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# Mitochondrial Genome Mutation in Cell Death and Aging

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This article reviews the concept, molecular genetics, and pathology of cell death and aging in relation to mitochondrial genome mutation. Accumulating evidence emphasizes the role of genetic factors in the development of naturally occurring cell death and aging. The ATP required for a cell's biological activity is almost exclusively produced by mitochondria. Each mitochondrion possesses its own DNA (mtDNA) that codes essential subunits of the mitochondrial energy-transducing system. Recent studies confirm that mtDNA is unexpectedly fragile to hydroxyl radical damage, hence to the oxygen stress. Cellular mtDNA easily fragments into over a hundred-types of deleted mtDNA during the life of an individual. Cumulative accumulation of these oxygen damages and deletions in mtDNA results in a defective energy transducing system and in bioenergetic crisis. The crisis leads cells to the collapse of mitochondrial trans-membrane potential, to the release of the apoptotic protease activating factors into cytosol, to uncontrolled cell death, to tissue degeneration and atrophy, and to aging. The total base sequencing of mtDNA among individuals revealed that germ-line point mutations transmitted from ancestors accelerate the somatic oxygen damages and mutations in mtDNA leading to phenotypic expression of premature aging and degenerative diseases. A practical survey of point mutations will be useful for genetic diagnosis in predicting the life-span of an individual.

**KEY WORDS:** mtDNA; mitochondrial; ATP; mutations.

## INTRODUCTION

A remarkable feature of mitochondria is their possession of an independent genetic system (Ephrussi *et al.*, 1949), now known to be necessary for the morphogenesis of the energy transduction system consisting of the respiratory chain and ATP synthase (Anderson *et al.*, 1981).

In contrast to one nuclear genome located on nuclear DNA (nDNA), thousands of copies of the mitochondrial genome on the mitochondrial DNA (mtDNA), a closed circular duplex, exist in a cell, as there are many mitochondria per cell and each contains 2–3 molecules of mtDNA (Robin and Wong, 1988). Hence, in addition to the genetic information located on each mtDNA, the ratio of the oxidatively damaged mtDNA to the wild-type mtDNA (ωmtDNA) signifies much of the oxygen stress to a cell. In contrast to nDNA, the mtDNA repair system is inefficient (Clayton *et al.*, 1974); thus, oxidatively modified bases easily accumulate leading to double-strand separation (Hayakawa *et al.*, 1992), to strand break by oxygen radicals (Deng and Fridovich, 1989), and to deleted mtDNA (ΔmtDNA) (Hayakawa *et al.*, 1995). The accumulation of oxidized nucleotide and the fragmentation of ωmtDNA into ΔmtDNA has been documented among many human tissues, especially in postmitotic cells in stable tissues, such as nerve and muscle. Up to 90% of ωmtDNA disintegrate into hundreds of types of ΔmtDNA in the aged heart (Hayakawa *et al.*, 1996), in the parkinsonian brain (Ozawa *et al.*, 1997), and in the young cardiomyopathy heart with inherited mtDNA point mutations (Ozawa *et al.*, 1995a). Recent studies clearly revealed that the mtDNA is extremely fragile under oxygen stress. Under 95% oxygen for

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tissue slice preservation, the ωmtDNA of cultured fibroblasts fragments into hundreds of types of ΔmtDNA within 3 days, leading to apoptosis in the majority of the cells (Yoneda *et al.*, 1995). When disintegration of ωmtDNA exceeds a certain point, it switches on the irreversible apoptosis cascade, like as a cell autonomous rheostat. The cascade starts with a defective electron transfer chain (ETC), enhanced production of reactive oxygen species (ROS), and the cellular bioenergetic deficit.

Mitochondrial control of apoptosis, through attenuation of mitochondrial membrane potential and release of apoptotic protease-activating factors into the cytosol, is documented by recent publications (Liu *et al.*, 1996; Ozawa, 1995a, Zamzami *et al.*, 1996): The suppression of mitochondrial respiration both by endogenous and exogenous factors leads mitochondria to deplete the electrochemical proton gradient ($\Delta\mu_{H^+}$), to collapse the membrane potential ($\Delta\Psi_m$), to open the permeability transition (PTP), to release apoptotic protease-activating factors into cytosol, which ultimately lead to protease digestion of the nucleus.

Cellular apoptosis, the naturally occurring cell death process, has been reported in human stable tissues, such as muscle and nerve, to be a major cause of geriatric processes, such as ataxia, heart failure, and mental weakness. On the other side of the coin, apoptosis can be induced by exogenous factors to eliminate damaged or transformed cells. Interaction of leukocytes' cytotoxic ligands (Fas-L) with the cytoplasmic membrane receptor (Fas) induces nitric oxide (NO) synthetase. NO inhibits cytochrome oxidase (Sakai *et al.*, 1996) leading ETC to a reduced state, thus enhancing ROS. Fragile mtDNA, as an important endogenous factor for apoptosis, can be regarded as an inevitable event during biological evolution from a single cell to a human.

Further study on this autonomous switching mechanism of the apoptosis cascade may pave the road to keeping humans from geriatric aging by protecting unwanted cell death and maintaining a healthy life by eliminating harmful cells.

## MITOCHONDRIAL DNA AND MUTATION

### Mitochondrial DNA

In 1949, Ephrussi (Ephrussi *et al.*, 1949) discovered a respiratory-deficient strain of yeast forming a small colony, called cytoplasmic "petite" mutants. Their studies showed that mutations resulting in the petite phenotype are inherited in a nonMendelian fashion. They, therefore, postulated the lesions to be in an extrachromosomal or cytoplasmic element, designated the rho (ρ) factor. Hence, cytoplasmic petite mutants are also referred to as ρ mutants. Although this was not known at the time of the discovery, the ρ factor was subsequently shown to be identical to mtDNA. Common ρ mutants arising from large deletions in mtDNA were called $\rho^-$ mutants (Tzagoloff, 1982).

In 1963, an electron micrographic study (Nass and Nass, 1963) indicated that the mitochondria of chick embryo cells contained threadlike structures that could be digested by DNase, but not by RNase. At the same time, Schatz *et al.*, 1964 first detected DNA and quantitated it in purified yeast mitochondria by biochemical procedures. The first circular genetic map of the yeast mitochondrial genome obtained by using the petite mutants was published in 1975 by Linnane's group (Molloy *et al.*, 1975). In 1981, a group at Cambridge (Anderson *et al.*, 1981) reported the gross genetic structure of human mtDNA: The 16,596 base-pair (bp) molecule of which each bp is numbered as nucleotide position (np) is commonly referred to as the Cambridge Sequence (Camb. seq.). The genes located on mtDNA encode some important subunits of both the ETC and of the ATP synthase; 7 subunits of complex I, 3 subunits of complex IV, 2 subunits of complex V, and one subunit of complex III. Each of the 13 structural genes encoding these subunits and 2 ribosomal RNA genes are tightly packed, being punctuated not by introns, but by 22 transfer RNA genes.

