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Fragile Mitochondrial DNA: The Missing Link in the Apoptotic Neuronal Cell Death in Parkinson's Disease

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The oxidative stress theory, the mitochondrial (mt) hypothesis, and the apoptosis hypothesis are proposed as the cause of neuronal cell death in Parkinson's disease (PD). However, the direct link between them has remained unknown. Recently, the mt control of nuclear apoptosis is documented that collapse of mt transmembrane potential due to energy crisis leads to release of apoptotic protease activating-factors into cytosol and subsequently nuclear DNA fragmentation. However, an endogenous factor responsible for the energy crisis under physiological conditions is missing. Here we report the missing factor as that mtDNA in the striatum of a parkinsonian patient fragments into 134 types of deleted pieces, being detected by the total detection system for mtDNA deletion. The system has documented that the mtDNA is extremely susceptible to hydroxyl radical damage, hence to oxidative stress, enough to cause the cellular energy crisis. The extensive fragility of mtDNA in brain stem could link the oxidative stress up with the apoptotic neuronal cell-death of PD.

The oxidative stress theory of neurodegeneration (1), the mt hypothesis of cellular energy crisis (2), and the apoptosis hypothesis (3) are proposed as the cause of neuronal cell death in PD. Experimental models and human brain studies suggest that oxidative stress with iron-catalyzed free radicals derived mainly from mt oxidative metabolites and nitric oxide may play an important role in neuronal degeneration in diseases such as PD and AD (1, 4). However, it is not yet established whether oxidative stress is a major cause of cell death or simply a consequence of an unknown pathogenic factor. The mt hypothesis proposes that cellular energy crisis induced by respiratory failure is the most important mechanism of nigral cell death in PD (2), as several groups (5, 6) have found a decrease in NADH:CoQ oxidoreductase (complex I) of the mt electron transfer complex in the substantia nigra of patients with PD. The DNA nick-end labeling (TUNEL) on human brain specimens documents nDNA fragmentation (7, 8, 9), suggesting that apoptosis may be involved in these disorders. However, the direct link between these theories has been eluded.

In the previous paper (10), we first reported that 5 kbp ΔmtDNA (common deletion) exists in the striatum of PD patients as well as aged controls, suggesting a possible involvement of ΔmtDNA in pathophysiological processes underlying PD. However, the absolute level of the common deletion detected by PCR, 0.01-to-0.3 % of the total thousand copies of cellular mtDNA, seems to be far low to account for the observed decline of complex I activity. Hence, a question arose whether the observed ΔmtDNA is the cause or the effect of ageing (11). However, the total number 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 (12). Hence, a PCR-detectable ΔmtDNA is suggested to be the "tip of the iceberg" of the spectrum of somatically acquired mutations (13).

Here we present the results that the previously detected common deletion (10) is really the "tip of the iceberg" of over hundreds of ΔmtDNA that could link the oxidative stress and the cellular energy crisis up with the apoptotic neuronal-cell death.

MATERIALS AND METHODS

Materials. The mtDNA specimen was obtained from the autopsied striatum of a female PD patient died at age 65 whose histological

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Abbreviations: PD, Parkinson's disease; AD, Alzheimer's disease; ALS, amyotrophic lateral scrolesis; CM, cardiomyopathy; HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; ND, NADH dehydrogenase; MPP^+, 1-methyl-4-phenylpyridinium ion; PCR, polymerase chain reaction; mt, mitochondrial; n, nuclear; Δ, deleted; ω, wild-type; kbp, kilo base-pair; np, nucleotide position; Or, replication origin; ·OH, hydroxyl radical; 8-OH-dG, 8-hydroxydeoxyguanosine.

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diagnosis including nigral degradation and existence of Lewy bodies was confirmed, and the existence of the common deletion was first reported as the case 8 in the previous paper (10). A serious point mutation, np14180 T-to-C replacing the conserved tyrosine to cystein in the ND6 gene, in the specimen detected by the entire mtDNA sequencing was also reported previously (14). As an age-matched control, another mtDNA specimen was obtained from the autopsied striatum of a female patient with amyotrophic lateral scrolosis (ALS) died at age 65.

