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Modelling the effects of age-related mtDNA mutation accumulation; Complex I deficiency, superoxide and cell death

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Abstract

Deleterious mitochondrial mutations accumulate during normal human aging in postmitotic tissues. How these mutations affect aging cells is currently unknown. This issue has been addressed in two ways. The first is to determine the likeliest effect of random mutations in the mitochondrial genome, and of the 4977 bp deletion and MELAS point mutation that rise in frequency with age. The results indicate that Complex I is statistically much more likely to be affected than any other product of the mitochondrial genome. We have also attempted to model Complex I deficiency in animals with the drug MPTP, a specific inhibitor of Complex I. We find that MPTP causes massive damage in brains of mice with a genetic deficiency in the mitochondrial superoxide dismutase, MnSOD, but less in mice that overexpress the enzyme. We conclude from these data that MPTP-induced cell death must be mediated through an increase in the steady-state concentration of superoxide anion in mitochondria. Since the likeliest target of mitochondrial mutation is Complex I, deficiency of which causes MnSOD-inhibitable lethality, we propose that rising mtDNA mutations with age will cause an increase in superoxide-mediated cell death. Such a mechanism for age-related cell death has the potential to explain several age-related phenotypes.

Keywords: Aging; Mitochondrion; Mutation; Superoxide; Apoptosis; Complex I

1. Introduction

Several degenerative diseases occur in the aged, and the molecular basis of most of these are still unexplained [1]. Many of the phenotypes of age-related degenerative disease are associated with deficiencies of function in postmitotic cells, including age-related hearing loss, progressive muscle weakness, ataxia, dementia, and cardiomyopathy. It is difficult to explain such deficiencies in the context of one popular model of aging called replicative senescence, in which cells reach a replicative barrier at a particular number of cell divisions, because the nuclear genomes of the cells which fail in these age-related degenerative diseases have usually not replicated since parturition.

By contrast, a mitochondrial model for aging may explain how aging phenotypes arise in postmitotic tissues [2–7]. Unlike the nucleus, the mitochondrial genome continues to replicate in postmitotic cells about once per month [8], and so the average mtDNA in a neuron of an 84-year-old may have undergone approx. 1000 replications. In addition to vulnerability to mutation that is the result of replication, mtDNA is located in the mitochondrial matrix, the site of generation of reactive and mutagenic oxygen species [9,10].

Such considerations prompted the inspection of mtDNA of normally aging humans for particular deleterious mutations which had previously only been described in persons with mitochondrial genetic disease [3–7]. It was shown that mitochondria exhibit more than 100-fold increase in mutation frequency with age in heart and brain [3]. This level of increase in a particular biomarker of aging was unprecedented in aging research. Mitochondrial DNA mutations occur with a strikingly characteristic tissue specificity, in postmitotic cells of aging humans [7]. Cells of the substantia nigra contain the highest level of mtDNA mutation in the brain [11–13], and these cells die earlier in Parkinsonian individuals than in normally aging humans. Death of nigral cells can be specifically induced by the mitochondrial Complex I poison MPTP [14].

The aim of work presented here is to determine what effect a rise in mitochondrial mutations with age may have on cells, by calculating which mitochondrially-encoded enzymes are most likely to be affected by random mutations, and by two age-associated mutations which have been reported to rise in frequency in aging human tissue.

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Preliminary attempts to model Complex I deficiency in animals are also described. A model for how mitochondrial superoxide may mediate death of Complex I-deficient cells is presented, as is a model for how early cell death mediated by mitochondrial superoxide could induce age-related degenerative disease.

2. Materials and methods

2.1. Calculation of mtDNA bases encoding mitochondrial proteins and UUR and CUN codon usage, mutant MnSOD mice and SOD activity measurements

Calculations were based on the Cambridge mtDNA sequence [15]. Spectrophotometric and gel measurements of MnSOD activity are as described [16]. All mice used were from the Jackson Laboratory.

3. Results

3.1. Random mutations in coding mtDNA are most likely to occur in Complex I

The mitochondrial genome encodes 13 proteins, ND 1-6, ND4L, COI, COII and COIII, ATPase 6 and 8, and cytochrome *b* [15]. Seven of the 13 proteins are subunits of Complex I. The number of mitochondrial base pairs directly encoding protein (counting the overlap of ATPase 6 and 8 messages once) is 11320. Of those 11320 bases, bases directly encoding Complex I subunits make up 6338 or 56%. Therefore a random mutation in any part of the mitochondrial genome encoding protein is more likely to occur in Complex I than any other mitochondrially encoded protein (Table 1). Mutations in ribosomal RNA and tRNA genes which partially decrease the efficiency of translation are also more likely to affect Complex I function because of the numerical dominance of Complex I transcripts.

