscle fiber (Fig. 5), which is the rule rather than the exception.[12]

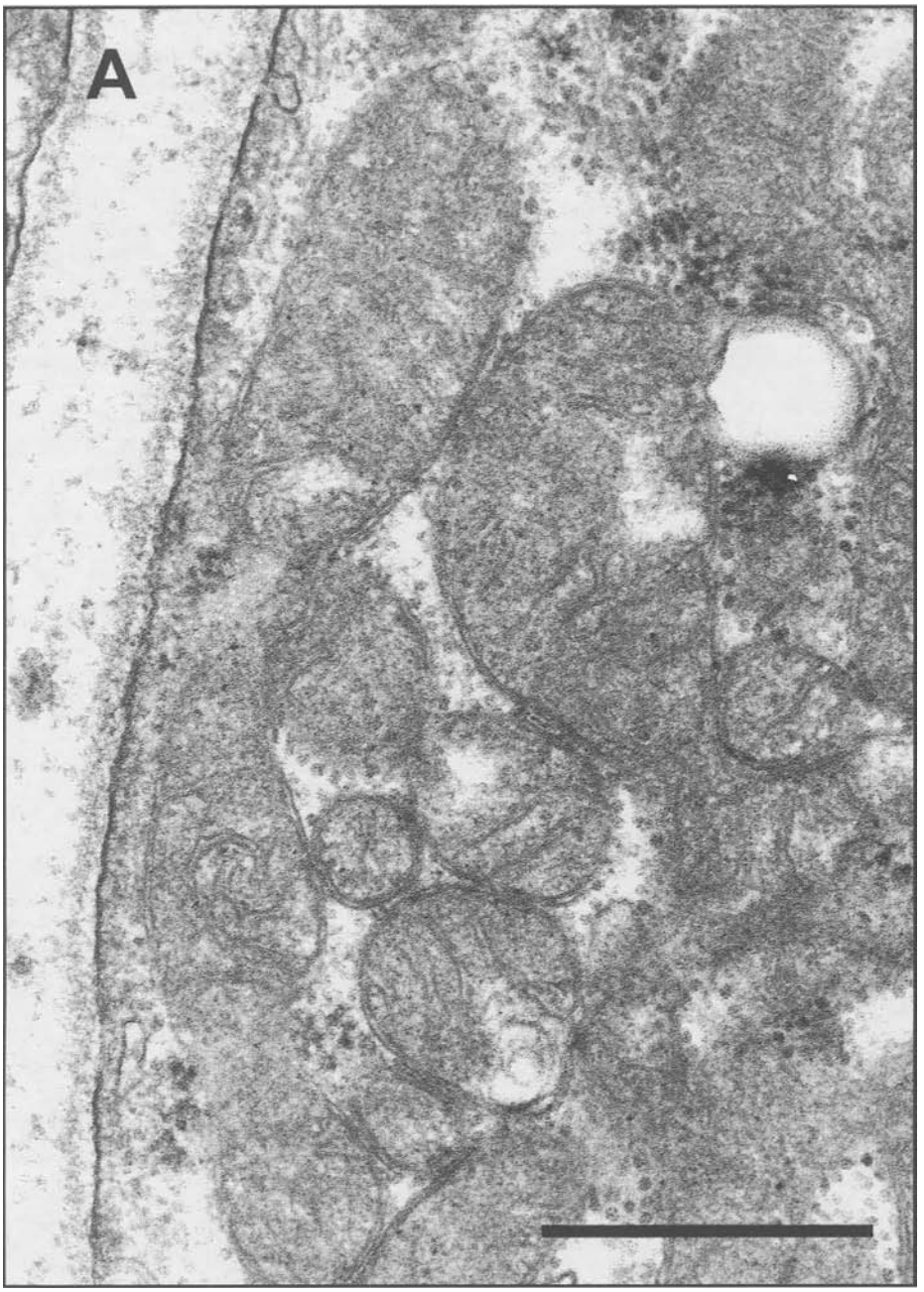
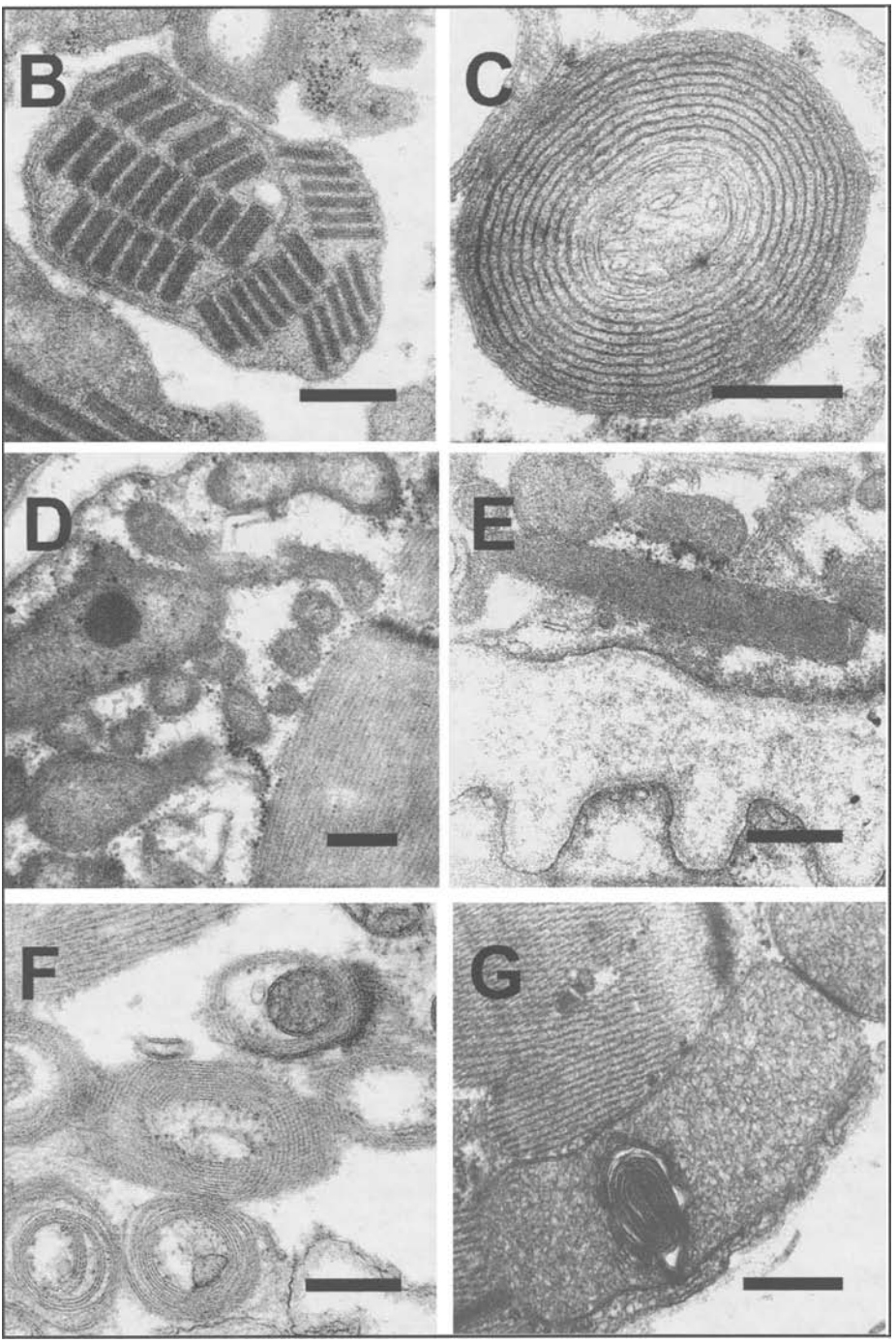


Figure 3. Normal (A) and pathological mitochondria (B-G). B) large mitochondrion with paracrystalline inclusions in parking lot formation. C) large mitochondrion with concentric cristae. D) mitochondria with cristae only peripherally; a dense, black inclusion in the largest mitochondrion, case 3. E) elongated mitochondrion with paracrystalline inclusion, case 4. F) mitochondrion located in a cylindrical laminated body (upper right), case 5. G) large degenerating mitochondrion with cristae like tubular structures and with a myeloid body, case 6. Bar= 500 nm.



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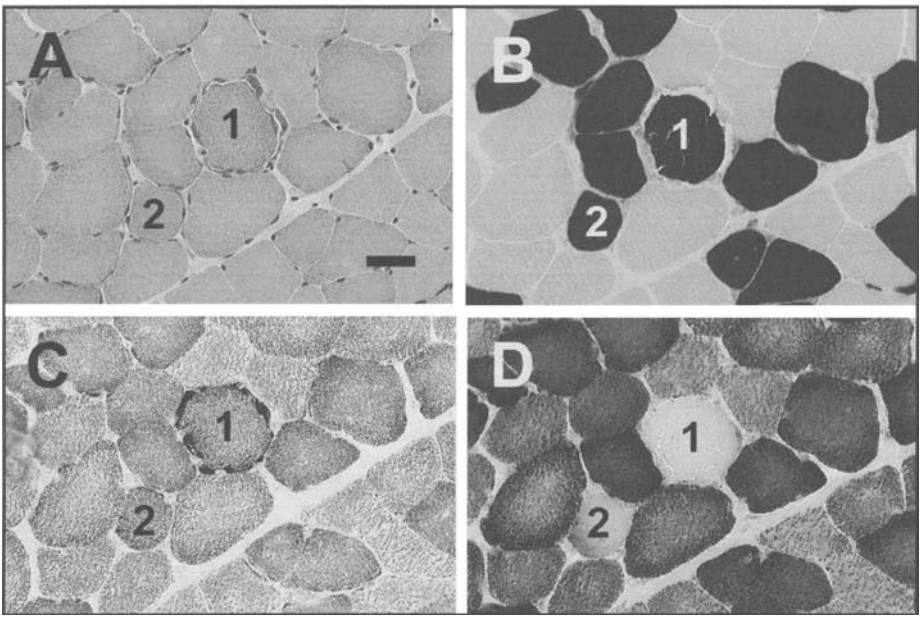

Figure 4. Two abnormal COX-deficient muscle fibers (fiber 1 and 2). Fiber 1 is an almost cytochrome oxidase (COX)-negative ragged-red fiber. Fiber 2 is a COX-deficient type I fiber. A) H-Phl stain; B) myofibrillar ATPase (preincubated at pH 4.2) stain; C) SDH stain; D) COX stain. Quadriceps muscle biopsy from a 22-year-old patient (case 1) with CPEO and a mitochondrial DNA deletion including the COX III region (50% heteroplasmy). Bar= 50 μm.

COX-Negative or COX-Deficient Fiber

Since the early 1980s the COX stain is gradually introduced in standard enzyme histochemical investigations of mitochondrial myopathies. Ragged-red fibers were frequently shown to be COX-negative or COX-deficient, but also normal looking fibers without mitochondrial proliferation in the same specimen were found to be COX-negative or COX-deficient; these fibers outnumbered the ragged-red fibers in transverse sections.[13] In longitudinally cut sections from muscle biopsies, COX-negative fibers were observed but also limited regions with a length till several hundred microns without COX activity but normal SDH activity;[14] corresponding cytochemical electron microscopy of the abnormal COX-stained regions confirmed light microscopic findings. In two cases with CPEO, COX-negative regions varying in length from 10 to 1200 micron were observed together with fibers with normal activity over their entire length; furthermore, irregularly alternating short COX-negative and longer positive regions were observed.[15] However, sparsely present COX-positive mitochondria could be present in the COX-negative regions.[16,17]

Apart from its association with ragged-red fibers, COX activity may also be absent or severely decreased in mitochondrial disease (Figs. 8 and 9) without the presence of ragged-red fibers.[10]

Paracrystalline Inclusions

These inclusions were first described by Luft et al[18] in their article on a female patient with mitochondrial myopathie with uncoupled oxidative phosphorylation. Paracrystalline inclusions are located in the intermembrane space of the mitochondria. Two types are discerned, viz. type I (Figs. 3B and 3E) located in the cristae and almost completely enclosed by the inner membrane and type II located at the border of the mitochondrion with the outer membrane

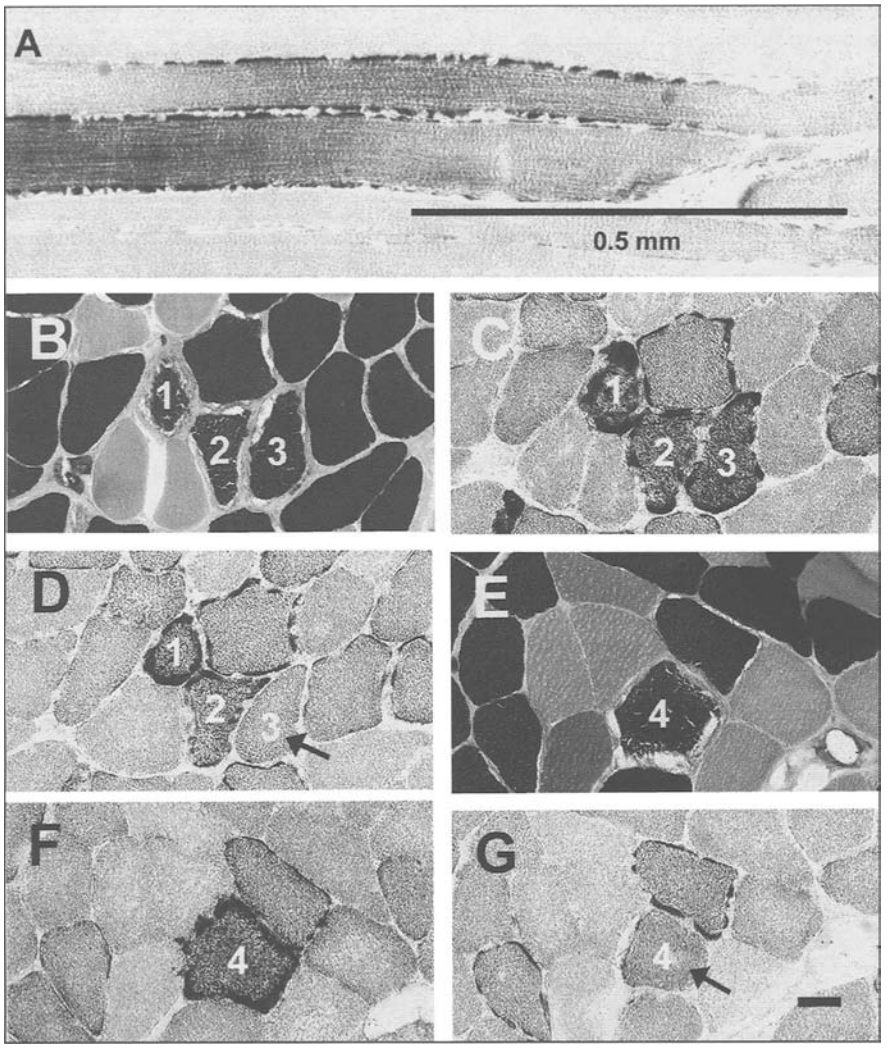

Figure 5. Ragged-red fibers with local differences in mitochondrial proliferation. Longitudinal (A) and transverse sections (B, C, D, E, F, G). In Figure A two fibers show dark staining regions only in a limited part of the fiber. Figures B, C and D, and Figure E, F and G show serial transverse sections of ragged-red fibers (1, 2, 3, and 4). Fiber 1 and 2 are ragged-red over their total investigated length. Fiber 3 and 4 show a ragged-red appearance in C and F, but not in D and G (D and G are at 240 μm distant from C and F). A, C, D, F and G) SDH stain; B and E) myofibrillar ATPase stain (preincubated at pH 4.6). Quadriceps muscle biopsy from a 14-year-old boy with Kearns-Sayre syndrome (case 2). Small bar= 50 μm.

always closely attached to the inclusion.[19] It was thought that these two types represented two expressions of an identical crystalline structure. However, the two types were shown to be basically different real crystal formations;[20] at least a considerable part of the paracrystalline inclusions (both type I and type II) is composed of mitochondrial creatine kinase.[21] In fact paracrystalline inclusions are unspecific and may be found in different neuromuscular disorders[22] and even in normal muscle.[19] Specificity for mitochondrial disorders increases as more inclusions are observed and it is impossible to give an exact range, the more so as an increasing

number of disorders are believed to be mitochondrial in origin or associated with defect mitochondrial functioning.

Discussion

The above mentioned morphological abnormalities for OXPHOS disorders are often simultaneously found. The isolated finding of one of these abnormalities may be a strong indication for mitochondrial disease. A decrease of COX activity in both type I and II fibers without ragged-red fibers is found in muscle with mitochondrial $tRNA^{Ser\ (UCN)}$ mutations associated with hearing loss.[23] Normally, ragged-red fibers are COX deficient and display abnormal mitochondria on electron microscopy; but the occurrence of scattered COX deficient fibers without ragged-red aspect is also an indication for mitochondrial myopathy. The same is true for the observation of diffuse COX deficiency without ragged-red fibers in children with benign or fatal COX deficiency. It is also possible that abnormal mitochondria with paracrystalline inclusions are clearly present, but without any evidence of ragged-red fibers.[24] The latter observation stresses the importance of applying EM research in cases with suspicion of a mitochondrial disorder but without presence of ragged-red fibers.

An intriguing point of observation concerns the many cases with apparent clinical and biochemical mitochondrial myopathy without the histological hallmarks for mitochondrial disease. It is not always possible to predict exactly the morphological picture from known mitochondrial or nuclear mutations. The degree of heteroplasmy, the nature of the mutation, the different threshold levels of particular mutations before being manifest, the generally unknown effects of mutations on biophysics and biochemistry in the various tissues, are some of the factors responsible for the diversity of phenotypes caused by identical mutations and for identical phenotypic representations caused by different mutations.[25]

Most mitochondrial point mutations concern tRNA genes.[25] Large scale deletions, as in PEO, include several tRNA genes. Since a single tRNA mutation disturbs the attachment of the respective amino acid at several locations in the various mitochondrial coded proteins, tRNA mutations might be expected to be more severe than similar point mutations of mitochondrial DNA coding for only a single structural protein component needed for oxidative phosphorylation. The morphological consequence may be the presence of both ragged-red fibers and COX negative or COX deficient fibers. Unfortunately, deficiency of the other complexes cannot be traced by the usual enzyme histochemical methods. For cases with large scale deletions (with loss of many tRNA genes) the former changes are expected to be more severe.

In muscle biopsies from patients with mutations in mtDNA coding for the various subunits, the histological hallmarks may be present or not. Hallmarks are not present in LHON (complex I, subunit 1, 4, and 6) and NARP (complex V, subunit ATPase 6), but ragged-red fibers were present in a case with exercise intolerance and complex I subunit 4 mutation,[26] in most cases with complex III mutations,[27] and in cases with complex IV mutation in subunit COX I [28] and in subunit COX III.[29]

Mutations in nuclear genes coding for the various OXPHOS complex protein subunits are only known for a complex II subunit[30] and for 6 different complex I subunits.[31] Most of these mutations concern patients with Leigh or Leigh-like syndrome. Muscle histology was only described for mutations in the NDUFS4- and the NDUFS2 gene of complex I: diffuse lipid accumulation was shown, but no hallmarks for OXPHOS disease were found in 2 cases with a NDUFS4 mutation. As a rule, ragged-red fibers seldom occur in Leigh or Leigh-like syndrome, which might be expected from the very low number of cases with mitochondrial tRNA mutations.[32]

Mutations in nuclear DNA coding for complex IV subunits are still unknown. However, mutations in nuclear genes responsible for the correct assembly of complex IV holoenzyme were shown to be responsible for severe COX deficiency.[33] Severe histochemical COX deficiency was present in the corresponding muscle biopsies for SURF-1-,[34] SCO2-,[35,36] and COX10-mutations,[37] the three so-called assembly genes that are known yet.[38] Again, ragged-red fibers were never observed in them.

In general, mitochondrial mutations do cause more morphological mitochondrial abnormalities in muscle than nuclear mutations do. Many of these abnormalities concern proliferative changes such as increase in numbers of mitochondria, larger mitochondria, mitochondria with tightly packed cristae, and extra protein production in mitochondria (paracrystalline inclusions). In principle, such changes may compensate for defects. However, ragged-red regions are mostly COX deficient and contain very high percentages of mutated mitochondriae which is in fact a rather ineffective way of compensation.

Till now, ragged red fibers as a consequence of mutations in nuclear genes coding for OXPHOS subunits or for their assembly are still unknown.

From the above mentioned data it seems clear that only mutated mitochondria may give rise to mitochondrial proliferation or ragged-red fibers. However, in two mouse models for mitochondrial myopathy ragged-red fibers can be induced by disrupting the nuclear gene for transcription factor A (mtTFA or Tfam)[39] or by knocking out the nuclear gene for adenine nucleotide translocator (Ant1).[40]

In short, ragged-red fibers point to mitochondrial myopathy. However, it is not known why mitochondrial proliferation in some cases is clearly present and is lacking in other cases where they are expected.

Mitochondrial Changes in Muscle Biopsies without OXPHOS Disorder

Ragged-red fibers and COX-negative muscle fibers are not completely pathognomonic for OXPHOS diseases, as they can be present in different other situations. With aging, some mitochondrial DNA-mutations may occur in individual fibers, so after the age of 50 one or a few individual COX-negative fibers or ragged-red fibers may be present in otherwise normal muscle tissue.[41,42] COX-deficient fibers and ragged-red fibers may also occur in other neuromuscular diseases,[43] such as inclusion body myositis, myotonic dystrophy, congenital nemaline myopathy, and other.

Ragged-red fibers also occur normally in several specialized muscles such as the cricopharyngeal and the eye muscles.

Finally ragged-red fibers or COX-negative fibers can be induced by several medications. Especially renown for this is zidovudine, a medication used in HIV-patients, which may ultimately even lead to a zidovudine myopathy.[44] Germanium, an antineoplastic agent also leads to a myopathy with ragged-red fibers with electron-dense granules in the mitochondria on EM.[45]

Muscle Biopsy in OXPHOS Disorders

From the previous section it will be clear that hallmarks for OXPHOS disorders are not always present in the muscle biopsy. Thus, normal biopsies may result from patients with biochemical proven respiratory enzyme complex deficiencies and from patients with maternally inherited OXPHOS mutations whose percentages of abnormal mitochondria are too low to cause morphological changes. It serves no purpose to present the morphology of such cases in this section.

Therefore, muscle biopsies will be shown from patients with successively CPEO, Kearns-Sayre syndrome, MELAS-, and MERRF syndrome (cases 1-4); in these cases the ragged-red fiber is the most important hallmark. Subsequently, four examples will be given of a muscle biopsy with COX deficiency as the main morphological hallmark without ragged-red fibers (case 5-8). In case 1 and 2 deletions of mitochondrial DNA were shown or probable; in case 3 and 4 point mutations were shown or probable and in cases 5-8 a nuclear DNA mutation is the most likely option.

Case 1: CPEO

Clinical data: A 22-year-old patient noticed an increasing ptosis of his right eye from age 12. At examination both eyes revealed ptosis and strabismus was present; the retina was normal. A CT-scan of cerebrum and orbita was normal. Arm and leg muscles were normotone.

There was no muscle weakness and no atrophy. Tendon reflexes were low. EMG and ECG were normal.

Muscle biopsy: The quadriceps muscle biopsy contained some atrophic fibers; the variability of the muscle fiber diameters was moderately increased. There were no internal nuclei in the fibers. The percentage of type I fibers was 22 (normally 35-50). The biopsy contained 1% ragged-red fibers (Fig. 4, such as fiber 1), which were type I and strongly COX-deficient or COX-negative. About 0.5% of the fibers in the biopsy showed no ragged-red aspect, but revealed decreased or deficient COX activity (Fig. 4D, like fiber 2). Some fibers showed regional COX deficiency transitions per fiber.

Ultrastructural examination revealed one fiber (probably a type I or IIA fiber) out of 20 with only abnormal mitochondria both subsarcolemmally and between the myofibrils. The cristae in these mitochondria were only peripherally located with the center devoid of cristae (like those in Fig. 4D). Some other scattered mitochondria in this fiber contained paracrystalline inclusions. The mitochondria in the other fibers were ultrastructurally normal.

Laboratory data: A deletion including the gene coding for subunit COX III was shown in 50% of the mtDNA molecules from muscle. Biochemical examination of muscle mitochondria was normal.

Remarks: The SDH stain of the COX-deficient non-ragged-red fibers showed variations of SDH activity ranging from normal to diffusely increased with subsarcolemmally a partial ragged aspect (maximally up to 50% of the fiber circumference); the latter fibers were not classified as ragged-red fibers.

Case 2: Kearns-Sayre Syndrome

Clinical data: A 14-year-old boy showed apparent somatic and mental retardation from age 4. At age 14 his weight was only 19 kg. There was serious muscle weakness, generalized muscle hypotrophy, and hyporeflexia. Further clinical examination revealed retinal pigmentary degeneration, ptosis, cardiomyopathy on echocardiographic examination, slight perception deafness, and epilepsy.[46]

Muscle biopsy: The quadriceps biopsy revealed a predominance of type I fibers (70%), a slight increase of fibers with internal nuclei, and a slight increase of muscle fiber diameter variation caused by the presence of both atrophic and hypertrophic fibers. About 10% of the fibers (9% type I and 1% type II) showed a clear ragged-red appearance as judged by ATPase- and SDH stain (Fig. 5B-5G). Another 10% of the fibers (most type I fibers but also some type II fibers) showed a minor ragged-red aspect because of diffusely increased SDH activity and the presence of a thin non-ragged rim of extra activity (see Fig. 5C and 5F). From Fig. 5 it appears that such observations are explained by regional differences in a particular fiber. In longitudinal sections (Fig. 5A) many transitional regions with regard to SDH activity were observed. The upper fiber shows a ragged-red region over a length of 0.65 mm; this fiber was continuous with normal regions of 0.65 mm both to the left and the right. Furthermore it was seen that both length and degree of maximal activity increase in an abnormal region, were different for various fibers. Another fiber with a length of 2 mm showed two SDH-increased regions with varying maximum intensity.

In transverse sections the ragged-red fibers showed an increase of lipid droplets (Fig. 2) and in some of these fibers (about 10%) cytoplasmic bodies were present.

Electron microscopy showed an excessive mitochondrial proliferation with a broad variety of qualitative and quantitative changes including paracrystalline mitochondrial inclusions (like Fig. 3B).

Laboratory data: Blood CK was normal. Serum and cerebrospinal fluid (CSF) contained excessively increased lactate levels after provocation by exercise. CSF protein was moderately increased. ATP production measured from a muscle homogenate was lowered.

Remarks: From the above observations it may be clear that the ragged-red appearance is not homogeneous for a single fiber. Furthermore, the degree of maximum SDH activity per

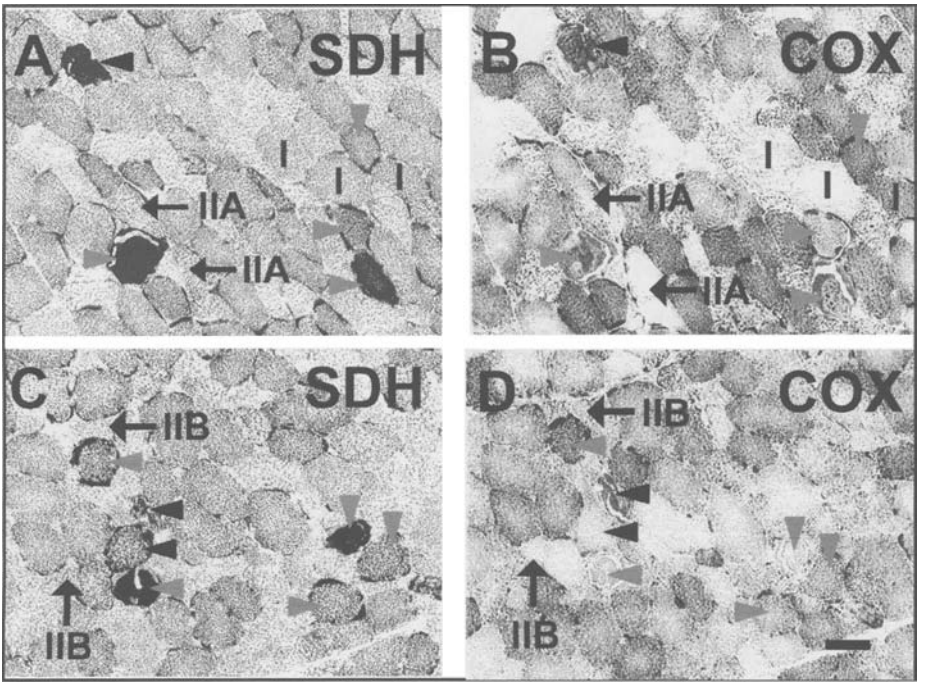

Figure 6. Ragged-red fibers in MELAS. Serial SDH- and COX-stained sections with both type I (gray arrow-heads) and type IIA ragged-red fibers (black arrow-heads). Ragged-red fibers show a COX activity ranging from normal or slightly increased to severely deficient with respect to SDH activity. Non-ragged-red fiber types (denoted by I, IIA and IIB) show the same pattern. Fiber typing was done from remote serial ATPase stained sections. Quadriceps biopsy from a 22-year-old patient with MELAS (case 3). Bar= 50 μm.

ragged-red region is different. It may be concluded that a particular fiber contains several abnormal regions with varying SDH activity of varying length.

Case 3: MELAS Syndrome

Clinical data: A 22-year-old patient presented with delayed development, exercise intolerance, perception deafness and periods with vomiting. At the time of investigation there was a temporoparietal infarction picture resulting in dysphagia, dyspraxia and central facial nerve paresis at the left. CT-scan and MRI investigations were indicative for an infarction at the temporal lobe and showed hypodense regions in both left and right caudate nucleus.

Muscle biopsy: The quadriceps biopsy contained normal percentages of type I, IIA, and IIB fibers (50, 25, and 25 respectively). There were hardly any fibers with internal nuclei (1%: normal). Muscle fibers were generally small, especially the type IIB fibers; some were atrophic. The biopsy contained 5% ragged-red fibers, which were provided with a clear ragged rim composed of mitochondria or were almost totally filled with mitochondria (arrow-heads in Fig. 6). Both type I and type IIA ragged-red fibers (80% and 20%) mostly displayed COX activity varying from comparable to the SDH activity level to almost negative. Besides the distinct ragged-red fibers another 5% of the fibers showed mild diffuse mitochondrial proliferation and were provided with a narrow and smooth rim of mitochondria; these fibers too showed varyingly decreased COX activity. Finally, the COX activity of normal looking fibers without any mitochondrial proliferation varied from normal to severely decreased for all fiber types (arrows in Fig. 6). Generally, many fat droplets and much glycogen could be found in

regions with mitochondrial proliferation. Cytoplasmic bodies did occur in the ragged-red fibers only.

Ultrastructurally, mitochondrial size varied from normal to increased with a varying presence of cristae per mitochondrion from tightly packed to almost empty (Fig. 3D). Circular dense inclusions were often present, but paracrystalline inclusions were not observed.

Laboratory data: Lactate levels were increased in serum; creatine kinase was slightly increased.

Mitochondrial DNA investigations in muscle revealed the A to G transition at nt-3243 in the $tRNA^{Leu(UUR)}$ gene with a heteroplasmy level of 60%.

Remarks: This case not only shows that besides distinct ragged-red fibers other abnormal fibers with ragged-red appearance are present; there are also gradations in COX decrease for most of the fibers, irrespective of fiber type.

Case 4: MERRF Syndrome

Clinical data: This 38-year-old patient developed normally till age 11. At this age the first epileptic seizures occurred. From age 20 there was a progressive deterioration in vision and hearing. Clinical examination revealed myoclonus of arms, head and neck, tapetoretinal degeneration, bilateral perception deafness, muscle atrophy in arms and legs, and dysdiadochokinesis.

Muscle biopsy: The percentage of type I fibers was slightly increased (56 versus 35-50 normally) and IIA and IIB fibers were present in equal percentages. There was no increase in fibers with internal nuclei (2%). Muscle fiber diameters were smaller than normal with only some atrophic fibers. About 2% of the fibers could be classified as ragged-red fibers (only one IIB fiber was observed). These were COX-negative or COX-deficient type I fibers (Fig. 7). The COX activity was also seriously decreased in a varying degree in many non-ragged-red fibers (Fig. 7); both type I, type IIA, and type IIB were concerned.

On electron microscopy clusters of mitochondria with paracrystalline inclusions were located beneath the sarcolemma of some fibers (Fig. 3E).

Laboratory data: Lactate was slightly increased in serum.

Remarks: Contrarily to the former case, COX activity in ragged-red fibers is much more decreased in this case with only 2% ragged-red fibers. Like the former case this decrease is also apparent in many non-ragged-red fibers of all fiber types.

Case 5: COX Deficiency and Encephalomyopathy

Clinical data: A 29-month-old girl was born after an uneventful pregnancy with maximum Apgar scores. Hypotonia and motor retardation were manifest at 6 months. At examination at the age of 15 months there was a generalised muscle hypotonia, weakness, hyporeflexia and a slight hepatomegalia. ECG and echographic analysis did not reveal hypertropic cardiomyopathy. EMG was not abnormal. EEG showed increased local delta-activity from the right occipital lobe and there were increased spaces filled with cerebrospinal liquid on CT-scan. A slight bilateral optical nerve atrophy was observed. At 19 months she still could not walk freely and from that time on, there was an increasing need for assisted ventilation. From the age of 31 months there was a fast improvement of her condition and motor development. She started to walk and at 36 months the respiratory problems were over.

Muscle biopsy: The quadriceps muscle biopsy contained near normal percentages of type I and type IIC fibers (30 and 10 versus 15-50 and 0-5 normally). The muscle fibers were too small (8-23 μm versus 15-35 μm). There were no ragged-red fibers. SDH activity was normal but COX activity (Fig. 8A) was severely decreased in both muscle and spindle fibers. Electronmicroscopical examination revealed most normal and some large mitochondria. A special finding concerned some cylindrical laminated bodies; in one of these bodies a mitochondrion was present (Fig. 3F).

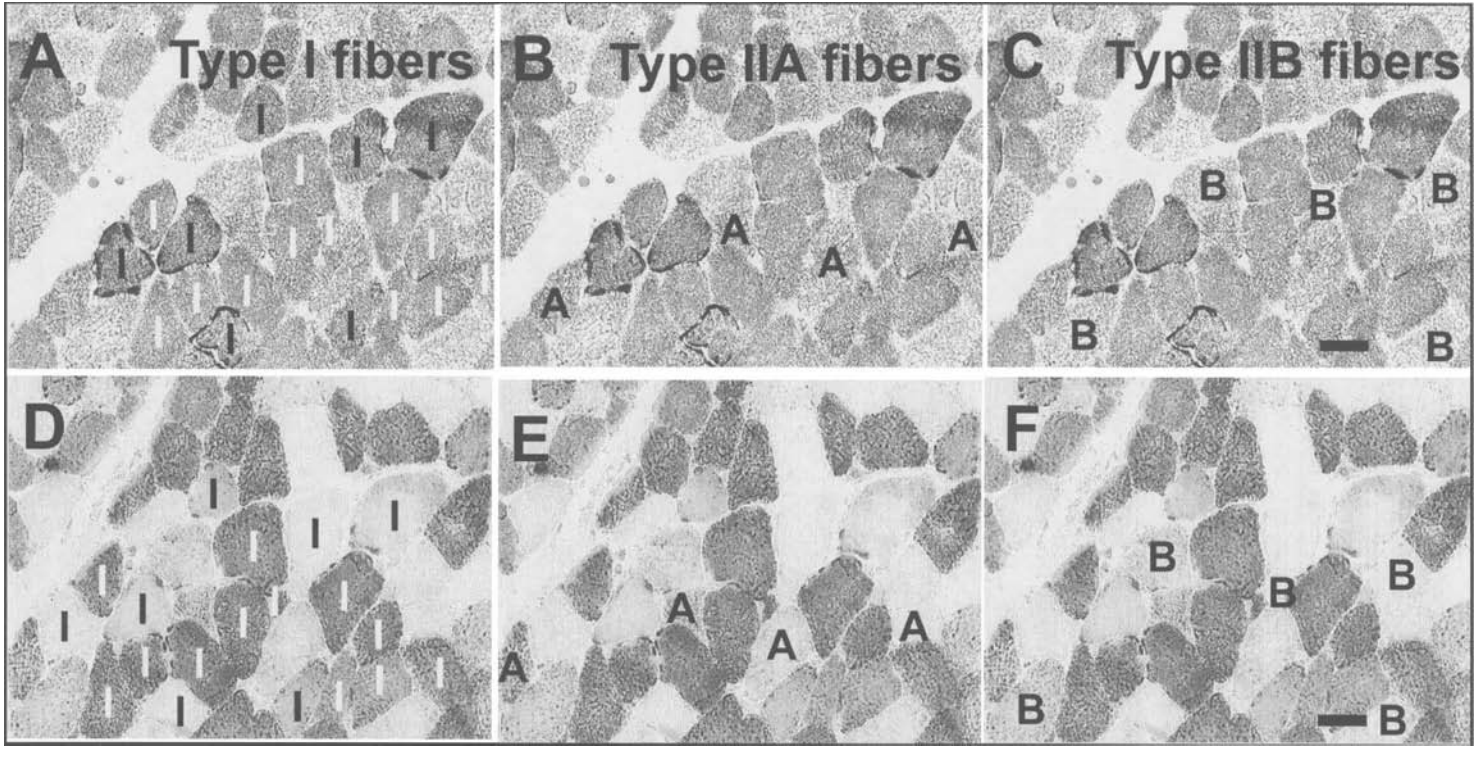

Figure 7. Variability of COX activity in MERRF. SDH (upper row) and COX (lower row) activity in serial sections are most discrepant in the three type I ragged-red fibers (at the left in the left column). This discrepancy is also present for many type I fibers (left column), type IIA (middle column), and type IIB fibers (right column), but in a less degree. There are no type IIA or type IIB ragged-red fibers in this picture. Quadriceps biopsy from a 39-year-old male with MERRF syndrome (case 4). Bar= 50 μm.

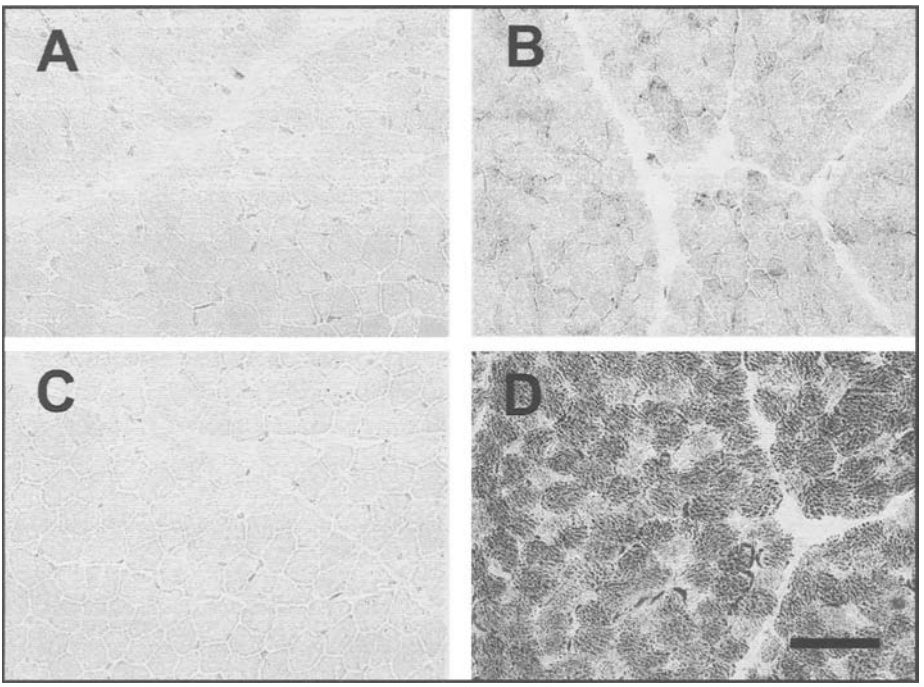

Figure 8. Severe COX deficiency in three patients (Figs. A, B, and C). Control COX stain (Fig. D; 11-month-old patient). Case 5 (Fig. A), case 6 (Fig. B), and case 7 (Fig. C). Bar= 50 μm.

Laboratory data: At 15 months lactate/pyruvate ratio was increased in serum; liquor was normal. At 17 months lactate was increased in both serum and liquor; protein content was increased in liquor. Examination of muscle homogenate (at 29 months) revealed severe complex IV deficiency and moderate complex I and III deficiencies. Mitochondrial DNA analysis from muscle and fibroblasts did not reveal point mutations nor deletions.

Remarks: the presence of cylindrical laminated bodies surrounding mitochondria was first observed by Luft et al[18] in their description of the muscle biopsy of a case with uncoupled oxidative phosphorylation. The structure of these bodies is not compatible with biological membranes and they supposed these bodies to consist of concentric sheaths of globular macromolecules with a crystal-like appearance. Since then, these bodies were also observed in various different clinical settings.

Case 6: COX Deficiency

Clinical data: A 1-month-old girl was born at term with Apgar scores of 5 and 7 after 1 and 5 minutes respectively. There was severe hypotonia and artificial ventilation was needed. Echographic analysis showed a hypertrophic cardiomyopathy which was disappeared after one month. Echographic and MRI-scan analysis of the cerebrum were normal. The child died at the age of 2.5 months.

Muscle biopsy: Fiber analysis from the quadriceps muscle showed only 10% type I and 45% type IIC fibers (normally 35-50% and 0-15% respectively at this age). There were no fibers with internal nuclei and muscle fiber diameters were smaller than normal (2-17 μm

versus 5-25 μm normally). Motor nerves were normal. SDH activity was slightly decreased but COX activity was hardly observable (Fig. 8B) in both muscle and spindle fibers. No ragged-red fibers were present.

On electron microscopy there was a clear increase of mitochondria; in most mitochondria the normal platelike cristae were replaced by clustered tubular structures. In some mitochondria clear myeloid figures pointing to cristae degeneration were apparent (Fig. 3G).

Laboratory data: Serum CK and liquor lactate were normal. Muscle mitochondria were deficient for complex I and complex IV.

Case 7: COX Deficiency and Encephalomyopathy

Clinical data: A 3-year-old girl showed hypotonia and delayed motor development after an uneventful neonatal period. Around the age of 14 months deterioration started. At clinical examination (36 months) the child was extremely hypotonic and seriously retarded. Heart rhythm was normal, but breathing was irregular. There was an extreme convergent strabismus. Muscle atrophy was conspicuous and there was areflexia. Sensibility was normal, but coordination was disturbed. The child died at the age of 5 years.

Muscle biopsy: The quadriceps biopsy was abnormal because of the severe deficiency of COX activity (Fig. 8C) and the moderately decreased muscle fiber diameters with the presence of only a few isolated atrophic fibers. There was a normal checkerboard pattern of type I and II fibers, and there were no fibers with internal nuclei. The percentage of type I fibers was larger than usual (65%). Electron microscopy showed normal presence of intact mitochondria; sporadically cylindrical laminated bodies were present beneath the sarcolemma.

Laboratory data: Serum lactate/pyruvate ratio was increased at 22 months and NMR spectroscopy revealed lactate acidosis in brain. Muscle homogenate examination revealed a severe and isolated complex IV deficiency. Fibroblast homogenate was also deficient for complex IV. Mitochondrial DNA analysis from blood, muscle, and fibroblasts revealed no point mutations nor deletions.

Case 8: COX Deficiency with Severe Spinal Muscle Atrophy (SMA) and Cardiomyopathy

Clinical data: This 4-month-old girl suffered from severe spinal muscular atrophy (SMA) and was born with severe hypotonia and severe axial and limb weakness.[47] There was hypertrophic cardiomyopathy and respiratory failure. EMG showed normal motor nerve conduction velocities and fibrillation potentials. EEG was normal. The child died at the age of 5 months.

Muscle biopsy: The quadriceps biopsy showed the typical picture of a severe SMA with hypertrophic type I fibers and both atrophic type I and II fibers (Fig. 9); the diameter histogram was bimodal. The percentage of type I fibers was increased (80% versus 35- 50%) and there were no fibers with internal nuclei (Fig. 9A). COX stain revealed a total absence of activity (Fig. 9D) with the exception of the muscle spindle fibers. Ragged-red fibers were not present and SDH activity (Fig. 9C) was normal. Ultrastructurally there was redundancy of muscle basal membranes indicative for a fast atrophying process, but mitochondria were normal.

Laboratory data: Lactate concentrations were increased in serum and liquor. Creatine kinase was normal. Complex IV activity from muscle homogenate was decreased and Western blot analysis showed decreased levels of all COX subunits. No mutation in the SMN gene for spinal muscular atrophy was found and investigations for mitochondrial DNA mutation were unsuccessful.

Remarks: In a recent publication[48] a similar case with clinical Werdnig-Hoffmann disease, hypertrophic cardiomyopathy, and severe COX deficiency was described with a novel SCO2 mutation. This is a COX-assembly gene on chromosome 22 coding for a protein required for correct copper assembly in the cytochrome oxidase.

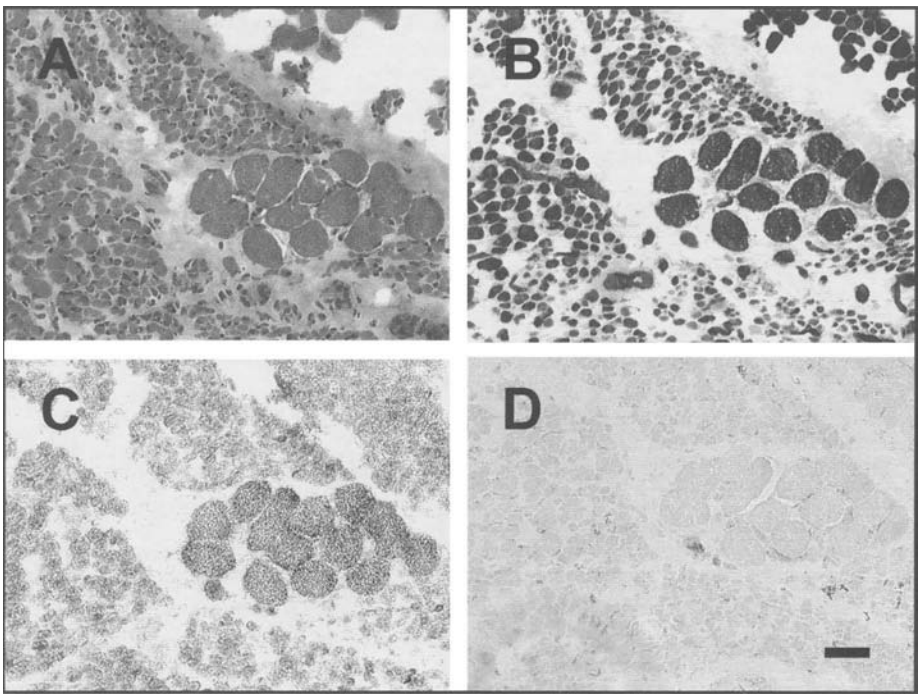

Figure 9. Spinal muscle atrophy with severe COX deficiency. Serial sections from quadriceps muscle biopsy. Fascicle with hypertrophic type I fibers (B); the other fascicles contain small and atrophic fibers. Note the severe COX deficiency (D). H-Phl (Fig. A), ATPase 4.2 (Fig. B), SDH (C), and COX (D). Quadriceps biopsy from a 4-month-old girl (case 8). Bar= 50 μm.

Pathological Findings in Other Organs

Central Nervous System

The most frequent OXPHOS disease, in which the brain is affected, is Leigh's disease (subacute necrotizing encephalomyelopathy). Most classic are the bilateral diencephalic lesions in the pallidum (Fig. 10A), but also the brain stem, the spinal cord, the cerebellum, and the optic nerves are frequently involved.[49] The lesions consist of more or less symmetric bilateral patchy areas of spongiosis (Fig. 10B), capillary proliferation with endothelial hypertrophy (Fig. 10C), and gradual necrosis of the neuropil with at first preservation of the neurons (Fig. 10C). There is some reactive gliosis and a few macrophages are present. Gradually also the neurons disappear, until finally pseudocystic (Fig. 10B and C) lesions remain.

Brain involvement in MELAS and MERFF disease usually consists of brain infarctions (Fig. 11A), which do not follow the usual vascular distribution pattern. Near these infarctions, capillary proliferation with very swollen endothelium may be prominent and more important than the necrosis would suggest (Fig. 11B). This phenomenon seen in a brain biopsy, taken because of suspicion of brain tumor in a 36-year-old male, was one of the clues leading to the diagnosis of MELAS, that was finally confirmed by mitochondrial DNA analysis. Several studies confirmed the presence of excess mitochondria in the capillary endothelial cells.

