Ageing Vulnerability: Causes and Interventions: Novartis Foundation Symposium 235. Volume 235
Edited by Gregory Bock and Jamie A. Goode
 ISBN: 0-471-49438-0

Mitochondrial DNA mutations in disease and ageing

D. A. Cottrell, E. L. Blakely, M. A. Johnson, G. M. Borthwick, P. I. Ince* and D. M. Turnbull

*Department of Neurology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH and *MRC University of Newcastle upon Tyne, Developmental Centre for Clinical Brain Ageing, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne, NE4 6BE, UK*

Abstract. The chronological accumulation of mitochondrial DNA mutations has been proposed as a potential mechanism in the physiological processes of ageing and age-related disease. We discuss the evidence behind this theory and relate some of the ageing mitochondrial changes to mitochondrial DNA disorders. In particular, we describe the aggregation of cytochrome c oxidase-deficient cells in both skeletal muscle and the CNS in normal ageing as seen in the mitochondrial DNA disorders. These mitochondrial enzyme-deficient cells have been shown to occur in significant quantities in both muscle and CNS in patients with mitochondrial DNA disorders. In both ageing and mtDNA disorder muscle these cytochrome c-deficient fibres contain high levels of a single mutant strain of mitochrondrial DNA. Whether these mutations are a primary or secondary event in the physiology of ageing remains to be determined.

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Mitochondria contain the only extra-nuclear DNA, consisting of a small (16.5 kb) double-stranded genome, the mitochondrial genome (mtDNA). Although mammalian mtDNA constitutes less than 1% of the total cellular nucleic acid, its role is essential for the normal survival of the mitochondria and hence the cell. Human cells contain several hundred to many thousand mitochondria, with each mitochondrion having 2–10 copies of mtDNA (Robin & Wong 1988). Thus, several thousand copies of the mtDNA genome can be present within a single cell. Both mutated and wild-type (normal) mtDNA can co-exist in any proportion, a situation termed as heteroplasmy. The level of mutant mtDNA can vary considerably between mitochondria, cells and even tissues within the same individual.

The mitochondrial genome is highly efficient in terms of expressed DNA, with virtually no introns. MtDNA contains 37 genes, all of which are involved in

synthesizing subunits of the respiratory chain complex, either directly as 13 essential polypeptide components, or indirectly as 22 transfer RNAs and two ribosomal RNAs of the mitochondrial protein synthesis machinery. When high levels of mutated mtDNA exist within a single cell the activity of mtDNA dependent respiratory chain enzymes, such as cytochrome c oxidase (COX), complex IV decreases. However the activity of the solely nuclear DNA-encoded enzyme succinate dehydrogenase (SDH) complex II remains intact. When tissue from a case with an mtDNA disorder is exposed to the sequential histochemistry of COX followed by the SDH, this alteration of respiratory chain enzyme activity produces a mosaic of COX-positive and COX-deficient, SDH-positive cells. This technique is often used for diagnostic purposes in suspected cases of mtDNA disease. Such a mosaic pattern of COX activity has been previously demonstrated in ageing muscle (Brierley et al 1998). We have recently observed a similar mosaic type appearance in neurons throughout the CNS of a patient with an mtDNA disorder, and in normal ageing human brain.

Hereditary mitochondrial disorders

First described over a decade ago, mtDNA mutations are now recognized as an important cause of human disease, with over 100 pathological mtDNA defects having been characterized (Chinnery & Turnbull 1999). Alterations in the mtDNA genome can occur either as point mutations involving the tRNA or protein-encoding genes, or rearrangements in the form of deletions or duplications. We believe an essential factor in our understanding of the role of defects of mitochondrial function in ageing, is knowledge of the pathogenic mechanisms involved in mtDNA defects and disease. In the presence of heteroplasmy there appears to be a critical ratio of mutant to wild-type mitochondrial genomes which is necessary before disease becomes both biochemically and clinically apparent. Whilst this threshold level is dependent upon both the nature of the mutation and the tissue affected, the vast majority of pathological mtDNA encoded tRNA mutations investigated are extremely recessive and biochemical dysfunction is only apparent when levels of mutant mtDNA exceed 70%.