Method. The total detection system for mtDNA deletions (TD system) was recently devised(15). The PCR primers (20 mer nucleotide) were designed using a primer analysis software "OLIGO" of National Biosciences (Macintosh Version), so that they should be highly specific for intended target sequences, and form stable duplexes with the template. A total of 30 L-strand primers distanced from 0.5 to 0.6 kbp each other were located to cover all around the mtDNA duplex. Each L-strand primer was paired with six H-strand primers, distanced 3 kbp each. Thus, overall 180 kinds of primer pairs could detect all ΔmtDNA over 0.5 kbp. Detected deletions were distinguished from the artifact derived from the misanealing of primers by the primer shift method (16), which is built in the system by the combination of these primers. Extracted mtDNA from the tissue was amplified by using the primers. The amplified fragments were separated by electrophoresis on agarose gel, stained with ethidium bromide. The gel images of multiple ΔmtDNA were captured in a digital format for accurate molecular weight and mass analysis by the electrophoresis documentation and analysis system of Kodak (Macintosh Version). MtDNA specimens were completely digested with DNase I, spleen exonuclease, snake venom exonuclease, and alkaline phosphatase. Deoxynucleosides, dG, and 8-OH-dG in the hydrolysate were quantitatively determined by using a micro-HPLC/mass spectrometry system (17). From the total amount of ωmtDNA and that of deoxynucleoside, ωmtDNA % to the total mtDNA was calculated.

RESULTS AND DISCUSSION

The TD system was applied to the same mtDNA specimen of the parkinsonian striatum of which the common deletion was first reported (10). The system visualizes the whole "iceberg" as 134 types of ΔmtDNA, including 97 types of 'minicircles' that lack either one Or or the both associated with decrease in ωmtDNA down to 63%, as shown in Figure 1 and Table 1. In the striatum of the age-matched control, a patient with ALS, 98 types of ΔmtDNA is detected (Table). As the oxidative damage in ALS cells is pointed to be dominant (1), the listed ALS case could not be a normal control, but a positive control of oxidative damage. Accelerated mtDNA fragmentation of the PD patient could be resulted by an inherited point mutation in the ND6 gene (14), being revealed by the base sequencing of the entire mtDNA.

Random distribution of size and location of the ΔmtDNA and their remarkable mirror image shown in Figure suggest random occurrence of ΔmtDNA without preferential site. Hence, it seems reasonable to presume random double-strand separation by the accumulation of ·OH adducts with dG, 8-OH-dG (18), single-strand breaks by ·OH attacks (19), and rejoining of mtDNA as a preferable mechanism for its fragmentation. Nitric oxide may also involve in the fragmentation of mtDNA, as oxidative DNA damage in form of 8-OH-dG in calf thymus DNA was reported to be induced by simultaneous generation of nitric oxide and superoxide radical (20), and nucleotide-selective cleavage of duplex DNA by nitric oxide was observed at G:C sites (21). The TD system has revealed that, with chronic/acute oxidative damage, the great majority of ωmtDNA in tissues/cells fragments into hundreds types of ΔmtDNA (Table 1), which is documented in autopsied

