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Aging- and smoking-associated alteration in the relative content of mitochondrial DNA in human lung

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Abstract mtDNA mutations and oxidative DNA damage has been observed to accumulate in the lung and other tissues in human aging. Thus, it is of interest to know whether the content of mtDNA is changed in aging tissues of the human. Using a competitive PCR method, we determined the relative content of mtDNA in the lung tissues of 49 subjects aged 16–85 years. The results showed that the relative content of mtDNA (with respect to the β-actin gene) in the lung tissues was significantly increased with age ($P < 0.005$). The average mtDNA content in the lung tissues of the subjects over 80 years of age was found to be about 2.6-fold higher than that of the subjects below age 20. However, the relative content of mtDNA was slightly increased in the lung tissues of light smokers but significantly decreased in heavy smokers. Moreover, we found a significant increase with age in the level of oxidative damage to DNA as indicated by the ratio of 8-OH-dG/dG in total DNA ($P < 0.0005$). These results together with our previous findings suggest that the increase in mtDNA content of aging tissues may be effected through a feedback mechanism to compensate for the functional decline of mitochondria in human aging and that smoking may modulate the mechanism.

Key words: Mitochondrial DNA; Competitive polymerase chain reaction; Lung; Aging; Smoking

1. Introduction

Mitochondrial respiratory function has been demonstrated to decline in various human tissues during the aging process [1–3]. This is thought to be caused, at least partly, by oxidative damage and mutation of mitochondrial DNA (mtDNA) in somatic tissues of aged individuals [3].

The mitochondrial mass and mtDNA copy number of individual cells vary with the type of cell and tissue, and are changed during cell differentiation, hormone treatment and exercise [4–6]. In addition, gene expressions of both nuclear and mitochondrial genomes have been demonstrated to respond in a complex manner to a variety of physiological and developmental signals [7]. Over-proliferation of mitochondria and an increased synthesis of mitochondrial respiratory enzymes are often observed in patients with mitochondrial myopathies [8]. However, it remains unclear how the tissue cells respond to the decline of mitochondrial function in human tissue during the aging process.

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Abbreviations: mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; ROS, reactive oxygen species; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine

Cigarette smoke contains more than 3800 compounds, including potent carcinogens, such as benzo[*a*]pyrene, and a substantial amount of free radical forming substances, such as hydroquinone. These organic radicals and toxic agents may cause extensive damage to DNA, proteins and lipids [9]. Moreover, human mtDNA is extremely compact and has no protective histones, and is replicated without efficient proofreading and DNA repair systems [10]. These unique characteristics render mtDNA susceptible to damage by reactive oxygen species (ROS) and genotoxic agents [11]. Accumulation of these toxic products in human lung may lead to an increase of DNA damage and impairment of mitochondrial respiratory function. In previous studies, we have shown that mtDNA mutations, oxidative DNA damage and lipid peroxidation are increased during aging in the human lung [12,13] and other tissues [14,15]. In addition, smokers were found to have more extensive damage to DNA and lipids in the lung tissues as compared with non-smokers [16]. However, the effect of smoking on the mtDNA content in the lung in response to oxidative DNA damage and mtDNA mutation is unclear.

In this study, we established a competitive PCR method for the quantification of mtDNA with respect to the β-actin gene of nuclear DNA in the lung tissues. The significance of the alteration of the relative content of mtDNA in the lung tissue during aging and smoking is discussed.

2. Materials and methods

2.1. Human lung tissues

The lung tissues examined in this study were obtained from 49 patients at the Division of Thoracic Surgery, Veterans General Hospital-Taipei, who underwent surgical resection for treatment of various pulmonary diseases, including spontaneous pneumothorax, metastatic lung tumors and bronchogenic carcinoma. None of the patients recruited in the study had a known history of industrial or occupational exposure to asbestos or organic solvent. A small piece of the lung tissue (ca. 350 mg) was collected from the grossly normal region of the resected specimen and quickly frozen in liquid nitrogen until analysis. The study subjects were 25 non-smokers and 24 smokers, who were between 16 and 85 years of age. The smoking index is expressed as pack-years (cigarettes or parts thereof smoked per day × years of smoking/20).