Whilst the phenotypes of mtDNA disorders are diverse and multi-system in nature, many have predominantly neurological and muscular symptoms. These include dementia, seizures, ataxic syndromes, peripheral neuropathies and myopathy which progress temporally. Higher levels of mutant mtDNA are found in post mitotic tissues such as muscle and CNS, possibly as they lack a mechanism for de-selection of cells with high mutant mtDNA that could occur during cell replication. CNS imaging of patients with mtDNA disorders often reveals moderate degrees of cerebral or cerebellar atrophy which is consistent

with neurodegeneration and often comparable with senescent brains or those with dementia. Limited neuropathological studies have confirmed massive neuronal cell loss, and demyelination indicating neurodegeneration (Sparaco et al 1993, Cottrell et al 2000a).

Mitochondria and ageing

In 1972, Harman was amongst the first to propose that mitochondria may have a central role in the process of ageing (Harman 1972). Chronologically mitochondria have been shown to become larger and less numerous with vacuolisation. Abnormalities in the cristae structure and the accumulation of intra-mitochondrial paracrystalline inclusions also appear (Feldman et al 1981, Frenzel et al 1984). Cardiolipin, an acidic phospholipid, which occurs only in mitochondria has also been shown to decrease with age (Paradies & Ruggiero 1990, 1991, Ruggiero et al 1992). This inner membrane lipid has excellent electrical insulating properties and is thought to contribute significantly to the transmembrane potential, which drives the formation of ATP via ATP synthase, the terminal complex of the respiratory chain. Physiological studies have indeed shown that there is a decrease in the mitochrondrial membrane potential in older animals (Linnane et al 1989, Hagen et al 1997).

In addition, mitochondria and mtDNA are particularly vulnerable to oxidative damage. Their high consumption of metabolic fuels, in the pursuit of ATP production via the inner membrane incorporated respiratory chain, releases the toxic by-products known as free radicals (Sohal & Sohal 1991, Hruszkewycz 1992, Sohal et al 1994). These highly unstable molecules are normally disposed of by free radical scavenging enzymes such as superoxide dismutase and catalase. It is unlikely that any free radical system will be 100% efficient and thus free radical damage is likely throughout life. In addition it has been reported that during ageing the activity of a number of free radical scavenging systems is decreased (Semsei et al 1991, Sohal et al 1990a,b) resulting in a more oxidative environment within the organelle. These free radicals can oxidize membrane lipids in close proximity to the respiratory chain, decreasing the fluidity and increasing the permeability of the inner mitochondrial membrane (Hruszkewycz 1992). Oxidation of mitochondrial proteins and DNA also increases markedly with age (Stadtman 1992), but the role of these damaged proteins has long been questioned. It has been assumed that any damaged protein would not accumulate, since cellular proteins are continuously overturned and damaged proteins more rapidly than normal. However functionally inactive forms of enzymes have been shown to accumulate with age and are thought to be a result of oxygen free radical-mediated damage. An age-related increase in the amount of oxidized protein is possibly the result of an accumulation of damage to DNA, which affects the

factors responsible for protein oxidation, and the degradation of oxidised protein (Stadtman 1992). An accumulation of oxidized protein may also result in cross-linking to other proteins, which would alter their biochemical and physiological function in mitochondria.

Levels of the oxidized nucleotide 8-hydroxy-deoxyguanosine (8-OH-dG), a biomarker of DNA damage, have also been shown to accumulate with age (Hayakawa et al 1991, 1992, Mecocci et al 1993). In several tissues, including the CNS and muscle, levels of 8-OH-dG in mtDNA exceed that in nuclear DNA (nDNA) some 16-fold (Richter et al 1988). Several studies indicate that 8-OH-dG most frequently codes correctly for cytosine but also has the ability to pair with adenine approximately 1% of the time (Cheng et al 1992, Kuchino et al 1987, Shibutani et al 1991, Wood et al 1990). It also has the ability to cause misreading at adjacent residues. Hayakawa et al (1991) found that increased levels of 8-OH-dG in human heart correlated with increases in levels of a 7.4 kb mtDNA deletion. Mecocci et al (1993) found a similar correlation in human brain.

