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Human Complex I deficiency: Clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect

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Human NADH-ubiquinione oxidoreductase deficiency can be present in a wide variety of biochemical and symptomatic phenotypes. In fact, the observed spectrum of severity varies from fatal infantile lactic acidosis to adult onset exercise intolerance or optic neuropathy. Since most genetic diseases display a much more narrow set of indices, perhaps this wide variety of clinical presentation requires some explanation. Many of the problems that revolve around the diagnosis of Complex I defects stem from the fact that Complex I is a huge multienzyme complex of 41 separately encoded gene products derived from both nuclear and mitochondrial genomes [1,2]. This dual coding system leads to a number of difficulties, misconceptions and pitfalls surrounding etiology and diagnosis. In addition, a further complicating factor related to oxygen free radical generation seems to be an important factor in the determination of pathology in affected individuals.

1. Basic principles—the biology of Complex I

Though Complex I (NADH-ubiquinone oxidoreductase) in mammalian mitochondria appears to contain at least 41 distinct proteins, it is not known how many of them are involved directly in transport of electrons coupled to vectorial proton movements. The proteins of Complex I are traditionally divided up into flavoprotein (FP), iron protein (IP) and hydrophobic protein (HP) fractions [1,2]. Mitochondria of higher animals have seven mitochondrially encoded proteins in Complex I that are intricately associated with the inner mitochondrial membrane and are part of the HP fraction [3]. Based on the elbow or boomerang shape of Complex I observed by electron microscopy for the *Neurospora crassa* complex, the subfractionation profiles of mammalian Complex I and the homology to bacterial hydrogenase and dehydrogenase complexes, a possible arrangement of the major subunits can be predicted (Fig. 1) [4–9]. The passage of electrons goes through an FMN centre located in the 51 kDa protein, then through a 4Fe–4S centre on the same protein, through a 2Fe–2S centre in the 24 kDa protein, a 4Fe–4S centre in the 75 kDa protein and then probably through iron–sulfur centres on the 20 kDa and 23 kDa subunits [9,10]. The final acceptor of the electrons is ubiquinone although there is evidence that at least two quinone acceptance loci exist with different sensitivity to quinone analogue inhibitors [11–13]. The reaction mechanism whereby electron transfer is coupled to vectorial proton translocation is not clear [10]. There is evidence that a high titre of semiquinone radical is produced within Complex I and that this semiquinone is an integral

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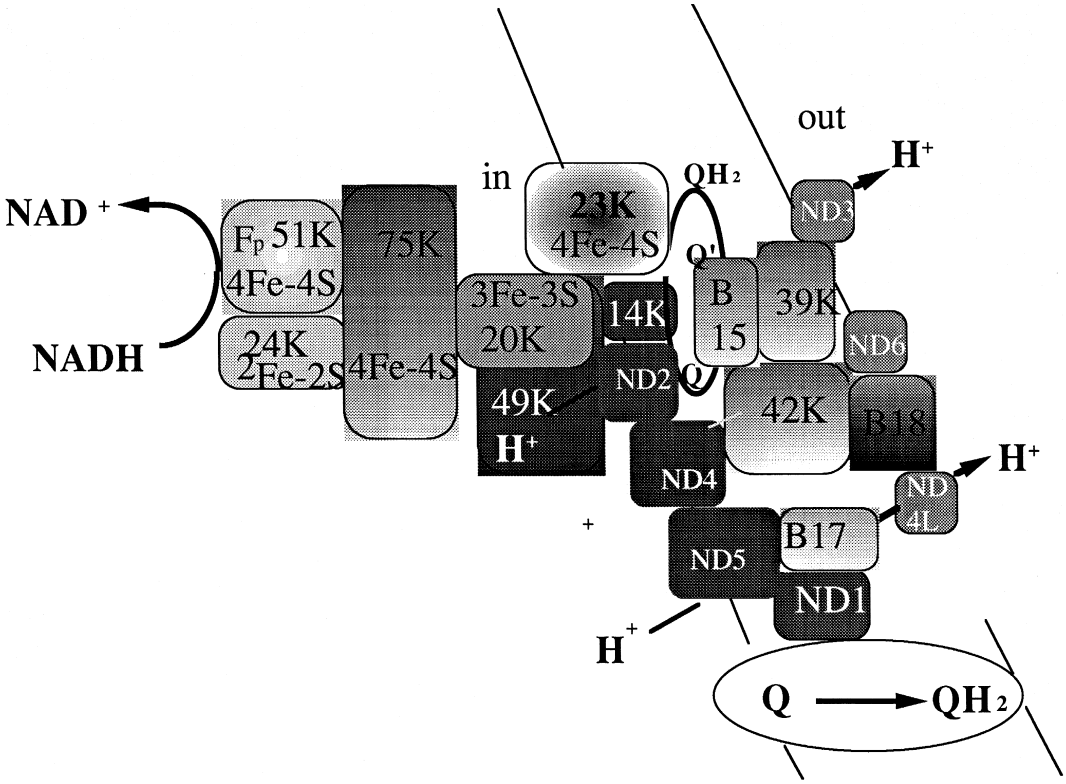

Fig. 1. Complex I of the mitochondrial respiratory chain. The complex is depicted as an elbow shaped assembly with one arm facing into the matrix and the other arm being associated with inner mitochondrial membrane. The subunits considered to be involved in the transport of electrons are the 51 K flavoprotein containing a 4Fe–4S centre, with the 24 K, 75 K, 23 K and 20 K iron sulfur proteins. Two quinone reduction sites are postulated. The mtDNA-encoded subunits are all part of the membrane arm of the complex and some of them are possibly involved in proton pumping functions.

part of the reaction mechanism [14,15]. In order to produce vectorial proton movement via the movement of electrons it is probable that the FeS centres associated with the membrane arm of the complex, i.e., the two centres in the 23 kDa TYKY subunit, catalyse a vectorial movement of protons coupled to semiquinone oxidation/reduction [10]. This becomes important later in the consideration of free radical production in patients with Complex I deficiency.