2.2. Competitive polymerase chain reaction

A competitive PCR method similar to that developed by Gilliland et al. [17] was used for the quantification of DNA. The competitor DNA was constructed by a method similar to that described by Celi et al. [18]. The nucleotide sequences of the primers used in this study are described in Table 1. To generate an internal DNA standard (Fig. 1), a plasmid was constructed by cloning the PCR product in the pGEM-T vector (Promega Co., Madison, WI). A known amount of the internal DNA standard was introduced with the lung DNA sample into the PCR reaction mixture. Each aliquot of 50 μl reaction mixture contained 200 μM of each dNTP, 5 μCi of [α-^{33}P]dATP, 20 pmol of each primer, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus,

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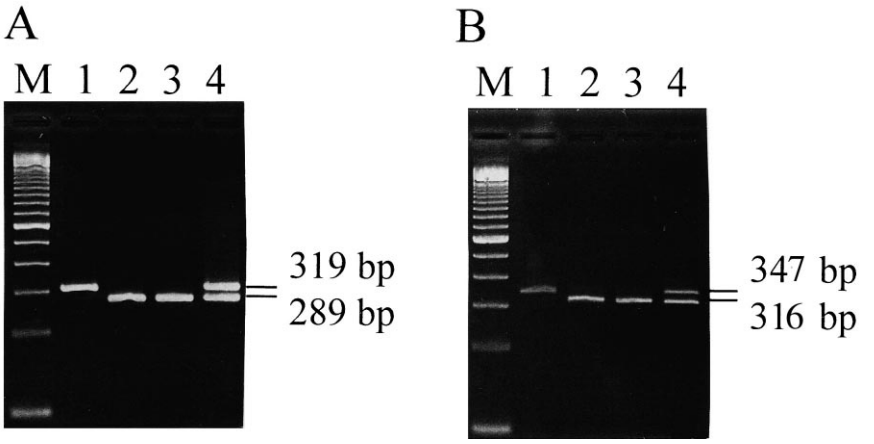

Fig. 1. The construction of the competitor DNA for competitive PCR. A: A 319 bp and a 289 bp PCR product were amplified from human β-actin gene with primers BA1 and BA2 (lane 1) and with primers BA1 and BA2–BA3 (lane 2), respectively. A plasmid was constructed by insertion to the pGEM-T vector of the PCR product amplified with primers BA1 and BA2–BA3. A 289 bp PCR product was amplified from the plasmid with primers BA1 and BA2 (lane 3). When a known amount of the internal DNA standard was introduced into the sample and amplified with primers BA1 and BA2, two products with sizes of 319 bp and 289 bp were generated, respectively, from the endogenous template and from the internal DNA standard (lane 4). Lane M is the 100 bp DNA marker. B: With the same approach, a 347 bp and a 316 bp PCR product were amplified from the ND1 gene of human mtDNA with primers L3540 and H3887 (lane 1) and with primers L3540 and H3887-h3836 (lane 2), respectively. A plasmid was constructed by insertion to the pGEM-T vector of the PCR product that was amplified with primers L3540 and H3887-h3836. A 316 bp PCR product was amplified from the plasmid with primers L3540 and H3887 (lane 3). When a known amount of the internal DNA standard was introduced into the DNA sample and amplified with primers L3540 and H3887, two products with sizes of 347 bp and 316 bp were generated, respectively, from the endogenous template and from the internal DNA standard (lane 4).

Norwalk, CT), 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). PCR was carried out with 30 s denaturation at 94°C, 20 s annealing at 58°C and 40 s extension at 72°C for 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler. The PCR products were separated electrophoretically in a 3% agarose gel. A Kodak X-ray film was exposed for 4–6 h to the gel after it had been vacuum dried. The intensities of the PCR products of the target and competitor DNAs were analyzed by scanning densitometry for each reaction. In each competitive PCR run, a standard curve was constructed by plotting the competitor/target DNA intensity ratio against the logarithm of the content of the internal DNA standard added to the reaction mixture (Fig. 2). Using this method, the relative content of mtDNA was cal-

