Age-related Fragmentation of Mitochondrial DNA Associated with Oxygen Damage

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1. Introduction

In 1989, we proposed the 'mitochondrial theory of ageing' that accumulation of mitochondrial (mt) DNA mutations during the life of an individual constitute an important contributor to general senescence and age-related diseases (20). This was based on the fact that the rate of somatic mutation of mtDNA in man is unknown, but in yeast is extremely high; the petite mutation of yeast, characterized by deletions of large sections of the mtDNA, leading to a respiratory deficient phenotype, occur spontaneously in growing cultures at a rate of 0.1 to 10% of the total cell population. Using timely invented polymerase chain reaction (PCR) technology (31), considerable publications (3,4,8,9,16,19) have documented an extensive array of age-dependent accumulation of mtDNA with deletions (ΔmtDNA). PCR quantitative data on a single ΔmtDNA among individuals with various ages indicated that there are four orders of magnitude fewer ΔmtDNA in infancy as compared to old age (34,36), and that a newborn harbors only very low amounts of the 5 kilo base-pair (kbp) deletion that is commonly observed in different tissues of adult (19), being not detected in the corresponding fetal tissues (4). Hence, PCR-detectable ΔmtDNAs seem to arise afresh with each generation. A progressive age-related increase in a hydroxyl-radical adduct of deoxyguanosine (dG), 8-hydroxy-deoxyguanosine (8-OH-dG), in mtDNA was documented in correlation with a single ΔmtDNA with 7.4 kbp deletion in human hearts (9). On the other hand, a cell harbors hundreds of mitochondria and thousand copies of mtDNA, and the fractional concentration of each ΔmtDNA detected by the conventional PCR using a single primer-pair is usually as low as 0.01-to-0.2% of the total mtDNA. Hence, a question arose whether an observed ΔmtDNA is the cause or the effect of the ageing (2). However, the total number (n) of ΔmtDNA visualized by PCR depends on a particular primer-pair used, such that the more distantly separated

primers enable to detect the larger deletions. Hence, a PCR-detectable ΔmtDNA was suggested to be the "tip of the iceberg" of the spectrum of somatic mutations (35).

To settle the problem, a recently devised total-detection system for deletions with 180 kinds of PCR primer-pairs (10), that enables to detect all possible ΔmtDNA over 0.5 kbp, was applied to mtDNA specimens from normal hearts of various ages. Surprisingly, the whole 'iceberg' in the oldest subject examined is visualized as 358 types of ΔmtDNA including 280 types of 'minicircles' that lack either one replication origin (Ori) or the both, associated with decrease in wild type mtDNA (ωmtDNA) down to 11%. Among normal subjects, a progressive age-related fragmentation of ωmtDNA into ΔmtDNA, predominantly 'minicircles', was demonstrated with a correlative increase in oxygen damage. In the patients with mt cardiomyopathy harboring severe point mutations in mtDNA, similar fragmentation and oxidative damage in mtDNA was documented at their age 7 to 19 equivalent to the normal subjects of age over 80. Exposure of a cultured cell line under oxygen stress, 95% oxygen, could mimic these changes within 3 days leading to an apoptotic cell death, whereas mtDNA lacking cells are relatively immune. These results were obtained by the following survey.

2. Specimens of mtDNA

Cardiac muscles from 21 human subjects, 8 males and 13 females aged 3 to 97 years, without cardiological symptoms were obtained at random, regardless of diseases at autopsy. As the positive controls, three specimens from the patients with mt cardiomyopathy harboring severe point mutations and two specimens of cultured cell exposed to oxygen stress were analyzed as listed in Table I; no. 1, excised heart at heart transplantation from a 7-years-old patient with dilated cardiomyopathy (27), no. 2 and 3, autopsied hearts from patients died of mt cardiomyopathy (25) at age 17 and 19, respectively, and no. 4 and 5, an immortalized human fibroblast cell line (ρ^+) before and after exposure to hyperoxia for 3 days (38). The ρ^+ carrying normal mtDNA, underwent an active cell death under 95% oxygen; 68% and 84% of the cells died on the 3rd and 4th days, respectively, as shown in Figure 1; by contrast, its derivative lacking mtDNA (ρ^0) exhibited a marked resistance to cell death. The total DNA was extracted from specimens with phenol and chloroform, precipitated with ethanol, and suspended in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 (10). MtDNA was extracted with phenol and chloroform from mt fraction that was prepared from 10 grams of heart muscles, purified with a discontinuous sucrose density-gradient centrifugation, and digested with proteinase K in 0.5% SDS, then with pancreas ribonuclease (10).

TABLE I
TYPES OF mtDNA AMONG SUBJECTS

Subject	Sex	Age	Disease	ΔmtDNA Type (n)	Sub-Type of ΔmtDNA				ωmtDNA (%)	8-OH-dG per 10^4 dG
					$OriL^+/H^+$	$OriL^-$	$OriH^-$	$OriL^-/H^-$		
A.K.	F	3	VSD	5	4	1	0	0	>99	<1
S.T.	M	24	Accident	49	16	15	8	10	85	<1
N.N.	F	28	Pul. Emb.	67	23	14	8	22	71	<1
Y.I.	F	48	Thymoma	43	13	8	4	18	73	5.9
Y.T.	M	60	Gastric ca.	49	13	23	1	12	71	17.5
Y.Y.	F	76	SAH	218	66	68	37	47	47	18.6
K.A.	M	85	Colon ca.	230	61	64	33	72	58	15.3
H.M.	F	97	Gastric ca.	358	78	88	63	129	11	148
1 M.K.	F	7	DCM	212	37	58	38	79	n.d.	n.d.
2 H.A.	F	17	mtCM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	21.5
3 T.K.	M	19	mtCM	235	48	59	31	97	16	20.1
4 ρ^+			normoxia	49	14	15	5	15	n.d.	n.d.
5 ρ^+			95% O_2	187	35	55	28	69	n.d.	n.d.

Abbreviations: ΔmtDNA, mtDNA with deletions; ωmtDNA, wild type mtDNA; Ori, replication origin; F, female; M, male; VSD, ventricular septal defect; Pul. Emb., pulmonary embolism; ca., cancer; SAH, subarachnoidal hemorrhage; DCM, dilated cardiomyopathy (27); mtCM, mitochondrial cardiomyopathy (17); ρ^+, a cultured human cell line, 701.2.8c (39); n.d., could not be determined due to the shortage of specimen.

