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Axonal degeneration in peripheral nerves in a case of Leber's Hereditary Optic Neuropathy

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Abstract

Background—Leber's hereditary optic neuropathy (LHON) is a mitochondrial DNA (mtDNA) genetic disorder characterized by profound bilateral loss of central vision due to selective loss of retinal ganglion cells. Most patients with LHON do not have complaints related to the peripheral nervous system. We investigated possible qualitative and quantitative histological changes in the peripheral nerve of a patient with LHON as compared to normal controls.

Methods—Brachial plexus specimens were obtained at necropsy from an LHON patient carrying the 3460/ND1 mtDNA mutation and age-matched controls without known history of neurological disease. The nerves were evaluated by light microscopy coupled to a digital camera based morphometric analysis and electron microscopy.

Results—Extensive axonal degeneration of the large heavily myelinated fibers was found in the brachial plexus from the LHON patient. In LHON nerve fascicles we counted over ten times as many degenerated profiles as found in control nerve fascicles.

Conclusion—Microscopic examination of the brachial plexus in this LHON patient clearly demonstrated a significant pattern of neurodegeneration. Our study suggests that peripheral neuropathy may be a subclinical feature associated with LHON.

Keywords

Leber's Hereditary Optic Neuropathy (LHON); peripheral neuropathy; mitochondria; mutation; degeneration; axon

Leber's hereditary optic neuropathy (LHON) is a maternally inherited form of bilateral subacute loss of central vision affecting predominantly young adults. Three pathogenic mtDNA point mutations, at positions 11778/ND4, 3460/ND1, and 14484/ND6, account for over 90% of cases (1). Optic atrophy with permanent and severe loss of central vision is the usual endpoint of the disease (1,2). Extensive efforts have been made to further elucidate the pathology and pathophysiology in LHON. It has been found that within the optic nerve there

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is a predominant involvement of the papillomacular bundle (PMB), represented by small caliber parvo-cellular axons (3). The disease is characterized by symmetrical dropout of retinal ganglion cell axons in the PMB in the absence of inflammation on fundus examination (4). Larger axons in the optic nerve are selectively spared. Dramatic loss of retinal ganglion cells and the axons that compose the nerve fiber layer are the main histopathological findings in LHON (1-3).

Biochemical evidence in LHON patients indicates that all three pathogenic mitochondrial mutations involving different subunits of complex I affect respiratory function by decreasing complex I-driven ATP synthesis (5). This in combination with an increase of reactive oxygen species (ROS) production may lead to tissue damage. It has been suggested that environmental factors, such as smoking and alcohol consumption might influence the penetrance of the LHON mtDNA homoplasmic mutation (1,2). An extensive investigation of a large Brazilian pedigree of 11778/ND4 on haplogroup J was conducted on 328 living family members; this study found a statistically significant association with drinking alcohol and especially tobacco use in LHON affected members as compared to asymptomatic carriers (6). Among affected LHON patients, smokers also had poorer visual acuity (6,7). Most recently, an extensive study on many LHON pedigrees confirmed the risk factor of smoking on the conversion of carriers to affected (8).

LHON has been classically thought to only affect the optic nerve. This tissue specificity is intriguing. Mitochondrial dysfunction might be expected to involve other systems since it plays a central role in all cellular functions through impaired oxidative phosphorylation (9). Indeed, evidence of some systemic involvement in LHON has been previously recognized. Rare, non-ophthalmologic manifestations have been reported including defective cardiac conduction, spinal cord disease, brain stem and basal ganglia involvement, dementia, tremor, Parkinsonism, migraine, epilepsy, myoclonus, dystonia, a multiple sclerosis (MS)-like disorder, Charcot-Marie-Tooth disease, progressive auditory neuropathy and peripheral nerve involvement (10-18). All these cases have been characterized as the “Leber’s plus” clinical phenotype. The fact that other metabolic mitochondrial optic neuropathies, like Cuban epidemic optic neuropathy, also involve the peripheral nerves (19) prompts the question as to whether patients with LHON may also have subclinical or histopathological evidence of a peripheral neuropathy. There have been associations of peripheral neuropathy with LHON (18,20) but none of that have provided strong evidence for a common etiology.