2. The supernumery subunits

Only one of the mtDNA encoded subunits appears to have a defined role. The ND1 subunit has been shown to be the binding site for both rotenone and for photoaffinity-labelled ubiquinone analogues, suggesting that this is the terminal acceptor for quinone reduction within the membrane portion of Complex I [13,16]. The function of the remaining six mtDNA encoded subunits remains obscure, though there are some important clues from the patients affected with mutations in the ND subunits. It has been demonstrated that patients with mutations in the ND1 gene have defective electron transport while patients with ND4 defects have no such defect but are unable to carry out ATP synthesis from NAD linked substrates in isolated mitochondria [17]. This implies that a coupling function such as proton translocation is impaired in ND4 mutations. Since ND2, ND4 and ND5 possess a certain amount of homology, it is not unreasonable to propose that these three subunits are all involved in proton transport in some way [18]. The ND6 subunit is probably also involved in electron transport events since mutations in this subunit affect electron flow in Complex I [18,19].

Of the 34 nuclear encoded subunits in the bovine complex, the 75, 51, 49, 30, 24, 23 and 20 kDa

subunits are equivalent to proteins present in bacterial Complex I [3]. There are another 27 proteins in the mammalian complex as identified by Walker and others [20–33]. These proteins are unusual in that nine of them have blocked N-termini and no leader sequence for import, while another nine proteins have no leader but an open N-terminus. Another nine have the more expected cleavable leader sequence designating import into the matrix. The majority of the leaderless peptides appear to be part of the hydrophobic membrane fraction (Table 1).

Few functions have been assigned to the supernumery subunits not held in common with *Paracoccus*. The SDAP protein, a small 10 kDa protein with homology to acyl carrier proteins, appears to have a function in mitochondrial phospholipid metabolism [40] and its removal in yeast and *Neurospora* seems to impair a number of mitochondrial functions as the intramitochondrial lysophospholipid content is increased. The phosphorylation of at least four subunits of Complex I have been described, namely the 42 kDa, 29 kDa, 18 kDa AQDQ subunit and the MWFE

Table 1
Nuclear-encoded subunits of Complex I

Name	M_r	Leader	N-terminus	Function	Chromosome	References
75 K FeS	75	+		4Fe–4S	2q33–34	[21,22]
51 K Fp	51	+		Flavin, 4Fe–4S	11q13	[25,34]
49 K	49	+		–	Chr 1	[26,35]
42 K	42	+		–	–	[30]
39 K	39	+		Phosphorylation	12p	[30]
30 K	30	+		–	–	[27]
24 K FeS	24	+		2Fe–2S	18p11.3	[27]
23 K FeS (TYKY)	23	+		4Fe–4S	11q13	[36]
B22	22	–	*N*-Acetyl	–	8q13.3	[37]
PSST	20	+		3Fe–3S	19p13.2	[29,38]
PDSW	22	–		–		[31]
PGIV	19	–		–	–	[28]
ASH 1	18	+		Phosphorylation?	–	[33]
SGDH	18	+		–	–	[33]
B18	18	–	*N*-Myristoyl	Phosphorylation?	–	[33]
18KIP (AQDQ)	18	+		Phosphorylation?	–	[33]
B17	17	–	*N*-Acetyl	–	–	[33]
B15	15	–	*N*-Acetyl	–	–	[33]
B14.5a (ASAT)	14.5	–		Q-binding	17	[24]
B14.5b (MMTG)	14.5	–		–	–	[24]
B14	14	–	*N*-Acetyl	–	–	[33]
B13	13	–	*N*-Acetyl	–	–	[33]
15 K(1P)	15			–	–	[33]
B12	12	–	Unknown Mod	–	–	[33]
B8	8	–	*N*-Acetyl	–	–	[33]
13 K (1P)(DDGD)	13	+		–	–	[33]
MLRQ	9	–		–	–	[33]
B9	9	–	Unknown Mod	–	–	[33]
AGGG	10	+		–	–	[33]
MWFE	6	–		Phosphorylation	Xq24	[33,39]
MNLL	5.5	–		–	–	[33]
KFY1	5	–		–	–	[33]
10 K (1P)(SHES)	10	+		–	–	[32]
5DAP	10	–		Acyl Carrier Protein	–	[22]

References are given for the subunit determined from *Bostaurus*, except for those with chromosomal allocations which have human cDNA clones reported. See text for details.

subunit [41]. Two of these proteins, namely the 18 kDa AQDQ and the MWFE have been shown to be phosphorylated by cyclic AMP dependent kinase [41,42]. While there is some evidence that there is a cyclic AMP dependent kinase activity which is mitochondrial in location, it almost certainly resides on the outer membrane [43]. It is not clear what phosphorylation does to the activity of Complex I. It seems that these subunits can be phosphorylated in an atractyloside sensitive manner in whole mitochondria, but to what purpose is not clear at this time [44,45].