The etiology of Alpers disease or progressive infantile poliodystrophy still remains controversial and is probably heterogeneous. Several cases of Alpers disease would nowadays be designated as MELAS or MERRF. Recently a few cases of Alpers disease revealed to be due to DNA depletion.[50] The pathology consists at first of elective loss of neurons, with spongiosis and

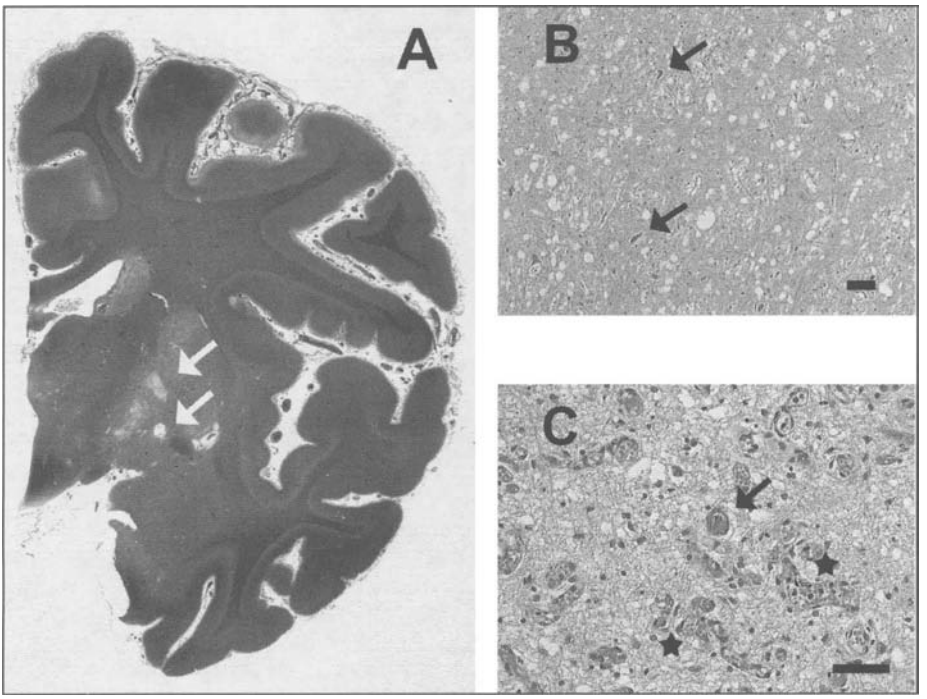

Figure 10. Morbus Leigh. A) right cerebral hemisphere with pseudocystic lesions in the pallidum (arrows) (Masson trichrome). B) microscopic detail of early lesion in the pallidum of a 14-month-old boy consisting of spongiosis and slight gliosis; neurons are remaining (arrows). C) spongiosis, capillary dilatation and proliferation (asterisks), and still remaining neuron (arrow) in an older lesion in a 3-year-old boy. Bar= 50 μm.

gliosis of the cortical laminae 2 and 4, especially from the striate cortex. Later the whole cortex is affected and becomes spongious, until all neurons have disappeared. This also leads to axonal loss and microcephaly (Fig. 12). Later other parts of the brain such as the cerebellar cortex, basal ganglia, and brain stem are involved.

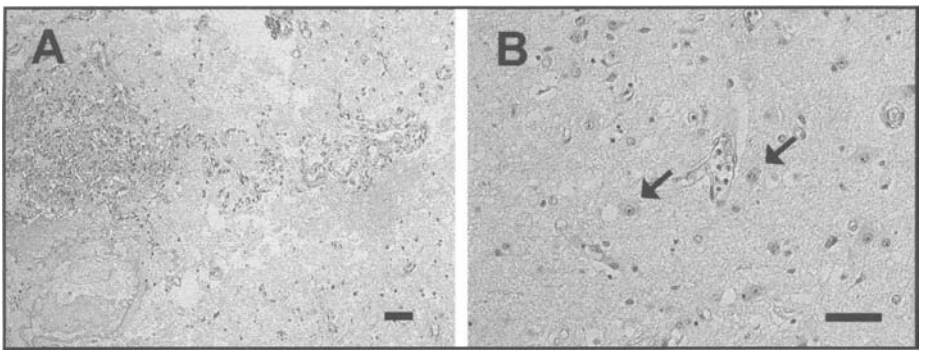

Figure 11. Biopsy of pseudo-tumoral occipital cortical lesion in a 36-year-old man, in whom the diagnosis of MELAS syndrome was made. A) infarcted, edematous brain tissue with capillary proliferation at the left. B) excessive accumulation of proliferated capillaries with very swollen endothelium at the edge of the infarction and remaining neurons (arrows) HE; Bar= 50 μm.

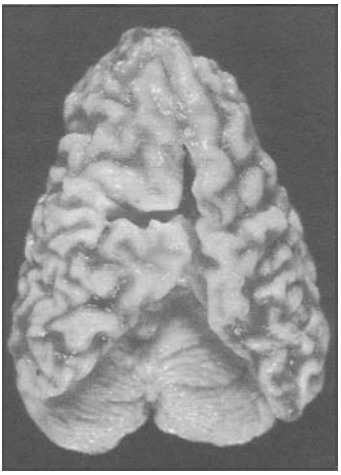

Figure 12. Superior view of extremely atrophic brain of a 3 year-old girl with Alpers disease.

Although several neuroradiological reports point to selective white matter involvement in some OXPHOS disorders,[51,52] the histopathological descriptions in the literature and personal experience point to relatively unspecific findings of diffuse periventricular white matter gliosis[53,54] or ischemic pseudocystic lesions.

Pyruvate dehydrogenase deficiency can be at the origin of early prenatal changes in the form of olivary heterotopia and absence of corpus callosum and pyramis.[55] Pontocerebellar hypoplasia[53] is another rare form of presentation of OXPHOS disorder.

Peripheral Nervous System

The peripheral nervous system is often involved in OXPHOS diseases. In NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa) there is peripheral nerve involvement by definition. LHON (Leber Hereditary Opticus Neuroretinopathy) is defined as Hereditary Motor Sensory Neuropathy type VI in the classification of Dyck et al.[56] A hereditary neuropathy is a prominent symptom in a mitochondrial disease with hereditary sensory neuropathy, progressive external ophthalmoplegia, ataxia and myoclonic status epilepticus (Fig. 13).[57]

A peripheral nerve biopsy is not the method of choice to diagnose an OXPHOS disease. It may be indicated to obtain a better insight in the disease process of an individual patient, but must then be performed in a well-equipped center with experience in the interpretation of nerve biopsies. The findings mostly are not specific. Mostly an axonal or neuronal neuropathy is seen (Fig. 12). Abnormal paracrystalline inclusions in the mitochondria can be observed in Schwann cells or axons of OXPHOS disease, but are not specific. Sometimes accumulations of large groups of mitochondria without paracrystalline inclusions are seen in the smooth muscle cells of the endo- and perineurial capillaries and arterioles.[58] Rarely axons with thin myelin sheets can be seen.[59,60] Signs of demyelination may accompany the hypomyelination in Leigh disease.[61]

Other Organs

Although OXPHOS diseases clearly are multisystem disorders and other important organs such as the heart, the kidneys, and the gut often are involved; the pathological alterations in these organs are mostly not very specific. A biopsy of these organs rarely leads to a pathological diagnosis of an OXPHOS disease. In case of suspicion of an OXPHOS disease it may be more rewarding to take a muscle biopsy than to take a biopsy of the most affected organ.

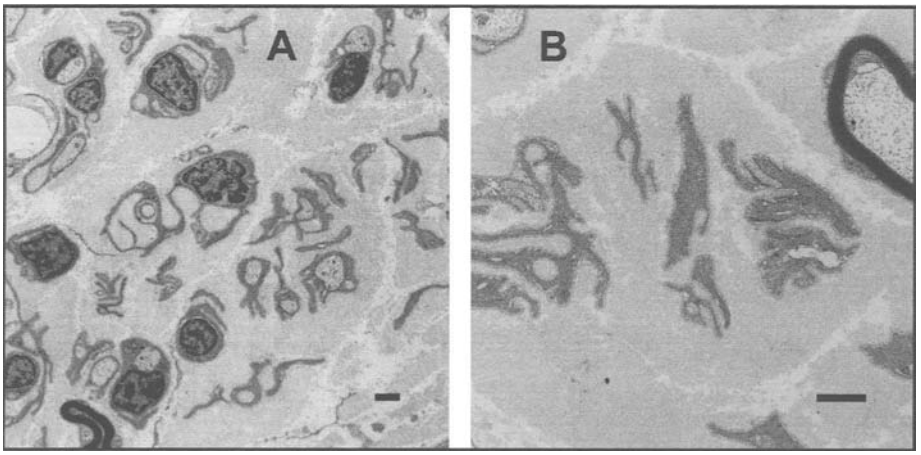

Figure 13. Electronmicroscopical pictures of peripheral nerve biopsy in a patient with hereditary sensory neuropathy with progressive external ophthalmoplegia, ataxia and fatal myoclonic epileptic status. A) severe diffuse loss of myelinated fibers and augmentation of collagen fibers. B) detail with rarefaction of unmyelinated fibers and empty Schwann cell processes. Bar= 1 μm.

However, some cases of hypertrophic cardiomyopathy have been diagnosed after biochemical examination of a myocardial biopsy.[62] The heart in OXPHOS disease shows concentric hypertrophy of the myocardium without an outflow tract obstruction. Hypertrophy of the myocardium appears to result from swelling of the cardiomyocytes caused by accumulation of mitochondria and by morphologically abnormal megamitochondria.[63] The latter is also present in Barth syndrome. In other cases mitochondrial depletion has been observed.

When the smooth muscle is an important target organ of the OXPHOS disease, rectum biopsy may offer diagnostic clues.[64] In these instances vacuolation of smooth muscle is present, on electron microscopy accumulation of mitochondria is observed. Ultimately some of the cells become necrotic.

Mitochondrial respiratory chain disorder may also cause liver failure. In these patients oncocytic transformation of hepatocytes has been found in combination with microvesicular steatosis, cholestasis, and focal cytoplasmic biliary necrosis.[65] These findings are not specific, but in an appropriate clinical context can lead to the diagnosis of a respiratory chain disorder.

The most common renal manifestation of OXPHOS diseases is a proximal tubulopathy.[66,67] Renal biopsy shows non-specific abnormalities of the tubular epithelium, but giant mitochondria are often observed.[66] Glomerular diseases with nephrotic syndrome and a tubulointerstitial nephropathy have also been described. In those cases the renal biopsies are not specific at all for an OXPHOS disease.[68]

Conclusion and Future Perspectives

This chapter reviewed the histopathological alterations of several organs in OXPHOS diseases. From the viewpoint of the pathologist, the muscle gives the most valuable information for the diagnosis of these disorders. As the muscle biopsy is an invasive diagnostic procedure, reducing the number of biopsies may be an important issue. Therefore, the search for non-invasive methods that could give more specific information about the energy metabolism and so reduce the necessity to perform muscle biopsies as a mere screening method, remains paramount. The use of magnetic resonance spectroscopy and new and faster methods in genetic testing on leukocytes for mitochondrial and nuclear encoded mitochondrial proteins may open new horizons. However, another goal for ongoing and future research, is to augment the sensitivity of

the methods and so reduce the *false* negative muscle biopsies. In this respect, the application of high pressure freezing of biopsies resulting in better preserved ultrastructural morphology,[69] may give new clues for distinctive electron microscopical features in OXPHOS-diseases, enabling earlier diagnosis. Furthermore, the introduction in the every day diagnostic practice of new immunohistochemical tools such as the already described antibodies against the subunits of COX and probably later on against nuclear encoded subunits of Complex I, II, III or V, and the application of molecular biological tools now used in research, give perspective. Finally, the research field of proteomics may augment the diagnostic yield of the muscle biopsy.

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CHAPTER 5

Biochemical Diagnosis of OXPHOS Disorders

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Abstract

In this chapter the biochemical diagnosis of OXPHOS disorders is presented. The laboratory investigations in suspected patients are started with the examination of body fluids. The most important metabolite to be measured is lactate, that is frequently found to be elevated in blood, urine and cerebrospinal fluid of patients with OXPHOS disorders. The next step in the diagnostic procedure consists of the examination of tissues. The biochemical diagnostic investigations are preferably performed in muscle tissue because in most patients the defect is expressed in muscle. Biopsy material is preferred above autopsy material. Biochemical examination of a fresh muscle sample is to be preferred because mitochondria are intact in fresh muscle thus allowing measurement of the overall oxidative capacity of the mitochondria. In a frozen muscle sample only enzyme activities of the OXPHOS complexes can be measured. In the latter case patients with a disturbance in the oxidative phosphorylation not localized in one of the OXPHOS complexes remain undiagnosed. Practical guidelines for the biochemical examinations of muscle are provided. In certain circumstances it is necessary to examine also fibroblasts. This is an absolute prerequisite in case prenatal diagnosis is requested. The interpretation of the biochemical investigations is discussed with special emphasis on the observed residual enzyme activities.

Introduction

The biochemical laboratory diagnosis of patients suspected to suffer from an OXPHOS disorder on clinical and morphological features consists of metabolic examinations of body fluids and tissues. This chapter will focus on:

- Examinations of body fluids (blood, urine and cerebrospinal fluid) on metabolites that may occur in abnormal quantities in suspected patients.
- Biochemical examinations of tissues (e.g., muscle, liver, fibroblasts) on disturbances in the mitochondrial OXPHOS system.

Selections of the patients to be investigated biochemically can be considered to be the most difficult aspect in the diagnostic procedure. The selection criteria might be too narrow leading patients to be undiscovered. Too wide criteria may give use to redundant biopsies and assays.

The difficulty with regard to the selection of patients to be thoroughly investigated is caused by the tremendous heterogeneity of the clinical pictures of the several OXPHOS disorders.

For a detailed description of the clinical features of these commonly severe diseases, the reader is referred to Chapter 3 of this book.

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Examination of Body Fluids

OXPHOS disorders are frequently associated with increased lactate concentrations in blood, urine and cerebrospinal fluid. The lactate concentration in body fluids is a secondary consequence of a defect in the mitochondrial respiratory chain leading to an increased redoxpotential, intra- and extramitochondrially, defined by the ratio $NADH/NAD^+$. The increased $NADH/NAD^+$ ratio exerts a feedback inhibition on the intramitochondrially localized pyruvate dehydrogenase complex (PDHc) resulting in an increased pyruvate concentration in the mitochondria and subsequently in the cytoplasm of the cell where it is converted to lactate by means of the lactate dehydrogenase (LDH) catalyzed reaction. The increased intracellular lactate and pyruvate concentrations finally result in increased concentrations of these compounds in the extracellular fluid.

So, raised lactate concentrations are an important pointer to OXPHOS disorders.

Concomitant determinations of pyruvate, especially in blood and cerebrospinal fluid may be of additional diagnostic value because many patients with OXPHOS disorders show increased ratios of lactate/ pyruvate (L/P) in blood and cerebrospinal fluid.

This phenomenon can be explained by a shift of the LDH catalyzed reaction towards lactate as a consequence of the increased $NADH/NAD^+$ ratio:

$$\text{Pyruvate} + NADH + H^+ \Leftrightarrow \text{Lactate} + AD^+$$

It should be emphasized, however, that OXPHOS disorders are not always associated with increased lactate concentrations in body fluids or increased L/P ratios. We found, for instance, in 15 percent of the patients with a proven, isolated complex I deficiency no lactate accumulations in body fluids, even not after oral glucose loading.[1]

The absence of lactate accumulations in the fasting state and after a meal, repeatedly established, may give rise to performance of an oral glucose loading test with concomitant measurement of the lactate concentration in blood in case of severe suspicion on an OXPHOS disorder. In some cases an abnormal increase of the blood lactate concentration will occur, necessitating further biochemical examinations of tissue(s) of the patients. No pathological increase does not exclude an OXPHOS- disorder completely. Further diagnostic measures are strongly dependent on the clinical presentation of the patient. Although lactate accumulation in body fluids is an important indication for the suspicion on OXPHOS disorders, other metabolic disorders associated with lactate accumulations have to be excluded, before biochemical examinations of tissues will be performed. The most important ones are pyruvate dehydrogenase complex deficiency, glycogen storage diseases, defects in gluconeogenesis and some organic acid disorders. Besides secondary causes of lactate accumulation should be excluded before, like hypoxia, sepsis, shock and muscle exercise.

Blood lactate concentration is influenced by physical exercise, feeding and stress.

In our experience, resistance to venipuncture, especially in children, can result in increased blood lactate levels. Reliable values for blood lactate in young children can be obtained by insertion of an indwelling intravenous cannula. After 45 minutes of bed-rest, the blood sample can be taken off.

Apart from measurement of lactate in body fluids, it is very worthwhile to measure the concentration of alanine in blood of suspected patients. Alanine concentration is frequently increased in blood of patients with an OXPHOS disorder. This can be explained by transamination of accumulated pyruvate into alanine. Other less frequently observed abnormalities in these disorders are generalized aminoaciduria, abnormal excretion of citric acid cycle intermediates in urine and ethylmalonic aciduria.

Examination of Tissues

Choice of Tissues

The specific biochemical examinations in patients, who are suspected to suffer from an OXPHOS disorder on clinical features and findings of the metabolic screening, are preferably performed in muscle tissue because in most patients the defect is expressed in muscle. Examination of fibroblasts could be considered to be a better alternative because performance of a skin biopsy is less invasive as compared to a muscle biopsy. However, in our experience and from other investigators, defects in the OXPHOS system are not expressed in fibroblasts in a substantial number of patients.

This has the consequence that normal findings in fibroblasts do not exclude a defect in the OXPHOS system in other tissues, like muscle. This so-called tissue-specific expression of these defects is a common feature in OXPHOS disorders. The first patient described with a cytochrome c oxidase deficiency in muscle tissue, showed a normal activity in liver.[2]

This finding has been criticized considerably at the time of publication, but has been accepted as a quite normal phenomenon at present. Several explanations are available to understand the tissue-specific expression of the OXPHOS disorders.

One explanation is based on the discovery of tissue-specific subunits of the complexes of the respiratory chain. This has been demonstrated for subunits VIa and VIIa of cytochrome c oxidase, which have both specific isoforms, a liver (L) and a muscle (M) form. A mutation in the M-form of one of these subunits may lead to a deficiency of cytochrome c oxidase in muscle but not in liver. Another explanation can be that a defect in one of the respiratory chain complexes is caused by a mutation in mitochondrial DNA (Chapter 7).

It has been discussed that the percentage heteroplasmy can differ considerably between several tissues within a patient. This implies that the percentage mutated DNA in a patient in muscle as compared to liver, may lead to an enzymic defect in a respiratory chain complex in case this percentage exceeds the threshold level. On the contrary in liver the percentage heteroplasmy can be below the threshold level, thus resulting in a normal enzymic activity of that complex. The phenomenon of tissue specific expression of OXPHOS disorders must be considered in the choice of the tissue to be investigated. Start of the diagnostic procedure with fibroblasts has the disadvantage that a delay in the diagnostic schedule of several months will be introduced in case no defect appears to be present. The consequence is that a muscle sample must be examined after all, because the defect may be expressed in muscle, but not in fibroblasts. It should be emphasized that the absence of a defect in one tissue doesn't exclude a defect in another tissue. This aspect is of considerable importance when prenatal diagnosis is requested by the parents of an affected child (see Chapter 12). Our criteria for performance of reliable prenatal diagnosis include that the defect in the OXPHOS-system must be present at least in two different tissues, one of them being fibroblasts.

Summarizing it can be stated that muscle tissue is the preferred tissue to be examined but in certain cases other tissues should be investigated as well.

Biopsy Versus Autopsy Material

In general a biopsy specimen is preferred. If only autopsy material is available it should be kept in mind that the activities of the respiratory chain complexes are decreasing after death. So it is very important to perform the autopsy as soon as possible after death. In our center we recommend to carry out the autopsy for biochemical diagnostic purposes within one to two hours after death.

Fresh Versus Frozen Tissue

Biochemical examination of a fresh muscle sample is to be preferred because mitochondria remain intact in this material in contrast to frozen muscle. We advocate examination of a fresh muscle sample because the diagnostic possibilities exceed those of a frozen muscle sample. A fresh muscle sample can be defined as a sample that has been taken off by general or local anaesthesia and is immersed immediately in a vessel with ice-cold medium (preferably SETH-medium, PH 7.4) and immediately transported on ice to the laboratory. The sample must arrive in the laboratory within 2 hours after the biopsy.

Otherwise the sample should be immediately frozen without medium in liquid nitrogen, stored at -80° C and transported as frozen tissue in dry-ice. A sufficient amount of dry-ice is necessary to prevent thawing of the sample before arriving at the laboratory.

The major advantage of investigating a fresh muscle sample is that the oxidative phosphorylation capacity can be measured in intact mitochondria, followed by determination of the activities of the OXPHOS complexes.

In a frozen muscle sample, in which the mitochondria have been destroyed, only enzyme activities can be measured.This means that no information becomes available about the total oxidative capacity of the mitochondria and on the coupling state.

Biochemical Diagnostic Investigations

Fresh Muscle Samples

Currently used methods for assessment of defects in the OXPHOS-system are measurement of oxygen consumption and measurement of the oxidation rates of several substrates by radiochemical methods in combination with the production rate of ATP from mitochondrial substrates.

Polarographic analysis of oxygen consumption by intact mitochondria is a widely used assay to measure several mitochondrial functions.

Measuring the rate of oxygen consumption by mitochondria in the presence of substrates, like malate, pyruvate, succinate and ascorbate plus TMPD reveals disturbances in the OXPHOS-system, in mitochondrial transport systems (including the ATP/ADP translocator), in pyruvate dehydrogenase complex and in the tricarboxylic acid cycle. For instance a diminished oxygen consumption in State 3 condition of mitochondria with pyruvate as substrate may point to a defect in complexes I, III, IV, V, pyruvate dehydrogenase complex and in the ATP/ADP translocator. In combination with other substrates (glutamate, succinate, ascorbate +TMPD) a defect may be approached but must be proven by measurement of the activity of the relevant enzyme complex(es).

In our center a radiochemical method to detect disturbances in the OXPHOS-system has been developed for several decades.[3] The method is based upon measurement of the oxidation rates of several mitochondrial substrates (Fig. 1). In Table 1 the combinations of substrates used have been summarized.

The oxidation rate of pyruvate can be determined by measurement of the $^{14}CO_2$ production with [1-^{14}C] pyruvate as substrate in the presence of malate (Fig. 1A). Malate will be converted during the incubation into oxaloacetate, which reacts with acetyl-CoA, produced from pyruvate, thereby forming citrate.

Malate is functioning as an acetyl-CoA acceptor to prevent accumulation of acetyl-CoA during the incubation. Acetyl-CoA can exert a feedback inhibition on the pyruvate dehydrogenase complex (PDHc) that is circumvented by addition of malate.

In the presence of a defect in the citric acid cycle, for instance in malate dehydrogenase, the production of oxaloacetate from malate will be insufficient to trap all acetyl-CoA produced from pyruvate.

For that reason we use in a parallel experiment an alternative acetyl-CoA acceptor, viz. carnitine (Fig.1B). Carnitine is converted with acetyl-CoA, a reaction catalyzed by carnitine-acetyl-CoA- transferase, into acetylcarnitine and CoA. In this case the acetyl-CoA acceptor is functioning independent of the citric acid cycle.

Table 1. Measurement of the oxidation rates of mitochondrial substrates in muscle tissue

Substrates	$^{14}CO_2$ Production Reduced in Case of a Deficiency in the:		
1 [1-^{14}C] pyruvate + malate	PDHc	citric acid cycle	OXPHOS system
2 [1-^{14}C] pyruvate + carnitine	PDHc		OXPHOS system*
3 [U-^{14}C] malate + pyruvate + malonate	PDHc	citric acid cycle	OXPHOS system
4 [U-^{14}C] malate + acetylcarnitine + malonate		citric acid cycle	OXPHOS system
5 [U-^{14}C] malate + acetylcarnitine + arsenite		citric acid cycle	OXPHOS system
6 [1,4-^{14}C] succinate + acetylcarnitine		citric acid cycle	OXPHOS system

* The $^{14}CO_2$ production does not necessarily be reduced in case of an OXPHOS system defect.

Application of two different acetyl-CoA-acceptors enables us to differentiate between reduced $^{14}CO_2$ production from pyruvate caused by a PDHc deficiency or a disturbance in the citric acid cycle.

In case of a PDHc deficiency both incubations 1 and 2 yield a reduced $^{14}CO_2$ production from pyruvate (Table 1). Is a disturbance present in the citric acid cycle, the $^{14}CO_2$ production from pyruvate in the presence of carnitine is in the normal range (incubation 2), whereas a reduced $^{14}CO_2$ production will be measured in the presence of malate (incubation 1).

It has to be mentioned here, that the PDHc does not only experience a feedback-inhibition by acetyl-CoA but also by NADH. This implies that in case of a defect in one or more of the respiratory chain complexes NADH will accumulate, thus resulting in inhibition of the PDHc, which is reflected by reduced $^{14}CO_2$ production in the presence of both acetyl-CoA-acceptors, malate and carnitine (incubations 1 and 2). In the presence of carnitine (incubation 2) however, the $^{14}CO_2$ production rate normally exceeds that in the presence of malate (incubation 1). The $^{14}CO_2$ production may be found even in the normal range in spite of a respiratory chain defect in the presence of carnitine (incubation 2). This can be explained in the following way.

In the presence of carnitine only one molecule NADH is produced pro molecule pyruvate oxidated. In the presence of malate, however, as acetyl-CoA acceptor, additional molecules of NADH will be produced as a result of the malate oxidation in the citric acid cycle (incubation 1). In the latter case the residual activity of the respiratory chain may be insufficient to reoxidize the NADH produced thus resulting in inhibition of the PDHc. In the presence of carnitine the lower amount of NADH produced can be reoxidized substantially by the respiratory chain resulting in a less pronounced inhibition of the PDHc, reflected by a higher $^{14}CO_2$ production (incubation 2) as compared to incubation 1. Depending on the residual activity of the disturbed respiratory chain complex the $^{14}CO_2$ production rate from [1-^{14}C] pyruvate in the presence of carnitine may even be normal.

Summarizing the biochemical findings obtained by measurement of the $^{14}CO_2$ production from pyruvate it can be concluded that reduced $^{14}CO_2$ production in the presence of both malate or carnitine points to a PDHc deficiency or a respiratory chain defect. In case of the latter the $^{14}CO_2$ production (incubation 2) in the presence of carnitine can be normal if a relatively high residual activity is present. A reduced $^{14}CO_2$ production in the presence of malate (incubation 1) but a normal one in the presence of carnitine points to a defect in the citric acid cycle or in the respiratory chain.

The second part of our diagnostic program is based on measurement of the oxidation of [U-^{14}C] malate in the presence of an acetyl-CoA-donor. The latter is necessary to convert oxaloacetate, formed from malate, together with acetyl-CoA into citrate.

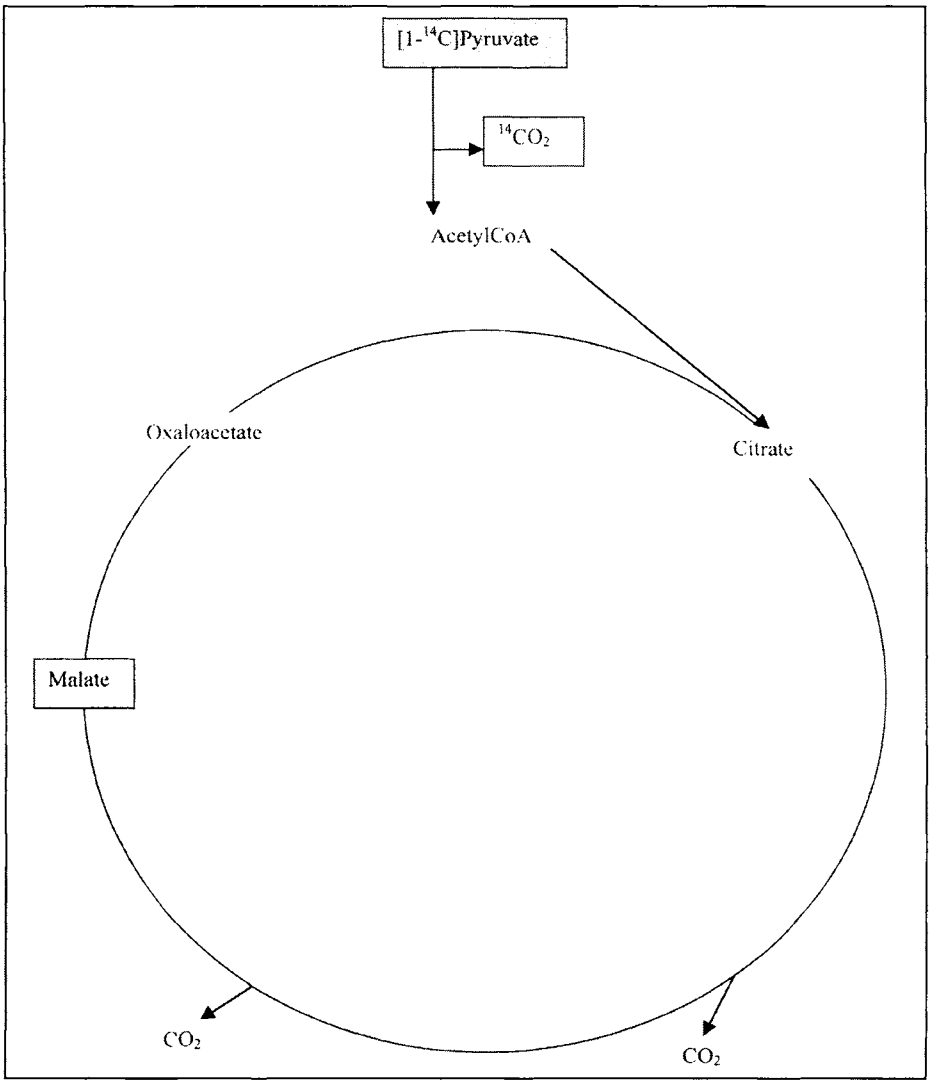

Figure 1A. A radiochemical method to detect disturbances in the OXPHOS system. The oxidation rate of pyruvate.

Pyruvate can be used as acetyl-CoA-donor (incubation 3, Table 1). Pyruvate is converted into acetyl-CoA by the PDHc (Fig. 1C). However, in case of a PDHc deficiency pyruvate does not yield sufficient acetyl-CoA to convert the produced oxaloacetate immediately into citrate.

For that reason an alternative acetyl-CoA-donor, that is functioning independent of the PDHc, is used, viz. acetylcarnitine (incubation 4, Table 1). Acetylcarnitine is converted into acetyl-CoA and carnitine, in the presence of CoA, by the enzyme carnitine-acetyltransferase (Fig.1D). In this way one can differentiate between a defect in the citric acid cycle or in the PDHc. In the presence of a respiratory chain deficiency the malate oxidation, measured by means of the $^{14}CO_2$ production from [U4-^{14}C] malate, is diminished in the presence of both pyruvate or acetylcarnitine as acetyl-CoA donor (incubations 3 and 4, Table 1).

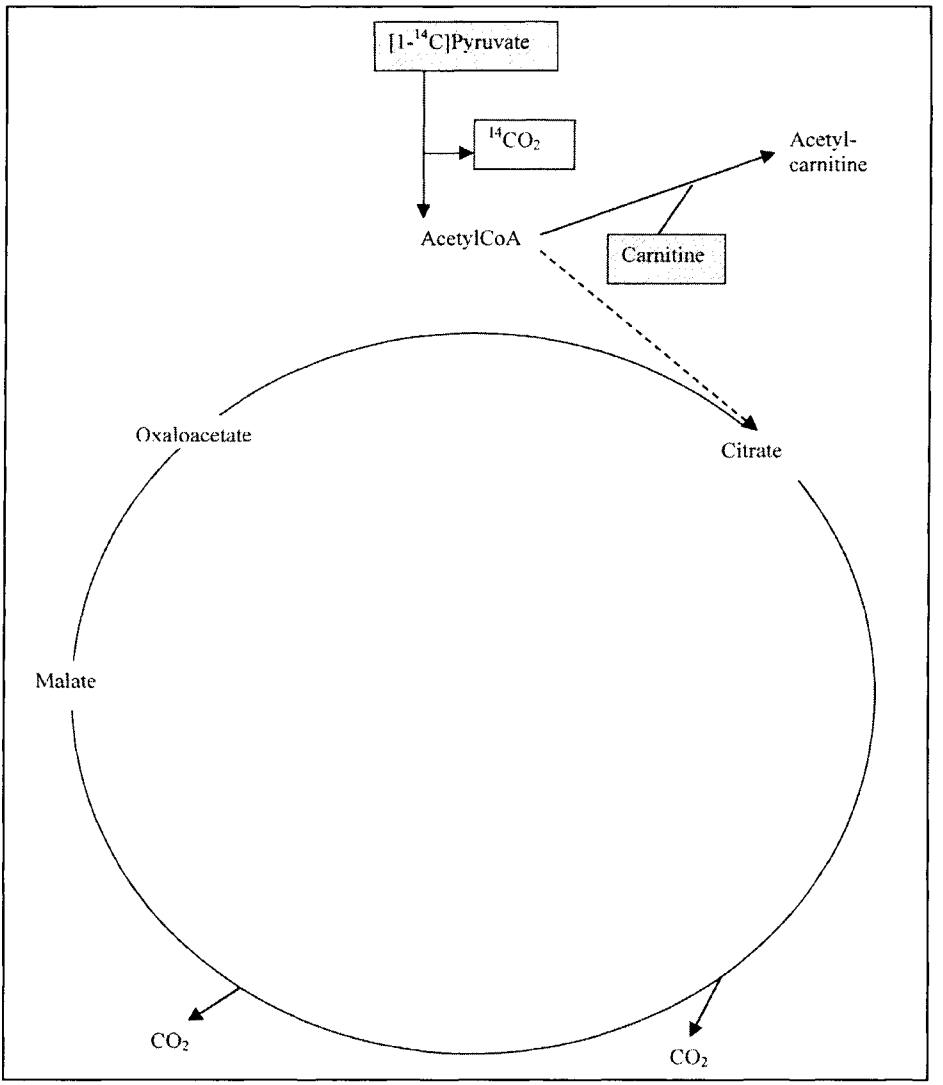

Figure 1B. A radiochemical method to detect disturbances in the OXPHOS system. Carnitine as an alternative acetyl-CoA acceptor.

The experiments with [U-^{14}C] malate as substrate are performed in the presence of malonate as a competitive inhibitor of succinate dehydrogenase. In this manner dilution of the specific activity of [U-^{14}C] malate by malate produced in the citric acid cycle is prevented. This implies that only disturbances in the citric acid cycle between malate and succinate are included in the diagnostic process.

To prove a defect in the conversion of succinate to malate, the $^{14}CO_2$ production from [1,4-^{14}C] succinate is measured (Fig. 1E). A reduced $^{14}CO_2$ production points to a defect in either succinate dehydrogenase (complex II) or fumarase when the [U-^{14}C]malate oxidations are normal.The incubation with [1,4-^{14}C] succinate is also performed in the presence of acetylcarnitine as acetylCoA-donor (incubation 6, Table 1).

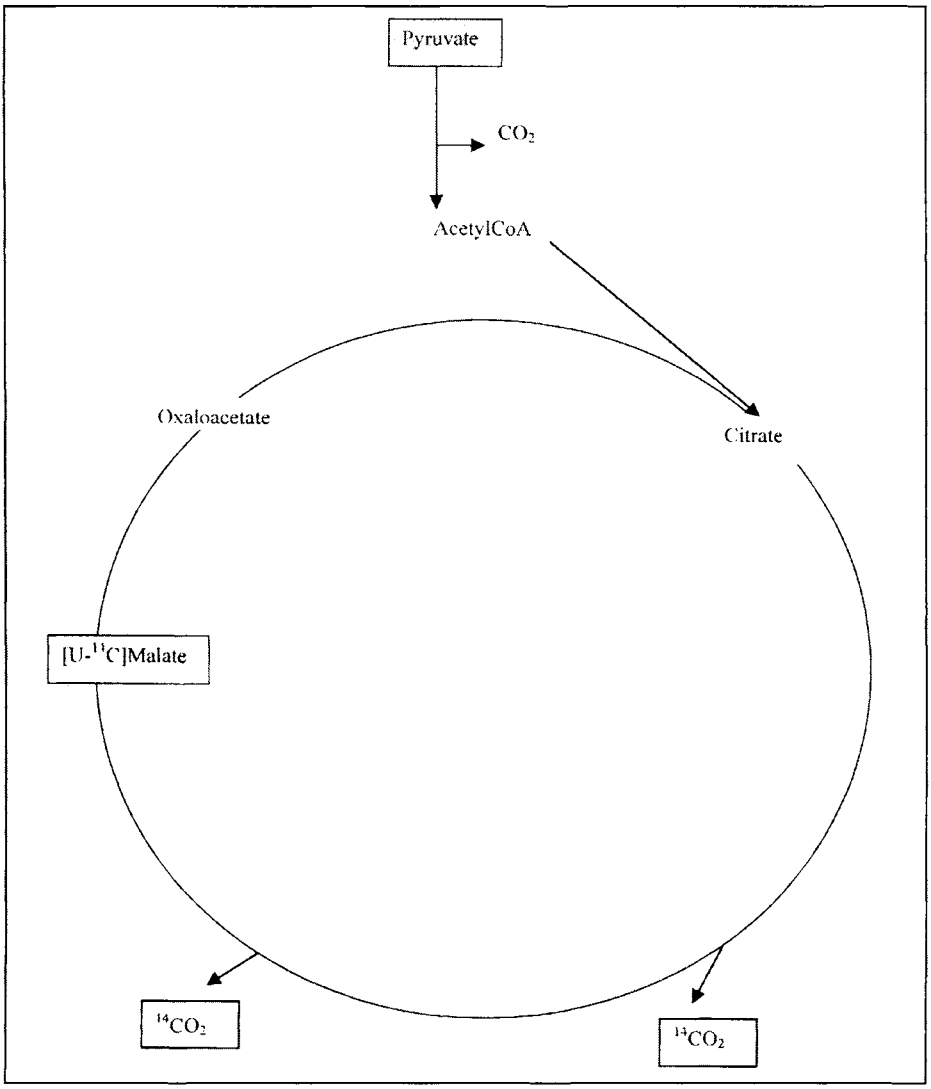

Figure 1C. A radiochemical method to detect disturbances in the OXPHOS system. Pyruvate is converted into acetyl-CoA by the PDHc.

In our diagnostic procedure we include also an experiment in which the oxidation of [U-^{14}C] malate in the presence of acetylcarnitine is measured with arsenite instead of malonate as inhibitor of the citric acid cycle (Fig. 1F). Arsenite inhibits in this case the 2-oxoglutarate dehydrogenase complex thus inhibiting the conversion of 2- oxoglutarate into succinylCoA.

Under normal conditions the $^{14}CO_2$ production with malonate as citric acid cycle inhibitor is twice as high as that in the presence of arsenite. In the latter case only one molecule $^{14}CO_2$ is produced from one molecule [U-^{14}C] malate oxidized whereas in the presence of malonate an additional molecule $^{14}CO_2$ is produced at the level of 2- oxoglutarate dehydrogenase. In case of a defect in 2- oxoglutarate dehydrogenase the ratio of the $^{14}CO_2$ production in the presence of

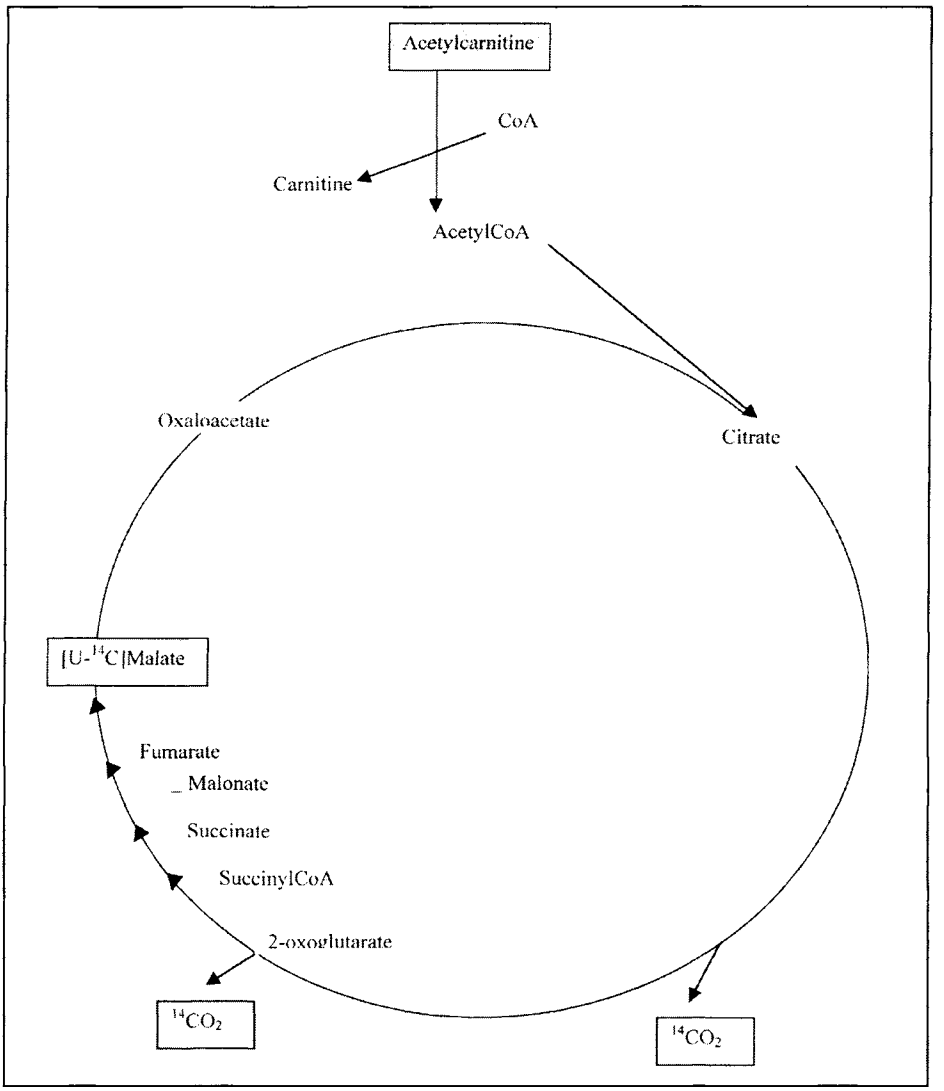

Figure 1D. A radiochemical method to detect disturbances in the OXPHOS system. Acetylcarnitine, an alternative acetyl-CoA donor independent of PDHc, is converted into acetyl-CoA and carnitine by the enzyme, carnitine acetyltransferase.

either malonate or arsenite is approximately 1. In this manner we can also include a 2-oxoglutarate dehydrogenase deficiency in our diagnostic procedure.

The aforementioned experiments are performed in the presence of ADP to measure the oxidative phosphorylation capacity under state 3 conditions.

The oxidation rate of [1-^{14}C] pyruvate+malate is measured both in the presence and absence of ADP to determine the stimulation of the oxidation rate by addition of ADP. Under normal conditions the stimulation by ADP varies between 3 to 8. In case the mitochondria are uncoupled or loosely-coupled the ADP stimulation is decreased or even absent.

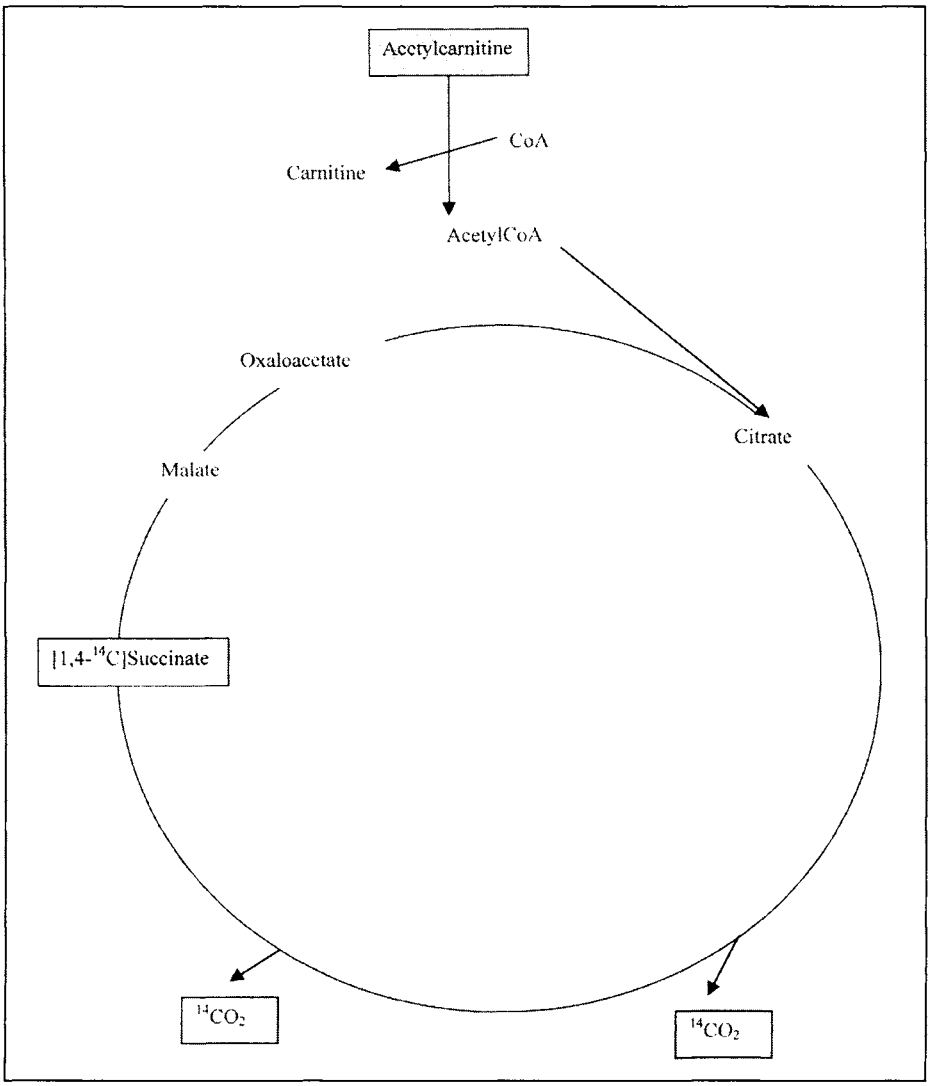

Figure 1E. A radiochemical method to detect disturbances in the OXPHOS system. To prove a defect in the conversion of succinate to malate, the $^{14}CO_2$ production from [1,4-^{14}C] succinate is measured.

Our diagnostic program in the examination of fresh muscle samples involves also the measurement of the production rate of ATP + CrP from pyruvate. This biochemical parameter provides also important information on the oxidative capacity of mitochondria. In our diagnostic procedure the production rate of ATP + CrP from pyruvate is determined in the presence of malate, ADP, creatine and AP_5A (di-adenosine-pentaphosphate). The latter is a specific inhibitor of myoadenylate kinase, the enzyme catalyzing the conversion of ADP into

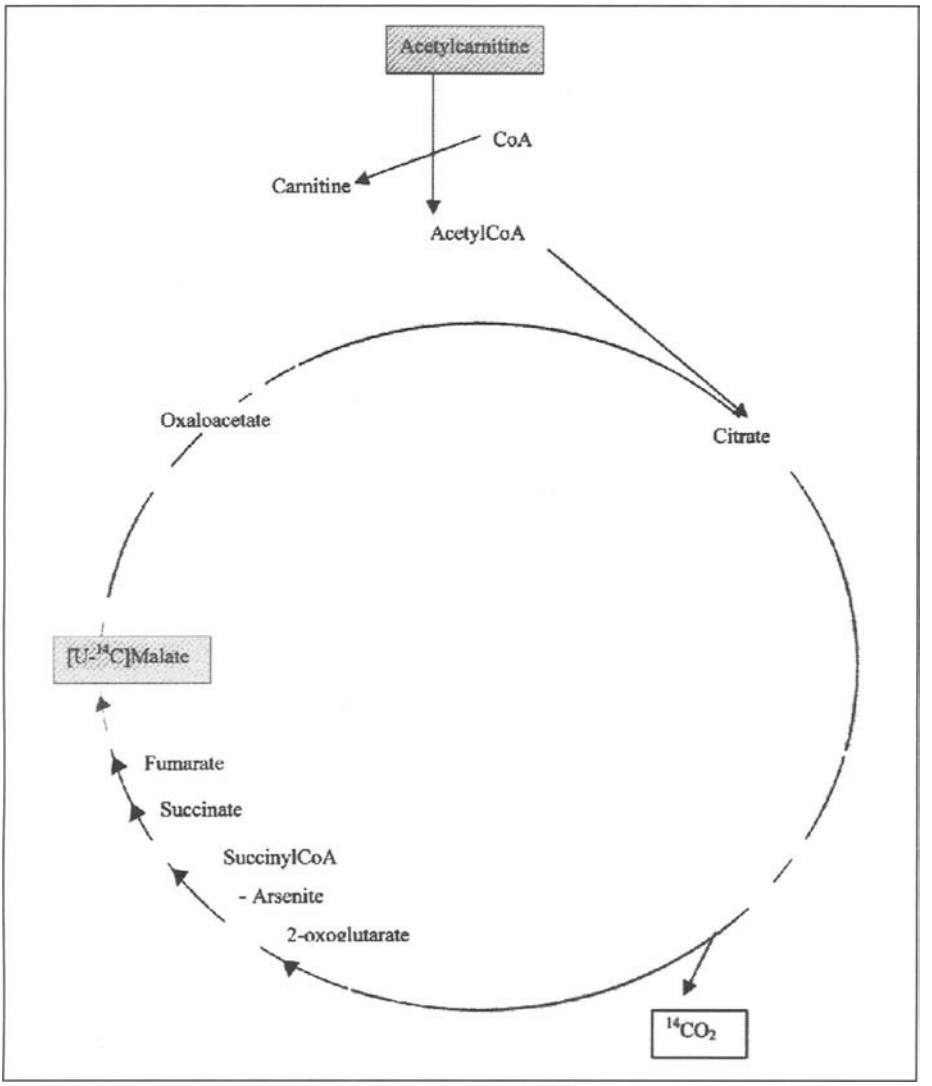

Figure 1F. A radiochemical method to detect disturbances in the OXPHOS system. The oxidation of [U-^{14}C] malate in the presence of acetylcarnitine is measured with arsenite instead of malonate as an inhibitor of the citric acid cycle.

