Roles of mitochondrial DNA mutation and oxidative damage in human aging

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One of the consequences of the age-dependent decline of respiratory function is the increase in the production of reactive oxygen species (ROS) and free radicals in mitochondria due to enhanced electron leak of the respiratory chain. It has been shown that the rate of production of superoxide anion and hydrogen peroxide in mitochondria increases with age. Moreover, the cellular levels of antioxidants and activities of free radical scavenging enzymes decrease during aging. These two concurrent events lead to an age-dependent increase of the oxidative stress, which may overwhelm the antioxidant defense system and cause oxidative damage to vital biomolecules in tissue cells. Abundant evidence now indicates that lipid peroxidation, protein modification, and DNA mutation are enhanced in aging tissues. The mitochondrial DNA (mtDNA) which is not protected by histones and yet exposed to ROS inside the mitochondria, is much more susceptible than nuclear DNA to oxidative insult during aging. In recent years, oxidative modification and mutation of mtDNA have been found to increase exponentially with age in human and animal tissues. The mutant mtDNA-encoded respiratory enzymes exhibit impaired respiratory function, and thereby increase the production of ROS and free radicals, which further elevate the oxidative stress and oxidative damage to mitochondria. In this article, we review the recent work pertaining to this 'vicious cycle' and discuss the role of mitochondria in human aging and age-related degenerative diseases.

AGING is a complex biological process that leads to the gradual loss of the ability of an individual to maintain homeostasis. It is accompanied by a general decline in the biochemical and physiological functions of most organs, a decrease in the ability of the individual to respond to challenges or stresses, and an increase in the susceptibility to age-associated diseases.

About four decades ago, Harman[1] first proposed that free radicals play a key role in the human aging process. He suggested that the accumulation of free radical-induced damage to vital molecules is an important cause of human aging. Subsequently, he re-shaped this 'free-radical theory of aging' and proposed that mitochondria are the major intra-cellular target of free radical attack that leads to human aging[2]. Over the years, this theory has received much attention and gained much support from the molecular and cellular biological research on aging. Miquel and coworkers[3] first provided significant support for this notion by showing that lipofuscin pigment formation and mitochondrial DNA (mtDNA) mutation in animal tissues increase during aging. On the basis of the previous findings that mitochondria are the major intracellular source of reactive oxygen species (ROS)[4] and free radicals, and that mtDNA is vulnerable to oxidative damage, Linnane *et al.*[5] proposed that accumulation of somatic mutations in mtDNA is a major contributor to human aging and degenerative diseases.

Recently, we proposed that the production of ROS and free radicals in tissue cells increases during aging as a result of enhanced electron leak from the defective respiratory chain, and that the ensued oxidative damage and mutation of mtDNA cause further impairment of the respiratory function[6]. Here we review experimental data gathered over the past few years that are relevant to this vicious cycle in human aging.

Respiratory function decline in aging – its cause and effect

It was first demonstrated in 1989 that the respiratory functions of mitochondria gradually decline in human liver[7] and skeletal muscle[8]. This phenomenon was confirmed in other human tissues[9]. In addition, it has also been found that the number of muscle fibers deficient in cytochrome *c* oxidase increases with age in the heart and skeletal muscle[10]. By histochemical staining of respiratory enzymes in the frozen sections of autopsied heart muscle, Müller-Höcker first showed that there are randomly distributed human cardiomyocytes which are deficient in cytochrome *c* oxidase activity. He further demonstrated that the density of muscle fibers deficient in cytochrome *c* oxidase activity in the heart, skeletal, and ocular muscles increases with age[11]. On the other hand, Nohl and Krämer[12] showed that the activity of adenine nucleotide translocase (ADP/ATP carrier) ex-