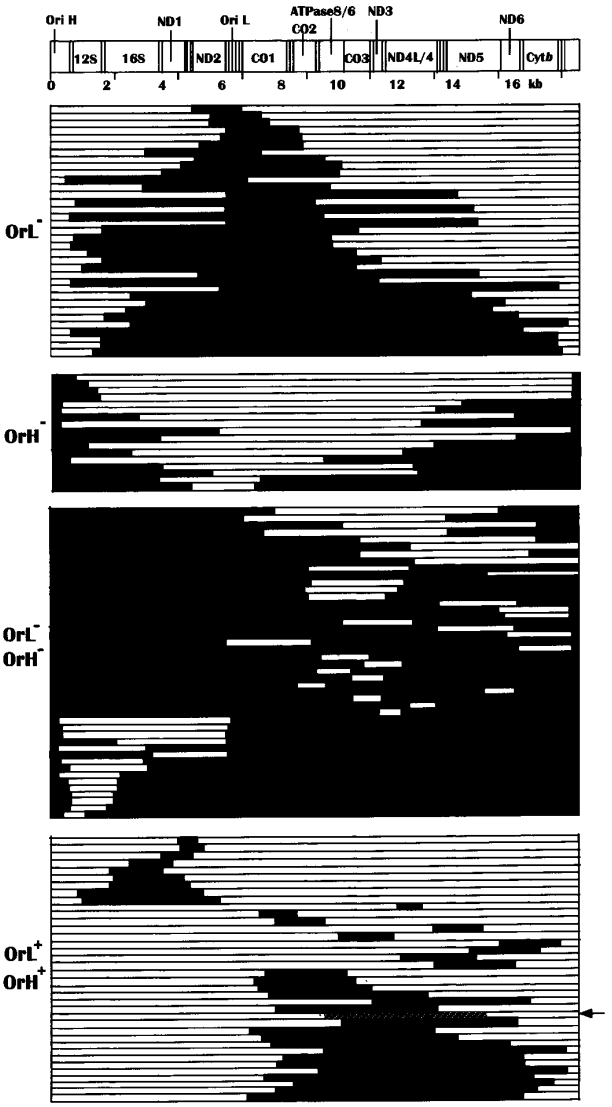

FIG. 1. Size distribution of ΔmtDNA in the striatum mtDNA of a patient with PD. To survey ΔmtDNA, the TD system (15) was applied to mtDNA specimen in the striatum of a patient with PD died at age 65 (10). Detected 134 types of ΔmtDNA were classified into four groups: OrL^-, OrH^-, OrL^-/OrH^- and OrL^+/OrH^+ according to the preservation of the Or of L-stand (L) or H-strand (H). White bars indicate ΔmtDNA, and dark shading bars indicate deleted regions. They are arranged according to their sizes. The hatched deletion indicated by an arrow is the common deletion reported in the previous paper (10). Genomes in mtDNA are schematically illustrated at the top of Panel.

TABLE 1

Types of Deleted mtDNA Among Subjects/Cells

Subject	Sex	Age	Disease/ stress	Tissue	ΔmtDNA type (*n*)	Sub-type of ΔmtDNA				ωmt DNA (%)	8-OH-dG/ 10^4 dG
						OrL^+/H^+	OrL^-	OrH^-	OrL^-/H^-		
S.K.	F	65	PD	Striatum	134	37	35	15	45	63*	17.1*
T.M.	F	65	ALS	Striatum	98	36	25	10	27	70*	9.4*
A.K.	F	3	VSD	Heart	5	4	1	0	0	>99	<1
S.T.	M	24	Accident	Heart	49	16	15	8	10	85	<1
Y.Y.	F	76	SAH	Heart	218	66	68	37	47	47	18.6
H.M.	F	97	Gast. ca.	Heart	358	78	88	63	129	11	148
M.K.	F	7	DCM	Heart	212	37	58	38	79	47*	38.8*
T.K.	M	19	mtCM	Heart	235	48	59	31	97	16	20.1
ρ^+			Normoxia	Fibroblast	49	14	15	5	15	80*	<1*
ρ^+			95% O2	Fibroblast	187	35	55	28	69	53*	29.9*

Note. DNA was extracted from the tissues/cells and analyzed by the total detection system for mtDNA deletion. Numbers of ΔmtDNA are listed. Mitochondrial DNA extracted from purified mt fraction was analyzed by microHPLC/MS for the determination of ωmtDNA, 8-OH-dG, and dG. Previously reported data of the subjects without cardiological findings (22), of the patient with DCM (24), of the patient with mtCM (23), of the cultured fibroblast, ρ^+, with and without oxygen stress (26) are summarized. Abbreviations: Or, replication origin; L, L-strand; H, H-strand; F, female; M, male; VSD, ventricular septal defect; SAH, subarachnoidal hemorrhage; Gast. ca., gastric cancer; ρ^+, a cultured human cell line, 701.2.8c; *, calculated from the regression formula (22).

