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Mitochondrial disease associated with complex I (NADH-CoQ oxidoreductase) deficiency

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Abstract:

Mitochondrial diseases due to a reduced capacity for oxidative phosphorylation were first identified more than 20 years ago, and their incidence is now recognized to be quite significant. In a large proportion of cases the problem can be traced to a complex I (NADH-CoQ oxidoreductase) deficiency (Phenotype MIM #252010). Because the complex consists of 44 subunits, there are many potential targets for pathogenic mutations, both on the nuclear and mitochondrial genomes. Surprisingly, however, almost half of the complex I deficiencies are due to defects in as yet unidentified genes that encode proteins other than the structural proteins of the complex. This review attempts to summarize what we know about the molecular basis of complex I deficiencies: mutations in the known structural genes, and mutations in an increasing number of genes encoding “assembly factors”, that is, proteins required for the biogenesis of a functional complex I that are not found in the final complex I. More such genes must be identified before definitive genetic counselling can be applied in all cases of affected families.

Introduction:

Until about 25 years ago the words ‘mitochondria’ and ‘mitochondrial diseases’ probably did not exist in the vocabulary of the majority of physicians. In 1988 several pioneering papers opened a new era of biomedical research resulting in an avalanche of publications with no end in sight (Holt et al 1988a, b; Wallace et al 1988a, b). The most novel and unexpected finding reported in these

publications was that severe and debilitating human pathologies could result from mutations in the mitochondrial DNA. The Wallace laboratory identified a mutation in the in the ND4 gene on mtDNA as the cause of Leber's hereditary optic neuropathy (LHON; Phenotype MIM #535000), while the group in London described deletions in mtDNA in patients with myopathies.
It had been known for some time that a mammalian cell contains hundreds of mitochondria and ~1000 mtDNAs (ex- act numbers depend on the tissue). Therefore, expressions of a distinct phenotype would only be expected if all or a large fraction of the mitochondrial genomes (our "25th chromo- some") carried the deleterious mutation. The technical achievement in these papers was the experimental proof for such a condition. Normally the population of mtDNAs in an individual is homoplasmic, i.e., consists of a collection of mtDNAs with identical sequences. Methods of the day did not detect a small fraction of random polymorphisms distrib- uted throughout the mitochondrial genome, probably generat- ed during somatic cell proliferation and differentiation. The patients with myopathies had mitochondria with a mixture of mtDNAs (heteroplasmy); a fraction had the wild type sequence, while the other large fraction had a substantial dele- tion. In the case of the LHON patients the even more surprising finding was that their mtDNA polulation was homoplasmic, with all mtDNAs having the pathological point mutation in the ND4 gene.

Two general conclusions were quickly accepted. When the patient is homoplasmic for the mutation, the mutation must be a "mild" mutation (missense mutation) resulting in a partially defective protein/function, since the patient is affected but still alive. In the case of a significant deletion in the mtDNA, a patient will be alive only in the heteroplasmic state. Because of the dense packing of genes on mtDNA any sizeable dele- tion will eliminate not only a protein coding sequence, but also one or more tRNA sequences, and mitochondrial translation would stop in the homoplasmic organelle. What is affected in heteroplasmy is not the nature of a single protein, but the total capacity of the electron transport chain required for oxidative phosphorylation. In short and simplified form, patients with mitochondrial diseases suffer from an energy insufficiency ("weak batteries") that can be caused by a single partially defective protein in the OXPHOS machinery, or by an overall partial

depletion of one or more complexes of this machinery. Subsequently patients were characterized where the efficiency of mitochondrial translation was reduced by point mutations in either the rRNA genes or one of the tRNA genes (Li et al 2004; Zheng et al 2012); Guan 2011; Zheng et al 2012).

Possible mechanisms responsible for the development of heteroplasmy or homoplasmy with close to 100 % mutated mtDNAs will be discussed briefly below. However, it became clear that heteroplasmy (the fraction of mutated genomes) can be variable, and as a first approximation the severity of the symptoms is correlated with the mutation load. Mitochondrial DNA is maternally inherited, and heteroplasmy typically varies from one generation to the next, and even within the siblings of a single generation. The mechanism and rules for this non-mendelian mode of inheritance are still not complete- ly understood, and as a consequence genetic counseling of such families constitutes a serious challenge. A stochastic mechanism for quality control (“bottleneck”) of mtDNA has been proposed to be in operation during oogenesis or very early embryogenesis (Shoubridge and Wai 2008; Wai et al 2010).

The sequence of human mtDNA was first established in 1981, and all the functional sequences were identified by 1986. They include sequences for the two ribosomal RNAs, 22 tRNAs, and 13 structural proteins found in the complexes of the OXPHOS system (seven polypeptides of complex I, one polypeptide/protein of complex III, three polypeptides of complex IV, and two polypeptides of complex V). One could therefore expect mutations in the rRNAs or tRNAs affecting the mitochondrial translation machinery, and therefore the abundance of complexes I, III, IV, and V. Alternatively, a mutation in a specific protein coding sequence would be expected to affect the activity of a single complex. Naively one might conclude that either way the result is a diminished energy supply (ATP production). A “dead battery” would most likely result in embryonic lethality, a very weak battery might permit a live birth followed by severe impairment of growth and other functions and low life expectancy, while less severe defects may lead to tissue-specific pathologies (e.g., LHON) with a delayed onset. A large literature has accumulated in the past 25 years

to elaborate on these aspects of mitochondrial disease(s). One should also consider that an impairment of electron transport at complex I or further downstream might cause single electrons to escape to molecular oxygen and lead to the formation of reactive oxygen species (ROS). These reactive species have a multitude of targets (lipids, proteins, DNA), and an accumulation of dam- age (chemical modifications in one or a combination of these) could potentially also be pathogenic.

