

Medicinal and Genetic Approaches to the Treatment of Mitochondrial Disease

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Abstract: Although great progress has been made in our understanding of the molecular bases of mitochondrial disorders due to defects in the respiratory chain, little exists in the way of rational therapy. Possible therapeutic approaches include: palliative therapy; removal of noxious metabolites; administration of artificial electron acceptors, metabolites, and free radical scavengers; genetic counseling; and gene therapy. There has been progress with each of these approaches, although much work remains to be done. Finally, a novel approach to treating a specific mitochondrial disorder, MELAS, is presented.

Keywords: MELAS, mtDNA, nitric oxide, nitroglycerin, oxidative phosphorylation, therapy, respiratory chain.

INTRODUCTION

Mitochondrial Genetics and Mitochondrial Disease

Mitochondrial encephalomyopathies, as defined here, are a group of disorders resulting from defects in the respiratory chain and oxidative phosphorylation. Because this is also the only biochemical pathway in the cell that is under dual genetic control (i.e. mitochondrial DNA [mtDNA] and nuclear DNA [nDNA]), we will review briefly the rules of mitochondrial genetics and how they differ from those of mendelian genetics [1].

The human mitochondrial genome (Fig. 1) is a 16,569-bp circle of double-stranded DNA [2]. It contains genes encoding two ribosomal RNAs (12S and 16S), 22 transfer RNAs, and 13 polypeptides, all of which are subunits of the respiratory chain/oxidative phosphorylation system (OXPHOS) (Fig. 2). Seven of the 13 polypeptides (ND1 - ND6) encode subunits of Complex I (NADH-CoQ oxidoreductase), one (Cyt *b*) encodes the cytochrome *b* subunit of complex III (CoQ-cytochrome *c* oxidoreductase), three (COX I - COX III) encode subunits of Complex IV (cytochrome *c* oxidase, or COX), and two (A6 and A8) encode subunits of Complex V (ATP synthase). The complexes also contain subunits encoded by nuclear genes, which are imported from the cytoplasm and assembled, together with the mtDNA-encoded subunits, into the respective holoenzymes located in the mitochondrial inner membrane. Complex II (succinate dehydrogenase-CoQ oxidoreductase) is encoded entirely by nuclear genes. There are also two mobile electron carriers: ubiquinone (also called Coenzyme Q10, or CoQ10), located in the inner membrane, and cytochrome *c*, located in the intermembrane space (see Fig. 2). Besides the structural components of the OXPHOS system, a number of mitochondrially-targeted nuclear-

encoded gene products are required for the proper assembly and functioning of these complexes, including proteins needed for the modification, importation, and insertion of co-factors, such as heme, biotin, metals, and iron-sulfur groups.

As all mtDNA are derived from the oocyte, the mode of transmission of mtDNA and of most (but not all) mtDNA mutations differs from that of mendelian inheritance. A mother carrying a mtDNA mutation will pass it on to all her children (boys as well as girls) but only her daughters will transmit the mutation to their children [3]. Recently, evidence has been obtained that there can be paternal inheritance, but such an event appears to be quite rare [4].

In normal tissues, all mtDNA molecules are identical, a situation known as homoplasmy. Each cell contains hundreds or thousands of organelles, each in turn containing multiple mtDNAs (approximately five [5]), which distribute randomly among daughter cells at cell division. When a mutation occurs, it usually affects only a subset of mtDNAs, resulting in the presence of two populations of mtDNA genotypes - normal and mutated - within a cell, tissue, or individual, a situation known as heteroplasmy. The clinical expression of a pathogenic mtDNA mutation is determined in large part by the relative proportion of normal and mutant genomes in different tissues. Furthermore, a minimum number of mutant mtDNAs is required to cause overt mitochondrial dysfunction in a particular tissue or organ, a phenomenon known as the threshold effect.

Organellar division and mtDNA replication are essentially unrelated to the cell cycle. Therefore, the proportion of mutant mtDNAs in daughter cells following cell division may shift (owing to random drift) and the clinical phenotype may change accordingly. This process, called mitotic segregation, explains how certain patients with pathogenic mtDNA mutations can have one clinical phenotype in infancy or youth and another phenotype later in life. For example, infants who survive a usually fatal hematopoietic disease (Pearson syndrome) may develop an encephalomyopathy (Kearns-Sayre syndrome) as the

