Mitochondrial DNA Deletions/Rearrangements in Parkinson Disease and Related Neurodegenerative Disorders

GUANGYU GU, MD, PATRICIO F. REYES, MD, GREGORY T. GOLDEN, PhD, RANDALL L. WOLTJER, MD, PhD, CHRISTINE HULETTE, MD, THOMAS J. MONTINE, MD, PhD, AND JING ZHANG, MD, PhD

Abstract. Inhibition of mitochondrial respiratory chain function may contribute to dopaminergic neurodegeneration in the substantia nigra (SN) of patients with Parkinson disease (PD). Since large-scale structural changes (e.g., deletions and rearrangements in mitochondrial DNA (mtDNA)) have been associated with mitochondrial dysfunction, we tested the hypothesis that increased total mtDNA deletions/rearrangements are associated with neurodegeneration in PD. This study employed a well-established technique, long-extension polymerase chain reaction (LX-PCR), to detect the multiple mtDNA deletions/rearrangements in the SN of patients with PD, multiple system atrophy (MSA), dementia with Lewy bodies (DLB), Alzheimer disease (AD), and age-matched controls. We also compared the total mtDNA deletions/rearrangements in different brain regions of PD patients. The results demonstrated that both the number and variety of mtDNA deletions/rearrangements were selectively increased in the SN of PD patients compared to patients with other movement disorders as well as patients with AD and age-matched controls. In addition, increased mtDNA deletions/rearrangements were observed in other brain regions in PD patients, indicating that mitochondrial dysfunction is not just limited to the SN of PD patients. These data suggest that accumulation of total mtDNA deletions/rearrangements is a relatively specific characteristic of PD and may be one of the contributing factors leading to mitochondrial dysfunction and neurodegeneration in PD.

Key Words: Aging; Energy metabolism; Free radicals; Mitochondria; Oxidative stress; Parkinsonism.

INTRODUCTION

Idiopathic Parkinson disease (PD) is one of the most important medical problems of modern Western society. Its pathological hallmarks are a loss of dopaminergic neurons from the substantia nigra (SN) associated with depletion of striatal dopamine and the presence of intraneuronal inclusions called Lewy bodies (1). The cause of the cell death underlying PD is unknown. Several groups of investigators have reported inhibition of mitochondrial respiratory chain function in PD patients (2–4), although there is controversy as to whether there is a specific inhibition of complex I alone or whether other complexes also are affected (5, 6). In addition, the pathogenesis of inhibited mitochondrial function in PD is unclear. It may result from an inherited defect either in one of the genes encoding the different components of the mitochondrial respiratory chain or in other genes involved in normal mitochondrial function (7, 8). Alternatively, mitochondrial dysfunction may result from inhibition of any of the complexes by non-specific free radical mediated damage or exposure to exogenous toxins during the aging process (9, 10), given that aging is the most important risk factor for PD development (11, 12).

Compelling evidence has suggested that an alteration in the genetic material within mitochondria, mitochondrial DNA (mtDNA), which encodes many proteins of mitochondrial complexes including several subunits of complex I (13), may be particularly important since cybrids made from individuals with PD show reductions in complex I activity (7, 9). mtDNA is particularly vulnerable to various insults from either sporadic mutation during the aging process or exposure to environmental toxins because it has no protection from histones and its repair system is much less efficient than those of nuclear DNA (14). In fact, the rate of mutation in mtDNA is approximately 10 times greater than that in chromosomal DNA (15), and mutations in mtDNA are more likely to have functional consequences because mtDNA has no noncoding sequences except for a small segment involved in the replication of mtDNA (13).

Numerous abnormalities such as single or multiple mutations, single or multiple deletions, duplications, and rearrangements have been described in mtDNA during aging and in association with numerous degenerative diseases, among which a 4,977-bp deletion has been investigated most extensively (16–20). This deletion was initially found in several neuromuscular diseases associated with mitochondrial dysfunction and is referred to as the “common deletion” (21). This common deletion has been reported in some studies to occur exclusively or with increased frequency in the midbrain of patients with PD. Other studies could not confirm these results; rather,
it was suggested that the mitochondrial common deletion is associated with aging in the midbrain and not PD. One possible explanation for these conflicting results is the difficulty in quantifying mtDNA deletions or mutations in the whole midbrain or the SN, while only a subset of midbrain neurons degenerate in PD. Using an in situ hybridization assay, we have demonstrated that the common deletion is not associated with loss of vulnerable neurons in PD (22). Here we hypothesized that it is not a specific mutation or deletion but accumulated total mitochondrial damage that is associated with PD. To test this hypothesis, we have used long-extension polymerase chain reaction (LX-PCR) in this study to compare total number of mtDNA deletions/rearrangements and determine whether there was any difference in the multiple deletions/rearrangements in the SN between age-matched controls and patients with PD. Then, we investigated whether the multiple mtDNA rearrangement was simply a reflection of neurodegeneration or specifically related to PD by analyzing the total mtDNA deletions/rearrangements in patients with multiple system atrophy (MSA), dementia with Lewy body disease (DLB) and Alzheimer disease (AD). All of these neurodegenerative diseases except for AD, though different both clinically and pathologically from PD, share prominent neuronal degeneration in the SN. Finally, we have assessed the regional specificity by comparing total mtDNA deletions/rearrangements in different brain regions in patients with PD.