### Mutation

Soon after the Camb. seq. was reported, our group using Northern blot analysis (Tanaka *et al.*, 1986, 1987) elucidated that the mitochondrial-gene encoded subunits, but not the nuclear-gene encoded subunits, are selectively defective in the ETC complexes of skeletal muscle from patients with mitochondrial myopathy, named after mitochondrial morphological and biochemical abnormalities (DiMauro *et al.*, 1985). The specific defect suggesting the existence of large deletions was demonstrated to arise from an inherited genetic abnormality by Southern blot analyses (Ozawa *et al.*, 1988) that documented large deletions in the patients' mtDNA; namely, the $\rho^-$ mutation as in the case of yeast. The $\rho^-$ mutation associated with several degenerative diseases was also reported by other

groups (Holt *et al.*, 1988; Rotig *et al.*, 1989). Expanding the findings of biochemical and molecular biological studies on human tissues as well as yeast, we (Linnane *et al.*, 1989) proposed that the somatic accumulation of mtDNA mutations during human life is a major cause of human aging and degenerative diseases: muscle weakness of senescence, declining mental capacity, age-related progressive decline of ventricular performance, and mitochondrial diseases. This *mitochondria theory of aging* stems from the following: the high frequency of gene mutations in mtDNA, analogous to the extremely high mutational rate of yeast; the small size of the mitochondrial genome and its known information content; the inefficient repair mechanism for mtDNA and the lack of protective histone, unlike nuclear DNA; and the somatic segregation of individual mtDNA during eukaryotic cell division. Since 1989, the timely discovery of polymerase chain reaction (PCR) technology (Saiki *et al.*, 1988) was brought into practical use, making it possible to amplify small quantities DNA. Considerable publications have documented an extensive array of age-dependent accumulation of ΔmtDNA resulting in the $\rho^-$ mutation among many human tissues, especially in postmitotic stable tissues, such as nerve and muscle. In addition, also aided by PCR, many point mutations, similar to the $mit^-$ mutation in yeast, were found in the patients with the degenerative diseases. These mtDNA mutations have been reported to be the cause of degenerative diseases, named as diseases of the mtDNA (Wallace, 1992). However, the cause–effect relation between the reported mutational genotype and clinical phenotype remained unclear in these early studies, mainly because the mutation survey is carried out within limited regions of mtDNA. Grivell (1989) cited that, curiously, there is no obvious correlation between the severity of the clinical symptoms or biochemical abnormality and either the location of the reported deletion or the number of deleted genes. In retrospect, the curiosity was derived from the survey of deletion within a limited region of mtDNA using a particular mtDNA probe for the Southern blot analyses or a single PCR primer pair. The problem has been settled by a newly devised system to detect the total deletions (TD system) using 180 PCR primer pairs covering the entire ωmtDNA duplex (Hayakawa *et al.*, 1995). The inconsistency of point mutational genotype and clinical phenotype was solved by the direct base-sequencing of the entire mtDNA (Tanaka *et al.*, 1996).

*Point Mutation*

It has been disclosed that mitochondria of patients with mitochondrial myopathies harbor point mutations either maternally inherited (Wallace *et al.*, 1988a,b; Yoneda *et al.*, 1989) or somatically acquired (Kovalenko *et al.*, 1996; also cf. Ozawa, 1997a). Accumulated data of the entire mtDNA base-sequence clarified the dominant feature of the mtDNA diseases was that their clinical signs and symptoms are triggered by maternally inherited or somatically acquired multiple point mutations locating over the entire ωmtDNA (Ozawa, 1995b). It has become clear that the mutational genotypes based on the severity of point mutations and on their combination correspond to the clinical phenotypes, ranging from the asymptomatic to incapacitating symptoms (for more details, see Ozawa, 1997a). The combination of point mutations synergistically accelerates the accumulation of somatic mtDNA lesions, large deletions, and oxidative damage (Hayakawa *et al.*, 1992).

In addition to the maternally inherited germline mutations, nucleotide substitution is documented to occur in a single generation of Holstein cows, probably due to a genetic "founder effect" during oogenesis (Hauswirth and Laipis, 1982)—that is, amplification of one or a few mtDNA molecules as template will yield one predominant genotype in the mature oocyte that contains 100 to 1000 times more mtDNA than is commonly found in somatic cells (Piko and Matsumoto, 1976). In humans, a somatically acquired point mutation at np3243 A-to-G transition was reported in the cells of an individual (Zhang *et al.*, 1993). The somatically acquired point mutation at np3243 A-to-G was also detected in the cloned skeletal muscle mtDNA from a MELAS patient (10 clones/60 clones), being significantly higher than in those from normal skeletal muscle (0/60) as well as in a normal placenta (2/60)(Kovalenko *et al.*, 1996).

*Deletions*

In human, two types of mtDNA deletions have been detected; (i) the Southern blot-detectable deletion and (ii) the PCR-detectable deletion.

(i) *The Southern blot-detectable deletion*. During early stage of studies on mitochondrial diseases, the Southern blot-detectable deletion was observed often among patients with chronic progressive ophthalmoplegia (Ozawa *et al.*, 1988), with early-onset mito-

chondrial myopathies (Holt *et al.*, 1988), or with Pearson's syndrome (Rotig *et al.*, 1989). A single ΔmtDNA of a large quantity, 20–85% of the total mtDNA, was detected by Southern blot analysis. The ΔmtDNA which *heteroplasmically* coexists with ωmtDNA, presumably originated from a clonal expansion of an initial deletion event occurring early in oogenesis.

(ii) *The PCR-detectable deletion*. Soon after the practical use of PCR, multiple populations of ΔmtDNA were detected in human myocardium using a single PCR primer pair (Sato *et al.*, 1989). Hence, the author noted (Ozawa *et al.*, 1991) that the PCR-detectable multiple forms of ωmtDNA *pleioplasmically* coexist with ωmtDNA in a tissue. The *mitochondria theory of aging* (Linnane *et al.*, 1989) predicts the somatic accumulation of mtDNA mutations during human life as a major cause of human aging and the geriatric process. Accumulation of PCR-detectable ΔmtDNA has been documented among many human tissues, especially in the postmitotic stable tissues, such as nerve and muscle (Corral-Debrinski *et al.*, 1992; Cortopassi and Arnheim, 1990; Hattori *et al.*, 1991; Hayakawa *et al.*, 1992; Ikebe *et al.*, 1990; Linnane *et al.*, 1990). Postmitotic cells tend to accumulate somatic mtDNA deletions during an individual's life span, being different from mitotic cells with a short lifespan, such as intestinal microvilli cells that are continuously replaced with newly divided cells. Quantitative data on a single ΔmtDNA detected by PCR among various aged individuals indicate that there are four orders of magnitude fewer ΔmtDNA in infancy as compared to old age (Simonetti *et al.*, 1992; Sugiyama *et al.*, 1991) and that a newborn contains only very low amounts of the 5 kbp deletion that is commonly observed in different tissues of adults (Linnane *et al.*, 1990), being not detected in the corresponding fetal tissues (Cortopassi and Arnheim, 1990). Hence, PCR-detectable multiforms of ΔmtDNA seem to newly arise with each generation and to accumulate with age.