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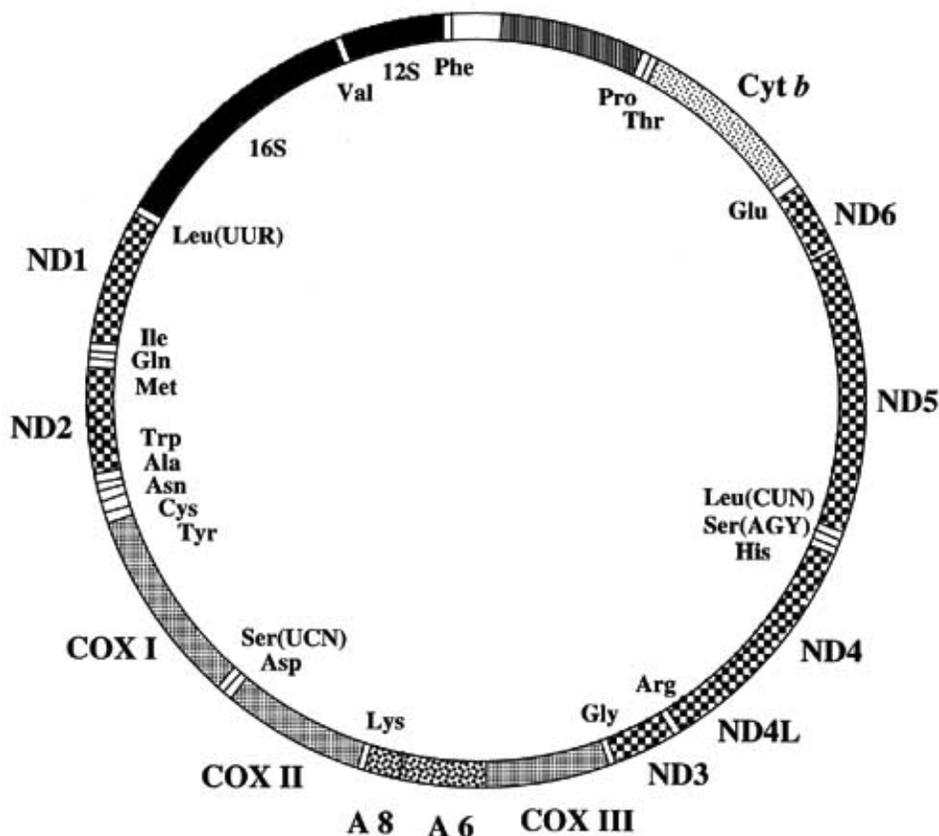


Fig. (1). Map of the 16,569-bp human mitochondrial genome (2). Polypeptide coding genes are shown outside the circle; rRNA and tRNA genes are shown inside the circle.

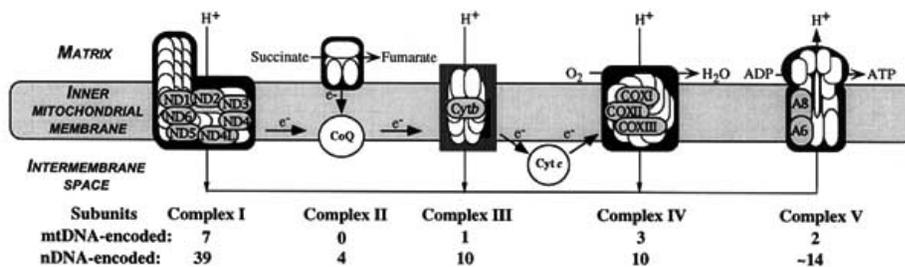


Fig. (2). The mitochondrial respiratory chain. Subunits encoded by mtDNA are in the solid gray ovals.

proportion of mutated mtDNAs decreases in blood cells while increasing in brain and muscle cells [6, 7].

Genetically speaking, mitochondrial encephalomyopathies fall into three major groups (Table 1):

1. Disorders due to mutations in mtDNA

More than 150 pathogenic point mutations have been identified [8], and even after more than fifteen years of investigation, more are still being found [9]. In addition, an even greater number of partial large-scale deletions and duplications of mtDNA, typically associated with sporadic Kearns-Sayre syndrome

(KSS), progressive external ophthalmoplegia (PEO), and Pearson syndrome (PS), have been identified [10].

2. Disorders due to mutations in nDNA

Given that the vast majority of respiratory chain subunits and all of their assembly proteins are encoded by the nuclear genome, mendelian defects of the respiratory chain account for a substantial, and growing, fraction of mitochondrial encephalomyopathies [11]. To date, mutations in 16 genes have been described (Table 1).

3. Defects of Intergenomic Signaling

Among the mendelian-inherited mitochondrial encephalomyopathies, a subset are due to alterations in mtDNA stability and copy number, arising from the fact that mtDNA replication, transcription, and translation are all processes that require nDNA-encoded gene products [12]. At least 7 mutated genes fall in this category. A comprehensive review on mitochondrial diseases can be found in the article by Ohta elsewhere in this volume.

Table 1. Mitochondrial Diseases and Mutations

| Disorder | Typical affected gene |
|--|--------------------------|
| Mutations in mitochondrial DNA | |
| Leber hereditary optic neuropathy (LHON) | ND4 |
| Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) | tRNA ^{Leu(UUR)} |
| Maternally-inherited diabetes and deafness (DAD) | tRNA ^{Leu(UUR)} |
| Maternally-inherited progressive external ophthalmoplegia (MI-PEO) | tRNA ^{Leu(UUR)} |
| Maternally-inherited cardiopathy (MI-PEO) | tRNA ^{Ile} |
| Myoclonus epilepsy with ragged-red fibers (MERRF) | tRNA ^{Lys} |
| Neuropathy, ataxia, retinitis pigmentosa | ATP6 |
| Maternally-inherited Leigh syndrome | ATP6 |
| Maternally-inherited deafness | tRNA ^{Ser(UCN)} |
| Aminoglycoside-induced deafness (AID) | 12S rRNA |
| Sporadic myopathy | Cyt <i>b</i> |
| Sporadic Kearns-Sayre syndrome | mtDNA deletion |
| Sporadic progressive external ophthalmoplegia (PEO) | mtDNA deletion |
| Sporadic Pearson syndrome (PS) | mtDNA deletion |
| Mutations in nuclear DNA affecting the respiratory chain | |
| Leigh syndrome with complex I deficiency | NDUFS1 |
| Leigh syndrome with complex I deficiency | NDUFS4 |
| Leigh syndrome with complex I deficiency | NDUFS7 |
| Leigh syndrome with complex I deficiency | NDUFS8 |
| Leigh syndrome with complex I deficiency | NDUFV1 |
| Leigh syndrome with complex II deficiency | SDHA |
| Pheochromocytoma with complex II deficiency | SDHB |
| Paraganglioma with complex II deficiency | SDHC |