3.2. The most frequent mtDNA deletion eliminates more subunits of Complex I than any other mitochondrial enzyme

The mtDNA deletion that occurs most frequently in Kearns-Sayres Syndrome [17] also occurs in postmitotic cells of aged humans [3–7]. This deletion erases coding information for four subunits of Complex I, but only one subunit of Complex IV, and one subunit of ATPase. Other mtDNA deletions also accumulate with age [18], which are centred in the region between the light and heavy-strand origins of replication. These deletions frequently erase coding information for the genes ND3, ND4, ND4L, and ND5, all subunits of Complex I.

Table 1
Bases of the mitochondrial genome devoted to protein

Enzyme	Genes	Bases	Percent
Complex I	ND1-6, ND4L	6338	56
Complex IV	COI-III	3003	27
Complex II	Cyt. *b*	1140	10
ATPase	ATPase 6,8	839	7
Total		11320	

Table 2
CUN and UUR codons in human mitochondrial genes

Complex I				Non-Complex I			
8iGene	UUR	CUN	Percent	Gene	UUR	CUN	Percent
ND1	6	57	10	COI	7	55	11
ND2	9	55	14	COII	5	28	15
ND3	10	18	36 *	ATPase8	2	8	20
ND4	9	87	9	ATPase6	5	39	11
ND4L	1	22	4	COIII	3	31	9
ND5	9	95	9	Total	22	161	12
ND6	14	3	82 *** †				
Total	58	337	15				

The usage of CUN and UUR codons in Complex I and non-Complex I proteins encoded on the mitochondrial genome. Single or double asterisks (*, **) signify Chi-square deviation from mean expectation of UUR codons in Complex I genes (15%) at the $P < 0.01$ level and $P < 0.001$ level, respectively. ND6 (†) is the only mitochondrial protein whose RNA is transcribed from the heavy strand.

3.3. The reported rise in mutant $tRNA^{Leu(UUR)}$ with age is more likely to affect decoding of Complex I mRNAs than others

One of the more common mitochondrial diseases is MELAS (Myoclonic Epilepsy, Lactic Acidosis, and Stroke), attributable to point mutations in the $tRNA^{Leu(UUR)}$ that decodes leucine codons [19,20]; the remaining $tRNA^{Leu(CUN)}$ is not associated with disease. Such $tRNA^{Leu(UUR)}$mutations could have the effect of decreasing translation efficiency of particular messages with high usage of UUR codons. The 3243 MELAS mutation rises in frequency with age [21,22]. One may inspect the sequence of the mitochondrial genome to assess which mitochondrially encoded genes are most likely to be affected by mutations in the UUR decoding tRNA. In fact, 73% of such codons are found in subunits of Complex I, more than twice the number found (27%) in non-Complex I encoding genes (Table 2).

As Table 2 shows, the ND6 gene has an extremely high frequency of UUR codons (82%), and might therefore be disproportionately affected by $tRNA^{Leu(UUR)}$ mutations that decreased the translation efficiency of messages bearing such codons. In fact, in vitro studies have shown that cells with a high fraction of a $tRNA^{Leu(UUR)}$ mutation are specifically defective in Complex I activity, and are specifically unable to correctly translate the ND6 message, a subunit of Complex I [23]. The inversion of UUR to CUN codon usage in the ND6 gene is most likely explained by the

unique inversion of the ND4 gene with respect to other protein encoding genes on the mtDNA. Because of its inverted orientation, the sense strand of the ND4 gene is the mtDNA light strand, which is deficient in cytosine. Low usage of cytosine in the light strand is better achieved by UUR codons, which contain no cytosine, than CUN codons, which may contain one or two cytosines. The evolutionary force which maintains low cytosine in the more often unpaired light strand is thought to be deamination of cytosine to uracil (W.K. Thomas, personal communication).

3.4. Drugs that induce Complex I deficiency cause cell death

If one desires to model the effect of mitochondrial DNA mutations on aging cells, a reasonable first step is to look at the effects of inhibitors of Complex I. Two specific inhibitors of Complex I (rotenone and MPP^+) have the effect of inducing cell death. The mechanism by which cell death is induced by these mitochondrial inhibitors has not been elucidated, but one possibility is that cell death is the result of a rise in mitochondrial reactive oxygen species. To address this issue, we attempted to identify experimental animals with excesses or deficiencies of the sole enzymic scavenger of mitochondrial superoxide, MnSOD.