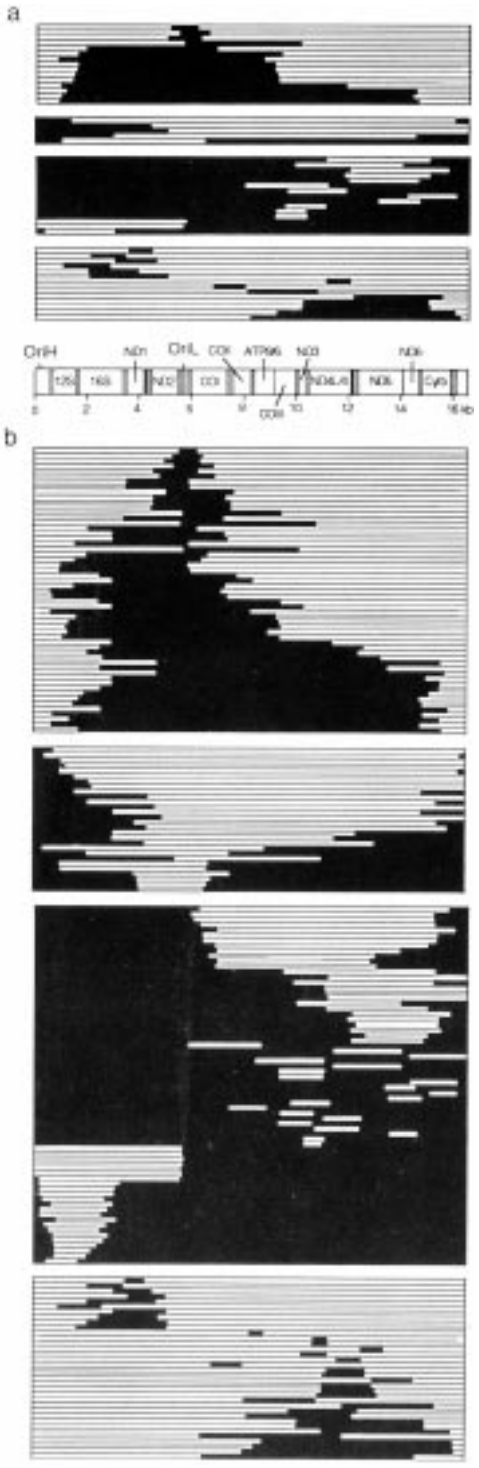
### *Disintegration into Fragments*

The clinically causative somatic mutations must contain the following characteristics: (i) The absolute level of accumulated mutations is accountable for age-related decline of mitochondrial function and bioenergetic deficit. (ii) The mutations accumulate age dependently, correlating closely with oxidative damage and cell death. (iii) The mutations causing cell death could be reproduced experimentally in human cultured cells.

(i) A cell contains hundreds of mitochondria and mtDNA copies and the fractional concentration of each ΔmtDNA, detected by the conventional PCR using a single primer pair, is usually 0.01–0.3% of the total mtDNA (Cooper *et al.*, 1992; Ozawa *et al.*, 1990; Remes *et al.*, 1994). Based on the low absolute level of a single ΔmtDNA, a question arises whether an observed ΔmtDNA is the cause or the effect of the aging (Cooper *et al.*, 1992; Remes *et al.*, 1994). However, the number and the size of ΔmtDNA visualized by PCR depend on a particular primer pair used, such that the more distantly separated primers enable the larger deletions to be detected (Zhang *et al.*, 1992). Hence, a PCR-detectable ΔmtDNA is suggested to be the "tip of the iceberg" of the spectrum of somatic mutations (Soong *et al.*, 1992). To settle the issue and to establish the total number of somatic deletions, the TD system that detects all possible ΔmtDNA over 0.5 kbp was recently devised (Hayakawa *et al.*, 1995) and applied to mtDNA specimens from human tissues of various age (Hayakawa *et al.*, 1996). Surprisingly, the whole "iceberg" in cardiomyocytes of age 97 shows 358 types of ΔmtDNAs including 280 types of "mini-circles," that lack either one of the replication origins (Ori) or both (cf. Fig. 1). This is associated with a decrease in ωmtDNA down to 11%.

(ii) The TD system documents extreme fragility of ωmtDNA with oxidative damage that leads the ωmtDNA molecule to disintegrate into hundred of types of ΔmtDNA fragments during normal aging (Hayakawa *et al.*, 1996), in patients with Parkinson's disease (Ozawa *et al.*, 1997), and even in young mtCM patients harboring severe point mutations at age 7 and 19, thus representing premature aging equivalent to normal subjects with an age over 80 (Fig. 2) (Hayakawa *et al.*, 1996; Katsumata *et al.*, 1994; Ozawa *et al.*, 1995a). Similar fragmentation [rearrangement that is shown to be the preceding step for the deletion (Poulton *et al.*, 1993)] and depletion of ωmtDNA below detection limit is demonstrated by applying long PCR to mtDNA in skeletal muscles of aged subjects (Kovalenko *et al.*, 1997).

(iii) The fragmentation of mtDNA and apoptotic cell death could be mimicked within 3 days in cultured human fibroblast cells under oxygen stress, as shown in Fig. 1 (Yoneda *et al.*, 1995). Under 95% oxygen for tissue slice preservation, the ωmtDNA of the cultured cells fragmented within 3 days leading to apoptosis of a great majority of the cells. The derivative




cells ($\rho^0$) without mtDNA and lacking functional ETC were relatively immune, with more than 80% of cells surviving. These results indicate that mtDNA is a critical cellular target for ROS leading to apoptosis of the cells.

## OXIDATIVE DAMAGE OF MTDNA

### Redox Mechanism of Aging

In parallel with a survey of inherited point mutations and the somatically acquired $\rho^-$ mutations, oxidative damage of mtDNA was surveyed. A hydroxyl radical ($^{\bullet}OH$) adduct of deoxyguanosine (dG), 8-hydroxydeoxyguanosine (8-OH-dG), was noted as a hallmark of oxidative damage of DNA (Richter *et al.*, 1988). Hence, 8-OH-dG in mtDNA specimens was quantitatively determined using a microHPLC/mass spectrometer (Hayakawa *et al.*, 1991a). It was disclosed that the increase in $^{\bullet}OH$ adduct of guanine in mtDNA correlates closely with the cumulative increase in ΔmtDNA associated with aging and degenerative diseases (Hayakawa *et al.*, 1991b, 1992; Ozawa, 1994). This fact implies that the oxidative damage due to ROS is the underlying cause of somatic $\rho^-$ mutations. Cellular oxidative damage was proposed as a main cause of aging by the *free radical theory of aging* (Harman, 1960). Unifying both ideas of the mitochondria and the free radical theory of aging, the author proposed the *redox mechanism of mitochondrial aging* (Ozawa, 1995a) as the molecular genetic and bioenergetic basis for the progressive decline of cellular activity leading to apoptosis of human cells. The mechanism is based on an age-related increase in somatic mtDNA mutations and oxidative damage among human tissue degeneration and atrophy, in nigrostriatum of parkinsonian brain (Ikebe *et al.*, 1990; Ozawa *et al.*, 1997), in