2.1. Chromosomal allocation

We and others have isolated human cDNA clones for the 51 kDa flavoprotein, the 75 kDa $Fe^{2+}S$ protein, the 39 kDa, 24 kDa, the B22, the 20 kDa $Fe^{2+}S$ protein, the 14.5 kDa and 6 kDa protein and mapped them by in situ hybridization to chromosome spreads [21–23,38,34,35,46,36,37,39]. Since there are few if any families with X-linked Complex I deficiency, these allocations are not particularly helpful, but it is possible that a patient with a chromosomal rearrangement and Complex I deficiency may lead to the definition of the gene(s) responsible for this defect.

3. Diagnosis of Complex I deficiency

3.1. Nuclear-encoded defects in Complex I

Isolated Complex I deficiency appears to be an autosomal, or possibly in a few cases, an X-linked disease in which patients can suffer from a wide constellation of symptoms [37,39,47]. In the first case reported, the case of Moreadith et al. [48], e.s.r. analysis of muscle mitochondria showed an $Fe^{2+}S$ protein to be reduced [49]. We showed that the defect was not as pronounced in cultured skin fibroblast mitochondria from this patient but was detectable by the altered redox state of the cells [35]. A second case described by Hoppel et al. [50] also died in infancy and was demonstrated to have deficient rotenone-sensitive NADH cytochrome *c* reductase and NADH duroquinone reductase in liver mitochondria, muscle mitochondria and skin fibroblasts. These two patients at autopsy showed evidence of mitochondrial proliferation, ventricular cardiomyopathy and spongy degeneration of the brain. Both had a chronically raised level of blood lactate with intermittent severe acidosis. Two very well documented patients described by Fujii et al. [51] with Leigh disease had muscle Complex I deficiency. Both infants displayed damage to the putamen, thalamus and substantia nigra on CT scan of the brain. This agreed with data we reported on 24 patients exhibiting Complex I deficiency, half of which had Leigh disease with possible associated cardiomyopathy [47,52]. More recently Turnbull's group in Newcastle and Thorburn's group in Melbourne has confirmed our findings that isolated Complex I deficiency is the most common mitochondrial respiratory chain defect [53,54].

Our ability to identify large numbers of patients with Complex I deficiency stems from a screening test we perfected some 12 yr ago. The screening test we invented to identify fibroblast cultures with respiratory chain defects depends on the measurement of lactate to pyruvate ratio after incubation of the cells with glucose for a one hour period [55–57]. We showed that for both cytochrome oxidase deficiency and Complex I defects there is a relationship between the residual activity of the enzyme and the L/P ratio [9,47]. This test does not detect mtDNA derived respiratory chain defects except in rather unusual circumstances (ρ^o or mtDNA depleted fibroblasts) and is particularly useful in the case of Complex I deficiency which is not easy to demonstrate in cultured cell systems by enzyme analysis. This is largely because of heteroplasmy so that the abnormal redox state is not expressed unless % mutant mtDNA goes above 85% or 90% [47,57,58].

Correlation of the L/P ratios and clinical data and data from clinical descriptions of Complex I deficiency diagnosed in muscle has led to the classifications shown below (Table 2) [47,52–54]. Our study of Complex I defects has also shown us that muscle mitochondria will often show severely deficient rotenone-sensitive NADH-cytochrome *c* reductase, while fibroblast mitochondria exhibit a less pronounced deficiency [47]. Despite the less pronounced deficiency in fibroblast mitochondria, the defect is usually manifest in a high L/P ratio [9,47]. The clinical spectrum of patients exhibiting Complex I deficiency as judged by the above criteria is a wide one. The clinical course we found to be most com-

Table 2
The clinical presentation of isolated Complex I deficiency in 60 patients

Incidence	Major features	Number of patients
Most common	Leigh's disease ± cardiomyopathy (WPW) or progressive deterioration—slow/fast	35
Fairly common	Fatal infantile lactic acidosis cystic changes in white matter	15
Uncommon	Hepatopathy and tubulopathy	4
	Cardiomyopathy and cataracts	2
	Cataracts and developmental delay	2
	Neonatal lactic acidemia	2
	Normal development	

mon was that of Leigh disease with onset from three months to ten years of age [47,56]. The patient's muscle exhibits mitochondrial hypertrophy but true ragged red fibres are rarely seen. We found a considerable incidence of ventricular cardiomyopathy (~ 40%) in patients with Complex I defects not seen in other patients with Leigh disease [47,56]. All of these patients were tested for the mtDNA mutations responsible for MERRF and MELAS and for mutations in other mitochondrial tRNAs but no cell lines were positive. The information gathered from families with Complex I deficiency showed that there were equal numbers of males and females affected, the pattern being suggestive of autosomal recessive disease with possibly a few cases being X-linked [47,56]. A second category frequently seen is fatal neonatal lactic acidosis, where the patient has such a severe defect that the acidosis that results from the lactic acid production exceeds the buffering capacity of the body [47,59,60]. Severe cases of Complex I deficiency were found by Western blotting not to assemble Complex I [47]. More recently we described three less common categories: the presentations of those patients with hepatopathy and tubulopathy, those with very mild symptoms such as exercise intolerance, and those with cardiomyopathy and cataracts [52]. In the first category there is an unusual involvement of the liver and kidney sometimes accompanied by neurodegeneration. In the second category there is normal development but with some exercise intolerance and sometimes congenital cataracts [52]. The third is described below. Western blots with anti-Complex I antibody failed to show reproducible differences specific to any one subunit, though the display of cross-reacting subunits is limited to eight or nine proteins [47,52]. Techniques using Native Blue PAGE followed by SDS/PAGE in the second dimension have been more recently developed which resolve more than 20 subunits of Complex I [61]. The technique was used to show a reduction in several Complex I subunits in a case of Complex I deficiency with lactic acidosis, cerebellar cystic lesions and hypertrophic cardiomyopathy [62]. There is some correlation between the redox state of cells measured by L/P ratio and the magnitude of depression of NADH-cytochrome *c* reductase (Fig. 2) for Complex I deficient patients but this is a rather loose association and does not account for clinical severity of the disease [52].