ATP + AMP. Addition of AP_5A prevents the rapid decrease of ADP as a result of this conversion. Creatine is added to the incubation mixture to regenerate the ADP used for ATP production, thus keeping ADP at optimal level for oxidative phosphorylation:

$$\text{Creatine} + \text{ATP} \overset{\text{CK}}{\leftrightarrow} \text{Creatinephosphate (CrP)} + \text{ADP}$$

In case of a deficiency in the OXPHOS-system (except for complex II deficiency), in the citric acid cycle or in the PDHc the production rate of ATP + CrP from pyruvate is always diminished. Besides a reduced production of ATP + CrP from pyruvate may point to a defect in the pyruvate transporter or a defect in ANT (adenine nucleotide translocator). When the mitochondria are uncoupled no ATP + CrP production from pyruvate will take place.

We observed a very good correlation between the measured subtrate oxidation rates and the production rate of ATP + CrP from pyruvate. This implies that both biochemical parameters are used to establish whether or not a defect is present in the OXPHOS-system.

Frozen Muscle Samples

It has been stated before that examination of a fresh muscle sample is highly preferred in the diagnostic procedure of OXPHOS disorders. The diagnostic possibilities in case only a frozen muscle sample is available are very limited because only measurement of the enzyme activities of the OXPHOS-complexes can be performed. In frozen samples the integrity of the mitochondrial innermembrane is destroyed resulting in an artificially induced disturbance of the oxidative phosphorylation system. It should be kept in mind that, at least in our experience, in a substantial number of patients, in which a defect in the oxidative capacity of the mitochondria has been shown, no defect in the complexes of the OXPHOS-system could be established.

This means that in such cases it is only justified to draw a conclusion about the absence of a defect in one or more of the OXPHOS-complexes. Normal values for the activities of these complexes do not rule out a defect in the OXPHOS-system. Unfortunately the geographic distance between the hospital, in which a suspected patient has to be biopsied, and the diagnostic center is frequently too large to guarantee arrival in that center in time. We recommend to admit such patients for a few days to a center to be biopsied, thus enabling us examination of a fresh muscle sample. This procedure is, however, rather expensive.

Measurement of the Enzyme Activities of the OXPHOS-Complexes

The final step in the biochemical diagnostic procedure of mitochondrial cytopathies consists of establishment of the specific defect(s) in one or more of the OXPHOS-complexes.

Numerous procedures for measurement of these enzyme activities have been reported in literature. In the majority of these assays spectrophotometric assays are used:

Complex I (NADH: Q_{10} Oxidoreductase)

The activity of complex I can be determined by measurement of the rotenone-sensitive NADH oxidation in skeletal muscle with the coenzyme Q_{10} analogue, Q_1, as final electron acceptor. Under these circumstances the specific oxidation of NADH by complex I is measured.[4] The NADH oxidation is measured both without addition of rotenone and in the presence of rotenone. The difference between these measurements yields the specific oxidation of NADH by complex I.

Complex II (Succinate Dehydrogenase Complex)

The activity of complex II can be measured by investigation of the succinate oxidation in skeletal muscle using 2,6 dichlorophenol indophenol (DCPIP) as final electron accceptor.[5] The electrons released during the conversion of succinate to fumarate are transferred to CoQ. The reduced form $CoQ_{10}H_2$ is reoxidized by DCPIP. The reduction of DCPIP is registrated at 600 nm. To correct for the aspecific DCPIP reduction the incubation is also performed in the presence of malonate, a specific inhibitor of complex II. The difference between both measurements yields the specific DCPIP reduction by complex II.

Complex III(Decylubiquinol Oxidoreductase)

Complex III activity can be determined using the artificial substrate decylubiquinone-H_2 (DUH_2) in the presence of oxidized cytochrome c and Na-azide. DUH_2 is oxidized by complex III, followed by reduction of cytochrome c. The increase in absorbance at 550 nm of

reduced cytochrome c is a measure for the activity of complex III. Na-azide is added to inhibit cytochrome c oxidase, thus preventing oxidation of reduced cytochrome c by cytochrome c oxidase.Antimycine, a specific inhibitor of complex III, is added to the blank incubation mixture.

Complex IV (Cytochrome c Oxidase)

The activity of complex IV can be determined by measurement of the oxidation of reduced cytochrome c (ref. 6).

Complex V

The activity of complex V (F_1F_0-ATPase) is usually measured in the reversed direction of the physiological function: the hydrolysis of ATP into ADP + Pi. So ATP is used as substrate in the presence of Mg^{2+}. The produced ADP is reconverted into ATP according to the following reaction:

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}$$

The produced pyruvate is in the final step reduced to lactate:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{NAD}^+ + \text{lactate}$$

(PEP= phosphoenolpyruvate; PK= pyruvate kinase; LDH= lactate dehydrogenase). The oxidation of NADH and the subsequent decrease of the absorbance of NADH at 340nm is a measure for the complex V activity. Other ATPases are specifically inhibited: Na^+-K^+-ATPase by ouabaïn and Ca^{2+}-ATPase by EGTA (ethyleneglycol tetraacetic acid).

AP_5A (adenosine-5^1-triphosphate) is added to inhibit the adenylate kinase (AK) catalyzed conversion of ADP into ATP and AMP:

$$2\text{ADP} \xrightarrow{\text{AK}} \text{ATP} + \text{AMP}$$

Rotenone is added to inhibit activity of complex I, thus preventing oxidation of NADH by the electron transport chain. Finally oligomycine is added to the incubation mixture. Oligomycine inhibits specifically complex V activity. The residual activity after addition of oligomycine does not reflect complex V activity and a correction must be made for this activity.

In our diagnostic procedure the assay for measurement of the succinate: cyt c oxidoreductase (SCC) activity is included. Performing this assay the metabolic route complex II →CoQ→ complex III is studied. The SCC assay forms an obligatory part of our diagnostic procedure especially in case only small muscle samples are available. In that case the sample amount is too small to prepare a 600xg supernatant for measurement of the individual activities of complexes II and III. This implies that only a total homogenate can be examined in which complex II and complex III can't be measured reliably. Both assays are vulnerable to disturbances by particles present in the homogenate. In the total homogenate the SCC assay can readily be performed. A diminished SCC activity may point to a deficiency of complex II, complex III or coenzyme Q. In certain cases complex II and/or complex III activity can consecutively be measured in a small aliquot of a 600xg supernatant prepared from the remaining total homogenate sample. Alternatively a new muscle biopsy has to be performed to measure both activities.

It is absolutely necessary to measure all complexes of the OXPHOS system in each muscle sample to be investigated because in many patients no isolated deficiency of one of the complexes is present but combined deficiencies.

In our experience various combinations of defects may occur.[7] Most frequently a combined deficiency of complexes I and complex IV has been found but numerous other combined defects have been reported, including defects of the OXPHOS complexes with a PDHc deficiency.[8] There are several explanations for the occurrence of multiple defects of respiratory chain complexes. There may be a deficiency of a mitochondrial protein that is involved in the assembly of more then one of the complexes or in the transport of the nuclear encoded subunits across the mitochondrial membranes. Mutations in the mitochondrial DNA, like the various tRNA mutations, may also account for the phenomenon of multiple defects especially in cases in which all complexes of the OXPHOS system are involved apart from complex II. The latter one consists only of nuclear encoded subunits, whereas the other complexes are encoded both by nuclear and mitochondrial genes. Whatever the nature of these multiple deficiencies, the whole diagnostic program should be performed in every patient, even when a specific defect has already been established.

Practical Guidelines for Biochemical Examinations of Muscle

Reliable diagnostic examinations of muscle tissue with regard to OXPHOS-disorders require performance under accurate, standardized assay conditions.[9]

The quadriceps or soleus muscle are the preferred muscle types to be investigated. It goes without saying that the reference values must originate from the same muscle samples. A major problem in the biochemical examinations of muscle samples from patients suspected to suffer from an OXPHOS-disorder consists of collection of reliable reference values. Muscle samples from healthy subjects are hardly available. In our center we gathered reference values by examination of muscle tissue obtained from patients who underwent orthopedic surgery or patients who were biopsied in view of diagnostic investigations on the presence of malignant hyperthermia.

It is very important to send a muscle sample of sufficient size, enabling us to perform the whole diagnostic program. In our center a sample of at least 300 mg is required for examination of a fresh muscle sample. In that case the whole program, consisting of measurement of the substrate oxidation rates, the production rate of ATP+CrP and determination of the activities of the OXPHOS complexes can be performed. In case a substantial lower amount is send to our laboratory, only enzyme activities of the OXPHOS complexes can be determined. The quality of the sample is also very important.

The sample must be free of connective tissue and fatty tissue as far as possible. The transport conditions are very critical. In case a fresh muscle sample has to be investigated, the muscle specimen must be placed immediately after the biopsy in a vial containing ice cold SETH-buffer, cooled by a sufficient amount of melting ice and sent to the diagnostic laboratory by courier. It is absolutely necessary that the sample does not arrive in the laboratory later than 2 hours after the biopsy. For frozen muscle samples the biopsy must be frozen immediately in liquid nitrogen and should be transported in a vial, without medium, that is placed in a sufficient amount of dry ice to keep the sample in a frozen state until arrival in the laboratory.

It is highly recommended to perform the muscle biopsy in a suspected patient at the age above one month because we established an age-dependent development of the activities of the OXPHOS complexes in the neonatal period, especially in preterm neonates.[10]

Recently[11] no correlation with age of these enzyme activities has been reported. The authors investigated skeletal muscle biopsies from 201 patients between 0 and 65 years old.

However, only very few (two to five) patients were included in this study in the age group below one month. It is therefore not justified to draw a conclusion about age dependency in this very young age group. In case of a life- threatening course of a disease in a patient, a muscle biopsy should be performed as soon as possible.

Medication must be avoided as much as possible before performing the biopsy because a defect may be masked by treatment (e.g., vitamins, coenzyme Q, etc.) or even induced (e.g., sodium valproate).

Table 2. Indications for biochemical examinations of fibroblasts

- If no muscle sample is available.
- If prenatal diagnoses is required.
- To confirm diagnostic findings obtained in muscle, especially if no clear-cut conclusion can be drawn.
- To perform molecular-genetic investigations.
- For research purposes.

Investigation of Fibroblasts

It has been stated before that biochemical diagnostic investigations focused on OXPHOS-disorders should preferably be performed in muscle tissue. However, there are indications to investigate fibroblasts (Table 2).[9]

There are several objections against performance of biochemical examinations in fibroblasts as a first diagnostic approach.

The main objection is that a defect present in muscle is not always expressed in fibroblasts due to the occurrence of tissue-specific expression of OXPHOS-defects. This implies that normal findings in fibroblasts do not exclude one or more defects in the OXPHOS complexes in muscle. An additional disadvantage of examination of fibroblasts consists of the observation that defects established in muscle are frequently less pronounced in fibroblasts. In case of multiple defects found in muscle only a single defect may occur in fibroblasts. In the latter case the molecular genetic approach may be inadequate. If prenatal diagnosis is required it is an absolute prerequisite to demonstrate the defect(s) both in muscle (or any other organ like liver, kidney) and in fibroblasts.

Absence of a defect in fibroblasts excludes performance of prenatal diagnosis in native chorionic villi (see Chapter 11) because normal findings in chorionic villi may be consistent with one or more defects in muscle.

It is usual in our policy to pay attention to the necessity of examination of fibroblasts if there are indications in a certain family on future pregnancies.

Fibroblasts are very suitable for molecular-genetic analysis. The advantage above muscle tissue as a diagnostic target for this purpose is that fibroblasts can be easily cultivated in sufficient amounts even decades after death of the patient. This has been demonstrated recently in our department in a patient with Leigh syndrome associated with a cytochrome c oxidase deficiency in skeletal and heart muscle but not in liver.

The clinical and biochemical findings of this patient have been reported in 1977.[2]

Recently the cytochrome c oxidase deficiency could be attributed in fibroblasts of the patient to a mutation in a SURF-1-gene.

We recommend to always store fibroblasts or a skin biopsy from every patient who might become a candidate for molecular-genetic and cell-biological studies. This holds also for every patient who is suspected of dying from a mitochondrial cytopathy because future diagnostics can be applied to subsequent relatives.[9]

Residual Enzyme Activity

Interpretation of the biochemical measurements in view of the diagnostic procedure of OXPHOS disorders is of utmost importance to draw a definite conclusion. In many cases a considerable residual activity is observed .In our diagnostic program to be performed in a fresh muscle biopsy the influence of a relatively mild deficiency of one or more complexes of the OXPHOS-system on the oxidative capacity in vitro can be established. In nearly all patients decreased oxidation rates and a reduced ATP+CrP production rate is observed in such cases, thus confirming the importance of a mild deficiency on the oxidative phosphorylation

capacity, at least under optimal conditions in vitro. Additional evidence has been obtained from our examinations of fibroblasts of suspected patients.[9]

We established an isolated complex I deficiency in 55 patients with a residual complex I activity varying between 18 and 89% of the lowest control value. We established in 13 of the 55 patients a mutation in one of the nuclear encoded subunits of complex I. In these 13 patients the residual complex I activity varied between 32 and 82%. From this observation it is justified to conclude that, at least in fibroblasts, patients with a proven mutation in a nuclear encoded complex I gene may exhibit a relatively high residual enzyme activity, even exceeding 80%. In addition we observed a high residual activity of complex I in chorionic villi of two pregnant women who gave birth to a child with a complex I deficiency. The residual activity in the chorionic villi amounted to 75% and 81%, respectively. Both pregnancies were continued and in both cases a severe complex I deficiency was found in the newborn child. These cases illustrate that also in chorionic villi a high residual activity has a pathological significance.

Our findings are not in agreement with those reported in literature.[11] which stated that only patients presenting extremely low activity of one or more of the respiratory chain complexes could be confidently diagnosed, if focusing on residual activity values only. We proved that even high residual activities can be used to make a final diagnosis.

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CHAPTER 6

Mitochondrial DNA and OXPHOS Disorders

Massimo Zeviani and Valerio Carelli

Abstract

Mitochondrial OXPHOS is at the crosspoint of two quite different genetic systems, the nuclear genome, and the mitochondrial genome (mitochondrial DNA, mtDNA). The latter encodes a few essential components of the mitochondrial respiratory chain, and has unique molecular and genetic properties that account for some of the peculiar features of mitochondrial disorders. Although mitochondrial disorders have been known for more than thirty years, a major breakthrough in their understanding has come much later, with the discovery of an impressive, ever increasing number of mutations of mitochondrial DNA. Partial deletions or duplications of mtDNA, or maternally inherited point mutations, have been associated with well-defined clinical syndromes. Given the complexity of mitochondrial genetics and biochemistry, the clinical manifestations of mitochondrial disorders are extremely heterogeneous. They range from lesions of single tissues or structures, such as the optic nerve in Leber's hereditary optic neuropathy, or the cochlea in maternally-inherited nonsyndromic deafness, to more widespread lesions including myopathies, encephalomyopathies, cardiopathies, or complex multisystem syndromes. The recent advances in genetic studies provide both diagnostic tools and new pathogenetic insights in this rapidly expanding area of human pathology.

General Background

Mitochondria are double-membrane cytoplasmic organelles, which provide most of the energy to eukaryotic cells. Modern mitochondria are thought to derive from primordial respiration-competent prokaryotes that were stably incorporated as "endosymbionts" into early respiration-less eukaryotic cells.[1] Respiration consists of a chain of sequentially ordinated redox reactions that utilize the reducing equivalents derived from the oxidative degradation of carbon substrates to convert molecular oxygen, a very active and harmful element, to water. The redox reactions are carried out by four multiheteromeric enzymes, the respiratory chain complexes I, II, III and IV. These structures are embedded in the inner mitochondrial membrane, in close contact with each other and with two small "shuttle" molecules, coenzyme Q (a lipoidal quinone) and cytochrome c. The active sites of the respiratory complexes contain heme and/or nonheme prosthetic groups, bound to the protein moiety. The energy liberated in the redox reactions is partially stored as a transmembrane proton gradient ($\Delta\Psi$), generated by active extrusion of protons from the inner mitochondrial compartment, and is ultimately utilized by complex V, the ATP-synthase, to phosphorylate ADP to ATP.[2] The entire process is known as oxidative phosphorylation (OXPHOS) and provides most of the ATP in the cell.

Mitochondrial respiration is also the main cellular source of toxic by-products, broadly defined as "reactive oxygen species" (ROS). ROS are generated by electrons that leak out of the respiratory chain and react with molecular oxygen, to produce highly reactive oxygen radicals.[3]

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Intramitochondrial antioxidant enzymes are specifically deputed to buffer these highly reactive molecules.[4] For instance, the superoxide anion ($O_2^{\cdot -}$) generated by the "leakage" of the respiratory chain, is converted into hydrogen peroxide (H_2O_2) by the manganese superoxide dismutase (MnSOD). H_2O_2 is further metabolized by glutathione peroxidase (GPx) into H_2O. Alternatively, H_2O_2 may also generate the hydroxyl radical ($OH^{\cdot}$) in the presence of reduced transition metals, through the Fenton reaction. Superoxide $O_2^{\cdot -}$ may also react directly with nitric oxide (NO) to produce peroxynitrite ($ONOO^{\cdot}$). ROS production seems to increase when the respiratory chain is defective, especially in defects of complex I and II, and this may contribute to the pathogenesis of several mitochondrial disorders. ROS can play a physiological role in intracellular signaling,[5] but their overproduction is toxic to the cell, by oxidatively damaging phospholipids, proteins and nucleic acids, including mtDNA.[6] In addition, ROS-mediated oxidative damage of mitochondrial membranes, and swelling of mitochondria caused by reduced ATP supply, can cause the opening of a nonspecific mitochondrial channel, the permeability transition pore (PTP), through which apoptotic factors inside mitochondria can reach the cytoplasm and initiate the apoptotic cascade.[7]

Mitochondrial Genetics

The origin of mitochondria as independent prokaryotic organisms is supported by the notion that biogenesis of mitochondria depends upon complementation of two separate genomes. Most of the mitochondrial proteins, including most of the subunits of the respiratory complexes, are encoded by nuclear DNA (nDNA) genes, and synthesized by cytoplasmic ribosomes, usually as precursors containing a N-terminal extension. The latter serves as a leader peptide, which precisely addresses the protein to mitochondria. Import into mitochondria is carried out by a complex, ATP-dependent transport system, and is followed by a series of posttranslational modifications, including cleavage of the leader peptide, that eventually produce a mature, functional protein.[8] However, mitochondria also contain their own genome, commonly called mitochondrial DNA, mtDNA.[9] The increased dependency of eukaryotic cells upon OXPHOS as the most efficient energy supply, the massive transfer of genetic information from the proto-mitochondrial genome to the nucleus, and, finally, the divergence between the universal and the mitochondrial genetic codes are thought to have determined the perdurance of mitochondria as stable components of modern eukaryotes, and the survival of mtDNA. In vertebrates, mtDNA retains the genetic information to encode a few essential components of the respiratory chain and the RNA apparatus that carries out their autochthonous translation.

The genetics of mtDNA differs from that of nDNA in the following unique properties.

1. The mitochondrial genome is maternally inherited.[10] The mother transmits her oocyte mtDNA to all of her offspring, and her daughters transmit their mtDNA to the next generation. Paternal mtDNA does not contribute to mitochondrial inheritance despite a few sperm mitochondria enter the egg.[11] Recent evidence suggests that paternal mitochondria are ubiquitinated, then recognized and destroyed at very early stages during embryogenesis.[12] Exceptionally, however, paternal transmission of mutant mtDNA has been documented in humans.[13]
2. Mitochondria are polyploid. Each human cell has hundreds of mitochondria, each containing 2 to 10 mtDNA molecules. At cell division, mitochondria and their genomes are randomly distributed to daughter cells.
3. The mitochondrial genome has a much faster evolution rate than that of the nuclear genome.[14,15] This is explained by the fact that, although the mitochondrial γ DNA polymerase retains a proofreading activity,[16] and certain types of repair enzymes have been identified in mitochondrial fractions,[17] mitochondria appear to lack an efficient DNA repair system, based, for instance, on homologous recombination. In addition, the mitochondrial genome lacks protective proteins like histones, and is physically associated with the inner mitochondrial membrane, where it is exposed to ROS-mediated oxidative damage.[18]

4. Normally, the mitochondrial genotype of an individual is composed of a single mtDNA species, a condition known as homoplasmy. However, the intrinsic propensity of mtDNA to mutate randomly can occasionally determine a transitory condition known as heteroplasmy, where the wild-type and the mutant genomes coexist intracellularly. Because of mitochondrial polyploidy, during mitosis the two mtDNA species are stochastically distributed to subsequent cell generations. Eventually, an intracellular genetic drift known as mitotic segregation determines the separation of the two mitochondrial genomes into two distinct cell lineages, each one containing only one mtDNA type.[19] To explain the rapid segregation observed in vertebrate mitochondrial DNA, despite its high copy number and mutational rate, a model based on a "bottleneck" effect has been proposed to occur during oogenesis.[20] Evidence has been provided in mice in support of the existence of this phenomenon as early as the formation of primary oogones.[21] During this stage of female germline development, the number of mitochondria and mtDNA copies is drastically reduced, to less than 200 segregation units per cell. Therefore, a few mitochondria with a reduced mtDNA copy number will segregate into the dividing cells of the female germline, and then expand enormously in the mature egg. This phenomenon can account for the drastic change in mutation loads, observed in different generations of families carrying heteroplasmic mtDNA, and increases the remarkable variability in the phenotypic presentations of mitochondrial disorders. High mutation rate, maternal inheritance, bottleneck-driven meiotic segregation, and absence of recombination cooperate to fix mutations, after a transient period of heteroplasmy, as homoplasmic changes in a given maternal lineage. Eventually, a distinct human mitochondrial haplotype may occur, characterized by the presence of a particular, sometimes unique set of mitochondrial polymorphisms. Furthermore, diagnostic clusters of ancient, well-established homoplasmic polymorphisms are used to define different mtDNA haplogroups, i.e., the major population-specific sublineages of human mtDNA.[22,23] By contrast, deleterious mutations may arise frequently, but are rapidly eliminated by negative selection: therefore, they do not become fixed in any specific mitochondrial haplogroup, but are rather found in different haplogroups, and are often heteroplasmic. Heteroplasmy, distribution among different human populations and specific segregation with the disease, are distinctive features of pathogenic mutations.

Because of mitotic mtDNA segregation and polyploidy, a threshold effect dictates the phenotypic expression of a mtDNA-associated character (Fig. 1). For a given heteroplasmic mutation, only when mutated gene copies accumulate over a certain threshold, the deleterious effects of the mutation will no longer be complemented by the coexisting wild-type (wt) mtDNA, and will be expressed phenotypically as a cellular dysfunction leading to disease.

In addition, the coexistence of wt and mutant mtDNAs within the same organelle can in principle lead to functional complementation (intra-organellar complementation). By contrast, the segregation of mutant vs. wt mtDNA in different organelles will give rise to a population of OXPHOS-incompetent organelles containing homoplasmic mutant mitochondrial genomes, which can then "parasitize" the cell by dissipating the ATP supplied by OXPHOS-competent mitochondria or by anaerobic energy pathways (Fig. 2).

Finally, phenotypic expression will depend upon the nature of the mutation, i.e., its intrinsic pathogenicity, its tissue distribution, and the relative reliance of each organ system on the mitochondrial energy supply. In general, the visual and auditory systems, the central and peripheral nervous systems, the heart, muscle, endocrine pancreas, kidney and liver are, in that order, the organs most sensitive to OXPHOS failure. The influence of nuclear genes, the age and sex of the individual, and environmental factors may also play an important, albeit poorly understood, role in the phenotypic expression of mtDNA mutations.

Sequence and Gene Organization of mtDNA

The sequence of human mtDNA and its genetic organization has been completely worked out.[9] The human mitochondrial genome is a 16569 base-pair (bp) long circle of double-stranded DNA. MtDNA molecules are thought to be physically associated in discrete polyploid "nucle-

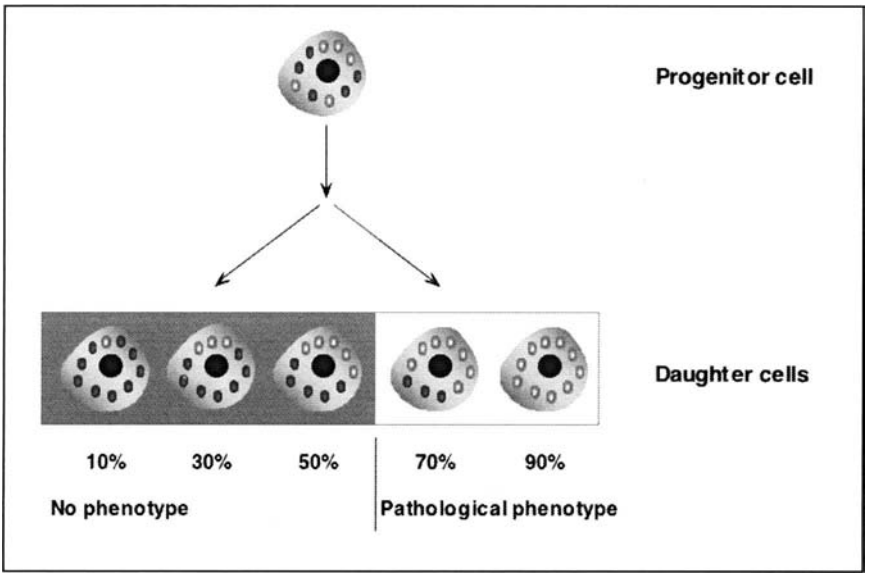

Figure 1. Schematic representation of the threshold effect in a cell lineage. Solid and "empty" organelles represent mitochondria containing "normal" or "pathological" mtDNA, respectively.

oids" anchored to the matrix side of the inner mitochondrial membrane.[24] The existence of these complexes may be important to understand the segregation patterns of mtDNA mutations and also the molecular pathogenesis of some mtDNA rearrangements, such as insertion-duplications or deletion dimers.[25]

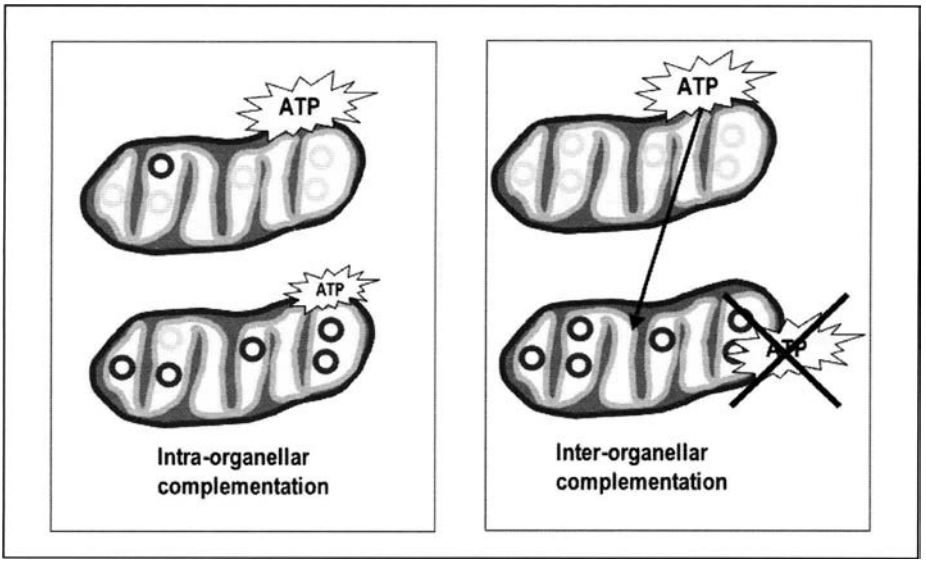

Figure 2. Schematic representation of intra- and inter-organellar complementation. Light and dark circles indicate wild-type and mutant mtDNAs, respectively.

As shown in Figure 3, human mtDNA contains 37 genes, which encode the RNA components of the mitochondrial translational apparatus, i.e., 22 transfer RNA (tRNAs) genes and the 12S and 16S ribosomal RNA (rRNA) genes, as well as 13 polypeptide-encoding genes (mRNAs). The two strands of mtDNA can be separated in alkaline cesium chloride gradients, according to a buoyant density difference due to a strand bias in the G+T content. The so-called heavy (H) strand has a greater density than the light (L) strand. The gene distribution in the H- and L-strands is highly asymmetrical: the H-strand contains 12 of the 13 polypeptide-encoding genes, as well as 14 of the 22 tRNA-encoding genes and both rRNA-encoding genes. All 13 polypeptides are essential components of four of the five complexes that form the mitochondrial OXPHOS pathway. Seven polypeptides, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, are subunits of complex I (NADH-ubiquinone oxidoreductase); cytochrome b, is part of complex III (ubiquinone-cytochrome c reductase); COI, COII and COIII, are the catalytic subunits of complex IV (cytochrome c oxidase), and ATPase 6 and 8 are subunits of Complex V. In order to produce functionally active complexes, the mtDNA-encoded subunits of each complex must interact with several subunits, which are encoded by nuclear DNA (nDNA), and then imported into mitochondria from the cytoplasm. All four subunits of complex II (succinate dehydrogenase-ubiquinone reductase) are nDNA encoded.

The mtDNA of mammalian and other vertebrate cells has an extraordinarily compact gene organization. There are no introns, all of the coding sequences are contiguous with each other,

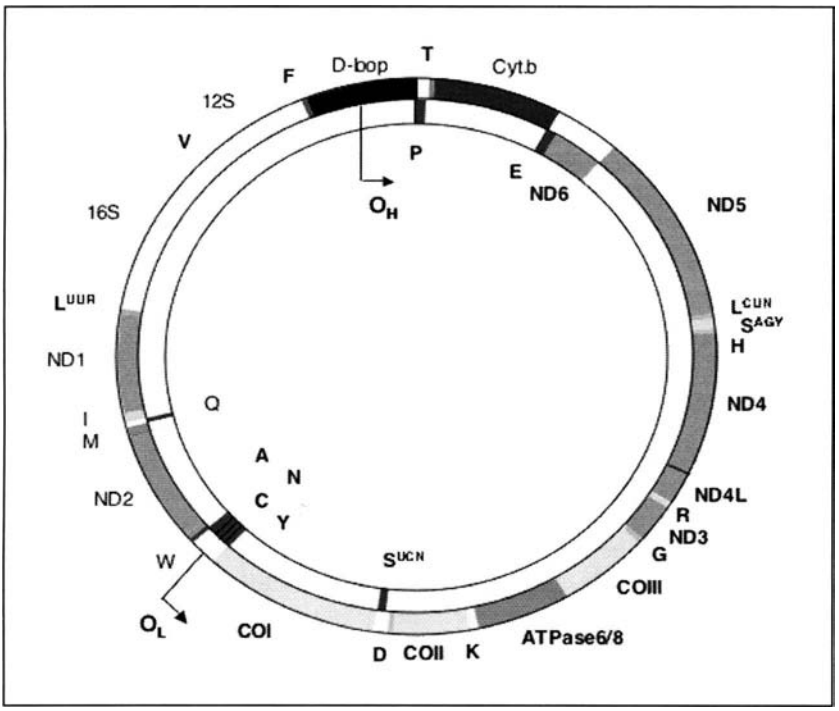

Figure 3. Schematic representation of human mtDNA. O_H, O_L indicate the origins of replication of the H-strand, L-strand; HSP, LSP denote the heavy-strand, light-strand transcriptional promoters; ND1-6 are the genes encoding the subunits of complex I; COI-III those for complex IV; ATPase 6, 8 are the genes encoding subunits 6, 8 of complex V; cyt *b* is the gene encoding cytochrome *b* (complex III); the tRNA genes are indicated by the single-letter code of the corresponding amino acids; 12S, 16S denote the genes encoding the small and large rRNAs.

or are separated by only a few nucleotides, and lack significant nontranslated flanking regions. In most cases, the polypeptide-coding sequences are incomplete, as they lack one or two nucleotides of the termination codon, which must be completed by post-transcriptional polyadenylation. There are only two noncoding stretches in mtDNA of functional significance.[26] One is the displacement-loop (D-loop), a region of about 1 kb which contains the promoters for L- and H-strand transcription and, according to the asymmetric/asynchronous model of mtDNA replication, the origin of replication of the H-strand (O_H). The other important noncoding sequence is a 30 nucleotide (nt)-long region, positioned at 2/3 of the mtDNA length from the O_H. This region, which is surrounded by a cluster of five tRNA genes, is able to form a stable hairpin structure, and was believed to serve as the origin of replication of the L-strand (O_L). However, the "orthodox" asymmetric/asynchronous model of mtDNA replication has recently been questioned and the exact roles of O_H and O_L are currently under investigation.[27] The two rRNA genes, and most of the polypeptide-encoding genes are flanked by regularly interspersed tRNA genes. The punctuation of the genome with tRNA genes is thought to allow the generation of mature RNA species through the action of processing RNA enzymes at the 5' and 3' ends of the tRNAs.[28]

To add uncertainty to the evaluation of mtDNA variations there are several mistakes found in the "Cambridge" mtDNA sequence, the human reference mtDNA sequence that was first published in 1981. In the last few years a number of differences from the Cambridge sequence has been repeatedly reported. Explanations given for these discrepancies include mistakes in the reading of the original sequence, and the presence of an unusual array of polymorphisms. The sequence published in 1981 was a mosaic of sequence from a number of human placentas and HeLa cells, and this could perhaps account for some of the differences. Recently, a revision of the Cambridge mtDNA sequence has been finally published, which corrects numerous errors but maintains for convenience the traditional numbering of the nucleotides.[29]

Mitochondrial Disorders Due to Mutations of mtDNA

General Considerations

Pathogenic mutations of mtDNA are associated with a bewildering variety of multisystemic as well as tissue-specific human diseases. It is indeed a unique case in human medical genetics, and an unexpected finding even for researchers devoted to the study of "mitochondrial genetic medicine", that the small circle of mtDNA has been shown to harbor so many illnesses.[30] A recent epidemiological study carried out in north-eastern England[31] has shown that, overall, 12.48 per 100,000 individuals in the adult and child population either had mtDNA disease or were at risk of developing mtDNA disease. It is of note that these results reflect the minimum prevalence of mtDNA disease and pathogenic mtDNA mutations, and demonstrate that pathogenic mtDNA mutations are a common cause of chronic morbidity.

Nevertheless, known mutations of mtDNA only account for approximately 40% of adult mitochondrial cases. This figure is substantially smaller for mitochondrial disorders of babies and children, where known mtDNA mutations can be found in less than 10% of the cases. A number of recent clinical and molecular observations indicate that many syndromes, especially in infants and children, are due to abnormalities in OXPHOS-related nuclear genes.

The complexity of the biochemical and genetic features of the respiratory chain accounts for the extraordinarily wide range of clinical presentations of mitochondrial disorders.[32] In general, organs with the highest aerobic demand, such as skeletal muscle, brain and heart, are the most severely involved, although many exceptions are reported. In addition, since these organs are composed of highly-specialized, post-mitotic cells, negative selection against cells containing faulty mitochondria is impossible. As a result, abnormal organelles proliferate in these organs, often at an accelerated rate, probably as a compensatory, but ineffective, mechanism against OXPHOS impairment. In the case of skeletal muscle, mitochondria of larger size and/or bizarre shape can ultimately fill discrete portions of the sarcoplasm, producing the well-known morphological hallmark of many mitochondrial myopathies, i.e., the ragged-red transformation of the muscle fibres.

Each tissue can be affected alone ("pure" mitochondrial myopathies, neuropathies, encephalopathies or cardiomyopathies), or more often in combination with each other (mitochondrial encephalo-myopathies and encephalo-cardio-myopathies). Hence, muscle weakness with or without exercise intolerance may be the sole clinical feature of mitochondrial disease, or it may be associated with chronic progressive external ophthalmoplegia (PEO) and ptosis, pigmentary retinopathy, optic atrophy, sensorineural hearing loss, and encephalopathic features including dementia, seizures, myoclonus, ataxia and stroke-like episodes. Cardiac conduction defects are common in mitochondrial disorders and symptomatic mitochondrial cardiomyopathy, with or without skeletal myopathy, has been reported at increasing frequency in the recent past.[33] However, virtually any organ or tissue in the body can be affected, including gastrointestinal tract, liver, kidney, haemopoietic and endocrine systems.

Some mitochondrial syndromes are well established and nosologically defined entities (Fig. 4). However, clinical data are not sufficient to provide a systematic classification of mitochondrial diseases, because overlap syndromes or unspecific phenotypes are possible. Likewise, identical biochemical (e.g., lactic acidosis) and morphological (e.g., ragged-red fibers, RRF) abnormalities, can be found in different clinical presentations, or be absent in patients with otherwise-proven mitochondrial diseases.

Until the discovery of the first mutations of mtDNA, the diagnosis of mitochondrial (encephalo)-myopathies was largely based on the detection of RRF in the muscle biopsy[34] and of enlarged and abnormal muscle mitochondria at the electron microscopy examination (Fig. 5). An important advancement towards the identification and characterization of these disorders was the set-up of methods to visualize histochemically (Fig. 5) and measure spectrophoto-

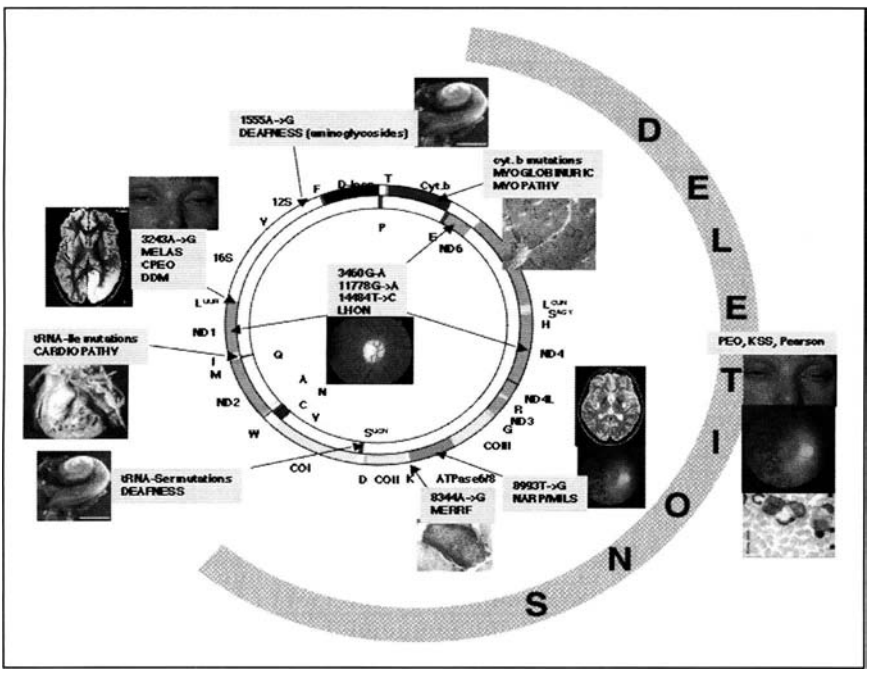

Figure 4. Morbidic map of mtDNA. The most frequent syndromes are shown in association with the corresponding mutations. Numbers and surrounding letters refer to position and type of the mutated nucleotide. For a complete list of mutations, see ref. 31. Phenotypes are denominated according to the acronyms of Table 1.

metrically the activities of individual respiratory enzymes, as well as the respiration rate in intact mitochondria, or whole cells, by polarography.

With the identification of mtDNA mutations, genetic studies have been providing further pathogenetic insights and new diagnostic clues on mitochondrial disorders. The recent discovery of the first mutations in nuclear genes responsible for OXPHOS abnormalities has ushered a "fourth era" of research. However, in several cases, mitochondrial disorders, defined on the basis of morphological or biochemical findings, still lack a molecular-genetic definition. Moreover, some OXPHOS defects identified biochemically, have no morphological counterpart in muscle (e.g., the NARP syndrome, see below). In a few clinical syndromes, such as Leber's Hereditary Optic Atrophy (LHON), the diagnosis is mainly based on the identification of mtDNA mutations, while morphological and biochemical analyses are less or noninformative. Therefore, an approach based on multiple diagnostic strategies is necessary and recommended.

In spite of the discouragingly high number of pathogenic mutations of mtDNA reported in the literature, it is important to emphasize that only a few of them are frequent enough to enter the routine diagnostic screening (Fig. 6). In particular, mtDNA deletions leading to PEO or KSS, the three most frequent point mutations associated with LHON, the 3243G->A MELAS mutation, the 8993T->G NARP mutation and the 8344G->A MERRF mutation account for over 90% of the cases in our cohort of mtDNA-mutant patients.

Mutations of mtDNA

Table 1 and Figure 7 report the genetically-defined defects of the mitochondrial genome.

From a molecular genetic standpoint, two categories of mtDNA mutations have been identified, i.e., large-scale mtDNA rearrangements, and mtDNA point mutations.

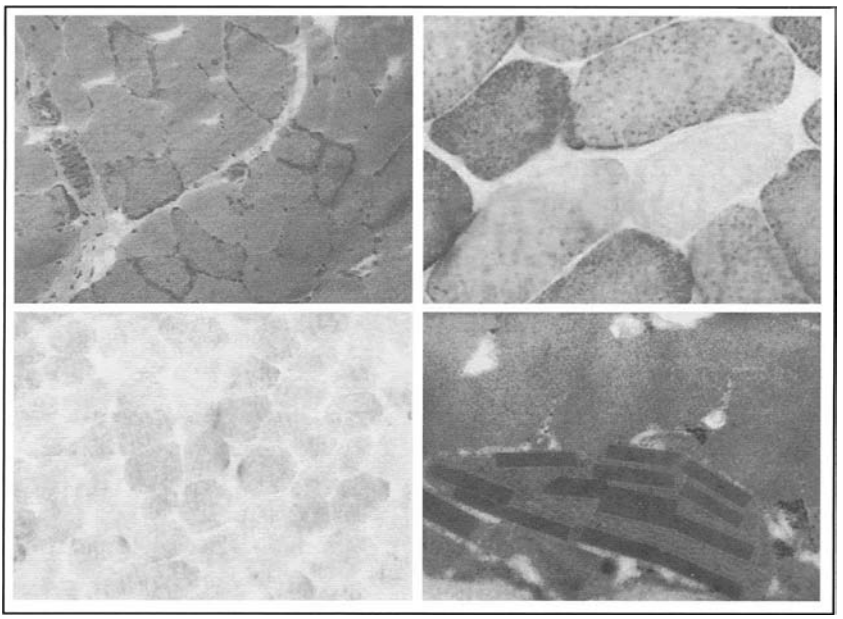

Figure 5. Morphological features in muscle. Upper left panel: ragged-red fibers, characterized by increased subsarcolemmal staining are indicated by arrowheads (modified Gomori trichrome stain). Upper right panel: histochemical reaction to COX showing a mosaic pattern (one fiber is COX-depleted). Lower left panel: histochemical reaction to COX in a case of severe infantile myopathy: COX reaction is virtually absent in all muscle fibers. Lower right panel: ultrastructural abnormalities of muscle mitochondria in an adult-onset mitochondrial myopathy. Enlarged mitochondria contain paracrystalline inclusions.

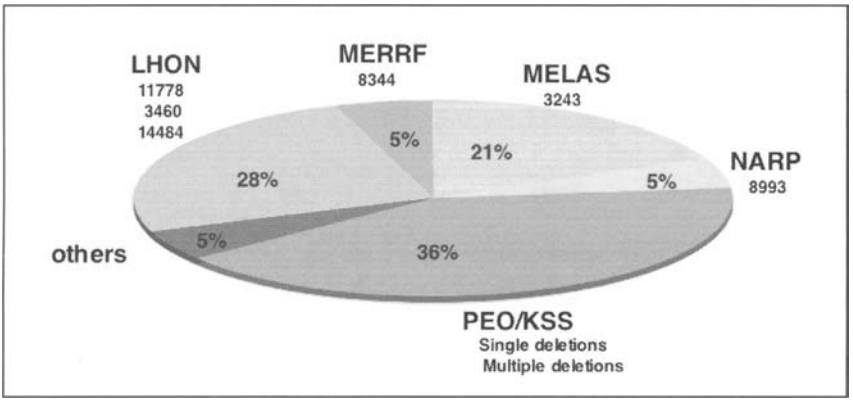

Figure 6. Distribution of mtDNA mutations in 200 Italian families with mitochondrial disease collected at the National Neurological Institute "C. Besta" from 1992 to 2002.

Large-Scale Rearrangements

Large-scale rearrangements consist of single partial mtDNA deletions ($mtDNA^{\Delta}$) or, more rarely, partial duplications.[35-38] Both typs of mutation are heteroplasmic since they coexist with variable amounts of wild-type mtDNA ($mtDNA^{wt}$). These mutations are associated with sporadic disorders (see below). Since deleted mtDNA species have been detected in human oocytes,[39] an unknown mechanism must prevent the vertical transmission or the expansion of $mtDNA^{\Delta}$-genomes. However, a single case of vertical transmission of a $mtDNA^{\Delta}$ species from an affected mother to an affected son has recently been documented.[40] The presence of a single mtDNA deletion (or partial duplication) in each patient is explained by the clonal amplification of a single mutational event. As for other mtDNA mutations, mitotic segregation can increase the variability of the tissue distribution of $mtDNA^{\Delta}$, hence influencing the clinical phenotype. Deletions are usually flanked by direct repeats of variable length,[41] suggesting that they are generated by a mechanism of slipped-mispairing[42] or illegitimate elongation[43] of the single mtDNA strands during replication (according to the orthodox replication model). All of the mtDNA deletions described so far encompassed more than one gene, including both mRNA and tRNA genes. The loss of tRNA genes contained in the deletion makes the $mtDNA^{\Delta}$ species translationally incompetent. Therefore, translation of these genomes can take place only through the complementation by $mtDNA^{wt}$ contained in the same organelle. The $mtDNA^{\Delta}$/$mtDNA^{wt}$ ratio can thus influence dramatically the functional consequence of the mutation.[44,45]

Three clinical phenotypes have been consistently associated with $mtDNA^{\Delta}$: Kearns-Sayre syndrome, Progressive External Ophthalmoplegia (PEO), and Pearson's bone marrow-pancreas syndrome.

Kearns-Sayre syndrome (KSS) is a sporadic, severe disorder characterized by the invariant triad of (1) PEO, (2) pigmentary retinopathy, (3) onset before age 20. Frequent additional symptoms are poor growth, a progressive cerebellar syndrome, heart block, and increased protein content in the cerebro-spinal fluid (CSF). RRF and COX-deficient fibers are the morphological hallmarks of the muscle biopsy. Cerebellar ataxia can for long be the only sign of CNS involvement in KSS, but additional pyramidal and extrapyramidal symptoms, and cognitive deterioration, usually ensue later in the course of the disease.

Progressive External Ophthalmoplegia (PEO), a partial and milder variant of KSS, is an adult-onset disease characterized by bilateral ptosis and ophthalmoplegia, frequently associated with variable degrees of proximal muscle weakness and wasting, and exercise intolerance. Again, numerous RRF and COX-deficient fibers are usually detected in the muscle biopsy. PEO is occasionally associated with signs of central nervous system involvement, including mild ataxia and parkinsonism, and peripheral neuropathy.