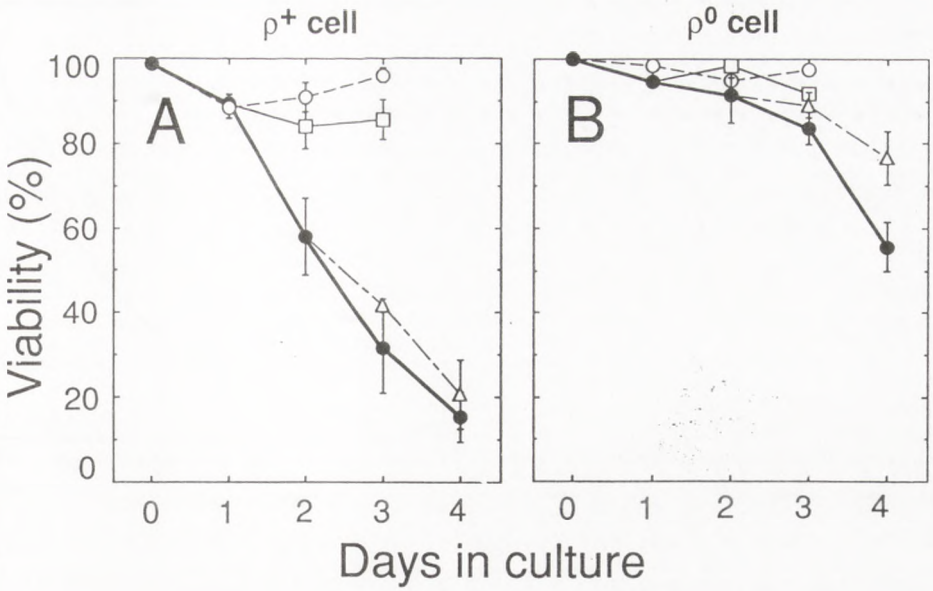

Fig. 1. The effect of oxygen stress on the viability of mtDNA-carrying 701.2.8c, on the mtDNA-lacking $\rho^0$701.2a, and the resistance against the death in the ρ^0 cell line.

A. The viability of 701.2.8c, a normal mtDNA-carrying cell line, is shown during an exposure to 95% oxygen or to 20% oxygen.

B. The viability of $\rho^0$701.2a, an mtDNA-lacking cell line derived from the 701.2.8c, is shown during an exposure to 95% oxygen or to 20% oxygen.

Both cells were cultured in a 95% (closed circle) or 20% (open circle) oxygen for 3-4 days. Some cells were cultured in 95% oxygen for one day and in 20% oxygen for two more days (open square), or in 95% oxygen for two days and in 20% oxygen for two more days (open triangle). The SD is expressed by the bar.

3. Oxygen damage in mtDNA

MtDNA specimens were completely digested with DNase I, spleen exonuclease, snake venom exonuclease, and alkaline phosphatase. The total amount of the ωmtDNA was determined on a 1% agarose gel, as described by Hengen (12). Deoxynucleoside, dG, and 8-OH-dG in the hydrolysate were quantitatively determined by using a micro-HPLC/mass spectrometry system (11). From the total amount of ωmtDNA and that of deoxynucleoside, ωmtDNA % to the total mtDNA was calculated. 8-OH-dG % among normal subjects (Fig. 2) increase exponentially associated with age [log(8-OH-dG %) = - 3.91 + 0.042 x age, r = 0.89]. The amount of ωmtDNA (Tab. I) decreased negatively correlating with 8-OH-dG % [log(ωmtDNA % = 1.90 -0.587 x (8-OH-dG %), r = 0.97] indicating oxygen damage as a responsible factor for the degradation of ωmtDNA. Overlaid plots of 8-OH-dG % in the positive control no. 2 and no. 3 is equivalent to age 78.

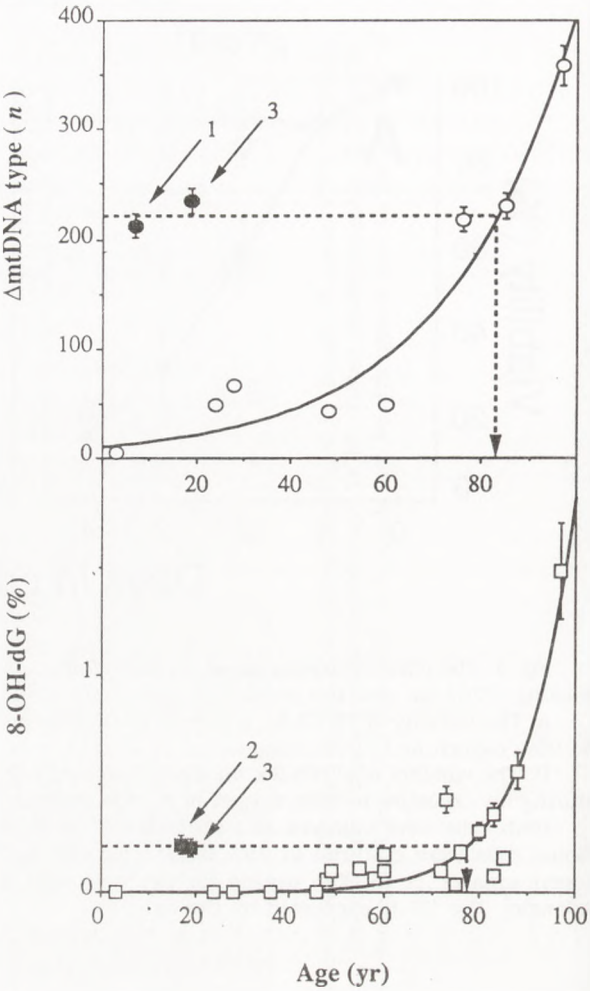

Fig. 2. Age-associated correlative increase in the total number of ΔmtDNA type and oxygen damage.