There are excellent reasons, however, to expect peripheral nerve involvement in LHON. The peripheral nerves are the longest axons in the body. Long (and unmyelinated) axons might be vulnerable in LHON as they have a higher energy demand (9).

To test this hypothesis, we examined the brachial plexus obtained at necropsy from an LHON patient with the 3460/ND1 mtDNA mutation, looking for morphological changes, fiber loss, degeneration, and morphometric changes.

METHODS

We obtained at necropsy a specimen of one brachial plexus nerve from a previously reported LHON female patient (3) carrying the homoplasmic 3460/ND1 mutation, who died of cardiac failure at 75 years of age. Her well documented history did not include the clinical features of peripheral neuropathy. She had lost her vision in both eyes at the age of 22. Her right and left eyes were affected within a one month interval from each other. At about the time of her visual loss, her neurological examination revealed extrapyramidal signs. Furthermore, the last 10 years of her life were characterized by a slowly progressive cognitive decline. The tissue was obtained with informed consent from the University of

Bologna, Italy. Control nerves from four age-matched subjects were obtained from the tissue bank of the National Disease Research Institute (NDRI) in Philadelphia, Pennsylvania. The controls had no history of neurological disease, diabetes, or infectious disease.

The tissues were formalin fixed within 24 hours postmortem. The peripheral nerves were separated from surrounding adipose tissue and remnants of the skeletal muscles, then cut in cross-sections. The nerves were post-fixed in a buffered aldehyde solution (2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffered saline (PBS)) and then processed for embedding into plastic blocks. This process involved further fixation of the nerves in 2% osmium tetroxide (OsO₄) in 0.1 M PBS for 3-4 hours for lipid stabilization, then rinsing first with PBS, then with 0.1 M sodium acetate buffer. Enblock staining was performed with 1.0% uranyl acetate in 50 mM sodium acetate overnight. The tissues were then dehydrated with increasing concentrations of ethanol to 100%, then propylene oxide.

Following dehydration, each specimen was infiltrated with a 1:1, then a 2:1 epon:propylene oxide mixture. Next the nerves were placed in 100% epon in vacuum overnight. Finally the tissue was placed in freshly made 100% epon, oriented in an embedding mold, labeled and placed in an oven at 60° C for 2 days to allow for complete polymerization of the epon into tissue blocks.

For light microscopic examination, semithin (1 µm) cross-sections of nerves embedded in epon were obtained using a diamond histoknife on an ultramicrotome. Tissue sections were then dried on glass microscope slides and stained with p-phenylenediamine (PPD) in absolute methanol (21). The slides were then cleared in xylene, mounted with a permanent mounting media, then coverslipped. The brachial nerves were evaluated using a Zeiss Axioskop light microscope (Carl Zeiss, Inc., Thornwood, New York) coupled to a Spot RTke digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) that allowed for capturing and saving of the images onto a computer. Approximately 8-10 areas were sampled from the nerve cross-sections at 630x magnification. Degenerated fibers were recognized as dark, opaque solid profiles. The average number of thin (< 2 µm), highly myelinated thick (> 2 µm), and degenerated axonal profiles were calculated per 1 mm² manually.

We counted and measured the calibers of normal appearing axons per mm² of the LHON and control brachial nerves using computer-assisted image analysis from a morphometry software program called Aphelion (Amerinex Applied Imaging, Inc., Monroe Twp, New Jersey).

For examination using transmission electron microscopy (TEM), ultra-thin sections of nerves were cut using a diamond knife on an ultramicrotome from selected areas chosen using a light microscope. The sections were placed on copper grids and stained with uranyl acetate and lead citrate for examination on a JEOL transmission electron microscope (JEOL USA, Peabody MA).

PPD stains lipid aggregates and membranes (22). We used the appearance of homogeneously opaque, thick, dark brown individual fibers and less commonly, larger singular profiles consisting of elements from many degenerated axons often surrounded by additional myelin (21,22) as defining degenerated axons. We defined the normal myelinated axon as containing a clear axoplasm surrounded by an intensely stained annulus of myelin.

The qualitative changes, average density of thin and thick myelinated fibers per 1 mm² and percentage of fibers undergoing axonal degeneration were then estimated from our calculations.