(Table 1) contd.....

| Disorder | Typical affected gene |
|---|-----------------------|
| Paraganglioma with complex II deficiency | SDHD |
| Tubulopathy with complex III deficiency | BCS1L |
| GRACILE syndrome with complex III deficiency | BCS1L |
| Tubulopathy/encephalopathy with complex IV deficiency | COX10 |
| Fatal hypertrophic cardiomyopathy with complex IV deficiency | COX15 |
| Hepatic failure with complex IV deficiency | SCO1 |
| Cardioencephalomyopathy with complex IV deficiency | SCO2 |
| Leigh syndrome with complex IV deficiency | SURF1 |
| Hepatopathy with complex IV deficiency | LRPPRC |
| Mutations in nuclear DNA affecting mtDNA plasticity and stability | |
| Autosomal dominant PEO with multiple mtDNA deletions (AD-PEO) | ANT1 |
| Autosomal dominant PEO with multiple mtDNA deletions (AD-PEO) | TWINKLE |
| Autosomal dominant PEO with multiple mtDNA deletions (AD-PEO) | POLG |
| Autosomal recessive PEO with multiple mtDNA deletions (AR-PEO) | POLG |
| Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) | TP |
| Mitochondrial DNA depletion syndrome (MDS) | DGK |
| Mitochondrial DNA depletion syndrome (MDS) | TK2 |
| Congenital Amish microcephaly (MCPHA) | DNC |

The huge diversity of etiologies for mitochondrial encephalomyopathies means that treatment strategies will have to be equally diverse: no single "magic bullet" will be able to treat (let alone cure) all mitochondrial disorders in one fell swoop. Upon some reflection, one can envision numerous approaches to treatment, ranging from the most simple (e.g. exercise) to the most complex (e.g. gene therapy), with many possible approaches occupying the middle ground between these two extremes. A list of possibilities, which is by no means all-inclusive, is shown in Table 2.

Table 2. Treatment Approaches for Mitochondrial Disorders

| Approach | Examples |
|--------------------------|-----------------------------|
| Physiological approaches | |
| Palliative approaches | |
| Drugs | Insulin; valproate |
| Prosthetics | Cochlear implant; pacemaker |

(Table 2) contd.....

| Approach | Examples |
|--|---|
| Surgery | Blepharoplasty; transplantation |
| Other | Blood transfusion; dialysis; hydration |
| Stimulation of muscle regeneration | |
| Exercise | Eccentric vs. concentric exercise |
| Controlled muscle destruction and regeneration | Bupivacaine; exercise |
| Biochemical approaches | |
| Supplementation of OXPHOS components | |
| Endogenous substrates | CoQ10; succinate |
| Related substrates | Idebenone; menadione; vitamin C and K |
| Metabolites and co-factors | Carnitine; Cu; folate; riboflavin; thiamine |
| Mitigation of ancillary toxicity | |
| Reduction of lactate | Bicarbonate; dichloroacetate |
| Reduction of thymidine in MNGIE | Dialysis; diuretics |
| Scavenging of free radicals | Lipoic acid; vitamin E; CoQ10; Mn-TBAP |
| Genetic approaches | |
| Genetic counseling | |
| Family screening | PCR/RFLP in available tissues |
| Prenatal screening | PCR/RFLP in amniocytes, chorionic villi |
| Ooplasmic transfer | IVONT |
| Shifting of heteroplasmy | |
| Inhibition of mutant DNA replication | Peptide nucleic acids; AZT |
| Selection for respiratory function | Galactose-oligomycin |
| Induced organellar fusion (homogenization) | Mitofusins; ethacrynic acid |
| Importation of exogenous molecules | |
| Importation of wild-type nDNA-encoded proteins | Nuclear structural and assembly proteins |
| Allogenic expression of related proteins | ND11 from <i>S. cerevisiae</i> |
| Allotopic expression of mtDNA-encoded proteins | ATP6, ND4 |
| Xenotopic expression of mtDNA-encoded proteins | ATP6 from <i>C. reinhardtii</i> |
| Importation of ancillary proteins | Aminoacyl tRNA synthetase, MnSOD |
| Importation of RNAs | tRNAs, ribozymes |
| Importation of DNAs | Peptide-DNA fusions |

PALLIATIVE TREATMENTS

Traditional approaches used by clinicians to ameliorate the symptoms of disease can sometimes be effective in treating mitochondrial disorders. For example, patients with sporadic mtDNA deletions causing KSS, PEO, and PS show many symptoms that can be treated with currently-

available techniques. In patients with ophthalmoplegia (not only sporadic PEO due to mtDNA deletions, but also maternally-inherited PEO due to mtDNA point mutations and mendelian-inherited PEO due to "intergenomic communication" mutations), severe ptosis can be ameliorated surgically by blepharoplasty. In sporadic Pearson syndrome due to mtDNA deletions, the sideroblastic anemia may respond to repeated blood transfusions from a normal donor. Note, however, that if a child with PS survives into childhood, it may well grow up to develop KSS [6, 7]. Exocrine pancreas dysfunction, also typical of PS, requires replacement of digestive enzymes. Patients with KSS often have life-threatening conduction block, which can be managed by surgical implantation of a pacemaker. Even in patients with multisystem disorders, such as KSS or mendelian-inherited PEO with multiple mtDNA deletions, cardiac transplantation should be considered [13, 14]. Diabetes mellitus is a feature of many mitochondrial disorders (most prominently KSS and MELAS), and should be treated as appropriate, depending on whether the diabetes is insulin-dependent or not.