Table 1. Some of the best-established pathogenic mutations associated with mitochondrial syndromes and clinical phenotypes

MELAS	MERRF	CPEO	ISOLATED MYOPATHY	CARDIO-MYOPATHY	DEAFNESS	NARP/ MILS/LS	LHON/ DYSTONIA
583A tRNA^{Phe}	***8344G tRNA^{Lys}***	3243G tRNA$^{Leu(UUR)}$	618C tRNA^{Phe}	*3243G tRNA$^{Leu(UUR)}$*	delT961Cn 12SrRNA	1624T tRNA^{Val}	***3460A ND1***
1642A tRNA^{Val}	*8356C tRNA^{Lys}*	3251G tRNA$^{Leu(UUR)}$	3250C tRNA$^{Leu(UUR)}$	*3254G tRNA$^{Leu(UUR)}$*	1095C 12SrRNA	1644T tRNA^{Val}	4171A ND1
3243G tRNA$^{Leu(UUR)}$	*8363A tRNA^{Lys}*	3256T tRNA$^{Leu(UUR)}$	3288G tRNA$^{Leu(UUR)}$	*3260G tRNA$^{Leu(UUR)}$*	***1555G 12SrRNA***	5537-insT tRNA^{Trp}	10663C ND4L
3252G tRNA$^{Leu(UUR)}$	7512C tRNA$^{Ser(UCN)}$	*4274C tRNA^{Ile}*	3302G tRNA$^{Leu(UUR)}$	*3303T tRNA$^{Leu(UUR)}$*	***3243G tRNA$^{Leu(UUR)}$***	8344G tRNA^{Lys}	***11778A ND4***
3260G tRNA$^{Leu(UUR)}$		*4285C tRNA^{Ile}*	3902-8inv7 ND1	*4269G tRNA^{Ile}*	*7445G tRNA$^{Ser(UCN)}$*	8363A tRNA^{Lys}	*14482A/GND6*
3271C tRNA$^{Leu(UUR)}$		*4298A tRNA^{Ile}*	4370-insA tRNA^{Gln}	*4295G tRNA^{Ile}*	*7472-insC tRNA$^{Ser(UCN)}$*	*8851C ATPase6*	***14484C ND6***
3291C tRNA$^{Leu(UUR)}$		*4309A tRNA^{Ile}*	4409C tRNA^{Met}	*4300G tRNA^{Ile}*	*7510C tRNA$^{Ser(UCN)}$*	***8993G/C ATPase6***	*14459A ND6*
5814G tRNA^{Cys}		5692G tRNA^{Asn}	5521A tRNA^{Trp}	*4320T tRNA^{Ile}*	*7511C tRNA$^{Ser(UCN)}$*	*9171C/G ATPase6*	*14495G ND6*
8316C tRNA^{Lys}		5703A tRNA^{Asn}	5874C tRNA^{Tyr}	8296G tRNA^{Lys}	*7512C tRNA$^{Ser(UCN)}$*	9537insC COX III	*14568T ND6*
9957C COX III		5877T tRNA^{Tyr}	6930A COX I	8363A tRNA^{Lys}	12258A tRNA$^{Ser(AGY)}$	14459A ND6	
13513A ND5		8342A tRNA^{Lys}	7671A COXII	9997C tRNA^{Gly}			
13514G ND5		12311C tRNA$^{Leu(CUN)}$	11832A ND4	15243A Cyt b			
14787del4 Cyt b		12315A tRNA$^{Leu(CUN)}$	12320G tRNA$^{Leu(CUN)}$				
		Single large deletion	*14846A Cyt b*				

Table continued on next page

Table 1. Continued

MELAS	MERRF	CPEO	ISOLATED MYOPATHY	CARDIO-MYOPATHY	DEAFNESS	NARP/ MILS/LS	LHON/ DYSTONIA
			15059A Cyt b				
			15084A Cyt b				
			15150A Cyt b				
			15168A Cyt b				
			15197C Cyt b				
			15242A Cyt b				
			15498del24 Cyt b				
			15615A Cyt b				
			15723A Cyt b				
			15762A Cyt b				

Table: The number of mtDNA pathogenic mutations is constantly growing and for an updated list refer to the MITOMAP website. In bold are indicated the most common pathogenic mutations which are part of the standard screening protocols; in italics are indicated the mutational hot spot-genes which are characteristically associated with specific clinical phenotypes; note that the 3243G mutation in the $tRNA^{Leu(UUR)}$ is associated with a wide spectrum of clinical manifestations ranging from the full blown MELAS syndrome to CPEO, isolated cardiomyopathy, and deafness/diabetes. MELAS= Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like syndrome. MERRF= Myoclonic Epilepsy, Ragged-Red-Fibers. CPEO= Chronic Progressive External Ophthalmoplegia. NARP= Neuropathy, Ataxia, Retinitis Pigmentosa. MILS= Maternally Inherited Leigh Syndrome. LS= Leigh Syndrome. LHON= Leber's Hereditary Optic Neuropathy.

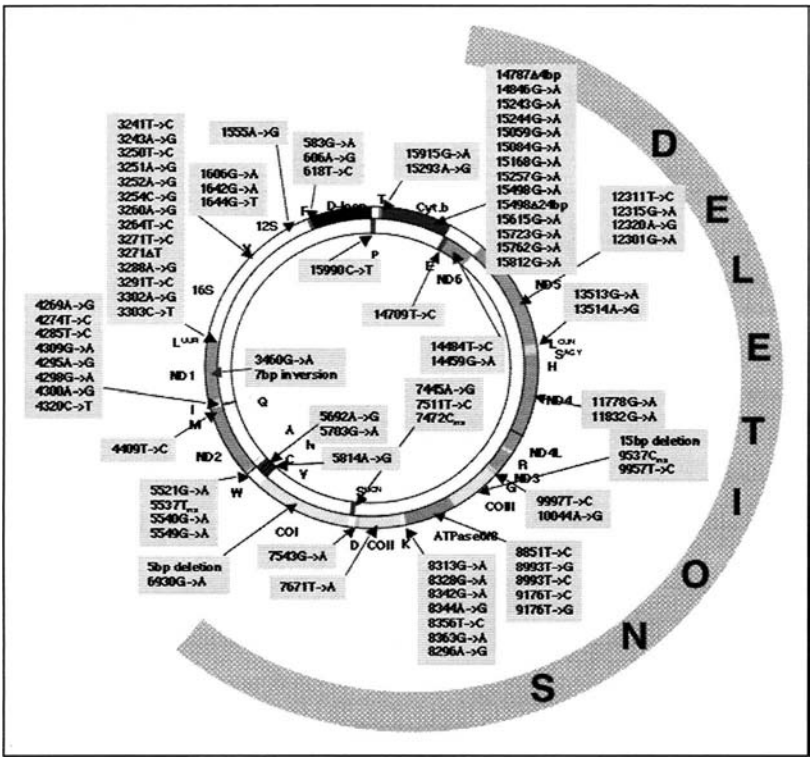

Figure 7. Mutational map of mtDNA. Numbers and surrounding letters refer to position and type of the mutated nucleotide. For a complete list of mutations, see ref. 30.

Pearson's bone marrow-pancreas syndrome (PS) is a rare disorder of early infancy characterized by sideroblastic anemia with pancytopenia, and exocrine pancreatic insufficiency. Infants surviving into childhood may develop the clinical features of KSS.[46,47]

KSS is characterized by distinctive neuroradiological abnormalities affecting the deep structures of the brain and the subcortical white matter.[48] In the brainstem, the mesencephalon can be affected diffusely, but occasionally the red nuclei are selectively involved. In the cerebellum, the most severely affected structures are the dentate nuclei and the dentate-rubral fibers in the superior cerebellar peduncle. Light microscopy examination can reveal neuronal degeneration and gliosis of the basal ganglia and spongy degeneration of the white matter, including the cerebellum.[49,50] Loss of Purkinje cells has been reported in KSS, along with severely reduced expression of mtDNA-encoded proteins in neurons of the dentate nucleus.[51] These findings are rather specific to KSS, and contribute to explain why cerebellar ataxia is a prominent, and occasionally the only CNS symptom.

Sporadic KSS, PEO and PS are all associated with large-scale heteroplasmic rearrangements of mtDNA. However, familial PEO has also been reported, and in this case it can be due to either a maternally-inherited point mutation of mtDNA, or to the accumulation of multiple mtDNA deletions caused by defects in nuclear genes (see Chapter 7 in this book). Deletions are easily detected by Southern-blot analysis of muscle DNA and, more recently, by quantitative PCR analysis, while they can be absent in lymphocyte or fibroblast DNA, especially in the milder cases (e.g., PEO).

Point Mutations

In contrast with large-scale rearrangements, mtDNA point mutations are usually maternally inherited, and can occur in mRNA, tRNA or rRNA genes. Given the very high mutational rate of mtDNA and the presence of numerous "private" or population-specific polymorphisms, it is essential to distinguish nondeleterious from pathogenic mutations. The latter are usually characterized by the following features: (1) high conservation; (2) segregation with phenotype; (3) quantitative correlation between phenotype and heteroplasmy, if present; (4) identification of the mutation in affected families from ethnically distinct human populations.

Point mutations involving tRNA genes cause a reduced availability of functional tRNAs that impairs the overall mitochondrial protein synthesis. Both mitochondrial protein synthesis and respiration are markedly reduced above a threshold of 80-90% of mutant mtDNA.[52] Mutations involving protein-encoding genes affect specifically the function of the respiratory chain complexes, which the corresponding protein belongs to.

As already mentioned, the clinical and biochemical variability of many mtDNA mutations may be due to different mitochondrial and/or nuclear "gene backgrounds". For instance, the fate and expression of mutations in cultures appears to be strongly influenced by the different nuclear backgrounds of the cell types.[53] It has also been proposed that nucleotide changes in mtDNA that are not intrinsically pathogenic may predispose to, modulate the effects of, or reflect a propensity for the occurrence of deleterious mutations. In turn, deleterious mutations may promote the accumulation of somatic changes, through the generation of OXPHOS-related mutagens.[54] This phenomenon could trigger a positive feed-back loop contributing to the progression of the mitochondrial dysfunction.

Given their different pathophysiology and genetic features, heteroplasmic and homoplasmic mtDNA point mutations will be discussed separately.

Heteroplasmic Point Mutations

Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes (MELAS) is defined by the presence of (i) stroke-like episodes due to focal brain lesions, often localized in the parieto-occipital lobes, (ii) lactic acidosis and/or RRF. Other signs of central nervous system involvement include dementia, recurrent headache and vomiting, focal or generalized seizures, pigmentary retinopathy and deafness. Ataxia can be observed in some patients.[55] Diabetes, intestinal pseudo-obstructions and cardiomyopathy may complicate single cases of MELAS patients.

Infarct-like lesions widespread in the cerebral cortex are associated with diffuse fibrillary gliosis in the cerebral and cerebellar white matter. Multiple focal lesions with demyelination and numerous spheroids have been reported in the pontocerebellar fibers, together with marked degeneration of the posterior columns and spinocerebellar tracts.[56] Electron microscopic examination shows accumulations of abnormal mitochondria in smooth muscle cells and endothelium of the cerebral and cerebellar blood vessels, suggesting a "mitochondrial angiopathy". However, the presence of diffuse, prominent white matter gliosis of the central nervous system and cerebellar cortical degeneration of granular cell type, may indicate morphologically widespread cellular dysfunction, not restricted to either neuronal or vascular derangement.[57] MRI examination typically shows that the signal abnormalities in the brain do not correspond to well-defined vascular territories. The stroke-like lesions may be transient and resolve after a few months. The recurrent occurrence of stroke-like episodes eventually leads to permanent lesions.

MELAS was first associated with a heteroplasmic point mutation in the $tRNA^{Leu(UUR)}$, an A->G transition at position 3243.[58] Many other MELAS-associated point mutations were later reported,[30] although the A3243G remains by far the most frequent one. The genotype-phenotype correlation of the A3243G mutation is rather loose since the observed clinical manifestations are not limited to the full-blown MELAS syndrome. For instance, the A3243G mutation has been detected in several patients (and families) with maternally-inherited PEO, isolated myopathy alone, cardiomyopathy, or in pedigrees with maternally inherited diabetes mellitus and

deafness. Biochemically, complex I is frequently the most affected respiratory chain activity in MELAS, while complex IV is often normal. This accounts for the observation that, in contrast with other mitochondrial syndromes, RRF in A3243G-MELAS (but not in A3243G-PEO) specimens display a robust histochemical reaction to COX.

Myoclonic-Epilepsy with Ragged-Red Fibers (MERRF) is a maternally inherited neuromuscular disorder characterized by myoclonus, epilepsy, muscle weakness and wasting with RRF, cerebellar ataxia, deafness and dementia.[59-61] Symmetric lipomatosis, especially in the trunk, is a frequent, intriguing sign in MERRF, that can anticipate the onset of neurological symptoms by several years.

Neuronal loss and gliosis of the cerebellar dentate nuclei and inferior olives have been reported in MERRF,[62,63] and confirmed by neuroimaging studies.[48]

The most commonly observed mutation of mtDNA associated with MERRF is an A->G transition at nt 8344 in the $tRNA^{Lys}$ gene.[64] A second mutation has been reported in the same gene, at position 8356.[65,66] Complex IV deficiency is the most prominent biochemical finding in MERRF muscle, although Complex I can be affected too. COX-depleted RRF are invariably detected in the muscle biopsy.

Clinical, biochemical and molecular investigation of large pedigrees showed a positive correlation between the severity of the disease, age at onset, mtDNA heteroplasmy and reduced activity of respiratory chain complexes in skeletal muscle. However, even though the genotype-phenotype correlation between MERRF syndrome and the A8344G mutation is tighter than that of other mutations, the A8344G transition has also been reported in phenotypes as different as Leigh's syndrome, isolated myoclonus, familial lipomatosis, isolated myopathy[67,68] and a variant neurologic syndrome characterized by ataxia, myopathy, hearing loss, and neuropathy.[69] MERRF must be considered in the differential diagnosis of progressive myoclonus epilepsies, including Ramsay-Hunt syndrome and Unverricht-Lundborg disease.[70]

Neurogenic weakness, Ataxia and Retinitis Pigmentosa (NARP) is a maternally-inherited syndrome in which the cardinal manifestations include ataxia, pigmentary retinopathy and peripheral neuropathy.

MRI examination of NARP patients has revealed the presence of moderate, diffuse cerebral and cerebellar atrophy, and, in the most severely affected patients, symmetric lesions of the basal ganglia.[71]

NARP is associated with a heteroplasmic T->G transversion at position 8993 in the ATPase 6 subunit gene.[72] A transition in the same position (T8993C) has later been described in patients affected by a mild variant of NARP.[73] RRF are consistently absent in the muscle biopsy. The degree of heteroplasmy is correlated with the severity of the disease. For instance, when the percentage of mutant mtDNA is more than 95% patients show the clinical, neuroradiologic and neuropathologic findings of maternally-inherited Leigh's syndrome (hence called MILS).[74] NARP/MILS phenotypes have been described in association with other mutations of the ATPase 6 gene, e.g., mutation 9176T->C.[75,76] NARP and MILS may coexist in the same family. Impairment of ATP synthesis has been reported in cell cultures harboring the T8993G mutation, as well as in tissue-derived mitochondria, showing a strict correlation with the mutation load.[77]

Hearing loss-Ataxia-Myoclonus (HAM) syndrome was originally reported in a large Italian pedigree.[78] The responsible mutation, 7472insC, affects the $tRNA^{Ser(UCN)}$ gene. This mutation has later been reported in several families, in which affected members showed a wide range of clinical manifestations, from isolated hearing loss,[79] to Epilepsia Partialis Continua and Ataxia,[80] to overt MERRF.[81] Given the increasing frequency at which the 7472insC has been found, the search for this mutation should become part of the routine screening of mitochondrial encephalomyopathies and/or maternally inherited hearing loss.

Other Syndromes

In spite of the enormous variability of the clinical presentations associated with heteroplasmic mtDNA point mutations, the accumulation of a remarkable amount of clinical and genetic data makes it possible now to establish a tentative correlation between specific mutations, or mutations clustered in specific mtDNA genes, and different clinical presentations.[32] For instance, several mutations in $tRNA^{Ser(UCN)}$, including the 7472insC, may present with hearing loss as the only or predominant symptom, suggesting an exquisite sensitivity of the cochlear receptor and auditory system to the functional impairment of this particular mt-tRNA gene.[82] The pathogenetic mechanisms underlying this well-established observation are presently unknown. Likewise, mutations in $tRNA^{Ile}$ are mainly associated with cardiomyopathy[83] or PEO, while mutations in cytochrome b are mainly associated with isolated myopathy with high serum CK or myoglobinuria.[84] Cytochrome b mutations are usually restricted to skeletal muscle and, in contrast with most of the other point mutations, are not transmitted maternally. A number of different clinical presentations, ranging from infantile Leigh syndrome to adult-onset motor neuron disease, to complex multisystem disorders have been reported with different point mutations of the three genes encoding complex IV, while point mutations of the genes encoding complex I subunits are usually associated with MELAS, LHON, or overlap syndromes.[32] However, an increasing number of Leigh-like, infantile encephalopathic syndromes have recently been reported in association with the 15313G->A mutation in ND5.[85]

Homoplasmic mtDNA Mutations

General Features

In contrast with many heteroplasmic mutations, the clinical expression of disorders associated with homoplasmic mutations is often stereotypical and mainly restricted to a single tissue. In this group of disorders, the presence of a pathogenic mtDNA mutation is necessary but not sufficient to induce disease. As a consequence, penetrance is incomplete[86] and possibly controlled by environmental factors, additional mitochondrial polymorphisms, or the effect of nuclear gene(s). However, the specific molecular mechanisms underlying these contributions are still largely unkown.

Leber's hereditary optic neuropathy (LHON)[87] was the first maternally inherited disease to be associated with a mtDNA point mutation.[88] LHON typically affects young adults, more often males. Visual acuity deteriorates over a period of days/weeks as a consequence of rapid, painless loss of central vision in one eye, usually followed by the other eye.[89] Stable residual values at or below 20/200 are reached in a few months, associated with a large centro-cecal absolute scotoma. Characteristic fundus changes include circumpapillary telangiectatic microangiopathy with tortuosity of peripapillary arterioles, swelling of the nerve fiber layer and hyperemic optic disc, and absence of leakage on fluorescein angiography.[90] Axonal loss in the papillomacular bundle, leading to an early and prevalent temporal atrophy of the optic disc, is a pathognomonic feature of LHON.[91]

Histopathological investigations show loss of retinal ganglion cell and nerve fiber layers, while the remaining layers appear virtually normal.[92] Ultrastructural investigations in genetically-proven LHON optic nerves[93] showed degenerative features in both axoplasm and myelin sheaths. Patchy accumulations of mitochondria suggested an impairment of axoplasmic transport.[94] Variability in myelin thickness was also evident, some axons being almost denuded of myelin sheath.[91] Morphometric investigation showed a preferential loss of the smallest axons, corresponding to the P-cell population which provides central vision.[94]

Approximately 90% of the worldwide LHON patients carry one of the three most frequent mtDNA mutations associated with LHON, namely the 11778G>A, 3460A>G and 14484T>C mutations.[88,95,96] A further group of rare, but well-established pathogenic mutations have been found only in a few families.[97-101] Other mutations, found only in single cases or families, still await confirmatory identification from multiple independent cases.

All the LHON mutations, which have been proved to be pathogenic, affect different mtDNA-encoded subunits of complex I. Mutations are usually homoplasmic, although heteroplasmy can be occasionally found in some families or singleton cases.

Variable expression of LHON may be due to the association of pathogenic mutations with specific mtDNA haplogroups. For instance, the European-specific haplogroup J is found more frequently in 11778- or 14484-positive LHON patients than in ethnically-matched control populations, suggesting that this haplogroup may increase the penetrance of the disease.[102-104] Environmental factors seem also to play a role as risk factors, in particular tobacco smoke.[105,106] Finally, a nuclear modifier is thought to be a major determinant for both disease expression and male prevalence. However, search for an X-linked nuclear modifier has been unsuccessful to date.[107-109]

Additional puzzling features of LHON are the exquisite tissue-specificity and the subtle and ill-defined biochemical abnormalities found in this condition. The unique anatomical and physiological features of the optic nerve[110] may explain its vulnerability to the decreased bioenergetic efficiency and increased oxidative stress associated with LHON mutations.[91,111]

LHON-like optic atrophy may be part of more complex syndromes including dystonia, Leigh syndrome,[112,113] and MELAS.[114] Private or infrequent mutations, again affecting complex I subunit genes, have been reported in these cases.

Nonsyndromic and aminoglycoside-induced sensorineural hearing loss (SNHL) have been both associated with a unique, maternally inherited point mutation at position 1555 (A to G) of the 12S rRNA gene.[115] Similar to LHON, this mutation is almost invariably homoplasmic,[116] and variable penetrance and clinical severity have been documented.[117] A two-locus model, including a primary mitochondrial mutation associated with a nuclear modifier gene, has been suggested to explain incomplete penetrance.[118] This hypothesis has recently been confirmed by the identification of a locus on chromosome 8 for a putative nuclear modifier gene.[119] In addition, a paraomomycin-resistance mutation in yeast, homologous to the human 1555 mutation, expresses a respiratory deficient phenotype only in the presence of a nuclear mutation in one of two genes, Mss1 and Mto1.[120] The human analogs of Mss1 and Mto1 are obvious candidates as nuclear modifier genes in the 1555-related SNHL.[121] The 1555 mutation affects a highly conserved region of the 12S rRNA gene, homologous to the bacterial domain that binds aminoglycosides and increases the similarity of the human 12S rRNA to its bacterial counterpart.[115] Growth rate of mutant cells was markedly reduced when exposed to aminoglycosides, confirming their sensitivity to this drug.[122] However, 1555-positive subjects who were never exposed to aminoglycosides can also become deaf. Therefore, the 1555 mutation is now considered as a frequent genetic cause of both nonsyndromic and aminoglycoside-induced post-lingual SNHL.[82] The hair cells of the cochlea are very energy-dependent and local gene expression may also play a relevant role in the strict tissue-specificity observed with the 1555 mutation and with other mutations of mtDNA which are predominantly characterized by hearing loss.

Other Homoplasmic Mutations

Homoplasmic mutations are frequently found during systematic screening of mtDNA in mitochondrial patients, but their pathogenic significance remains uncertain. A well documented case is a recently reported mutation at position 1624 in the $tRNA^{Val}$ gene.[123] This homoplasmic mutation was found in a clinically normal woman, who had six stillbirths and one surviving child with Leigh syndrome, from different partners. Biochemical investigations demonstrated a profound respiratory chain deficiency in both the apparently healthy woman and her child.

The nuclear gene background might play an important role in defining the pathological expression of the mitochondrial defect.

Genetic Counseling

The peculiar genetics underlying mtDNA-related defects has relevant consequences on genetic counseling and evaluation of the recurrence risk. The following rules-of-thumb can be used on this difficult matter.

1. Males do not transmit the disease.
2. Deletions are sporadic and not transmitted.
3. Homoplasmic point mutations are transmitted through the maternal lineage, but penetrance is incomplete.
4. Heteroplasmic point mutations may or may not be transmitted through the maternal lineage, but the mutation load may vary unpredictably.
5. Only for specific mutations (e.g., the 8993T->G NARP mutation), but not for others (e.g., the 3243A->G MELAS mutation) the mutation load found in chorionic villi or amniocytes seems to faithfully reflect the mutation load present in the fetus's tissues. Therefore, prenatal diagnosis can be proposed only in selected cases.

Conclusions

The identification of a broad spectrum of mtDNA-associated human diseases has revolutionized our concepts and approach to mitochondrial disorders. On the other hand, the establishment of a rational diagnostic strategy and pathogenetic interpretation of mtDNA abnormalities is based on the knowledge of the principles regulating mtDNA genetics, expression and control. A better understanding of mitochondrial biogenesis, cytoplasm-mitochondria trafficking, and mtDNA metabolism is also important for a genetic approach to therapy, for which the first experimental strategies have just started.[124,125]

On a practical ground, clinical presentation, inheritance pattern, and biochemical and morphological clues such as the presence of lactic acidosis or RRF on muscle biopsy, usually lead to the genetic tests. If common mutations are excluded, then a systematic screening for rare or new mtDNA mutations is based on automated nucleotide sequence or heteroduplex detection by DHPLC. In other cases, more complicated approaches, such as the preparation of transmitochondrial cybrids, are carried out in order to exclude the nuclear origin of a defect. It is important to emphasize that a morphologically "negative" muscle biopsy does not exclude a mitochondrial disorder, which can still be diagnosed either biochemically, or genetically, or both. A difficult decision to be taken is to attribute a pathogenic significance to the several homoplasmic changes that are invariably found in individual mtDNA sequences. With the exception of homoplasmic LHON mutations, heteroplasmy has for long been considered a major feature of pathogenic mutations, but the recent discovery of other homoplasmic mutations that are clearly associated with disease challenges this notion and opens new avenues to the clinical genetic investigation of mitochondrial disorders.

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CHAPTER 7

Nuclear DNA and Oxidative Phosphorylation

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Summary

The ubiquitous nature of mitochondria, the dual genetic foundation of the OXPHOS system in mitochondrial and nuclear genome, and the peculiar rules of mitochondrial genetics all contribute to the extraordinary heterogeneity of clinical disorders associated with defects of oxidative phosphorylation (mitochondrial encephalomyopathies). Here, we review recent findings about nuclear gene defects in OXPHOS enzyme complex deficiency. This information should help in identifying patients with mitochondrial disease and defining a biochemical and molecular basis of the disorder found in each patient. This knowledge is indispensable for accurate genetic counseling and prenatal diagnosis, and is a prerequisite for the development of rational therapies, which are still, at present, woefully inadequate.

Introduction

Mitochondria, the cellular energy plants, generate ATP through oxidative phosphorylation (OXPHOS). OXPHOS, defined as the oxidation of fuel molecules by oxygen and the concomitant transduction of this energy into ATP, is the final process of the complicated biochemical network involved in cellular energy production. The OXPHOS molecular system, which is embedded in the lipid bilayer of the mitochondrial inner membrane, consists of electron acceptors, coenzyme Q and cytochrome c, and five multisubunit protein complexes (complexes I-V). The OXPHOS system comprises about 75 nuclear gene products and 13 mitochondrial gene products.

Numerous patients with enzyme deficiencies, isolated or combined, have been reported. The estimated incidence of OXPHOS deficiencies is approximately 1:10,000 living births. Complex I deficiency is the most frequently observed OXPHOS disorder. Due to the bi-genomic origin of the OXPHOS complexes (except for complex II), the genetic cause of OXPHOS complex deficiency can be located on either the mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). The percentage of patients with mitochondrial DNA abnormalities is relatively low. In our experience, screening for common mtDNA mutations in patients with an established OXPHOS disorder is unsatisfactory: Common mtDNA mutations are encountered in less than 5% of our (mainly pediatric) patients. For more detailed clinical, morphological and biochemical aspects of OXPHOS disorders as well as mitochondrial DNA in relation to OXPHOS disorders, the reader is referred to the numerous excellent reviews.[1-8]

This review summarizes the current knowledge of nuclear gene defects in OXPHOS disorders characterized by an (isolated or combined) enzyme complex deficiency. With only a few exceptions, all human nuclear cDNAs/genes encoding structural OXPHOS subunits have been characterized recently making a review of these genes and the clinical effects of their mutants timely. The present knowledge about the contribution of various human structural and nonstructural nuclear encoded OXPHOS subunits to isolated or combined enzyme complex deficiency will be reviewed. As stated before, in most patients displaying an OXPHOS disor-

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der, a nuclear gene defect must be responsible for the enzyme deficiency. The finding of mutations in these genes is not only of importance for genetic counseling and prenatal diagnosis but will also extend our knowledge about the functional properties of the individual subunits. This information should also be helpful in generating animal models which, in turn, should be of value in designing and testing new treatment modalities.

Biochemistry and Molecular Biology of the OXPHOS System

Energy generation in mitochondria occurs primarily through oxidative phosphorylation, a process in which electrons are passed along a series of carrier molecules called the electron transport chain. These electrons are generated from NADH (reduced nicotinamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotide) which are produced by oxidation of nutrients such as glucose, and are ultimately transferred to molecular oxygen. The electron transport chain consists of four respiratory enzyme complexes arranged in a specific orientation in the mitochondrial inner membrane (Fig. 1). The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane and is then used by ATP synthase to make ATP from ADP (adenosine 5'-diphosphate) and phosphate by a fifth complex. The synthesized ATP is used for energy- requiring reactions in the matrix and is exported to the cytosol by the adenine nucleotide translocator, in exchange for cytosolic ADP.

In patients suspected of an OXPHOS deficiency, diagnosis can be made by enzymatic measurement of activity of the different OXPHOS complexes. The tissue of choice is a (fresh) skeletal muscle biopsy sample. Cultured fibroblasts can also be used. Noteworthy in this context is that not all deficiencies observed in skeletal muscle specimens are present in fibroblasts.[9] In nearly all patients, a considerable residual enzymatic activity is observed. Only very few patients have been described with an immeasurable low enzymatic OXPHOS complex activity.

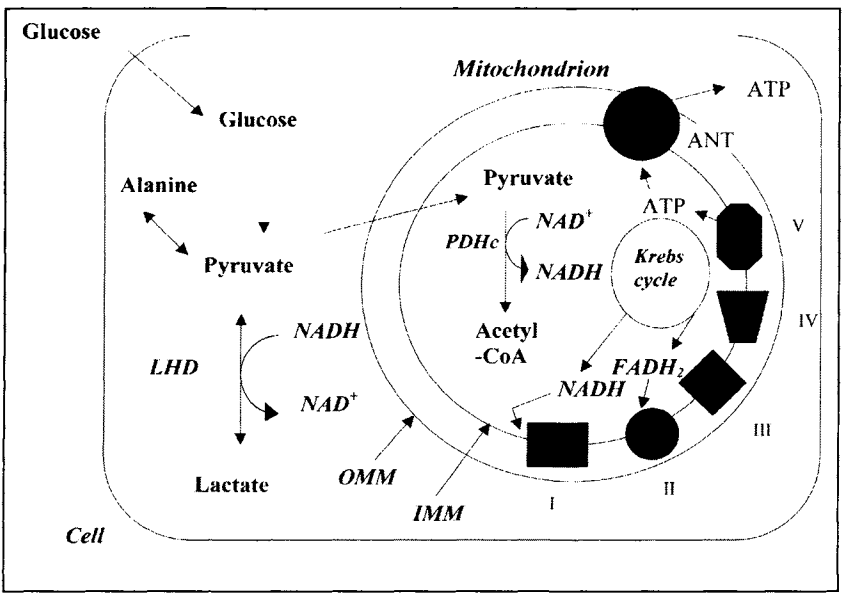

Figure 1. Schematic representation of the biosynthesis of ATP via the oxidation of pyruvate. LDH= lactate dehydrogenase; I – V= complex I to V of the oxidative phosphorylation (OXPHOS) system; ANT= adenine nucleotide translocator; PDHc= pyruvate dehydrogenase complex; CoA= coenzyme A; OMM= outer mitochondrial membrane; IMM= inner mitochondrial membrane.

Patients with a mitochondrial disorder may present their first signs and symptoms at any age, in any organ or tissue, and with any mode of inheritance. The majority of patients, however, present within the first five years of age. In general two phenotypes can be found: (1) pure myopathic patients, a disease presentation that is relatively mild and (2) a multi- system disorder affecting organs and tissues with a high energy demand such as brain and heart. The latter is the most frequently encountered phenotype and the disease course is progressive often with a fatal outcome. Most cases have as discriminative laboratory parameters increased lactic acid and alanine concentrations in body fluids as well as increased lactate/pyruvate ratios.[10] The latter is caused by the abnormal cytoplasmic redox state. One of the most frequently encountered phenotypes is Leigh syndrome, a progressive, subcortical encephalopathy affecting specific brain areas in a symmetrical pattern.[11-12] Frequently this diagnosis is a pathological anatomical one and, for that reason, we termed patients with a similar phenotype in which no brain autopsy has been/could be performed as Leigh-like syndrome.

Structural OXPHOS Genes: General

Each complex of the OXPHOS system consists of multiple components. Apart from the complex II subunits, which are exclusively encoded by the nuclear genome, the subunits of complexes I and III-V are encoded either by the nuclear DNA or by the mitochondrial DNA (mtDNA). Each mitochondrion possesses multiple mtDNA copies. The mtDNA is a 16,569 base-pair, double-stranded circular molecule that encodes seven subunits of complex I, one subunit of complex III, three subunits of complex IV and two subunits of complex V. In addition to protein-encoding genes, mtDNA also codes for 22 transfer RNAs and 2 ribosomal RNAs (for review see Zeviani').

Approximately 75 structural nuclear gene products constitute, together with the mitochondrial contribution, the building blocks of the OXPHOS system and participate directly to the formation of the five OXPHOS complexes: complex I 39, complex II 4, complex III 10, complex IV 10 and complex V $\geq$ 12. The approximately 75 nuclear-encoded cDNAs have been characterized in man and genes appear to be randomly distributed over the chromosomes with no obvious clustering. Certainly at present, little is known about the exact stochiometry and the function of the individual subunits of a particular complex.

Nuclear DNA Mutations

Defects in Structural OXPHOS Genes

Since 1995 nuclear gene mutations that affect various protein subunits of complex I, II and complex III have been identified. This knowledge is not only important for genetic counseling and prenatal diagnosis, but has also contributed to a better understanding of the functional properties of the subunits that are affected.

The first structural OXPHOS-gene mutation was reported in two sisters with Leigh syndrome and isolated complex II deficiency.[13] The pathogenic mutation was in the gene that encodes the flavoprotein, one of only four proteins that make up the smallest OXPHOS complex. Subsequently, another family was found to have mutations in this subunit.[14] Very interestingly in 2000 two groups independently reported mutations of the complex II subunit D and C genes (*SDHD* en *SDHC* respectively) in hereditary paraganglioma- usually benign, vascularized tumours in the head and in the neck.[15,16] Also *SDHB* appeared to have a major role in the pathogenesis of familial phaeochromocytomas.[17] This work has uncovered a new and surprising association between mitochondrial defects and carcinogenesis.

In 1997, the members of the Nijmegen Centre for Mitochondrial Disorders (NCMD) started to genetically characterize complex I, the Goliath of the OXPHOS system, which contains 39 nuclear-encoded subunits. In a patient with a Leigh-like presentation, we found a 5-base-pair (bp) duplication in *NDUFS4* (NADH dehydrogenase (ubiquinone) Fe-S protein 4)) that destroys the consensus phosphorylation site in the gene product and extends the length of the protein by 14 amino acids.[18] Further studies have revealed that this duplication abolishes

cyclic-AMP-dependent phosphorylation of NDUFS4, thereby impairing activation of the complex. These findings showed for the first time that human complex I is regulated through phosphorylation.[19] Further complex I mutations have been identified and approximately 40% of complex I deficiencies in children, in which the defect is detected both in muscle as well as in cultured skin fibroblasts, can now be explained by mutations in structural nuclear genes.[2,20-27]

The group of Uli Brandt used the obligate aerobic yeast *Yarrowia lipolytica* as a eukaryotic model to analyse mutations in the *NDUFS7* and *NDUFS8* genes associated with Leigh syndrome in humans.[28] Mitochondrial membranes from *Y. lipolytica* strains that carry any one of the three point mutations observed in complex-I-deficient patients also showed complex I deficiency. These results show that *Y. lipolytica* will be a valuable model system to study the cell biological consequences of human complex I deficiency.

Disorders of complex III are comparatively rare but are nevertheless present as a clinically heterogeneous group of diseases. To date only one mutation in a nuclear-encoded structural subunit has been described. Recently, a homozygous deletion in the nuclear gene *UQCRB* encoding the human ubiquinone-binding protein of complex III (QP-C subunit or subunit VII) was reported in a consanguineous family with an isolated complex III defect.[29] Low temperature spectral studies performed on isolated mitochondria of the index patient showed a decreased cytochrome b content suggestive of a role for the QP-C subunit in the assembly or maintenance of complex III structure.

Disorders of mtDNA Maintenance

The replication and maintenance of the mtDNA require a large number of nuclear encoded enzymes and balanced nucleotide pools. Mitochondrial nucleotide synthesis (Fig. 2) is of major importance because of the impermeability of the mitochondrial membranes to charged deoxyribonucleosides and the constant need for nucleotides for mtDNA maintenance even in quiescent cells. As de novo enzymes are not present in the mitochondria, synthesis is accomplished via the salvage pathway. Defective mtDNA synthesis and maintenance manifest by multiple deletions or by depletion of the mitochondrial genome. Patients with multiple deletions typically present with progressive external ophthalmoplegia, ptosis and exercise intolerance after the first decade of life. mtDNA depletion is usually an infantile disease characterized by severe muscle weakness, hepatic failure, or renal tubulopathy with fatal outcome. These molecular aberrations are usually tissue specific, dictating invasive procedures for final diagnosis. The etiology of some of these disorders resides in the above mentioned nuclear factors which participate in mtDNA synthesis and maintenance. However, recent findings indicate that the mitochondrial nucleotide metabolism is also a central player in these disorders. Clues in this direction were already provided by the adenine nucleotide translocator (ANT1) -/- mouse which suffered from multiple mtDNA deletions in both muscle as well as heart,[30] and from yeast strains mutated in the nucleotide metabolizing enzymes thymidylate synthetase, thymidylate kinase or ribonucleotide reductase, which exhibited deleted or depleted mtDNA.[31]

Multiple mtDNA Deletions

Linkage analysis was performed in several families with adult onset progressive external ophthalmoplegia (PEO), ptosis, exercise intolerance and multiple mtDNA deletions transmitted in either autosomal dominant (ad) or recessive manner. Heterozygosity for missense mutations in the polymerase domain of DNA polymerase gamma (POLG) resulted in adPEO whereas similar disease, transmitted in a recessive manner, was caused by compound missense mutations in nonenzymatic coding regions of the POLG.[32] Mutations in the *C10orf2* (Twinkle) gene, encoding for a mitochondrial protein with homology to the helicase domain of a bifunctional (helicase/primase) enzyme of T7 bacteriophage, were associated with adPEO and multiple mtDNA deletions.[33] AdPEO and multiple mtDNA deletions in muscle were also associated with missense mutations in the gene encoding the heart- and skeletal muscle-specific isoform of the ANT1. This protein is an isoform of ADP/ATP translocator regulating adenine nucleotide concentrations in the cytoplasm and within the mitochondria.[34]

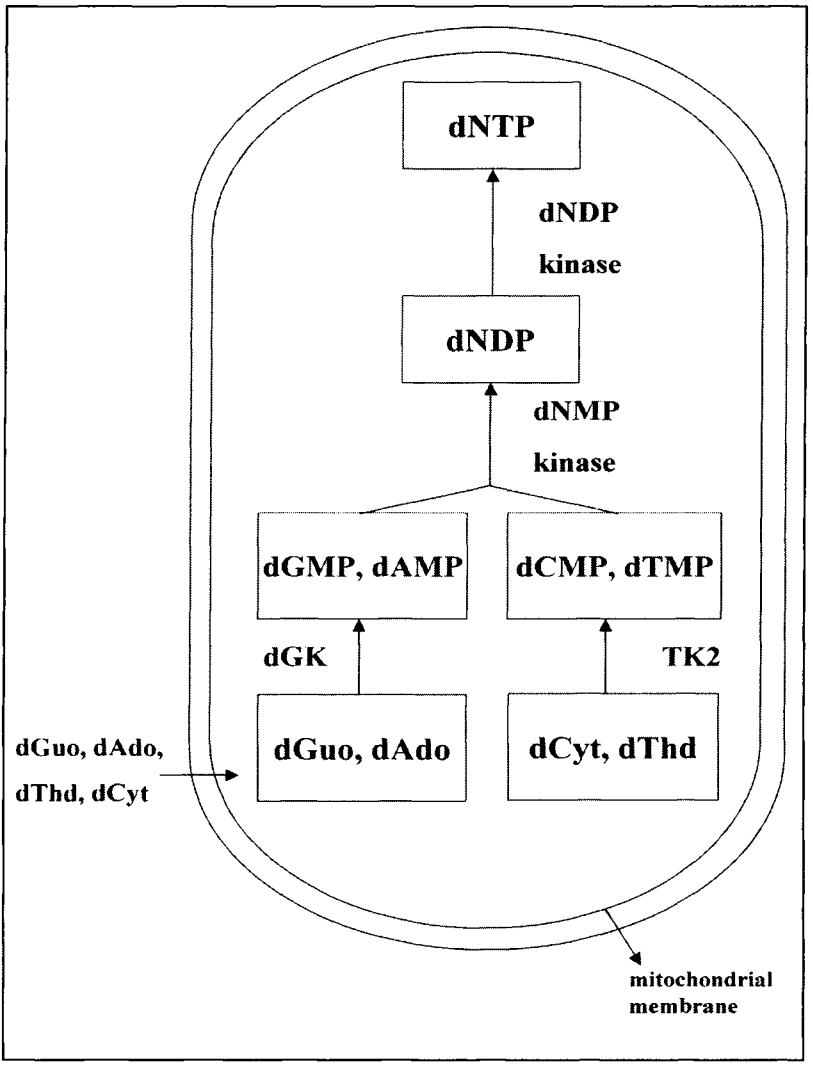

Figure 2. Schematic representation of the deoxyribonucleotide synthesis in human mitochondria.[86] The deoxynucleoside transporter(s) across the mitochondrial membrane has not been defined. The substrate specificity and the number of the dNMP and dNDP kinases are currently unknown. Abbreviations used: dGK= deoxyguanosine kinase; TKs= thymidine kinase 2; dGuo= deoxyguanosine; dAdo= deoxyadenosine; dCyt= deoxycytidine; dThd= deoxythymidine; their monophosphate (dNMPs), diphosphate (dNDPs) and triphosphate (dNTPs) derivatives.

mtDNA Depletion

Disturbances of the mitochondrial deoxyribonucleoside triphosphate (dNTP) pool does not necessarily lead to multiple deletions and may also result in mtDNA depletion. The clinical features of the mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) include ophthalmoparesis, peripheral neuropathy, leucoencephalopathy and gastrointestinal symptoms (chronic diarrhoea and intestinal dysmotility). Muscle biopsy shows RRFs (ragged-red fibres) and COX-negative fibres and either partial isolated complex IV deficiency or combined

OXPHOS-complex deficiencies.[35] Mitochondrial DNA analysis in this autosomal recessive syndrome showed mtDNA deletions, depletion, or both. The MNGIE locus was mapped to chromosome 22qI3.32-qter, a region that contains the thymidine phosphorylase (TP) gene (gene symbol *ECGF1).* Studies on patients showed that TP activity was markedly decreased, whereas the plasma thymidine levels were increased about 50-fold. Nishino et al[36] found various homozygous as well as compound heterozygous *ECGF1* mutations in the genomic DNA of MNGIE patients. The precise mechanism by which TP deficiency leads to mtDNA rearrangements has still to he explained, but imbalance of the mitochondrial nucleotide pool is likely to have a role.

Additional syndromes associated with mtDNA depletion have been extensively reported in the past decade. Most affected individuals presented in the neonatal period with muscle weakness, hepatic failure, or renal tubulopathy accompanied by lactic acidemia and died during the first year of life; others presented with isolated myopathy associated with motor regression or with a slowly progressive encephalomyopathy that began in early childhood.[37-42] Patients with these and other phenotypes displaying similar extent of mtDNA depletion demonstrate severely decreased activity of the respiratory chain complexes which contain mitochondrial encoded proteins.

Because the putative imbalance in the mitochondrial dNTP pool in patients with TP deficiency was associated not only with multiple mtDNA deletions but also with mtDNA depletion, and because of the occurrence of mtDNA depletion in nucleoside analog treated patients, a role for this pool in the pathogenesis of the other mtDNA depletion syndromes has been proposed.[43,44] Homozygosity mapping in 3 Druze kindreds with fatal liver failure, encephalopathy and mtDNA depletion confirmed this concept, identifying deleterious mutation in the *DGUOK* gene which encodes deoxyguanosine kinase (dGK), an essential member of the mitochondrial dNTP salvage pathway.[45] Further evidence for the involvement of the mitochondrial nucleotide pool was offered by the finding of mutations in the second mitochondrial deoxyribonucleoside (dN) kinase gene, thymidine kinase 2 *(TK2),* in infants with isolated skeletal myopathy and mtDNA depletion.[46]

Given the ubiquitous expression of *DGUOK* and *TK2,*[47,48] the sparing of certain tissues is puzzling. This is exemplified by a normal mtDNA content in the blood, skin and infantile muscle of dGK deficient individuals and in the lack of liver and CNS involvement in TK2 deficiency. Perturbations of dNTP pools either by decreased level of one of the four dNTPs or by an imbalance among them can cause serious disease.[49] dNTP imbalance has been associated not only with an - increased mutation rate but also with breakage of mature DNA and inhibition of its repair.[50,51]

OXPHOS Assembly

Enzyme complex I and IV deficiencies are by far the most frequently observed abnormalities of the OXPHOS system. In sharp contrast to isolated complex I, II and III deficiencies, no mutations have been found as yet in the ten or twelve nuclear genes that encode the structural proteins of complex IV or V.[52] Complex III (cytochrome bc1 complex) consists of 11 subunits, 10 encoded by the nuclear genome. Until recently, only mutations in the mitochondrial cytochrome b complex gene have been described. Mutations in the cytochrome b gene are associated with different clinical phenotypes.[53] As expected, not all complex III-deficient patients bear a mutation in the mitochondrial-encoded subunit.[54] To date, mutation detection studies in the 10 structural nuclear complex III genes has resulted in one mutation in a nuclear-encoded subunit (QP-C subunit or subunit VII).[29] The only characterized human gene which may play a role in complex III assembly is *BCS-1.*[55] Yeast *BCSI* mutations affect the amount of the Rieske iron-sulphur subunit of complex III, and complementation experiments indicate that BCS1 protein is involved either in forming the active site iron-sulphur cluster or in providing a chaperone-like function in assembling the Rieske protein with other subunits of the complex. Very recently, de Lonlay et al [56] reported on *BCS1* mutations in six patients from four unrelated families, presenting at neonatal age with proximal tubulopathy and hepatic involvement.

The relative number of *BSC1* mutated complex III-deficient patients found by the French group warrants further analysis of other isolated complex III-deficient patients.

The discovery of mutations in a nuclear assembly gene that is associated with COX deficiency resulted from chromosomal transfer experiments, in which chromosomes can be identified that complement the mitochondrial defect in patient cell lines. The inference is that the chromosome contains a functional copy of the gene that has been mutated in the patient. Once the chromosome has been identified, the gene is localized more precisely by introducing deleted versions of this chromosome. Candidate genes can then be identified and tested for complementation on the basis of information from human and model organism genome projects. In particular, many yeast genes are already known to participate in the assembly of complex IV, and human gene orthologues of these yeast genes have been identified. The first success of this approach was the identification of mutations in the *SURF1* (surfeit 1) gene in patients with COX- deficient Leigh syndrome.[57,58] *SURF1* is part of a cluster of unrelated housekeeping genes and is the only gene of this cluster that is known or believed to be involved in COX assembly.[59-62] The *SURF1* mutations were found in COX- deficient families by using genetic linkage studies combined with candidate gene approaches. In a relatively short period of time after the discovery of mutations in *SURF1,* mutations were found in genes that encode four other complex IV assembly factors.[63-66] Patients with a mutation in the *SCO2* gene, another complex IV assembly gene, show clinically infantile cardioencephalomyopathy.[64] *SCO2* is thought to function in the copper trafficking of complex IV, as is also true for the functional homologue *SCOI.* Even though *SCOI* and *SCO2* are functional homologues, mutations in these genes give a different clinical outcome.[65] *COX10* encodes for heme A: farnesyltransferase, which is also required for the biogenesis of complex IV.[63] Finally, mutations in *COX15* produce a defect in the mitochondrial heme A biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy.[66] This study establishes COX15 as an additional cause, along with SCO2, of fatal infantile, hypertrophic cardiomyopathy associated with isolated COX deficiency.

Additional studies with human cells and yeast[62,67-71] have identified the function of these assembly proteins of complex IV and their relationship to each other. Genes involved in the delivery of copper to complex IV are *COX17, SCO1* and *SCO2.*[72,73] *COX10, COX15* (and also *COX 11)* are involved in the maturation of heme. *COX10* is necessary for the proper incorporation of heme into complex IV. COX15 is a protein involved in the synthesis of heme A, the heme prosthetic group for COX. Other assembly genes for COX in yeast are *COX18, COX20, PET197* and *COX14.*[74,75]

Nuclear gene defects that are associated with isolated complex V deficiency have not yet been discovered. So far only mutations in *ATPase 6,* one of the mitochondrial-encoded subunits of the complex have been found. However, a nuclear defect related to complex V has been suggested.[76,77]

Transcription and Translation of OXPHOS Genes

Saguenay-Lac-Saint-Jean cytochrome oxidase (COX) deficiency (Leigh syndrome French-Canadian type (LSFC)), an autosomal recessive form of congenital lactic acidosis, presents with developmental delay and hypotonia. It is an Leigh syndrome- variant found in a geographically isolated region of Quebec. Using high-resolution genetic mapping the disease-causing gene was mapped to a 2 cM region between D2S119 and D2S2174.[76] Integrative genomics was used to identify the gene causing LSFC. *LRPPRC* appeared to cause LSFC.[77] *LRPPRC* encodes a mRNA-binding protein likely involved in mtDNA transcript processing, suggesting an additional mechanism of mitochondrial pathophysiology.

OXPHOS Homeostasis and Import Defects

In recent years, four inherited neurodegenerative diseases, Friedreich ataxia, hereditary spastic paraplegia, human dystonia deafness syndrome and dominant optic atrophy have also been shown to be mitochondrial disorders that are caused by nuclear DNA mutations in the genes

for frataxin, paraplegin, DDP and OPA1, respectively. Mitochondria obtained from heart biopsies of Friedreich ataxia patients have disclosed specific defects in the citric-acid cycle enzyme aconitase, and complex I-III activities.[80] The causative Friedreich ataxia protein, dubbed frataxin, has an essential role in mitochondrial iron homeostasis, and Friedreich ataxia can therefore be considered as an OXPHOS homeostasis defect.[81] Muscle biopsies from the autosomal recessive form of patients with hereditary spastic paraplegia revealed histochemical signs of a mitochondrial disorder, namely RRFs, COX-negative fibers and succinate dehydrogenase-positive hyperintense fibres.[82] Linkage and subsequent mutation analysis revealed large deletions in a gene dubbed paraplegin.[82] Owing to the homology with a yeast mitochondrial ATPase with both proteolytic and chaperone-like activities, it has been suggested that this form of hereditary spastic paraplegia could be a neurodegenerative disorder due to OXPHOS deficiency, attributing a putative function in the assembly or import of respiratory chain subunits or cofactors to paraplegin.[82,83]

The dystonia deafness syndrome, an X-linked recessive disorder also known as the Mohr-Tranebjaerg syndrome, is associated with a novel defect in mitochondrial protein import.[84] The defective gene is homologous to the yeast protein Tim8, which belongs to a family of proteins that are involved in intermembrane protein transport in mitochondria. Therefore, the dystonia deafness syndrome should be considered as the first example of a new group of mitochondrial-import diseases.[84] Finally, optic atrophy (OPA) is caused by defects in a dynamin-related protein that is targeted to mitochondria and might exert its function in mitochondrial biogenesis and in stabilization of mitochondrial membrane complexes.[85]

The variability in clinical presentation of mitochondrial disorders is a recurring theme in the field. However, in the case of nuclear gene mutations (Table 1), there is at least the following general correlation between genotype and disease: *ECGF1, SURF1, DDP* and OPA1 are involved in MNGIE, Leigh syndrome, deafness-dystonia syndrome and optic atrophy, respectively. Although this is not an infallible rule, it should at least be helpful for the molecular screening of patients.