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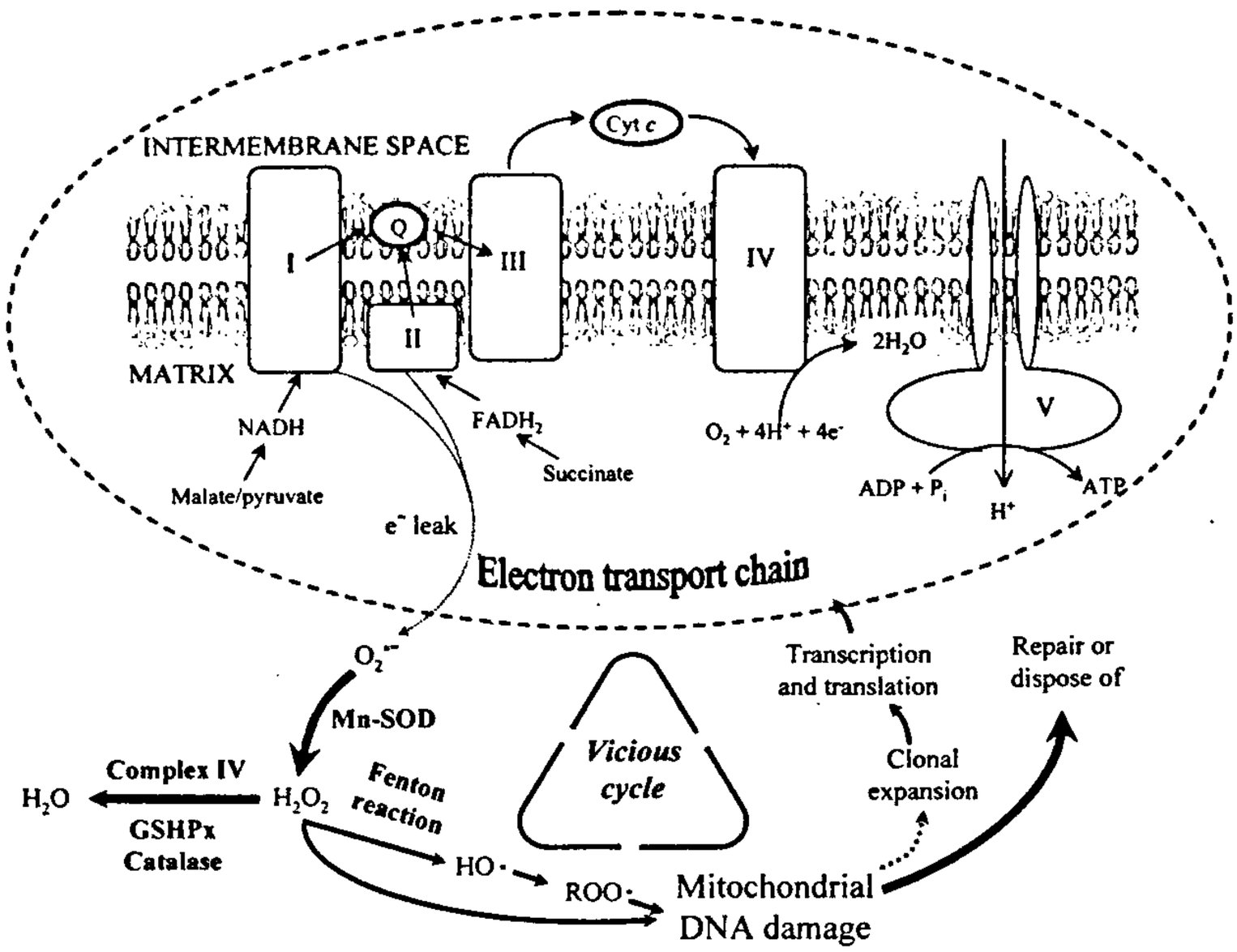

Figure 1. The 'vicious cycle' that occurs in the human aging process. In the electron transport chain, a small fraction of oxygen is incompletely reduced by 1-electron transfer to generate ROS and free radicals, which are usually disposed off by the free radical scavengers in mitochondria. When overproduced or inefficiently removed, they may cause oxidative damage and mutation of the nearby mtDNA molecules that are located in the vicinity of the inner membrane[51]. The mutated or oxidatively modified mtDNA may be transcribed and translated to produce dysfunctional protein subunits that are assembled to form defective respiratory enzymes. The impaired respiratory chain not only produces ATP less efficiently, but also generates more ROS, which further enhances the oxidative damage to various biomolecules in mitochondria. This vicious cycle operates in an age-dependent manner and results in the aging-associated decline of the bioenergetic function of tissues.

hibited a 30% decrease in the mitochondria of aged animal tissues.