Other mitochondrial symptoms can also respond to palliative treatment. For example, patients with seizures often respond to conventional anticonvulsant therapy; however, if valproic acid is used, it should always be administered together with L-carnitine, because of the inhibition of carnitine uptake by valproate [15]. Cataracts can be dealt with surgically. In patients with isolated cardiomyopathy, cardiac transplantation is a potentially attractive, albeit extreme, possibility. Hearing aids can be useful in patients with neurosensory hearing loss, probably the most common symptom of mitochondrial encephalomyopathy, and deafness due to isolated cochlear dysfunction in MELAS patients has been treated successfully with cochlear implantation [16]. Kidney problems (e.g. renal tubular acidosis and Fanconi syndrome) require readjustment of electrolyte balance. Similarly, recurrent myoglobinuria in patients with CoQ10 deficiency or in patients with protein-coding gene mutations [17] requires vigorous hydration or renal dialysis to avoid renal failure. Children with Leigh syndrome (LS), and especially those with LS associated with COX deficiency, often develop severe feeding problems, typically due to recurrent vomiting and gastroesophageal reflux. These symptoms can be relieved by drugs or by surgery (gastric fundoplication). Intestinal dysmotility with pseudo-obstruction (a feature of MNGIE and of MELAS) often requires emergency surgery.

REMOVAL OF TOXIC METABOLITES

A block in the respiratory chain will result in the accumulation of pyruvate in the cytosol, and the consequent increase in lactate, pyruvate's reduction product, and alanine, its transamination product. Thus, all three compounds, and lactate in particular, tend to increase in blood from patients with mitochondrial encephalomyopathies. The severity of lactic acidosis varies considerably among different conditions, and blood lactate can be normal in some mitochondrial disorders. Also, lactate may be elevated in cerebrospinal fluid (CSF) but not in blood. Elevated CSF or brain lactate can be documented by proton magnetic resonance spectroscopy (MRS), especially in MELAS.

Since elevated lactate in the central nervous system (CNS) is neurotoxic, it seems reasonable to reduce it in patients. Unfortunately, the most common treatment for high lactate - bicarbonate - has only a transient buffering effect and may even exacerbate the cerebral symptoms [18]. Dichloroacetate (DCA), on the other hand, is a much more specific compound for reducing lactate. DCA inhibits the phosphorylation of pyruvate dehydrogenase (PDH), thereby keeping the enzyme in the dephosphorylated, active state [19]. There is anecdotal evidence that DCA improves the clinical outcome in children with MELAS (increased growth, reduced frequency of stroke-like episodes) [20-23] and in COX deficiency [24]. One drawback of DCA is that it may cause peripheral neuropathy unless it is administered together with thiamine [25, 26]. A double-blind, placebo-controlled trial in MELAS patients carrying the A3243G mutation is currently underway at Columbia University.

While lactate accumulation is a feature of many mitochondrial diseases, some disorders are associated with the accumulation of highly specific harmful compounds. A case in point is MNGIE, a devastating autosomal-recessive multisystemic disorder [27] caused by mutations in thymidine phosphorylase, an enzyme required to maintain nucleotide homeostasis in the cytoplasm and in mitochondria (and located, interestingly, not in mitochondria but in the cytosol [28]). Patients with MNGIE have extremely high levels of thymidine in the blood (about 50 times normal) [29, 30]. Thus, an obvious approach to treating this disorder would be to reduce blood levels of thymidine. In fact, thymidine can be reduced by dialysis, but the benefit is only temporary (M. Hirano, personal communication). A more useful approach may be to administer a diuretic that would increase the excretion (or decrease the retention) of circulating thymidine.

CIRCUMVENTING THE BLOCK IN ELECTRON TRANSPORT BIOCHEMICALLY

If mutations in mtDNA (or nDNA for that matter) cause defects in the respiratory chain/OXPHOS system, one can imagine that strategies designed to get around a presumed "block" in the respiratory chain might be useful clinically. One of the earliest approaches to circumventing such a block was to administer artificial electron acceptors, in the hope that improved electron transport would allow for an increase in proton pumping across the mitochondrial inner membrane, thereby causing an increase in ATP synthesis.

Many years ago, the idea arose to treat children with lactic acidosis with methylene blue, based on the rationale that this compound would "discharge" electrons from a presumably "over-reduced" electron transport chain, but the efficacy of this approach was never demonstrated [31, 32].

A more rational approach was tried in 1984, in a patient with myopathy and isolated complex III deficiency [33]. Because the problem was localized specifically to complex III, Eleff *et al.* [34] tried to bypass the respiratory block by providing artificial electron acceptors whose redox potentials matched closely that of cytochrome *b* (the mtDNA-encoded electron transfer subunit in complex III). In particular, they gave the patient vitamin C and menadiol diphosphate, a precursor of vitamin K. There was a dramatic improvement,

documented by ^{31}P nuclear magnetic resonance (NMR), which lasted for more than one year [35], but unfortunately, the benefit was not sustained (N. Kennaway, personal communication). The mutation in this patient was ultimately found to be, indeed, in the cytochrome *b* gene [36], thus justifying this first "rational" approach to treatment. In the last few years, a number of patients with isolated myopathy and sporadic mutations in cytochrome *b* have been described [37]. Several of these patients have now been treated with vitamins C and K, but without any objective or subjective improvement.