Concluding Remarks

An important spin-off of the genetic studies of OXPHOS disorders has been the development of reliable molecular prenatal diagnosis for increasing numbers of families that are affected by nuclear DNA mutations, most of which have lost at least one child to encephalomyopathies, such as Leigh syndrome. Prenatal diagnosis is only rarely possible for mtDNA mutations, but the need for it is made more urgent by the lack of effective therapies for these devastating diseases. The final goal of investigating OXPHOS disorders is the prevention or treatment of these mostly devastating, and often fatal, inborn errors of metabolism. Knowledge concerning the approximately 75 human nuclear genes creating most of the essential building blocks of the five multisubunit protein complexes of the OXPHOS system has expanded greatly in the past years. Detailed knowledge of the numerous human proteins involved in the regulation of transcription, translation, post-translational modification, mitochondrial signaling, import and assembly of the OXPHOS system, however, is still rather scanty. The information that is being obtained from the various genome projects will greatly enlarge our insight concerning (mitochondrial) proteins involved in cellular energy homeostasis. A careful evaluation of the results of fundamental research groups studying lower eukaryotes and prokaryotes is warranted to extend our insights into the biogenesis of OXPHOS complexes. It is expected that the application of direct (candidate gene identification by comparison between genes in lower species and the human EST database) and indirect (chromosome transfer, linkage analysis and positional cloning) genetic strategies will rapidly change.

In parallel with the identification of disease-causing genes responsible for OXPHOS disorders, extensive cell biological studies have to be initiated. An additional major area of research will be the generation of animal models to study the regulation and function of structural and

Table 1. Classification of nuclear mutations in OXPHOS disorders

Structural OXPHOS defects:	
Defects in structural OXPHOS genes:	
a) Complex I deficiency:	
* Leigh and Leigh-like syndrome	NDUFS1, NDUFS4, NDUFS7, NDUFS8
* Hypertrophic cardiomyopathy and encephalomyopathy	NDUFS2, NDUFV2
* Macrocephaly, leucodystrophy and myoclonic epilepsy	NDUFV1
b) Complex II deficiency:	
* Leigh and Leigh-like syndrome	FP
c) Complex III deficiency:	
* Hypoglycemia and lactic acidosis	QP-C subunit
Non-structural OXPHOS defects:	
mtDNA maintenance:	
a) Multiple mtDNA deletion	
* Autosomal dominant progressive external ophthalmoplegia (adPEO)	ANT1, POLG
* Autosomal recessive progressive external ophthalmoplegia (arPEO)	POLG, C10orf2
b) mtDNA depletion	
* Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)	ECGF1
* Hepatic failure and encephalopathy	DGUOK
* Isolated skeletal myopathy	TK2
Assembly defects:	
a) Complex III	
* Neonatal proximal tubulopathy and hepatic failure	BCS1
b) Complex IV	
* Leigh syndrome	SURF1
* Neonatal-onset hepatic failure and encephalopathy	SCO1
* Cardioencephalomyopathy	SCO2
* Leigh and De Toni-Fanconi-Debre syndrome	COX10
* Fatal infantile hypertrophic cardiomyopathy	COX15
Homeostasis and import:	
* Friedreich's ataxia	Frataxin
* Hereditary spastic paraplegia	Paraplegin
* Deafness-dystonia syndrome	DDP
* Dominant optic atrophy	OPA1
Transcription and translation of OXPHOS genes:	
* Leigh syndrome French-Canadian type (congenital lactic acidosis, developmental delay and hypotonia)	LRPPRC

nonstructural OXPHOS genes. These animal models may also be used for therapeutic intervention studies, which are almost impossible in patients due to the great clinical variability. Clearly, much work still has to be done with regard to the pathogenesis and improvement of treatment of OXPHOS disorders. It goes without saying that different scientific disciplines are crucial to these investigations and will continue to play vital roles in these processes.

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CHAPTER 8

Cell Biological Consequences of OXPHOS Disorders

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Abstract

During the past century mitochondria have been recognized to play a central role in many cellular functions. Apart from producing cellular energy in the form of ATP (adenosine 5'-triphosphate) this organelle harbors essential parts of the urea cycle and is crucial for the breakdown of fatty acids, heat generation and the biosynthesis of heme, pyrimidines, amino acids, phospholipids and nucleotides. In addition to these 'classical' functions, mitochondria are also key players in cellular signaling through their involvement in apoptosis, generation of reactive nitrogen- and oxygen species (ROS/RNS), transduction of electrical signals and calcium homeostasis. This chapter summarizes current insights concerning the consequences of oxidative phosphorylation (OXPHOS) dysfunction at the cellular level. We will start with illustrating how mitochondrial and cellular metabolism is intertwined during ATP generation, calcium transport and ROS production. Moreover, the relation between mitochondrial morphology and function will be addressed. Next, we will summarize how OXPHOS deficiency and cellular functioning have been analyzed using pharmacological model systems and patient-derived cell lines. Also results of mathematical modeling, applied to integrate and understand the complex experimental data, will be treated. Finally, we will discuss possible adaptive mechanisms that counterbalance OXPHOS deficiency in the living cell.

Mitochondrial Function in the Living Cell

Production of ATP

Mitochondria are present in virtually all eukaryotic cells and in higher animals they produce ~95% of the principal carrier of chemical energy, ATP.[1,2] These cellular power plants are fueled not only by the pyruvate produced from sugars by glycolysis in the cytosol but also by fatty acids. Pyruvate and fatty acids are transported from the cytosol into the mitochondrial matrix, where they are used as carbon sources for the tricarboxylic acid cycle (TCA) and fatty acid oxidation (β-oxidation), respectively. The products of these pathways, NADH and $FADH_2$, subsequently feed reducing equivalents into the electron transport chain (ETC) embedded within the mitochondrial inner membrane (MIM; Fig. 1). The ETC consists of four respiratory chain enzyme complexes (complex I to complex IV) and a transport system (ubiquinone and cytochrome c). Together they transfer electrons from the hydrogens on NADH and $FADH_2$ to oxygen. The electrons start with very high energy and lose it in small steps as they pass along the ETC. At three locations within the chain, complex I, complex III and complex IV, these quanta of energy are used to expel 4, 4 and 2 protons per electron respectively from the matrix

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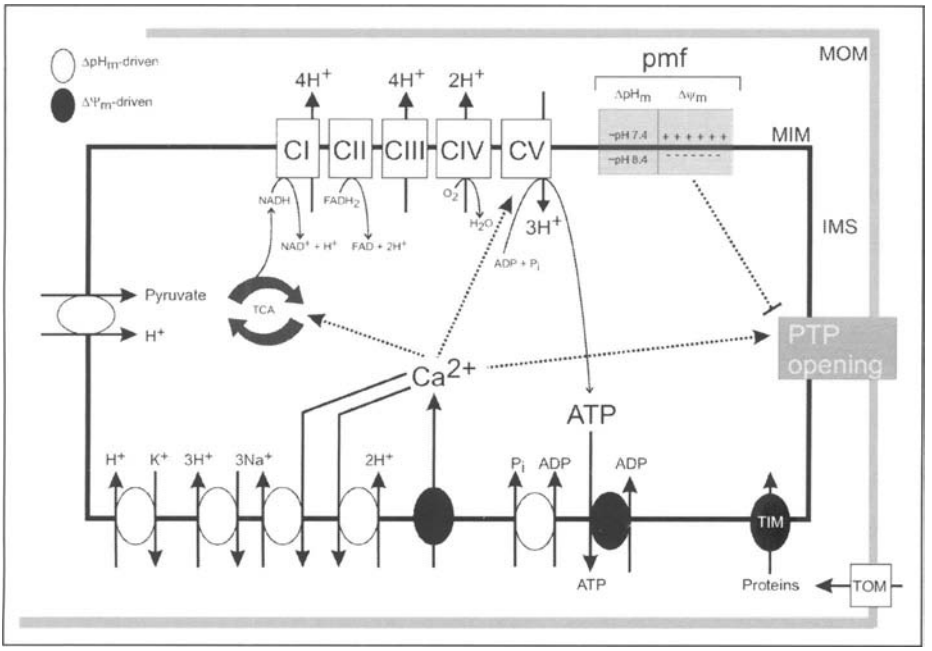

Figure 1. Central role of oxidative phosphorylation in mitochondrial metabolism. By expelling H^+ from the mitochondrial matrix across the mitochondrial inner membrane (MIM), Complex I, III and IV generate a proton motive force (pmf) that is used for the production of ATP and as an energy source by several mitochondrial transporters. The latter are driven by the pH gradient (ΔpH_m) or mitochondrial membrane potential ($\Delta\psi_m$) across the mitochondrial inner membrane (MIM). In this way the pmf is used to supply pyruvate to the tricarboxylic acid (TCA) cycle, to transport ions and other small solutes, and to import nuclear-encoded mitochondrial proteins via the TIM/TOM import machinery. Moreover, the export of ATP and inhibition of the permeability transition pore (PTP) require $\Delta\psi_m$. Ionic calcium (Ca^{2+}), once taken up, increases the production of ATP by stimulating several key-enzymes of the TCA cycle and complex V. Ca^{2+} overload will eventually lead to opening of the PTP. If the duration of this opening is above a certain threshold, apoptosis will be triggered. Abbreviations: IMS= inter membrane space, TIM= translocase of the inner membrane; TOM= translocase of the outer membrane.

space to the inter-membrane space. The H^+ ejection results in the establishment of a H^+ electrochemical gradient ($\Delta\mu_H$ or pmf: proton motive force) which can be written as:

$$\Delta\mu_H = z \cdot F \cdot \Delta\psi + R \cdot T \cdot \ln\left(\frac{[H^+]_{matrix}}{[H^+]_{cytosol}}\right)$$

where Δy denotes the membrane potential difference ($\psi_{in} - \psi_{out}$) in millivolts; z is the charge of the ion, F is the Faraday constant, R is the gas constant and T is the temperature in Kelvin.[3] Because the first term is about -160 mV and the second equals -60 mV, the total pmf value is -220 mV (inside of mitochondrial inner membrane negative). At complex V (F_0/F_1-ATPase), protons are allowed to flow back into the mitochondrial matrix to drive the synthesis of ATP from ADP and inorganic phosphate (P_i). In intact mitochondria, the ETC and ATP synthesis are effectively coupled and together constitute the oxidative phosphorylation (OXPHOS) system. It is important to realize that the primary event is not the generation of ATP but proton pumping and maintenance of $\Delta\mu_H$.[3] This is illustrated by the fact that complex V, rapidly turns from the main producer into main consumer of ATP during anoxic conditions in an effort to preserve $\Delta\mu_H$.[4]

Mitochondrial Transporters

In addition to ATP synthesis, $\Delta\mu_H$ is also utilized by a variety of other mitochondrial processes (Fig. 1). Most of these processes are driven by $\Delta\psi$ or ΔpH. Mitochondrial exchangers are responsible for antiport of H^+/K^+, H^+/Na^+, $3Na^+/Ca^{2+}$ and $2H^+/Ca^{2+}$ (all dependent on ΔpH), channels for Na^+ and K^+ (important for regulating mitochondrial volume), and a uniporter Ca^{2+} uptake mechanism (discussed in more detail in Mitochondrial Participation in Cellular Calcium Signalling).

In addition to dedicated transporters, Ca^{2+} and other solutes of low molecular weight can also exit the mitochondrial matrix via the permeability transition pore (PTP) complex, which consists of a voltage-dependent anion channel (VDAC or mitochondrial porin), the adenine nucleotide translocase (ANT) and cyclophylin-D (CyP-D). This complex resides at contact sites between mitochondrial inner and outer membranes, and is involved in the apoptotic pathway.[5] The PTP is closed by ATP and $\Delta\psi$ and opened by oxidative stress, Pi and Ca^{2+} in the mitochondrial matrix.[3,5-7] It also interacts with several proteins like hexokinase, glycerol kinase and the pro-apoptotic protein Bax.[5] Creatine kinases, present between MIM and MOM, also interfere with PTP functioning in a Ca^{2+}-dependent fashion.[8] In isolated mitochondria, PTP opening was shown to be modulated by complex I and specifically blocked by ubiquinone analogs.[9] PTP opening on its turn also affects $\Delta\psi$, especially under conditions of prolonged oxidative stress and/or cellular Ca^{2+} overload. It has been suggested that this mechanism serves as a rescue pathway that allows partial dissipation of $\Delta\psi$, fast release of accumulated mitochondrial Ca^{2+} and a decreased production of reactive oxygen and nitrogen species.[10]

The mitochondrial genome contains only a small number of genes (37 in mammals). Thirteen of these are encoding proteins of the OXPHOS system (seven for complex I, one for complex III, three for complex IV and two for complex V). Therefore the vast majority of mitochondrial proteins, adding up to ~730 in humans,[11] are derived from nuclear DNA and synthesized as cytosolic precursors or preproteins. Mitochondria possess dedicated import pathways for these proteins,[12] which have been largely characterized in *S. cerevisiae* and the nematode *C. elegans*. The import machinery consists of at least three large multi-subunit protein complexes, one in the outer membrane and two in the inner membrane that cooperate with soluble proteins from the cytosol, intermembrane space and the mitochondrial matrix.[13] According to their localization, these translocases are designated as a TOM (translocase of the outer membrane) or TIM (translocase of the inner membrane). In relation to OXPHOS functioning, it is important to realize that transport across the inner membrane utilizes the mitochondrial inner membrane potential ($\Delta\psi$) and the hydrolysis of ATP whereas translocation across the outer membrane is governed by electrostatic interactions. Also in the mitochondrial matrix the mitochondrial heat shock protein 70 (mtHSP70), which acts as an import motor, requires ATP.[14]

Cellular Calcium Signalling

How Calcium Signals Are Evoked

In the cell, ionized calcium (Ca^{2+}) not only represents the most common signal transduction element relaying information within cells to control a wide array of activities, including secretion, contraction and cell proliferation, but also is invariably involved in cell death.[15-17] To coordinate all of these functions, the cytosolic calcium concentration ($[Ca^{2+}]_C$) needs to be precisely controlled in space, time and amplitude. Normal $[Ca^{2+}]_C$ levels at ~100 nM are 20,000-fold lower than the 2 mM concentration found extracellularly. Maintenance of such low resting Ca^{2+} levels requires the continuous action of the plasma membrane Ca^{2+}-ATPase (PMCA) compensating for the influx of Ca^{2+} along the steep electrochemical gradient (Fig. 2). The transduction of many external stimuli, including hormones and neurotransmitters, is mediated by rises in $[Ca^{2+}]_C$[15-17] that is either derived from the extracellular medium or the endoplasmic reticulum (ER). Release from the ER can be mediated by inositol-(1,4,5)-

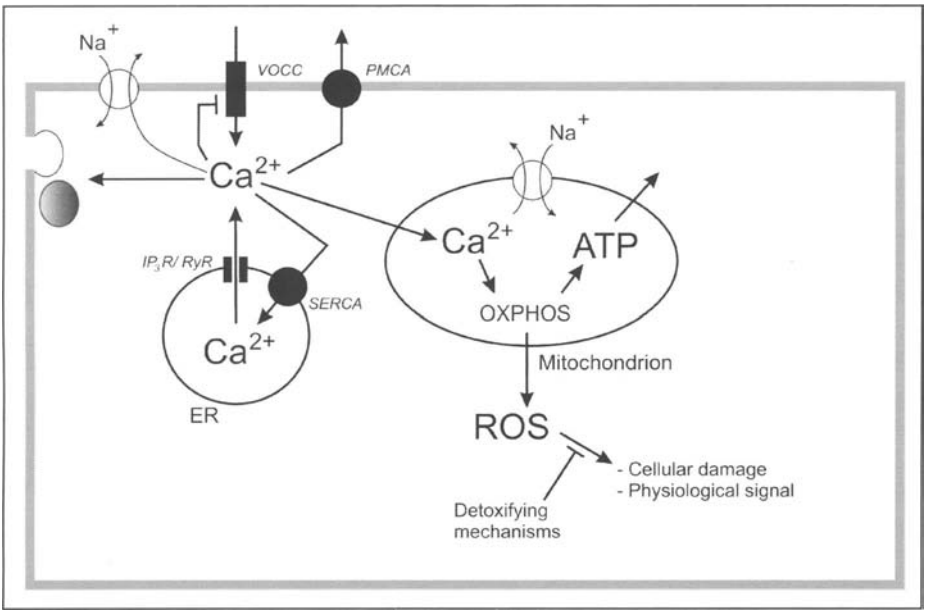

Figure 2. Calcium homeostasis in mammalian cells. In non-excitable cells, hormones interact with their cell surface receptors to promote the production of inositol 1,4,5-trisphosphate (IP_3). IP_3 is an intracellular messenger that interacts with IP_3-operated Ca^{2+}-channels (IP_3-receptors: IP_3R) to trigger the release of Ca^{2+} from the endoplasmic reticulum (ER). Once released into the cytosol, Ca^{2+} is rapidly pumped back into the ER by the action of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) or translocated to the extracellular medium by the plasma membrane Ca^{2+}-ATPase (PMCA) or Na^+/Ca^{2+} exhange. In excitable cells a similar mechanism is used that induces Ca^{2+} release via ryanodine-sensitive receptors (RyR) from the ER or Ca^{2+} influx across the plasma membrane via voltage-operated Ca^{2+} channels (VOCCs) upon arrival of electrical signals. Interestingly, several cell types use a combination of both mechanisms to elevate cytosolic Ca^{2+} levels. In the cytosol, Ca^{2+} might for example regulate exocytosis or the inactivation of VOCCs. Mitochondria rapidly take up and slowly release Ca^{2+} from and into the cytosol. In this way, they buffer cytosolic Ca^{2+} signals and thereby profoundly affect the signaling function of this ion. Inside the mitochondrial lumen, Ca^{2+} stimulates the production of ATP by the OXPHOS system. This stimulation might also enhance the production of reactive oxygen species (ROS), which can cause cellular damage or serve as a physiological messenger.

trisphosphate (IP_3)-operated Ca^{2+} channels (IP_3R), ryanodine-sensitive receptors (RyR), nicotinic acid adenine dinucleotide phosphate ($NAADP^+$)-dependent release or a combination of the three.[18] Which mode of release is used depends on the cell type, location within the cell and the nature of the stimulus. In non-excitable cells, extracellular stimuli (like hormones) generally trigger Ca^{2+} release from the ER via IP_3-mediated release. In excitable cells like skeletal muscle, Ca^{2+} is released from the sarcoplasmic reticulum (SR) via opening of the RyR. This process is triggered through a physical interaction between the RyR and the DHPR (dihydropyridine receptor) in the sarcolemma, which acts as a voltage sensor.

Other excitable cells, like pituitary melanotropes, display Ca^{2+} influx across the plasma membrane by means of voltage-operated Ca^{2+} channels (VOCCs) that transiently open upon depolarization.[19]

The open probability of both the IP_3R and RyR depends on $[Ca^{2+}]_C$ in a bell-shaped fashion, meaning that low $[Ca^{2+}]_C$ stimulates the release of Ca^{2+} whereas high $[Ca^{2+}]_C$ acts inhibitory. This property allows Ca^{2+}-induced Ca^{2+}-release (CICR), which is responsible for the propagation of Ca^{2+} waves in many cell types. Ca^{2+} release is terminated by closure of IP_3R/RyR/VOCCs, after which Ca^{2+} is rapidly removed from the cytosol by the concerted action of

the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and PMCA.[20,21] In most non-excitable cells, a dedicated Ca^{2+} entry mechanism ('capacitative Ca^{2+} entry' or Ca^{2+}-release activated Ca^{2+} entry: CRAC), that serves to refill the ER, becomes activated upon depletion of the ER.[22]

Calcium as a Local Messenger

Cytosolic Ca^{2+} signals can display a complex spatio-temporal arrangement which is independent of the underlying mechanism.[21] It is generally observed that in the continuous presence of an external stimulus cells display short-lasting increases (oscillations) in $[Ca^{2+}]_C$ (temporal aspect). These pulsatile increases can be extremely localized or global, spreading as a wave over the entire cell (spatial aspect). Inside the ER, Ca^{2+} also appears to function as a signaling molecule[23] that regulates protein synthesis, apoptosis[24] and the folding, sorting and/or degradation of proteins.[25] Cytosolic Ca^{2+} signals also propagate into the nucleoplasm where they not only initiate gene expression and cell cycle progression, but also can activate degradative processes during programmed cell death, or apoptosis.[26] Prolonged high $[Ca^{2+}]_C$ promotes DNA digestion by direct stimulation of endonucleases, or indirectly by activation of Ca^{2+}-dependent proteases, phosphatases, and phospholipases, resulting in a loss of chromatin structural integrity.[27] Obviously, the pulsatile nature of the hormone-induced $[Ca^{2+}]_C$ rises allows Ca^{2+} signaling with a minimal risk of cell damage. It is evident that changes in cell metabolism that alter cytosolic Ca^{2+} handling will compromise the coordinated control of cellular activities.

Mitochondrial Participation in Cellular Calcium Signalling

Mitochondria can accumulate Ca^{2+} at up to 0.5 mM in the mitochondrial matrix.[20,28] Ca^{2+} uptake is mediated by a $\Delta\psi_m$-dependent electrogenic uniporter, whereas Ca^{2+} efflux, being 100-1000 times kinetically slower than Ca^{2+} uptake[28,29], occurs by either Na^+/Ca^{2+} or H^+/Ca^{2+} exchange.[1] Because the uniporter displays a low affinity for Ca^{2+} ($K_{0.5}$ = 5-10 μM[3]), mitochondrial Ca^{2+} uptake at resting $[Ca^{2+}]_C$ is neglectible. Several hormones and neurotransmitters that induce Ca^{2+} release from the ER also rapidly increase the Ca^{2+} concentration in the mitochondrial matrix ($[Ca^{2+}]_M$). This means that mitochondria have to be closely juxtaposed to the ER Ca^{2+} release sites to allow $[Ca^{2+}]_C$ to rise sufficiently high for activation of the uniporter.[15,30-35] A similar local communication between ER Ca^{2+} release channels and adjacent mitochondrial Ca^{2+} uptake has been demonstrated in cardiac cells, where Ca^{2+} that was released via a single RyR induced miniature Ca^{2+} signals inside the mitochondrial matrix ('Ca^{2+} marks'[36]).

The mitochondrial matrix contains several components involved in mitochondrial ATP production that are Ca^{2+}-sensitive (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+-isocitrate dehydrogenase and complex V itself[1,30,37-39]). Indeed, several external stimuli have been demonstrated to stimulate mitochondrial metabolism in a Ca^{2+}-dependent manner.[30,34,37] This tight coupling between $[Ca^{2+}]_C$, $[Ca^{2+}]_M$ and mitochondrial metabolism ensures that ATP is only produced at those times and places in the cell where it is needed (i.e., to fuel PMCA/SERCAs or other energy consuming processes). Also during $[Ca^{2+}]_C$ oscillations, the activity of CSMDHs (Ca^{2+}-sensitive mitochondrial dehydrogenases) is regulated by the frequency of the oscillating $[Ca^{2+}]_M$.[40] In addition, the slow rate of Ca^{2+} efflux from the mitochondrion allows a prolonged metabolic activation of the mitochondrion following stimulation.[41]

The kinetic properties of mitochondrial Ca^{2+}-uptake and -release result in a significant buffering of cytosolic Ca^{2+} signals[37,42] and indicate that mitochondria can actively participate in Ca^{2+} homeostasis under physiological conditions.[43] It is evident that this participation has not only consequences for the control of mitochondrial function but also for the modulation of the complexity of cytosolic calcium signals themselves. The latter has been observed in mathematical models, which predict that mitochondria maintain constant amplitudes of cytosolic Ca^{2+} oscillations.[44] Other modelling studies predict that mitochondrial Ca^{2+} uptake is essential to prolong the recovery time of IP_3Rs from a refractory state.[45] This crucial role of mitochondrial uptake, for instance in exocytosis, inactivation of VOCCs and neuronal excitability, has also been demonstrated in several experimental studies.[46-48]

Generation of Reactive Oxygen Species

Reactive oxygen species (ROS) can be generated intracellularly by several pathways (Fig. 3). In higher organisms, mitochondria are the quantitatively most important source of superoxide ($O_2^{\cdot -}$), which is generated by the reduction of molecular oxygen (O_2) by electrons leaking from the ETC.[49-51] This leak, totaling to about 1-2% of total electrons passing the ETC, mainly occurs at complex I (NADH:ubiquinone-oxidoreductase) and complex II (ubiquinol:cytochrome-c-oxidoreductase; Robinson, 2000). Within the respiratory chain, a major site for the univalent reduction of O_2 to $O_2^{\cdot -}$ is ubisemiquinone, that is generated in iron-sulfur cluster N2 of complex I at the interface between the NDUFS7 (PSST) and NDUFS2 (49-kDa) subunits in the electron transfer reaction from NADH to ubiquinone[52] The resulting ubisemiquinone radicals provide a constant source of $O_2^{\cdot -}$ and therefore are specifically detoxified by quinone reductase (QR).[53,54]

The net amount of $O_2^{\cdot -}$ produced depends on the balance between production and detoxification and on the electron influx (at complex I and complex III) and the consumption of electrons (at complex IV). The latter is typically low if the pH gradient is high, i.e., if only a few protons are being consumed by the ATP-generating system as a result of relatively high ATP and low ADP concentrations within the mitochondrial matrix.[50] This condition normally inhibits glycolysis and the influx of energy substrates in the mitochondrial matrix. $O_2^{\cdot -}$ itself can be toxic, especially through inactivation of proteins that contain iron-sulfur clusters such as aconitase, succinate dehydrogenase and several subunits of complex I.

$O_2^{\cdot -}$ is rapidly detoxified by the action of superoxide dismutases converting it into hydrogen peroxide (H_2O_2). Mammalian cells contain an intramitochondrial Mn^{2+} superoxide dismutase (SOD2) and a cytosolic Cu^{2+}, Zn^{2+} superoxide dismutase (SOD1) in the inter membrane space (IMS). A third Cu^{2+}, Zn^{2+} superoxide dismutase (SOD3) is present extracellularly. H_2O_2 generated on either side of the mitochondrial inner membrane is processed to H_2O by glutathione peroxidase (GPX). In this latter reaction, reduced glutathione is converted to oxidized glutathione. Cytosolic H_2O_2 can also be removed by the action of catalase (CAT). By reacting with endogenous nitric oxide (NO) that is generated from L-arginine by nitric oxide synthase (NOS), $O_2^{\cdot -}$ can be converted into peroxynitrite ($ONOO^-$). This reactive nitrogen species (RNS) is particularly capable, similar to H_2O_2, of inactivating mitochondrial aconitase.[55,56]

Fe^{2+}, released from damaged iron-sulphur proteins can promote the formation of the hydroxyl radical ($OH^{\cdot}$) from H_2O_2 (Fenton reaction). This $OH^{\cdot}$ is very reactive and causes peroxidative damage to proteins, DNA and lipids. The latter is especially important for mitochondrial and cellular functions because the integrity of mitochondrial outer membrane prevents release of cytochrome c, and thereby, mitochondria-dependent caspase activation and apoptotic cell death.[57] The Fenton reaction is inhibited by ferritin and heme oxygenase (HOx). Recently it has been shown that iron-deficiency also stimulates oxidant-induced damage to mitochondria in liver.[58] It therefore appears that both iron deficiency and moderate iron excess induce functional deterioration of mitochondria and that iron-levels need to be tightly controlled.

In contrast to $OH^{\cdot}$, some of the $O_2^{\cdot -}$ and H_2O_2 generated may diffuse from the mitochondrion to damage distant cellular components.[59] Trivially, mitochondrial proteins are essentially all susceptible to inactivation by $OH^{\cdot}$ but, surprisingly, are rather resistant to the effects of H_2O_2.[60]

In analogy to Ca^{2+}, ROS can also function as a physiological messenger, for example during cell growth and differentiation.[51] It appears that consequences of ROS generation on the cellular level depend on the magnitude and duration of the change in ROS and RNS concentration.[50] In this mechanism, a temporary imbalance acts regulatory whereas persistent changes lead to pathological conditions. Interestingly, two bacterial proteins, the transcription factor OxyR and the chaperone heatshock protein 33 (Hsp33[61]), are activated by the oxidation of cysteine residues to disulfide bonds.[62] This allows functioning of redox-operated genetic switches that sense (mitochondrial) stress by disulfide bond formation.[63] In this context it is relevant

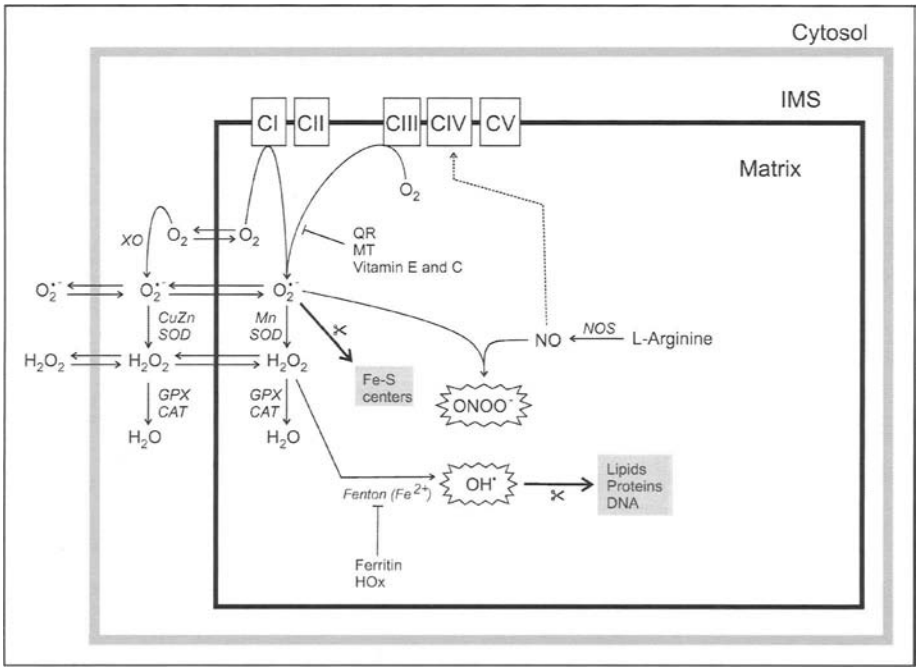

Figure 3. Generation and detoxification of reactive oxygen species. Superoxide ($O_2^{\cdot-}$) is mainly generated at complex I and III from molecular oxygen (O_2). This production is inhibited by several antioxidant systems. Quinone reductase (QR) detoxifies quinone compounds, metallothioneins (MT) trap metal cations and vitamins E and C trap free radicals. Via an intermediate conversion (catalyzed by superoxide dismutase; SOD) to hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$ is converted into H_2O by glutathione peroxidase (GPX) and catalase (CAT). There are two types of mitochondrial SOD, a manganese SOD in the mitochondrial matrix and a copper-zinc SOD between the mitochondrial inner and outer membrane in the inter membrane space (IMS). In an alternative reaction, $O_2^{\cdot-}$ can react with nitric oxide (NO, formed from L-arginine by nitric oxide synthase; NOS) to yield the reactive and harmful reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$). By reaction of H_2O_2 with iron (Fe^{2+}) in the Fenton reaction, the extremely reactive (lifetime: ~1 nanosecond) hydroxyl radical ($OH^{\cdot}$) is formed, which can damage (mitochondrial) lipids, proteins and DNA. The Fenton reaction is inhibited by ferritin and heme oxygenase (HOx). $O_2^{\cdot-}$ can also attack proteins containing Fe-S centers thereby releasing additional Fe^{2+}.

that Nuclear Factor Kappa B, a protein transcription factor that is required for maximal transcription of a wide array of pro-inflammatory mediators, is also redox-regulated.[64] Additionally, the nuclear $NAD^+/NADH$ ratio can be used to control transcription via the redox-sensing transcriptionfactor CtBP (carboxyl-terminal binding protein[60]). On the other hand, gene expression can also be repressed by oxidative stress.[65]

ROS can also more directly affect cellular signaling pathways. For example by inhibiting receptor protein-tyrosine phosphatase α by modifying its structure[66] or by reversibly inactivating KGDH (α-ketoglutarate dehydrogenase) and SDH (succinate dehydrogenase[56]). Interestingly, $O_2^{\cdot-}$ activates mitochondrial uncoupling proteins (UCP1). This diverts energy from ATP synthesis to thermogenesis in the mitochondria of brown adipose tissue by catalyzing a regulated leak of protons across the inner mitochondrial membrane.[67]

OXPHOS functioning can also be controlled by ROS, as demonstrated by the finding that endogenous generation of low steady state levels of NO by eNOS (endothelial NOS) dynamically regulates mitochondrial respiration, which provides protection against H_2O_2-mediated injury and cell death.[68] Moreover, NO can be a substrate, inhibitor or effector of complex IV,

depending on cellular conditions.[69] As far as ROS production is concerned, recent evidence suggests that electronflow through complex I and the production of ROS can be regulated by PKA-mediated phosphorylation of the NDUFA1 (MWFE) and NDUFS4 (AQDQ) subunits of this complex.[53] This might provide additional flexibility in respiratory control.

Cross links have also been demonstrated between Ca^{2+} handling and ROS. This is not surprising since various oxidants cause Ca^{2+} influx into the cytoplasm, and consequently into both mitochondria and the nucleus.[70] Moreover, mitochondrial damage reduces ATP levels and mitochondrial Ca^{2+} uptake and thereby directly affects cytosolic Ca^{2+} signalling. Of relevance in this context is the observation that oxidation of Ca^{2+}-ATPases inhibits their ability to extrude Ca^{2+} out of the cell or into the ER.[70] Additionally, a number of studies indicate that Ca^{2+} release through the IP_3R is stimulated by oxidants.[71] In astrocytes, increased mitochondrial ROS generation initiates a destructive cycle involving Ca^{2+} release from intracellular stores and mitochondrial Ca^{2+} loading, which further increases ROS production. This amplification of oxidative stress and Ca^{2+} loading induced opening of the permeability transition pore (PTP) and necrotic cell death.[72] In endothelial cells, a bolus of H_2O_2 or oxygen metabolites generated by hypoxanthine oxidase increased the $[Ca^{2+}]_M$.[73]

OXPHOS and Mitochondrial Morphology

Based upon light- and electron-microscopy studies, mitochondria were originally described as kidney-bean-shaped structures.[74] In recent years, however, the development of powerful imaging techniques in combination with mitochondrion-specific fluorescent dyes[75] and mitochondria-targeted spectral variants of GFP (green fluorescent proteins) has altered this view (Fig. 4).[76] It has become clear that, depending on the cell type and/or physiological condition of the cell, mitochondria can exist as shorter or longer filaments, more or less branched reticula, or as a continuous interconnected reticulum.[77] The morphological plasticity is presumably coupled to variations in energy demand and essential for local regulation of cellular processes.[36] This is supported by a recent study in which a perinuclear localization of mitochondria was required to support transport across the nuclear membrane.[78] Mitochondrial proliferation and degradation appears to depend on functional states of the organelles or energetic states of the cell.[79] This makes sense because disorganization of these processes is often associated with abnormal accumulation of mitochondria in various models of cell death.[80,81] Of note, the number of mitochondria, as well as their form and volume are closely related to both the cell cycle phase and functional state of the cell.[82] It has been proposed that the increase in mitochondrial mass observed during chronic exercise lowers the rate of respiration per mitochondrion for any given workload, thus reducing the level of potentially damaging ROS.[83]

A mitochondrial network can act as an intracellular cable that locally delivers ATP,[77] transmits electrical signals[84,85] or functions as a Ca^{2+} transport system.[86] Recently, this view has been challenged by Bootman and coworkers, who observed in various cell lines that mitochondria within individual cells were morphologically heterogeneous, unconnected and possessed distinct functional properties.[87]

The morphology of the mitochondrial reticulum depends on abundant fusion and fission events between mitochondrial tubules.[35,88-91] Fusion of mitochondria likely serves to mix and unify mitochondrial compartments. This is important for the inheritance and maintenance of the mitochondrial genome.[92] In human cells, mitochondrial fusion can protect against mitochondrial dysfunction by complementation of DNA products.[93] This complementation however, appears to depend on the used experimental system.[94,95] At present, only limited information is available concerning the molecular players involved in the competing fission and fusion processes that determine mitochondrial structure.

Most research on the regulation of mitochondrial fusion and fission has been performed in yeast. It was discovered that a dynamin-related protein, referred to as Dnm1p, is involved in mitochondrial division.[89,90,96,97] In analogy to the role of genuine dynamins in the final stages of vesicle formation, Dnm1p is thought to wrap around constricted parts of mitochondria where it helps to sever the mitochondrial outer membrane. Two recently discovered factors in

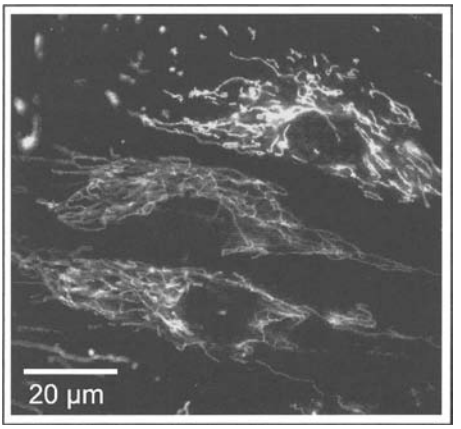

Figure 4. Mitochondrial morphology in human skin fibroblasts. Staining living human fibroblasts with the lipophilic cation rhodamine 123 reveals extensive mitochondrial structures (W. Koopman, unpublished observation).

yeast, Mdv1p (alias Fis2, Gag3) and Fis1p (Mdv2), appear to assist Dnm1p in scission of the mitochondrial membrane.[98-100] From these studies it was concluded that defective fusion causes mitochondrial networks to become overly fragmented[89,90] and probably leads to a decrease in mitochondrial function. It is still unclear how division of the outer membrane and division of the inner membrane are coordinated. The existence of a separate inner membrane division apparatus, acting in concert with the external Dnm1p complex, has been hypothesized.[101] This idea implies the presence of a signaling mechanism linking the inner and outer membrane division machineries. Another dynamin, Mgm1p, could be a suitable candidate for this role since in yeast it was proposed to be responsible for mitochondrial inner membrane division.[101]

In mammalian systems, the homologs of Dnm1p (Drp1 and DLP1) and Mgm1p (OPA1) contribute to mitochondrial distribution and function.[96,102,103] Additionally, two human genes encoding potential mediators of mitochondrial fusion, Mfn1 and Mfn2 ('mitofusins'), have been identified.[91] These GTPases are homologs of the fuzzy onion (fzo) protein, first identified in Drosophila, and are opposed by dynamin related GTPases. Recently, it has been shown that both mitofusins are ubiquitous mitochondrial proteins and that Mfn2 resides in the outer mitochondrial membrane.[104] Moreover, this protein recruited mitochondria to the perinuclear region independently of the cytoskeleton and was capable of modifying mitochondrial structure without affecting the integrity of the inner and outer membranes.

The MIM and MOM appear to be linked by several classes of contact sites.[105] These sites play a role in bidirectional metabolite channeling between mitochondrial matrix and cytosol, coordination of fusion and fission and translocation of proteins.[106] Electron microscopy, has revealed another distinct class of morphological contact sites that are not involved in any of these processes but extremely stable.[105] It are the latter sites that might transfer morphological changes of the MOM to the MIM. Because the morphology of the MIM (co)determines the relative localization of its embedded proteins it likely affects the efficiency of the OXPHOS system, for example by changing the efficiency of substrate channeling between individual complexes[107] or altering the viscosity of the mitochondrial matrix as a function of respiratory state.[108] In such a mechanism, the bilayer of the MIM may shape the structure of the OXPHOS complexes or the individual proteins of which they are composed. It is likely that mitochondrial morphology is at least co-regulated from within the matrix. This is illustrated by studies in yeast that demonstrated that loss of mitochondrial Hsp70, involved in protein import and folding, causes aggregation of mitochondria.[109] The authors of this study proposed the presence of an unknown matrix protein responsible for the maintenance of mitochondrial mor-

phology that requires Hsp70 for its function. In relation to OXPHOS, evidence has been presented for the existence of a functional link between dimerization of complex V and the morphology of the MIM.[110]

Cellular Consequences of OXPHOS Deficiency

Pharmacological Studies

Earlier studies have demonstrated that pharmacological inhibition of mitochondrial activity by for example uncouplers, induces signaling pathways involving the cAMP-responsive element-binding protein,[111] cytosolic calcium[112] and MAP kinases.[113] Chemical inhibition of complex I by rotenone leads to altered redox-potentials, excessive radical production, enhanced lipid peroxidation,[114] alterations of the mitochondrial membrane potential, apoptosis and decreased ATP production.[115] Also the kinetics of cytosolic Ca^{2+} signals and oscillations is affected.[116-118] In other studies, complex I inhibition by MMP (1-methyl-4-phenylpyridinium) induced caspase-3 activation demonstrating a link with the apoptotic machinery.[119]

Recent evidence has accumulated pointing toward the mitochondrial membranes as the key targets for lipid and glycolipid mediators of stress-induced apoptosis. These membranes may thus act as sensors of cellular stress by quantization of the local accumulation of specific lipids and glycolipids.[120] Alternatively, the resulting alterations in mitochondrial membrane composition might affect their structure and consequently OXPHOS function.

A profound effect on mitochondrial functions has already been demonstrated for ceramides, involved in apoptosis,[31] and GD3 ganglioside (actively synthesized in the early phases of apoptosis). Anti-apoptotic proteins like Bcl-2 play an important role in the regulation of mitochondrial physiology and mitochondria-dependent caspase activation.[57,121] It has been suggested that the anti-apoptotic effect of Bcl-2 is related to its ability to maintain a threshold level of $[Ca^{2+}]_M$ and $\Delta\psi$, while the pro-apoptotic protein Bik has the opposite effect.[122] Also the Bcl-2-induced reduction of $[Ca^{2+}]$ inside the ER, appears to lower the sensitivity of cells to apoptotic insults.[24] In larger cells like myotubes, mitochondrial Ca^{2+} uptake during apoptotic insults leads to traveling mitochondrial Ca^{2+} waves that are blocked by a Bcl-2 family member (Bcl-x_L), involve PTP opening and yield cytochrome c release, caspase activation and nuclear apoptosis.[123] In this way, apoptotic agents transform the mitochondria into an excitable state by sensitizing the PTP to Ca^{2+}.

Interestingly, B16.6 (alias GRIM-19), a cell death regulatory protein, was recently identified as the 43rd subunit of complex I, providing a new link between the mitochondrion, the ETC and apoptotic cell death.[124] When cells are induced to undergo apoptosis in the general presence of caspase inhibitors and then returned to their normal growth environment, there follows an extended period during which the entire population of mitochondria (including their mitochondrial DNA) disappears from the cell.[125] This widespread phenomenon is blocked by Bcl-2 overexpression and has been attributed to ROS-induced mitoptosis[126] (mitochondrial apoptosis). It is suggested that injury accumulation is monitored by a special system that actuates a mitoptotic program when the number of injuries reaches some critical level.

Changing nuclear gene expression is another cellular response to alterations in mitochondrial activity. This process of retrograde communication[127] has been shown to be activated by mitochondrial stress or compromised ATP synthesis. Results obtained in human endothelial cells demonstrated that the synthesis of a subset of mitochondrial proteins (among which the NDUFV1 and complex III-core 2 protein) was stimulated after exposure to hydroperoxide stress. This suggests that ROS might constitute (part of) the retrograde signal that signals mitochondrial damage to the nucleus.[128] A similar treatment in HeLa cells, induced expression of ferritin (an iron sequestering protein) which reduced ROS accumulation in response to oxidant challenge.[129] In again another model system (mouse liver mitoplasts), multiple isoforms of mitochondrial glutathione S-transferase were differentially induced under oxidative stress.[130]

From this it can be concluded that antioxidant and pro-oxidant mechanisms are important regulators of redox-sensitive transcription factors and other signal transduction pathways.[131,132]

As far as mitochondrial morphology is concerned, current information is limited. Optical scatter imaging was recently used to quantify calcium-induced alterations in mitochondrial morphology. It was found that mitochondria transformed from elongated organelles into spherically shaped particles, which likely is caused by Ca^{2+} overload-induced PTP opening and loss of mitochondrial membrane integrity.[7] Another biophysical technique, Fourier imaging correlation spectroscopy, was used to follow the dynamics of the mitochondrial reticulum in osteosarcoma cells.[133] This study demonstrated long-range jump movements that were associated with the action of cytoskeletal components. The latter is important because the distribution of mitochondria in many mammalian cells is regulated by interphase microtubules. Depolarization of microtubules by nocodazole or colchicine arrested asynchronously cultured cells in G_2/M phase of cell cycle and at the same time inhibited mitochondrial mass increase and mtDNA replication.[82] Thus far, a temporarily fragmented mitochondrial phenotype was observed during the S phase of the cell cycle.[77] Furthermore, fragmentations could be artificially induced in human lung fibroblasts and osteosarcoma ρ0 cells in which the mitochondrial DNA was eliminated by ethidium bromide treatment.[134]

Results in Patient-Derived Cell Lines

Abnormalities of mitochondrial metabolism causing human disease comprise defects of transmembrane transporters, fatty acid oxidation, the pyruvate dehydrogenase (PDH) complex, tricarboxylic acid cycle enzymes and disturbances of the OXPHOS system.[135]

A recent study demonstrated for the first time that mitochondrial Ca^{2+} homeostasis was disturbed in cells derived from patients with the $tRNA^{Lys}$ mutation associated with MERRF[136] (myoclonic epilepsy with ragged red fibers). Importantly, treatment of MERRF cells with drugs affecting organellar Ca^{2+} transport mostly restored both the agonist-dependent mitochondrial Ca^{2+} uptake and the ensuing stimulation of ATP production. Agonist-induced mitochondrial Ca^{2+} uptake was not changed in cells derived from patients with the ATPase mutation of NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa). This contrasts to other studies in which skin fibroblasts from MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes) patients had elevated cytosolic Ca^{2+} levels and could not normally sequester Ca^{2+} influxes due to a decreased $\Delta\psi_m$.[137] Moreover, in complex IV-deficient fibroblasts from patients suffering from Leigh disease CRAC channels were not sufficiently activated.[138] The latter could indicate that mitochondrial Ca^{2+} uptake is required to prevent inactivation of these channels. Taken together, these results emphasize the differences in the cellular pathogenesis of the various mitochondrial DNA defects and indicate specific pharmacological approaches to the treatment of some of the mitochondrial diseases.

In complex I-deficient patients, an excessive formation of hydroxyl radicals and aldehydic lipid peroxidation products was observed in cultured skin fibroblasts.[113] Robinson and co-workers measured O_2^- production in mitochondria isolated from skin fibroblasts and found that it was markedly increased in patients with the mildest symptoms (complex I deficiency associated with cataracts and developmental delay; CD), whereas it was even lower than control values in mitochondria from patients with severe defects[139] (complex I deficiency associated with cardiomyopathy with cataracts; CC). In contrast, SOD2 expression was highest in CC and equal to control in CD. Residual complex I activity was slightly lower in CC than in CD. These data show that a similar decrease in complex I activity may or may not be accompanied with an upregulation of SOD2 and a consequent decrease in $O_2^{\cdot -}$ production. It was concluded that mutations in complex I subunits are of two types (i) those that block the flow of electrons before the quinone reduction centre (cluster N2) and (ii) those that block after. Those that block before will result in impairment solely of oxidative phosphorylation while those that block at or after will result in both impairment of oxidative phosphorylation and generation of excess O_2^-. If O_2^- production is high induction of SOD2 may occur. As a result, excess H_2O_2

will be produced. This H_2O_2 will be converted to the highly reactive OH· by the action of Fe^{2+} released during O_2^- induced inactivation of proteins containing iron-sulfur clusters. Of note, a layer of insulating protein surrounding the electron pathway prevents the capture of electrons by other redox acceptors present in the aqueous and membrane phases.[140]

Indeed, high levels of SOD2 were found to be accompanied with high rates of OH· production and lipid peroxidation.[113,139] Cell cultures with high levels of SOD2 expression appeared to be hypersensitive to toxicity from menadione, paraquat and other free radical amplification agents.[113] Apparently, upregulation of SOD2 is not accompanied by upregulation of H_2O_2 metabolizing enzymes (glutathione peroxidase and/or catalase). An intriguing observation in this context is that transgenic Drosophila with increased SOD1 did not show increased life span, unless accompanied by increased expression of catalase to remove H_2O_2. When taking into account that only SOD2 is upregulated as a consequence of complex I deficiency this finding suggests that the severity of the clinical symptoms may, at least in part, reflect the patient-specific expression pattern of the H_2O_2 metabolizing enzyme systems. In other words the symptoms may be considerably milder in patients with a genetically determined high expression level of these activities.