3.2. The syndrome of cardiomyopathy and cataracts

McKusick (entry 212 350) lists a number of cases in which bilateral congenital cataracts are associated with hypertrophic ventricular cardiomyopathy (HC), exercise-induced hyperlactatemia and mitochondrial myopathy [64]. Three unrelated sibships produced seven affected children out of 22 in which symptoms of varying severity were apparent [65–67]. Again, there was no CNS involvement but many affected family members had chronic progressive cardiomyopathy with early death as the endpoint. We have studied a kindred from Alberta who produced three such affected children in a double cousin marriage. They had congenital cataracts, cardiomyopathy and chronic lactic acidemia [68]. Inheritance pattern in these families is consistent with an autosomal recessive gene, and paternal transmission rules out any role of mitochondrial DNA. We have shown that in skin fibroblasts from affected family members, there is a problem affecting the redox state of the mitochondria at the level of Complex I (NADH-CoQ reductase) [69]. We also have good evidence that this

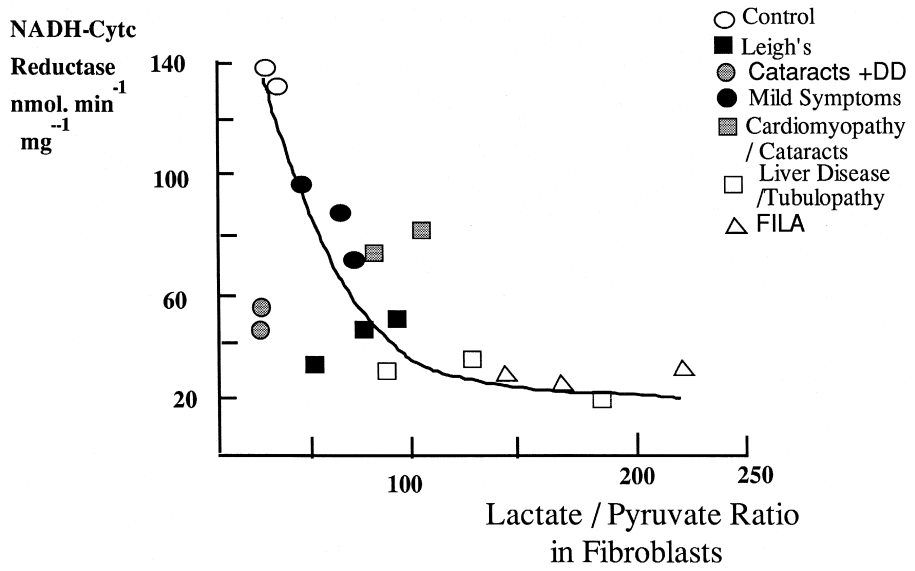

Fig. 2. Dependence of lactate to pyruvate ratio on the residual activity of complex I + III. Fibroblast mitochondrial NADH-cytochrome c reductase (rotenone-sensitive) is plotted as a function of the observed lactate to pyruvate ratio in the whole cell cultures. Data from Ref. [63].

effective blockage within Complex I leads to an excessive rate of free radical production and hypersensitivity of the cell cultures to toxicity from menadione and other free radical amplification agents [59]. We showed that the presence of unusual lipid alcohols resulting from peroxidation of lipids in these cells is also another indicator of free radical damage [64]. We recently showed that a massive induction of the mitochondrial Mn^{2+} superoxide dismutase takes place in these cells [66].

3.3. Defects encoded in mtDNA

There has been a substantial increase in our knowledge of how deletions, duplications and point mutations in mtDNA can influence the expression of the components of the respiratory chain [9]. There are basically two ways in which mitochondrial DNA defects can produce disease: by interference with the process of mitochondrial protein synthesis, and by interference at specific sites in the process of oxidative phosphorylation. Both can give rise to measurable defects in Complex I in muscle mitochondria.

In the former group the proliferation of subsarcolemnal mitochondria gives muscle histology a characteristic appearance with the Gomori trichrome stain such that a proportion of the fibres appeared to be stained red around the periphery and the fibre margin appears to be convoluted [70–72]. These fibres are called ‘ragged red fibres’ and only a small group of diseases characteristically display this form of muscle pathology. The differential diagnosis for these ragged red fibre diseases is complex because they usually involve systems other than skeletal muscle, usually components of the central nervous system [72]. The ragged red fibre diseases that have been reported as having respiratory chain defects are: mitochondrial encephalomyelopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonus epilepsy with ragged red fibres (MERRF), Kearns–Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), myopathy and cardiomyopathy (MMC) and mitochondrial myopathy (MM) [70].