It was noted that the age-dependent decline in the rate of the glutamate-malate-supported (Complex I-mediated) respiration is much more pronounced than that of the succinate-supported (Complex II-mediated) respiration[7]. Moreover, the activity of Complex II was not so much decreased as the other respiratory enzymes in aging tissues[9]. These observations led us to surmise that mutation(s) in some of the 13 genes of mtDNA coding for subunits of Complexes I and IV may be involved in the age-dependent decline in respiratory function. We confirmed this idea by showing that both the frequency of occurrence and the proportion of the mtDNA with the 4,977 bp deletion increase with age in liver[13] and other human tissues[14]. Many other large-scale deletions, and point mutations of mtDNA have also been detected in aging human tissues[6].

The mtDNA molecules with the 4,977 bp deletion are transcribed and translated in cultured human cells[15]. It is conceivable that the fusion transcript and expressed defective proteins exert some deleterious effect on mitochondrial functions in aged human tissues. The notion that mtDNA mutations contribute to aging is furthe supported by recent findings that the extent of mtDN mutation is strongly correlated with the degree of de crease in the electron transport activities of cytochrom *c* oxidase and other respiratory enzymes[16]. The impair ment in the respiratory function not only causes a de cline in the efficiency of ATP synthesis but als increases the electron leak from the respiratory chain o mitochondria. This constitutes a part of the recentl proposed 'vicious cycle', which is aggravated durin aging in human tissues (Figure 1). Recently, we culture skin fibroblasts from individuals of different ages wh had none of the known mitochondrial disorders or de generative diseases. The activities of respiratory en zymes of the fibroblasts from old donors were found t be significantly lower than those of the young donor (Table 1), a finding consistent with the previous obser vation that the ATP/ADP ratio and energy charge of th fibroblasts decline in an age-dependent manner[17]. In recent study, Luo *et al.*[18] showed that a defect in Com plex I of the respiratory chain results in excessive pro duction of hydroxyl radicals and enhanced lipi peroxidation in human skin fibroblasts. This findin

Table 1. Comparison of electron transport activities of the respiratory enzyme complexes of mitochondria of the fibroblasts from subjects in different age groups

Age group (year)	Electron transport activity (nmol/min/mg)		
	NCCR	SCCR	CCO
10–30	720 ± 84 (2)	221 ± 29 (2)	103 ± 24 (2)
3–60	590 ± 52 (2)	55 ± 23 (7)	23 ± 12 (7)
7–80	340 ± 152 (2)	44 ± 19 (6)	29 ± 6 (6)

Values in parentheses represent the number of subjects whose fibroblasts were used for enzyme assays. NCCR, NADH-cytochrome *c* reductase; SCCR, succinate-cytochrome *c* reductase; CCO, cytochrome *c* oxidase.

demonstrates that age-dependent decline of mitochondrial respiratory functions may elicit higher oxidative stress, which in turn may lead to a wide spectrum of oxidative damage. It is possible that oxidative damage and mtDNA mutation further impair the function of electron transport chain synergistically and increase the rate of ROS generation. Although it is generally accepted that mtDNA mutations are associated with human aging[6], a clear causal relationship between oxidative damage and mutation of mtDNA, decline in mitochondrial function and aging remains to be established.