It became immediately obvious that a partially defective OXPHOS machinery (the electron transport chain and complex V, the ATP synthase) could also result from nuclear mutations, since the entire system is made up of 80 or more proteins, most of which are encoded by nuclear genes. It did not take long to find patients suffering from nuclear mutations causing OXPHOS deficiencies and symptoms typical of energy insufficiency, particularly affecting the nervous system and muscle. The genetic basis of mitochondrial diseases was thus broadened to include nuclear genes and their gene products contributing to the capacity for oxidative phosphorylation. As more attention was focused on patients with the typical symptoms of a mitochondrial disease (neuropathy, myopathy, cardiomyopathy, lactic acidosis, etc.), even more genes were implicated in the origin of these diseases. The discovery and characterization of these genes and their protein functions goes hand in hand with our increasing understanding of the many proteins/activities required for the assembly of a functional electron transport chain. Furthermore, mitochondria have long been recognized to be the “hub of metabolism”, and mitochondrial dysfunctions can become linked to metabolic diseases (hypertension, obesity, diabetes), neurodegenerative diseases (e.g., Parkinson’s disease, hearing loss, blind- ness, and more) and even aging (Wallace et al 2010). As a result, the definition of a “mitochondrial disease” has become very fluid. Partially defective mitochondria have become implicated in many diseases, caused either directly as a result of an energy deficiency, or more indirectly because of the intricate integration of mitochondria into metabolism, signal transduction, apoptosis, maintenance and quality control, and even gene expression. The relative importance of these functions is also highly organ and tissue specific.

The following discussion will concentrate on the composition, structure and function of complex I of the electron transport chain (NADH-ubiquinone oxidoreductase), and the diseases associated with deficiencies of complex I activity. The term “isolated complex I deficiency” has been introduced when a biochemical analysis of the electron transport chain and complex V reveals normal levels of activity for all complexes except complex I. However, the picture may not be so simple, because complex I assembly and steady state levels may ultimately also influence the assembly of the respirasome (supercomplexes) and thus affect the levels of other complexes (III and IV) as well (Moreno-Lastres et al 2012).

Complex I: composition, structure and function

The recycling of NADH to NAD+ is absolutely essential to sustain the operation of the Krebs cycle (and glycolysis) under aerobic conditions. The mammalian complex I (NADH-CoQ oxidoreductase) was first characterized by the classical studies of the Hatefi laboratory as a multisubunit complex responsible for the oxidation of NADH, with co-enzyme Q (ubiquinone) as the ultimate electron acceptor (Hatefi 1985; Hatefi et al 1979). It was isolated from beef heart and shown to contain a large (>>25) number of subunits, although its final composition was not established until 2003 by the laboratory of Walker in Cambridge (Carroll et al 2003). The number of subunits in the mammalian complex I is now accepted to be 44. Fourteen of these subunits have homologues in the prokaryotic complex I, and of these 14 subunits seven are encoded by nuclear genes (NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFV1, and NDUFV2) and seven are encoded by the mtDNA (ND1-6 and ND4L). The 14 subunits constitute the “catalytic core”, and in prokaryotes they alone are fully capable of carrying out the function of complex I: to oxidize NADH, transfer electrons to CoQ, and to pump pro- tons across a membrane. This leaves 30 subunits in search of a function in mammalian mitochondria; they have been variously referred to as “supernumerary”, “accessory” or “ancillary” subunits, and their role in the assembly, stabilization, and activity of the complex is still under active investigation (see below).

Numerous studies have shown that complex I has more than 40 subunits in mitochondria of all eukaryotes, including fungi and plants (Scheffler 2008). However, it is completely absent in the yeast Saccharomyces cerevisiae, which for de- cades has been one of the favored model systems for the biochemical and genetic studies of oxidative phosphorylation. In this organism the recycling of NADH to NAD is accomplished by an enzyme consisting of a single polypolypeptide/ protein (Ndi1p) encoded by the NDI1 gene. Although this protein is imported into the mitochondria and associated with the inner membrane, no protons are pumped out of the matrix, and therefore baker's yeast lacks one of the coupling sites for OXPHOS. As described in more detail below, the yeast Ndi1p protein can be imported into mammalian mitochondria lacking complex I and restore respiration in such a mammalian mutant cell.

The elucidation of the structure of complex I became a great challenge, and a complete, high resolution structure for a eukaryotic complex has not yet been achieved. The complex is firmly embedded in the inner mitochondrial membrane, making it technically difficult to purify in an intact form and to obtain suitable crystals for x-ray crystallography. Biochemical approaches succeeded in separating the complex into a peripheral membrane subcomplex and an integral membrane subcomplex, and some further subfractionations have been achieved. Relatively low resolution cryo-electron microscopy revealed that the complex had the shape of an "L" or a "boot" where the long arm of the L represents the integral membrane subcomplex with a large number of alpha-helical transmembrane segments of the various subunits, while the short arm represents the peripheral subcomplex extending into the matrix. More detailed biochemical fractionations and in- creased resolution by cryo electron microscopy has led to the current schematic structure (Fig. 1). Figure 1 shows the localization of the mtDNA-encoded proteins in the β,γ subdomains and the iron-sulfur centers in the λ subdomain.