This group of diseases has now been well characterized at the molecular level and involves point mutations in mitochondrially-encoded tRNA species for leucine and isoleucine in the case of MELAS, tRNA for lysine in the case of MERRF and major mtDNA deletions in the case of Kearns–Sayre or CPEO [73–77]. This group of diseases has recently been reviewed by Wallace [73] and many of them show either a decrease in Complex I activity, Complex IV activity or both [78–80]. The mechanism

related to the relative decrease in respiratory chain complexes depends very much on the species of tRNA affected and the contribution of that tRNA to the synthesis of mtDNA encoded polypeptides of the respiratory chain. Thus mutations in the $tRNA^{Leu(UUR)}$ produce major Complex I defects in the muscle of affected patients because of the high titre of leucines encoded in the ND subunits of Complex I [81]. On the other hand, mutation of $tRNA^{Gly}$ at 9997 produces mainly Complex IV deficiency because of the high titre of glycines in the cytochrome oxidase subunits I, II and III [82]. Such influences plus the distribution of heteroplasmy of the mutation in various tissues leads to rather different phenotypes which depend on the tRNA involved. Several $tRNA^{Leu(UUR)}$ mutations give rise to MELAS while the $tRNA^{Gly}$ mutation produces a hypertrophic ventricular cardiomyopathy (HVCM). Some $tRNA^{Leu(UUR)}$ mutations can produce also an HVCM or a mitochondrial myopathy alone and the reasons for this are not completely understood [73,83].

A number of point mutations in the mtDNA regions encoding respiratory chain polypeptides have been found to be present in patients with Lebers Hereditary Optic Neuropathy (LHON). While the majority of patients have a mutation at 11 778 in ND4 which changes an Arg to a His [73], other mutations have been found which affect ND1 (3460) or ND6 (14 484) [84], cytochrome *b* [73] and cytochrome oxidase [85]. While none of these patients show ragged red fibre disease or lactic acidemia, they show that the same clinical phenotype can result from the impairment of the respiratory chain at several different sites. It is also established that LHON is maternally inherited. In contrast to the mutation affecting ND4 which does not produce any measurable change in NADH-CoQ reductase activity, both the ND6 and the ND1 mutation shows considerably reduced activity [86]. All three defects, however, show impaired rates of ATP synthesis by isolated mitochondria indicating that ND4 is probably involved in coupling electron flow to ATP synthesis [86].

The implications of these findings are that: (1) there can be deleterious mutations in Complex I which are subtle and are without detectable changes in enzyme activity and without the patient displaying lactic acidemia, (2) there may be Complex I genetic defects that phenotypically do not resemble other mitochondrial diseases. Two more deleterious mutations have now been described, one in ND4 at 11 696 and one in ND6 at 14 459 which in addition to LHON produce in some family members the clinical symptoms of dystonia with accompanying lesions in the caudate and putamen visible on MRI [87,88]. These mutations present in the heteroplasmic state can cause depressed rates of ATP synthesis and electron transport in isolated mitochondria [87,88]. Those family members with high percentages of mutant mtDNA showed more severe symptoms and an earlier age of onset.

3.4. Free radical generation by the respiratory chain

It has been known for some time that free radicals, especially the superoxide ion O_2^-, are generated by respiratory chain activity. Indeed, it has been estimated that the fate of 1%–2% of all electrons passing down the electron transport chain is to be diverted into the formation of superoxide radicals [89]. Interest has been stimulated by the discovery that free radical generation may be involved in selective cell death (apoptosis) and that radicals generated from the respiratory chain contribute to this [90]. Indeed mitochondrial changes are central to several modes of apoptosis. Superoxide itself probably arises from interaction between molecular oxygen and a species of semiquinone (UQ_{10}) which is a natural intermediate in one electron reduction events in electron transport through Complexes I and III [89]. Little is known about the processes that are involved in this generation of superoxide though it is reasonable to guess that it occurs at the juxtaposition of an electron carrier such as an FeS protein and a site of quinone reduction within the respiratory chain complexes [91,92]. Semiquinone radicals generated in the vicinity of the TYKY 23 kDa FeS protein would be a prime candidate as electron donors for superoxide formation. It is not known how such a process is controlled at high and low respiration rates, what the sidedness is of the process with respect to the mitochondrial membrane and how excessive superoxide production is set in motion in response to cytokines. These questions are awaiting resolution.

Removal of free radicals generated in the course of normal electron transport is obligatory if cellular damage is to be avoided. Superoxide is removed by

the enzyme Mn^{2+} superoxide dismutase which is mitochondrial, and Cu^{2+} Zn^{2+} superoxide dismutase which is extramitochondrial and also exists in an extracellular form [79,63]. Both superoxide dismutases produce hydrogen peroxide which unlike superoxide is probably diffusable across biological membranes and is reasonably stable. Thus hydrogen peroxide produced both inside and outside of the inner mitochondrial membrane can be removed by the activity of either catalase or glutathione peroxidase (Fig. 3). Dependence on the latter enzyme may then be linked to the availability of glutathione. While we have concentrated our efforts on defining the role of MnSOD [63] it is possible that respiratory chain free radical production is relevant to other genetic defects where superoxide dismutase is involved, such as trisomy 21 and Amyotrophic Lateral Sclerosis. It has been suggested that pathophysiological events result in these cases from over-activity of CuZnSOD particularly in the reverse direction where mutant CuZnSOD can catalyse formation of hydroxyl radical from hydrogen peroxide generated in the mitochondria. We therefore found it of interest to investigate: a) whether superoxide is produced in excess in patients who have defects within Complex I, and b) whether these free radicals generated actually participate in producing cell damage and cell death in the individuals affected [69,63].