Oxidative stress in aging

Mitochondria are the major intracellular producers of ROS and free radicals in animal and human cells. The electron leak from the impaired respiratory chain may elicit enhanced production of ROS through one electron reduction of molecular oxygen in mitochondria[4,6]. Under normal physiological conditions, approximately 1–5% of the oxygen consumed by mitochondria of human cells is converted to superoxide anion, hydrogen peroxide, and other ROS[4–6]. It was estimated that each mitochondrion in rat liver produces about 3×10^7 superoxide anions per day[4]. Human cells can dispose off ROS by coordinated expression and functioning of an array of free radical scavenging enzymes including manganese-superoxide dismutase (MnSOD), copper/zinc-superoxide dismutase (Cu/ZnSOD), catalase, and glutathione peroxidase[6,19]. However, these antioxidant defense systems are not perfect and are subjected to alterations during aging. As a result, there is an age-dependent increase in the fraction of ROS and free radicals escaping these cellular defense systems and damage cellular constituents including lipids, proteins, and nucleic acids[20]. Recently, Xu *et al.*[21] demonstrated that the amount of oxygen consumed in the electron leak route increases in aged mice. The rate of production of superoxide anion and hydrogen peroxide in mitochondria was found to increase with age in various mammalian tissues[22]. The enhanced production of ROS inevitably elevates the oxidative stress and oxidative damage of the cell.

On the other hand, it was observed that *Drosophila melanogaster* with homozygous mutations in either Cu/ZnSOD or catalase gene exhibit increased sensitivity to oxidative stress, and have reduced viability and shorter lifespan[23]. Since glutathione peroxidase is absent in *D. melanogaster*, Cu/ZnSOD and catalase provide the main enzymatic antioxidant defenses[24]. The fruit flies that overexpress Cu/ZnSOD alone or in combination with the overexpression of catalase were found to exhibit higher tolerance to oxidative stress, and have significantly less oxidative damage to proteins and have longer lifespan[25]. It is established that the activities and capacities of antioxidant systems of cells decline with age, which in turn leads to a gradual upset of the prooxidant/antioxidant balance and accumulation of oxidative damage, most notably in DNA, in the aging tissues[17,20]. Asuncion *et al.*[26] recently showed that the mitochondrial glutathione is markedly oxidized during aging in animals. The ratio between oxidized and reduced glutathione, which is a good index of oxidative stress, was found to increase with age in the liver, kidney, and brain of the rat and mouse. In the same study, the 8-hydroxy-2′-deoxyguanosine (8-OH-dG) content of mtDNA in these tissues was also found to increase with age in the rat and mouse. Moreover, oral administration of antioxidants such as α-tocopherol was found to protect rats and mice against glutathione oxidation and mtDNA damage. These observations have lent support to the notion that oxidative stress and ensued oxidative damage to vital biomolecules in tissue cells play an important role in the aging process of human beings and animals.

Aging-associated oxidative damage to mitochondria

The age-dependent increase in the production of ROS and free radicals (e.g., ubisemiquinone and flavosemiquinone) in mitochondria inevitably elevates the oxidative stress and oxidative damage to mitochondria and the cell as a whole[6]. The rate of mitochondrial hydrogen peroxide release is important in determining the extent of oxidative damage sustained by mitochondria. It was found that the level of 8-OH-dG, a specific product of oxidative damage to DNA, increases in an age-dependent manner in animal tissues[27,28]. Furthermore, dietary restriction, which extends the average and the maximum lifespan of mammals, decreases the rate of age-dependent accumulation of 8-OH-dG in various tissues of mice[29].

Mitochondrial DNA is naked and is located in the vicinity of mitochondrial inner membrane where ROS and free radicals are generated. Hence oxidative damage to