3.5. Mice with deletions and duplications of the Tme locus have deficiencies and excesses of MnSOD

Mice with deficiencies at the Tme (T-associated maternal effect) locus are also deficient in activity of the mitochondrial superoxide dismutase, MnSOD [16]. Deficiencies at the Tme locus are inherited in a mode that is unique in all of mouse genetics, in that deletions at Tme inherited from the father (i.e., the haploid sperm) result in viable progeny, but such deletions inherited from the mother (i.e., the haploid egg) are lethal [24]. This maternal effect has been attributed to genomic imprinting [25]. Deletions of the Tme locus remove the coding region for MnSOD, the mitochondrial superoxide dismutase [26]. Animals bearing heterozygous Tme deletions have 50% of normal MnSOD activity, and animals with heterozygous duplications of Tme have 150% of normal MnSOD activity [16]. Haploid eggs that bear a deletion of MnSOD should have zero MnSOD activity, and mitochondrial oxidative damage may explain the lethality observed in fetuses derived from these eggs [16]. Deficiencies of MnSOD in haploid male sperm are less likely to lead to fetal lethality because male sperm do not contribute mitochondria to the zygote.

3.6. Mice with deficiencies in MnSOD activity are hypersensitive to the inhibitor of Complex I, MPTP

MnSOD-deficient mice exhibit massive brain toxicity when treated with MPTP, a level of toxicity that is not observed in littermates with 100% MnSOD and animals with 150% and 200% MnSOD (Fig. 1 and [27]). A dose of 100 mg/kg MPTP is lethal to MnSOD-deficient animals, but not to animals with normal or excess MnSOD [27]. Thus, massive MPTP toxicity occurs in animals with deficiencies but not excesses of the enzyme that metabolizes mitochondrial superoxide.

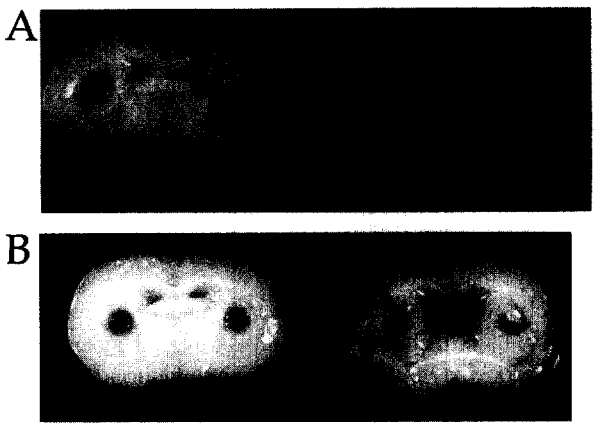

Fig. 1. Mice were treated with 15 mg/kg MPTP i.p. four times at intervals of 2 h, then sacrificed 21 days post-treatment, and brains were sectioned. Panel A, left side, 100% MnSOD; right side 50% MnSOD. Panel B, 50% MnSOD animals, left side no MPTP, right side plus MPTP. Massive enlargement of the lateral ventricle is observed in MnSOD-deficient animals treated with MPTP. Holes in the striatum are the result of samples punched out for dopamine measurements.

4. Discussion

4.1. Random and observed somatic mutation of the mitochondrial genome is more likely to affect Complex I activity than other mitochondrial enzymes

Somatic mutations of the mitochondrial genome occur and increase with age (reviewed in [28]). Both point mutations and deletion mutations have been reported. The statistical analysis presented here demonstrates that random mutations of mtDNA are much more likely to occur in genes encoding Complex I than any other mitochondrial protein. A random mtDNA mutation is about twice more likely to occur in a gene encoding Complex I than in a Complex IV gene, and eight and six fold more likely than mutations in ATPase or cytochrome *b*, respectively, (Table 1). Because of the inversion of ND6 and the coincidence of ND3-5 with the region of mtDNA most frequently deleted in aging cells, mutations that have been demonstrated to increase with age may disproportionately affect Complex I genes.

4.2. Complex I deficiency may increase the concentration of mitochondrial superoxide

Animals with deficiencies in the sole mitochondrial scavenger of superoxide, MnSOD, exhibit much more cell

death in the brain than animals with normal or excess levels of this enzyme when treated with MPTP (Fig. 1 and [27]). In vivo, MPTP is metabolized to MPP^+, a selective inhibitor of Complex I [14]. The simplest explanation of the inhibitability of MPTP-induced cell death by MnSOD is that such death is mediated through an increase in mitochondrial superoxide concentration.