Upper panel: The ΔmtDNA type *n*, detected by the total-detection system, is plotted against subjects' age. A positive control of premature ageing (arrow **1**) is 7-year-old female who received heart transplantation (27), and another one (arrow **3**) is 19-year-old male with mt encephalomyopathy who died of mt cardiomyopathy (25). Mean ΔmtDNA type *n* of the controls is equivalent to the normal subject of 82-year-old.

Lower panel: The 8-OH-dG % is plotted against subjects' age Specimens of mtDNA were enzymatically hydrolyzed into nucleosides, and analyzed using the micro-HPLC/mass spectrometry system (9). A positive control (arrow **2**) is 17-year-old female who died of mt cardiomyopathy associated with MELAS. Mean 8-OH-dG content of the controls is equivalent to the normal subject of 78-year-old.

4. Total detection of ΔmtDNA

Among the 21 specimens for the oxygen damage measurements, eight specimens (3 males and 5 females, as listed in Tab. I) of enough quantity for the comprehensive analyses were selected, and the *n* of ΔmtDNA types was determined using the total-detection system for deletions (10). A progressive age-related increase in ΔmtDNA type *n* up to 358 [log(ΔmtDNA type *n*) = 1.00 + 0.016 x age, r = 0.90, P < 0.01] with a linear decrease in ωmtDNA % with age [ωmtDNA % = 102.9 – 0.729 x Age, r = 0.89] was documented in the mtDNA from normal subjects (Fig. 2, Tab. I). A strong negative correlation between ωmtDNA % and ΔmtDNA type *n* [ωmtDNA % = 90.1 – 0.201 x (ΔmtDNA type *n*), r = 0.94] indicates extensive fragmentation of ωmtDNA mainly into ΔmtDNA of closed circular duplex, but not much amount into linear DNA fragments that could not be detected by the system (cf. Fig. 5).

Overlaid plots of ΔmtDNA type *n* from the positive control patients, no. 1 and no. 3, are nearly equivalent to age 82 of normal subject (Fig. 2), indicating premature ageing of the patients' hearts. The 49 types of ΔmtDNA in the ρ^+ cell before the oxygen stress, equivalent to normal heart mtDNA of age 24, increased up to 187 types, the level of age 79, after 3 days of oxygen stress (Tab. I). Namely, the accumulation process of oxygen damage and ΔmtDNA during 80 years of the normal subjects is compressed into 19 to 7 years by the hazardous point mutations of the patients, and into 3 days by the oxygen stress.

5. Deletion type

The types of ΔmtDNA, distinguished from each other by their deletion sizes and locations, were classified into 4 sub-genotypes according to the preservation of replication origin (Ori): One is $OriL^+/OriH^+$, preserving both Ori for L-strand (OriL) and for H-strand (OriH), and other three are 'minicircles', lacking either one Ori or the two; *viz.*, $OriL^-$ lacks OriL, $OriH^-$ OriH, and $OriH^-/OriL^-$ both Oris. A remarkable mirror image between $OriL^-$ and $OriH^-$, and that between $OriH^-/OriL^-$ and $OriL^+/OriH^+$ in mtDNA size distribution were observed among normal subjects and positive controls, as shown in Fig. 3. A strong linear correlation between minicircles' type *n* and that of $OriL^+/OriH^+$ type [minicircle type $n = -21.3 + 3.27 \times (OriL^+/OriH^+$ type $n)$, $r = 0.97$] indicates random occurrence of minicircles without preferential site for deletions. Similar fragmentation of mtDNA and ΔmtDNA size distribution were mimicked in cultured cells within 3 days of oxygen stress (Fig. 4).

Types of ΔmtDNA visualized by the total detection system were thoroughly verified by the followings; **a)** no possible artifacts derived from misannealing of primers by shift of PCR products linked with the primer shift (Fig. 5-A), namely by the primer shift PCR method (32) built in the system (10). **b)** PCR-predicted restriction-site loss by the enzyme digestion (Fig. 5-B): Using *Eco*RI digested mtDNA as template, not a fragment from ωmtDNA, but two fragments from ΔmtDNA with restriction site-loss were visualized. PCR could amplify the fragment from a closed circular duplex DNA, but not from a linear one because the direction of the primer-pair becomes reverse. Hence, visualized mtDNA with restriction site-loss are closed circular duplex DNA. c) PCR-predicted Ori site-loss by Southern blot analysis (Fig. 5): All the mtDNA fragments, amplified using a particular PCR primer-pair out of 180 kinds putting OriL site in-between, hybridized with the probe for non-deleted region. On the contrary, not fragments from mtDNA, but a fragment from ΔmtDNA hybridized with the probe targeting OriL.

Somatic mt genome mutations as an important contributor to ageing, proposed by the 'mt theory of ageing' (20), have to satisfy following qualifications: **i)** The mutations arise afresh with each generation and accumulate