3.5. Free radical production in Complex I deficiency

We then went on to examine mitochondria isolated from cultured skin fibroblasts from healthy individuals (controls) and patients with Complex I (NADH-CoQ reductase) deficiency of the mitochondrial respiratory chain. The patients included those with fatal infantile lactic acidosis (FILA), cardiomyopathy with cataracts (CC), hepatopathy with tubulopathy (HT), Leigh disease (LD), cataracts and developmental delay (CD), and lactic acidemia in the neonatal period followed by mild symptoms (MS). Production of superoxide radicals were measured using the luminometric probe lucigenin with isolated fibroblast mitochondrial membranes on addition of NADH [69,63]. Superoxide production rates were highest with CD and decreased in the order CD ≫ MS > LD > CONTROL > HT > FILA = CC [63] (Fig. 4). Quantity of Mn superoxide dismutase (MnSOD), as measured by Elisa techniques, MnSOD activity and Northern blots, however, was highest in CC and FILA and lowest in CD [63]. The induction of MnSOD appeared to be a function of the disturbance in

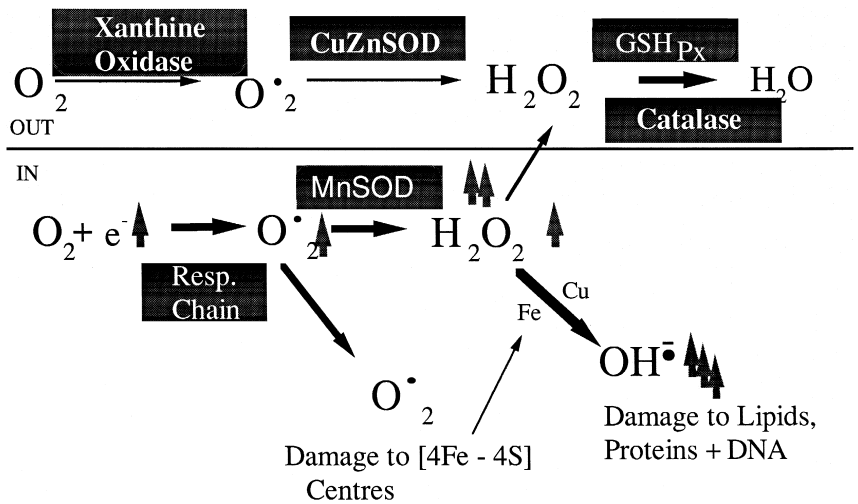

Fig. 3. Relationship between superoxide production by the respiratory chain and xanthine oxidase to its disposal. Superoxide can be removed by either manganese superoxide dismutase (MnSOD) or copper zinc superoxide dismutase (CuZnSOD) to produce hydrogen peroxide which is then removed by catalase or glutathione peroxidase. The two compartments are depicted as the inside of the mitochondrial matrix space (in) and the space outside of the mitochondrial matrix (out). When superoxide production exceeds the capacity of MnSOD to remove it, damage to FeS centres may result in releasing free iron. More severe damage to DNA, lipids and proteins can occur if hydrogen peroxide is not removed and forms hydroxyl radicals by the Fenton reaction.

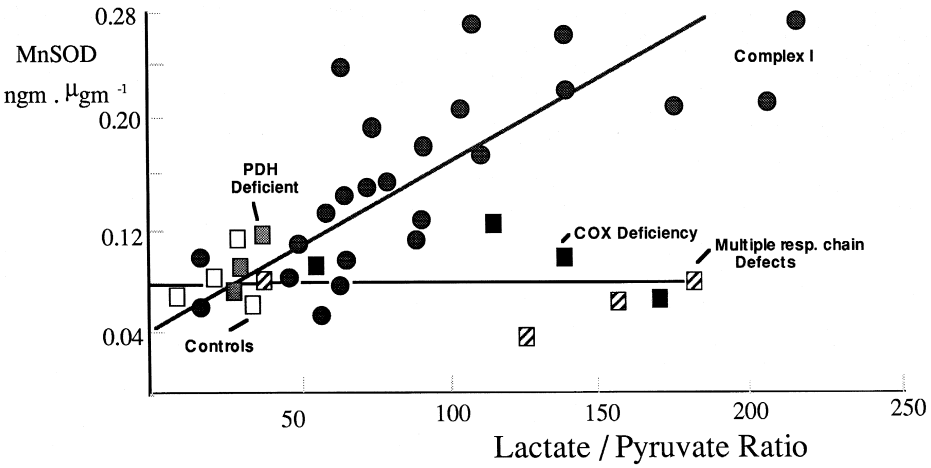

Fig. 4. The relationship of induction of MnSOD to the redox state of skin fibroblasts as measured by lactate to pyruvate ratio in patients with complex I deficiency. Plots of MnSOD quantity versus L/P ratio showed an inverse relationship for patient cell lines with complex I deficiency. For patient fibroblast mitochondria with pyruvate carboxylase, pyruvate dehydrogenase, complex III or complex IV there was no correlation. Data taken from Ref. [63].

redox state in these cell lines but was only seen in Complex I deficiency and not in other respiratory chain defects involving Complex III or Complex IV [63] (Fig. 4).

We hypothesized that oxygen radical production is increased when Complex I activity is compromised. However, the observed superoxide production rates are modulated by the variant induction of MnSOD