**Fig. 1.** Types and size distribution of deletions detected in mtDNAs from the $\rho^+$ cells in the presence or absence of oxygen stress. The TD system (Hayakawa *et al.*, 1995) was applied to determine the *n* of ΔmtDNA types acquired during a 3-day exposure of the 701.2.8c to 95% oxygen. The deleted regions were marked with the black bars, arranged according to their sizes. Genomes in mtDNA are schematically illustrated between Panel (a) and (b). The ΔmtDNAs lacking either of OriL and OriH, or both, or having both were shown in the bulk of the bars in respective order. (a) ΔmtDNAs are shown before the exposure to 95% oxygen. (b) ΔmtDNAs are shown after the exposure for 3 days. (Modified from Yoneda *et al.*, 1995.)

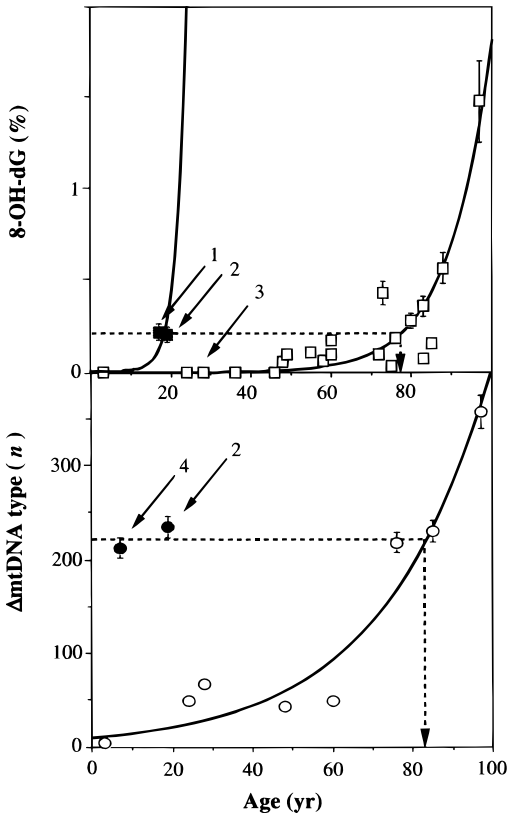



**Fig. 2.** Age-associated correlative increase in the total number of ΔmtDNA type and oxygen damage. (Upper panel): mtDNAs were extracted from autopsied cardiac muscles of 21 human subjects, 8 males and 13 females aged 3 to 97 years, without cardiological symptoms and obtained at random. Samples of mtDNA were enzymically hydrolyzed into nucleosides, subjected to precolumn concentration, and analyzed by the microHPLC/MS system (Hayakawa *et al.*, 1991a). Both selected ion monitoring and ionization MS spectra of 8-OH-dG and dG were recorded. The percentage of 8-OH-dG to the total guanine increases exponentially with the ages of subjects ($r = 0.84$). Overlaid plots are the 8-OH-dG % of mtCM patients who died by heart failure at age 17 (female, a closed square pointed by arrow 1) and at age 19 (male, MCM P-1, a closed square pointed by arrow 2) (Katsumata *et al.*, 1994), respectively. The 8-OH-dG % of a negative control (female, ID119 an open square pointed by arrow 3) harboring nonsevere base substitutions is a marginal level. An arrow with dashed line indicates that the 8-OH-dG % of mtCM patients is equivalent to that of normal 78-year-old subjects. (Lower panel): The number ($n$) of ΔmtDNA types was determined by the total-detection system (Hayakawa *et al.*, 1995) on 8 mtDNA specimens (from 3 males and 5 females) of sufficient quantity out of 21 samples after the 8-OH-dG analyses (shown in the upper panel). ΔmtDNA type *n* increases exponentially with the ages of subjects, whereas there is a resulting decrease of the wild-type mtDNAs, down to 11%, with a strong negative correlation with age ($r = 0.89$). Overlaid plots are ΔmtDNA type *n* of a female DCM patient (Ozawa *et al.*, 1995a) who received a heart transplant at age 7 (DCM P-3, a closed circle pointed by arrow 4), and that of a male mtCM patient who died at age 19 (MCM P-1, a closed circle pointed by arrow 2, as shown in the upper panel). Mean ΔmtDNA type *n* of the positive controls is equivalent to a normal 82-year-old subject. (Modified from Hayakawa *et al.*, 1996).

human skeletal muscle (Hayakawa *et al.*, 1991b, 1992) and in decreased oxygen utilization of skeletal muscle (Sugiyama *et al.*, 1995) leading to extensive tissue oxygenation (Ozawa *et al.*, 1995b).

## ROS

In ETC, the majority of electrons reduce molecular oxygen in cytochrome oxidase (CO), forming $H_2O$. However, part of the electrons eluted from the ETC perform one or two-electron reduction of oxygen forming ROS. At the time of CO discovery, Warburg (1924) insisted that the oxygen molecule, activated by CO, directly accepts four-electron reduction forming water without $H_2O_2$ formation. However, Michaelis (1946) proposed that the oxidation of bivalent organic molecules proceeds in two obligatory univalent steps, the intermediate being a free radical, viz., one-electron $O_2^-$ and two-electron reductant, $H_2O_2$. Thus, it has been a matter of great interest to discover the production site of such intermediates in ETC. In 1975, on the basis of optical studies on oxy- and peroxy-CO by Chance (Chance *et al.*, 1975), it became clear that the intermediates remain within the active site of CO until the final four-electron reduction of oxygen to form water is achieved, probably for protection against cellular intoxication. From general properties of the mitochondrial generation of $H_2O_2$ and effect of hyperbaric oxygen, it was postulated (Boveris and Chance, 1973) that besides the well-known flavin reaction, formation of $H_2O_2$ may be due to interaction with an energy-dependent component of ETC at the cytochrome (cyt) *b* level. These findings indicated that the active sites of the complex IV and III, cyt *a* and *b*, play a crucial role not only for the cellular energy production, but also for protection against cellular oxidative damage. Hence, genetically defective cyt *a* and *b*, even at low absolute levels, could result in a serious outcome in cellular viability. Actually, severe deleted cardiomyopathy or hypertrophic cardiomyopathy is clinically expressed in mitochondrial cardiomyopathy patients who harbor hazardous point mutations in CO and *b* and/or in transfer RNA genes that affect the transcription of the cyt genes (Ozawa *et al.*, 1995a).

