Neuropathological and Histochemical Changes in a Multiple Mitochondrial DNA Deletion Disorder

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Abstract. The identification of cytochrome c oxidase (COX)-deficient/succinate dehydrogenase (SDH)-positive cells using sequential histochemistry has proved important in the identification of cells with high mitochondrial DNA (mtDNA) mutant load. We demonstrate large numbers of COX-deficient/SDH-positive neurons in a mosaic pattern throughout the CNS of a patient with a multiple mtDNA deletion disorder. This patient had prominent central and peripheral nervous system involvement with marked cerebellar ataxia, a parkinsonian extra-pyramidal movement disorder, external ophthalmoplegia, dysphagia, and a severe peripheral neuropathy. There was degeneration of myelin tracts in the cerebellum and dorsal spinal columns, diffuse astrocytosis, and selective neuronal degeneration particularly in the midbrain and cerebral microvacuolation. The proportional distribution of the COX-deficient neurons did not always correlate directly with the degree of neuropathological damage with regions of high neuronal loss having relatively low proportions of these cells. Other clinically affected CNS regions have high levels of COX-deficient neurons without significant cell loss. The role of these COX-deficient neurons in causing neuronal degeneration and clinical symptoms is discussed.

Key Words: Cytochrome c oxidase; Deficient; Deletions; DNA; Mitochondrial; Multiple; Neurons.

INTRODUCTION

Mitochondrial respiratory chain defects are an important cause of disease and morbidity. They can present with a diverse range of clinical symptoms, often dominated by neurological features. These can take many forms including ataxia, movement disorders, stroke-like episodes, deafness, seizures, and dementia (1). While there have been many advances in our understanding of the molecular basis of these disorders, involving either the mitochondrial or nuclear genome, we still have very limited information on the underlying mechanisms involved in the selective neurodegeneration observed.

Few autopsy studies of patients with known mitochondrial defects with both biochemical and neuropathological studies have been reported. Without such studies it is difficult to address the associations between biochemical abnormalities, neuropathology, and the clinical features. Understanding these processes of selective neuronal degeneration will lead to an understanding of these diverse clinical phenotypes and the development of effective therapies.

One category of mitochondrial disease that exhibits prominent progressive CNS involvement includes cases with multiple mitochondrial DNA (mtDNA) deletions. Patients with this disorder do not exhibit the classical mitochondrial maternal inheritance; instead they follow autosomal dominant or recessive Mendelian inheritance patterns (2, 3). This indicates that a primary nuclear genetic defect can cause an accelerated accumulation of high levels of multiple mtDNA deletions and, in muscle, to cytochrome c oxidase (COX) deficiency in individual muscle fibers.

Mitochondria contain their own DNA, a double-stranded 16.5-Kb structure that encodes for 13 polypeptides of the respiratory chain, 22 tRNAs, and 2 RNAs required for their translation. This DNA is particularly susceptible to mutation partly because of its close proximity to the mitochondrially located respiratory chain, the main source of free radical production within the cell. Three major catalytic subunits of COX (complex IV) are encoded for by mtDNA, the other 10 are nuclear encoded. Consequently, damage to mtDNA can impair the activity of COX that contrasts with succinate dehydrogenase, (complex II), which is entirely encoded within the nuclear genome. Sequential histochemistry of these 2 enzymes has been used to show cells containing impaired mtDNA in the study of skeletal muscle in mitochondrial disorders (4) and normal aging (5). More recently the technique has been applied in the CNS of patients with amyotrophic lateral sclerosis (6).

The cellular and molecular pathology underlying CNS involvement in cases with multiple mitochondrial deletions has not previously been studied, both in terms of the neuropathological changes and the presence or absence of biochemical abnormalities between individual CNS cells. Understanding the connections between biochemical defects and neuronal death or dysfunction may have important implications not only for known mitochondrial diseases, since there has been growing speculation of the
Possible involvement of mitochondrial dysfunction in both aging (7) and neurodegenerative diseases (8, 9).