MATERIALS AND METHODS

Patients

Brain tissue was obtained from autopsies performed at Vanderbilt University Medical Center (Nashville, TN), Creighton University Medical Center (Omaha, NE), Coatesville Department of Veterans Affairs (Coatesville, PA), University of Pennsylvania (Philadelphia, PA), and Duke University Medical Center (Durham, NC). Controls were cognitively normal at enrollment with no clinical evidence of a movement disorder. Neuropathological examinations of control individuals showed age-related changes only. All patients had been diagnosed during life with an extrapyramidal movement disorder or dementia. Final diagnoses were established by neuropsychological examination according to established criteria. Autopsy protocols, approved by each individual institution, were very similar and included flash freezing of tissue followed by storage at −80°C until analyzed. The material analyzed consists of 8 cases of PD, 6 cases of AD, 6 cases of DLB, 4 cases of MSA, and 4 controls.

Extraction of Deoxyribonucleic Acid

All frozen tissue (100–200 mg from each case) was fractured while frozen in liquid nitrogen, and total deoxyribonucleic acid (DNA) was extracted via Puregene DNA extraction kits (Gen- tra, Minneapolis, MN).

Long Extension Polymerase Chain Reaction

Long extension polymerase chain reaction (LX-PCR) uses primers that are homologous to the mitochondrial genome with the 5' end of the primers in close physical proximity to each other, but with the 3' ends facing in opposite directions. Typically, after the use of 10-min extensions during the PCR, nearly the entire mitochondrial genome is amplified, ~16.3 kb in humans (23, 24). When there are mutational events that bring the 2 primers closer together, i.e. when there are single and multiple deletions or rearrangements in the mtDNA, truncated PCR products result (24). This assay has been used to map mitochondrial DNA deletions/rearrangements from patients suffering from mitochondrial disease (25), as well as in numerous aging studies (24, 26). This technique has the advantage of not being biased toward any one mutational event, such as the 5-kb common deletion (27). However, while this assay assesses the spectrum of mtDNA deletions/rearrangements present within a sample, it is not quantitative for the relative amounts of specific products amplified in a given sample (28).

The method used in this study was similar to the one described by Anderson et al with minor modifications (29). In brief, the LX-PCR employed 2 opposing primers FLFor (5’-TGAGGCCAAATATCATTCTGAGGGGCC-3’) and FLRev (5’-GTTTTCATCATGCGGAGATGGTGGATG-3’) synthesized by the Molecular Biology Core of Vanderbilt University Medical Center and a LX-PCR kit (Roche, Indianapolis, IN) to generate either a full-length mtDNA (16.3 kb) or mtDNA with deletions/rearrangements. PCR reaction with biphasic hot start was performed with the following components: PCR gem 50 (Perkin Elmer, Foster City, CA), 0.2 μM primers (final concentration), 300 μM dNTPs, 1× buffer, 1 unit supplied enzyme, and 10 picograms (pg) of mtDNA (normalized by Southern blotting and hybridization with alkaline phosphatase (AP)-labeled full-length mtDNA followed by densitometry). The amplification was carried out in a total volume of 50 μl in thin walled microamp tubes (MJ Research, Watertown, MA) in a PTC-100 TM thermocycler (MJ Research). The PCR profile involved initial denaturation for 2 min at 94°C, followed by a 2-step amplification of 35 cycles at 94°C (denaturation) for 10 s, and annealing/extension at 68°C for 11 min, and a final extension for 10 min at 72°C. To avoid variations of PCR reactions among different runs of experiments, each reaction included cases from all the experimental groups.

Field Inversion Gel Electrophoresis (FIGE)

A 5 μl aliquot of the PCR products was electrophoresed on 0.8% agarose gel containing ethidium bromide (EtBr) in 0.5× TBE for 12 h using field inversion gel electrophoresis (FIGE). The FIGE settings were as follows: 180 V forward direction and 120 V reverse direction at switch time ramp 0.1 ~ 0.4 s in a linear shape. FIGE gels were photographed in a UV transilluminator first, then denatured, neutralized, and finally blotted onto hybridization transfer membrane (NEN TM Life Science Products, Boston, MA).