Mitochondrial DNA mutations in ageing

Linnane in 1989 proposed that the accumulation of mtDNA mutations during life is a major cause of age related disease (Linnane et al 1989). The compact and efficient mitochondrial genome is a particularly vulnerable piece of DNA. Not only does it lack introns but it is believed to have a 10-fold higher mutation rate than nuclear DNA (Richter et al 1988, Merriwether et al 1991, Fraga et al 1990). There are several reasons for this higher susceptibility to mutate. Firstly mitochondria are reported to have limited nucleotide excision and recombination DNA repair mechanisms (Richter et al 1988). Secondly mtDNA lacks the structurally protective histone proteins. Thirdly mtDNAs reside and replicate close to the inner mitochondrial membrane and hence are exposed to the enriched free radical milieu produced by both the respiratory chain and monoamine oxidases located within the membrane.

There is a large body of evidence to show that mtDNA mutations increase temporally, with the highest levels seen in the CNS and muscle. To date over 20 different types of deletions have been shown to accumulate in ageing human tissues. The first published report of an age-related increase in a mtDNA deletion, was the so-called common deletion, found in elderly brain tissue and patients with Parkinson's disease (Ikebe et al 1990). The common deletion occurs between two 13 bp sequence repeats beginning at nucleotides 8470 and 13447, removing a region of almost 5 kb of mtDNA between ATPase 8 and the ND5 genes. The deletion is thought to occur during replication of the mtDNA. This absent arc encodes for six essential polypeptides of the respiratory chain and five tRNAs. It has been associated with several different clinical entities, including

chronic progressive external opthalmoplegia (CPEO) and Kearns Sayre syndrome (KSS). The common deletion has also been shown to increase with age in skeletal muscle (Cooper et al 1992, DiDonato et al 1993, Lee et al 1994, Lezza et al 1994, Simonetti et al 1992), cardiac muscle (Simonetti et al 1992, Cortopassi & Arnheim 1990, Corral-Debrinski et al 1991, Cortopassi et al 1992), diaphragm (Cortopassi et al 1992), retina (Barreau et al 1996), skin (Pang et al 1994, Yang et al 1994), ovary (Kitagawa et al 1993) and sperm (Kao et al 1995). However it is undetectable in fetal tissues. Another mutation widely reported to increase with age in skeletal and cardiac muscle, brain and skin is a 7.4 kb deletion between np 8649 and np 16084. This deletion is also found in the muscle of some patients with CPEO and KSS.

However the overall levels of individual mutations observed in ageing human tissue are low ($<1\%$), and by analogy to the situation in patients with mtDNA diseases it is difficult to see how individual mutations could affect mitochondrial function. The low levels of mtDNA mutation reported have been observed in homogenate samples of tissue. Work by Brierley et al (1998) in individual aged skeletal muscle fibres demonstrated that a clonal expansion of a single mutation (the common deletion) occurs to very high levels in a few of the COX-deficient fibres. High levels of clonally expanded deleted mtDNA were not observed in fibres with normal COX activity. They went on to suggest that other mutations could be responsible for the COX-deficient fibres not containing this one particular deletion. Recently Khrapko et al (1999) have used long PCR techniques in individual cardiomyocytes from old patients to study mtDNA deletions at the single cell level. Their results indicate that various different deletions occur within different cells, but that clonal expansion of only one particular mutation occurs within an individual cell. Therefore in homogenate samples the overall level of a single mutation would be expected to be low, if many different mutant strains are occurring in different cells.