SUPPLEMENTATION OF COFACTORS AND METABOLITES

The lack of a clear understanding of the underlying mechanisms of pathogenesis has led to relatively simple, and often naive, approaches to therapy, based upon the idea that defects in the respiratory chain and in oxidative phosphorylation can somehow be compensated for by "boosting" the system with vitamins and/or cofactors that are known to participate in oxidative energy metabolism, even when those factors themselves are not apparently deficient. Since many of these factors are relatively non-toxic and can even be purchased over the counter, the perceived potential benefit of administering such agents compared to the relatively low risk inherent in using them has encouraged both physicians and patients to employ a panoply of compounds.

Administration of some factors may well be beneficial if they are found to be reduced in a particular disorder. For example, riboflavin, a component of complex I, was helpful in patients with mitochondrial myopathy and complex I deficiency due to a mutation in the tRNA^{Leu(UUR)} gene [38]. Similarly, folic (or folinic) acid is given to patients with KSS because it is lower than normal in the blood and CSF of patients with this condition [39]. It seems reasonable to supplement free L-carnitine because levels of free carnitine in the blood from patients with mitochondrial encephalomyopathies tend to be below normal, whereas esterified carnitines tend to be higher. Such a shift could be due to an impairment in β -oxidation, whose reducing equivalents enter the respiratory chain at the level of coenzyme Q10 via the electron-transfer flavoprotein (ETF). Our group usually combines L-carnitine supplementation with CoQ10, not so much because CoQ10 is decreased in mitochondrial encephalomyopathies - which it is, to a modest extent [17, 40-42] - but rather to take advantage of the oxygen-scavenging action of this compound (see below). Administration of carnitine and CoQ10 has been used with partial success [43], especially in patients with sporadic KSS or PEO.

Notably, in cases where carnitine or CoQ10 deficiency is the primary defect, administration of the "missing" compound can have dramatic beneficial effects. In primary systemic carnitine deficiency, an autosomal recessive disorder due to mutations in the plasma membrane carnitine transporter [44, 45], progressive cardiomyopathy is the most common presentation. In these patients, cardiac function responds dramatically to carnitine supplementation, with progressive normalization of cardiac function indices within

a few months [46, 47], underlying the critical importance of measuring blood carnitine concentrations in infants and young children with unexplained cardiomyopathy.

Similarly, in primary CoQ10 deficiency (also an apparently autosomal recessive and clinically heterogeneous trait where no culprit genes have yet been identified) CoQ10 administration has been extremely beneficial [48, 49]. There are three described clinical variants: (1). myopathy with recurrent myoglobinuria, lipid storage and ragged-red fibers (RRF) in muscle, and CNS involvement with seizures, ataxia, or mental retardation [42, 48, 50]; (2). a multisystemic disease of infancy, with encephalopathy, hepatopathy, and nephropathy [49]; and (3). ataxia and cerebellar atrophy, often associated with weakness, pyramidal signs, seizures, or mental retardation [51, 52].

REDUCTION OF FREE RADICAL DAMAGE

Besides reduced ATP synthesis, impaired respiratory chain function can have other adverse consequences, such as reduced calcium buffering [53] and increased production of reactive oxygen species (ROS), which can damage proteins, lipids, and mtDNA [54]. To counteract the effects of oxidative stress, oxygen radical scavengers have been used, most commonly CoQ10 or idebenone, a quinone compound similar to CoQ10, and dihydrolipoate [55-58]. Encouragingly, in a trial with patients with Parkinson disease, CoQ10 slowed the progression of the disease [59].

A novel genetic approach to scavenging free radicals has been to overexpress antioxidant enzymes normally found in mitochondria, such as Mn-superoxide dismutase (*SOD2*). Overexpression of human Mn-SOD in transgenic mice protected them from lung injury from exposure to hyperbaric oxygen [60]. Encouragingly, there are chemical compounds that mimic the effect of Mn-SOD (e.g. Mn-TBAP) which, when administered to SOD-deficient transgenic mice, can improve the course of the disease and extend lifespan [61, 62].

GENE THERAPY

The identification of genes responsible for mitochondrial disorders - both nDNA- and mtDNA-encoded - has led to a number of ideas aimed at gene therapy. For nuclear-encoded genes, such as those responsible for autosomal dominant and recessive-inherited PEO, MNGIE syndrome, Leigh syndrome associated with COX deficiency, and the like, gene therapy approaches have followed the "mainstream" technologies that have been pioneered by researchers outside the mitochondrial field (e.g. cystic fibrosis, adenosine deaminase deficiency, and the hemoglobinopathies). These approaches are geared toward replacing the defective gene (e.g. *ANTI* or *POLG* in the case of mendelian-inherited PEO; *TP* in the case of MNGIE [see Table 1]), with all the problems - mainly revolving around efficient delivery of vectors, tissue-specificity, immunological responses, and inappropriate vector insertion into the nuclear genome - inherent in attempts at "standard" gene therapy.

With respect to mutations in mitochondrial genes, a different set of problems present themselves [63]. First and

foremost among these is our inability to transfect mammalian mitochondria stably and heritably with exogenous DNA. Although stable and heritable transfection of organelles with DNA has been accomplished successfully in other organisms - most notably yeast mitochondria [64] and plant chloroplasts [65] - using biolistic transformation (the "gene gun"), there has been no report of any similar success using mammalian cells. Thus, in the absence of any transformation capability, gene therapy approaches have taken a number of different tacks.

Reducing the Load of Mutant mtDNAs

An approach based upon our understanding of heteroplasmy and the threshold effect has been pioneered by Turnbull's group in Britain and by Shoubridge's group in Canada. Since almost all mtDNA-related disorders are recessive (i.e. only a relatively small number of normal mtDNAs can overcome the deleterious effects of a large number of mutated mtDNAs, which is why the threshold for mutated mtDNAs in most mitochondrial diseases exceeds 70%, and in some cases 90%), any strategy that could reduce the proportion of mutated mtDNAs, even by a small amount, could have disproportionately large, and beneficial, effects in affected tissue.