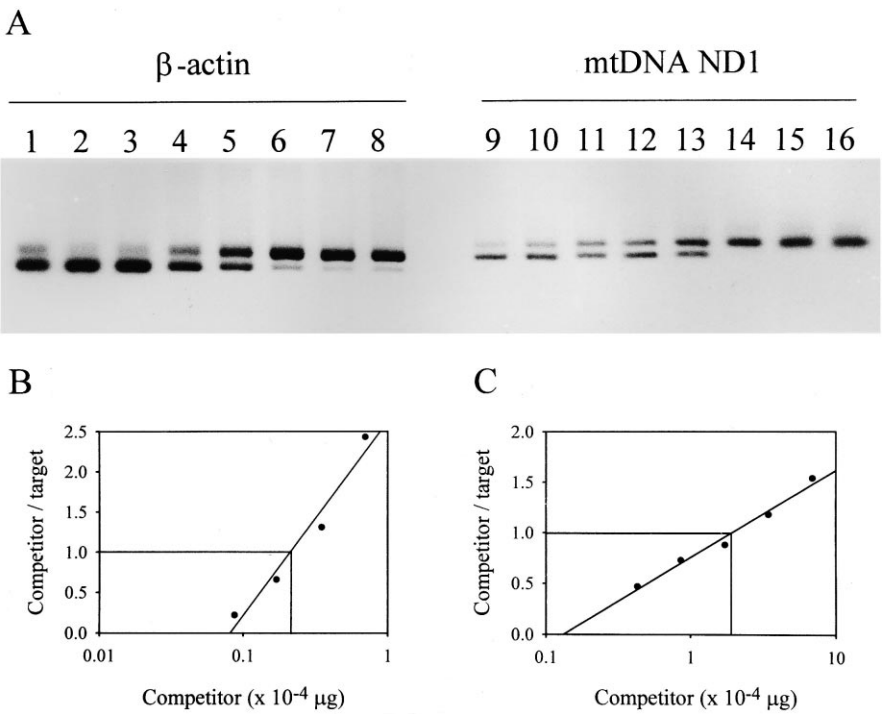

Fig. 2. Competitive PCR. A: The amount of unknown wild-type target DNA was determined by comparison of the amount of the wild-type PCR product with the amount of the PCR product derived from an internal DNA standard. The band intensities of the wild-type and competitor PCR products were analyzed by scanning densitometry for each reaction. B and C: Standard curves constructed by plotting the ratio of competitor/target DNA intensity against the logarithm of the amount of the internal DNA standard added to the reaction to quantify the amount of nuclear DNA (β-actin gene) and mtDNA (ND1 gene), respectively. The best-fit line was established and the corresponding algebraic equation was used to calculate the relative amount of target DNA.

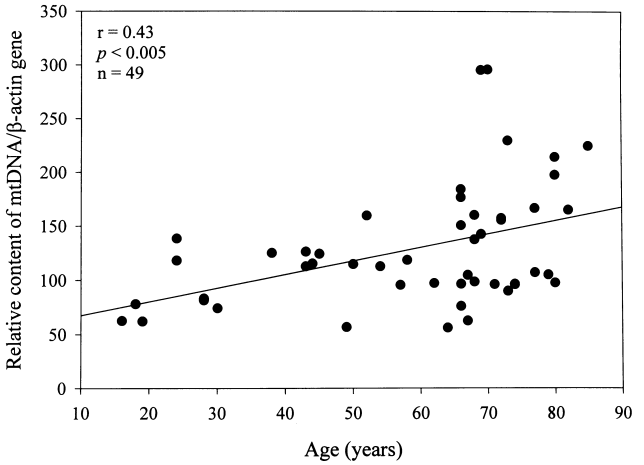

Fig. 3. Increase of the relative content of mtDNA in human lung. The relative content of mtDNA of each of the 49 lung samples was determined by the competitive PCR method illustrated in Fig. 2. The relative content of mtDNA (with respect to the β-actin gene) in the lung tissues was found to significantly increase with age ($P < 0.005$).