**MATERIALS AND METHODS**

**Case Report**

The patient was the second of 3 children of unrelated parents with no family history of mitochondrial disease. Both parents are alive and clinically unaffected in their ninth decade. Neither sibling is affected. It is presumed that the syndrome in this patient was inherited in an autosomal recessive manner or as a spontaneous mutation. His childhood was unremarkable although in retrospect the patient recalled difficulty with intricate dextrous work at school and a tendency to tire easily. At the age of 17 he survived a road traffic accident remarkably unscathed. The following year he noted some slurred speech, problems with balance, increasing fatigue, and jerky uncontrolled movements of the limbs. He completed school with sufficient grades to enter college and study for a civil engineering and music degree. His academic progress was less successful than expected, although he did complete his degree. At age of 26 he had developed bilateral ptosis and a progressive external ophthalmoplegia, with gradually progressive problems of imbalance and fatigue. At ages 40 and 42, he had surgical elevation of his eyelids. At age 46 his prominent ataxia culminated in neurological referral, when a muscle biopsy confirmed the diagnosis. At this time his poor balance was attributed mainly to a peripheral neuropathy. Following this referral he continued to deteriorate and developed dysphagia and an extra-pyramidal movement disorder predominantly consisting of bradykinesia. At age 50, he suffered a fall and fractured the neck of his left femur. After surgical fixation, he developed postoperative respiratory insufficiency necessitating admission to ITU. Over the next few weeks he deteriorated, eventually requiring ventilation for the development of tonic-clonic seizures and respiratory insufficiency. He continued to decline and died due to suppurative meningitis.

On his terminal admission, at age 50, serum lactate measurement were consistently elevated. MRI showed further progression with some high signal intensity involving the right hippocampus and parahippocampal gyrus and some patchy high signal intensity within the pons. Both of these changes were reported at the time as being consistent with ischemia. Nerve conduction studies showed a severe sensory neuropathy. An EEG recorded when the patient had no clinical evidence of seizure activity displayed a diffusely abnormal pattern with numerous multi-focal discharges.

**Neuropathology**

Autopsy was performed within 4 h of death with full consent for organ retention for research including the brain, spinal cord, dorsal root and paraspinal sympathetic ganglia, multiple peripheral nerves, and multiple skeletal and visceral organs. Samples of these tissues were either snap frozen in dichloro-difluoromethane (Arcton 12, I.C.I) cooled to −150°C with liquid nitrogen or fixed in 10% formalin and paraffin embedded. Routine histological studies to characterize the extent and morphology of pathological changes used a panel of conventional stains and immunocytochemistry. The latter included GFAP and CD68 (macrophage/microglial marker) to detect nonspecific foci of tissue damage. Neuronal loss was assessed in Luxol fast blue stained sections. Stains for cytoskeletal markers (tau, neurofilament and ubiquitin) were consistently negative throughout.

**Histochemical Analysis**

The sequential demonstration of COX and SDH activities was performed on brain and muscle fresh frozen sections of 20 and 30 μm thickness, air dried at room temperature for 1 h. COX activity was demonstrated using 4 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 100 μM cytochrome c (Sigma-Aldrich Co. Poole, UK) in 0.1 M phosphate, pH 7.0 at 37°C. This medium is based upon the one used by Seligman and coworkers (10) modified for use in quantitative histochemistry (11). SDH activity was assayed using 1.5 mM Nitro Blue tetrazolium (NBT), 130 mM sodium succinate, 0.2 mM phenazine methosulfate (PMS) and 1.0 mM sodium azide in 0.1 M phosphate, pH 7.0, at 37°C. Incubation time for the COX reaction was 45 min; this prolonged incubation was designed to detect all histochemically demonstrable COX activity with maximal deposition of the reaction product. Under these conditions, only mitochondria in sites of abnormally low COX activity will not be saturated with COX reaction product and will allow the demonstration of SDH activity by the reduction of NBT to a blue formazan end product. Incubation time for SDH was 40 min to allow all SDH in COX-deficient/SDH-positive sites to be demonstrated. Between successive COX and SDH incubations, sections were washed for 5 min × 3 in distilled water. Specificity controls for COX activity were reacted in incubation medium plus the respiratory chain inhibitor, sodium azide (2.5 mM), and for SDH, sections incubated in medium minus the substrate, sodium succinate, with the addition of 50 video fluoroscopy revealed a reasonably well-coordinated swallow but incomplete clearance of the bolus from the pharynx with aspiration.