From the above mechanism of oxygen reduction, the elution of electrons from ETC and the generation of ROS is expected to result not only by genetic defects but also by physiological attenuation of the cellular redox state. Hyperoxia (Turrens *et al.*, 1982) increases $H_2O_2$ release by lung mitochondria, because the excess

oxygen supply exceeds the enzymic capability to dispose of ROS. Skulachev (1996a) pointed out that mammalian uncoupled respiration or plant's noncoupled respiration is an effective device to prevent oxidative damage and cell death by maintaining safely a low level of oxygen and its one-electron reductants. Boveris and Chance (1973) demonstrated that $H_2O_2$ production by animal mitochondria, negligible in active respiration (State 3) or in the presence of uncouplers, becomes quite measurable in resting respiration (State 4). State 4 increases reduced electron carriers, such as flavins, NADH oxidoreductase, CoQ, cyt *b*, and nonheme iron proteins (Cross and Jones, 1991; Ksenzenko et al., 1983; Massey, 1994), which are the main target for one-electron oxidation by oxygen. State 4 mitochondria from rat liver or from pigeon heart generate about 0.3 to 0.6 nmol $H_2O_2$/min/mg of protein (Boveris and Chance, 1973). This $H_2O_2$ generation represents approximately 2% of the total oxygen utilization under these conditions. Thus, during the life of an individual, a vast sum of the redox energy is consumed for the generation of ROS. In addition, we (Hayakawa *et al.*, 1989) found that cyt *c*, an essential component of ETC, is a twenty times more efficient catalyst than ferrous ion in promoting the formation of the most reactive oxygen radical, $^{\bullet}OH$, by the Fenton reaction. Namely, ETC is an efficient factory for $^{\bullet}OH$ production (Fig. 3).

The $^{\bullet}OH$ production was demonstrated by interaction of $O_2^-$ with NO (Hogg *et al.*, 1992), the synthase (NOS) of which was extensively induced by cytolytic factors, such as tumor necrosis factor (TNF) (Dong *et al.*, 1996, Geng *et al.*, 1996) and interleukin-1β (IL-1β) (Ankarcrona *et al.*, 1994; Dunger *et al.*, 1996) leading to apoptosis of the target cells. In this respect, $^{\bullet}OH$ could be regarded as not only a mere byproduct of mitochondrial respiration, but an active one with an important bioenergetic role to cause cell death under physiological condition and to eliminate unwanted/transformed cells.

## Oxidized Nucleotide and Mutations

mtDNA is located inside the mitochondrial inner membrane where ROS continuously leaks from the respiratory chain (Chance *et al.*, 1979), hence, becoming directly susceptible to attack by ROS, despite cellular defenses against damage from ROS (Yu, 1994). During evolution from yeast to mammals, mtDNA has downsized to one-fifth losing its intron, in which mutations are inert. Therefore, human mtDNA becomes more susceptible to oxidative damage and large deletions than a single-cellular organism. It seems reasonable that the genes coding the cellular energy factory have to be manageably fragile in order to be the primary target of the apoptotic process (Ozawa, 1997a).

The underlying mechanism for the formation of the large deletions is oxidized nucleotide-induced double-strand separation resulting in the generation of stretches of single-stranded DNA, in which breaks form endonuclease-sensitive sites susceptible to oxygen radical attack (Deng and Fridovich, 1989) and rejoining of mtDNA forming ΔmtDNA. In the nucleus, where an efficient repair system operates, the oxidized nucleotides in nDNA are rapidly excised and excreted into urine (Fraga *et al.*, 1990). Thus, the level of 8-OH-dG in nDNA occurs in one out of $10^5$ dG. However, in mitochondria where the repair system is inefficient, the oxidized nucleotides accumulate up to 1% dG in mtDNA with age, especially in postmitotic stable tissue. A study (Kamiya *et al.*, 1992) on the mutagenesis of 8-OH-dG in a mammalian cell clearly demonstrated that a synthetic protooncogene containing 8-hydroxyguanine induces random point mutations at the modified site and adjacent positions during gene replication. The defective ETC, encoded by the mutated mtDNA, enhances the $^{\bullet}OH$ formation, resulting in more accumulation of 8-OH-dG and large deletions. Such a vicious cycle of the $^{\bullet}OH$ damage and mutations in mtDNA seems to be synergistic and exponential among aged subjects (shown in Fig. 2).

## Oxidized Nucleotide and Fragmentation of mtDNA

In human heart mtDNA specimens, quantitative determination of 8-OH-dG demonstrated that an age-related progressive increase of 8-OH-dG, up to 1.5% of the dG at age 97, correlating closely ($r = 0.93$) with the accumulation of a ΔmtDNA with 7.4 kbp deletions in up to 7% of mtDNA (Hayakawa *et al.*, 1992). In human diaphragm muscle, the accumulation of 8-OH-dG reached levels of 0.5% dG in an 85-year-old individual with the association of multiple deletions. Consistent with heart and diaphragm, a similar accumulation of 8-OH-dG was reported in human brain mtDNA, up to 0.87% at age 90 (Mecocci *et al.*, 1993). The TD system revealed that a progressive age-related increase in the total number ($n$) of ΔmtDNA