In parallel, studies on the prokaryotic complex have yielded a high resolution x-ray structure of the complex from Thermus thermophilus (Baradaran et al 2013). This structure reveals the organization of the 14 polypeptide making up

the bioenergetically functional complex I of prokaryotes and the most likely structure of the core of the eukaryotic complex. The peripheral subcomplex includes the active site for binding the substrate NADH, the immediate electron acceptor FMN+, and eight non-heme iron sulfur centers which form an electron transport chain from FMN+ to the Q-binding site. The integral membrane subcomplex contains the seven hydrophobic poly- peptides which are encoded by the mtDNA in eukaryotes. They must therefore be responsible for the proton pumping. Although the x-ray structure suggested a mechanism that couples electron transport within one subdomain to conformational changes in the other, a more detailed description of the coupling mechanism is still incomplete. In the mammalian complex I the 30 accessory subunits have to be placed around this core structure. By a variety of approaches most of these have by now been localized to subdomains of the complex. Although they have been referred to as accessory or supernumerary, studies from the Scheffler laboratory (Scheffler et al 2004) first demonstrated that at least two of these, MWFE and ESSS (NDUFA1 and NDUFB11), are absolutely essential for the assembly of an active complex I (see below). Detailed descriptions of the structure and function of complex I can be found in numerous recent reviews (Carroll et al 2003; Hirst 2009; Hirst 2011; Angerer et al 2011; Hoefs et al 2012).

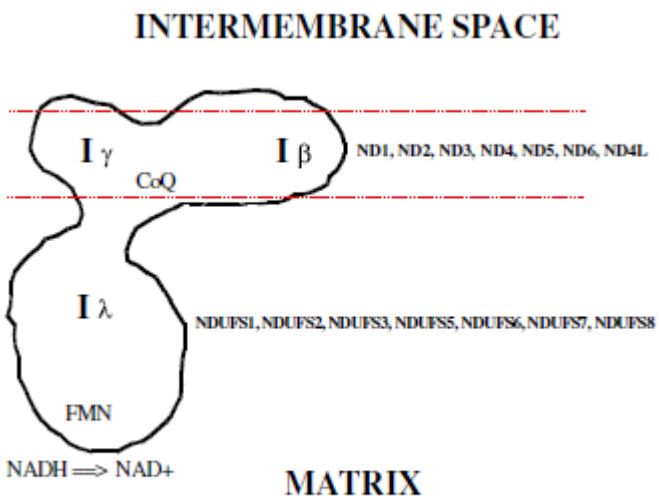

Fig. 1 Localization of the mtDNA-encoded proteins in the β,γ subdomains and the iron-sulfur centers in the λ subdomain

The subunit composition of the complex I defined by the Walker laboratory (Carroll et al 2003) was accompanied by a determination of the amino acid sequence of each polypeptide/protein by mass spectroscopy, and thus nucleotide sequences of the respective coding sequences. In combi- nation with the human genome sequence the position of the genes for each of the subunits could be mapped on human chromosomes, confirming also a number of independent genetic mapping data from previous studies. The stage was set for the molecular genetic investigation of human patients with isolated complex I deficiencies.

Complex I-deficient mammalian cells in tissue culture

The first mutant mammalian cell line with a complete deficiency in complex I activity was described in 1976 by the Scheffler laboratory (DeFrancesco et al 1976). Its isolation was serendipitous, but its characterization lead to the development of an enrichment scheme which subsequently yielded additional respiration-deficient Chinese hamster cells in culture, all characterized to have almost no capacity for respiration and oxidative phosphorylation (Breen and Scheffler 1979; Ditta et al 1976). The ability of mammalian fibroblasts to grow in tissue culture media using glycolysis exclusively for their energy supply was quite unexpected at the time. The mutant cell lines were grouped into seven complementation groups by pairwise fusion and complementation tests (Soderberg et al 1979). One mutant was defective in complex II, subsequently characterized to have a chain terminating mutation in the SDHC gene (Soderberg et al 1977; Oostveen et al 1995). A second single mutant was completely deficient in mitochondrial protein synthesis (Ditta et al 1977), formally resembling the rho− (ρ−) yeast mutants characterized before. The mutated gene in this mutant has only recently been identified to be in the mRPS6 subunit of the small mitochondrial ribosome (paper submitted). In the absence of this very small polypeptide/protein hydrodynamically normal ribosomal subcomplexes are assembled, but the initiation of translation is apparently arrested (Burnett and Scheffler 1981).