These data were surprising in that it has been assumed by many that because superoxide is short-lived and a relatively weak oxidant it may be a less physiologically significant toxin than its product hydrogen peroxide. Our data support the view that excess mitochondrial superoxide concentration has a very significant deleterious effect (cell death) from which animals with more hydrogen peroxide and less superoxide are protected.

4.3. How could Complex I inhibition lead to excess superoxide?

There is ample in vitro support for the finding that the inhibitors of Complex I rotenone and MPP^+ induce the formation of mitochondrial superoxide [29–33]. Several authors have claimed that the site of superoxide production in Complex I inhibited mitochondrial is Complex I itself [29–33]. By contrast, Ramsay and Singer claim that superoxide resulting from rotenone treatment is not produced at the Complex I site [34].

We note that whether MPP^+ treatment induces superoxide formation directly at Complex I or not, that partial inhibition of Complex I would still leave electron transport from Complex II unaffected (Fig. 2). Electrons channelled via Complex II produce about 4 times more mitochondrial superoxide than electrons channelled via Complex I [35]. Thus the partial inhibition of Complex I by MPP^+ should favor the transfer of electrons by Complex II, and hence a higher production of superoxide anion.

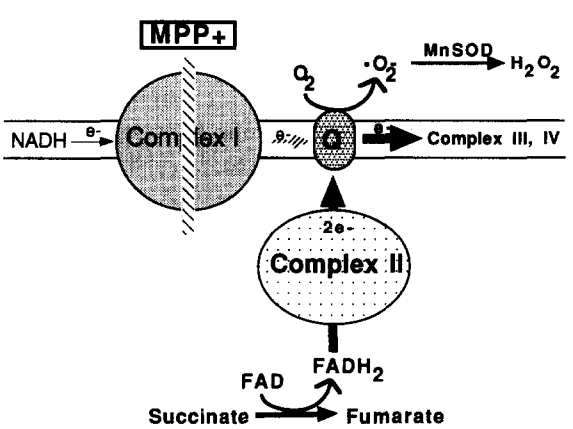

Fig. 2. How a partial inhibition of Complex I may favor the production of mitochondrial superoxide.

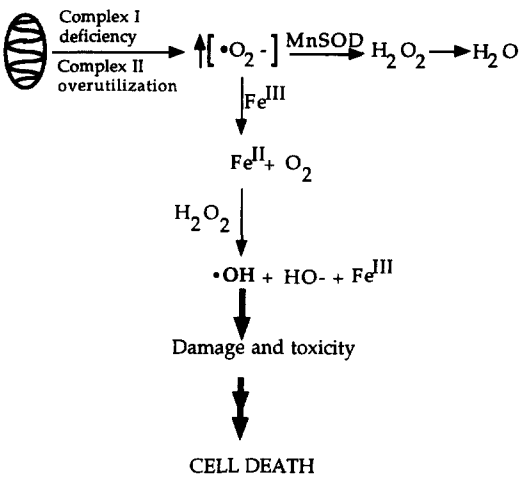

Fig. 3. An increased level of mitochondrial superoxide may lead to cell death by promoting hydroxyl radical formation.

4.4. How could an increased level of superoxide cause cell death?

As explained above, the combination of impairment of Complex I and deficiency of superoxide removal results in massive cell death. How could superoxide cause mitochondrial toxicity? One possibility is that superoxide anion may promote the Fenton reaction. Superoxide can reduce Fe III (the state in which iron is normally found in the cell), to Fe II. Fe II can react with hydrogen peroxide to form the highly toxic hydroxyl radical (Fig. 3). Damage by this radical may be a signal for cell death.

Also, Complex I deficiency causes lactic acidosis, which should favor the protonation of superoxide (pKa = 4.6) to the very reactive hydroperoxyl radical, $HO_2\cdot$. A drop in the pH of the mitochondrial matrix from 8 to 6 would result in 100-fold more hydroperoxyl radical. Either or both of these mechanisms could provide a basis for how superoxide can mediate toxicity.