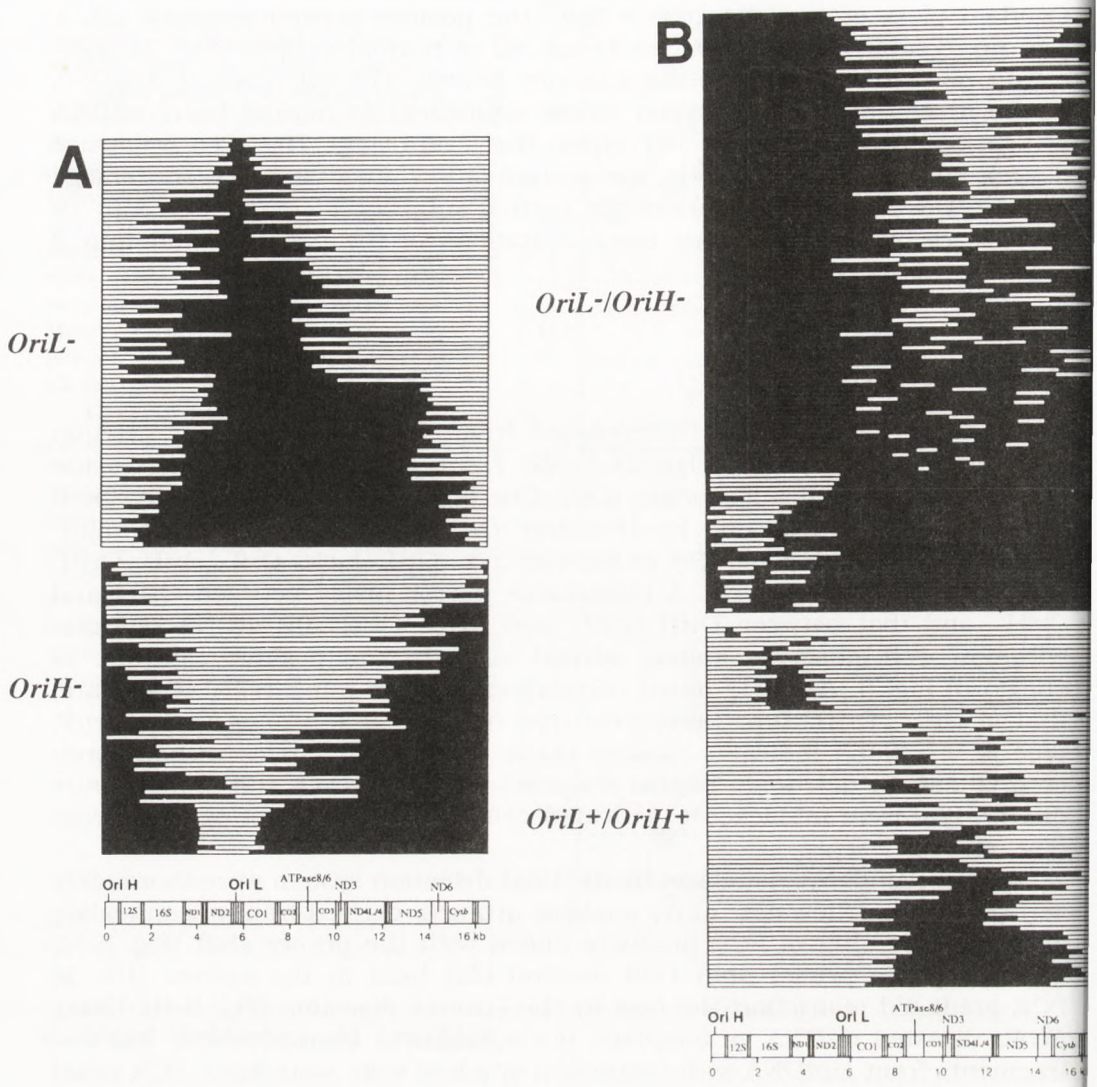

Fig. 3. Size distribution of ΔmtDNA in the heart mtDNA of a 97 year-old subject.

White bars indicate ΔmtDNA, and dark shading bars indicate deleted regions. Using the total-detection system for deletions (10), 358 types of ΔmtDNA were detected, and classified into four groups: *OriL*$^-$, *OriH*$^-$ (Panel A), *OriL*$^-$/*OriH*$^-$ and *OriL*$^+$/*OriH*$^+$ (Panel B) according to the preservation of Ori(s). They are arranged according to their sizes. Genomes in mtDNA are schematically illustrated at the bottom of each Panel.

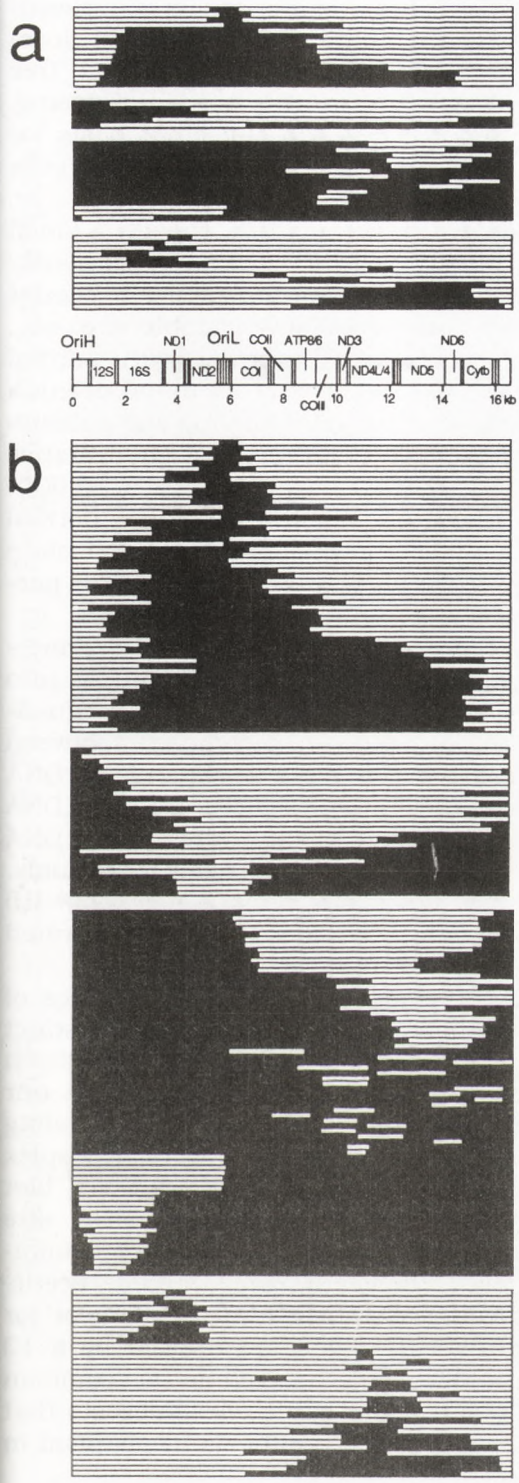

Fig. 4. Types of deletions detected in mtDNA from the ρ^+ cells in the presence or absence of oxygen stress.

PCR analyses using 180 primer pairs were performed for detecting the total number of the deleted mtDNAs during 3-day exposure of the 701.2.8c to 95% oxygen. The deletions were marked with the black bars. The deleted mtDNAs lacking either of Ori_L and Ori_H, or both, or having both were shown in the bulks of the bars in the respective orders. *a*, Deleted mtDNA is shown before the exposure to 95% oxygen. *b*, Deleted mtDNA is shown after the exposure for 3 days.

age-dependently. **ii)** The absolute level of accumulated mutations is accountable for age-related decline of mt function. **iii)** The mutations correlate closely with oxidative damage as proposed by a major theory of ageing, 'free radical theory of ageing' (7). Unifying ideas of these two theories of ageing, 'redox mechanism of ageing' (26) was proposed as the molecular basis for the progressive decline of cellular activity especially in post-mitotic cells such as muscle and brain.