Mathematical Modelling Studies

Because the OXPHOS system plays an important role in mitochondrial and cellular functioning, it is not surprising that defects in this system can have profound effects. Although the cell biological analysis of these effects has been the subject of several studies during the last years, it is difficult to come up with a model that unifies the diverse experimental results. This is caused by the fact that mitochondrial function has been assessed under diverse experimental conditions using many model systems. Furthermore one should realize that (I) the consequences of (subtle) changes in respiratory chain capacity differ between tissues, cells, individuals and organisms, and (II) mitochondrial and cellular signalling pathways are intertwined by numerous regulatory cross links.

During the last years, considerable energy has been put into the construction of mathematical models to understand OXPHOS functioning and control in different tissues.[141-143] Among these models, only that developed by Korzeniewski and co-workers has been tested for a broad range of experimentally-measured parameter values and system properties.[144] It predicts that different OXPHOS complexes are activated simultaneously to increase the resistance of the system to a decrease in concentration/activity of its constituting complexes.[145] The physical factor that activates different OXPHOS complexes still remains to be identified, although Ca^{2+} activates at least some of these complexes. The 'parallel-activation mechanism' helps to keep intermediate metabolite concentrations as constant as possible, increases the capacity of OXPHOS and shortens transition times between different steady states. Interestingly, such a mechanism also delays the effect of inborn enzyme deficiencies, physiological inhibitors, external poisons and substrate shortage.[145]

Some of the results obtained experimentally or by modelling are understandable in using the framework of metabolic control analysis.[146-148] These studies demonstrate that the respiratory rate (and phosphorylation potential) depends on the relative activity of particular OXPHOS complexes. This dependence displays a characteristic threshold value of enzyme activity/concentration, above which the respiration rate is not affected significantly and below which it decreases rapidly.[149] The values of the obtained control coefficients can therefore be used to predict the effect of deficiencies during OXPHOS diseases. Letellier and co-workers have proposed that the control coefficient for individual OXPHOS complexes varies between tissues. This means that individual tissues/organs will display different sensitivity to an OXPHOS defect.[150] In brain mitochondria this approach revealed that complex I activity has a major control of OXPHOS.[151] Korzeniewski hypothesized that every mechanism which leads to an increase in the capacity of OXPHOS and to stability of the intermediate metabolite concentrations (especially of phosphorylation potential), is very profitable for the compensation of the

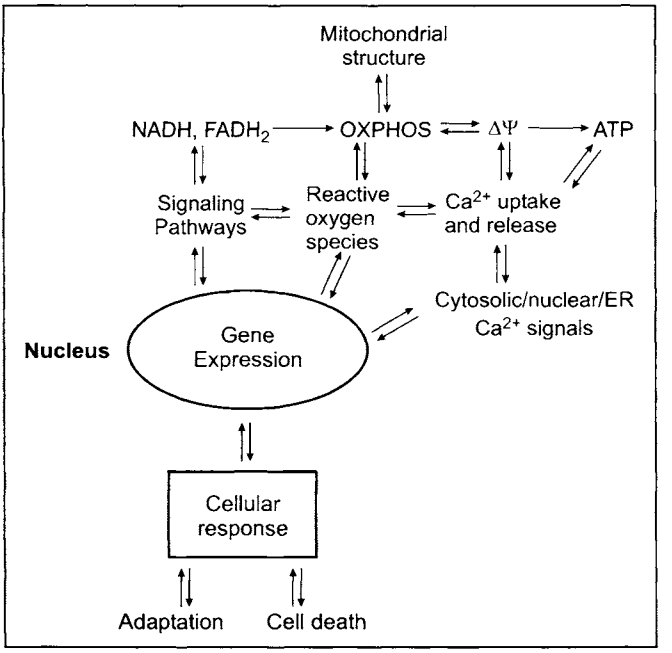

Figure 5. Effects of OXPHOS deficiency at the cellular level. The mitochondrial OXPHOS system is central to mitochondrial functioning. It maintains the mitochondrial membrane potential ($\Delta\psi$) and thereby the production of ATP. Moreover, OXPHOS is the main generator of reactive oxygen species (ROS) which can function as signaling molecules or cause cell damage. Mitochondrial Ca^{2+} uptake, which is also driven by OXPHOS, stimulates mitochondrial ATP production and affects the dynamics of cytosolic, nuclear and intra-ER Ca^{2+} signals. In this way, mitochondrial (dys)function can significantly affect cellular physiology, for example by modulating gene expression. The effectiveness of the cellular response determines whether the cell dies or successfully adapts to OXPHOS deficiency.

effect of inborn enzyme deficiencies.[145] Such a mechanism would delay the manifestation of mitochondrial diseases by decreasing the threshold value in the relative activity of OXPHOS.

Adaptive Mechanisms

In general, mitochondrial metabolism adapts to cellular requirements. For example in muscle, where mitochondrial mass increases as a result of exercise[83] or in rapidly proliferating cells where gene transcription of some mitochondrial-encoded proteins is increased (Fig. 5).[152]

Also during pathological conditions, for example arising from OXPHOS dysfunction, mitochondria are expected to (partially) compensate for the defect by activation of retrograde pathways. The latter is crucial because mitochondrial biogenesis and function depends on the coordinated balancing of two genomes.[153]

Indeed, an increased expression of 'energy genes' appears to a general adaptive phenomenon that was observed during mitochondrial dysfunction in uncoupler-treated PC12 cells,[113] tissues of patients carrying the MELAS A3243G mitochondrial DNA mutation[154] and mice deficient in the heart/muscle specific isoform of the adenine nucleotide translocator[155] (ANT1). P53, a homotetrameric transcription factor that senses DNA damage, might be an important constituent of the retrograde pathway because it has a direct positive effect on mitochondrial biogenesis and function.[156]

Moreover, moderate ROS-induced oxidative stress triggers adaptive responses during which antioxidant defenses are activated and ROS-producing systems are repressed.[65] Similar differential gene induction was also observed for glutathione S-transferase isoforms.[130]

Recent evidence demonstrates that mitochondria possess an organelle-specific stress response, which increases the expression of mitochondrial matrix chaperones (chaperonin 60, chaperonin 10, mtDNAJ) and a protease (ClpP) via activation of the CHOP (C/EBP homology protein) transcription factor.[157]

An important adaptive mechanism involves the selective degradation of defective mitochondria, either by autophagy, specific degradation of aberrant protein molecules, or mitochondrial apoptosis.[79] The latter is an example of the 'samurai law of biology' which states that "it is better to die than to be wrong".[85] Especially the accumulating ROS-induced damage resulting from OXPHOS deficiency might trigger selective mitoptosis to prevent cellular dysfunction.

Summary

Almost all mitochondrial functions require a functional oxidative phosphorylation (OXPHOS) system. Because cellular and mitochondrial metabolism is tightly interconnected, OXPHOS deficiency will manifest itself both at the mitochondrial and cellular level. We have illustrated this in this chapter using ATP generation, calcium homeostasis, ROS production and mitochondrial morphological plasticity as examples. At present, the cellular consequences of OXPHOS deficiency have mainly been analyzed using pharmacological model systems and patient-derived cell lines. Mathematical modeling of the obtained results, applied to integrate and understand the complex experimental data, support the presence of adaptive mechanisms, which counterbalance the deficiency.

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CHAPTER 9

Animal Models of OXPHOS Disorders

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Abstract

Dysfunction of the mitochondrial respiratory chain has been associated with a wide range of human diseases ranging from diabetes to cardiomyopathy. Mutations in a number of nuclear as well as mitochondrial genes have been implicated in causing these diseases. Several animal models have now been created which reproduce some of the clinical pathology observed in human patients suffering from OXPHOS disorders. In this chapter we review some of these animal models of OXPHOS disorders and how they have led to a further understanding of both mitochondrial respiratory chain function and dysfunction.

Introduction

The link between mitochondrial dysfunction, due to an impaired respiratory chain, and human disease has been well documented within the last decade. There are between 80-100 protein subunits comprising the respiratory chain and only 13 of these are encoded by mitochondrial DNA (mtDNA) with the remainder being nuclear DNA encoded. It is not surprising, therefore, that both nuclear and mitochondrial DNA mutations can directly affect the function of mitochondria. A large number of mtDNA point mutations and rearrangements have been identified as the primary cause of OXPHOS disease in affected patients. In addition, an increasing number of nuclear DNA mutations are being discovered in mitochondrial disorders.[1-3] Despite this increasing knowledge in the underlying gene defects causing OXPHOS disorders, the molecular pathogenesis events that link the mutated gene to the observed clinical phenotypes are largely still undetermined.

In addition to the well-defined genetic disorders linked to mitochondrial dysfunction, there is circumstantial evidence that mitochondrial dysfunction perhaps plays a role in common disorders such as neurodegeneration (Parkinson and Alzheimer diseases), diabetes mellitus and heart failure. There have also been reports that an age-dependent accumulation of somatic mtDNA deletions may contribute to ageing.[4] To be able to ameliorate or cure these disorders, it is necessary for us to understand in more detail the mechanisms leading to the phenotypes observed. An ATP deficiency in affected cells is often assumed to be the main cause of pathology, however, no conclusive evidence has yet been presented to confirm this. Along with an ATP deficiency, an impaired respiratory chain also leads to alterations of cellular reduction-oxidation (redox) status, induction of the mitochondrial pathway for apoptosis or increased production of reactive oxygen species (ROS), all of which can lead to the pathogenesis observed in patients.

Whilst there is limited availability of human tissues, the ability to produce model organisms with specific nuclear or mtDNA mutations gives us the necessary tissue to study in-depth the resulting biochemical defects. Basic biochemical defects due to nuclear and mtDNA mutations have been studied in lower model organisms such as budding yeast, worms and fruit flies (one model of which is discussed here), however, it is clear that many physiological differences

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between these organisms and humans exist. Hence, there are a number of advantages in creating mouse models of OXPHOS disorders since both mice and humans display similar gene content, along with comparable types of internal organs and physiology. In this chapter we discuss a Drosophila model and a number of mouse models in which nuclear genes have been modified to reproduce mitochondrial respiratory chain disorders and also describe some of the most recent advances in directly manipulating mouse mtDNA to recreate symptoms observed in humans carrying mtDNA point mutations or mtDNA rearrangements. Table 1 provides a summary of the animal models discussed here.

A Drosophila Model of Mitochondrial Deafness

In the fruit fly *Drosophila melanogaster*, a mutant phenotype of bang sensitivity (*technical knockout, tko*) i.e., temporary paralysis resulting from mechanical vibration, was isolated and has been extensively studied.[5-7] This temporary paralysis is associated with a failure of signalling from mechanoreceptor neurons and a comparison was made between this phenotype and that of sensorineural deafness observed in humans resulting from a signalling failure in the mechanosensory receptor cells of the inner ear. It was found that by transgenically complementing a 3.2-kb fragment of nuclear DNA it was possible to rescue the *tko* phenotype.[8] This segment of genomic DNA encodes a single transcript for a homologue of bacterial ribosomal protein S12. The N-terminal sequence of this protein contained features commonly observed in mitochondrial targeting presequences indicating a probable mitochondrial localization for this protein.[9] Sequencing of the gene revealed a single amino acid change in the *tko* mutants which affects a conserved leucine residue.

A more detailed study of these mutant *tko* flies was undertaken to more clearly establish the phenotype.[10] A severe hearing deficiency was discovered along with developmental delays and behavioural and sensory abnormalities. Decreased activity of the mitochondrial complexes I, III, and IV were observed in mutant larvae, along with a reduction in the level of small subunit (12S) to large subunit (16S) mitochondrial rRNA, when compared with controls. Conclusive evidence that it is the point mutation L85H that causes the mutant phenotype was obtained by transgenically complementing homozygous mutants with the wild type gene for *tko* and consequently rescuing the mutant phenotype. Homozygous mutants complemented in the same way with the *tko* gene carrying the L85H mutation maintained the mutant phenotype. These results indicate a role for the *tko* protein in mitochondrial protein translation and a potentially useful model for human mitochondrial disease. Many mitochondrial mutations resulting in a dysfunction of mitochondrial protein translation are known to have a phenotype of sensorineural deafness, however, the reasons why a mitochondrial translation defect causes such a tissue-specific disorder is not understood. The availability of this model organism is of huge benefit to study the evidently essential role of mitochondria in the mechanosensory cells, and will aid the understanding of diseases leading to human sensorineural deafness.

Mouse Models of Nuclear DNA Mutations

Mammalian mitochondrial DNA encodes 13 polypeptides, all of which are involved in oxidative phosphorylation, along with 22 tRNAs and 2 rRNA subunits. Hence, the majority of proteins that make up the respiratory chain and all the proteins responsible for mtDNA maintenance and transcription are nuclear encoded, translated in the cytoplasm and imported into mitochondria. In more recent years, mutations in these nuclear encoded mitochondrial proteins have been found to be connected with mitochondrial disorders e.g., Leigh syndrome caused by mutations in the SURF1 protein.[11-12] The ability to reproduce phenotypic symptoms of these diseases by mutating the associated nuclear genes would be a major breakthrough for not only understanding the mechanisms of these disorders but also for developing effective treatments. To date no such mouse models have been successfully created. However, a number of nuclear genes encoding mitochondrial proteins have been knocked out to create either germ line or tissue specific knockout mice. These genes include *Ant1* connected with mitochondrial

Table 1. Animal models of OXPHOS disorders

Model	Biochemical Defect	Phenotype
Knockout fly		
Technical knockout, *tko*	Decreased activity of complex I, III and IV, reduction in levels of mitochondrial small-subunit rRNA in larvae.	Severe hearing impairment, developmental delays, behavioural and sensory abnormalities
Knockout mice		
Adenosine nucleotide translocator, *Ant1*	Impaired mitochondrial transport of ATP/ADP, defect in coupled respiration	Cardiac hypertrophy, mitochondrial myopathy
Glutathione peroxidase, *Gpx1*	Inability to reduce mitochondrial H_2O_2, reduced ATP production and respiration rates	Growth retardation
Mitochondrial superoxide dismutase, *Sod2*	Loss of mitochondrial superoxide dismutase activity, reduced aconitase activity	Dilated cardiomyopathy, lipid accumulation in liver and skeletal muscle, metabolic acidosis
Mitochondrial transcription factor A, *Tfam*		
Germ line *Tfam* knockout	mtDNA depletion, respiratory chain deficiency	Embryonic lethality at E9.5, mutant phenotype with absence of heart and optic disc, delayed neural development, caspase dependent apoptosis
Heart-specific *Tfam* knockout	mtDNA depletion, respiratory chain deficiency	Dilated cardiomyopathy, atrioventricular heart conduction blocks, caspase dependent apoptosis, induction of ROS defenses
β-cell-specific *Tfam* knockout	mtDNA depletion, respiratory chain deficiency	Diabetes, insulin deficiency, impaired stimulus-secretion coupling in β-cells in young knockouts, loss of β-cells in older knockouts
Neuronal *Tfam* knockout	mtDNA depletion, respiratory chain deficiency	Vulnerable to excitotoxic stress, induction of apoptosis and massive neuronal cell death precedes death at ~5 months
Frataxin, *Frda*		
Germ line *Frda* knockout	Loss of frataxin	Embryonic lethality at E7-8
Tissue-specific *Frda* knockout	Loss of frataxin, decreased activity of complex I, II, III and aconitase	Ataxia, loss of proprioception, hypertrophic cardiomyopathy, weight loss

Continued on next page

Table 1. Continued

Model	Biochemical Defect	Phenotype
Transmitochondrial mice		
Heteroplasmic mice	None	Maternal transmission of a neutral mtDNA polymorphism
Chloramphenicol resistance (CAP-R)	Not demonstrated	Ocular abnormalities, mitochondrial myopathy and cardiomyopathy, growth retardation, perinatal lethality
ΔmtDNA mice	Respiratory chain deficiency	Growth retardation, kidney failure, mitochondrial myopathy
Spontaneous mutants		
Defective nuclear-mitochondrial communication	Not demonstrated	Deafness

bioenergetics, Tfam a mtDNA maintenance factor, the antioxidant genes *GPx1* and *Sod2*, and a gene associated with the Frataxin syndrome, *Frda*.

Tissue Specific Adenine Nucleotide Translocator Knockout

The main function of the adenine nucleotide translocator (ANT) is to exchange mitochondrial matrix ATP for cytosolic ADP across the inner mitochondrial membrane, utilising the electrochemical gradient. In humans there are three isoforms of this protein, ANT1, ANT2 and ANT3, whereas there are only two isoforms in the mouse (Ant1 and Ant2). The gene *Ant1* is predominantly expressed in heart and skeletal muscle and has been designated as a heart/muscle specific isoform. By knocking out *Ant1* in mouse, an ATP deficiency would result in the heart and skeletal muscle tissues, effectively creating a tissue specific knockout. It was hoped pathological features arising in this mouse model would be similar to those observed in patients suffering from mtDNA mutation syndromes. Perhaps there would be a correlation between ATP deficiency and clinical manifestation giving further insight into the mechanism behind the diseases. More recently, two heterozygous missense mutations in *Ant1* were identified in patients suffering from adPEO (autosomal dominant progressive external ophthalmoplegia).[13] Large-scale mtDNA deletions are characteristic of this disorder, suggesting ANT1 may play a role in mtDNA maintenance.

Mice lacking *Ant1* were created by removing exons 1-3 producing a mutant allele that is null for Ant1 activity.[14] Homozygous mutants were viable, fertile and showed comparable growth to wild-type animals, however, there was a complete absence of the Ant1 protein and no *Ant1* gene expression. Skeletal muscle from mutant mice showed ragged-red fibres (RRF) as seen in mitochondrial disorder patients, along with increased succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX) activities. Hearts from mutant animals 4-6 months old showed cardiac hypertrophy and were enlarged when compared with wild-type hearts, however, no difference in heart size between mutants and wild-types was observed in the younger 6-8 week old mice. Again there was a marked mitochondrial proliferation in older mutant hearts. Mitochondrial respiration rates in skeletal muscle were compared in mutant and wild-type age-matched individuals and found to be significantly reduced in the *Ant1* knockouts, with a lack of ADP stimulated respiration; this was expected due to the mutants inability to transport ADP into or ATP out of it's skeletal muscle mitochondria. Consistent with the drop in respiration levels, homozygous mutant animals had an exercise intolerance, only being capable of

completing 54% of an exercise program that all wild-type mice completed. A metabolic profile from blood was compiled and the *Ant1* mutant mice showed a profile associated with OXPHOS deficiency. In conclusion, by knocking out the *Ant1* gene in mice it was possible to create an animal model presenting features commonly associated with mitochondrial myopathy and cardiomyopathy. In doing so it was demonstrated that mitochondrial ATP deficiency can lead to at least some of the clinical phenotypes manifested in humans with mitochondrial disorders.

As one of the morphological phenotypes of the *Ant1* mutant mice was mitochondrial proliferation in both skeletal muscle and heart it was hoped that by looking at gene expression profiles of these tissues it would be possible to identify factors involved in mitochondrial biogenesis as well as genes up-regulated due to OXPHOS dysfunction. By using differential display reverse transcription-polymerase chain reaction techniques 17 genes were found to be up regulated in the muscle of homozygous mutant mice. This included both mtDNA and nuclear DNA encoded respiratory chain components, some mitochondrial tRNA and rRNA genes and intermediary metabolism genes. Clearly, this is an attempt by the cells to increase energy metabolism. Additionally, genes connected with apoptosis (Mcl-1) and some genes potentially involved with mitochondrial biogenesis (SKD3) were increased in expression.

Another proposed mechanism for the onset of symptoms due to mitochondrial disease is the overproduction and toxicity of mitochondrial reactive oxygen species (ROS). Mitochondrial ROS are by-products of energy production by the respiratory chain and include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$). Manganese superoxide dismutase (*Sod2*) and glutathione peroxidase-isoform 1 (Gpx1) are antioxidant enzymes located in the mitochondria to convert ROS to harmless products. Due to the close proximity of mtDNA to the site of production of ROS it is essential that they are dissipated fast and efficiently to eliminate DNA damage. However, over the course of a lifetime a gradual build-up of ROS damage to mtDNA has been observed.[15,16] Skeletal muscle and heart from *Ant1* mutant mice were analysed for their levels of production of the ROS H_2O_2.[17] Both tissues were found to have maximal production when compared to age-matched wild-type individuals. Along with increased H_2O_2 was a concomitant increase in mRNA levels of *Sod2* as well as increased steady state protein levels of both *Sod2* and *Gpx1*. A greater number of mtDNA rearrangements were to be found in heart and to a lesser extent in the skeletal muscle of *Ant1* mutant animals as would be expected from the increased ROS production. Why do *Ant1* mutant mice produce more mitochondrial ROS? By inhibiting ADP/ATP transfer, proton transport through the ATP synthase complex is blocked and consequently inhibits the electron transport chain (ETC). Electrons would build up in the ETC complexes and be transferred to oxygen, generating $O_2^{\cdot -}$ and hence H_2O_2 levels will rise.

What conclusions can be drawn from the creation of the *Ant1* knockout mouse model? By inhibiting ADP/ATP transfer it is possible to recreate some of the symptoms observed in humans with mitochondrial disorders with a striking similarity to patients suffering from adPEO. Consistent with the phenotype of these animals is the dysfunctional OXPHOS system and an increased production of ROS with a concomitant rise in antioxidant levels. The increased mitochondrial ROS may well have precipitated the accumulation of rearranged mtDNA molecules leading to a negative feed back system; increasing proportions of damaged mtDNA due to ROS, giving rise to a more dysfunctional ETC and increasing ROS production. The mitochondrial manganese superoxide dismutase (MnSOD) knockout mouse model, discussed later, shows the role that mitochondrial ROS play in the pathophysiology of mitochondrial diseases.

Mice Deficient in Cellular Glutathione Peroxidase

Reactive oxygen species (ROS) are produced during cellular processes involving oxygen and include highly reactive molecules such as singlet oxygen ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Aerobic organisms have a defence mechanism to prevent oxidative damage to tissues and cells and includes an array of antioxidant enzymes, such as superoxide dismutases, glutathione peroxidases and catalase. In mammals there are five known glutathione

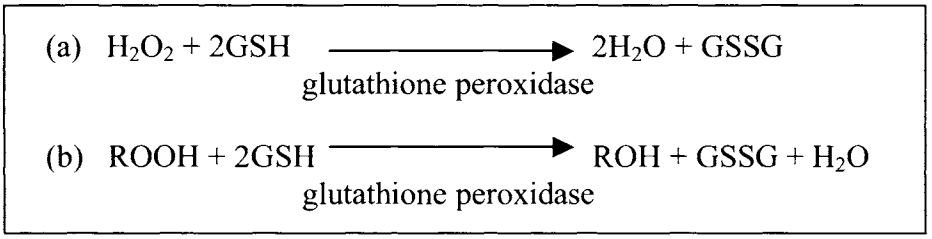

Figure 1. Reactions catalysed by glutathione peroxidase (a) reduction of hydrogen peroxide to form water; b) reduction of various hydroperoxides to form corresponding alcohols. GSH= reduced glutathione; GSSG= glutathione disulfide; ROOH= organic hydroperoxide.

peroxidase (Gpxs) isoenzymes that vary in expression levels between tissues. The function of Gpxs is to catalyse the reduction of H_2O_2 to water as well as a number of hydroperoxides e.g., DNA and lipid peroxides, to alcohols (Fig. 1). However, it is uncertain whether the primary role of these enzymes is to protect against oxidative damage under normal physiological conditions or if it has a more protective role during oxidative stress.

In order to map the expression pattern of glutathione peroxidase isoform 1 (Gpx1) a 'knock-in' mouse model was created by homologous recombination to insert the β-galactosidase gene into exon 1 of Gpx1.[18] In situ detection of β-galactosidase activity was performed by tissue staining with X-gal to reveal expression of Gpx1. In heterozygous mutant adult mice, high levels of Gpx1 expression were detected in liver and kidney cortex, with a small amount detected in heart, skeletal muscle, lung and spleen.

The gene encoding Gpx1 was first knocked out in mice in 1997 by Ho et al[19] by disruption of exon 2 with an inserted neo cassette. Although there were no detectable mRNA levels of the gene in brain, heart, kidney, liver or lung and virtually no Gpx1 activity, there appeared to be no physiological differences between homozygous knockouts ($Gpx1^{-/-}$) and wild-type mice. $Gpx1^{-/-}$ mice grew normally and were apparently healthy up to 20 months of age. In addition, no protein or lipid oxidation was found and there was no increased sensitivity to hypoxia. Whilst glutathione peroxidase activity has been diminished in certain tissues of the $Gpx1^{-/-}$ mouse, indicating the large role this isoform plays, there is still sufficient activity to prevent accumulation of H_2O_2 under physiological conditions. Replacing exons 1 and 2 with a PGKneo cassette created a second Gpx1 knockout mouse where again homozygous mutants $Gpx1^{-/-}$ were viable and fertile, although by eight months they exhibited growth retardation.[18] Mitochondria were isolated from liver, kidney, heart, skeletal muscle, and brain of $Gpx1^{-/-}$ animals and found to contain no Gpx1 activity. In addition the ability of $Gpx1^{-/-}$ liver mitochondria to reduce H_2O_2 was down to 1.5% compared to wild-type liver mitochondria, suggesting Gpx1 is the only liver mitochondrial Gpx. Interestingly, there was no detectable Gpx activity at all in control heart mitochondria, implicating that Gpx1 plays a role in protecting liver mitochondria from oxidative stress but does not function in heart mitochondria. Quantification of ATP production and respiration rates by $Gpx1^{-/-}$ liver mitochondria showed a reduction by about one-third compared to wild-type controls. Meanwhile heart mitochondria from both knockout and wild-type animals showed comparable respiration rates. Livers from $Gpx1^{-/-}$ mice showed an increase in lipid oxidation and isolated liver mitochondria appeared to produce a greater amount of H_2O_2. Results from this animal model clearly show the importance of Gpx1 activity in mouse liver mitochondria, which appears to be a location that lacks any of the other glutathione peroxidase isoforms. The role of Gpx1 in heart mitochondria does not seem to be as important, at least under physiological conditions. This may be due to the presence of catalase, another H_2O_2 oxidoreductase, in heart mitochondria.

To determine the role Gpx1 plays during oxidative stress, de Haan et al[20] treated $Gpx1^{-/-}$ mice to the oxidant paraquat. Paraquat is known to generate free radicals within cells. Within

five hours of injecting paraquat intraperitoneally, the $Gpx1^{-/-}$ mice died, contrasting greatly with the wild-type controls that showed no lethality even up to 10 days later. Activity of Gpx1 in the lungs of wild-type mice was found to be elevated 2-fold following paraquat treatment that was subsequently found to be due to increased transcription of the Gpx1 gene. $Gpx1^{-/-}$ mice showed no Gpx1 activity in the lungs either before or after paraquat injection. Cultured neuronal cells derived from $Gpx1^{-/-}$ mice also showed a higher sensitivity to low doses of H_2O_2 added to the culture medium when compared to wild-type derived neuronal cultures that were completely viable under such conditions. These results suggest that Gpx1 plays a role in protection against oxidative stress induced by certain factors, however, its' role under physiological conditions may be limited. The role Gpx1 takes in protection of cells and tissues under conditions of oxidative stress induced by disease has yet to be elucidated.

Two Mitochondrial Superoxide Dismutase Knockout Mouse Models

Manganese superoxide dismutase (MnSOD) is one of several oxygen radical scavengers and has been located within mitochondria, a major cellular source of free radicals. MnSOD converts the mitochondrial superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2), which is subsequently broken down to water by glutathione peroxidase-isoform 1 (Gpx1) (Fig. 2). To further establish the role mitochondrial ROS play in the pathophysiology of diseases especially age-related disorders, Li et al[21] created a knockout mouse of the *Sod2* gene, which encodes MnSOD. *Sod2* was inactivated by deletion of exon 3, resulting in a loss of enzyme activity. Heterozygous mutant animals showed no abnormal phenotype whilst the deletion proved to be lethal in the homozygous knockouts with death occurring 4-10 days postnatally. These results are in sharp contrast to similar mouse models where inactivation of the cytosolic Sod1 or extracellular Sod3 genes had little effect on viability or fertility of the animals.[22-23] This suggests that mitochondria are not only a major source of $O_2^{\cdot -}$ but also the toxicity of $O_2^{\cdot -}$ is far greater in mitochondria than in the cytosol or extracellular matrix.

A detailed characterisation of the *Sod2* mutant mice showed dilated cardiomyopathy, significant lipid accumulation in liver and skeletal muscle, a marked deficiency in succinate dehydrogenase (SDH or complex II) in the heart and skeletal muscle, and a partial deficiency of complex I and citrate synthase in heart mitochondria.[24] In addition, activity of the mitochondrial enzyme aconitase (a component of the citric acid cycle) was shown to be greatly reduced in isolated heart and brain mitochondria. This iron-sulphur containing enzyme has previously been shown to be particularly sensitive to $O_2^{\cdot -}$ damage.[25] Evidently the increased levels of mitochondrial $O_2^{\cdot -}$ has resulted in disruption of the iron-sulphur containing enzymes leading to a dysfunctional electron transport chain (ETC) and inhibition of the tricarboxylic acid cycle. To confirm this conclusion, a study of the rate of respiration of mitochondria isolated from the liver of homozygous mutant mice showed a 40% reduction of state III respiration, indicative of an ETC dysfunction. This defective respiratory chain system may lead to an increased production of mitochondrial ROS further weakening a system unable to process its harmful free radicals. As would be expected from an accumulation of mitochondrial ROS, there was a concomitant rise in oxidative damage to total DNA isolated from heart, liver and brain of *Sod2* mutant mice.

The above *Sod2* knockout mouse was created in the CD1 mouse background and at the same time a similar *Sod2* knockout mouse was produced in the C57BL/6 mouse background,

$$2O_2^{\bullet -} + 2H^+ \xrightarrow[\text{MnSOD}]{} H_2O_2 + O_2$$

Figure 2. MnSOD (manganese superoxide dismutase) catalyses the reduction of the mitochondrial superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2).

giving rise to a different set of phenotypes.[26] Inactivation of the *Sod2* gene was carried out by excision of exons 1 and 2, resulting in the removal of the transcription and translational start sites. Mutant and wild type offspring from heterozygous *Sod2* mutant crosses were indistinguishable at birth with a typical Mendelian ratio of 1:2:1 being achieved. However, growth retardation of the homozygous mutants was obvious from postnatal day 2 up until their death on day 18. Electron microscopy carried out on the brain and spinal cord of 10 day old mutant animals showed signs of degenerative injury including extensive mitochondrial damage, especially to the neurons of the basal ganglia and brainstem. This result contrasted with electron microscopy data obtained from brain, heart, liver and skeletal muscle of 4-5 day old *Sod2* mutant mice in the CD1 background where no mitochondrial damage was observed. Phenotypic similarities between the two *Sod2* knockout mouse models include a dilated cardiomyopathy, lipid accumulation in the liver and progressive motor abnormalities, including early onset of fatigue. Clearly MnSOD is an important mitochondrial antioxidant enzyme which plays an essential role in the removal of mitochondrial ROS in tissues with a high energy demand. These are the first animal models where increased mitochondrial ROS can be associated with onset of mitochondrial disease. Increased mitochondrial $O_2^{\cdot -}$ results in disruption of certain Fe-S containing enzymes leading to inactivation of the ETC and TCA with a subsequent loss in mitochondrial fatty acid oxidation and accumulation of fat in the liver. Energy starvation in the heart due to the dysfunctional ETC then leads to cardiac myopathy and heart failure.[15]

Due to mounting evidence that accumulation of mitochondrial ROS leads to mitochondrial defects and tissue pathologies, it is of importance to test antioxidant treatments, which will hopefully scavenge the harmful mitochondrial free radicals. Using the *Sod2* mutant mice in the CD1 background, Melov et al[27,28] tested a number of such superoxide dismutase mimetics. Treatment of *Sod2* mutant animals with MnTBAP (manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin), a SOD mimetic, was capable of increasing the life-span of these animals from 10 days to 3 weeks along with ameliorating their cardiac myopathy and diminishing lipid accumulation in the liver. However, MnTBAP treatment revealed a neuropathology, previously undetected in these mice, which was characterised by a spongiform degeneration of the cortex and brain stem nuclei. Whilst MnTBAP is unable to cross the blood brain barrier, it is clearly able to slow the rapid accumulation of mitochondrial ROS in peripheral tissues. In addition, these results suggest that accumulation of mitochondrial ROS is extremely toxic to the brain.[27] Three more catalytic antioxidant drugs were successfully used to rescue the pathologies seen in *Sod2* mutant mice.[28] All three salen manganese complexes (EUK-8, EUK-134 and EUK-189) contain SOD2 and catalase activities and were administered daily from postnatal day 3, at various doses to both mutant and wild type animals. Treatment of *Sod2* mutants greatly increased their life-span, not only to 3 weeks as was the case with MnTBAP but up to 44 days with the cause of death at this age still undetermined. None of the neurobehavioral phenotypes revealed in the MnTBAP treated mice were observed, although mice over the age of 25 days developed a progressive movement disorder. This class of SOD-catalase mimetics can evidently cross the blood brain barrier and rescue the spongiform encephalopathy along with protecting peripheral tissues from mitochondrial ROS overproduction. Perhaps these drugs will provide a therapeutic approach to combating neurodegenerative diseases that have been linked to oxidative stress such as Alzheimer's and Parkinson's diseases as well as spongiform encephalopathies.

Characterisation of the Sod2 Heterozygous Knockouts

The homozygous *Sod2* knockout mouse proved to be a useful animal model for acute mitochondrial $O_2^{\cdot -}$ damage, however, the heterozygous *Sod2+/-* mice proved to be just as useful in the study of chronic $O_2^{\cdot -}$ toxicity. A lifetime of oxidative phosphorylation exposes mammalian cells to an ever increasing amount of reactive oxygen species (ROS) which eventually leads to oxidation of lipids, proteins and DNA. This is the basis for the free radical theory of ageing which claims that age-related physiological decline is a result of oxidative damage to tissues and

cells by ROS. Since the majority of ROS are produced in mitochondria it are the mitochondria that are the most affected by ROS toxicity, leading to an age-related accumulation of mitochondrial damage. To further investigate the link between accumulated mitochondrial ROS damage and ageing, Kokoszka et al[29] characterised in more depth the heterozygous *Sod2* knockout mice, which are subjected to a deficiency of the antioxidant enzyme MnSOD and potentially are exposed to greater levels of $O_2^{\cdot-}$ toxicity. Protein levels of MnSOD were determined to be reduced ~50% in *Sod2+/-* compared to wild-type mice. Mitochondrial membrane potential ($\Delta\Psi$) was found to be reduced in *Sod2+/-* mice but interestingly there was also an age-related decline of $\Delta\Psi$ in both wild-type and *Sod2+/-* animals suggesting chronic oxidative damage could cause an increase in mitochondrial inner membrane proton permeability. Protons are pumped from the mitochondrial matrix across the mitochondrial inner membrane; creating an electrochemical gradient or $\Delta\Psi$ which can be dissipated when protons are allowed to leak back across the inner mitochondrial membrane. Since the $\Delta\Psi$ is used by ATP synthase (complex V of the ETC) to make ATP, a direct link can be made between oxidative phosphorylation and $\Delta\Psi$. Hence, a reduction in $\Delta\Psi$ will result in reduced oxygen consumption, slowing down of mitochondrial oxidative phosphorylation with a concomitant increase in ROS production from electrons stranded in the inhibited ETC. A suggested reason for the increase in proton leakage was that oxidative damage to lipids in the mitochondrial inner membrane was rendering it leaky. Indeed it was shown that middle aged *Sod2+/-* animals had twice the level of lipid hydroxides compared to controls.

Chronic oxidative stress is known to activate the mitochondrial permeability transition pore (mtPTP) which when open allows the passage of molecules less than 1,500 Da between the mitochondrial matrix and cytosol.[30-31] Opening of the mtPTP channel results in a drop of $\Delta\Psi$, a loss of mitochondrial matrix solutes and the release of some apoptosis inducing factors such as cytochrome *c*. Middle to old age *Sod2+/-* mice were found to have a highly sensitised mtPTP compared with age matched controls and this was linked to the three fold increase in apoptotic hepatocytes observed in aged *Sod2+/-* livers. The decline in mitochondrial function observed in the *Sod2+/-* mice was accompanied by a rise in respiratory chain enzyme activity, a common phenomenon seen in mitochondrial disorders in a futile attempt to compensate for the energy deficiency. Throughout this study it was noted that the mitochondrial oxidative damage seen in tissues of *Sod2+/-* individuals was also accumulated in aged controls, although at a much later stage. In brief, this study has shown that increased mitochondrial ROS can result in a decrease in $\Delta\Psi$, leakage of protons across the mitochondrial inner membrane, oxidation of mitochondrial lipids, sensitisation of the mtPTP and subsequent initiation of apoptosis. In addition, it further supports the hypothesis that increased mitochondrial ROS leads to a decline in mitochondrial function and the initiation of apoptosis, a common feature in the ageing process.

Manipulation of Mitochondrial Transcription Factor A Expression in Mice

Regulation of mitochondrial gene expression in a mouse model was achieved by disrupting the nuclear encoded mitochondrial transcription factor A (*Tfam*).[32] As illustrated in Figure 3, two *loxP*-sites were introduced either side of exons 6 and 7 of the *Tfam* gene by homologous recombination in embryonic stem cells creating *TfamloxP* mice. *TfamloxP* mice were then crossed with mice ubiquitously expressing *cre*-recombinase under the β-actin promoter, which excised exons 6 and 7 of the *Tfam* gene by *cre*-mediated recombination. Heterozygous knockout animals were viable even though levels of Tfam transcripts and protein were reduced by approximately 50%. A reduction of mtDNA copy number of up to 34% was observed in all tissues analysed, however, there were no significant reductions in mtRNA transcripts or respiratory chain complex activities with the exception of the heart. These results were interesting considering the reduced mtDNA copy numbers and suggest a compensatory increased stability of mitochondrial mRNA transcripts and polypeptides. Homozygous knockouts of *Tfam* were

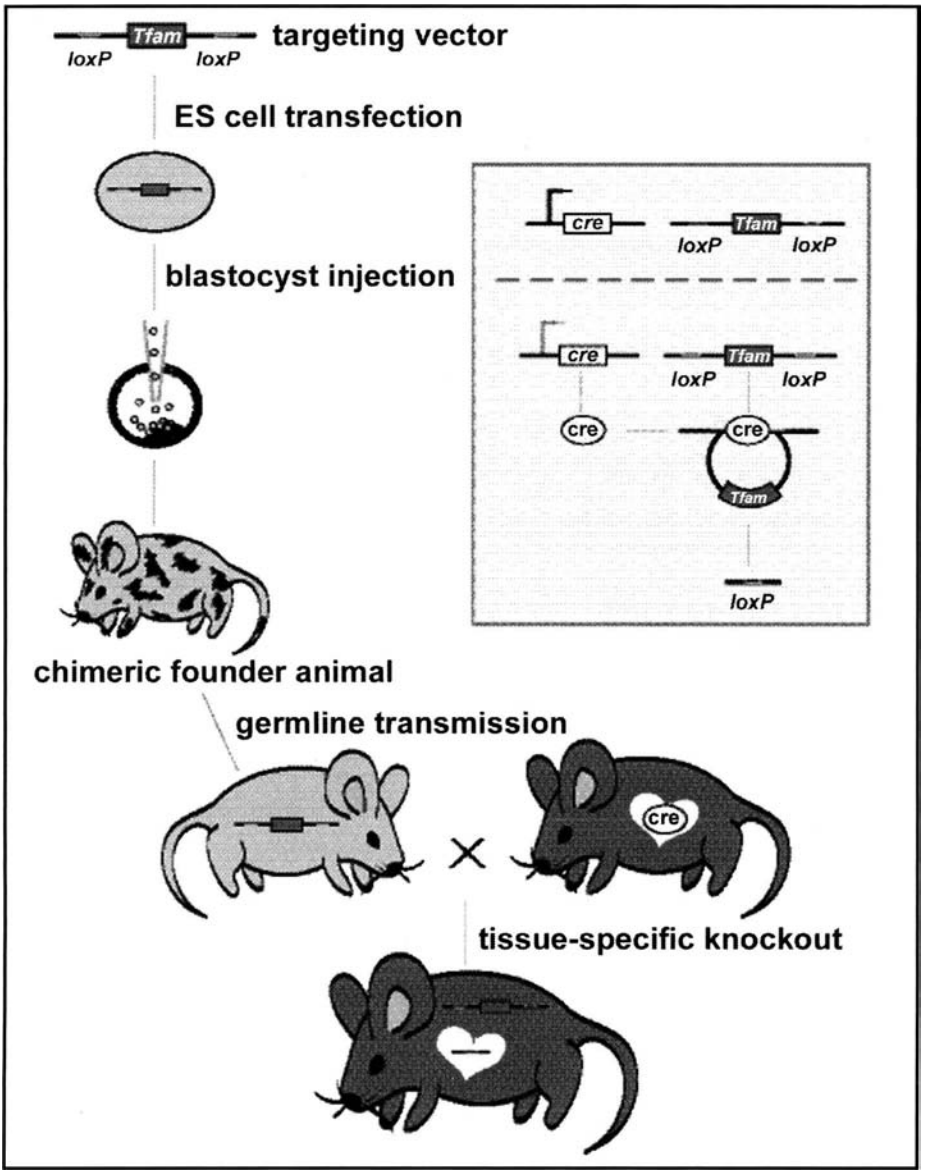

Figure 3. Schematic drawing of the construction of conditional *Tfam* knockout mice. Initially a targeting vector containing loxP-flanked *Tfam* was transfected into ES cells, which were then injected into blastocysts. Chimeric founder animals were mated to obtain germ line transmission of the loxP-flanked *Tfam* allele and mated with *cre*-transgenic animals to obtain tissue-specific knockout animals. The inserted box shows how *cre*-recombinase specifically recognizes the loxP-sequences flanking two crucial exons of the *Tfam* gene to generate the knockout allele.

embryonic lethal with death occurring between embryonic day (E) 8.5 and 9.5 at which stage no mtDNA was detectable. These results confirmed that Tfam is essential for mtDNA maintenance in vivo and is an essential protein during embryogenesis in the mouse.

Germ-line disruption of *Tfam* confirmed the efficiency of the *cre-loxP* recombination system in generating knockout models and this system was then used to create a series of tissue-specific *Tfam* knockout mice. *TfamloxP* mice were mated with transgenic mice carrying cell type specific expression of *cre* recombinase.

Heart Specific Disruption of Tfam

Two mouse models of a heart specific knockout of the Tfam protein have been generated using either the muscle creatine kinase (*Mck*) promoter or the α-myosin heavy chain (*Myhca*) promoter to control *cre* recombinase expression.[33-34] *Tfam* expression was disrupted in both heart and skeletal muscle of homozygous *TfamloxP* mice expressing *cre* recombinase by the *Mck* promoter from E13. These mutant animals (*TfamloxP/TfamloxP, +/Mck-cre*) had a normal respiratory chain function in the heart at birth, however, they had a mean survival of just 20 days and at death exhibited a dilated cardiomyopathy. The *Mck* promoter is active only in cardiomyocytes of the heart where a reduction of Tfam protein levels was observed, as well as in skeletal muscle. There was a concomitant drop in steady-state levels of mitochondrial transcripts and mtDNA levels in both heart and skeletal muscle tissues but not in any other tissues analysed. Several cardiomyocytes lacked COX activity confirming the breakdown in respiratory chain function. Morphological analysis of the hearts by electron microscopy revealed abnormally enlarged mitochondria containing tubular cristae. This animal model has reproduced many pathophysiological features of human diseases resulting from either deletions or point mutations in mitochondrial tRNA genes: (i) impaired mtDNA expression leading to a dysfunctional respiratory chain in a tissue specific manner; (ii) a mosaic pattern of respiratory chain-deficient cells in affected tissues; (iii) appearance of morphologically abnormal mitochondria; and (iv) a time dependent deterioration of respiratory chain function.

A second heart specific knockout of Tfam was created with *cre* recombinase expression being controlled by the *Myhca* promoter that results in *cre* expression from E8. The resulting knockouts (*TfamloxP/TfamloxP, +/Myhca*) had onset of cardiomyopathy during embryogenesis with the majority (75%) of knockouts dying in the neonatal period. Interestingly, the 25% of mutant animals that survived lived for several months before dying from dilated cardiomyopathy. This suggested modifying gene(s) played a role in determining the lifespan of the knockouts. Both the *Mck* and *Myhca* animal models demonstrate that by regulating *cre* expression in the heart it is possible to control the age of onset of cardiac respiratory chain dysfunction and will therefore be a very useful tool for the study of other tissue specific knockouts of *Tfam*.

A Mouse Model for Mitochondrial Diabetes

A tissue-specific disruption of *Tfam* in mouse pancreatic β-cells was achieved by crossing mice with a *loxP*-flanked *Tfam* with transgenic mice expressing *cre* recombinase by the rat insulin-2 promoter.[35] Seven week old mutant mice showed a severe mtDNA depletion in islets along with a COX deficiency. Pancreatic sections of mutant mice were analysed by electron microscopy and revealed abnormally appearing mitochondria with tubular cristae in the insulin-producing cells, a morphology commonly found in mitochondria with a dysfunctional respiratory chain. Along with the drop in mtDNA expression and concomitant respiratory chain deficiency in pancreatic β-cells, mutant mice from the age of just 5 weeks developed diabetes with decreased blood insulin concentrations.

At 7 weeks of age mutant mice showed a normal distribution of β-cells within the pancreas, yet were diabetic, suggesting an impaired β-cell stimulus-secretion coupling. Metabolism of glucose in the β-cell is directly coupled to insulin secretion. During mitochondrial oxidation of glucose metabolites, there is an increase in the concentration of ATP that results in closure of the ATP-dependent potassium channels (K_{ATP} channel). Depolarisation of the plasma membrane occurs when the K_{ATP} channels are closed and a subsequent opening of the voltage-sensitive L-type Ca^{++} channels occurs allowing Ca^{++} to enter the cytosol. The incoming Ca^{++} activates fusion of the insulin-containing vesicles, causing release of insulin. Hence, it is clear that mi-

tochondrial production of ATP plays a key role in the release of insulin by regulating the K_{ATP} channel and that to block the ETC will subsequently lead to inhibiting plasma membrane depolarisation and insulin secretion. Islets isolated from both mutant and control mice were subjected to glucose stimulation and the levels of intracellular Ca^{++} were measured. Mutant animals showed a decreased rise in Ca^{++} levels compared with controls. These results confirm that mitochondrial dysfunction in the β-cells of the mutant mice are the principle cause of the diabetic phenotype observed. Not only do these mice reproduce pathophysiological features of mitochondrial diabetes but also give evidence that the respiratory chain plays a central role in normal glucose-induced insulin secretion.

Disrupting Oxidative Phosphorylation to Produce Mitochondrial Late-Onset Neurodegeneration (MILON) Mice

By crossing *TfamloxP/TfamloxP* mice with mice heterozygous for a transgene expressing *cre* recombinase from the calcium-dependent calmodulin kinase II promoter it was possible to create MILON mice.[36] The *Tfam* gene was knocked-out in neocortex with maximum *Tfam* recombination having occurred by 1 month of age. At 2 months mutant mice had much reduced levels of Tfam protein, however, there was no overt phenotype at this age. From 5-6 months of age, mutant (MILON) mice showed abnormal behaviour with a physical deterioration that degenerated rapidly towards death approximately one week after the first symptoms appeared. By 2 months of age there was a ~40% drop in mtDNA levels and by 4 months the levels of mtRNA transcripts were also reduced by ~40%. As would be expected with the reduction in mtDNA transcripts, the activities of complex I and IV in neocortical samples were shown to be reduced in 4- and 5-months old MILON mice. Cell death, as detected by TUNEL-positive nuclei, was only observed in the brains of symptomatic animals, no TUNEL-positive cells were seen in 2-4 months old presymptomatic MILON mice. Massive neurodegeneration was displayed in end-stage symptomatic MILON mice. To determine if MILON mice were more susceptible to stress-induced neuronal death both controls and MILON mice were injected with kainic acid which induces seizures. Both sets of mice exhibited high level seizures but only the MILON mice developed an increased number of TUNEL-positive cells, as assessed by performing TUNEL staining on brains harvested 24 hours after kainic acid injection, indicating an increased sensitivity to neuronal stress in these animals. Human neurodegenerative disorders generally develop later in life, as is the case in this animal model where MILON mice are healthy well into adulthood. Interestingly, before onset of neurodegenerative symptoms MILON mice are more vulnerable to excitotoxic challenges indicating that perhaps patients with mitochondrial encephalomyopathies may exhibit a clinical deterioration in response to moderate stress, e.g., viral infections. In addition, it was observed that neurons could survive for at least one month after oxidative phosphorylation was inhibited. Such a time lag has been observed in human patients with mitochondrial neurodegeneration and possibly reflects the ability of neurons to withstand long periods of OXPHOS deficiency. As there are many similarities between this mouse model of mitochondrial dysfunction in neurons and human sufferers of mitochondrial neurodegeneration, these mice will prove useful in future studies of the molecular pathways leading to neuronal cell death and in the testing of pharmacological treatments for these disorders.