RESULTS

We estimated the qualitative changes, average density of thin and thick myelinated fibers per square millimeter, and percentage of fibers undergoing axonal degeneration in a sample of brachial plexus nerve from a patient with LHON. We found extensive axonal degeneration at various stages in cross-sectional profiles of nerves. The typical spectrum of this degeneration observed appeared to range from axonal swelling to increasing levels of condensation of axoplasm and myelin thickening (Figs 1B and 3C). There were focal areas within the fascicles demonstrating higher densities of axonal degeneration. In the control peripheral nerves, a few degenerated profiles were also observed. Figure 1 compares cross-sections from normal and LHON brachial nerves.

The magnitude of axonal degeneration was calculated as a ratio of degenerated profiles to normal axons. We previously demonstrated that in later stages of axonal degeneration, a single large profile can consist of elements from many degenerated axons (Fig 3B)(21,22). However, most of the degenerated axons in our counts were observed to be at an early, one axon stage (Fig 3C). We manually counted an average of 7,000 normal appearing axons per square millimeter in the peripheral nerves of controls and about 6,000 in the LHON nerve. However, the number of degenerated profiles in LHON was increased by a factor of 13. Approximately 20% of the profiles were degenerated in the LHON nerve, compared to only 1-3% in the controls (Fig 2).

Computer-assisted image analysis of the nerve fiber spectra showed that the mean axonal diameter was similar in LHON and controls. Although the computerized study may mistakenly identify the same axon twice for external and internal diameter or overlook the smallest fibers, we manually corrected for these issues before counting.

The degeneration in the nerve of the LHON case was seen slightly more frequently in axons with medium to large diameters (not shown). Examination by electron microscopy permitted better qualitative assessment of normal and degenerated profiles in normal control and LHON peripheral nerves (Fig 3). The brachial plexus axons from the controls demonstrated a wide variety of axon calibers, most of which had medium thick myelin sheaths. The axoplasm was not electron dense and contained a normal distribution of neurofilaments and other axoplasmic constituents (Fig 3A). In contrast, the brachial plexus from the LHON patient displayed numerous individual axonal profiles that possessed thick electron dense myelin with a condensed axon (Fig 3C) and less often, a large degenerated profile consisting of globular subelements, each representing the degeneration of a separate axon (Fig 3B). These larger single profiles consisted of remnants from 4-8 axons (21,22). In addition to extensive axonal degeneration, muscle histopathology showed wide variability of muscle fiber caliber, hypotrophic fibers and angulated fibers.

CONCLUSIONS

This is the first qualitative histologic and quantitative morphometric description of degeneration in peripheral nerves from a patient with Leber's hereditary optic neuropathy (LHON). Extensive neurodegenerative morphological changes affecting many fibers in the peripheral nerve were seen in this case of 3460/ND1 mutation but not in controls. Calculations quantitated this degeneration to be at a rate approximately 13 times higher than the age-matched controls. This finding suggests that LHON does affect peripheral nerves at a subclinical level probably as a part of a common pathogenetic mechanism. The fact that the total number of fibers in the LHON brachial plexus was not significantly reduced suggests that compensatory regeneration may maintain fiber number leading to a steady state and subclinical condition.

Non-specific axonal degeneration (up to 3%) and segmental demyelination were seen in the peripheral nerves of our controls and this has been observed with normal aging as a part of other morphological changes (23,24). Limited morphological degenerative changes in fibers of peripheral nerves have been reported as due to repeat micro-traumas, pressure on the nerve, but also simply as age related (23). Indeed the number of myelinated axons in peripheral nerves decreases with age in normals, especially in the distal regions, but overall, fiber morphology abnormalities are mild (24).

We observed larger scale degeneration in the LHON brachial plexus specimen that greatly exceeded expected age related changes. The peripheral nerves in the present LHON case demonstrated pathological features of neurodegeneration. Mitochondrial dysfunction from LHON might have at least two deleterious consequences for neurons. The first is a reduction in ATP production.

Cybrid studies have shown that LHON mutations can impair complex I-driven ATP synthesis but total cellular ATP content is not significantly affected (5,25). This explains why, even in symptomatic patients, most tissues do not manifest pathology (1). A second consequence of LHON mutations concerns the complex I-driven increase of reactive oxygen species (ROS). ROS may act as an intracellular messenger and lead to changes in the membrane potential of mitochondria, predisposing to the opening of the mitochondrial permeability transition pore (MPTP), releasing cytochrome C and thus activating the apoptotic cascade (26-29). Neurons consume ATP at the highest rate, and most of this energy requirement is for axonal membrane polarization and axonal transport. Hence, neurons are sensitive to mitochondrial dysfunction particularly these with long axonal segments and less myelin. This includes the retinal nerve fiber layer and optic nerve (1) and perhaps other neuronal systems with long axons, including peripheral nerves.