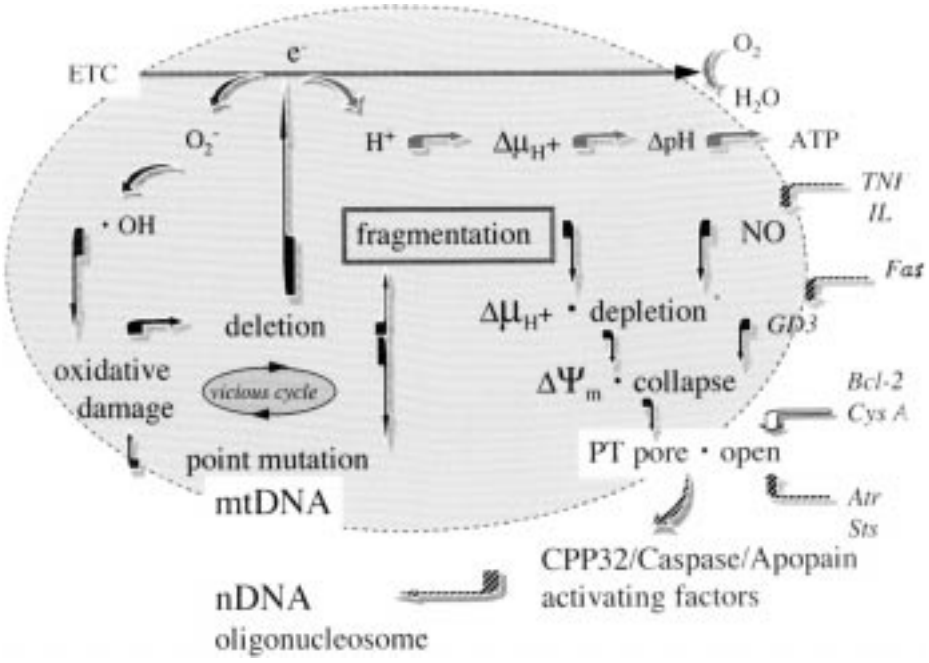



**Fig. 3.** Bioenergetics and the cascade of cellular apoptosis are schematically illustrated, based on the reports that follow. In mtDNA, each individual contains unique base substitutions diverged from the common ancestor of modern human, and include point mutation(s), in the case of mitochondrial diseases (Ozawa, 1995a). A particular base substitution occurs in mtDNA of tissues of aging humans (Zhang *et al.*, 1993). Somatic nucleotide substitutions accumulate in mtDNA of mitochondrial diseases (Kovalenko *et al.*, 1996). Point mutations accelerate oxidative damage and fragmentation of mtDNA of premature aging (Katsumata *et al.*, 1994; Ozawa *et al.*, 1995a). Extensive tissue oxygenation and focal hyperoxia are associated with mtDNA mutations and with age (Ozawa *et al.*, 1995b). Hyperoxia induces an apoptotic cell death associated with fragmentation of mtDNA (Yoneda *et al.*, 1995). Oxidative damage and deletions of mtDNA synergistically increase in human tissues with age (Hayakawa *et al.*, 1991b, 1992). Oxidative damage and extensive mtDNA fragmentation are associated with age (Hayakawa *et al.*, 1996). Age-associated accumulation of oxidative damage and deletion, lead to the loss of mitochondrial ETP activities (Hayakawa *et al.*, 1993; Sugiyama *et al.*, 1993) in tissues. NO inhibits CO activity (Sakai *et al.*, 1996) and its synthase (NOS) is extensively induced by cytolytic factors, such as TNF (Dong *et al.*, 1996; Geng et al., 1996) and IL-1β (Ankarcrona *et al.*, 1994, Dunger *et al.*, 1996) leading the target cell to apoptosis. Anoxia or a respiratory inhibitor causes acute apoptosis (Jones, 1995; Shimizu *et al.*, 1995). A drop in $\Delta\Psi_m$ is one of the first events in apoptosis (Petit *et al.*, 1995). The uncouplers of oxidative phosphorylation or divalent cations cause the collapse of $\Delta\Psi_m$ leading to apoptosis (Zamzami *et al.*, 1996). Apoptosis is prevented by Bcl-2 (Korsmeyer *et al.*, 1995) or by *cys* A (Zoratti and Szabo, 1995), a specific ligand of ANT. The PT induction in response to the ANT ligand Atr is inhibited by a specific ANT ligand bongkreakic acid or by hyperexpression of Bcl-2 (Zamzami *et al.*, 1996). An apoptosis-inducing reagent *Sts* results in the opening of PT pore and mitochondrial swelling eluting intramitochondrial solutes such as dATP and cyt *c* that activate the inactive form of an ICE family protease CPP32 (Liu et al., 1996). CPP32 cleaves various substrates, including nuclear lamin (Lazebnik et al., 1995), exposing nDNA to $Ca^{2+}$-endonuclease digestion (Gaido and Cidlowski, 1991) into nucleosomes. The hatched line represents outer or inner mt membrane. The shaded area represents mitochondrial matrix.

types correlates with accumulation of 8-OH-dG in heart mtDNA (Table I; Fig. 2) reflecting a long-term accumulation of oxidative damage during human life. A remarkable mirror image of the size distribution of ΔmtDNA (cf. Fig. 1) (Hayakawa *et al.*, 1996), and a strong linear correlation ($r = 0.97$) between minicircles and ΔmtDNA preserving both Oris (Table I), suggest a random occurrence of ΔmtDNA without a preferential site. Thus, it seems reasonable to presume that random double-strand separations accompanying accumulated 8-OH-dG (Hayakawa *et al.*, 1992), single-strand breaks by ·OH attacks (Deng and Fridovich, 1989),

**Table I.** Types of mtDNA among Subjects/Cells[a]

| Subject | Sex | Age | Disease | ΔmtDNA type (*n*) | Subtype of ΔmtDNA | | | | ωmtDNA (%) | 8-OH-dG (per $10^5$ 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 cancer | 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 cancer | 230 | 61 | 64 | 33 | 72 | 58 | 15.3 |
| H.M. | F | 97 | Gastric cancer | 358 | 78 | 88 | 63 | 129 | 11 | 148 |
| M.K. | F | 7 | DCM | 212 | 37 | 58 | 38 | 79 | 47* | 38.8* |
| T.K. | M | 19 | mtCM | 235 | 48 | 59 | 31 | 97 | 16 | 20.1 |
| $\rho^+$ | | | Normoxia | 49 | 14 | 15 | 5 | 15 | 80* | <1* |
| $\rho^+$ | | | 95% $O_2$ | 187 | 35 | 55 | 28 | 69 | 53* | 29.9* |

[a] All samples of mtDNA are extracted from autopsied heart muscle, except cultured cell line. Key to abbreviations: ΔmtDNA mtDNA with deletions; ωmtDNA, wild-type mtDNA; Ori, replication origin; F, female; M, male; VSD, ventricular septal defect; Pul. emb., pulmonary embolism; SAH, subarachnoidal hemorrhage; DCM, dilated cardiomyopathy (Ozawa, 1994); mtCM, mitochondrial cardiomyopathy (Katsumata *et al.*, 1994); $\rho^+$, a cultured human cell line, 701.2.8c (Yoneda *et al.*, 1995);*, calculated from the regression formula (Hayakawa *et al.*, 1996).

and rejoining of mtDNA is a preferable mechanism for its fragmentation into hundreds of ΔmtDNAs, which further accelerates the oxygen damage. Experimentally, these changes in mtDNA were correlated with a decline in the ETC activity in the laboratory animals (Hayakawa *et al.*, 1993; Takasawa *et al.*, 1993). Extensive oxygenation of skeletal muscle that indicates mitochondrial dysfunction causing suppressed oxygen utilization and, hence, relative tissue hyperoxia, is demonstrated noninvasively among senescent individuals and patients with mitochondrial cardiomyopathy and/or myopathy harboring hazardous point mutations (Ozawa *et al.*, 1995b). Similar reduced oxidative metabolism is reported in the cortex of Alzheimer-type dementia (Hoyer, 1986). Therefore, the vicious cycle of progressive oxidative damage, fragmentation of ωmtDNA, defective ETC, and relative tissue hyperoxia seems to result in synergistic and exponential changes associated with age (Fig. 2).