Southern Blotting

Probes for Southern Blotting: To further confirm the specificity of the PCR products, instead of using the original primers, a pair of nested primers HNFor (5’-CTAGGAAATCA-CCTCCCCATTCCGATAA-3’, 15,369–15,395 bp) and HNRev (5’-GTTCCTTGTAATGGAATCCATACGATGTT-3’, 14,742–14,715 bp) was used to amplify a 15.9-kb fragment from human...
muscle tissue, which was isolated and purified from low gelling temperature agarose (FMC, Rockland, ME). This product was used as an mtDNA probe to hybridize the PCR products generated from the original opposing primers. Finally, both original and nested PCR products were digested by 3 restriction enzymes with different restriction sites. The resulting fragments were consistent with the expected mtDNA restriction map (data not shown).

**Southern Blotting:** Southern blotting was performed using a standard method with the agarose gel presoaked in 0.25M HCl and in 0.5 M NaOH. DNA was transferred to a GeneScreen plus nylon membrane (NEN TM Life Science Products, Inc.) and UV-linked. Then, mtDNA fragments labeled with alkaline phosphatase direct labeling and detection system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) were hybridized to nylon filters by Southern blotting, and hybridization and wash conditions were carried out under high stringency.

**Statistical Analysis**
Excluding full-length mtDNA, the number of LX-PCR products detected by ethidium bromide staining and confirmed by Southern blotting was tabulated and averaged for each group of patients by investigators who were not aware of the pathological diagnoses. Statistical analysis was performed with commercially available software, GraphPad Prism (GraphPad, San Diego, CA).

**RESULTS**
**Patient Data**
A total of 28 individuals were included in this study. There was no significant difference in age, gender ratio, or postmortem interval among the 5 groups (Table).

<table>
<thead>
<tr>
<th>Number</th>
<th>Control</th>
<th>PD</th>
<th>MSA</th>
<th>DLB</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Male/female</td>
<td>3/1</td>
<td>5/3</td>
<td>2/2</td>
<td>4/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Postmortem interval (h)</td>
<td>6.1 ± 2.7</td>
<td>8.1 ± 3.9</td>
<td>10.2 ± 11.4</td>
<td>11.7 ± 6.8</td>
<td>7.7 ± 5.2</td>
</tr>
</tbody>
</table>

-age and postmortem intervals are expressed as means ± SEM. One-way analysis of variance for age, gender ratio, and postmortem intervals were not significantly different when the 5 groups were compared (p > 0.05). Abbreviations: PD, Parkinson disease; MSA, multiple system atrophy; DLB, dementia with Lewy body disease; and AD, Alzheimer disease.

probe. It was apparent that most of the PCR products seen by ethidium bromide staining were also found to hybridize to the mtDNA probe, confirming that they are derived from the mitochondrial genome (Fig. 1B).

Comparison of the mobility of the smaller mtDNA products with the molecular weight standards revealed that each individual had a distinctive array of rearrangements without any single predominant mtDNA deletion/rearrangement in any of the diseases. To confirm quantitatively the difference in mtDNA deletions/rearrangements among PD, AD, MSA, DLB, and control patients, the number of smaller molecular weight products seen in the Southern blotting membrane after LX-PCR was counted for each subject. The average number of rearranged forms of mtDNA in patients with PD, AD, MSA, DLB, and controls was 10.75, 5.67, 4.75, 4.83, and 6.00, respectively (Fig. 2). The number of mtDNA deletions/rearrangement in SN of PD was significantly higher than that in other groups (p < 0.05).

To study whether increased mtDNA deletions/rearrangements in the SN of PD patients were also present in other brain regions, the numbers of mtDNA deletions/rearrangements were compared among the SN, cerebral cortex, hippocampus, and cerebellum in 5 cases. The results demonstrated that there was no statistical difference in the number of mtDNA deletions/rearrangements among PD, AD, MSA, DLB, and control patients, the number of smaller molecular weight products seen in the Southern blotting membrane after LX-PCR was counted for each subject. The average number of rearranged forms of mtDNA in patients with PD, AD, MSA, DLB, and controls was 10.75, 5.67, 4.75, 4.83, and 6.00, respectively (Fig. 2). The number of mtDNA deletions/rearrangement in SN of PD was significantly higher than that in other groups (p < 0.05).