Age-dependent accumulation of PCR-detectable ΔmtDNA, though a small quantity of each, was reported in many human tissues. As pointed previously (29), PCR-detectable ΔmtDNA with multiple deletions pleioplasmically coexist with ωmtDNA in a tissue, in contrast to Southern blot-detectable one; *viz.*, a single ΔmtDNA of a large quantity heteroplasmically coexists with normal mtDNA, presumably originated from a clonal expansion of an initial deletion event occurring early in oogenesis, being detected often among the patients with early-onset mt myopathies (15) or Pearson's syndrome (30). Quantitative data on a single PCR-detectable ΔmtDNA indicated that there is a 10,000-fold increase in heart muscle and in skeletal muscle during the normal human life span (9,34), satisfying the qualification **i)**. However, the absolute level of a ΔmtDNA detected by the conventional PCR detection using a particular PCR primer-pair is far low to meet the qualification **ii)**.

In this study, the total-detection system using 180 kinds of PCR primer-pairs documented a progressive age-related fragmentation of ωmtDNA into mtDNA correlating with oxygen damage (Tab. I, Fig. 3), fulfilling the qualifications above mentioned: **i)** A strong negative correlation between ωmtDNA % and age ($r = 0.89$), and that between ωmtDNA % and ΔmtDNA type *n* ($r = 0.94$) indicate that somatically acquired deletions lead ωmtDNA to extensive fragmentation into ΔmtDNA. **ii)** The absolute levels of ωmtDNA and ΔmtDNA could account for mt dysfunction in the senescent individuals, hence, age-related progressive decline of ventricular performance (23). **iii)** Correlative increase in 8-OH-dG % with ΔmtDNA types *n* is demonstrated in normal subjects as well as positive controls (Fig. 2).

It was our surprise that 358 types of ΔmtDNA including 280 types of minicircles exist in the oldest subject examined (Fig. 3), because two distinct Oris were reported to regulate mtDNA replication (1), and there have been few reports on a deletion including Ori site of human mtDNA. Hence, our results were thoroughly verified by confirming **a)** no possible misannealing of PCR primers (Fig. 5-A), **b)** restriction-site loss and closed circular duplex of ΔmtDNA (Fig. 5-B), and **c)** Ori site-loss in minicircles by Southern blot analysis (Fig. 6). A remarkable mirror image of the entire ΔmtDNA' size distribution (Fig. 3) and a strong linear correlation ($r = 0.97$) between minicircle and ΔmtDNA preserving both Oris (Tab. I) suggested random occurrence of deletions without preferential site, in contrast to the hotspot for Southern blot-detectable deletions; *viz.*, a 5 kbp deletion bridged by a 13 bp direct repeat was found so frequently that being referred as the 'common deletion' (33). A progressive age-related increase in minicircles suggests that a replicative mechanism similar to a discontinuous synthesis mechanism in

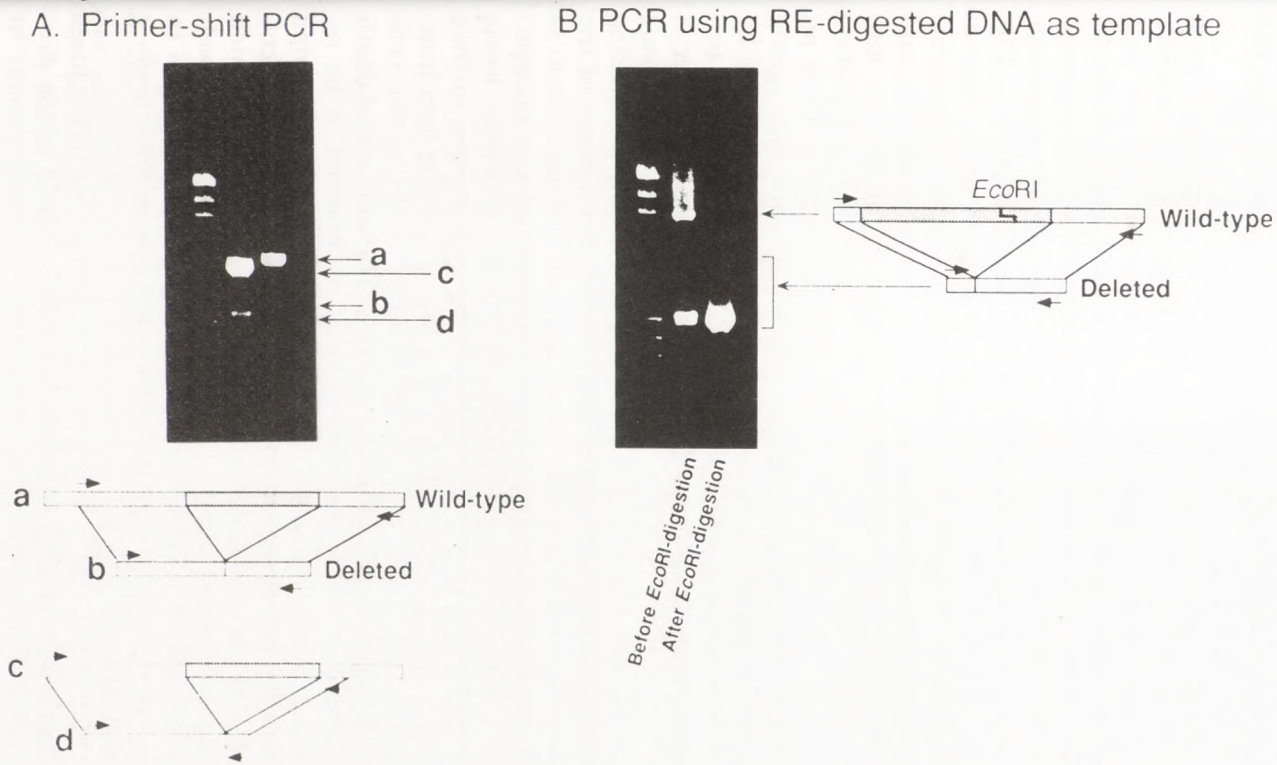

Fig. 5. Verification of ΔmtDNA.