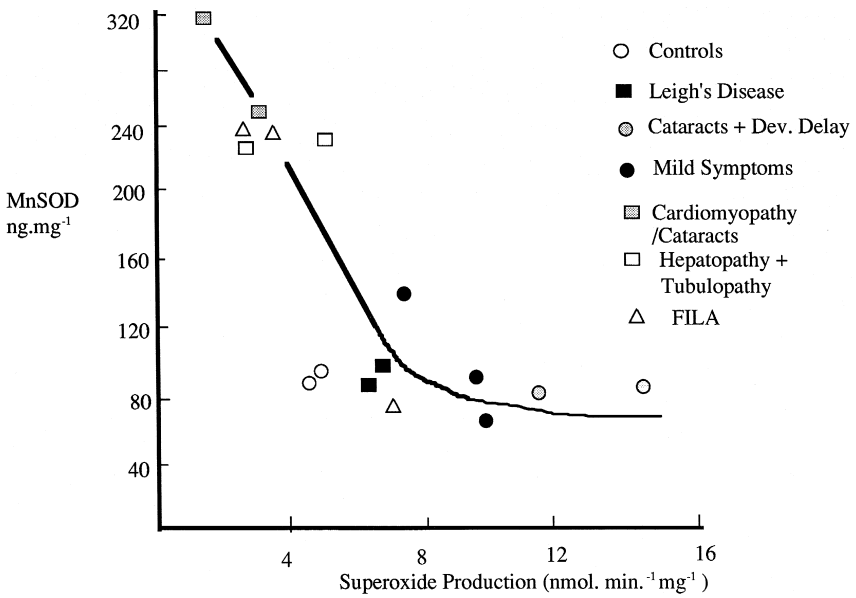

Fig. 5. The reciprocal relationship between superoxide production and manganese superoxide dismutase for mitochondria prepared from patient skin fibroblasts. Manganese superoxide dismutase measured by immunoreactivity is plotted as a function of the observed rate of superoxide production as measured by lucigenin fluorescence. Data is from Ref. [63].

which decreases the rate, sometimes below that seen in control fibroblast mitochondria. The variant induction of MnSOD in turn we show is most likely a function of the change in redox state of the cell experienced as a result of the Complex I defect. Thus a plot of MnSOD present in mitochondria as a function of redox state estimated by L/P ratio is a linear one (Fig. 4). Plots of MnSOD quantity versus superoxide production showed an inverse relationship for most conditions with Complex I deficiency [63] (Fig. 5).

3.6. Relationship of MnSOD status to clinical severity

What was important from the patient prognostic point of view is that while we could find little correlation between the severity of symptoms and the severity of the defect in Complex I activity, there was a definite tendency for the patients with high induction of MnSOD to be more severely affected than those with a low induction [63]. This suggests the following scenario: mutations in Complex I subunits are of two types—those that block the flow of electrons before the first quinone reduction centre and those that block after. Those that block before will result in impairment solely of oxidative phosphorylation while those that block after will result in both impairment of oxidative phosphorylation and generation of excess superoxide. If the superoxide production is high and the redox state is fairly reduced, induction of MnSOD occurs. This produces excess hydrogen peroxide which can in turn act as a substrate for the Fenton reaction (Fig. 3). The final result is the production of damaging hydroxyl radicals which in affected individuals leads to high rates of morbid-

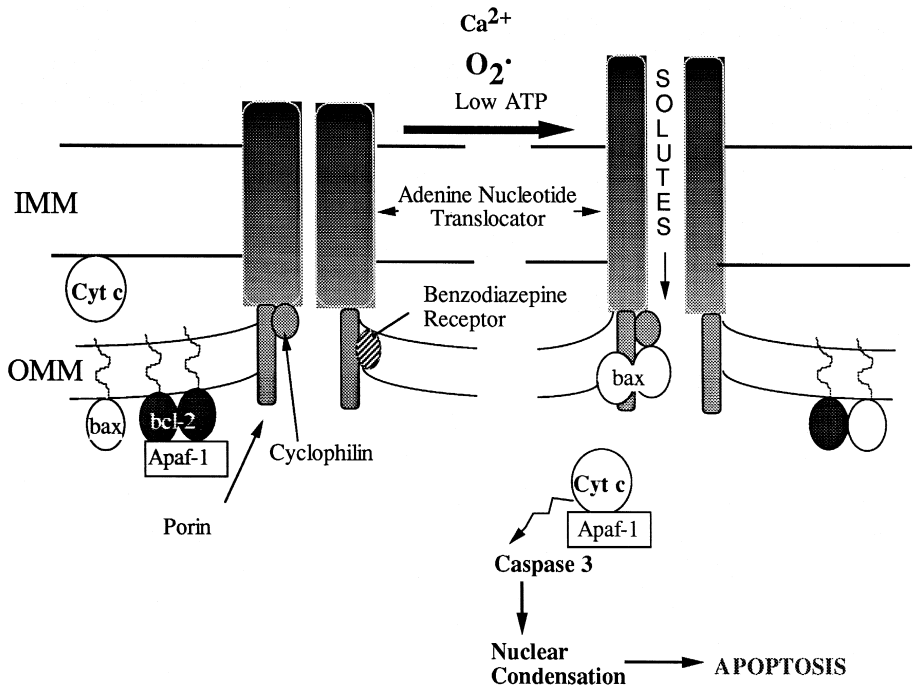

Fig. 6. The mitochondrial permeability transition and bcl-2. The adenine nucleotide translocator in the inner mitochondrial membrane undergoes a reconfiguration triggered by oxygen free radicals or low ATP or increased Ca^{2+} so that a pore develops allowing solutes to exit from the matrix space. The transition is prevented by cyclosporin A so it is thought to involve a *cis–trans* prolidyl transpeptidase (cyclophilin). The transition is also prevented by the activity of the bcl-2 protein. This protein is anchored in the outer mitochondrial membrane at its C-terminus. Bcl-2 forms dimers with itself or with the bax protein and the bcl-2 dimers bind the apaf-1 protein. When bax is in excess the pore formation is favoured, when bound to bcl-2 it is prevented. Pore opening is thought to be an early event in apoptotic cell death. Concomitant with the opening of the pore or as a result of it, cytochrome c is released to the cytosol where in conjunction with the apaf-1 protein it activates Caspase 3. This in turn promotes apoptosis by precipitating nuclear condensation and inactivation of DNA repair enzymes.

ity and mortality. When we tested this hypothesis in whole cells it was found that high levels of MnSOD did indeed lead to high rates of hydroxyl radical production and lipid peroxidation [69].