At least four LHON patients with the 11778/ND4 mutation have been reported with diminished or absent sensation (i.e. light touch and vibration sense) and reflexes in their extremities and nerve conduction velocities were slightly attenuated (18). Fiber loss and chronic axonal degeneration were noted in peroneal nerve biopsies from patients with mitochondrial disorders other than LHON such as: Mitochondrial Encephalomyopathy Lactic Acidosis Stroke (MELAS), Myoclonic Epilepsy Ragged Red Fibers (MERRF), Mitochondrial NeuroGastroIntestinal Encephalopathy (MNGIE) (20). One further patient with 11778/ND4 LHON has been described with progressive sensory complaints and electrophysiological evidence of sensorimotor demyelinating polyneuropathy but without peripheral nerve biopsy (30). Hence, to date, there has not been strong evidence for LHON associated peripheral neuropathy. The present study, in showing marked degeneration of fibers in LHON peripheral nerve, provides further support for the notion that long axons of peripheral nerve may also be vulnerable to the same pathophysiological mechanism (1).

We observed extensive degeneration in peripheral nerve (brachial plexus) from a patient with 3460/ND1 LHON, not seen in controls. Though not previously described, this finding was predicted by the pathophysiological mechanisms that our group has previously proposed (1). Long and less myelinated axons, such as the retinal nerve fiber anterior to the optic disc, may be vulnerable to mitochondrial dysfunction. However, while the clinical manifestation of blindness is common in LHON, clinical evidence of peripheral neuropathy is not. The difference is probably due to the capacity of the peripheral nerve system to regenerate.

Many processes can cause peripheral neuropathy and brachial plexus damage. However, based on the well-documented history, there were no motor or sensory symptoms or signs suggesting the presence of other neurological conditions. There was no history of compression, transection, or ischemia of the brachial plexus. No risk factors such as

radiation therapy were noted. Cervical disc disease is a common condition affecting the elderly. Since this is a retrospective study, we cannot completely rule out cervical disc disease. There was no history of pain, sensory loss, or weakness involving the shoulder, arm, or hand, and cervical disc disease was not likely present. LHON, confirmed by genetic analysis, was the only neurological condition that could be supported. Furthermore, postmortem histopathology of muscle samples demonstrated variable fiber size most consistent with a neurogenic myopathy. All of our findings suggest that the basis of the peripheral neuropathy in our patient due to axonal damage.

Although our histopathological sampling was extensive, we only studied one LHON and four control cases. Such limitations often occur in conditions as rare as LHON. We look forward to confirmation in other cases of molecularly characterized LHON, as well as other mitochondrial optic neuropathies.

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Figure 1. Plastic embedded semi-thin sections of human brachial plexi, cross-sectional profiles, p-phenylenediamine stain, light microscopy; (A) Normal nerve demonstrating a typical spectrum of thick and thin axons. (B) LHON nerve demonstrating axonal degeneration. Note examples of axonal swelling (asterisks) and various stages of axonal condensation and myelin thickening (arrows). (A and B x630; scale bars: 8 μ m)

Figure 2. Average counts of the thin ($< 2\mu$), thick ($> 2\mu$), and degenerated axons per 1 mm^2 of the brachial plexus nerve in control and LHON are shown. The degenerated fibers only account for 0.8%-2.8% of the control nerves as compared to approximately 20% of the LHON nerve.

Figure 3.

Plastic embedded thin sections of human brachial plexi, cross-sectional profiles, transmission electron microscopy; (A) Example of a typical myelinated fiber from a normal control. (B) Example of a degenerated profile consisting of a cluster of electron-dense material representing multiple individual degenerated axons such as those seen in C. (C) Note small, condensed axons (arrows) in two of these degenerated fibers with thickening of electron-dense myelin. (scale bar in A, 0.5 μm , B, 1 μm , and C, 3 μm)