It has been found that muscle stem cells ("satellite" cells) and myoblasts often contain lower amounts of the pathogenic mtDNA mutations than does the mature muscle itself [66-70]. This observation implies that one could reduce the overall amount of mutated mtDNAs by "forcing" the proliferation of "mutation-poor" satellite cells in muscle. One way to accomplish this is by causing limited muscle necrosis by injection of myotoxic agents, such as bupivacaine [71], followed by muscle regeneration. A second approach is to induce muscle damage, and subsequent satellite cell proliferation, through isometric exercise: both conditions would be followed by regeneration of muscle fibers harboring lower mutational loads [66]. This type of approach might be particularly useful in patients with isolated myopathy due to mutations in mtDNA protein-coding genes [9, 37].

For patients with systemic mtDNA mutations - the vast majority - such an approach would most likely not be helpful. However, the concept of "heteroplasmic shifting" might still be useful if the agent could be delivered to all affected cells. For example, in patients with the T8993G mutation in the *ATP6* gene causing NARP and MILS [72] (see Table 1), there is a reduction in the amount of ATP synthesized [73]. The mutation is located in the same transmembrane domain of ATPase 6 that confers sensitivity to oligomycin. Growth of cybrids harboring the T8993G mutation in medium containing galactose instead of glucose (to force the cells to rely on oxidative metabolism for ATP) plus low levels of oligomycin resulted in a subtle but significant, and irreversible, reduction in the amount of mutation, coupled with an increase in ATP synthesis in these cells [74]. While no one is proposing to treat MILS infants with oligomycin, a metabolic poison, efforts are currently underway to see if other, less toxic compounds, or more "benign" treatment conditions, could achieve the same result.

The problem of heteroplasmy is exacerbated by the heterogeneous distribution of mutated mtDNAs within cells and tissues. This is most clearly exemplified by the clinical difference between MELAS and maternally-inherited PEO, both of which are caused by the same A3243G mutation in tRNA^{Leu(UUR)}. The differences between those two disorders, at least in muscle, are believed to be due mainly to differences in the localized concentration and distribution of mutated mtDNAs in individual muscle fibers [75]. Thus, in terms of heteroplasmic shifting, it might not be necessary to reduce the amount of the mutation at all. If one could redistribute the proportion of mutant and wild-type mtDNAs within mitochondria so that, instead of having "homoplasmic mutant" and "homoplasmic wild-type" organelles in the cell, one could encourage interorganellar fusion so that each organelle had at least one or two wt-mtDNAs (which could then complement the function of the mutant mtDNAs in that organelle), one would have a population of organelles and/or cells that were all below the threshold for dysfunction. In the context of muscle, such "homogenization" of heteroplasmy would likely slow down, or even halt, the inexorable increase in ragged-red fibers.

A number of methods to enhance mitochondrial fusion and homogenization of heteroplasmy can be envisioned, including genetic, biochemical, and physiological strategies. The genetic approach could entail the modulation of the machinery already known to exist in cells that controls mitochondrial fission [76], such as the dynamin-related protein (gene *DNM1L*), and mitochondrial fusion [77, 78], such as the genes mitofusin 1 (*MFN1*), mitofusin 2 (*MFN2*), and the dynamin-related GTPase gene *OPA1*, which, when mutated, causes autosomal-dominant optic atrophy [79].

Biochemically, one might be able to administer a drug that enhances mitochondrial fusion. One such compound, ethacrynic acid, appears to cause mitochondria to fuse into "megamitochondria" [80], perhaps by disrupting the attachment of the organelles on cytoskeletal elements, such as microtubules. While the toxicity of this specific compound [81] might mitigate against its use, the general concept is nevertheless a useful one.

Physiologically, the exercise regime that was reported to improve muscle strength in patients with mtDNA mutations showed in some cases either no change in mutant load or even a paradoxical increase in mutant load following exercise [66]. This result might be explained by postulating that exercise induced not only an increase in satellite cell recruitment, but also a homogenization of heteroplasmy within the muscle, thereby causing the improvement in muscle performance. An exercise trial geared towards examining this hypothesis might prove fruitful.

In another strategy to reduce mutational loads, Lightowlers' group also considered the use of peptide nucleic acids (PNAs) to inhibit selectively the replication of complementary mutant, but not wild-type, mtDNAs [82-84]. While this approach has worked *in vitro*, delivering the PNAs *in vivo* has turned out to be surprisingly difficult.

Replacement or Addition of Gene Products

If the block in electron transport is located in a specific complex (as is the case with cytochrome *b* above), perhaps one could provide genes encoding one or more related

proteins whose function could substituted for, or bypass, the block due to the mutated protein, and allow the respiratory chain to function normally ("allogenic" therapy). Even better, perhaps one could provide the gene encoding the normal form of the mutated protein ("isogenic" therapy). In fact, both approaches have been tried, and at least *in vivo*, both show promise.

Allogenic Therapy

An elegant and clever genetic approach was used to circumvent a block in electron transport in Chinese hamster cells due to complex I deficiency. As opposed to the situation in mammals, the yeast respiratory chain does not contain a "classic" rotenone-sensitive complex I. Yeast do indeed oxidize NADH, but this function is performed by three rotenone-insensitive polypeptides, located in the cytosol (Nde1p, also called the "external" dehydrogenase), the mitochondrial intermembrane space (Nde2p), and the mitochondrial matrix (Ndi1p, also called the "internal" dehydrogenase).