Embryonic Lethality Due to Loss of Frataxin

Frataxin is a 210 amino acid nuclear-encoded mitochondrial protein that has been highly conserved through evolution and is thought to play a role in mitochondrial iron homeostasis. Analysis of frataxin in mouse has shown expression of the gene from E10.5. Tissue specific expression has been observed in humans and mice with frataxin being expressed in heart, dorsal root ganglia, pancreas, liver and skeletal muscle.[37,38] A large GAA triplet repeat expansion in the first intron of the gene encoding frataxin has been associated with the most common hereditary ataxia, Friedreich ataxia (FRDA). FRDA is a neurodegenerative disorder characterised

by progressive ataxia of the limbs, dysarthria and other coordination disorders. These symptoms are believed to be a result of neuronal degeneration, however, other manifestations such as cardiomyopathy and diabetes are also common. Tissue biopsies from patients reveal activities of mitochondrial iron-sulphur (Fe-S) containing enzymes to be greatly impaired as well as both mitochondrial and cytosolic aconitase.[39] It was believed that a loss of functional frataxin protein led to an accumulation of mitochondrial iron which is a potent oxidising agent, hence resulting in oxidation of the iron-sulphur containing enzymes with a subsequent loss of respiratory chain function.

Cossée et al[40] created a frataxin knockout mouse by deleting exon 4, a highly conserved sequence in the frataxin gene, by homologous recombination. Deletion of exon 4 results in a frameshift and a truncation of the frataxin protein. Heterozygous knockouts were crossed but no homozygous mutant offspring were obtained and subsequent analysis of the embryos from such a cross revealed gross morphological abnormalities in approximately one third of all embryos. Abnormal embryos between E7.5 and E8.5 were genotyped and discovered to be either homozygous or heterozygous knockouts for frataxin. TUNEL staining (to detect apoptotic cells) was carried out on E6.75 embryo sections and abnormal embryos showed substantial positive staining indicating extensive apoptosis compared with virtually no staining in normal embryos. Reabsorption of abnormal embryos occurred by approximately E9.5. Contrary to the belief that loss of functional frataxin results in mitochondrial iron accumulation, no iron deposits were observed in any of the abnormal embryos analysed. This knockout mouse model shows that frataxin plays an important role in development with complete loss of the protein being lethal in mice. No FRDA patients have been described with a truncated point mutation in both alleles of the frataxin gene possibly due to this early lethality. The length of the intronic GAA triplet repeat expansion observed in FRDA patients can be directly correlated with severity and age of disease onset. Smaller repeats produce milder symptoms and a later onset of disease as well as enabling the synthesis of residual levels of frataxin when compared with large expansions.

To try and mimic the symptoms observed in FRDA patients two conditional knockout mice for the frataxin gene were created, one muscle and the other neuron specific.[41] These conditional knockouts were produced by flanking exon 4 in the gene for frataxin (*Frda*) with loxP sites. Mice expressing the *cre* recombinase gene in specific tissues can then, by homologous recombination, excise out exon 4 of *Frda* creating a knockout. Homozygous mice for the conditional knockout allele of *Frda* were mated with mice that carried the Cre transgene either under the control of muscle creatine kinase (MCK) or the neuron-specific enolase (NSE) promoters. Upon expression of cre in these specific tissues there would subsequently be a tissue specific loss of frataxin. The skeletal muscle and heart specific (MCK) mutants begin to lose weight at 7 weeks and die at about 11 weeks of age. There is an absence of full-length transcript and protein in both skeletal muscle and heart of these knockouts and cardiomyopathy is observed in the latter stages, a similarity with the human FRDA disease. By contrast, the neuron-specific enolase (NSE) knockout mice have a low birth weight and develop ataxia and a progressive neurological phenotype with death occurring at about 24 days of age. Expression patterns show a lack of full-length frataxin transcript in not only neuronal tissues but also heart and liver. There is an earlier manifestation of cardiomyopathy in the NSE knockouts in addition to degeneration and necrosis of the nervous system, another common feature seen in FRDA patients. The hearts of both animal models revealed large-scale mitochondrial abnormalities suggestive of a mitochondrial dysfunction which was confirmed with biochemical analysis of heart tissue showing deficiencies in the Fe-S containing enzymes of the respiratory chain complexes I-III and aconitase. There were limited amounts of mitochondrial iron accumulation in MCK animals but no iron deposits were observed at all in the NSE mice suggesting that damage to Fe-S containing enzymes is not due to the accumulation of iron as previously thought. Frataxin is located in the mitochondrial inner membrane and its main role was believed to be transport of iron out of mitochondria. Since loss of function of frataxin does not

primarily lead to mitochondrial iron accumulation but instead to a deficiency of Fe-S centre enzymes it is more likely that frataxin plays a larger role in synthesis of Fe-S clusters or protection from oxidative stress. It is hoped that these mouse models of FRDA will reveal the role that frataxin plays in iron metabolism and the biogenesis of Fe-S containing enzymes.

Transmitochondrial Mouse Models

With the exception of both sperm and mature oocytes, between 10^3-10^4 copies of mtDNA are present in each cell. Mitochondrial DNA sequences are known to differ between unrelated individuals by approximately 0.3% (~50 nucleotides), however, it is normal for a given individual to contain just one variant of the mtDNA sequence. Heteroplasmy occurs when a cell contains more than one sequence variant of the mitochondrial genome and many known pathogenic mtDNA mutations are heteroplasmic in presentation. Therefore it would be of great value to have a mouse model with which to study transmission and tissue distribution of mutant mtDNA, along with a comparison between mutant load and observation of a pathogenic phenotype.

A number of techniques have been employed to develop a way of creating mice containing a heteroplasmic population of mtDNA. From these experiments two approaches succeeded in producing transmitochondrial mice, i.e., mice carrying two different types of mtDNA. The first method involves fusion of a cytoplast containing one form of mtDNA, with a one-cell embryo, which after a brief culture can be implanted into the fallopian tubes of a pseudopregnant female mouse as a two cell embryo.[42] The second approach again involves a cytoplast containing one form of mtDNA but this time it is fused to an undifferentiated female mouse embryonic stem cell with subsequent injection into a mouse blastocyst and implantation into a foster mother.[43]

Maternal Transmission of a Neutral mtDNA Polymorphism

Under the first method, fusing cytoplasts from either NZB/BINJ or BALB/cByJ zygotes to embryos of the opposite origin produced chimeric embryos carrying two variants of mtDNA, as illustrated in Figure 4. From the resulting offspring, nine were found to contain a mixed population of mtDNA and of these, five females were used for backcrossing. These females carried between 3-7% donor mtDNA independent of the source of this donor mtDNA (either NZB/BINJ or BALB/cByJ). Proportions of the donor genotype in each female were equivalent to the mean proportion of donor mtDNA in all of her offspring, however, individual offspring varied from 0-30%. Clearly, rapid segregation of mtDNA genotypes was occurring between these two generations of mice through maybe one of two hypothesised mechanisms: (1) Expansion of mtDNA copy number from 10^3 to 10^5 occurs during maturation of primary oocytes.[44] Replication of a subset of mtDNA molecules during this process could easily shift the proportions of a mtDNA genotype within one generation. (2) Before an embryo initiates mtDNA replication it undergoes a number of cell divisions with partitioning of mtDNA being completely random to each daughter cell.[45] The early blastocyst consists of an inner cell mass which gives rise to the three germ layers of the embryo, whilst the remaining cells become extraembryonic tissues. Hence, an unequal distribution of mtDNA molecules within embryonic cells at this stage of development could also lead to a shift in mtDNA genotypes between generations.

However, neither of these previously proposed hypotheses were found to be true for this animal model. Segregation of mtDNA was found to occur in oogonia, which are the precursors of primary oocytes, and contain a relatively small number of mtDNA templates, hence a possible explanation for the rapid segregation between generations. Both the NZB and BALB mtDNA genotypes should be completely neutral variants and any segregation of these genotypes could only occur by random drift as opposed to selection. Contrary to this belief a follow up study on this mouse model revealed a different rate of segregation in different tissues of the adult mice, with the BALB mtDNA genotype increasing in blood and spleen whilst the NZB genotype increased in liver and kidney.[46] No conclusion has been reached as to why this should

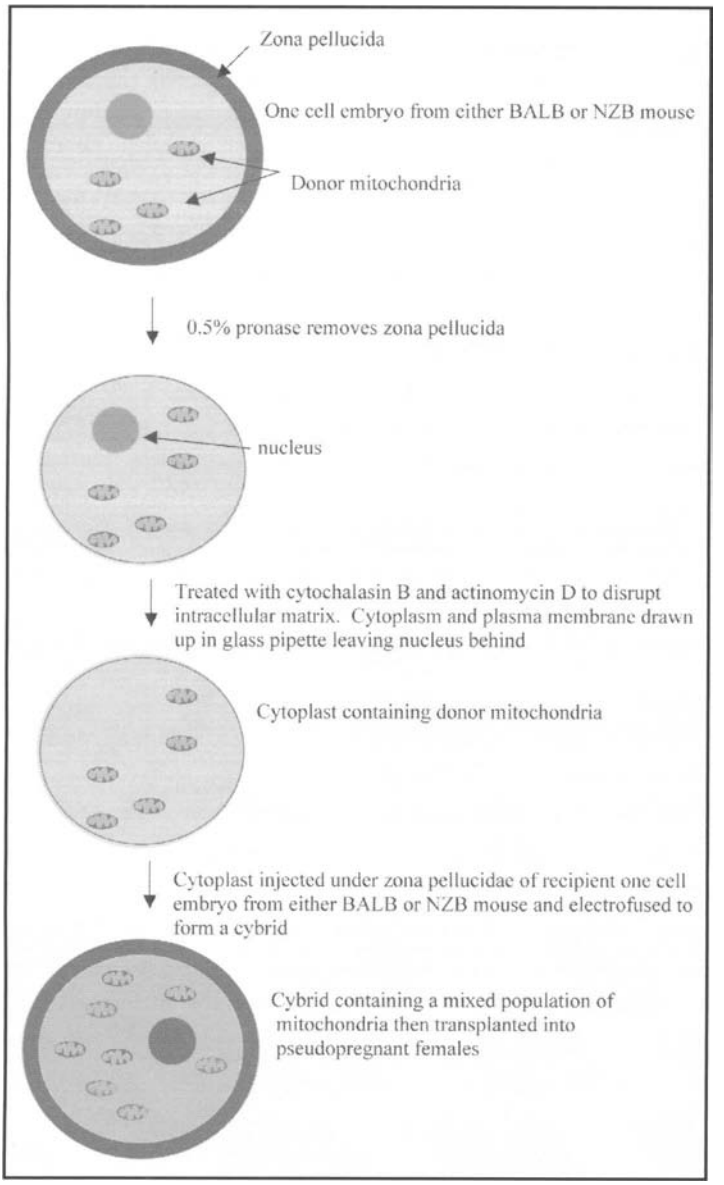

Figure 4. Diagram to illustrate the method used to create chimeric embryos containing a mixed population of mitochondria from either the BALB or NZB genetic background.

occur, however, it is clear there are strong tissue-specific selective pressures for different mtDNA genotypes in these animals. A more specific study of hepatocytes from these heteroplasmic mice revealed the selection for NZB mtDNA in the liver was not biased due to an increased oxidative phosphorylation capacity.[47] In addition the sequences known to be important in the regulation of mtDNA replication are identical in both NZB and BALB mtDNA genotypes eliminating a difference in replication efficiency. This leaves nuclear encoded factors that function in mtDNA maintenance e.g., proteins involved in expression, replication and mtDNA

structure, as likely candidates involved in segregation of different mtDNA genomes. Identification of these factors would certainly help in understanding the segregation patterns observed in different tissues of patients suffering from pathologies due to mtDNA mutations.

Transmitochondrial Mice Harbouring Mutated mtDNA

Embryonic stem (ES) cell cybrids have been successfully used to produce transmitochondrial mice carrying mtDNA from a partially respiratory-deficient chloramphenicol-resistant (CAP^R) cell line in two separate experiments.[48,49] In both cases, CAP^R cell lines were enucleated and fused with ES cell cybrids. The mtDNA population of these cybrids were made homoplasmic by Levy et al[49] (i.e., carrying exclusively CAP^R mtDNA) by treatment with the dye rhodamine-6-G to eliminate CAP^S mtDNA. Injection of CAP^R ES cell cybrids into blastocysts and subsequent implantation into foster mothers resulted in offspring with a low level of chimerism in various tissues along with detectable levels of CAP^R mtDNAs.

In order to create a viable and fertile female transmitochondrial mouse that could generate transgenic mice with altered mtDNA a slightly different approach was taken, as illustrated in Figure 5.[43] A female ES cell was chosen that would produce fertile oocytes. This ES cell line CC9.3.1 was first treated with R-6G to inactivate mitochondrial function, then fused with synaptosomes from the brain of an NZB mouse that would act as a neutral mtDNA variant. Selected ES cell cybrids were then injected into B6 cytoplasts and chimeric animals obtained. Using this technique it was possible to maternally transmit the NZB mtDNA variant through multiple generations with a heteroplasmic distribution for the NZB and common haplotype mtDNA throughout the animal.

Using the same procedure, CAP^R mtDNA was introduced into the CC9.3.1 cell line and ES cytoplasts fused with blastocytes to produce chimeric mice. Characterisation of the CAP^R chimeric mice showed ocular abnormalities, including congenital cataracts, decreased retinal function and hamaratomas of the optic nerve. Chimeric females were successful in producing offspring carrying CAP^R mtDNA. With the exception of just one pup, all animals born with the CAP^R genotype (either homoplasmic or heteroplasmic) died within 12 hours of birth, whilst many more died in utero. The failure to establish mouse lines with maternal transmission of the CAP^R mutation makes it difficult to critically evaluate the phenotype.

Mice with Mitochondrial Dysfunction Carrying Rearranged mtDNA

A transmitochondrial mouse model was produced by Hayashi and coworkers[50] to replicate the human pathology caused by deletions within the mtDNA genome. In humans large-scale mtDNA deletions (ΔmtDNA) directly affect respiratory chain function resulting in mitochondrial diseases as well as being associated with age-related disorders such as diabetes and neurodegenerative diseases.[51] Single deletions of mtDNA generally occur spontaneously with ΔmtDNA not being maternally transmitted and are always as a heteroplasmic mixture with wild-type mtDNA.[52,53] Levels of ΔmtDNA vary greatly between different tissues and indeed between individual cells within an organ. As with most pathogenic mtDNA mutations a minimum threshold of mutant mtDNA is required before respiratory chain dysfunction is detected.

To generate mice carrying ΔmtDNA, respiratory deficient mouse cybrid cells harbouring deletion mutant mtDNAs were cultured and a single clone containing a 4,696 bp deletion was isolated and determined to contain 30% ΔmtDNA which later increased to 83% before remaining stable. This deletion spanned 6 tRNA genes and 7 structural genes. High percentage mutant cybrids were enucleated and electrofused to pronucleus-stage embryos before being transferred to the oviduct of pseudopregnant females. Out of the resulting F0 offspring 24 mice contained ΔmtDNA at a level of 6-42% and transmission of ΔmtDNA was demonstrated through the female germline from F0 progeny to their offspring. Interestingly, these mice all contained a partially duplicated form of mtDNA consisting of one full-length mtDNA and one ΔmtDNA, this was in addition to the wild type and deleted mtDNA molecules that are present in the original mouse cybrid clone. Maternally transmitted human mitochondrial dis-

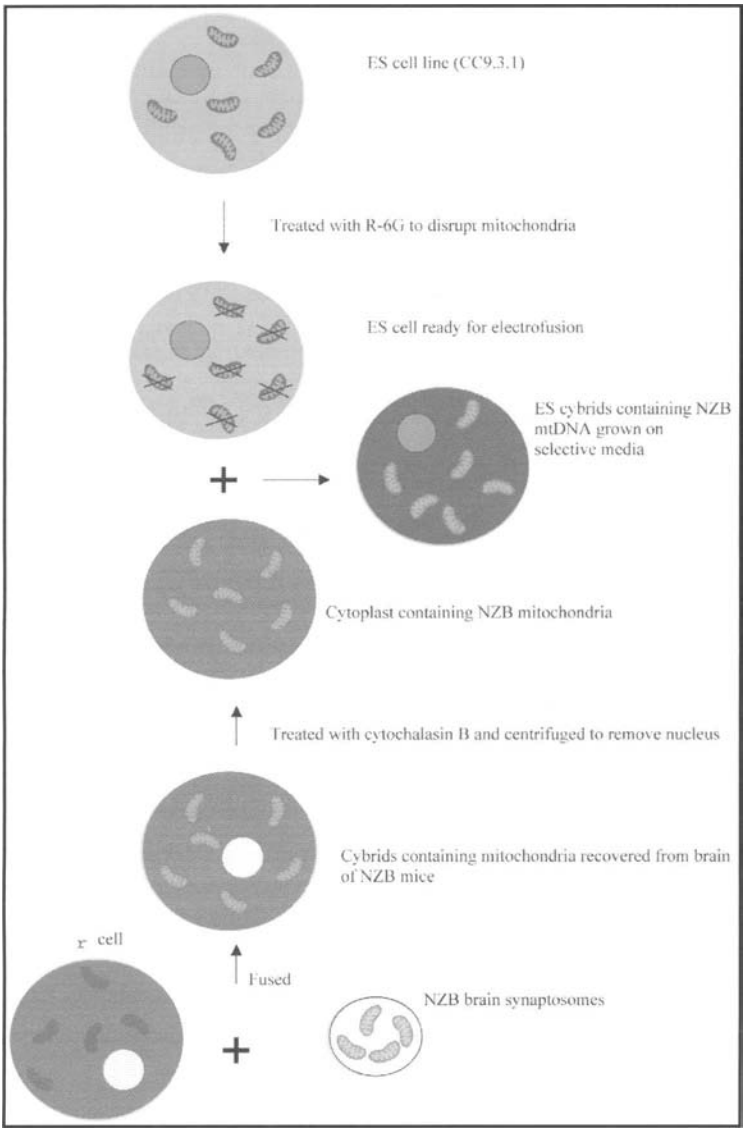

Figure 5. Diagram showing the creation of ES cells containing mtDNA from the NZB origin. These ES cells were then injected into B6 blastocysts and implanted into pseudopregnant females to produce chimeric mice containing a heteroplasmic distribution of NZB and common haplotype mtDNA.

eases are commonly associated with partially duplicated mtDNA and not ΔmtDNA so it raised the possibility that maybe ΔmtDNA was not being directly transmitted from one generation of these mice to the next. Instead perhaps ΔmtDNA was interacting with wild-type mtDNA generating partially duplicated mtDNA, allowing transmission to progeny and then later being rearranged to yield ΔmtDNA again.

Analysis of the ΔmtDNA mice revealed COX negative fibres only where ΔmtDNA was predominant with a threshold of 85% mutant mtDNA molecules being necessary. However,

the typical RRFs observed in muscle samples from human patients of mitochondrial diseases were not found. The majority of F1-F3 mice died within 200 days of birth due to renal failure with COX activity reduced to 28% that of a wild-type kidney. Mitochondrial dysfunction in the kidney causing renal failure has been reported in children with multisystem disorders due to wide-spread tissue distribution of ΔmtDNA.[54-56] A more in-depth analysis of individual skeletal muscle fibres to determine the COX activity of individual mitochondria revealed an interesting result.[57] Skeletal muscle from a mouse with overall 85% ΔmtDNA had 41% COX negative fibres which were subsequently shown to contain 89% ΔmtDNA whilst the remaining 59% COX positive fibres carried just 80% ΔmtDNA. Electron micrographs showed a complete lack of coexistence of COX positive and COX negative mitochondria within the muscle fibres. Evidently, there must be some form of complementation between ΔmtDNA and wild-type mtDNA with a threshold of more than 85% ΔmtDNA being required before COX negative mitochondria are observed, correlating with the onset of disease phenotypes.

Clearly a very useful tool for the study of human mitochondrial disorders has been established, opening up the possibility to investigate transmission of rearranged mtDNA and the subsequent tissue distribution of ΔmtDNA. In addition, the mechanism of in vivo mitochondrial complementation can be further investigated. This method of introducing mutant mtDNA molecules into a mouse germ line will hopefully aid in the generation of mouse strains which reproduce the phenotypes observed in human sufferers of mitochondrial diseases.

Defective Nuclear-Mitochondrial DNA Interactions Resulting in Hearing Loss

Mitochondrial DNA mutations associated with age-related hearing loss (AHL) are generally homoplasmic in presentation, however, not all individuals with these mutations suffer with a hearing loss whilst others with identical mtDNA mutations will develop a severe hearing loss. These differences in phenotypic expression have lead to the conclusion that nuclear genes must determine whether or not an individual carrying a specific mtDNA mutation will develop symptoms of the disease. Furthermore, a number of inbred mouse strains exhibiting AHL have been shown to share a common gene (designated *Ahl*) on mouse chromosome 10.[58]

To test the involvement of mtDNA in AHL three of these inbred mouse strains (A/J, NOD/LtJ and SKH2/J) were used in backcrosses with a wild-derived inbred mouse strain (CAST/Ei), that does not exhibit AHL.[59] Progeny from the first cross (F1) between each of the three hearing impaired strains and CAST/Ei mice did not develop AHL. When the F1 mice were backcrossed only mice originating from the A/J hearing impaired strain developed AHL, mice from the other two backcrosses had normal hearing. It was observed that only those mice homozygous for the A/J allele at the *Ahl* locus developed AHL supporting the hypothesised link between the *Ahl* gene and AHL. In addition, mice carrying A/J mtDNA as opposed to CAST/Ei mtDNA had a more severe hearing impairment. By sequencing mtDNA from all three AHL mouse strains the only difference that was observed was a single adenine nucleotide insertion in the D-loop of the tRNA-Arg gene in A/J mtDNA. This insertion is in an adenine repeat where the published sequence contains 8 adenine repeats, NOD/LtJ and SKH2/J strains carry 9 adenines and A/J mtDNA shows 10 repeated adenines. There is no direct evidence showing that the extra adenine renders the mitochondrial tRNA-Arg dysfunctional and it is particularly curious that mice carrying A/J mtDNA but are not homozygous for the A/J *Ahl* gene have normal hearing. However, the results from these backcrosses do support a link between development of AHL and an interaction between nuclear and mitochondrial DNA. As yet no biochemical analysis on these mice has been carried out so there is no evidence of dysfunction in oxidative phosphorylation. Many human patients with AHL have shown a number of acquired mtDNA mutations in their auditory tissue, which suggests a link between respiratory chain dysfunction and the onset of hearing loss.[60] It would be interesting to compare the respiratory chain function of mitochondria isolated from both normal and AHL mice created by the A/J x CAST/Ei backcrosses.

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CHAPTER 10

Therapeutic Options in OXPHOS Disorders

Rob C.A. Sengers, J.M. Frans Trijbels, Carolien C.A. Boelen, Eva Morava and Jan A.M. Smeitink

Abstract

No curative treatment of OXPHOS disorders is currently available, despite great progress in our understanding of the molecular bases of these diseases.

We review available and experimental therapeutic approaches that fall into the following categories:

(a) Palliative therapy; (b) removal of noxious metabolites; (c) administration of artificial electron acceptors; (d) administration of metabolites and cofactors; (e) administration of oxygen radical scavengers and f-gene therapy.

Progress in each of these approaches provides some glimmer of hope for the future, although much work remains to be done.

Introduction

Mitochondriocytopathies and especially OXPHOS disorders may be considered as inborn errors of metabolism, i.e., genetic diseases resulting from inherited abnormalities in the complex systems that program normal development and physiological homeostasis.

A few decades ago the diagnosis mitochondrial cytopathy was mainly based on electron-microscopical findings. Gradually, results of biochemical examinations of biopsied tissue, mainly skeletal muscle, became more and more important. Recently, in an increasing number of patients, abnormalities in mitochondrial or nuclear DNA are identified.

However, it must be realized, that an accurate diagnosis, based upon at least a biochemical diagnosis, confirmed by molecular biological data, is made only in a minority of the patients.

The pathogenetic mechanism of OXPHOS disorders is almost unknown. The same clinical phenotype can be associated with deficiencies of different enzymes or enzyme complexes, whereas the same biochemical abnormality can be associated with different clinical entities. Furthermore relatively little is known about the natural history of these disorders and collecting enough patients with a similar phenotype in one centre is extremely difficult.[3] This means that clinical trials are difficult to perform, necessitating multi centre trials. Incomplete knowledge of pathogenesis hampers our attempts at therapeutics.

When it is assumed, that an accurate diagnosis, early intervention and knowledge of the pathogenesis is required for the treatment of diseases in general and also for the treatment of OXPHOS disorders, then it may be concluded that a rational therapeutic for these diseases is hardly available.

Nevertheless, an increasing number of classical inborn errors of metabolism can successfully be treated. It should be investigated which approach is applicable in case of OXPHOS disorders.

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Therapeutic Approaches

The therapeutic approach to patients suffering from an inborn error of metabolism basically follows that of,[1] summarized in Table 1.

- Treatment at the clinical phenotype level covers a variety of conventional medical practices. Anticonvulsive drugs are given in case of an encephalopathy complicated by convulsions. In the treatment of patients some drugs should be withheld (vide infra). An acute crisis should be symptomatically treated. The basic genetic defect is not corrected, but the patients' problems are ameliorated.
- Treatment at the metabolite level often involves nutritional or pharmacological approaches. Dietary alteration may be effective if the pathophysiology involves an accumulation of toxic precursors. It is sometimes possible to promote the conversion of an offending metabolite to a readily removable substance. This approach includes the use of carnitine to conjugate with accumulated CoA esters in various defects of fatty acid and organic acid metabolism. Supplementation of a non synthesized product is sometimes possible.
- Treatment at the protein level implies activation or replacement of the mutant enzyme. Those mutations that either decrease the affinity of the enzyme for its cofactor or destabilize the protein may be partially overcome by substantially increasing the cofactor concentration in the surrounding milieu. Replacing the deficient protein is the ultimate therapeutic approach, but unfortunately this has yet been achieved in exceptional cases, for example in the treatment of nonneuronopathic Gaucher disease.
- Radical scavengers. Oxygen is reduced to mono- and tetravalent forms within mitochondria. Pathological amounts of oxygen radicals are formed in case of a disordered respiratory chain. Scavenging these radicals has, at least theoretically a beneficial effect.
- Somatic cell therapy. Several single gene disorders are candidates for gene therapy. The number, however, of diseases treatable with this appraoch is extremely limited.

Which of the approaches can be applied in case of OXPHOS disorders?

It should be noted, that OXPHOS disorders form a special group of inborn errors:

Firstly, we are dealing with enzyme-complexes. These complexes are fixed in membranes with spatially a typical arrangement.

Secondly, in the classical inborn errors of metabolism the catalytic activity is often decreased due to the absence of a specific protein or the presence of an abnormal protein; in the respiratory chain there are enzyme complexes composed of a few to a great number of subunits in which diverse abnormalities may cause decreased enzymatic activities. In the so-called primary OXPHOS disorders the defects are caused by mutations in genes, encoding subunits of the respiratory chain. This will include mutations of mtDNA as well as of nuclear DNA genes, that encode respiratory chain subunits. Nuclear mutations have been linked to multiple mtDNA deletions or mtDNA depletion, in both instances causing OXPHOS dysfunction.[7,9]

There furthermore are a number of abnormalities causing respiratory chain disturbances, such as mutations of nuclear encoded proteins involved in the assembly of OXPHOS complexes.

An individual complex may be deficient, but a number of combined deficiencies are well recognized.

In contrast to the classical inborn errors of metabolism, where a deficiency of a product is the consequence of a deficient enzyme, in mitochondrial abnormalities only the 'status' of the product is changed, in terms of changed redox status.

Putting all the enzyme-complexes in a black box (OXPHOS system) there is not seldom a decreased oxidation rate of a mitochondrial substrate and a decreased production rate of ATP, without a demonstrable deficiency of one of the complexes.

Mitochondria are formed by the concerted action of two genetic systems: the nucleocytoplasmic system and the intrinsic mitochondrial system. The genetic contribution of the mitochondria is modest because the genetic potential of mitochondrial DNA is restricted to the equivalent of 16,569 base pairs, coding for 13 proteins of the oxidative phosphorylation system.

Shortly: all mitochondrial disorders result from the progressive decline in the ability to supply cellular energy demands in the form of available ATP. The respiratory chain is also a potent source of free radicals.

Table 1. Strategies for the treatment of inherited metabolic diseases

1. Treatment at the clinical phenotype level
2. Treatment at the metabolite level
   a. Substrate reduction
   b. Utilization of alternative pathways
   c. Metabolite inhibitors
   d. Replacement of deficient product
3. Treatment at the protein level
   a. Activation with vitamin co-factors
   b. Protein replacement therapies
4. Radical scavengers
5. Somatic cell therapy

Table 2. Some causes of non-primary mitochondrial pathology

Cause	Example
Deficiency	Carnitine
	Copper
Intoxication	Drugs
Disturbed regulatory control	Hypothyroidism
Disturbed interaction with other cell organelles	Zellweger
Unidentified causes	

Thus a respiratory chain defect can result in ATP deficiency and an excess production of free radicals.

In case of OXPHOS disorders the therapeutic strategy might be specificated:

- Increase the production of ATP by bypassing the block in electron transfer using artificial electron acceptors.
- Enhance residual enzyme activity.
- Minimise the free radical induced damage.

A number of causes of non-primary mitochondrial pathology are recognized (Table 2).

Practical Approaches

Supportive Measures

Supportive care may significantly improve a patient's quality of life and help to counteract the progressive, disabling course in some patients. Examples:

- the appropriate treatment for diabetes and other endocrine disorders
- the management of seizures using anticonvulsant drugs
- ophthalmic splints or corrective surgery in PEO; surgery can improve quality of life of patients with ocular complications such as cataracts
- treatment of dysphagia by gastrostomy
- cochlear implantation for patients with hearing loss associated with mtDNA defects such as MELAS Syndrome
- cardiac dysrhythmias can be treated appropriately with either drugs or pacemaker
- load lowering medication should be considered in hypertrophic cardiomyopathy.

In the treatment of patients some drugs should be withheld:

- Valproate inhibits fatty acid oxidation and oxidative phosphorylation.
- Chloramphenicol inhibits mitochondrial protein synthesis.

Table 3. A variety of pharmacological agents for treatment of mitochondrial defects[1]

Ubiquinone	(CoQ_{10}; ubidecarenone) • role in electron transfer • an anti-oxidant both in lipid membranes and within mitochondria	(60-150 mg/d)
Idebenone	Synthetic analogue of CoQ_{10}	(90-290 mg/d)
Menadione	Lipid soluble analogue of ubiquinone – K3 (bypasses complex III)	(10 mg/d x 4 plus Vit C 4 dd 1 gr
Dichloroacetate	Inhibits the protein kinase that reversible inactivates the PDHC, maintaining PDHC in a catalytically active form	(25 mg/kg/d)
Riboflavin	Flavin moieties are present in complexes I and II	(100 mg/d)
Creatine	Supplementation	(5-10 g/d)
Carnitine	Supplementation	(1-3 g/d)
Succinate	Potential treatment of CI deficiency.	(6 g/d)

[1] Recommendations differ between metabolic centres in the world and are evolving continuously. We cannot guarantee the figures and recommend to read the manufacturer's drug information carefully.

- Antiviral agents inhibit the mitochondrial DNA polymerase γ and may cause fatal encephalopathy or myopathy.
- Aminoglycoside antibiotics may enhance the penetrance of mitochondrial 16S rRNA mutation, associated with inherited nonsyndromic deafness.
- Anaesthetic complications may arise in patients with enhanced sensitivity to both etomidate and thiopentone which may play a role in patients with KSS.

Based on anecdotal observations a number of variable therapeutic approaches may be mentioned (see Table 3; see also ref. 8). Administration of vitamins C and K may bypass a block in electron transfer through complex III of the respiratory chain. Thiamine and riboflavin have both been employed to enhance residual respiratory chain enzyme activity. Coenzyme Q_{10} (ubiquinone) is a component of the respiratory chain. It accepts electrons, transferring them sequentially to complex III; it also is an antioxidant. Patients with a CoQ-deficiency respond well to supplementation. It may also minimise free-radical induced damage that often occurs as a result of respiratory chain deficiency. Improvements, described are not always apparent in larger clinical trials.[5] Creatine supplementation increases strength and aerobic power in patients with mitochondrial disorders[6] and recent studies have shown, that exercise may be of therapeutic benefit for some patients.[10]

Future Therapies

The above mentioned treatment assumptions are based on current understanding of the pathophysiology, on anecdotal clinical reports and a limited number of clinical trials, which have not been encouraging.[2]

The lack of pharmacological treatments provoked a search for realistic gene therapies and other experimental strategies. According to[11] various approaches could be explored.

- Selective inhibition of mutated mtDNA replication: the specific inhibition by suitable antigenomic agents coupled to the selective propagation of the wild-type molecule could correct the imbalance of the ratio of mutant to wild-type mtDNA, that determines the expression of a mitochondrial biochemical defect.
- Antigenomic therapy.

The sequence specific inhibition of mutant mtDNA replication by antigenomic peptide nucleic acids (PNA's) is demonstrated in vitro. PNA's are uncharged, nuclease resistant DNA mimics. The antigenomic approach has been successfully extended to explore molecules that

might selectively bind and inhibit the replication of mtDNA molecules containing pathogenic deletions.

- Induced muscle regeneration in mitochondrial DNA disorders.

Some patients harbour very high levels of pathological mutation in mature muscle, and the same mutations at very low or undetectable level in satellite cells. The stimulation of muscle regeneration in vivo might restore in these patients a normal mitochondrial DNA genotype and biochemical activity.

Very recently data on in vitro gene therapy using human mtDNA, cybrid formation and micro-infections are presented by Kasawa et al. Mitochondrial DNA in the cytoplast is transferred into mutant cells via the formation of cybrids; once inside the cell the mtDNA complements the defect correctly.

Evaluation of Treatment

The evaluation of the therapeutic regimens used in the treatment of patients with a mitochondrial encephalomyopathy is another difficult problem. In literature the following criteria are mentioned:

1. Clinical observation including muscle power, anaerobic threshold, and exercise testing.
2. Laboratory examination including estimation of serum lactate in relation to exercise, CK, organic acids in urine, and carnitine levels in plasma.
3. Electrophysiological examinations.
4. Magnetic resonance imaging and spectroscopy.[14,32]
5. Histopathological examination.
6. Biochemical examination of biopsied muscle.

It must be stressed that only double blind, placebo-controlled studies can give an unambiguous answer concerning the validity of a therapeutic approach. Also it must be stressed that in view of the limited number of patients it is almost impossible to compile a homogenous group of patients.

When the treatment is evaluated based on the variables mentioned by Beaudet: lifespan, reproductive capability and social adaptation, one can only decide that evaluation of therapy is hardly possible since no data are available.

Concluding Remarks

Unfortunately, in patients with a mitochondrial cytopathy all therapy is as yet palliative. The clinical, biochemical and genetically heterogeneous nature of OXPHOS disorders, the relatively low prevalence and the unpredictable natural history make accurate analysis difficult. Nevertheless, symptomatic and supportive aids are available. Comfort, functional capacity and even life expectancy may be increased. Apart from dietetic approaches a few therapeutic manoeuvres may be of some value.

Insight in pathogenesis may open new ways for future therapies.

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CHAPTER 11

Prenatal Diagnostics in Oxidative Phosphorylation Disorders

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Abstract

In this chapter we describe about 18 years of experience with prenatal diagnosis in oxidative phosphorylation (OXPHOS) diseases in our centre. We start diagnostics of OXPHOS disorders in patients with a mitochondrial (encephalo)myopathy by preference by measuring oxidation rates of pyruvate, malate and succinate and ATP production rates from oxidation of pyruvate in a "fresh" muscle biopsy. In the same biopsy activities of the mitochondrial respiratory chain enzymes complex-I, complex-II, complex-III and complex-IV are also measured. When decreased substrate oxidation rates and ATP production rates give indication for suspicion on a complex-V or a pyruvate dehydrogenase complex (PDHC) deficiency, activities of these enzymes are also measured. In frozen muscle biopsies we only can measure the respiratory chain enzymes. In which cases now can we offer prenatal diagnosis? In about 30% of the muscle biopsies with clearly decreased substrate oxidation rates and ATP production rates, all respiratory chain enzymes, complex-V and PDHC show normal activities. In these cases it is impossible at the moment to offer prenatal diagnosis. In the remainder of the biopsies with clearly reduced substrate oxidation- and ATP production rates, decreased activities are measured of one or more of the afore mentioned enzymes. The most frequently occurring deficiencies in fresh as well as in frozen muscle biopsies are complex-I, complex-IV or combined deficiencies of these enzymes. The next step is to search if the deficiency is also expressed in cultured fibroblasts and to exclude a mtDNA mutation as a cause of the deficiency. If the deficiency is also expressed in cultured fibroblasts and mtDNA mutations have been excluded we are willing to offer prenatal diagnosis. This chapter is aggravated on prenatal diagnosis for complex-I, complex-IV or a combined deficiency of these enzymes because the majority of the total number of requests for prenatal diagnosis that reach us concerns pregnancies in families in which the index patient was suffering from a deficiency of one of these (or both) enzymes.

Introduction

Mitochondrial (encephalo)myopathies are a group of diseases with a very heterogeneous clinical picture. The clinical picture of patients varies from very severely affected with early onset and often fatal outcome at young age to only mildly affected, with sometimes late onset and reaching adult age. In some patients only one tissue seems to be affected and others suffer from a multisystem disorder. In some patients only one enzyme involved in OXPHOS is deficient, in others a combined deficiency of two or more enzymes is found. In the majority of the patients muscular and/or neurological complaints are the main presenting symptoms. There

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are age-dependent symptoms as failure to thrive in neonates and exercise intolerance in adults, other symptoms as hypotonia and retardation can present at any age. No single clinical feature is specific for one clinical phenotype and in some patients the clinical picture of a specific phenotype can gradually change into another. A patient is suspected to suffer from a mitochondrial disorder if he/she suffers from at least two chronic and unexplained symptoms as described in Table 1, preferably occurring in two different organs.[1,2] As soon as a child has been diagnosed to suffer from a mitochondrial disorder, many parents raise the question if prenatal diagnosis is possible, especially if the parents have more affected children or when the mother had one or more spontaneous abortions before or after birth of an affected child.

Prerequisites for Offering Prenatal Diagnosis in OXPHOS Disorders

Prenatal diagnostics in mitochondrial disease can be a delicate case and susceptible to many pitfalls. The human mitochondrion contains at least 1000 different proteins[3] but only a relatively small number of them is directly involved in the primary task of the mitochondrion: production of the energy-rich compounds ATP and creatine phosphate from oxidation of several substrates. Only these proteins can be evaluated by measuring their enzymatic activity. Other proteins are involved in the import, folding and defolding and assembly of proteins in the mitochondrion. The majority of the mitochondrial proteins is encoded by the nuclear DNA, synthesized in the cytoplasm and transported into the mitochondrion. Only 13 mitochondrial proteins, all subunits of the complexes of the mitochondrial respiratory chain and complex-V, are encoded by the mitochondrial genome (mtDNA). These are the seven ND-subunits of Complex-I (ND1-ND6 and ND4L), the cytochrome-b subunit of Complex-III, the cox-1, 2 and 3 subunits of Complex-IV (cytochrome c oxidase) and the Complex-V (F_1F_0 ATP-ase) subunits 6 and 8. In all these subunits mutations have been described causing mitochondrial disease.

Also mutations in the tRNA-genes of the mtDNA and deletions in the mtDNA have been described that can cause mitochondrial disease. If prenatal diagnosis for OXPHOS disorders is requested, it is important to exclude mtDNA mutations as a possible cause for the disorder. Mutations in the mtDNA have a very specific feature: the mutations can show heteroplasmy. This means that besides the mutated DNA there still is a part of normal (wild type) DNA present. If all the mtDNA is mutated, the mutation is called homoplasmic . The percentage of heteroplasmy varies between different tissues, mutant load in blood and cultured fibroblasts being mostly low as compared to high energy demanding tissues like muscle, liver and brain. For many mtDNA mutations a relation exists between the percentage heteroplasmy and the clinical phenotype, for instance MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke like episodes), MERRF (myoclonic epilepsy, ragged red fibers) and NARP (neuropathy, ataxia, retinitis pigmentosa).[4-7] Only when the percentage mutated DNA exceeds a certain threshold value, the patient is affected and shows the clinical phenotype. Other mutations like LHON (Leber's hereditary optic neuropathy) lack such correlation.[8] Several mutations in the mitochondrial ND genes as well as mutations in the tRNA genes cause isolated Complex-I deficiency. Mutations in the mitochondrial Complex-IV genes and the cytochrome-b gene cause Complex-IV and Complex-III deficiency, respectively. The Leigh/NARP 8993 mutation, causing NARP or Leigh syndrome (subacute necrotizing encephalomyelopathy) is the major source of Complex-V deficiency (for an up to date overview of mtDNA mutations and matched clinical phenotypes look at MITOMAP: http://www.mitomap.org on the internet). So, if a patient with a mitochondrial (encephalo)myopathy is suffering from a Complex-I, III, IV or V or a combined deficiency of some of these complexes, searching for mtDNA mutations is inevitable when prenatal diagnostics is required. In case of an enzyme deficiency caused by a mtDNA mutation, prenatal diagnostics should strongly be dissuaded at this moment. When normal activities of the affected enzyme are measured in native chorionic villi, cultured chorionic cells or cultured amniotic cells of such fetus, enzymatic activity can still be decreased in muscle, liver or brain tissue of the fetus. The

Table 1. Clinical symptoms most frequently found in mitochondrial disorders

Central Nervous System	Seizures
	Hypotonia/hypertonia
	Spasticity
	Transient paraparesis
	Lethargy/coma
	Psychomotor retardation/regression
	Extrapyramidal signs
	Ataxia (episodic)
	Dyspraxia
	Central hypoventilation
	Deceleration/acceleration of head growth
	Blindness (cortical)
	Deafness (perceptive)
Skeletal Muscle	Exercise intolerance/easy fatiguability
	Muscle weakness
Heart	Cardiomyopathy (hypertrophic or dilated)
	Conduction abnormalities
Eyes	Ptosis
	Restricted eye movements
	Strabismus
	Cataract
	Pigmentary retinopathy
	Optic atrophy
Liver	Hepatic failure
	Hypoglycaemia
Kidney	Tubular disfunction
Endocrine	Diabetes insipidus
	Diabetus mellitus
	Delayed puberty
	Hypothyroidism
	Exocrine pancreas dysfunction
	Primary ovarian dysfunction
Gastrointestinal	Diarrhoea (villous atrophy)
	Intestinal pseudoconstriction
Other	Failure to thrive
	Short stature
	Pancytopenia
	Anaemia
	Migraine

percentage of mutated mtDNA can be very low or even undetectable in the prenatally investigated cells, whereas high amounts of mutated mtDNA in muscle, liver or brain tissue can be present and therefore the fetus may be affected. Only if in native or cultured chorionic cells a severe deficiency of one or more respiratory chain enzymes has been measured, caused by a mtDNA mutation, one could conclude that the fetus would probably be affected because the percentage heteroplasmy increases from tissues like fibroblasts or chorionic cells towards muscle, liver or brain. However, more study has to be done to prove this conclusion.

Dahl et al[9] performed a first study to come towards reliable prenatal diagnosis for both the LEIGH/NARP 8993 mutations (8993T>G and 8993T>C) and concluded that there is no substantial tissue variation in mutant load between fetal and adult tissue, implicating that the mutant load in a prenatal sample will represent the mutant load in other fetal tissues. Each of

the two nucleotide 8993 mutations shows a strong correlation between mutant load and severity of the clinical picture. Their empirical data for calculating recurrence risk and predicting the clinical outcome of a given mutant load can be used cautiously for genetic counselling and prenatal diagnosis of nucleotide 8993 mutations. Harding et al[10] performed prenatal diagnostics in two pregnancies of a mother with a severely affected child with LEIGH/NARP 8993 T>G mutation. Compared with the mutant load in the probandus, the native chorionic villi showed a higher proportion of mutant mtDNA on both occasions and this was reflected in several fetal tissues including muscle and brain.

It is important that in patients with an (encephalo)myopathy and decreased activities of one or more enzymes of the OXPHOS system extensive examinations on the presence of mutations in mtDNA are performed to exclude mtDNA mutations. One method is sequencing the whole mitochondrial genome but this is expensive and time consuming. Besides the mtDNA contains a lot of polymorfisms. A convenient method to test whether an enzyme deficiency is caused by a mutation in the mtDNA or by a mutation in the nuclear genome is the ρ^0 test as described by King and Atardi.[11] Patient cells are enucleated and fused with a ρ^0 cell line (a human cell line lacking mtDNA). If the enzyme deficiency still remains in the transmitochondrial cell line, the deficiency is caused by a mtDNA mutation. If the deficiency disappears it is caused by a mutation of nuclear origin. A rapid and sensitive method to search for mutations in the whole mitochondrial genome could be the Conformation Sensitive Gel Electroforesis (CSGE) as described by Finnilä et al.[12] After analysis of the mtDNA haplogroup of the patient to exclude polymorfisms, the whole mitochondrial genome is analysed for mtDNA mutations. CSGE is based on the separation of heteroduplexes containing single base-pair mismatches from homoduplexes in a polyacrylamide gel. Heteroduplexes are formed by heat denaturation and reannealing PCR fragments of mtDNA from patients and a control.

An other very important prerequisite for performance of prenatal diagnosis is testing the obtained chorionic villi on maternal contamination. Immediately after the chorionic biopsy has been taken the tissue has to be dissected accurately under a microscope to separate fetal from maternal tissue. Especially when chorionic cells must be cultured from native chorionic villi this has to be done very precisely. However this precaution is not enough because when only a few maternal cells are left in the material, they can overgrow the fetal cells especially when the fetal cells, for instance, show a complex-I deficiency. In that case the "healthy" maternal cells may overgrow the fetal cells because of their advantage in oxidative energy metabolism. So, especially in cultured chorionic cells there is a substantial risk for maternal cell contamination (decidual cells) which can lead to false diagnosis. Milunsky and Cheney[13] proved the presence of maternal cells in 3 of 24 chorionic villus samples and concluded that microscopic examination of chorionic villi for apparent presence of maternal cells is not rigorous enough. Ledbetter et al[14] found a rate of 1.8% maternal cell contamination in chorionic cell cultures. Wang et al[15] reported a rate of 1.7% maternal cell contamination. In 10 long term cultures of chorionic cells we found one culture which consisted totally of maternal cells and this culture led to a wrong prenatal diagnosis for complex-I deficiency (Schuelke et al[16]). The probandus in this family was a girl, the first child of healthy Caucasian first-degree cousins (family 1). She suffered from acrocyanosis, muscular hypotonia, nystagmus, bitemporal retinal depigmentation and severe lactic acidosis (plasma lactate concentration 24.1mmol/l, reference interval 0.5-2.2mmol/l, cerebrospinal fluid lactate concentration 9.6mmol/l, reference values <2, and a plasma lactate-to-pyruvate ratio of 57, reference value <20). In muscle tissue and cultured fibroblasts respiratory chain complexes I, II+III and IV were measured and in both tissues an isolated complex-I deficiency was found. The child died at the age of 4 weeks from respiratory failure in the course of an uncontrollable metabolic crisis. As the mother became pregnant again the parents asked for prenatal diagnosis and we measured complex-I activity in native chorionic villi and cultured chorionic cells. Complex-I activity in native chorionic villi was normal, but decreased in cultured chorionic cells. This policy was adopted after we performed a prenatal diagnosis in an other family (family 2) where the

probandus also suffered from complex-I deficiency in muscle tissue and cultured fibroblasts. In the next pregnancy we measured a normal complex-I activity in native chorionic villi but a severely decreased activity in cultured chorionic cells of the fetus. The parents decided to continue the pregnancy and the child died at the age of 18 months from severe lactic acidosis and a hypertrophic cardiomyopathy. In cultured fibroblasts of the girl we found an isolated complex-I deficiency. Some years later we found a homozygous 683G>A mutation in the *NDUFS2* gene of Complex-I in cultured fibroblasts of this patient and the probandus of this family. Until now the reason of this discrepancy between native chorionic villi and cultured chorionic cells has not been cleared.

In the second pregnancy of family 1 the parents opted for a high degree of security and decided to terminate the pregnancy. In muscle tissue from the aborted female fetus, however, we measured a normal Complex-I activity. At the time of the third pregnancy in family 1 we could offer prenatal diagnosis on base of enzymatic measurement of complex-I activity as well as on mutation screening of complex-I genes. In the probandus we had proven a homozygous C>T transition at nucleotide 632 of the *NDUFV1* cDNA. In native chorionic villi as well as in cultured chorionic cells of this fetus normal complex-I activity was measured. In both fractions of this fetus and in cultured chorionic cells and muscle tissue from the fetus of the second pregnancy we searched for the mutation in the *NDUFV1* gene and both fetuses were found to be heterozygous for the mutation. Next we investigated the genetic identity of the cultured chorionic cells and the muscle tissue from the fetus of the second pregnancy, native chorionic villi and cultured chorionic cells of the third pregnancy and DNA from peripheral blood lymphocytes from both parents by analysing nine different polymorphic short tetranucleotide repeat loci and the X-Y homologous gene amelogenin with the AmpF/STR™ Profiler Plus Kit (Applied Biosystems). Native chorionic villi as well as cultured chorionic cells from the fetus of the third pregnancy were totally of fetal origin without any maternal contamination. However the cultured chorionic cells of the second pregnancy seemed to be totally of maternal, probably decidual origin. Because both parents were found to be heterozygous for the 632C>T mutation in the *NDUFV1* gene and cultured fibroblasts of both parents showed normal complex-I activity, it was strange that the decidual cells of the mother showed a decreased complex-I activity. In an attempt to clear up this discrepancy we measured complex-I activity in 3 control decidual cell cultures but also when compared to these control cells, complex-I activity in the decidual cells of the mother was clearly decreased (cultured decidual cells mother: 120 mU/U cytochrome c oxidase, controls: 220-300). Complex-I activity in cultured decidual cells of controls was comparable with control cultured chorionic cells, 220-300 (n=3) and 186-330 mU/U cytochrome c oxidase (n=13), respectively. So despite all efforts we could not find a satisfactory explanation for the enzymatic results obtained in the second pregnancy of this family.