the mitochondrial genome occurs easily at this site. Indeed, Ames and coworkers[27] first reported in 1988 that oxidative damage in mtDNA is much more extensive than that in the nuclear DNA of animal tissues. They discovered that the 8-OH-dG content in mtDNA was about 16 times higher than that of nuclear DNA of the rat liver. Furthermore, the 8-OH-dG content in liver mtDNA of the 24-month-old rat was 3 times higher than that of the 3-month-old rat[27]. In a recent study, Yakes and van Houten[30] demonstrated that human fibroblasts treated with 200 μM hydrogen peroxide for 15 or 60 min exhibited 3-fold more damage to mtDNA as compared with the damage to nuclear DNA. Moreover, they found that after 1 h treatment of the fibroblasts with H_2O_2, the damage to nuclear DNA was completely repaired within 1.5 h but no repair in the mtDNA was observed. This indicates that under oxidative stress, which may be elicited by aging, mtDNA damage is more extensive and persists longer than nuclear DNA. Recently, Ozawa and colleagues showed that oxidative modification of mtDNA, as indicated by 8-OH-dG, increases in an age-dependent manner in the human diaphragm, heart muscle, and brain tissues[31,32]. Furthermore, the levels of oxidatively modified proteins and lipids in mitochondria have been shown to increase during aging[33,34]. In the past decade, Stadtman and coworkers have provided convincing evidence that oxidatively modified proteins accumulate in the cells during aging[34]. Sohal *et al.*[35] discovered that protein oxidation and DNA damage in mitochondria increase concurrently in various tissues of aging fruit flies. These observations suggest a close relatonship between oxidative stress as indicated by glutathione oxidation, and oxidative damage to proteins, lipids, and DNA in mitochondria of cells during the aging process. However, further investigation is warranted to determine whether these oxidative damages are the cause or effect (or both) of aging.

Human mtDNA mutations in aging

Each human cell contains several hundred mitochondria, each carrying 2 to 10 copies of mtDNA. Human mtDNA is a 16,569 bp circular double-stranded DNA molecule (Figure 2)[36]. This extra-chromosomal genetic element contains genes that code for 13 polypeptides involved in respiration and oxidative phosphorylation, and 2 rRNAs and 22 tRNAs that are essential for protein synthesis in mitochondria[37]. Human mtDNA does not bind to histones, and replicates rapidly by a D-loop mechanism without proof-reading and lacks efficient DNA repair systems[6,37]. It is transiently attached to the mitochondrial inner membrane in which a considerable amount of ROS is generated by the respiratory chain[4,20]. These characteristics have rendered mtDNA vulnerable to free radical attack and mutation. The observation that mtDNA has