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mM malonate, a competitive inhibitor of SDH. Thirty micrometer sections of quadriceps muscle and various regions of the CNS were examined microscopically to determine the proportion of COX-deficient, SDH-positive muscle fibers or neurons.

**Southern Blot Analysis**

Southern blot was performed using standard techniques (12). A total of 3 μg of DNA from skeletal muscle, pancreas, kidney, liver, heart, extra-ocular muscle, blood, duodenum, esophagus, and various regions of the CNS were isolated. Each sample was digested with Pvu II for 90 min at 37°C and separated by electrophoresis through 0.7% agarose with ethidium bromide at 30 volts for 16 h. Following transfer to GeneScreen Plus™ membrane the samples were probed with a [32P]dCTP labelled purified PCR product of the mitochondrial DNA D-loop complementary to position 15782 across the D-loop to position 1298. Probed filters were exposed in a PhosphoImager cassette and analyzed using Molecular Dynamics software (Pharmacia).

**Controls**

The CNS findings in the neocortex, hippocampus, and cerebellum were compared with tissues from 3 neurologically normal controls and 5 cases with non-mitochondrial neurological disorders (Table). The case of hereditary spastic paraparesis had a mutation in the spastin gene.

**RESULTS**

**Postmortem Results**

**CNS Gross Pathology:** The brain showed moderate atrophy with significant loculation of CSF over the cerebral convexities. The brainstem and cerebellum were reduced in size. No focal lesions were identified in the cerebral hemispheres, spinal cord, brainstem, or cerebellum.

**CNS Histology:** The optic nerves were normally myelinated with no significant gliosis. The frontal, temporal, and parietal cortex showed no significant intra-neuronal cytopathology, and neuronal loss was not detectable on routine examination. Reactive glia, both astrocytes, and microgliosis were prominent in cerebral white matter associated with diffuse myelin pallor. There was astrocytic gliosis in superficial laminae of the occipital cortex with sparing of layers 5 and 6, which was not present in the frontal, temporal, and parietal lobes (Fig. 1d). Throughout the cerebral cortex there was some fine micro-vacuolation of the outer molecular layer (Fig. 1e) of uncertain significance.

The hippocampus showed severe changes of neuronal loss throughout the pyramidal cell population of CA sectors 1-4, associated with intense astrocytic gliosis and microglial permeation (Fig. 1f). The dentate granule cell layer, subicular subdivisions, and entorhinal cortex were unaffected. Sections of the basal ganglia, thalamus, and hypothalamus showed astrocytic gliosis of adjacent white matter only.

Sections of the midbrain showed severe neuronal depletion from the substantia nigra but there was no specific neuronal cytopathology or inclusion body formation. There was a moderate infiltrate of microglia that was also present within the red nucleus and in the region of the pedunculopontine tegmental nucleus. In the lower midbrain the inferior colliculus showed astrocytic and microglial reaction. Microgliosis was also conspicuous in the superior cerebellar peduncle. In the pons the locus ceruleus, pontine nuclei, and motor nuclei V and VI and white matter appeared normal.

In the medulla, the inferior olivary nucleus showed massive neuronal depletion with associated loss of myelin from the hilus and the inferior cerebellar peduncles (Fig. 1b). This degeneration was not associated with siderosis or microgliosis and may represent transneuronal degeneration secondary to cerebellar changes. The pyramids, Xth, XIth nuclei, solitary tract, and vestibular complex were unaffected.