Evidence for increase in mtDNA mutations in age-related disorders

Several age-related disorders have been shown to harbour higher levels of mtDNA mutations than their age-matched counterparts. In one study, the common deletion in cardiac muscle from patients with ischaemic heart disease contained up to 240 times the level of deletion observed in normal cardiac muscle (Corral-Debrinski et al 1991). In another study by Pang et al (1994), sun exposed skin from one individual contained 30 times the amount of common deletion than non-sun-exposed skin. In the CNS, Ikebe et al (1990) found 17 times the level of the common deletion in the striatum of Parkinson's disease patients compared with age-matched controls. There is also evidence showing increased percentages of the common deletion in patients with Alzheimer's disease versus age-matched controls (Corral-Debrinski et al 1994). Levels of the oxidized nucleotide

8-OH-dG are also higher in the brains of Alzheimer's patients (Mecocci et al 1994). Whether these changes are a primary or secondary event in the pathogenesis of the disease requires further single-cell and pathological studies.

An age-related increase in cells deficient in cytochrome c oxidase

Measurements of the activity of the respiratory chain complexes can reveal the presence of mtDNA dysfunction. Complex IV of the respiratory chain, COX is composed of 13 subunits, which are partly encoded by the mitochondrial genome and partly by nuclear DNA. The larger subunits, I, II, and III are encoded by mitochondrial DNA and are synthesized within the mitochondria; the remaining 10 subunits are encoded by the nuclear genome. Therefore mtDNA integrity is essential for the successful synthesis of active COX.

Many studies have reported the finding of cytochrome c oxidase deficiency at a single cell level. Muller-Hocker et al (1990) first demonstrated COX-deficient cardiomyocytes in human heart that increased with age. COX-deficient cardiomyocytes were regularly present from the sixth decade of life onwards, only occurring sporadically prior to this. It was observed that the loss of enzyme activity was always confined to single randomly distributed cardiomyocytes. The density of COX deficient cardiomyocytes increased from an average in the third decade of life of 3 defects/cm^2 to 50 defects/cm^2 in heart aged over 70 years.

Histochemical analysis of COX activity in various extraocular muscles also revealed randomly distributed COX-deficient fibres (Muller-Hocker et al 1993). Defects were observed in the second decade of life in some subjects and were consistently apparent from the third decade onwards. In limb muscle and diaphragm, an almost ten fold increase in the incidence of defective fibres was noted for those in the 8th and 9th decade (54–60 defects/cm^2) compared to those between the 3rd and 6th (5–7 defects/cm^2). The affected isolated muscle fibres showed normal SDH activity (Muller-Hocker 1990). These observations were confirmed by Byrne & Dennett (1992), who reported an accumulation of COX-deficient fibres in diaphragm from the 4th decade onwards, with an exponential increase in later life.

Our recent studies have concentrated on COX activity within the CNS. This, like muscle consists predominantly of post-mitotic cells and previous studies have shown low levels of mtDNA deletions in aged brains. We have already shown that COX-deficient neurons exist in abundance in the CNS of a patient suffering from a multiple mtDNA deletion disorder (Cottrell et al 2000). Interestingly, in this case the proportion of COX-deficient neurons seems to be inversely proportional to the degree of neuropathological damage. In both the cerebellum and hippocampus which exhibited marked neuronal loss, low levels of COX-deficient neurons were observed. In addition we have completed a comprehensive study of over 25 human

brains exploring whether there are COX-deficient neurons in specific CNS regions in brains with no evidence of neuropathological abnormality. An age-related increase in COX-deficient CA hippocampal pyramidal neurons and choroid plexus epithelial cells occurs (Cottrell et al 2000b). Furthermore preliminary findings suggest that these COX-deficient neurons are even more prevalent in cases with Alzheimer's disease. In the choroid plexus the COX-deficient cells exhibit marked swelling similar to that seen in the chroid plexus of patients with mtDNA disorders.