4.5. Hydrogen peroxide's toxicity is repressed by the mitochondrial repressor of cell death BCL2

Other support for a mitochondrial signal for programmed cell death has recently become available. The repressor of programmed cell death (apoptosis) BCL2 is primarily a mitochondrial protein [36]. Intriguingly, apoptosis induced by hydrogen peroxide can be rescued by overexpression of BCL2 in neuronal cells and lymphocytes, from which one may infer that the signal for cell death is mitochondrial [37,38]. We interpret these data in light of the model presented in Figs. 3 and 4, i.e., that hydrogen peroxide might short-circuit the toxic effect of intramitochondrial superoxide, by providing one of the two reagents critical for the Fenton reaction. The high level of mitochondrial mutation in dopaminergic cells [11–13]

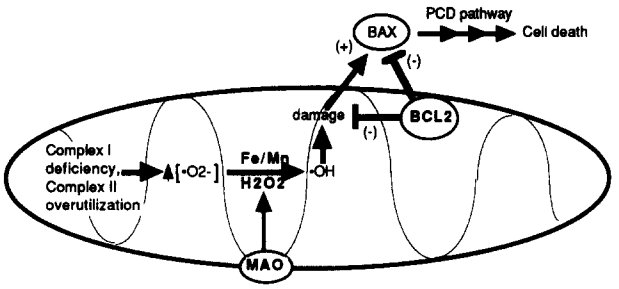

Fig. 4. Legend. A model to explain increased apoptosis as a result of inhibition of mitochondrial Complex I. MAO = monoamine oxidase, Fe = Fe II, Mn = Mn II, BCL2 = B cell lymphoma/leukemia locus-2 protein, BAX = B cell death acceleration protein, PCD = programmed cell death.

might then be the result of a relatively high concentration of Fenton reagents (Fig. 4). If the model presented in Fig. 4 is correct, one would expect the toxicity of mitochondrial superoxide-generating agents to be rescued by overexpression of BCL2.

4.6. How could mitochondrially signalled cell death mediate aging phenotypes?

A common theme among many age-related degenerative diseases is early or inappropriate cell death of postmitotic cells. For example, there is death of dopaminergic neurons in normally aging people [39]. If that loss exceeds 80% of dopaminergic neurons present at birth, Parkinsonism will develop. Similarly, in normal aging there is death of sensorineural hair cells of the inner ear, leading to presbycusis. The dementia associated with Alzheimer's disease is the result of death of cholinergic neurons, and late-onset diabetes is usually the result of death of pancreatic islet cells. Intriguingly, all of the same phenotypes mentioned above (deafness, dementia, ataxia, and diabetes) occur in persons with mitochondrial genetic disease; these phenotypes may have a common basis.

4.7. The age-related decline in tissue function might be the result of crossing an apoptotic threshold rather than a bioenergetic one

It has been proposed that the accumulation of mitochondrial mutation may lead to bioenergetic decline, and that such bioenergetic decline will affect some specific tissues that have a high threshold requirement for energy like neurons and muscle cells before other cell types like liver or kidney [40].

An alternative model is that apoptosis occurs at different rates in postmitotic cells with different mitochondrial superoxide levels, and that tissue dysfunction in mitochondrial disease and in aging humans is the result of loss of a number of cells sufficient for tissue function (Fig. 5). Unlike the bioenergetic threshold model, under the superoxide-mediated apoptosis model the bioenergetic requirement of the different tissues may be identical. The superoxide-mediated apoptosis model predicts that cells with higher levels mitochondrial superoxide, or of compounds that induce Fenton Chemistry (i.e., Hydrogen Peroxide, Fe, and Mn), should undergo more mitochondrial oxidative damage and more cell death, consistent with the findings of higher mitochondrial DNA damage and mutation in dopaminergic neurons.

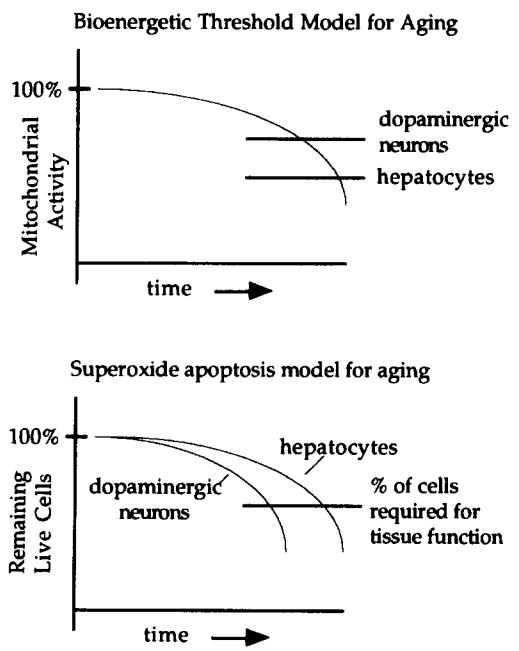

Fig. 5. Two mitochondrial models for aging: bioenergetic threshold and superoxide apoptosis.

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