Yagi's group in California decided to use the "internal" yeast NADH-quinone oxidoreductase, Ndi1p, to bypass defects in hamster complex I, thus enabling electrons from NADH to enter the respiratory chain. Transfection of the *ND11* gene encoding rotenone-insensitive internal NADH-quinone oxidoreductase into complex I-deficient Chinese hamster cells resulted in the functional expression of the enzyme and catalyzed electron transfer from NADH in the matrix to CoQ10 in the inner mitochondrial membrane [85]. Seo *et al.* then extended this work by showing that yeast *ND11* could be expressed and could function in human cells [86]. Moreover, Ndi1p was shown to rescue a defect in human cells due to a mutation in the mtDNA-encoded ND4 gene [87].

Hypothetically, a similar approach could be used to circumvent mutations in other complexes, such as complex IV, or COX. As with NADH dehydrogenase, the COX protein is most complex in mammals (13 subunits in human, 3 of which are mtDNA-encoded), especially when compared to yeast (approximately 10 subunits) and bacteria (2 - 4 subunits). In fact, the basic proton-pumping function of human COX is conferred only by the 3 mtDNA-encoded subunits (COX I, II, and III), which are the direct evolutionary descendants of the 3 prokaryotic subunits; the remaining 10 subunits, all nDNA-encoded, appear to be required for the modulation of COX function. In bacteria, such as *Rhodobacter sphaeroides*, the COX genes are typically encoded in a single operon [88] (sometimes including subunit IV, which is nDNA-encoded in humans), and there are bacteria in which some COX subunit genes are fused and expressed in a single polypeptide (e.g. COX I and III in *Thermus thermophilus* [89]). It can be imagined how, analogously to what was done with yeast Ndi1p, one might be able to target these "simpler" COX polypeptides (and we use the term advisedly) to human mitochondria. Bacteria also express quinol oxidases that are highly similar to COX [90], and presumably these, too, might be candidates for allogenic therapy in COX deficiency.

Isogenic Therapy

Although we do not yet know how to transfect foreign mitochondria with exogenous DNA, we do know how to

"transfect" mitochondria with exogenous proteins. This knowledge has led to a rather baroque strategy of trying to reduce the load of mutant polypeptides by importing a normal version of a mutated mtDNA-encoded polypeptide from a gene that has been "relocalized" to the nucleus. The basic strategy, which is called "allotopic expression" [91], is shown in Fig. 3, using *ATP6* as the example [92]. In brief, a construct encoding an N-terminal mitochondrial targeting signal (MTS) appended to a recoded *ATP6* gene (made compatible with the universal genetic code by *in vitro* mutagenesis) was transfected into the nucleus of human cells containing the T8993G mutation in *ATP6* causing NARP/MILS. Upon expression of the nuclear gene, the precursor polypeptide was targeted to mitochondria (by virtue of the MTS), was imported into and processed within the mitochondria, and was incorporated into complex V. Even in the presence of "endogenously-expressed" mutated *ATPase 6* polypeptides inside cybrids containing the patient's mitochondria, the allotopically-expressed and -imported normal *ATPase 6* was able to increase ATP synthesis in these cells [92]. In a variation of this approach,

called by us "xenotopic expression," the *ATPase 6* polypeptide in the green alga *Chlamydomonas reinhardtii*, which is encoded by a *nuclear* gene and which is imported into mitochondria in this organism, was also able to increase cell growth and ATP synthesis when transfected into T8993G cybrids [93]. Finally, allotopic expression of the human *ND4* gene was able to rescue defects due to a different mutation - that at position 11778 in the *ND4* gene causing maternally-inherited Leber hereditary optic neuropathy (LHON) [94].

GENETIC COUNSELING

For known mendelian-inherited disorders, prenatal diagnosis can be an important tool in predicting whether or not a fetus will have a mitochondrial disease, and appropriate family counseling can be offered at that point. However, prenatal diagnosis poses special problems for mtDNA-related diseases, for two key reasons: (1). the mutational load in amniocytes or in chorionic villi does not

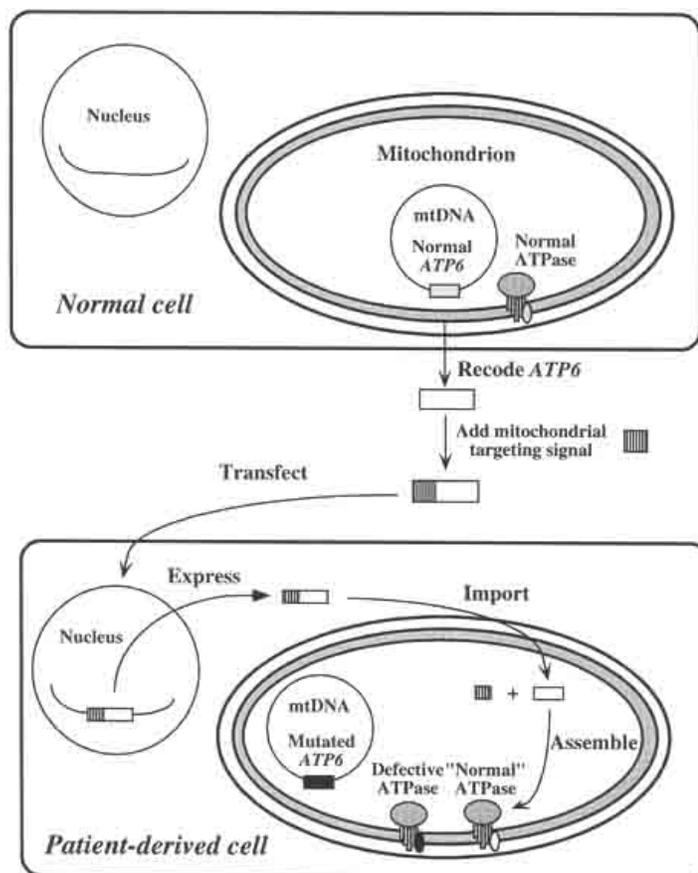


Fig. (3). Allotopic expression of mtDNA-encoded *ATP6* in cells from a patient with NARP/MILS (92).