A. Primer shift PCR method (32) that was automatically built-in with the combination of primers used in the total-detection system (10) demonstrated the shift of PCR products from the heart mtDNA of a 97 year-old subject (a-to-c derived from ωmtDNA, b-to-d derived from a ΔmtDNA) interlocking with the shift of primer-pairs L1095/H4222-to-L549/H3370.

B. The restriction-site loss predicted by the system was confirmed by the PCR using a primer-pair (L9561/H15755) before and after *Eco*RI digestion. The PCR could amplify fragments from ΔmtDNA of a closed circular duplex, but not from linearized ωmtDNA after the digestion because the direction of the primer-pair becomes reverse.

sea urchin oocytes (21), to that observed in stable DNA replication mutants of *Esherichia coli* without oriC (5), or to a endonucleolytic priming *rolling-circle* pathway of the replication (18) may operate not only during rapid mtDNA replication such as oogenesis, but somatic replication of ΔmtDNA *per se* during normal life span.

Deng et al. (6) demonstrated single strand breaks in a plasmid as a consequence of oxygen radical attack on DNA. In human heart mtDNA, Hayakawa et al. (9) demonstrated a progressive age-related increase in a single ΔmtDNA with 7.4 kbp deletion, correlated with increase in 8-OH-dG ($r = 0.93$) up to 1.5% at age 97; in human brain, Mecocci et al. (22) reported a similar accumulation in 8-OH-dG up to 0.87% at age 90. A progressive age-related accumulation in 8-OH-dG correlating with ΔmtDNA type *n* (Fig. 2) reflects a long-term accumulation of the oxygen damage during human life, analogous to sedimentary rocks in a glacier. Hence, it seems reasonable to presume random double-strand breaks by hydroxyl-radical attacks and rejoining of mtDNA as a referable mechanism for its fragmentation into ΔmtDNA, that accelerates further the oxygen damage. These changes leading to progressive decline of bioenergetic activities of cells and organs imply an underlying control mechanism of the cell death relating with the ageing process, as schematically illustrated in Fig. 7. This view is supported by the fact that a prominent apoptotic cell death of a human fibroblast cell line (ρ^+) was associated with the similar fragmentation of mtDNA after 3 days' exposure to hyperoxia, mimicking accumulation of the oxygen damage and ΔmtDNA during human life (Fig. 4), whereas its derivative lacking mtDNA (ρ^0) is relatively immune. Recent advances in the mechanism of the programmed cell-death provided the information that the mitochondrion is the major target of the *apoptosis* regulating protooncogen products manipulating the oxygen free radical damage, Bcl-2/Bax (13,24), of which levels are differentially affected by p53 protein (37). Revelation that human mtDNA has delicate susceptibility to the hydroxyl radical attack breaking into hundreds' pieces of minicircle (Fig. 3,4) will give an insight to the active cell-death machinery, as its major target would be mtDNA. The active cell-death essential for human morphogenesis and development is reported to be related with oxygen damage of macromolecules being blocked by Bcl-2 located in the mt inner membrane (14). However, the block seems to be not perfect, as there is cumulative oxygen damage and deletions in mtDNA associated with age (Fig. 3). From this stand point of view, normal ageing and development seem to be each side of a coin. The cell-death machinery seems to be easily imbalance by the point mutations of the patient's mtDNA leading to the premature ageing (Fig. 2).

Extensive oxygenation of skeletal muscle *in vivo* in senescent individuals was demonstrated non-invasively (28). Therefore, a *vicious cycle* of the progressive oxygen damage and fragmentation of ωmtDNA seems to result in those changes to be synergistic and exponential associated with age (Fig. 7). The similar premature fragmentation of mtDNA (17, 27) and extensive tissue oxygenation (28) was demonstrated in the patients with mt cardio-