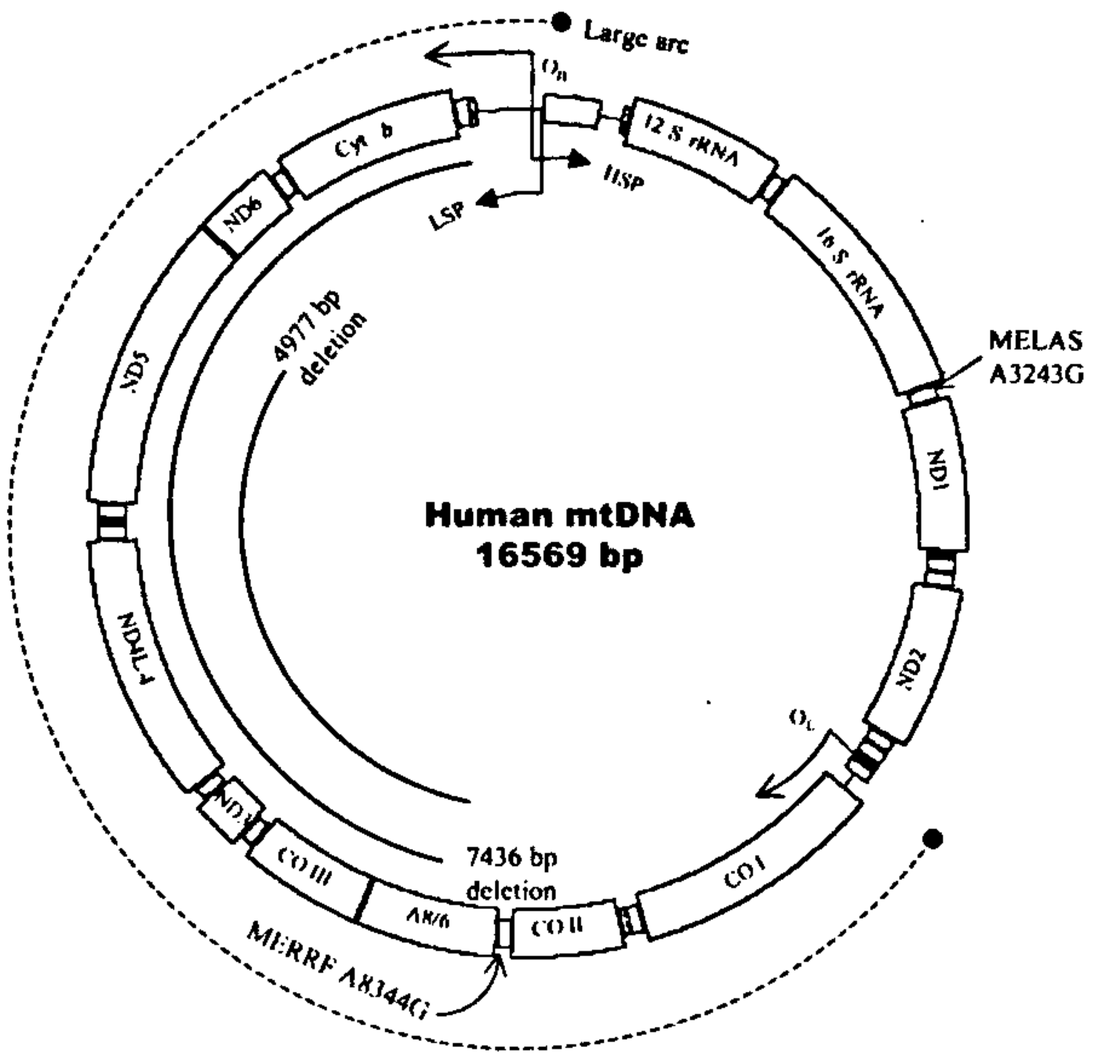

Figure 2. The genetic map and some commonly seen aging-associated mutations of human mitochondrial DNA. The 4,977 bp and 7,436 bp deletions, which occur within the large arc between the replication origins O_H and O_L, are the two most commonly found aging-associated large-scale deletions of human mtDNA. The A3243G and A8344G transitions in the $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes are the two well-established aging-associated point mutations of human mtDNA. Many other large-scale deletions, point mutations, tandem duplications, and rearrangements of mtDNA that have been detected in aging human tissues are not shown here[6,46]. They occur stochastically in an age-dependent manner.

about 100-fold higher 8-OH-dG content than nuclear DNA[27] implies that aging human and animal tissues may have abundant mutations in their mtDNAs.

More than two dozen point mutations, large-scale deletions, tandem duplications, and rearrangements of mtDNA have been found in various tissues of old humans[6]. Large-scale deletions occur almost exclusively within the large arc between the replication origins of heavy strand (O_H) and light strand (O_L), and most of the point mutations are located in the tRNA genes of mtDNA (Figure 2). Mutant mtDNA molecules often coexist with the wild-type mtDNA within a cell, a condition termed as heteroplasmy, and the degree of heteroplasmy varies in different tissues of the same individual[38]. It has been well established that many of the mtDNA mutations accumulate in an exponential manner with age in post-mitotic tissues[39]. Although some mtDNA mutations might occur in oocytes (germline mutation), it is generally believed that most of them arise and accumulate with time in somatic cells. The most wide-spread mtDNA mutation is the so-called 'common deletion', which removes a 4,977 bp DNA segment of mtDNA by a mechanism that involves a

Table 2. The age-dependent increase in the proportions of the 4,977 bp deleted mtDNA in various human tissues