Tissues to Be Used for Prenatal Diagnosis in OXPHOS Disorders

For a reliable prenatal diagnosis three kinds of fetal tissues are considered: native chorionic villi, cultured chorionic cells and cultured amniotic cells. Chorionic villi can be taken transcervical after 9 weeks of pregnancy or transabdominal after 11-13 weeks of pregnancy. Amniotic cells can be taken after 16 weeks of pregnancy.

In our institute we use the following policy. After the native chorionic villi sample has been taken the material is carefully made free from maternal contaminating tissue under a microscope. Always a blood sample of the mother is taken simultaneously for the test on maternal contamination. The pure fetal sample is centrifuged in an Eppendorf tube and the supernatant is discarded. Part of the material is cultured in Amnionmax tissue culture medium and the rest of the sample is immediately frozen in liquid nitrogen. This material can be used for DNA isolation for all kind of prenatal diagnosis on molecular genetic base and/or can be homogenized in 0.1M potassium phosphate buffer, pH 7.4 and used for enzymatic analysis of complex-I, complex-IV or both. Part of the homogenate is used for testing on maternal

contamination and for measuring Citrate Synthase (CS) activity as a mitochondrial marker enzyme and protein content. If whatever goes wrong with one or more of the measurements or if there are doubts about the reliability of a certain test we still have the cultured chorionic cells in reserve to repeat the measurements. Especially these cultured cells must be tested on maternal contamination for reasons as mentioned before. From cultured chorionic cells a mitochondrial enriched fraction is prepared as described by Attardi et al[17] with modifications as described by Bentlage et al.[18] This fraction can be used for enzymatic studies. We routinely measure complex-I, complex-III, Succinate: cytochrome c oxido reductase (SCC; Complex-II+III) and complex-IV activity in this fraction. CS activity and protein content are also measured. If something might go wrong with one of the assays on these cells or there is still doubt about the reliability of the results or when maternal contamination is proven in these cells there still can be done an amnioncentesis to get amniocytes. From cultured amniocytes we also prepare a mitochondrial enriched fraction and the same enzymes are measured as in cultured chorionic cells. When native chorionic villi are used for prenatal diagnostics on enzymatic base a diagnosis can be made within one week at most. With cultured chorionic cells or amniotic cells it takes between one or two weeks, beyond the time that is needed to culture the required amount of cells.

Methods for Prenatal Diagnosis in OXPHOS Disorders

To perform prenatal diagnostics on fetal tissue in general two methods can be used: the molecular genetic way based on searching for mutations that have already previously established in the index patient, in the DNA of fetal tissue, or the enzymatic way based on measuring the activity of those enzymes proved to be deficient in the index patient, in fetal tissue. It is beyond doubt that the first mentioned way is the most reliable method for prenatal diagnostics. However, this method is only possible if a mutation has been established in a nuclear gene that encodes for the enzyme or protein found to be deficient in the index patient. For a major number of deficiencies in enzymes involved in the OXPHOS the genetic defect is still unknown. In those cases only the enzymatic approach is possible. A thorough explanation and information from the biochemist towards the doctor about the possibilities and limitations of this method is of utmost importance. In our opinion it is not correct to go for absolute certainty because in that case a lot of parents will be left out in the cold. Besides every new case is a precept and has an added value to the reliability of the method. In our institute we offer prenatal diagnostics, especially when it has to be done on enzymatic base, only when the enzyme deficiency in the proband has been proven in at least two different tissues and by preference one of them being cultured fibroblasts. The necessity of a proven deficiency in cultured fibroblasts of the proband is generally accepted.[19] A deficiency in cultured fibroblasts is never an absolute deficiency, but although relatively high residual activities can be measured, for complex-I for instance up to 80% of the lowest control value, our experience is that even in those cases prenatal diagnosis can still reliably be performed.

At the moment about 26 mutations in nuclear genes, directly or indirectly involved in the enzymatic activity of one or more enzymes of the OXPHOS, are known. These mutations vary from mutations in genes encoding for subunits of complex-I, complex-II or complex-III[20] of the mitochondrial respiratory chain, to mutations in genes causing multiple mtDNA deletions or depletion, mutations in genes encoding for proteins involved in the assembly of complex-III and complex-IV, mutations in genes encoding for proteins involved in homeostasis and import of proteins over the mitochondrial inner membrane and one gene involved in transcription and translation of OXPHOS genes. For a review of these mutations see Chapter 7: Nuclear DNA and Oxidative Phosphorylation (OXPHOS) disorders, by van den Heuvel and Smeitink in this book. A mutation in one of these genes always leads to a severe disturbance in OXPHOS. If a mutation in one of these genes has been proven in a patient suffering from a deficiency in one or more enzymes of the OXPHOS, prenatal diagnosis can be offered on molecular genetic

base. Special attention must be paid to some patients with complex-I deficiency. Bénit et al[21] described 4 patients with hypotonia, ataxia, psychomotor retardation, or Leigh syndrome with complex-I deficiency in muscle tissue or in muscle and liver tissue but normal oxidation rates of NADH-generating substrates in cultured fibroblasts, suggesting a normal complex-I activity in cultured fibroblasts. Complex-I activity was not measured in cultured fibroblasts. Three of them were compound heterozygous for mutations in the *NDUFV1* gene encoding for the 51 kDa subunit of complex-I and one of them was compound heterozygous for mutations in the *NDUFS1* gene encoding for the 75 kDa subunit of complex-I. So possibly our rule that in patients with complex-I deficiency in muscle tissue, the deficiency must also be proven in cultured fibroblasts before prenatal diagnostics can be offered, does not always fit.

In patients with this phenotype and complex-I deficiency in muscle or muscle and liver tissue we currently advice to sequence the *NDUFV1* and *NDUFS1* genes, especially when complex-I activity in cultured fibroblasts is normal. Besides it is advisable to screen both parents for mutations in these genes. When a mutation is found, prenatal diagnosis is probably only possible on molecular genetic base.

Results of Prenatal Diagnosis for OXPHOS Disorders in Our Center

Complex-IV Deficiency

In 1985 we started our investigations for prenatal diagnosis for complex-IV deficiencies and in 1988 we published for the first time two cases of prenatal diagnosis in a family where the index patient suffered from Leigh syndrome and died at the age of three years.[22] In cultured fibroblasts of this patient we measured a clear complex-IV deficiency. In native chorionic villi and cultured amniocytes of the first fetus we measured a normal complex-IV activity. After an uneventful pregnancy a healthy boy was born that showed no symptoms of Leigh syndrome. In the second pregnancy we measured a clearly decreased complex-IV activity in native chorionic villi and cultured amniocytes. The parents decided for an abortion and in cultured fibroblasts of the aborted fetus also a markedly decreased complex-IV activity was measured.

From 1986 till June 2003 we diagnosed 47 pregnancies in 33 families for complex-IV deficiency. In 35 cases a normal complex-IV activity was measured in native chorionic villi, cultured chorionic cells, cultured amniocytes or a combination of one or more of these tissues, which resulted in an unaffected new-born in 24 cases; in 11 cases the outcome of the pregnancy is currently unknown for us. In 9 cases a clearly decreased complex-IV activity was measured in native chorionic villi, cultured chorionic cells, cultured amniocytes or a combination of two of these tissues. In 3 of these cases the complex-IV deficiency could be confirmed in fetal cultured fibroblasts from abortion tissue (2 cases) or in placenta tissue. In 3 cases no cultured fibroblasts of the aborted fetus were available. In 2 cases the parents decided to continue the pregnancy and in both cases an affected child was born which died at the age of 3 and 5 years, respectively. However no cultured fibroblasts from both children were available. In one case a decreased complex-IV activity was measured in cultured amniocytes. The parents decided to continue the pregnancy and a clinically unaffected boy was born. No cultured fibroblasts of the boy were available. In one case, apart from the 9 cases with clearly decreased complex-IV activity in fetal cells, only a mildly decreased Complex-IV activity was measured in native chorionic villi (complex-IV on CS 89% of the lowest control value). So we could not make a definite conclusion with respect to the fetus, affected or not. The parents decided to terminate the pregnancy. In cultured fibroblasts of the aborted fetus a normal Complex-IV activity was measured.

Two other cases of prenatal diagnosis for complex-IV deficiency resulted in an unexpected outcome of the pregnancy. In one case a normal complex-IV activity was measured in native chorionic villi, cultured chorionic cells and cultured amniocytes of the fetus but an intra-uterine death appeared, possibly a consequence of the amniocentesis. No cultured fibroblasts of this fetus were available. In one case a normal complex-IV activity was measured in native chori-

onic villi, however, a clinically affected boy was born. In muscle tissue of the boy a normal complex-IV activity but surprisingly a decreased pyruvate dehydrogenase complex activity was measured.

Complex-I Deficiency

In the same period 36 pregnancies in 24 families were diagnosed for complex-I deficiency. The first 23 pregnancies in 15 families have already been published.[23] In 27 pregnancies a normal Complex-I activity was measured which resulted in an unaffected new-born in 25 cases. In one case the parents decided not to continue the pregnancy and the fetus was aborted. No tissue of this fetus was available. In one case a spontaneous abortion appeared after intra-uterine death of the fetus and also from this fetus no tissue was available for further examinations. In 9 cases a decreased Complex-I activity was measured in native chorionic villi (7 cases) or cultured chorionic cells (2 cases). In one case the pregnancy ended in a spontaneous abortion after intra-uterine death of the fetus; no tissue was available to confirm the deficiency. In 3 cases out of 9 the parents decided to continue the pregnancy in spite of the complex-I deficiency established in fetal cells and 3 affected children were born. The Complex-I deficiency could be confirmed in muscle tissue (first case), muscle tissue and cultured fibroblasts (second case) and cultured fibroblasts (third case) of the children. In 2 cases out of 9 the pregnancy was terminated. From one fetus no tissue was available, the other fetus was from the second pregnancy in the family that we already described[16] and complex-I activity in fetal muscle tissue was normal. In the index-patient of the latter family we found the homozygous 632 C>T mutation in the *NDUFV1* gene as well as a severely decreased complex-I activity in muscle tissue and cultured fibroblasts. In one case out of 9 the outcome of the pregnancy is unknown. Recently we diagnosed a pregnancy of twins. The index patient of this family suffered from a complex-I deficiency in muscle tissue and cultured fibroblasts and we found a homozygous 686C>A mutation in the *NDUFS2* gene of complex-I. In native chorionic villi of one fetus a normal complex-I activity was measured and the mutation was not present. The other fetus showed a decreased complex-I activity in native chorionic villi and the same homozygous mutation was proven in this tissue. The outcome of this pregnancy is still unknown. In one case out of 9, mentioned already before in this chapter, a normal complex-I activity was measured in native chorionic villi but a decreased one in cultured chorionic cells. The pregnancy was continued and an affected girl was born. Complex-I activity was decreased in cultured fibroblasts of the girl and in her cultured fibroblasts and those of the index patient in this family we later found a homozygous 683G>A mutation in the *NDUFS2* gene of complex-I (family A in ref. 24)

Combined Deficiencies of Complex-I and Complex-IV

In 14 pregnancies in 10 families we performed prenatal diagnosis for combined deficiency of complex-I and complex-IV. In 11 cases normal activities were measured for complex-I as well as complex-IV in native chorionic villi (10 cases) or amniocytes (one case). In 6 cases a healthy unaffected child was born, in 5 cases the outcome of the pregnancy is still not known. In one case a decreased complex-IV with normal complex-I activity was measured in native chorionic villi. The pregnancy was terminated but no tissue of the fetus was available. In one case a decreased complex-IV activity was measured in native chorionic villi as well as in cultured chorionic cells. Complex-I activity was normal in both fractions. The pregnancy ended with an intra-uterine death of the fetus in the 17th week of the pregnancy. No tissue of the fetus was available. In one case a decreased complex-I activity was measured in native chorionic villi as well as in cultured chorionic cells. Complex-IV activity was normal in both fractions. The pregnancy was terminated and in cultured fibroblasts of the fetus we measured normal activities for complex-I as well as for complex-IV. Until now we have no explanation for this discrepancy between chorionic tissue and fetal fibroblasts.

Prenatal Diagnosis Only on Molecular Genetic Base

In 3 pregnancies in 2 families we performed prenatal diagnosis only on molecular genetic base. In the index patient of family one we measured an isolated complex-I deficiency in cultured fibroblasts and afterwards a homozygous 316 C>T mutation was found in the *NDUFS4* gene of Complex-I. The fetus was found to be heterozygous for the mutation as measured in native chorionic villi. The pregnancy was continued and a healthy unaffected child was born. In the other family we measured a decreased complex-IV activity in cultured fibroblasts of the index patient and afterwards we found a homozygous 688 C>G mutation in the *SURF1* gene. Both parents were found to be heterozygous for the mutation in blood. In 2 pregnancies in this family the fetuses were found to be heterozygous for the mutation, the pregnancies were continued and resulted in two healthy unaffected new-borns.

General Considerations

A diagnosis is considered to be 100% correct when after measuring a normal activity for complex-I and/or complex-IV in fetal tissue, a healthy unaffected child is born, or in case a decreased activity of complex-I and/or complex-IV, measured in fetal tissue, has been confirmed in fetal abortion tissue or in tissue of an affected new-born. We made a 100% correct prenatal diagnosis for complex-I in 30 out of 36 pregnancies (83%). For complex-IV we made a 100% correct diagnosis in 27 out of 45 pregnancies (60%). For combined deficiency of complex-I + complex-IV we made a 100% correct diagnosis in 6 out of 14 pregnancies (43%) but in this group the number of pregnancies with unknown outcome was relatively high (5 of 14 or 36%). Considering only the group of combined deficiencies with known outcome of the pregnancy the score was 6 of 9 (67%). In our laboratory most of the demands for prenatal diagnosis are coming from abroad and in our experience most of the chorionic villi samples and amniocytes are taken in a university clinic after we made thorough appointments with the sender. However, after the surgery the patients return to their peripheral hospital and

Table 2. Control values for respiratory chain enzymes in native chorionic villi, cultured chorionic cells and cultured amniocytes

Native chorionic villi		**Mean ± SD**	**Range**
Complex-IV *	(n= 29)	530 ± 200	270-920
Complex-I*	(n= 21)	160 ± 60	80-260
Cultured chorionic cell	(n= 13)		
Complex-IV*		688 ± 119	500-860
Complex-I**		264 ± 43	186-330
Complex-III**		2591 ± 542	
1620-3605			
SCC**		334 ± 105	190-530
Cultured amniocytes	(n= 10)		
Complex-IV*		510 ± 173	250-840
Complex-I**		344 ± 93	220-530
Complex-III**		2644 ± 701	
1770-3600			
SCC**		332 ± 100	240-590

*Values expressed in mU/U Citrate synthase. **Values expressed in mU/U Cytochrome oxidase. Activities in native chorionic villi were measured in crude homogenate. Activities in cultured cells were measured in a mitochondrial enriched fraction.

if relevant the abortion is also done there. In those cases it is mostly impossible to get cultured fibroblasts of the aborted fetus and it takes a lot of time and efforts to ascertain the outcome of the pregnancy.

Conclusion

Considering all the aspects we met in the last 18 years since we started with prenatal diagnosis for OXPHOS disorders, we think that with strict observance of some precautions, we can offer reliable prenatal diagnosis, especially for complex-I and complex-IV. We gathered control values for complex-I and complex-IV in native chorionic villi, cultured chorionic cells and cultured amniocytes. Recently we also gathered control values for complex-III and SCC in cultured chorionic cells and cultured amniocytes to be able to offer also prenatal diagnosis for these enzymes (for reference values of complex-I, III, IV and SCC, see Table 2). Until now we did not receive a request for prenatal diagnosis for these enzymes.

However, mitochondrial respiratory chain enzyme deficiencies are not the only cause of mitochondrial disease. The already mentioned mutations that have been proven in genes as those encoding for proteins involved in homeostasis and import of proteins over the mitochondrial inner membrane, can lead to disturbances in the mitochondrial energy metabolism that can not be detected with an enzymatic assay. Especially for these cases an assay that measures the overall capacity of the mitochondrial oxidative phosphorylation would be suitable and could also be used as a screening method for disturbances of the mitochondrial energy metabolism. Chowdhury et al[25] described a polarographic method on digitonin-permeabilised cultured amniocytes that looks suitable. However, they did not test the method on amniocytes with for instance a proven defect in one of the mitochondrial respiratory chain enzymes. Finally the whole mitochondrial proteome will probably be solved in the near future by proteomics and this will probably also be a valuable tool in prenatal diagnosis for mitochondrial disease.

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CHAPTER 12

Future Developments in the Laboratory Diagnosis of OXPHOS Disorders

David R. Thorburn

Abstract

Laboratory diagnosis of OXPHOS disorders has evolved substantially since mitochondrial DNA mutations were first shown to cause human disease in 1988. Traditional approaches such as skeletal muscle OXPHOS enzyme analysis and histochemistry remain among the most important diagnostic tools. However, molecular diagnosis of mitochondrial DNA mutations and, more recently, nuclear gene mutations are responsible for an increasing number of diagnoses. This trend will continue over the next two decades with new genomic approaches such as mutation chips eventually likely to become front-line diagnostic tools. In order to completely replace traditional approaches though, much work needs to be done identifying novel OXPHOS disease-causing genes and distinguishing pathogenic mutations in such genes from polymorphisms. In the interim, a number of other approaches will improve the ease and certainty of OXPHOS diagnosis. These are likely to include increased use of spectroscopic and other methods for assessing in vivo OXPHOS function, use of minimally invasive tissue biopsies, improved assays of OXPHOS function using immunocapture antibodies or fluorescent probes, and methods for assessing expression of OXPHOS genes and proteins using antibody chips, proteomics and cDNA microarrays. All these methods will require extensive validation studies to distinguish primary OXPHOS defects from other disease states and development of improved algorithms for defining how much evidence is needed for a definite diagnosis of OXPHOS disease.

Introduction

Dr. Bones McCoy waves his medical scanner over the patient and announces "He's got a mitochondrial myopathy with Complex I deficiency and it looks like compound heterozygosity for two nonsense mutations in the NDASS60 gene. Just give me a minute to make the gene therapy construct, then he'll need 30 minutes in the BioRegeneration Chamber."

Unfortunately that Star Trek scenario is not on the near, and perhaps not even far, horizon. However, there are many promising new techniques in use or under development in research laboratories. Some of these will make their way into routine use and improve our ability to give rapid and accurate answers to the two key diagnostic questions. Does a particular patient have an OXPHOS disorder? If so, what is the genetic basis of the OXPHOS disorder? At some stage in the future genomic methods will allow us to give rapid accurate answers to both questions for virtually all patients without needing to resort to invasive tissue biopsies of muscle, liver or other organs. By then we should also have gathered sufficient knowledge about OXPHOS pathogenesis that the answers will be able to guide effective approaches to prognosis and therapeutic and reproductive options.

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In the interim though, a range of methods has the potential to improve the speed, accuracy and information content of diagnostic approaches to OXPHOS disorders. Some will also help us to achieve diagnoses without having to resort to organ biopsy. The potential applicability of key emerging methods is described in the following sections.

In Vivo Assessment of OXPHOS Function

Magnetic Resonance Spectroscopy (MRS) and other spectroscopic approaches are becoming part of the routine clinical evaluation of patients suspected of an OXPHOS disorder. These and other screening techniques have been mentioned in Chapter 3, but will be briefly discussed here as they are an increasingly important component of deciding whether or not a particular patient has an OXPHOS disorder. When an OXPHOS disorder is suspected, a physician will typically want to perform a number of (nonspecialist) investigations before deciding whether or not to organise a muscle biopsy on their patient. Traditionally, the only easily accessible tests that were available and likely to be informative were imaging and metabolic investigations such as plasma lactate and urine organic acid analysis. Many centres expanded this approach by performing exercise tests or carbohydrate loads in conjunction with serial profiles of plasma lactate, pyruvate and ketone bodies,[1] and by measuring lactate in cerebrospinal fluid. A few specialist centres have greatly increased the information content of physiological exercise testing by incorporating methods such as gas exchange and cardiac output to quantify muscle oxygen extraction.[2]

More recently, many centres have had access to noninvasive methods that provide metabolic information. Magnetic Resonance Spectroscopy (MRS) is proving to be extremely useful in investigating patients with suspected OXPHOS disorders. Proton MRS can quantify lactate levels while phosphorus MRS provides information on the ratios of ATP to ADP, phosphocreatine to creatine plus phosphate and pH. Proton MRS is particularly useful in conjunction with magnetic resonance imaging of brain and can demonstrate elevated lactate levels in brain regions even when lactate levels are normal in cerebrospinal fluid.[3,4] OXPHOS patients often show symmetrical lesions in the basal ganglia or brainstem, but similar lesions can also be seen in ischemia. Disproportionate increase of lactate in such regions shown by proton MRS lends substantial weight to the likelihood of an OXPHOS disorder. Phosphorus MRS is used mainly for studying muscle metabolism, for example in assessing the recovery rate of phosphocreatine or pH after a standard exercise protocol.[5,6] A more recent trend is to combine the use of MRS with approaches such as Near-Infra Red Spectroscopy, which can measure muscle oxygenation status,[7] respiratory calorimetry to assess whole body oxygen consumption,[8] or stable isotope studies of lactate turnover.[9] These in vivo methods will become increasingly important in deciding which patients warrant more invasive investigation for an OXPHOS disorder. In the near-future, the major advances are likely to be in availability, increased resolution, ability to quantify additional markers, and in refinement of combining several of these approaches for patient investigation.

Minimally Invasive Tissue Samples

It would clearly be preferable to obtain a definite OXPHOS diagnosis by studying a sample such as blood, hair or mouthwash, than to subject a patient to an invasive biopsy of muscle or other tissues. This is already the case for a substantial proportion of adults whose clinical history is strongly suggestive of a classic mitochondrial cytopathy, where testing for a small number of common mtDNA mutations has quite a high diagnostic yield. However, perhaps half of all adults with OXPHOS disorders still require a muscle biopsy in order to obtain a diagnosis. This can be because their disease is either not caused by a common mtDNA mutation or it is caused by a common mtDNA mutation that is present in muscle but not in some other tissues such as blood.

For children with a suspected OXPHOS defect, mtDNA mutation screening of blood or other samples has a very low diagnostic yield compared with adults.[10,11] Enzyme or functional analysis of blood or cultured skin fibroblasts can provide a definite diagnosis, but also has a

poor diagnostic yield, except in cases with persistent lactic acidaemia. For example, only about half of OXPHOS enzyme defects expressed in muscle or liver are expressed in cultured skin fibroblasts.[12] This can sometimes be caused by varying levels of mtDNA heteroplasmy but is probably due to a tissue-specific nuclear defect in most cases.

How can we improve our ability to make diagnoses without needing to subject patients to invasive tissue biopsies? Several new and future approaches are outlined below.

1. Efficient high throughput screening and diagnostic methods. Most current OXPHOS enzyme and functional assays are laborious. Many centres, including my own, focus on studying muscle and liver biopsies because they can't afford the resources to study blood or cultured cells on all suspected patients, due to the lower diagnostic yield. Availability of simple, fast, effective functional assays would make it more practical to screen such samples, meaning that only patients with tissue-specific defects would require invasive tissue biopsies. Some promising immunochemical and FACS screening methods are described in the following sections. Similarly, high throughput assays for screening large numbers of mtDNA and nuclear gene mutations could avoid the need for functional studies of tissue biopsies in many patients.
2. Selection of the optimal sample for mtDNA analysis. One of the most common pathogenic mtDNA point mutations is the A3243G MELAS mutation. Like most other mtDNA point mutations, it appears that the mutant load of the A3243G mutation is similar in all tissues at birth. However, the A3243G mutation, and some other tRNA mutations, are selected against in blood where the levels of mutant mtDNA can decline at rates of over 1% per year.[13] Thus a symptomatic patient with this mutation can have a high mutant load in muscle but undetectable levels in blood, and the likelihood of this discrepancy increases with patient age. Two approaches to this problem show promise. Newborn screening of neonates has been standard practice in many countries for over three decades. Some centres have retained most of the original birth "Guthrie" dried blood spot cards, and these samples are usually suitable for PCR studies. If available, they may be regarded as preferable to fresh blood for analysis of mtDNA mutations. Dubeau et al[4] reported that urine sediment appears to retain higher levels of the A3243G mutation than blood, and further studies by that group and elsewhere (Eric Shoubridge, Sara Shanske, personal communications) suggest that urine may replace blood as a preferred sample for mtDNA analysis. mtDNA deletions, "muscle-specific" mtDNA point mutations in the cytochrome b gene and the recent report of paternally inherited mtDNA are also examples of mtDNA mutations that can be present in high mutant loads in muscle but absent in (adult) blood.[14,15] However, these mutations have probably been selected for in muscle rather than against in blood. It is likely that such mutations will not be present in significant amounts in urine or newborn screening cards.
3. Expressing muscle-specific genes in cultured fibroblasts. Some OXPHOS defects are caused by mutations in nuclear genes that are normally expressed in muscle but not in more easily accessible tissues such as fibroblasts. Functional studies of fibroblasts from such patients would thus be expected to show normal OXPHOS function, but what if we could "trick" the fibroblasts into expressing muscle-specific genes? MyoD is a master regulatory gene for myogenesis, and retroviral expression of MyoD has allowed the detection of dystrophin expression in amniocytes, chorionic-villus cells and fibroblasts from patients with suspected muscular dystrophy.[16] Thus expressing MyoD in patient fibroblasts could potentially allow the detection of a muscle-specific OXPHOS defect. The utility of this technique to date has been limited by its efficiency and universality. Initial studies with retroviral expression resulted in only about 2% of cells progressing to late-stage myogenesis. More recently, adenoviral expression has achieved much higher conversion efficiencies[17] and it now remains to be shown whether induction of muscle-specific gene expression includes genes causing OXPHOS defects. A number of centres are attempting to validate whether this approach can obviate the need for a muscle biopsy for functional studies of OXPHOS defects.

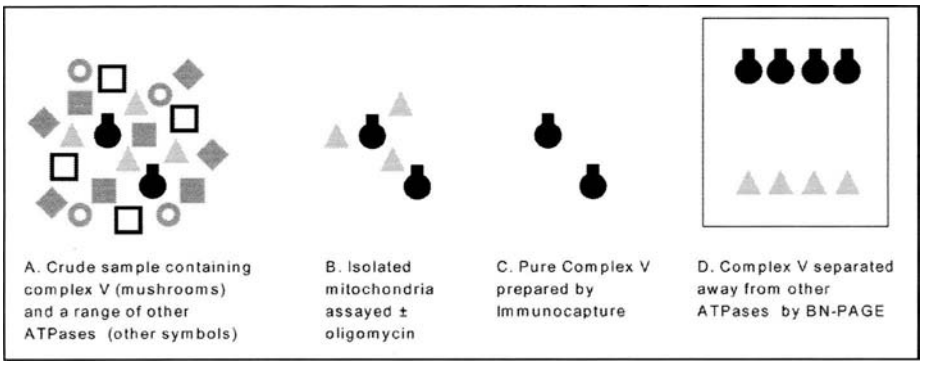

Figure 1. Overcoming interferences in OXPHOS enzyme assays. OXPHOS complex V is typically assayed in the reverse direction of ATP hydrolysis but (A) tissues and whole cells contain a large number of other ATPase activities that can potentially interfere with this assay. Complex V is depicted as a mushroom shape and other contaminating ATPases as different shapes. (B) The standard approach for assaying complex V is to minimise background interference by studying isolated mitochondria (to remove most of the nonmitochondrial ATPases) and to assay in the presence and absence of the specific complex V inhibitor oligomycin. An alternative simpler approach is to detergent-solublise complex V and purify it away from contaminating ATPase activities prior to assay using (C) a specific immunocapture monoclonal antibody[18] or (D) Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE).

OXPHOS Function

OXPHOS enzymology and studies of O_2 consumption, substrate oxidation or ATP synthesis have been by far the major methods used to diagnose OXPHOS functional defects in patient biopsies (see Chapter 5). More recently a wide range of other functional studies have been reported, although mostly in a research setting rather than in routine diagnosis. Recent improvements and prospects for the traditional methods and some of the more promising functional assays are described below.

Respiratory Chain Enzyme Assays

Several OXPHOS enzyme assays are made more problematic by interference from other enzymes that contribute a high background activity (Fig. 1). This problem could be avoided by rapidly purifying the OXPHOS enzyme away from background interferences, and such an approach has recently been described for the complex V assay. Capaldi and colleagues[18] generated a monoclonal antibody suitable for isolating human complex V in a functionally active form. They described an immunocapture assay in which detergent-solubilised cell extracts were applied to a 96-well microplate containing immobilised antibody. After washing the wells, immunoprecipitated complex V was shown to contain the full complement of subunits and displayed ATP hydrolysis that was fully sensitive to oligomycin and inhibitor protein. Similarly to complex V, background interferences of 30%-50% are typically present in assays for complex I, complex II+III and the marker enzyme citrate synthase in liver biopsies.[19] Immunocapture antibodies have recently been described for complex I[20] and the pyruvate dehydrogenase complex,[21] and at least the latter is suitable for assaying enzyme activity. If suitable antibodies can be generated and validated for all the OXPHOS complexes, then immunocapture assays have enormous potential for diagnosis. Advantages include high specificity and sensitivity plus simple protocols that would be amenable to screening large numbers of samples and be less prone to inter-laboratory variation. The lower background of such assays would also make them suitable for assaying at sub-saturating concentrations of substrates, so that kinetic defects (e.g., increased K_m) could be detected in addition to the V_{max} defects identified by standard assays.

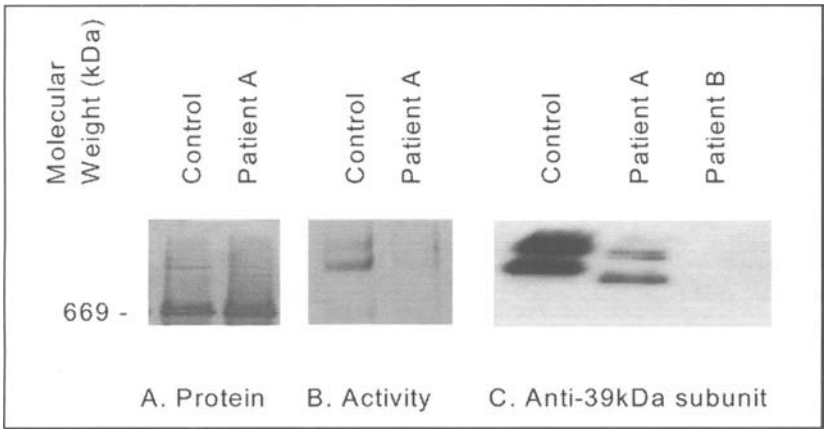

Figure 2. Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE) analysis of complex I. Fibroblast mitochondria from a control and two patients with lethal infantile complex I deficiency were solubilised in Triton X-100 and analysed by BN-PAGE. The three panels show (A) protein staining with Coomassie blue, B) enzyme histochemical staining of complex I (performed as described elsewhere),[23] C) Western blotting with an antibody to the 39 kDa subunit of complex I. Panels B and C show two bands for complex I, with the higher molecular weight band presumably representing a "supercomplex" of complex I.[75] Both patients showed essentially absent complex I activity on BN-PAGE (patient B not shown). The Western blotting results were dramatically different, however, implying different genetic defects as patient B had undetectable amounts of assembled complex I while patient A did show some assembled complex I, but of a smaller molecular weight than the full-size complex in the control. Note that the image in panel C was from a different gel and has been resized to be of comparable scale to panels A and B.

An alternative method for separating OXPHOS enzymes away from interfering enzyme activities prior to their assay is the use of Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE). BN-PAGE has become increasingly popular in recent years and involves detergent solubilisation of mitochondria followed by separation on BN-PAGE.[22-24] The OXPHOS complexes remain intact during BN-PAGE and retain their catalytic activity. Enzyme histochemical staining can be used to identify the relative activities of complexes I, II, IV and V, and is a useful adjunct to the conventional OXPHOS enzyme assays (Fig. 2).[25]

Other Functional Micro-Assays

The traditional accompaniment or alternative to assay of muscle OXPHOS enzymes was measurement of oxygen consumption using the Clark electrode. This approach has become less popular in recent years due to the need for large quantities of fresh muscle (typically >0.5g) for analysis by the conventional Clark electrode. The recent development of a much more sensitive (Oxygraph) oxygen electrode has meant that oxygen consumption can be measured even in single muscle fibres or a few milligrams of liver tissue.[26,27] This has led to some revival of the popularity of this approach, but the relatively high cost and laborious methodology currently limit its uptake.

A number of other aspects of mitochondrial function can also be assessed using sensitive micro-assays that require relatively small numbers of cells, typically 10^4 to 10^5 compared with 10^6 to 10^7 used in conventional assays. Such assays often utilise fluorometric or luminometric probe molecules. Fluorometric probes are potentially suitable for either (i) high throughput analysis using flow cytometry or microtitre plate readers or (ii) combining functional and morphological information using fluorescence or confocal microscopy. Luminometric probes can also be used in high throughput analysis with microtitre plate readers. Table 1 lists the most commonly used probes for measuring mitochondrial membrane potential, reactive oxygen species

Table 1. Fluorometric and luminometric probes used for measuring aspects of OXPHOS function

OXPHOS Functional Parameter	Probe*	References
Mitochondrial Membrane Potential	JC-1	76, 77
	CMXRosamine	77, 78
Mitochondrial Protein	MitoTracker Green FM	77
Mitochondrial Phospholipid (cardiolipin)	Nonyl acridine orange	77
Reactive Oxygen Species	H_2-rhodamine 123	28, 77
	H_2-CMXRosamine	77
	H_2-ethidium	29
	Carboxy-DCFDA	78
	Lucigenin (luminometric)	79
ATP	Luciferase (luminometric)	78

*All except lucigenin and luciferase are fluorometric probes

and ATP generation. These methods are currently used primarily in research studies on limited numbers of patient samples, but there are a few reports showing strong correlations between flow cytometric analysis and enzyme activity in patient cell lines.[28,29] Most micro-assays require more detailed studies to determine if they are sufficiently robust and sensitive for routine diagnostic use, but they do offer the potential to become simple, rapid methods suitable for screening large numbers of (noninvasive) tissue samples.

OXPHOS Constituents

Quantitation of mitochondrial components (other than enzyme activities) is becoming an increasingly important part of OXPHOS diagnosis. Traditionally the main components studied were cytochromes and individual OXPHOS proteins. Cytochrome spectroscopy is now seldom used since it requires large sample volumes and specialised equipment for low temperature analysis. Western blotting and immunohistochemical analysis of OXPHOS proteins have played a relatively minor diagnostic role for many years but are now becoming much more popular. Previous Western blotting studies tended to be limited to separating proteins on a one-dimensional SDS-PAGE gel, and probing with a polyclonal antibody, usually generated in-house. However, the introduction of BN-PAGE and the increasing commercial availability of OXPHOS monoclonal antibodies have revolutionised immunochemical approaches. It is now feasible to separate out the individual OXPHOS complexes on a BN-PAGE gel and probe with monoclonal antibodies against one or more proteins from each complex (Fig. 1). This offers a powerful and quite simple screening method for assessing the relative amounts of each complex in a patient sample, and is likely to become a more wide-spread approach in the next few years. In the future, there may be monoclonal antibodies developed against most OXPHOS subunits and related proteins involved in import, assembly and regulation. It is feasible that such antibodies could be used in a chip-based system, similar to a DNA micro-array that could be used for comprehensive screening of most OXPHOS disease-related proteins.[30]

Proteomics is one of the recent buzz words of cell biology. It relies on separating out all the proteins in complex biological samples, usually by two dimensional PAGE (2D-PAGE), and comparing the protein profile with other samples. Unknown protein spots can be identified by mass-spectrometric analysis. This approach can be highly automated and has obvious potential

Table 2. Potential causes of altered expression in expression analysis studies of skeletal muscle

Variations in RNA or Protein Expression Pattern Between Patient and Control Samples
1. Primary genetic abnormality causes a specific change in amount, size or charge of mutant species
2. Primary genetic abnormality causes a specific pattern of changes in other OXPHOS or mitochondrial stress response species
3. Downstream (pathological) changes that may be common to other causes of muscle disease
4. Changes due to differences in level of exercise or training
5. Changes due to different diet
6. Changes due to other environmental effects such as climate, altitude, smoking, etc
7. Changes due to inter-individual differences in genetic background
8. Changes due to varying purity of mitochondrial protein fractions or to quality of RNA preparations

for use in OXPHOS diagnosis by comparing the protein profiles of patient tissues or cell lines with normal controls.[30,31] Until recently though, 2D-PAGE of mitochondrial extracts has been very problematic because many of these hydrophobic membrane proteins tended to precipitate in the first dimension iso-electric focusing gel. Recent studies have been more successful in generating reproducible 2D-PAGE profiles of mitochondrial extracts, sometimes by using BN-PAGE in the first dimension instead of isolectric focusing.[32]

Three major problems have to be resolved before mitochondrial proteome analysis could be used as a front line diagnostic tool. The first relates to sensitivity and reproducibility. It will be critical to ensure that different mitochondrial preparations from the same patient tissue or cell line analysed on different occasions give an essentially identical profile and allow detection of even low abundance proteins. Variation in the purity of the mitochondrial extracts would introduce a variable number of nonmitochondrial protein spots that could befuddle interpretation. One way to ensure greater reproducibility and sensitivity may be to prefractionate mitochondria prior to 2D-PAGE analysis, for example by density centrifugation[33] or affinity chromatography.[34] Sucrose gradient fractionation, 1D-PAGE and mass spectrometry were recently used to identify 615 distinct proteins in the human heart mitochondrial proteome, including >90% of known OXPHOS subunits,[35] but the reproducibility of this approach was not reported. A second problem with diagnostic mitochondrial proteome analysis will be interpretation. For example, the mitochondrial proteome of skeletal muscle from a patient with a mitochondrial myopathy is likely to show a large number of differences from that of healthy muscle. Only some of these will relate to the primary genetic abnormality, while others will be caused by inter-individual (genetic) variation, environmental factors such as diet and exercise levels, or be secondary pathological effects that may be common to other myopathies (Table 2). The third problem with proteome analysis (and also immunochemical studies) is that some genetic mutations may not have sufficient effect on the amount, size or charge of an individual protein to be detectable.

Deficiency of nonprotein constituents of mitochondria, especially lipids, is being recognised as an increasingly prevalent class of OXPHOS disorders. A single case of coenzyme Q deficiency was recognised in 1989[36] but in the last five years this disorder has been recognised much more frequently.[37,38] The genetic basis of Barth syndrome was identified in 1996,[39] but it has only recently been recognised to represent a second class of mitochondrial lipid disorder, specifically affecting the synthesis or turnover of cardiolipin.[40,41] It is likely that other disorders affecting nonprotein mitochondrial constituents may be found in the future. Quantitative biochemical techniques such as HPLC and mass spectrometry are thus likely to become more important in investigation of OXPHOS disorders in the next few years.

OXPHOS Genetics

OXPHOS disorders can potentially be caused by mutations in 37 mtDNA-encoded genes and perhaps several hundred different nuclear genes. This genetic complexity is a clear impediment to rapid genetic diagnosis. Screening for a limited number of "common" mtDNA mutations and mtDNA rearrangements is widely available. Improvements in fluorescent sequencing technology mean that mtDNA sequencing is now relatively routine in a limited number of centres. However, this approach is still quite expensive, time-consuming and can potentially miss mutations present at low levels of heteroplasmy. A range of methods of screening for rare and novel mtDNA point mutations have thus been used such as SSCP,[42] DGGE[43] and DHPLC.[44] Other high-throughput methods of mutation screening are under investigation, including high density micro-arrays and mass spectrometric detection methods[45] that may be well suited to mutation analysis of mtDNA and nuclear OXPHOS genes.

A mtDNA sequencing chip was reported seven years ago, in which a series of up to 135,000 different 15- to 25-nucleotide oligomers were arrayed representing every possible sequence variation from a reference sequence.[46] Despite the potential of this approach there appears to have been no further development, apparently due to unclear sequence "footprints" or "bubbles" around any sequence change (that required conventional sequencing of substantial amounts of the genome) and the perceived lack of a suitable market. The highly polymorphic nature of mtDNA creates extra problems for all mutation detection strategies[12] and in the next few years direct mtDNA sequencing and DHPLC seem likely to remain the most efficient approaches for patients with a high suspicion of a mtDNA mutation. Southern blotting is the traditional method of testing for mtDNA rearrangements but many centres now use long-range PCR analysis as a faster and more sensitive method. Several recent reports have begun using RealTime PCR for this purpose and this robust quantitative method is likely to become standard for mtDNA deletion testing and should also prove suitable for quantitating mtDNA depletion.[47,48]

Over 20 nuclear genes have now been shown to cause OXPHOS disorders[49] and many more remain to be discovered. Deciding which gene(s) to test in an individual patient is difficult since most genes do not cause a unique clinical phenotype. For example, complex I-deficient Leigh disease can be caused by mutations in a range of nuclear and mtDNA-encoded complex I subunit genes including NDUFV1, NDUFS1, NDUFS4, NDUFS7, NDUFS8, ND6, ND5 and ND3.[25,50-53] Targeted analysis of such genes one at a time is possible by either sequencing or mutation screening approaches such as DHPLC, but patient demand is likely to exceed the capacity for service provision. A more efficient approach to screen for OXPHOS disease mutations would be to create an OXPHOS mutation chip containing oligonucleotides representing all known mutations in nuclear and mtDNA OXPHOS genes. There are a number of problems in making a suitable chip. It would need regular updating as new genes and mutations were identified, and would clearly not detect novel "family-specific" mutations. However, there are a limited number of common or recurrent mtDNA mutations reported in multiple, apparently unrelated families with OXPHOS disorders such as MELAS, MERRF, NARP and LHON that appear to account for the majority of such cases. Similarly, several nuclear OXPHOS genes, including SURF1, SCO2 and LRPPRC have common mutations present in at least one allele of many patients.[54-56] A number of practical problems have hindered the implementation of mutation chips into diagnostic practice, in particular issues about intellectual property, as described elsewhere.[57] An OXPHOS mutation chip would not be a diagnostic panacea, but is technically feasible now. It could provide a tool suitable for use in routine molecular genetics laboratories that would allow rapid diagnosis of a substantial proportion (probably more than a third) of OXPHOS patients without the need for an invasive biopsy or specialist testing.

Microarrays for studying gene expression offer another potential approach for screening for dysfunction of nuclear or mtDNA genes. Two groups have reported preliminary results using cDNA microarrays to study expression of approximately 600 genes involved in OXPHOS or related pathways.[58,59] Analysis of mRNA from cell lines of patients with known mutations in OXPHOS genes showed that one patient with a homozygous nonsense mutation in the SURF1

gene showed the expected marked reduction of the SURF1 transcript. Not surprisingly, other patients with missense mutations in complex I subunit genes did not show a dramatic change in the amount of the mutant transcript.[59] In both studies, individual patients tended to show up- or down-regulation of a large number of nuclear- and mtDNA-encoded transcripts.[58,59] Some transcriptional responses were common to multiple patients (e.g., upregulation of metallothioneins), while others varied between patients, and further work needs to be done on characterising transcriptional clusters. As with proteome analysis, there are potential problems with reproducibility and in distinguishing primary responses to the genetic abnormality from inter-individual, environmental and nonspecific pathological changes, particularly if patient tissues rather than cell lines are studied (Table 2). For example, microarray analysis of rat skeletal muscle showed that caloric restriction and high fat diets led to substantial changes in expression levels of a range of genes involved in OXPHOS, free radical scavenging and stress responses.[60,61] None the less, the preliminary results with patient cell lines clearly warrant more detailed studies in larger numbers of patient samples to determine whether expression analysis can become a primary approach for diagnosis of OXPHOS defects in general, and of specific causative genes. RNA expression analysis is potentially a more reliable approach than proteomic analysis, since it samples the same set of RNA species in each sample and is not reliant on fractionating samples in a highly reproducible manner.

Prenatal Diagnosis & Prevention

Options for prenatal diagnosis and prevention of OXPHOS disorders have been extremely limited until recently but have improved dramatically in the last five years and will continue to do so, as reviewed in detail elsewhere[62-64] and discussed in Chapter 11. Families with OXPHOS disorders can be thought of in the following three separate groups:

1. Families with an OXPHOS enzyme defect in whom no pathogenic mutation has been identified. Prenatal diagnosis of OXPHOS defects can sometimes be performed in these families by enzyme analysis of Chorionic Villus Sampling (CVS) cells or amniocytes.[62,63,65,66] Such studies are available in only a handful of centres internationally and have a number of limitations. They can usually be offered only if the defect is thought to be both systemic (i.e., expressed in all tissues) and nuclear-encoded. Even then it is often not possible to provide definitive exclusion of an affected pregnancy.[65,67] As more nuclear gene defects are identified, mutation analysis will replace the less robust enzyme analysis in an increasing number of families.
2. Families with a known pathogenic mutation in a nuclear-encoded gene. Identification of mutations in nuclear OXPHOS genes means that an increasing number of families can be offered reliable prenatal diagnosis by mutation analysis of CVS or amniocytes.[67,68] It is also becoming increasingly practical to offer preimplantation genetic diagnosis for (nuclear) single gene disorders.[69] Availability of preimplantation genetic diagnosis should increase in coming years, allowing families the option of avoiding the risk of pregnancy termination.
3. Families with a known pathogenic mutation in a mtDNA-encoded gene. Prenatal diagnosis of mtDNA mutations is problematic due to the complexity of mtDNA genetics. It is technically straightforward to measure the mutant load of a mtDNA mutation in a CVS or amniocyte sample. However, concerns about whether such measurements could be used to predict outcome have restricted the use of mtDNA analysis of CVS and amniocytes.[62] A consensus statement recently concluded that this approach could be recommended for women with low mutant loads of the T8993G mutation, which shows an especially strong correlation between mutant load and phenotype.[70] As we learn more about genotype/phenotype correlation for other mtDNA mutations, it is likely that the range of mtDNA mutations that will be tested for in CVS or amniocytes will increase substantially. We are also now recognising that a substantial proportion, perhaps a quarter, of patients with mtDNA point mutations appear to have de novo or sporadic mutations.[53,71] The low recurrence risk in such families means that CVS or amniocyte mtDNA analysis may be suitable reproductive options.

Preimplantation genetic diagnosis is a logical and promising approach for diagnosing the mtDNA mutant load of embryos and is likely to be used increasingly for preventing transmission of pathogenic mtDNA mutations. Indeed, the high copy number of mtDNA (>10^4 copies per cell in an 8-cell embryo) means that analysis of mtDNA mutations in embryos should be much more robust than analysis of nuclear gene defects. Unless the number of oocytes retrieved and the pregnancy rate per cycle can be increased substantially though, it will remain suitable only for women with low mtDNA mutant loads.[62]

The inherent limitations of CVS/amniocyte analysis and preimplantation genetic diagnosis make it imperative that other approaches are developed for preventing transmission of mtDNA mutations. Two approaches that may prove useful in the future are nuclear transfer (i.e., transferring the nucleus of the mother's unfertilized oocyte into an enucleated donor oocyte, followed by IVF with the partner's sperm) and cytoplasmic transfer (i.e., diluting any mutant mtDNA in an oocyte to a level below the pathogenic threshold by transfer of healthy donor mitochondria).[62] Both these approaches require substantially more research in animal models before it is clear if they will be safe and effective for clinical use.

Conclusions

In coming years an increasing proportion of patients with OXPHOS disorders will be diagnosed by genomic analysis of blood or urine, using micro-arrays or some other form of high-throughput mutation detection method. There will, however, be an ongoing need for tissue biopsies and functional analyses for the foreseeable future. Even when all the nuclear genes causing OXPHOS defects are known, some patients will have disease caused by mtDNA mutations that are present in significant amounts only in muscle, brain or other organs. Other patients may test negative for all known nuclear and mtDNA OXPHOS mutations but still have a high degree of clinical suspicion that demands further functional studies. Diagnostic methods such as immunocapture enzyme assays, functional micro-assays using fluorescent probes, and antibody micro-arrays seem likely to become increasingly important approaches. RNA expression analysis and proteomics are also very promising approaches but require substantially more research and validation before they can be widely used.

Finally, one aspect of OXPHOS diagnosis that demands more serious attention in coming years is how we interpret and integrate different modes of investigation in order to establish the certainty of diagnosis. This requires a determined effort by all investigators to clarify whether all patients with data outside a "normal range" for various investigations have truly unambiguous OXPHOS defects. A number of recent studies have addressed this issue[72-74] and will hopefully prompt greater levels of consensus about diagnostic criteria.

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