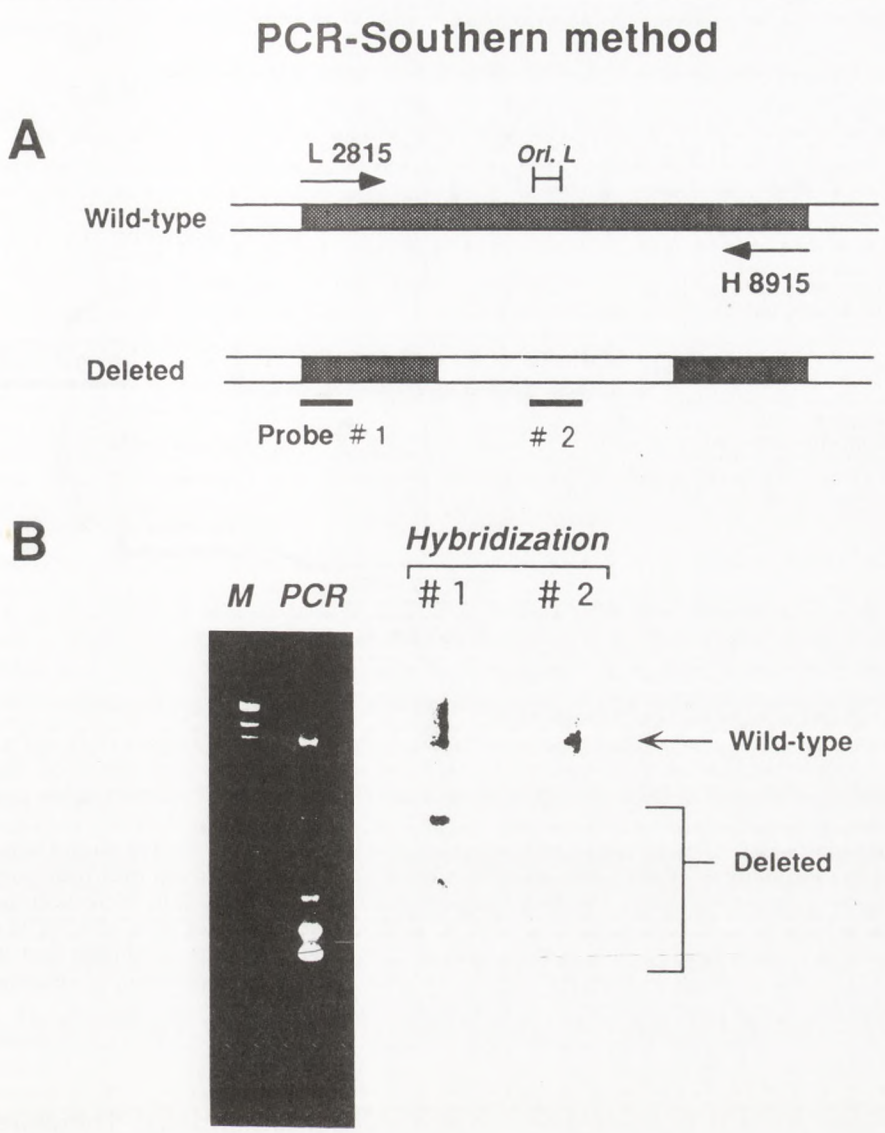

Fig. 6. Confirmation of OriL site-loss among ΔmtDNA.

A. PCR with a primer-pair (L2815/H8915) amplified fragments from the heart mtDNA of a 97 year-old subject. Probe #1, mtDNA fragment (np 2815-3251) containing the non-deleted region, and probe #2, the fragment (np 5668-6069) containing the OriL region, were amplified by PCR, purified by subcloning, and labeled with α-[^{32} P] dCTP.

B. Etidium bromide staining of the PCR products (PCR) demonstrated ωmtDNA and ΔmtDNA with several sizes, shown by the size marker (M). The duplicated PCR products were electrophoresed on a 1% agarose gel, blotted onto a GeneScreen Plus membrane, hybridized independently with the above probe #1 and #2, and the radioactive signals were visualized. The principle of this PCR-Southern blot method was reported previously (8).

The Southern blot by probe #2 clearly indicated the loss of OriL site among ΔmtDNA.

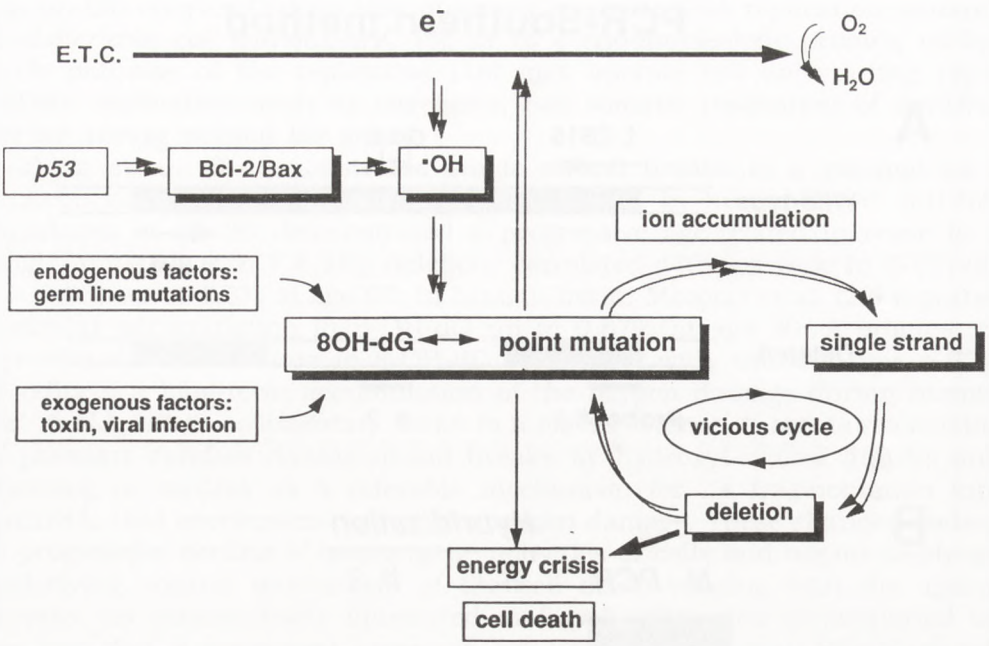

Fig. 7. Scheme for mtDNA mutations.

Production of reactive oxygen spices is enhanced by both endogenous factors such as inherited point mutations and exogenous factors such as toxins, drugs or viral infections. Damages of macromolecules due to reactive oxygen species are regulated by the protooncogene products, Bcl-2/Bax, of which levels are differentially affected by *p53* protein (37). Hydroxyl-radical adduct of guanosine, 8-OH-dG, resulted in random point mutations and the double-strand separation leading to a long stretch of single-stranded DNA, then deletion. Defected electron-transport chain encoded by ΔmtDNA enhances the hydroxyl-radical formation resulting in more accumulation of 8-OH-dG and ions in mitochondrial matrix. High concentration of ions also facilitate the double-strand separation. Such a *vicious* cycle of the oxygen free radical damage and deletion in mtDNA seems to result in those changes to be synergistic and exponential, as demonstrated in Fig. 6.

myopathy harboring hazardous germ-line point mutations. Therefore, the mt diseases seem to be derived from premature ageing in tissues where the tissue oxygenation and the somatic mutations of mtDNA are abnormally accelerated by the germ-line mutations.

The extensive fragmentation of mtDNA into ΔmtDNA, predominantly minicircles, with oxygen damage demonstrated here supports the 'redox mechanism of ageing' (26).