Recent findings (Liu *et al.*, 1996; Shimizu *et al.*, 1996a; Zamzami *et al.*, 1996) allow an apoptosis cascade to be outlined (Fig. 3). The active ETC creates an electrochemical proton gradient ($\Delta\mu_{H^+}$) that can be in the form of a mitochondrial transmembrane potential ($\Delta\Psi_m$) or ΔpH that drives ATP synthesis (Mitchell, 1979). Oxidative mtDNA damage and/or exogenous ligands leads cells to depletion of $\Delta\mu_{H^+}$ and then to the collapse of $\Delta\Psi_m$ (Skulachev, 1996a). Subsequently, the $\Delta\Psi_m$-dependent permeability transition pore (PTP) opens releasing intramatrix apoptotic protease-activating factors into the cytosol (Liu *et al.*, 1996), then to nDNA digestion into oligonucleosomes, and finally to cell death (reviewed by Ozawa, 1997a,b).

$$\text{oxidative damage} \rightarrow \text{mtDNA}$$

$$\text{fragmentation} \rightarrow \Delta\Psi_m \text{ collapse} \rightarrow \text{apoptosis}$$

The cascade links oxidative mtDNA damage to apoptosis.

As mentioned above, it was demonstrated in the cultured human cells that oxidative stress causes the fragmentation of mtDNA and cell apoptosis (Yoneda *et al.*, 1995). Recently, a similar line of evidence on oxidative damage of mtDNA was presented. In the presence of 200 $\mu M$ $H_2O_2$ features of both senescencelike growth arrest and apoptosis were observed in a primary human fibroblast cell line (Bladier *et al.*, 1997). When quantitative PCR was used to examine the formation and repair of hydrogen peroxide-induced DNA damage, Simian virus 40-transformed fibroblasts treated with 200 $\mu M$ $H_2O_2$ for 60 min exhibited threefold more damage to the mitochondrial genome compared with the nuclear fragment (Yakes and Van Houten, 1997). Following a 60-min treatment, damage to the nuclear fragment was completely repaired within 1.5 h, whereas no mtDNA repair was observed. Mitochondrial respiration also showed a sharp decline. These cells displayed arrested cell growth and morphological changes consistent with apoptosis.

## APOPTOSIS

The term *apoptosis* was named from morphological observations (Kerr *et al.*, 1972), and functionally termed as programmed cell death or naturally occurring cell death under physiological conditions. Apoptosis is considered to play an important role in the elimination of unnecessary cells in human morphogenesis and harmful cells, such as radical producing or transforming cells. Recently, with the advance of a technique to document DNA strand break on microscopic specimens, cell apoptosis has been reported to be prominent in many types of cells as a possible pathophysiological cause of age- or disease-related tissue degeneration and atrophy, e.g., as cardiac myocytes in the failing heart (Katz, 1995), in chronic heart failure (Sharov *et al.*, 1996), in dilated cardiomyopathy (Yao *et al.*, 1996), in sinus node cells in patients with complete heart block and fatal arrhythmias (bousituJames et al., 1996), and in neuronal cells in neurodegenerative diseases (Simonian and Coyle, 1996). However, the proposed mechanism of apoptosis had remained unclear and even controversial until recently, because of the existence of many apoptosis-inducible factors and survival factors that affect different steps in the apoptosis cascade, and, moreover, because the extreme fragility of mtDNA was not known.

The most readily measurable morphological features of apoptotic cell death are nuclear; namely, chromatin condensation and endonuclease-mediated nDNA fragmentation producing a "ladder" of oligonucleosomal-sized nDNA fragments visible by gel electrophoresis (Wyllie, 1980), which were considered to be the hallmark of apoptosis. To the contrary, no obvious morphological changes of mitochondria or of other organelles were observed until the end stage of apoptosis. Thus, it has been tempting to consider that nDNA plays a primary and causative role in apoptotic cell death. However, no clear evidence existed to support it. Conversely, the fact that the target cell nucleus is not required for cell-mediated granzyme or Fas-based cytotoxicity (Nakajima *et al.*, 1995) raised the possibility that apoptotic nuclear damage may be an epiphenomenon with respect to cell death. A series of reports (Jacobson *et al.*, 1994; Nakajima *et al.*, 1995; Schulze-Osthoff *et al.*, 1994) indicated that anucleate cytoplasts can undergo apoptosis and that the antiapoptotic protein Bcl-2 (Tsujimoto and Croce, 1986) and other extracellular survival signals can protect them. These facts indicated that the nuclear signaling is not required for apoptosis or for Bcl-2/survival factor protection.

### Apoptosis Program

The cell suicide program is best illustrated by genetic studies in the nematode *Caenorhabditis elegans* (Hengartner and Horvitz, 1994). Two genes involved in the control of apoptosis in *C. elegans* have been well characterized. One gene, *ced-9*, encodes a protein that prevents cells from undergoing apoptosis, while another gene, *ced-3*, encodes a protease whose activity is required to initiate apoptosis. The *bcl-2* family of genes are the mammalian counterparts of *ced-9* (Hengartner, 1994). The ced-3 protein is a cysteine protease related to the IL-1β-converting enzyme (ICE) family of proteases (also called caspases) in mammalian cells (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Schlegel *et al.*, 1996; Wang *et al.*, 1996; Yuan *et al.*, 1993). The closest mammalian homolog of ced-3 protein is CPP32, in terms of sequence identity and substrate specificity (Xue and Horvitz, 1995). Like ced-3 protein, CPP32 normally exists in the cytosolic fraction as its inactive form of 32 kDa. In cells undergoing apoptosis, the protease is activated proteolytically to be the 17/11 or 20/11 kDa active form (Nicholson *et al.*, 1995; Wang *et al.*, 1995), named apopain (Schlegel *et al.*, 1996). Apopain cleaves several substrates, including death substrates, poly (ADP-ribose) polymerase (Tewari *et al.*, 1995) and nuclear lamin (Lazebnik *et al.*, 1993), exposing the nucleus to $Ca^{2+}$-endonuclease (Gaido and Cidlowski, 1991) and/or DNase I (Peitsch *et al.*, 1993) digestion. However, the intracellular factors and/or bioenergetics that initially activate CPP32 had remained unknown.

Recently, a cell-free system was established to duplicate the features of the apoptotic program, including activation of CPP32 and nDNA fragmentation (Liu *et al.*, 1996). The system consisted of nuclei added to the cytosol from normally growing cells and intact mitochondria, carefully prepared using our method (Hayakawa *et al.*, 1993), of which the PT pore is well sustained. By adding an apoptosis-inducing reagent, staurosporine (*Sts*) or atractyloside (*Atr*), which disrupts the intact PT pore, nuclear apoptosis could be initiated by the release of apoptotic protease-activating factors from mitochondria into cytosol (Fig. 3). A phosphocellulose column separates the protease activating-factors into two types: deoxyATP and cyt *c*. Depletion of cyt *c* with anti-cyt *c* antibody causes loss

of the dATP-dependent activation of CPP32 and nDNA fragmentation. Conversely, without dATP, cyt *c* could not activate CPP32. Nuclear apoptosis in the presence of the intact mitochondria is induced by the external addition of dATP and cyt *c* to the system. Hence, both substances are required and sufficient for the activation of CPP32 to apopain.