Age group	Muscle (%)	Liver (%)	Testis (%)	Skin (%)	Lung (%)		Hair follicle (%)
					Non-smoker	Smoker	
0–9	0.0000 ($n = 4$)	N.A.	0.0000 ($n = 3$)	N.A.	0.0000 ($n = 2$)	N.A.	N.A.
10–19	0.0002 ($n = 5$)	0.0000 ($n = 1$)	0.0000 ($n = 4$)	0.0000 ($n = 1$)	0.0244 ($n = 4$)	0.0000 ($n = 1$)	N.A.
20–29	0.0015 ($n = 1$)	0.0008 ($n = 2$)	0.0000 ($n = 2$)	0.0000 ($n = 2$)	N.A.	0.0000 ($n = 1$)	0.0732 ($n = 2$)
30–39	0.0106 ($n = 6$)	0.0025 ($n = 4$)	0.0000 ($n = 6$)	0.0000 ($n = 1$)	0.0105 ($n = 7$)	0.0081 ($n = 3$)	N.A.
40–49	0.0203 ($n = 11$)	0.0025 ($n = 8$)	0.0000 ($n = 1$)	0.0000 ($n = 6$)	0.0254 ($n = 6$)	0.0504 ($n = 2$)	0.5615 ($n = 5$)
50–59	0.0252 ($n = 4$)	0.0021 ($n = 1$)	0.0000 ($n = 1$)	0.0206 ($n = 8$)	0.1506 ($n = 9$)	0.1038 ($n = 2$)	0.6049 ($n = 8$)
60–69	0.0346 ($n = 16$)	0.0032 ($n = 8$)	0.0141 ($n = 12$)	0.0052 ($n = 5$)	0.2075 ($n = 7$)	0.3751 ($n = 25$)	0.8321 ($n = 12$)
70–79	0.0600 ($n = 12$)	0.0076 ($n = 6$)	0.0275 ($n = 12$)	0.0561 ($n = 5$)	0.3256 ($n = 3$)	1.0661 ($n = 15$)	1.4124 ($n = 13$)
80–89	N.A.	N.A.	0.0529 ($n = 6$)	N.A.	0.0000 ($n = 1$)	0.0000 ($n = 2$)	2.1184 ($n = 9$)

The proportions of the 4,977 bp deleted mtDNA in each of the human tissues were determined by a semi-quantitative PCR method as previously described[14]. Total DNA from each of the tissue samples was used for quantitative analysis of the mtDNA with the 4,977 bp deletion. The number in each parenthesis indicates the number of subjects examined in the specified age-group, and the mean values of the proportions of the 4,977 bp deleted mtDNA are presented for each type of the tissues examined in each group. N.A., not available.

13 bp direct repeat flanking the 5′- and 3′-end breakpoints at nucleotide position (np) 8470/8482 and np 13447/13459, respectively[13]. This mtDNA deletion was originally observed in the muscle tissues of patients with mitochondrial myopathies, including the Kearns–Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), and Pearsons' syndrome[40]. Multiple large-scale deletions of mtDNA have also been found in various tissues of aged individuals[41]. Moreover, an A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene of mtDNA, which is associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome[42], and another A8344G mtDNA mutation that is associated with myoclonic epilepsy and ragged-red fibres (MERRF) syndrome[43] have also been detected in the muscle of elderly subjects[44,45]. Additionally, at least 5 different types of tandem duplications were found in the D-loop region of mtDNA in several tissues of normal subjects, and the incidence and abundance of mtDNA with these tandem duplications increase with age[46].

It is important to note that each of these aging-associated mtDNA mutations including deletions, point mutations, and tandem duplications rarely exceeds 1% of total mtDNA in old human tissues (Table 2)[6,14]. The aging-associated mutant mtDNAs are usually only detectable by PCR and are frequently quantified by the choice of primers and PCR conditions. It is noteworthy that there may exist some other mutations in aging human tissues that have not yet been revealed by the available detection system[6]. Recently, a more detailed detection system was designed for extensive screening of mtDNA deletions in human tissues[47]. The use of 180 kinds of PCR primer pairs allows one to detect all the possible deletions of mtDNA over 500 bp in size. Hayakawa *et al.*[47] applied this molecular technique to analyse large-scale deletions in mtDNAs of normal hearts of subjects of various ages. They observed an extensive fragmentation of mtDNA into minicircles with various sizes of deletions, and the incidence and abundance of mutant mtDNAs increase with age and are well-correlated with oxidative damage to mtDNA. The long-range PCR technique was recently adopted to amplify the entire mitochondrial genome to facilitate the detection of mtDNA deletions in old human tissues. Kovalenko *et al.*[48] demonstrated with an XL-PCR system that in skeletal muscle of subjects over 80 years of age most of the mtDNA molecules are truncated into smaller fragments and only very few full-length mtDNA molecules are detected. It is noteworthy that the reported mutations and the aforementioned oxidative damage of mtDNA represent only a small proportion of all the mtDNA damages that occur in the aging process, and that multiple mutations of mtDNA often co-exist in various tissues of aged individuals[6,49].