References

1. Clayton D. A., (1982), Cell, 28, 693-705.
2. Cooper J. M., Mann V. M., Schapira A. H., (1992), J. Neurol. Sci., 113, 91-98.
3. Corral-Debrinski M., Shoffner J. M., Lott M. T., Wallace D. C., (1992), Mutat. Res., 275, 169-180.
4. Cortopassi G. A., Arnheim N., (1990), Nucleic Acids Res., 18, 6927-6933.
5. De Massy B., Fayet O., (1984), J. Mol. Biol., 178, 227-236.
6. Deng R. Y., Fridovich I., (1989), Free Radical. Biol. Med., 6, 123-129.
7. Harman D., (1981), Proc. Natl. Acad. Sci. USA, 78, 7124-7128.
8. Hattori K., Tanaka M., Sugiyama S., Obayashi T., Ito T., Satake T., Hanaki Y., Asai J., Nagano M., Ozawa T., (1991), Am. Heart J., 121, 1735-1742.
9. Hayakawa M., Hattori K., Sugiyama S., Ozawa T., (1992), Biochem. Biophys. Res. Commun., 189, 979-985.
10. Hayakawa M., Katsumata K., Yoneda M., Tanaka M., Sugiyama S., Ozawa T., (1995), Biochem. Biophys. Res. Commun., 215, 952-960.
11. Hayakawa M., Ogawa T., Tanaka M., Sugiyama S., Ozawa T., (1991), Biochem. Biophys. Res. Commun., 176, 87-93.
12. Hengen P. N., (1994), Trends Biochem. Sci., 19, 93-94.
13. Hockenberg D. M., Oltvai Z. N., Yin X.-M., Milliman C. L., Korsmeyer S. J., (1993), Cell, 75, 241-251.
14. Hockenbery D., Nunez G., Milliman C., Schreiber R. D., Korsmeyer S. J., (1990), Nature, 348, 334-336.
15. Holt I. J., Harding A. E., Morgan-Hughes J. A., (1988), Nature, 331, 717-719.
16. Ikebe S., Tanaka M., Ohno K., Sato W., Hattori K., Kondo T., Mizuno Y., Ozawa T., (1990), Biochem. Biophys. Res. Commun., 170, 1044-1048.
17. Katsumata K., Hayakawa M., Tanaka M., Sugiyama S., Ozawa T., (1994), Biochem. Biophys. Res. Commun., 202, 102-110.
18. Kornberg A., Baker T. A., (1980), *DNA replication*, W. H. Freeman and Company, New York.
19. Linnane A. W., Baumer A., Maxwell R. J., Preston H., Zhang C. F., Marzuki S., (1990), Biochem. Int., 22, 1067-1076.
20. Linnane A. W., Marzuki S., Ozawa T., Tanaka M., (1989), Lancet, i, 642-645.
21. Matumoto L., Kasamatsu H., Piko L., Vinograd J., (1974), J. Cell Biol., 63, 146-159.
22. Mecocci P., Mac Garvey U., Kaufman A. E., Koontz D., Shoffner J. M., Wallace D. C., Beal M. F., (1993), Ann. Neurol., 34, 609-616.
23. Miller T. R., Grossman S. J., Schectman K. B., Biello D. R., Ludbrook P. A., Ehsani A. A., (1986), Am. J. Cardiol., 58, 531-535.
24. Oltvai Z. N., Milliman C. L., Korsmeyer S. J., (1993), Cell, 74, 609-619.
25. Ozawa T., (1994), Herz, 19, 105-118.
26. Ozawa T., (1995), Biochim. Biophys. Acta, 1271, 177-189.
27. Ozawa T., Katsumata K., Hayakawa M., Sugiyama T. M. S., Tanaka T., Itoyama S., Nunoda S., Sekiguchi M., (1995), Biochem. Biophys. Res. Commun., 207, 613-620.
28. Ozawa T., Sahashi K., Nakase T., Chance B., (1995), Biochem. Biophys. Res. Commun., 213, 423-438.
29. Ozawa T., Tanaka M., Hayakawa M., Sugiyama S., Sato W., Ohno K., Ikebe S., Yoneda M., (1991), *Mitochondrial DNA mutations: Types, mechanism and expression*, in: *Mitochondrial Encephalomyopathies, Progress in Neuropathology* (Eds. T. Sato, S. DiMauro), Ravan Press, New York, 141-151.
30. Rotig A., Colonna M., Binnefont J. P., Blanche S., Fisher A., Saudubray J. M., Munnich A., (1989), Lancet, i, 902-903.
31. Saiki R. K., Gelfand D. H., Stoffel S., Scharf S. J., Higuchi R., Horn G. T., Mullis K. B., Erlich H. A., (1988), Science, 239, 487-494.
32. Sato W., Tanaka M., Ohno K., Yamamoto T., Takada G., Ozawa T., (1989), Biochem. Biophys. Res. Commun., 162, 664-672.

33. Schon E. A., Rizzuto R., Moraes C. T., Nakase H., Zeviani M., DiMauro S., (1989), Science, 244, 346-349.
34. Simonetti S., Chen X., DiMauro S., Schon E. A., (1992), Biochim. Biophys. Acta, 1180, 113-122.
35. Soong N. W., Hinton D. R., Cortopassi G., Arnheim N., (1992), Nat. Genet., 2, 318-323.
36. Sugiyama S., Hattori K., Hayakawa M., Ozawa T., (1991), Biochem. Biophys. Res. Commun.,180, 894-899.
37. Wyllie A. H., (1994), Nature, 369, 272-273.
38. Yoneda M., Katsumata K., Hayakawa M., Tanaka M., Ozawa T., (1995), Biochem. Biophys. Res. Commun., 209, 723-729.
39. Yoneda M., Miyatake T., Attardi G., (1994), Mol. Cell. Biol., 14, 2699-2712.

Age-related Fragmentation of Mitochondrial DNA Associated with Oxygen Damage

Summary

In normal human hearts, a progressive age-related fragmentation of mitochondrial (mt) DNA into various-sized deleted (Δ) mtDNA up to 358 types was documented by a novel total-detection system for deletions. The ΔmtDNA lacking replication origin(s), minicircles, accumulated up to 280 types out of the 358, suggesting a yet unknown replication mechanism in human. Wild-type mtDNA decreased linearly down to 11% of the total with age negatively correlated with ΔmtDNA and oxidized nucleoside, 8-hydroxy-deoxyguanosine. A remarkable mirror image observed in ΔmtDNA size distribution as well implies that random hydroxyl-radical attacks resulted in double-strand break and rejoining of mtDNA as a preferable mechanism to form various ΔmtDNA of closed circular duplex. In the patients with mitochondrial cardiomyopathy, similar fragmentation and oxidative damage in mtDNA was documented at their age 7 to 19 equivalent to the normal subjects of age over 80. Exposure of a cultured cell line under oxygen stress, 95% oxygen, cold mimic these changes in mtDNA[1] within 3 days leading an apoptotic cell death, whereas mtDNA lacking cells are relatively immune. These facts support the 'redox mechanism of ageing'.

Key words:
mitochondrial DNA, fragmentation, oxygen, damage.

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