## Redox Factors for Apoptosis

The intact mitochondrial inner membrane is quasi-impermeable for small solutes allowing the generation of $\Delta\mu_{H^+}$ in form of $\Delta\Psi_m$, thus keeping these apoptotic protease-activation factors inside of the membrane. The collapse of $\Delta\Psi_m$-dependent PT pore and mitochondrial swelling releases the factors into one cytosol. By associating with PT, small molecules, such as glutathione, are rapidly released before large-amplitude swelling (Reed and Savage, 1995), while the apoptosis-inducing protein is released with consequent mitochondrial swelling and disruption of the mitochondrial outer membrane (Skulachev, 1996b).

The precise biochemical mechanism of cyt *c* function in the activation of CPP32 remains to be determined. However, cyt *c* is a twenty times more efficient catalyst than ferrous ion in promoting the formation of $^{\bullet}OH$ by the Fenton reaction, both in NADPH-driven respiration of leukocyte plasma membranes and in non-enzymic $H_2O_2$ solution (Hayakawa *et al.*, 1989). Thus, the efflux of cyt *c* from mitochondria into cytosol would be highly cytotoxic due to $^{\bullet}OH$ formation. Sensible cells translocate apo−cyt *c*, which is translated on cytoplasmic ribosomes, into the mitochondrial intermembrane space through a unique pathway (Mayer *et al.*, 1995). Here, it is loosely attached to the surface of the inner membrane (Gonzales and Neupert, 1990). Finally, heme is installed by cyt *c* heme lyase, a peripheral protein of the inner membrane, and the holo-protein folds into its native structure. Thus, catalytically active cyt *c* locates exclusively in mitochondria, as it is shown by using radiolabeled cyt *c* (Beinert, 1951).

The release of apoptotic protease-activating factors from mitochondria into the cytosol is caused by the bioenergetic crisis and the collapse of $\Delta\Psi_m$. Cyclosporin (Cys) A, a specific ligand of adenine nucleotide translocator (ANT) that is believed to be a component of the PTP, prevents PT and apoptosis (Skulachev, 1996a; Zoratti and Szabo, 1995). Mitochondrial control of nuclear apoptosis is demonstrated from the fact that PT constitutes a critically early event in the apoptotic process (Zamzami *et al.*, 1996). In a cell-free system combining purified mitochondria and nuclei, mitochondria undergoing PT in response to an adenine nucleotide transportor (ANT) ligand, atractyloside (Atr), is prevented by another specific ligand, bongkrekic acid, or by hyperexpression of Bcl-2. $\Delta\Psi_m$ is also disrupted by a ganglioside derived from stimulated CD95 (called also Fas or APO-1) and by anti-Fas immunoglobulin (De Maria *et al.*, 1997). However, other than these exogenous ANT ligands or respiratory inhibitors, the endogenous factors that caused the bioenergetic crisis and the collapse of $\Delta\Psi_m$ in naturally occurring chronic cell death had remained unknown until the extreme fragility of mtDNA was recently disclosed, as shown in Fig. 1 and 2 (Hayakawa *et al.*, 1996; Katsumata *et al.*, 1994; Ozawa *et al.*, 1995a; Yoneda *et al.*, 1995).

Bcl-2 and a splice variant of Bcl-x (Boise *et al.*, 1993) have been shown to heterodimerize with other members of the Bcl-2 protein family, including Bax. These oncoproteins are proposed to manipulate the oxygen free radical damage and cell death (Korsmeyer *et al.*, 1995). Consistent with this proposal, it was shown that the antioxidant suppressed dopamine-induced apoptosis in mouse thymocytes (Offen *et al.*, 1995). Several studies showed that Bcl-2 can protect cellular membranes from oxidative damage (Hockenberg *et al.*, 1993; Kane *et al.*, 1993) and the generation of ROS (Zamzami *et al.*, 1995). The NO-induced apoptosis is effectively protected by overexpression or transfection of antiapoptosis gene *bcl-2* (Albina *et al.*, 1996; Messmer *et al.*, 1996). In the case of hypoxia-induced apoptosis, ROS are not essential for apoptosis, and Bcl-2 protects against this process in ways that do not depend on the inhibition of ROS production or activity (Jacobson and Raff, 1995, Shimizu *et al.*, 1995). These facts could be interpreted as indicating that both the hypoxia-induced apoptosis and the naturally occurring cell death with age are triggered by the bioenergetic crisis of cells, the decrease in $\Delta\Psi_m$, and PT pore opening.

## Apoptosis Cascade

From the previous reports, the apoptosis cascade, based on molecular genetics and bioenergetics, can be illustrated as in Fig. 3. At the point of $\Delta\Psi_m$ collapse in the upstream of the cascade, one of main tributaries of apoptosis with aging, hyperoxia, and mtDNA fragmentation, merges with other tributaries of apoptotic/

necrotic acute cell death with hypoxia (Jacobson and Raff, 1995; Shimizu *et al.*, 1995), respiratory inhibitors (Reed and Savage, 1995; Shimizu *et al.*, 1996b), depletion of reductants (Jones, 1995; Meister, 1995; Reed and Savage, 1995), oxidative-phosphorylation uncouplers (Zamzami *et al.*, 1996), divalent cations (Reed and Savage, 1995; Zamzami *et al.*, 1996), or NO (Albina *et al.*, 1996; Messmer *et al.*, 1996). In the bioenergy-dependent apoptotic cascade, apparently contradictory apoptosis-inducible factors, e.g., hyperoxia and hypoxia, could be localized intelligibly, as schematically illustrated in Fig. 3.

## CONCLUSION

The comprehensive analyses of the entire mtDNA, including the detection of the inherited/acquired point mutations, of the somatic oxidative damage, and of the total deletions, could reveal the mutational genotype unique to an individual. The analyses disclosed that the types and combination of point mutations decide the severity of somatic oxidative damage and deletions. Hence, there is the definite correspondence between the point mutational genotype and the phenotype of the patients. The practical survey of point mutations will be useful for genetic diagnosis predicting the patients' life-span and for the management of patients, such as for cardiac transplantation and/or gene therapy.

The survey of the somatic oxidative damage and the total deletions in mtDNA disclosed that mitochondrial genes coding the cellular energy factory are unexpectedly fragile to the $^{\bullet}OH$ damage, and, hence, to oxygen stress. Cellular ωmtDNA easily fragments into over a hundred types of ΔmtDNA resulting in a defective mitochondrial energy-transducing system and in a cellular bioenergetic crisis. This could be the missing link in the cascade of naturally occurring cell death under physiological conditions without vascular involvement. Namely, the fragmentation of ωmtDNA because of oxidative stress leads to cellular bioenergetic crisis, to the collapse of $\Delta\Psi_m$, to the release of the apoptotic protease activating-factors into cytosol, to uncontrolled cell death, to tissue degeneration and atrophy, and to aging and degenerative diseases.

Further elucidation of precise mechanism of this apoptotic cascade will enable us to protect unwanted cell death, and, hence, to arrest degenerative diseases, and to accelerate harmful/transformed cell death.

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