Although the proportion of the mutated mtDNA molecules is found to correlate with the 8-OH-dG content of mtDNA[31], it is not understood how oxidative stress or ROS causes mtDNA mutations. Adachi *et al.*[50] have recently demonstrated that ROS may cause large-scale deletion of mtDNA in the rodents. They detected a 4 kb deletion of mtDNA in the heart of Balb/c mice that had received chronic intraperitoneal injection of doxorubicin which is known to induce cardiomyopathy and elicit profound lipid peroxidation of heart mitochondria. Moreover, they found that administration of coenzyme Q_{10} (a free radical scavenger) to mice effectively prevents deletion in mtDNA and decreases the lipid peroxide content of heart mitochondria. This finding provides the first direct evidence to support the notion that ROS and free radicals are involved in the deletions in mtDNA. In addition, it has been demonstrated that genotoxic intermediates of lipid peroxidation may have a role in eliciting age-associated mtDNA mutations[51]. The region of mtDNA that is attached to the ROS-generating sites in the mitochondrial inner membrane is

likely to be more susceptible to oxidative damage, strand breakage, and point mutation. It is conceivable that the frequency of occurrence and the type of mtDNA mutation are determined, at least in part, by the interaction between mitochondrial DNA polymerase and the ROS-damaged hot spots or hot regions of the mtDNA molecules during DNA replication[52].

In addition, exogenous sources of free radicals may cause mutation and oxidative damage to mtDNA in human tissues. Indeed, many types of mtDNA mutations are reported to occur more frequently and more abundantly in sun-exposed skin as compared with the non-exposed skin[53,54]. Recently, we demonstrated that aside from age, smoking is an exogenous factor that significantly increases the incidence and proportion of the mtDNA molecules with large-scale deletions in human lung tissues[55,56]. Moreover, the lipid peroxide contents in the lung tissues of smokers are significantly higher than those of the age-matched non-smokers[56]. These observations suggest that free radicals and/or organic radicals generated by environmental insults such as light irradiation, gaseous phase of cigarette smoke, and air pollutants may act as add-on factors in eliciting the formation and accumulation of mtDNA mutations during the aging process in humans.

Conclusion

Age-dependent decline in the function of the mitochondrial respiratory system increases the production of ROS and free radicals in mitochondria. On the other hand, the levels of the small-molecular-weight antioxidants and the activities of the free radical scavenging enzymes are insufficient in aging tissues[6,24]. Therefore, the balance between oxidants and antioxidants is gradually upset during the aging process due largely to the excess production and inefficient disposal of the ROS and free radicals[6,20]. This condition imposes an elevated oxidative stress on the cells, and causes a broad spectrum of oxidative damage to vital biomolecules in tissues[6,22,34]. Since mtDNA is not protected by histones and is continually exposed to the high-oxidative-stress environment of the mitochondria, it is extremely vulnerable to damage by ROS and free radicals[30]. Recent work from several laboratories have demonstrated that both the incidence and amount of specific mutations (point mutations and large-scale deletions) and random oxidative damage (indicated by the level of 8-OH-dG) of mtDNA increase exponentially with age[13,14,44–49]. The transcription and translation of the mtDNA-encoded polypeptides in the cells harbouring mutated mitochondrial genome get inevitably impaired both qualitatively and quantitatively[15,17]. The respiratory enzymes synthesized by such mitochondria are defective and function not only less efficiently in energy metabolism but also produce more ROS and free radicals which further damage the respiratory system of the mitochondria. This vicious cycle operates in cells at a rate dictated by the metabolic rate and physiological condition of the tissue[6,21]. We suggest that the ever-increasing levels of ROS and free radicals accelerate this cycle in the later part of life of an individual and lead to aging and age-related diseases. This mechanism also explains the well-documented observations that patients with mitochondrial disseases usually age much faster and often culminate in premature death.

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