heart of the normal subject over age 80 (22), of a mtCM patient died at age 19 (23), of a HCM patient died at age 20 (24), of a recipient of heart transplantation at age 7 with severe DCM (24). Consistent with our findings, extensive decrease in human skeletal muscle ωmtDNA down below 10% associated with multiple deletions and rearrangements was reported recently (25). The mtDNA fragmentation and the bioenergetic cell death could be mimicked by cultured cell line under hyperoxia (26): The exposure of a cultured fibroblast cell line (ρ^+) under hyperbaric oxygen stress for 2 days led majority of cells to apoptotic death at 3rd day with mtDNA fragmentation into 187 types of ΔmtDNA (Table 1), whereas the derivative cells (ρ^0) without mtDNA lacking a functional respiratory chain were relatively immune, more than 80% of the ρ^0 cells survived.

Experimentally, glutamate-induced apoptosis results in a loss of striatal neurons in the parkinsonian rat (27). Complex I inhibitors, both MPP^+ and rotenone, induce dose-dependent apoptosis/necrosis in dopaminergic cell cultures (28). These results suggest that the oxidative stress induces apoptotic neuronal cell death in PD. It is also reported that there was a significant increase in the amount of oxidative damage in form of 8-OH-dG in mtDNA in parietal cortex of AD patients compared with controls (29), and that there was in situ evidence for apoptosis with TUNEL in AD temporal lobes. However, direct link between the oxidative stress and the cellular apoptosis has been eluded. By contraries, a requirement of reactive oxygen species for an acute nuclear apoptosis is excluded *in vitro* cell free system (30, 31).

The proposed mechanism of apoptosis has remained unclear, even controversial until recently, because of the existence of many apoptosis inducible-factors and survival-factors that affect at different points among the apoptosis cascade-reaction, and because of unsettled cellular bioenergetics to kill cells under physiological conditions. The most readily measurable morphological features of apoptotic cell-death are nuclear; namely, chromatin condensation (32) and endonuclease-mediated nDNA fragmentation producing oligonucleosomal-sized fragments visible by gel electrophoresis (33), which is considered to be the hallmark of apoptosis. However, recent studies on anucleate cytoplasts (34, 35) demonstrate that cell nucleus and nDNA fragmentation are not required for apoptosis and for an apoptosis survival-factor, Bcl-2, protection. Quite recent studies disclose the mitochondrial control of nuclear apoptosis using the cell free system (31, 36): The mt bioenergetic crisis induced by adding the *exogenous* inhibitory factor leads mitochondria to the collapse of electrochemical proton gradient ($\Delta\mu_{H^+}$) in form of transmembrane potential ($\Delta\Psi_m$), to the opening of permeability transition pore, to the release of apoptotic protease CPP32 (37) activating-factors into cytosol, subsequently nDNA fragmentation. However, the *endogenous* factor responsible to the mt bioenergetic crisis and the collapse of $\Delta\Psi_m$ leading to the cell-death under physiological conditions has been missing.

Recently, we demonstrated non-invasively an extensive oxygenation of skeletal muscle that indicates mt dysfunction causing suppressed oxygen utilization among aged individuals and the patients with mt myopathy harboring point mutations or large deletions (38). Therefore, the progressive oxidative damage and the fragmentation of ωmtDNA seems to result in those changes to be synergistic leading cells to the bioener-

getic crisis, to the collapse of $\Delta\mu_{H^+}$ in form of $\Delta\Psi_m$, hence to chronic apoptosis under physiological conditions without vascular involvement. The fragmentation of ωmtDNA into hundreds of ΔmtDNA in the PD patient (Table 1) enough to cause the cellular energy crisis could link the oxidative stress up with apoptosis in the striatum. These changes would be more dominant in the patient's nigra where the most severe cellular degeneration took place and only microscopic fragments remained probably because of the breaking up of the cell into apoptotic bodies that are phagocytosed by macrophages. Comparably, in the case of complete heart block and fatal arrhythmias, apoptosis is recently proposed (39) to be responsible for nearly destroyed atrial-ventricular node, sinus node, and internodal pathways by a noninflammatory degeneration with no abnormal fibrosis or infiltrate.

In conclusion, the extensive fragility of mtDNA documented here could be the missing link between the oxidative stress and the apoptotic neuronal cell death of the parkinsonian brain stem.

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