Conclusions

Over the past two decades we have come a long way in the understanding of the role of mtDNA mutations in human disease. Numerous different mutations of both the mitochondrial and nuclear genome have been associated with mtDNA disorders, and the complex interactions between the mitochondria and the cell are slowly being unravelled. We have shown that the biochemical defects that occur commonly in mtDNA disorders at the cellular level can also occur, although in lower levels, in muscle and brain at the cellular level. What the effects of these changes within the mitochondria have on the cells they support remains to be elucidated. In cybrid studies the ATPase-dependent mitochondrial transmembrane potential has been shown to fall once the threshold level of 50–55% deleted mtDNA is exceeded (Porteous et al 1998). It may be expected that when lack of ATP synthesis reaches a threshold the cell would cease to function and die. In contrast, in our morphological studies COX-deficient muscle fibres and neurons look identical to COX-positive cells. Further studies are needed at the cellular level too look for evidence of neurodegeneration within these COX-deficient cells. In particular, do these cells exhibit evidence of apoptosis, in which mitochondria and their contents appear to play such a pivotal role (Green & Reed 1998)? The evidence that mtDNA damage occurs and accumulates with age is compelling, but the consequences are still elusive.

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DISCUSSION

Shay: In the choroid plexus, what was the percentage of cells that were COX deficient?

Cottrell: It varies. In patients above the age of 70 the percentage rises to 5 to 7%, whereas in the hippocampal pyramidal neurons it is lower, around 1%.

Shay: What percentage of the mitochondria have to be COX deficient in order to shift the colour from brown to blue?

Cottrell: This histochemical method demonstrates only cells with very high levels of mutant mtDNA and therefore shows only the tip of the iceberg of all mitochondrial DNA mutations. It supports the evidence that mtDNA mutations do accumulate in age and to levels which can cause a biochemical deficiency in some cells. It is not a uniform picture. It doesn't occur in every cell, and occurs at different rates in different tissues. COX-deficient cells usually have mtDNA mutations in excess of 80% of total DNA.

Shay: Presumably none of these cells in any of these parts of the brain have any basic proliferation, like the choroid plexus.

Cottrell: Interestingly the choroid plexus is not post-mitotic. Unfortunately there doesn't appear to be any literature on its mitotic rate although it would appear that its turn over rate is slow.

Shay: This was intriguing when you said that these cells get big. They seemed to have similarities to senescent cells. Perhaps in this part of the brain there is increased cellular turn over, and perhaps the cells are getting old.

Cottrell: Yes, it would be interesting to look at the telomere length of these cells and their mitochrondrial content *in situ*.

Wallace: In severe cases of the mtDNA deletion syndrome, chronic progressive external ophthalmoplegia (the Kearnes–Sayre syndrome) one of the interesting

phenotypes is a very high CSF protein level. This indicates that the choroid plexus is under great distress, which is consistent with the cells being lost.

The turnover rate of the cell is also important. For a slowly replicating cell, mitochondrial energy demand might not be high and mutant mtDNAs may not cause a replicative disadvantage. Thus, the mutant mtDNAs might be sustained or even accumulate. By contrast, cells that are rapidly replicating require more mitochondrial energy. Hence, those cells might have a reduced replication rate giving the cells with normal mitochondria a replicative advantage. Hence, cells containing mutant mitochondrial DNAs would be lost.

To augment what David has said, a number of years ago we analysed the deletion levels in normal brain and found they increased exponentially with age. In the brains of patients with Alzheimer's disease (AD), there is about a 15-fold higher level of deletion than in age-matched controls. This is true for younger patients, but as the AD patients aged after 75 years the amount of the deletion declined at the same time. To account for this, we proposed that as the deletion level in the cell rises, it ultimately reaches a point where the cell undergoes apoptosis and the neurons with the most deletion are removed. This would result in the decline in the average level of deletion in the brain tissues of the AD patients with the most pathology.

We also looked at the deletion levels in Huntington's disease. The deletion level was very elevated in the non-affected tissues, but in the basal ganglia, which suffers the most severe pathology, the deletions had been lost. This was consistent with the fact that in the basal ganglia all of the affected cells had been lost.

Cerami: Is there a pattern of how the tissues are affected? In the case you gave you were looking in the brain. If you looked in muscle or liver, could you observe mitochondrial changes? What else was wrong with the person you described?