The vast majority of the mutant cell lines isolated by our scheme were lacking complex I activity, and they could be sorted into five complementation groups. All mutations are on nuclear genes. Based on somatic cell genetic experiments, three complementation groups were predicted to represent mutations in X-linked genes (Day and Scheffler 1982). They were isolated in multiple and independent experiments, as expected for genes present in a single copy in pseudo- diploid Chinese hamster cells derived from a male animal. Complementation was possible by a normal X chromosome in somatic cell hybrids. Finally, some years later, two of the genes were identified on the X chromosome of mammals: NDUFA1 and NDUFB11. The corresponding cDNAs were produced by molecular cloning and shown to be able to complement the mutations in transfected cells (see below). The amino acid sequences were highly conserved in mammals, but it is noteworthy that in the complementation tests severe species-specific restrictions were observed. These nuclear-encoded polypeptides presumably interact with poly- peptides encoded by mtDNA, and these genomes have co- evolved such that the human MWFE protein cannot be substituted for the corresponding hamster protein. A small number of amino acid changes can restore compatibility (see (Scheffler et al 2004) for a summary and review).

A third mutation was also postulated on the X chromo- some, but when all 37 human nuclear-encoded subunits were identified and their genes mapped on the human genome, there was no additional X-linked gene for a known structural protein of the complex I, and the identity of this gene and its function in complex I assembly and/or activity is currently under investigation (see below).

The biochemical characterization of these mutant cell lines was relatively straightforward. Measurements of respiration (oxygen consumption) with an oxygen electrode using a va- riety of substrates were initially most revealing and definitive. When blue native gel electrophoresis was introduced to the field (Schagger 1995) the absence of intact complex I could be demonstrated directly after fractionations on gels and detec- tion with complex I-specific

antibodies.

To survive, these mutant cell lines require an abundant supply of glucose in the medium. Most of that glucose is converted to lactate. When the glucose is exhausted, the cells die. Galactose substituted for glucose in the medium does not sustain these cells, because the Leloir pathway is too slow to convert this hexose into a precursor for the required high rate of glycolysis.

In the absence of complex I it appears that the NADH levels in the mitochondria become highly elevated, leading to[3] a pronounced feedback inhibition of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The Krebs cycle is completely inhibited, as measured by [14C]CO_2 evolution from labeled precursors. The cells become auxotrophs for CO_2/HCO^- in the tissue culture medium, and they require the presence of the non-essential amino acids aspartate and asparagine in the medium, because the conversion of glutamate/glutamine to these four-carbon amino acids via the Krebs cycle is blocked. It is worth emphasizing these biochemical characteristics, which became crucial in the development of an enrichment scheme for the selection of mammalian mutant cell lines of this phenotype, and they are also critical for genetic selections and complementation assays in efforts to identify other unknown genes. These cells in culture represent the extreme case of an isolated complex I deficiency and essentially zero activity. Their behavior should be contrasted with similar studies employing human cell lines from patients with partial, or even severe complex I deficiencies. Such patients must have some significant residual activity to be alive, at least at birth, to present themselves to physicians.

The characterization of two specific mutants in our collection is relatively advanced. The CCL16 B2 mutant cells were shown to be mutated in the NDUFA1 gene encoding the MWFE subunit of complex I. Several independent mutations were isolated in this gene, causing this polypeptide/protein to be truncated and undetectable in the cells. The polypeptide/ protein belongs to the group of supernumerary/accessory sub- units, but it is definitely essential. The protein is 70 amino acids in length and forms a single transmembrane

helix in the α subcomplex, with the N-terminal facing the matrix (Yadava and Scheffler 2004). Most of its domain (~45 aa) is in the intermembrane space, where it serves as a scaffold or anchor in the assembly. In the absence of this subunit no complex I is assembled. When the protein was provided from an inducible complementing expression vector, the kinetics of assembly of an active complex I could be followed, and it was perhaps surprising that it required 48–72 h following the addition of the inducer before full activity was restored (Yadava et al 2004).

Respiration and oxidative phosphorylation in the CCL16- B2 mutant cells can be restored completely by transfection with the single yeast gene NDI1 and the targeting of the Ndi1p to mammalian mitochondria (Seo et al 1998). The yeast protein functions as an NADH-CoQ oxidoreductase without pumping protons out of the matrix. The recycling of NADH to NAD+ releases the feedback inhibition of the TCA cycle. It is insensitive to rotenone, which distinguishes its activity from any endogenous complex I activity. An initial suggestion was to use this gene for gene therapy (Yagi et al 2006). This has been accomplished in animal studies (e.g., (Marella et al 2008; Marella et al 2010; Sanz et al 2010)) but not attempted in humans for a variety of complex reasons.

A second X-linked mutation was identified in the NDUFB11 gene encoding the ESSS subunit, a relatively small integral membrane protein (122 amino acids) found in the β subcomplex. It also belongs to the ‘supernumerary’ group of subunits, but again proved to be absolutely essential for the assembly of an active complex I (Potluri et al 2004).

Both subunits (MWFE and ESSS) have been subjects of investigations of the potential role of phosphorylation in the control of complex I activity (Yadava et al 2008). In fibro- blasts no support for such a control mechanism was found, but tissue specific mechanisms cannot be excluded (Kim et al 2012). In general, the question whether protein phosphorylation of complex I subunits plays a major role in the control of complex I activity in vivo is still an open question.