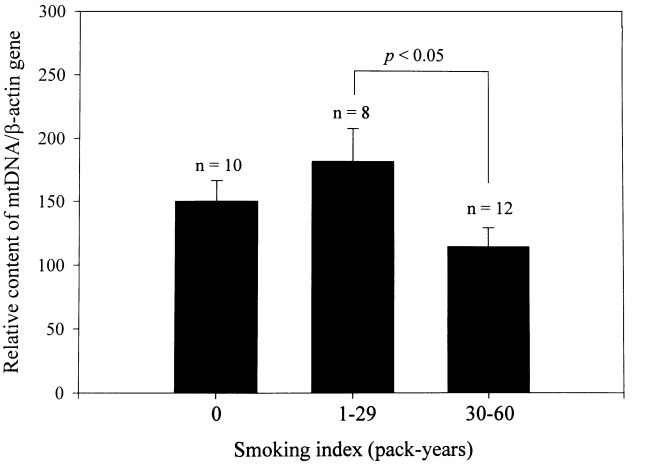

Fig. 4. The effect of smoking on the relative content of mtDNA in the lung tissues of aged individuals. In the group of subjects over 60 years of age, the average relative contents of mtDNA in the lung tissues of non-smokers ($n = 10$), light smokers (smoking index < 30 pack-years, $n = 8$) and heavy smokers (smoking index ≥ 30 pack-years, $n = 12$) were found to be 150.1 ± 16.4, 181.9 ± 26.1 and 114.4 ± 14.9 (mean ± S.E.M.), respectively. The average content of mtDNA in the lung tissues of heavy smokers was lower than that of light smokers ($P < 0.05$, Student's t-test). The average mtDNA content in the lung of light smokers was higher than that of non-smokers, but the difference did not reach statistical significance ($P = 0.15$, Student's t-test).

culated as an equivalent of one copy of β-actin gene and normalized with a factor of 25 for the difference in amplification efficiency between the two pairs of primers for β-actin and ND1 genes, respectively.

2.3. Analysis of the 8-hydroxy-2′-deoxyguanosine (8-OH-dG) content in lung DNA

The 8-OH-dG content of each lung DNA sample was determined according to the procedure described by Lu et al. [15]. The 8-OH-dG and dG contents in the total DNA of lung tissues were determined by an HPLC-ECD/UV detector system after sequential enzyme digestions at 37°C of freshly isolated DNA with DNase I, nuclease P1 and alkaline phosphatase, respectively. Deoxyguanosine (dG) (Sigma Chemical Co., St. Louis, MO) and 8-OH-dG (Cayman Chemical Co., Ann Arbor, MI) were used as standards.

3. Results

By use of the competitive PCR method, we determined the relative content of mtDNA in the lung tissues of 49 subjects who were 16–85 years of age. The results showed that the relative content of mtDNA (with respect to the β-actin gene) in the lung tissues was significantly increased with age ($P < 0.005$) (Fig. 3). The average relative content of mtDNA (179.5 ± 22.8, mean ± S.E.M., $n = 5$) of subjects in the group over 80 years of age was found to be about 2.6-fold higher than that (67.5 ± 5.3, mean ± S.E.M., $n = 3$) of subjects in the group younger than 20 years of age.

In the group of subjects over 60 years of age, the average relative contents of mtDNA in the lung tissues of non-smokers ($n = 10$), light smokers (smoking index < 30 pack-years, $n = 8$) and heavy smokers (smoking index ≥ 30 pack-years, $n = 12$) were 150.1 ± 16.4, 181.9 ± 26.1 and 114.4 ± 14.9 (mean ± S.E.M.), respectively. The average relative content of mtDNA in the lung tissues of light smokers was slightly higher than that of the non-smokers ($P = 0.15$, Student's t-test). However, the average content of mtDNA in the lung tissues of heavy smokers was significantly lower than that of light smokers ($P < 0.05$, Student's t-test, Fig. 4).

In order to assess the oxidative damage to DNA, we determined the relative content of 8-OH-dG in total DNA of lung tissues from subjects of different ages. The results showed a significant increase with age in the tissue level of the 8-OH-dG/dG ratio ($P < 0.0005$, Fig. 5).

4. Discussion

The lung tissue is continually subjected to ROS and free radicals generated by electron leak from the respiratory chain as well as exogenous sources such as cigarette smoke and air pollutants. The lung may serve as a good system to study the impact of environmental factors on human tissues during aging. Exogenous sources of ROS and free radicals, such as those derived from cigarette smoke, may aggravate the oxidative damage and mutation of mtDNA in aging human tissues.

Table 1
Sequences of the primers used in this study

Primer	Sequence (5′ to 3′)	Target DNA
BA1	CATGTGCAAGGCCGGCTTCG	nDNA, β-actin
BA2	CTGGGTCATCTTCTCGCGGT	nDNA, β-actin
BA2–BA3	CTGGGTCATCTTCTCGCGGTGCAGCACGGGGTGCTCCTC	nDNA, β-actin
L3540	TCTCACCATCGCTCTTCTAC	mtDNA, ND1
H3887	TTGGTCTCTGCTAGTGTGGA	mtDNA, ND1
H3887-h3836	TTGGTCTCTGCTAGTGTGGAGGCAGGAGTAATCAGAGGT	mtDNA, ND1