Cottrell: The majority of his symptoms were neurological. He had a mild generalized myopathy. Patients with mitochondrial disorders exhibit all sorts of multiorgan problems. For some reason the distribution of mtDNA mutations is higher in some tissues than in others. The proportion of mutation versus wild-type within a particular organ will determine what the clinical features of that patient will be. Even patients with the same mtDNA point mutation can present with all manner of different symptoms.

Bush: Looking at this issue another way, have you looked at the muscle fibres of people with AD?

Cottrell: No, that would be very interesting to do.

Wallace: We have done muscle biopsies on AD and Parkinson's disease (PD) patients. Sometimes we see oxidative phosphorylation enzyme deficiencies.

Bush: Do these reflect the changes in the brain?

Wallace: It is hard to say that they are the same, but they correlate. We have found a mtDNA mutation at nucleotide position 4336 in the $tRNA^{glu}$ gene that is

associated with about 5% of AD patients. In muscle biopsies from these individuals, there is a partial oxidative phosphorylation defect in older patients.

James: Doug Wallace is being very careful in his response: the reason is because in couch potatoes, old people who don't move around, these COX-positive fibres are also seen. Patients with PD don't move around much. The choice of controls is therefore terribly important. This is one of the problems that have bedevilled this area in respect to muscle mtDNA alterations with age. When we were doing our earlier studies we got muscle biopsies from active senior citizens who were running half marathons in order to get comparable controls in terms of physical activity.

I have a question. In the liver, there are apoptotic bodies which we now know are tombstones for cells that have just undergone apoptosis. Are there similar apoptotic bodies in the brain? If the hypothesis that you and Doug Wallace have been putting forward, which is that the place where there is the lowest number of blue fibres is the one where the worst damage is going on are correct, then you might expect to see apoptotic bodies if they exist.

Cottrell: The problem with that is you need to look at approximately 10 000 neurons to find one which is undergoing apoptosis. Also, this sequential histochemistry damages the tissue, and it is a problem doing anything after this procedure. Perhaps the way to look at it from the mechanism point of view is that the choroid plexus cells may be more useful, because of the swelling effect. In AD patients there are more of these COX-deficient cells in the choroid plexus than in elderly controls. If you do cross-sections of old choroid plexus, you can look at those swollen cells and see what is taking place inside them. We would like to look at apoptotic markers here.

Campisi: I have a question about mutation, which of course is fixation of damage. Can you explain how those numbers were arrived at? Does it take into account the replication of mtDNA?

Wallace: I am glad you made this point. There are a number of ways to address this question. There are two factors: the DNA mutation rate and the mutation fixation rate.

We compared the DNA sequence differences of mtDNA genes and nuclear DNA genes whose products function in the mitochondria between different species. This gave us the ratio of the mtDNA to nuclear DNA mutation levels. Since the two sets of genes diverged at the same time, this gives the ratio of the overall mutation rates. In our original studies, this ratio came out to 17. However, this ratio can vary significantly for different pairs of genes.

Since this approach is actually looking at final mutations it combines both the mutation and fixation rates. The number I was discussing was for the fixation of germline mutations. This encompasses the whole gamut of selection acting at any tissue or in the individual as a whole.

The second question concerns the somatic mutations, which is a whole different thing. This is the accumulation of spontaneous mitochondrial DNA mutations over the lifespan of an individual within cells and tissues. The accumulation and ultimate percentage of damaged mitochondrial DNA is highly tissue specific, as David Cottrell indicated. This could be due to mutation rate, cell replication rate, and cell loss rate by apoptosis. We don't know all of the factors involved in this. For instance, the cerebellum accumulates very little of the common 5 kb mtDNA with age, and yet we know that cerebellar cells become COX-negative quickly.

What is probably happening is that the cerebellum is undergoing atrophy rapidly, so as those cells that accumulate mtDNA mutations are being eliminated by apoptosis. The cortex by contrast, accumulates significant levels of mutant mtDNA presumably because apoptosis is not removing the cells harbouring mtDNA mutations as rapidly.