The hamster cell mutant with a null NDUFA1/MWFE mutation has proved to be useful in characterizing a mutation in the same gene in a patient with progressive mitochondrial complex I-specific neurodegenerative disease (Potluri et al 2009). A missense mutation segregating with the disease converted a highly conserved glycine in the MWFE polypeptide/protein at position 32 to arginine. The substitution was extremely non-conservative, but it remained to be proved that it was the pathological mutation. Therefore, the same mutation was made in a hamster cDNA, and the mutated MWFE protein was expressed in the NDUFA1 null hamster cell line. A significant decrease in the complex I assembly and activity was observed compared with wild type MWFE cDNA. However, to account for the relatively large deficiency measured in biopsied material from the patient it was speculated that the novel G32R mutation in NDUFA1 may be causing complex I deficiency either by itself or in synergy with additional mtDNA variants.

In summary: five Chinese hamster cell lines with a complete complex I deficiency are available as potential model systems (Scheffler et al 2004). The defective genes in two of these have been identified, while the others still await identification. The third X-linked gene is of particular interest, because so far it has not been recognized in human patients with isolated complex I deficiencies. It is to be noted, however, that in the majority of such patients the defective gene has not been identified, and all known nuclear and mitochondrial structural genes were found to be normal (see below).

When human patients were characterized, it became possible to establish cell lines in tissue culture (fibroblasts, lymphocytes from biopsies) as mutant cell lines with OXPHOS defects. They also represent useful model systems for further biochemical studies, but investigators should be aware that the defect is partial and the phenotype may be less clear cut, for example, for use under selective conditions in complementation studies and selection of somatic cell hybrids. Further- more, it has been found that in a fraction of patients the activity in fibroblasts may be normal, while a deficiency can be recognized in muscle biopsies (see below).

Complex I deficiency and mitochondrial disease

The path-breaking publication by the Wallace laboratory describing the first “mitochondrial disease” identified a point mutation in the ND4 gene on mtDNA encoding a core subunit of complex I (Wallace et al 1988a, b). In the following decades the number of well characterized patients with complex I deficiencies has expanded into the hundreds. With 44 sub- units, and therefore 44 structural genes, the number of targets for genetic defects causing complex I deficiencies is very large. An immediate distinction can be made between mutations in mtDNA, subject to the “rules” of non-mendelian inheritance, and mutations in nuclear genes.

For mutations in mtDNA the only certain prediction is transmission through the matrilineal lineage. A mother heteroplasmic for a mtDNA mutation may not show any symptoms, but her male or female offspring may exhibit symptoms if the fraction of mutated mtDNA exceeds a certain threshold. How heteroplasmy arises in earlier generations of a normal lineage may be clouded in history. For isolated com- plex I deficiencies one expects to find missense or nonsense mutations in one of the seven genes, ND1-6 and ND4L. Missense mutations may have a more or less severe effect, and may be found in homoplasmic individuals, i.e., individuals in which close to 100 % of the mtDNA carries the mutation. Nonsense mutations and frame-shift mutations in such genes would be expected to be found only in heteroplasmic cells containing a fraction of wild type mtDNA. How such genotypes segregate is difficult to study in humans with a limited number of offspring and no controlled matings, but some progress has been made with mouse models. A considerable effort and technically challenging approaches were necessary to create heteroplasmic mice (Battersby and Shoubridge 2001; Fan et al 2008; Sharpley et al 2012; Shoubridge and Wai 2007; Sligh et al 2000; Cao et al 2009; Inoue et al 2002). The animal model could address two fundamentally important questions: 1) what is the mechanism for the “bottleneck” that must be invoked to describe the observed variation in heteroplasmy in a number of siblings from a single heteroplasmic mother? 2) Is there further variation in heteroplasmy in different

somatic tissues originating from an early embryonic cell? There are still conflicting hypotheses about the bottleneck. At one extreme the postulate is a very significant reduction in mtDNA copy number in embryonic stem cells and in the germ cell lineage. Direct studies refute this idea and point to the selection of a small subset of mtDNAs for replication, or the transmission of a small subset of mtDNAs to the progeny (Wai et al 2010; Cao et al 2009). There is evidence for a further segregation and variation in heteroplasmy in somatic tissues. The selective pressure in early embryogenesis may originate from differences in OXPHOS efficiency between mitochondria, or in small selective advantages in mtDNA replication. For example, it has been argued that mtDNA with a substantial deletion would be more rapidly replicated and thus have an advantage over normal mtDNA.

The transmission of mutations in nuclear DNA affecting complex I activity is subject to the laws of Mendelian genetics. A priori one can make several generalizations. Mutations in X-linked genes would be expressed in males, and when the mutation is severe (deletion, frameshift, highly non- conservative substitution) the resulting OXPHOS deficiency is likely to cause embryonic lethality. Only milder forms of mutations can be expected to be detected in live-born males. Although X-linked structural genes for complex I have been identified (NDUFA1 and NDUFB11), the number of male patients with mutations in these two genes is very limited (Potluri et al 2009; Fernandez-Moreira et al 2007). As de- scribed below, there is an increasing number of genes encoding “assembly factors” for complex I.

Autosomal mutations would be expected to be detected in patients only in homozygous or compound homozygous individuals. Precise numbers for allele frequencies in different populations are not (yet) available to predict the incidence of homozygotes, but the number of such patients has swelled significantly in recent decades due to increased alertness of physicians and recognition of the symptoms, improved and reliable biochemical protocols to measure the individual components of the OXPHOS system, and rapid and cheaper sequencing of relevant DNA (or exome) sequences. It has been estimated that mitochondrial disorders occur with an incidence of one in

approximately 5000 births (Hoefs et al 2012).