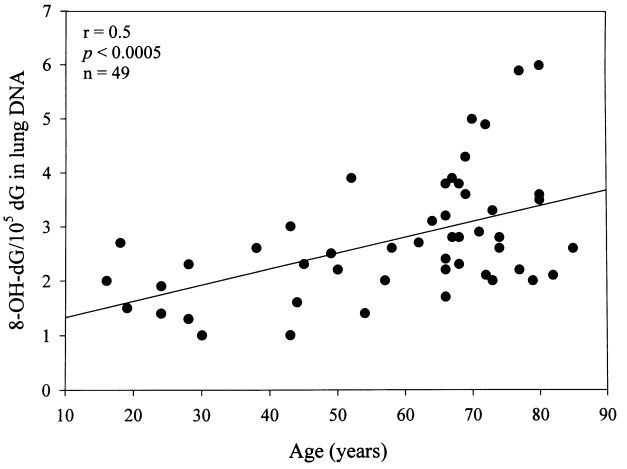

Fig. 5. Increase of oxidative damage to DNA in human lung during aging. The relative contents of 8-OH-dG in the 49 lung tissues were determined by the HPLC-ECD system. The ratio of 8-OH-dG/10^5 dG in the lung tissues was significantly increased with age ($P < 0.0005$).

In the past decade, a number of mtDNA mutations have been found to accumulate in various tissues of aged individuals [3]. Oxidative damage and mutation of mtDNA may be facilitated by cigarette smoke and synergistically cause the age-dependent decline of the respiratory function of mitochondria [3,13]. These earlier observations have prompted us to investigate whether the mtDNA content is altered in the lung tissues as a function of age or smoking index.

In this study, we demonstrated for the first time that the relative content of mtDNA in human lung is increased with age. This is consistent with the previous observation that the mtDNA copy numbers in liver, heart and brain of senescent rats are higher than those of the adult counterparts [19]. Moreover, it was reported recently that the content of mtDNA in the HeLa S3 cells is increased about 2-fold after treatment with 0.1 μM rotenone, which inhibits complex I activity [20]. These results support the idea that as respiratory function declines with age, the tissue cells are able to manage to compensate for the reduced ATP synthesis by inducing the proliferation of mitochondria and/or increasing expression of the OXPHOS genes.

The rate of production of superoxide anions and hydrogen peroxide in mitochondria was shown to increase with age in animal tissues [21]. Since the mitochondrial genome is more susceptible to free radical attack [3,11,22], it would be of interest to determine if the oxidative damage to mtDNA in human lung is more extensive than that to nuclear DNA during aging and upon smoking. However, due to the limited amount of lung tissues available from each of the study subjects, we could only determine the content of 8-OH-dG in the total DNA of human lung tissues. The results showed that oxidative DNA damage, indicated by the 8-OH-dG/dG ratio, in the lung is increased with age ($P < 0.0005$, Fig. 5). ROS and free radicals have been established to be able to act as secondary messengers to activate the transcription factors including NF-κB and AP-1 [23]. We conjectured that oxidative stress and oxidative DNA damage may elicit activation of replication and/or transcription of mtDNA in lung tissues. Recently, it was observed that human cells could respond to the impairment of electron transfer by promoting the expression of both nuclear and mitochondrial genes, through an H_2O_2-dependent signaling pathway [24]. Therefore, the increased production of superoxide anions and hydrogen peroxide from mitochondria in aging human tissues may be one of the factors involved in the feedback mechanisms of the compensation for the age-dependent increase of the oxidative damage and functional deterioration of mitochondria. This will allow tissue cells to adapt to the decline of mitochondrial respiratory function during the aging process.

On the other hand, it has been demonstrated that increased DNA adducts and/or single-strand breaks of mtDNA can not only increase mtDNA damage but also inhibit mtDNA replication in the lung tissues of smokers [25]. This is consistent with our finding that the relative content of mtDNA was decreased in the lungs of heavy smokers (Fig. 4). These results suggest that cigarette smoking could modulate, in a negative manner, the mtDNA content in the lung tissues of the smokers.

In summary, this study clearly demonstrated that oxidative DNA damage and the relative content of mtDNA in human lung tissues are increased concurrently during aging. Moreover, long-term heavy cigarette smoking causes a significant decrease in the mtDNA content in the lung tissues of aged individuals. We thus suggest that the increase in mtDNA content may be effected through a feedback mechanism to compensate for the respiratory function decline of the mitochondria harboring mtDNA with oxidative damage or mutation in aging lung tissues, and that cigarette smoking may modulate this mechanism in a negative manner.

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