necessarily correspond to that of other fetal tissues; and (2). the mutational load determined in a prenatal sample may shift *in utero* or after birth due to mitotic segregation. Taken together, it is extremely difficult to predict the clinical outcome for a fetus harboring a pathogenic mtDNA mutation, especially when the mutational load is at the "borderline" (e.g. in the 50-80% range). The best correlation between prenatal and postnatal mutant load appears to be for the NARP/MILS mutation at nt-8993 [95, 96].

For women with a mtDNA mutation who still want unaffected children, one therapeutic possibility is "ooplasmic transfer" (also called "*in vitro* ovum nuclear transplantation," or IVONT [97]). In theory, a woman carrying a mtDNA mutation could have the nucleus from one of her oocytes transferred into an enucleated oocyte from a donor (containing, of course, normal mitochondria); this "hybrid" oocyte, containing the nucleus of the patient but the mitochondria of the donor, could be fertilized *in vitro* and implanted in the woman's uterus. While ooplasmic transfer has not yet been performed on a woman with a known mtDNA mutation, the procedure has been performed successfully on women who have had a history of miscarriages [98, 99].

SOME THOUGHTS ON THE TREATMENT OF MELAS

In many ways, MELAS is a paradoxical disorder. Clinically, it causes an unusual symptom not found in most other mitochondrial disorders, the eponymous strokelike episodes. In fact, although MELAS is a multisystem disorder, the high morbidity and mortality of MELAS is due in large part to its angiopathic nature. The role of blood vessels in MELAS is underscored by a striking, and paradoxical observation at the biochemical and morphological level: in most mtDNA disorders associated with mitochondrial proliferation (e.g. ragged-red fibers), the RRF contain high mutation loads and are almost always negative for COX activity. This is the case for a related mtDNA disorder, MERRF (due mainly to mutations in tRNA^{Lys}). Patients with MELAS (due mainly to mutations in tRNA^{Leu(UUR)}) also have RRF in muscle, but these are typically COX-positive, in spite of their exceedingly high amounts of mutation (around 95%). Moreover, there is mitochondrial proliferation in the blood vessels - called "strongly SDH-positive vessels," or SSV's [100] - in both MERRF and MELAS, but again, the vessels (or more precisely the vascular smooth muscle cells) are COX-negative in MERRF whereas they are COX-positive in MELAS. Both MERRF and MELAS are maternally-inherited tRNA mutation disorders, and yet the mortality due to MELAS - with COX-positive RRFs and SSVs - is far greater than MERRF, which has COX-negative RRFs and SSVs. Shouldn't it be the other way around?

Perhaps the resolution of this paradox (which will be explored elsewhere in greater detail) lies in the very nature of the biochemical observation, namely, that the elevated COX activity in blood vessels contributes to the pathology. How might this be? In other words, why would increased COX activity harm, rather than help, a tissue? One possible answer lies in the biochemistry of COX and the physiology

of blood vessels. COX is a heme-containing protein that binds oxygen much in the same way that hemoglobin does. And like hemoglobin, COX can bind other molecules in place of oxygen, including carbon monoxide, cyanide (a classic respiratory poison), and, most importantly for our discussion here, nitric oxide (NO) [101]. Interestingly, NO, via the enzyme nitric oxide synthase (NOS), is a key player in controlling blood vessel diameter, as elevated NO participates in a biochemical pathway that ultimately causes dilation of blood vessels.

We hypothesize that under conditions where the body senses a need to dilate blood vessels via the NO pathway [102], the elevated amount of COX in MELAS blood vessels "titrates out" NO so that too little NO is available to initiate vasodilation (interestingly, under normal circumstances, COX may in fact modulate NO levels to mitigate NO toxicity [103]). We propose that the temporary relative constriction of blood vessels, at least in the brain, is the proximal cause of the strokelike episodes and cortical blindness that are the features of MELAS. This would explain why, as a rule, MERRF patients do not suffer from strokes, for the simple reason that although they also have SSVs, the SSVs are COX-negative and therefore cannot bind NO. In this scenario it is not strictly necessary to specify which of the NOS isoforms - neuronal, endothelial, or a hypothesized mitochondrial-targeted NOS (mtNOS) [104] - generates the NO, since it is diffused rapidly.

If this hypothesis is correct, one could envision an approach to treating (or perhaps even preventing) the strokes by increasing the transient level of NO in MELAS brain above the "titrated-out" threshold. In fact, it was reported recently that administration of L-arginine, a NOS substrate that generates NO, to three MELAS patients resulted in some clinical improvement [105]. An even simpler way to increase NO transiently would be to administer nitroglycerin (NG) orally. Nitroglycerin is a known vasodilator that has long been used in the treatment of cardiac angina. Of course, giving NG to MELAS patients may have negative effects, especially since many patients (and their oligosymptomatic mothers) have migraine headaches, which could be exacerbated by NG. Nevertheless, we believe that the hypothesis of an interaction between NO and COX in MELAS has merit, and is worthy of further exploration.

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