Major laboratories in the world publish regular reviews on the subject which should be consulted for access to the primary literature, and detailed descriptions of the symptoms, the biochemical and genetic characterizations, and their interpretations (Hoefs et al 2012; Nouws et al 2012; Swalwell et al 2011; Mimaki et al 2012; Pagniez-Mammeri et al 2012a, b). Each of these laboratories has reported on a cohort of 100+ patients, allowing some statistically meaningful conclusions and generalizations. Complex I deficiency is the most frequently observed cause of OXPHOS disorders, varying widely in the severity of symptoms and clinical presentations. Typical pathologies include cardiomyopathy, pure myopathy, leukoencephalopathy, hepatopathy with tubulopathy, and the most common phenotype is Leigh syndrome or Leigh-like syndrome (Phenotype MIM #256000). A telling characteristic is pronounced lactic acidosis that is often fatal. Clinical phe- notypes can have an early or a delayed onset, and an early onset is frequently followed by a rapid progression and death within a few years after birth.

The prevalence of complex I defects may at first sight be attributed to the large number of structural genes (44); how- ever, the most challenging finding is that among hundreds of patients investigated by DNA sequencing in the various lab- oratories, pathogenic mutations in mitochondrial and nuclear structural genes have been detected in less than half of the patients (Hoefs et al 2012; Pagniez-Mammeri et al 2012a, b). Thus, genetic counseling based on genomic data from pediatric patients and their parents is still severely limited in a majority of affected families.

Complex I deficiency caused by mtDNA mutations

A recent summary and review of mtDNA mutations responsible for complex I deficiency has been published (Swalwell et al 2011). The pediatric patient population now includes cases with mutations in each of the seven mtDNA genes encoding complex I subunits (ND1-6, 4 L). More intense screening and sequencing has revealed that in contrast to earlier estimates their prevalence

is greater than 10 %. A less obvious or expected finding was that isolated complex I deficiency could also arise from mutations in mt-tRNA genes (e.g., tRNALeu(UUR)). MtDNA sequencing is routinely a first step, and the effect of the mutation (or polymorphism?) can be tested in cybrid fusions (one nucleus, two mtDNA populations) or heterokaryons derived from the fusion of patient cells with ρo cells (cells with no mtDNA).

The majority of patients were diagnosed with Leigh or Leigh-like syndromes; children with a more delayed onset presented with MELAS (Phenotype MIM #540000), and others suffered from encephalomyopathies, pure mitochondrial myopathy, or lethal infantile mitochondrial disease. It is now clear that complex I deficiencies can cause a very heterogeneous set of clinical disorders. The ages of onset varied over a wide range. For the non-specialist it may not be surprising that symptoms appear within the first days or weeks of life and life expectancy is short. The severity of the mutation and the percentage of heteroplasmy undoubtedly are major factors, and measuring heteroplasmy in easily obtained biopsies (fibroblasts, muscle, lymphocytes) may not provide definitive answers. Furthermore, complex I deficiencies measured biochemically in one tissue (e.g., muscle) may not be seen in another (e.g., fibroblasts); Hoefs et al provide specific examples and distinctions made for different groups of patients (Hoefs et al 2012). It is more of a puzzle to find patients with a very late onset of the disease, for example, bilateral loss of vision (LHON). LHON is the most common disease caused by mtDNA mutations. The delay in this and other neurodegenerative diseases has stimulated much discussion and speculation about a biological clock mechanism responsible for an age dependent accumulation of mutations and the deterioration of the mitochondrial genome (Wallace 1997, 2001, 2005; Loeb et al 2005).

Complex I deficiency caused by nuclear DNA mutations

Several recent reviews by leading laboratories have summarized the current status of the clinical, biochemical and genetic characterization of a large number of patients with isolated complex I deficiency due to nuclear mutations (Hoefs et al 2012; Pagniez-Mammeri et al 2012a, b; Nouws et al 2012; Mimaki

et al 2012). Patients present with symptoms similar to those described for mtDNA mutations (Leigh and Leigh-like syndrome, encephalomyopathy, lethal infantile mitochondrial disease, leukoencephalopathy). In contrast to many mtDNA mutations, nuclear DNA mutations are expressed in the neo- natal phase, shortly after birth or in early infancy.

Thirty seven structural proteins are encoded by nuclear genes, and pathogenic mutations have now been found in more than a dozen of these genes. A detailed description of the mutations and the clinical phenotype associated with them can be found in the review by Pagniez-Mammeri et al (2012a, b). Seven of those are genes encoding subunits of the catalytic “core”: NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8. Pathogenic mutations have also been found in several genes encoding supernumerary” sub- units: NDUFS4, NDUFS6, NDUFA10, NDUFA12, NDUFA11, NDUFA2, and NDUFA1. Most of these mutations are affecting proteins in the modules (N,Q) of the complex I responsible for electron transfer from NADH via the flavin mononucleotide and the iron sulfur centers to coenzyme Q. The supernumerary subunits NDUFA1 and NDUFA10 have not been precisely localized, but they are integral mem- brane proteins near the interphase of the peripheral subcomplex and the integral membrane subcomplex responsible for proton pumping.