3.7. Bcl-2 expression in Complex I deficiency

The bcl-2 family of proteins is intimately associated with the process of apoptosis [93,94]. Apoptosis is a form of 'cellular suicide' which comes into play usually when a cell is not needed morphologically or when a cell is damaged to a point where it will not recover [94,95]. Such mechanisms are also operative when cells are attacked by viruses so that the cell can be put out of action before replicating large numbers of virus particles. One of the major pathways for induction of apoptosis is set in motion by a mitochondrial event. Evidence is growing that one of the early events in apoptotic cell death involves the opening of the mitochondrial transition pore (MTP) perhaps triggered by a phosphorylation event or by action of oxygen free radicals [94,95]. The functioning of the transition pore complex is thought to be mediated by a major change in the configuration of the adenine nucleotide translocator protein, which occurs when $\Delta\psi$ is decreased or oxygen free radicals, high levels of Ca^{2+} or low ATP (>1 mM) conditions prevail such that mitochondrial solutes can leak out [94–97] (Fig. 6). This pore opening is sensitive to Cyclosporin A and thus involves a *cis–trans* Peptidyl-prolyl isomerase. Bcl-2 somehow delays the opening of these MTPs by some process unknown, perhaps delaying the decay of the membrane potential [96,97]. Further research is required to fully understand the connection between free radical appearance, which seems to trigger opening of MTP, bcl-2 expression and apoptosis.

This opening of the MTP also seems to be associated with a process that allows cytochrome *c* to dissociate from the inner membrane to leak in to the cytosol [96]. Once the MTP has occurred and cytochrome *c* is released into the cytosol, in conjunction with the Apaf-1 protein [98] it activates the cytosolic Caspases (ICE proteases) which proteolytically cut DNA repair enzymes such as PARP and nuclear DNA undergoes condensation [96,98] and degradation. This cascade can be prevented by the bcl-2 gene product, a protein which is attached to the outer membrane of mitochondria. The bcl-2 protein itself is thought to be protective under most circumstances except under certain conditions of phosphorylation [99]. Several important observations have suggested a role for mitochondria in the control of apoptosis [100]. The bcl-2 protein is well situated in this regard as a protective agent. It is localized to the outer mitochondrial membrane [101,102], and without its C-terminal transmembrane (TM) domain, which acts as a membrane anchor, the anti-apoptosis role of bcl-2 is non-functional [103]. Increased expression of bcl-2 prevents the release of cytochrome *c* and has effects on the release of Ca^{2+} from the mitochondria [104,105] which in turn delay or prevent induction of the mitochondrial permeability transition.

Oxygen free radical production by mitochondria induced either by cytokines or by cellular damage may be one of the major triggers of the apoptotic process [104–107]. Increased apoptotic death can be induced by free radical amplifiers such as menadione [63]. Paradoxically bcl-2 appears to be induced by increased oxygen free radical production at the same time as it has a diminishing effect on apoptosis [104–107]. Bcl-2 may also decrease production of free radicalsin certain circumstances [105]. Thus bcl-2 is induced by the Complex I inhibitor rotenone [108] and is also increased in cells with respiratory chain defects involving Complex I [109,110]. It is especially increased in cell lines with poor MnSOD in-

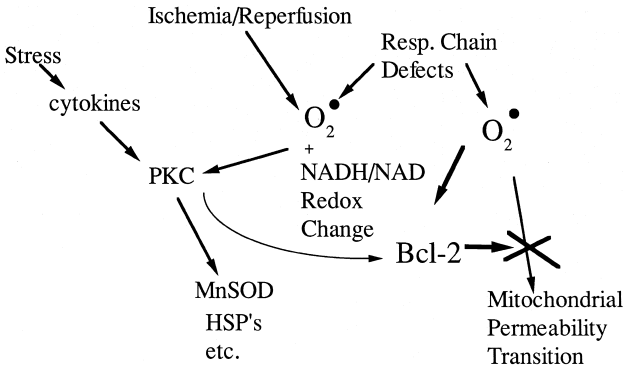

Fig. 7. Mechanisms for the induction and modulation of manganese superoxide dismutase and bcl-2. Superoxide produced as a result of respiratory chain defects or ischemia/reperfusion injury will induce either MnSOD (if the NAD/NADH redox state is changed) or bcl-2. Alternatively cytokines through a protein kinase C-dependant mechanism can induce MnSOD or modulate bcl-2 by phosphorylation.

duction [109]. MnSOD induction by superoxide and redox state is probably mediated through one of the species of protein kinase C [111] while bcl-2 induction and phosphorylation proceeds through a similar though different route involving the stress activated kinases [112–114]. Thus as with the protective up-regulation of MnSOD, the bcl-2 gene family may be induced to rescue these cells from the continuous exposure to increased ROS and their potential to induce cellular damage. The difference between the two systems, bcl-2 and MnSOD, seems to depend on relative changes in redox state such that cell lines showing big increases of superoxide with small disturbances of the NAD/NADH ratio have a higher tendency to induce bcl-2 (Fig. 7).

4. Conclusion

Many groups are trying to find the mutations in the nuclear and mitochondrial genomes that give rise to Complex I deficiency and that may give some insight into the molecular etiology of the pathogenetic mechanisms involved. At the same time information about the defense mechanisms that operate in these compromised cells is beginning to emerge that may be actually as useful as knowledge of the mutation in terms of future attempts at therapy of these disorders.

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