**DISCUSSION**
The demonstration of mitochondrial complex I deficiency in the SN of PD patients has brought about the speculation that mtDNA mutations, inherited or acquired during the aging process, may play a significant role in the pathogenesis of this disease. Our previous study indicated that the common deletion of mtDNA, which involves genes that code for several complex I subunits, is not specifically related to the dopaminergic neuronal loss seen in PD or during the aging process (22). Here we have tested the hypothesis that it is not a specific deletion but accumulated mitochondrial damage that is associated with PD.
In this study, using a LX-PCR technique, we have analyzed the total mtDNA deletions/rearrangements in the SN from patients with various neurodegenerative diseases as well as the SN from age-matched controls. In addition, we have compared the total mtDNA deletions/rearrangements in different brain regions of PD patients. Our results demonstrated that there were significantly more mtDNA deletions/rearrangements in the SN of PD patients compared to patients with other neurodegenerative disorders (Figs. 1, 2). Furthermore, increased mtDNA deletions/rearrangements were found in other brain regions, including those that do not usually demonstrate neurodegeneration in PD patients (Fig. 3).

What causes the increased total mtDNA deletions/rearrangements in PD is currently not known, although increased oxidative stress is likely to be an important contributor. There is a steady increase in free-radical production during aging, which is the most important risk factor in the development of PD (30, 31). Aging is also associated with increased mtDNA deletions/rearrangements in human cortex (28) and progressive impairment of oxidative phosphorylation (15). Support for this hypothesis can also be found in the experiments showing that there is significantly higher oxidative damage in the midbrain of PD patients compared to age-matched controls, as well as patients with other types of movement disorders (32, 33). The fact that more mitochondrial deletions/rearrangements are observed in PD than in DLB patients is entirely consistent with an early report showing that there is significantly more oxidative damage to nigral mitochondrial DNA in PD than in DLB patients (32). This difference is particularly interesting because involvement of a similar pathogenesis for both PD and DLB has been speculated, but our data suggest that the mechanisms for dopaminergic neurodegeneration may actually be different despite the fact that there is formation of Lewy body in the SN in both settings.
The mechanisms underlying the selective increase in oxidative stress in PD have not been fully characterized; however, it has been speculated that exposure to environmental toxicants like rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as well as genetic vulnerability may play a role. Rotenone, a lipophilic pesticide, is a mitochondrial toxin that increases oxidative stress and results in parkinsonism in rats with chronic exposure (34). MPTP is a contaminant of synthetic opioids that led to an outbreak of parkinsonism in young patients with PD (35). It is now recognized that MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP⁺), which also produces free radicals and inhibits complex I of the electron transport chain (2, 36, 37). The contribution of an individual's genetic background to mitochondrial dysfunction is exemplified by a family with parkinsonism reported initially to carry an inherited mutation at 11778 of mtDNA, which produces a complex I defect (38). Subsequent direct sequencing of mtDNA complex I and tRNA genes, however, failed to show homoplasmic mutations, suggesting that the genetic background of the family may confer its vulnerability to environmental exposures that lead to complex I inhibition (7).

The finding of increased mtDNA deletions/rearrangements in all the brain regions analyzed for PD patients is consistent with earlier reports showing that mitochondrial inhibition is not limited to the SN (39) and that rotenone affects complex I uniformly throughout the brain despite the fact that there is highly selective neurodegeneration in the nigrostriatal dopaminergic system (34). The mechanisms underlying this phenomenon are not understood fully; however, catechols contained in these vulnerable neurons may play an important role, given that neurodegeneration in PD patients as well as in animals exposed to MPTP and rotenone is largely restricted to neurons containing dopamine (40–45). Dopamine is metabolized to produce hydrogen peroxide (H₂O₂) and dihydroxyphenylacetate (DOPAC). Also, like the neurotoxin 6-hydroxydopamine, dopamine and related o-catechols are unstable molecules that can oxidize in the presence of transition metals to form reactive oxygen species (ROS) and quinones that may modify proteins, lipids, and nucleic acids (41, 42). Furthermore, it is known that products of catechol oxidation inhibit mitochondrial function and mediate neuronal death in experimental model systems (44, 46, 47). Of note, other mechanisms besides metabolism of catechols may also contribute to the selective vulnerability of these neurons. For instance, a recent study demonstrates that the expression of glutamate subunits in the vulnerable neurons appears to be different from those that are resistant to neurodegeneration in parkinsonian animals (48).

In summary, in this study we have analyzed total mtDNA deletions/rearrangements within the SN of patients with PD, MSA, DLB, and age-matched controls. In addition, we have compared the total mtDNA deletions/rearrangements in different brain regions of PD patients. The results demonstrated that both the number and variety of mtDNA rearrangements increased significantly in the brain of PD patients. The increase in mtDNA deletions/rearrangements may contribute to mitochondrial dysfunction and consequently neurodegeneration in patients with PD.

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