As explained above, these non-lethal pathogenic mutations cause a partial (more or less severe) loss of complex I activity as typically measured in muscle biopsies. Analyses by gel electrophoresis suggest that in most cases the lower activity is the result of a reduced steady state level of active complex rather than a reduced specific activity of the complex (Smeitink 2003; Smeitink et al 2004). This observation may have some implications in the search for cures, for example, efforts to speed up assembly or attempts to stabilize the complex by providing cofactors or other small molecules.

Mutations in the NDUFS4 gene/protein have received particular attention. The C-terminal of this highly conserved polypeptide/protein (175 aa) has a consensus site for a cAMP- dependent protein kinase. The majority of the

NDUFS4 mutations cause a loss of this phosphorylation site, prompting the authors to propose that phosphorylation is a positive control mechanism for complex I activity (Papa et al 2001). A later publication (De et al 2008) makes the suggestion that phosphorylation of the NDUFS4 protein regulates mitochondrial import. The issue remains unresolved, and an animal model with this or related relevant mutations created by knock-in methods would be quite desirable.

Complex I deficiency caused by nuclear DNA mutations in non-structural genes

The combined efforts of many laboratories have contributed extensively to our understanding of the biochemical and genetic basis of isolated complex I deficiencies and associated pathologies. At the same time there has been a realization that knowing all 44 structural genes of complex I is far from sufficient to identify the molecular basis of such deficiencies in a large fraction of patients. The number is obviously in flux, because of on-going efforts, but it is estimated that the genetic defect in more than 50 % of the patients is not in one of these structural genes, and that additional genes/proteins must contribute to the assembly of an active complex I.

Diverse approaches over the past decade have led to the identification of at least ten of such assembly factors and their genes (Nouws et al 2012; Pagniez-Mammeri et al 2012a, b). Assembly factors, also referred to as chaperones, are proteins that interact transiently with the structural proteins during their assembly but are not found in the final complex isolated by conventional fractionation techniques. The first such factor was found by homology cloning, after some pioneering re- search on complex I structure and assembly in the fungus Neurospora crassa had defined two required genes, CIA30 and CIA84 (Kuffner et al 1998). The human orthologue of CIA30, NDUFAF1, was shown to be essential for complex I assembly (Vogel et al 2005).

Several of the other putative assembly factors were found in studies aimed at characterizing assembly intermediates in complex I biogenesis. Such

intermediates were visualized on blue native gels when assembly was arrested by a missing structural protein or by synchronized assembly in inducible systems. They could be isolated by tandem affinity purification and subjected to mass spectrometric analysis. The involvement of the factors Ecsit and ACAD9 that were bound to the NDUFAF1 protein during assembly was discovered by this approach (Nouws et al 2010; Vogel et al 2007). Homozygosity mapping in consanguineous families with complex I disease directed attention to homozygous regions in which subsequently all candidate genes were sequenced. C20orf7 and NDUFAF4 were implicated in the assembly pathway by this path of investigations (Saada et al 2008; Sugiana et al 2008). Purely bioinformatics methods have contributed other candidates. For example, a comparison of the genomes of the yeast S. cerevisiae, which has no complex I, and other fungi has led to the recognition of NDUFAF2 (Ogilvie et al 2005). Pagliarini et al used bioinformatics, protein mass spectroscopy, microscopy, and machine learning to assemble the proteome of mitochondria and screened it with the help of phylogenetic information from affected families to focus on 18 genes with a potential requirement for complex I assembly/ activity (Pagliarini et al 2008). The gene C8orf38 was subsequently shown to be relevant by homozygosity mapping.

A list that will undoubtedly grow in the future includes the following assembly factors/chaperones: NDUFAF1 (CIA30), Ecsit, ACAD9, NDUFAF2 (NDUFA12L/B17.2 L), NDUFAF4 (C6orf66), NDUFAF3 (C3orf60), C8orf38, C20orf7, HuInd1, TMEM126B (Heide et al 2012), and FoxRed1 (Fassone et al 2010). HuInd1 (alias NUBPL in humans) has been identified as an assembly factor by Calvo et al (2010). Pagniez-Mammeri et al (2012a, b) and Nouws (Nouws et al 2012) have published more detailed reviews on many of these factors. The apoptosis inducing factor (AIF), a caspase-independent death effector, has been associated with mitochondria, and there were suggestions for its role as an assembly factor. In its absence defects in mitochondrial metabolism and in the electron transport chain have been noted (Vahsen et al 2004; Joza et al 2009; Ghezzi et al 2010). A form called AIF(mit) is targeted to the mitochondrial intermembrane space as a FAD-dependent NADH oxidase with no known function.

In many cases the precise function of these factors is not clear, but their critical role is indicated by 1) their co-isolation with assembly intermediates, 2) the genetic and biochemical characterization of patients/families with complex I deficiencies and mutations in the matching gene, and 3) by in vitro experiments including knock-down of specific factors causing the accumulation of assembly intermediates. Alternatively, epitope tagging of such factors has also demonstrated directly their association with assembly intermediates.

Since the complex contains eight iron-sulfur centers, one would expect to find mutations in proteins functioning in the assembly of these clusters (Sheftel et al 2010). At least 15 proteins are required for the formation of the clusters (Lill and Muhlenhoff 2008), and additional specific proteins may be crucial for their final incorporation into the mature complexes of the electron transport chain (CI, CII, and CIII). Mutations in this pathway may affect more than one complex, but Sheftel et al (Sheftel et al 2009) have reported that knock down of HuInd1 in HeLa cells resulted in complex I deficiency, the accumulation of a 450 kDa assembly intermediate, and a depletion of several subunits including NDUFS1 and NDUFS3. The HuInd1 protein is a mitochondrial P-loop NTPase with a highly conserved CXXC motif responsible for transiently binding an Fe/S cluster. The protein has sequence homology to two cytosolic P-loop NTPase proteins, Nbp35 and Cfd1, previously characterized in yeast (Lill and Muhlenhoff 2008). Previously it had been shown in the yeast Yarrowia lipolytica to be an iron-sulfur protein required for NADH dehydrogenase activity, named Ind1.

An intriguing case is the story of ACAD9. Pathogenic mutations in the ACAD9 gene were described in ten patients by three independent research groups investigating isolated complex I deficiencies in humans (Nouws et al 2012). ACAD9 was found initially by using the known assembly factors NDUFAF1 and Ecsit as “baits in a fishing expedition”, i.e., co-precipitation experiments with specific antibodies (Nouws et al 2010). The ACAD9 protein shows extensive sequence homology to a very long chain fatty acid dehydrogenase (VLCAD), a membrane-associated mitochondrial enzyme. However, ACAD9 does not have

any significant β oxidation activity in vivo, and it appears to function exclusively as an assembly factor for complex I. It is speculated that it arose from a gene duplication at the time of the evolution of the vertebrates. Thus, ACAD9 is an assembly factor for complex I in vertebrates, but is absent in all lower eukaryotes which also have a multi-subunit complex I.

Conclusion

Complex I has earned its name in more ways than one. As an integral membrane complex with 44 subunits it presented a major challenge to elucidate its composition, and it continues to challenge the crystallographers to complete a high resolution three dimensional structure. Even with a structure emerging, the problem of understanding the mechanism which links electron transport from FMN to co-enzyme Q to the conformational changes responsible for proton pumping is still a formidable one.

The complete high resolution structure of a eukaryotic complex I can be expected in the not too distant future. It will undoubtedly raise more questions about the role of the large number of accessory subunits. Do they perform the function of a scaffold during assembly or in maintaining stability, or are they performing other biochemical functions, for example in the regulation of complex I activity under different conditions? Are they required for respirasome assembly or maintenance? The excess of more than 30 highly conserved subunits in all eukaryotic complexes compared to the prokaryotic complex is certainly intriguing. Studies in model systems such as the one represented by the yeast Yarrowia lipolytica (Abdrakhmanova et al 2004) may have to blaze a path before insights can be applied to the human/mammalian complex.

From a human genetic point of view one can first consider that the 44 structural genes present a relatively large target for a variety of mutagenic insults, or in other words, there are many ways in which this complex can be inactivated or reduced in level and/or activity. Once mutations are identified, additional complications arise for genetic counselors because seven structural

genes are encoded by the mitochondrial genome, and their inheritance in a pedigree does not follow Mendel’s Laws. A majority of mitochondrial diseases are a consequence of a complex I deficiency, they are often fatal, and generally no effective pharmacological treatments are available. When a child is diagnosed to suffer from isolated complex I deficiency, genetic counseling of the family is clearly desirable, if other children are wanted. The technology is now advanced and economical to sequence all the structural genes of complex I in the patient and the parents. If two heterozygous carriers with a mutation in a nuclear structural gene can be identified, the genetic counseling can be expected to be relatively straightforward, and prenatal diagnosis in a pregnancy may become an option. On the other hand, if the mutation is on the mtDNA, such counseling becomes more of a guess work, since the change in heteroplasmy from an unaffected mother to her offspring is unpredictable.

Concentrated efforts in many laboratories have led to remarkable progress in the diagnosis and characterization of the pathology resulting from isolated complex I deficiency, but, perhaps as a surprise to some, the molecular genetic analysis in more than half of the patients investigated to date has found no mutations in these structural genes. Instead, a dozen or more genes have been identified in such a cohort of patients that, when defective, cause a serious and pathological depletion of complex I activity. The corresponding proteins have been classified as assembly factors or chaperones, since they are clearly needed for the assembly of this complex in the inner mitochondrial membrane, but are not found associated with the mature complex. Even with those additional genes included in a molecular genetic analysis, the primary defect in many patients remains to be found. Thus, the successful, high powered genetic, genomic, bioinformatics approaches perfected in the recent decade promise to lead to the discovery of additional genes (“factors”) in patients already registered or still to be identified.

Exome sequencing may in some cases not reveal the nature of the defect, if alterations in promoters or enhancers affect gene expression without resulting in a defective protein. Chip technology may have to be perfected to assess the mRNA levels from all the genes implicated in determining normal complex I

activity, but as mentioned above, tissue-specific information may be required to make the determination.

While it is impressive that mutations responsible for pathological phenotypes have been found in slightly more than half of the nuclear structural genes, questions can be raised with regard to the other known structural genes. Why have no pathogenic mutations been found so far in these genes? Is the population of patients that has been screened so far still too small? Are mutations in these genes generally lethal because other functions are affected? Do mutations in these genes lead to phenotypes/pathologies that are not recognized as “mitochondrial diseases”? Support for both clinical and molecular- biological investigations of diverse patients is clearly justified and required if genetic counseling in all families with “isolated complex I deficiencies” is to be more definitive and successful.

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