The cerebellar hemispheres showed moderate to severe loss of Purkinje cells affecting the whole cortex. The dentate nucleus showed neuronal depletion and both central and foliar white matter showed myelin pallor (Fig. 1c), severe microgliosis, and astrocytic gliosis. Changes were also present within the vermis, although to a milder degree.

In the cervical spinal cord there was massive myelin loss and associated axonal loss in a dying-back pattern.
Fig. 1. a: Severe myelin pallor in the dorsal columns of the mid-cervical spinal cord. b: Medulla oblongata showing loss of myelin from the inferior olivary nucleus compared with preservation in the pyramid and medial lemniscus. c: Irregular myelin pallor of the central and foliar cerebellar white matter. d: Diffuse astrocytic gliosis of the occipital cortex with sparing of the lower laminae. e: Microvacuolation of superficial cortical layers was prominent in all neocortical regions. f: Reactive astrocytes
from the whole of the dorsal columns, whereas the ventral and lateral myelin was intact. The dorsal column changes were present throughout the cord down to lumbar levels (Fig. 1a) and were associated with a severe astrocytic gliosis and a mild increase in microglia. The thoracic nucleus, lateral grey horn, and anterior horn cells were normal. Dorsal root ganglia and paraspinal sympathetic ganglia showed degenerative changes in the ganglion cells associated with macrophage permeation (Fig. 1g–i). A sample from quadriceps muscle showed severe disseminated fiber atrophy and many internal nuclei. About 40% of fibers showed “ragged-red” accumulations of mitochondria and a similar number were COX deficient.

Southern Blots

Analysis clearly revealed multiple mtDNA deletions ranging up to 6 Kb in whole DNA particularly in the frontal cortex, hippocampus, upper pons, and spinal cord. Detectable levels were also seen in cerebellum, motor cortex, and optic nerve.

Histochemical Analysis

Neurons that were COX-deficient/SDH-positive were found in all the CNS regions examined in varying quantities (Fig. 1j–p). They are compared with the proportion of COX-deficient muscle fibers in Figure 2. The CNS cell percentages in Figure 2 are for neurons only and are only semiquantitative because of the difficulty in identifying individual normal COX-positive neurons on a dense background of neuropil. Each region is an aggregate score of at least 3 different sections. The highest quantities of COX-deficient/SDH-positive neurons were found in the reticular formation, nucleus ambiguus, caudate nucleus, putamen, globus pallidus, and pontine nuclei. The lowest levels were found in the cerebellum, hippocampus, motor cortex, and spinal cord. There were no COX-deficient/SDH-positive neurons found in the frontal cortex, hippocampus, and cerebellum of 2 cases of Friedreich’s ataxia (age 25 and 28) and 1 case of hereditary spastic paraparesis (age 70).

A few COX-deficient neurons (<0.25%) were observed in the hippocampal CA regions of 2 cases that had died from ischemic heart disease (aged 45 and 55). One of these cases also underwent artificial respiration prior to death. Slightly greater numbers of COX-deficient hippocampal CA neurons, (0.5%–0.97%) were observed in the hippocampus of 2 cases of Alzheimer disease (ages 80 and 85) and 1 elderly patient who had died from a myocardial infarction (age 79). However, no COX-deficient cells were observed in the frontal cortex of any of these cases.

DISCUSSION

The neuropathological findings in this case are similar to those previously observed in other mitochondrial disorders. Another mitochondrial syndrome that shares large
numbers of mtDNA rearrangements, often in the form of a deletion, is the Kearns-Sayre syndrome (KSS). However in contrast, KSS is associated in each individual case with a single rearrangement. The findings in this case have many similarities to the reported findings in KSS with microvacuolation in the cerebral cortex, widespread astrocitosis, neuronal loss of the cerebellar purkinje cells and substantia nigra, and demyelination of the cerebellum and posterior columns of the spinal cord. We have also found some striking discrepancies with lack of basal ganglia involvement in the form of siderosis and basal ganglia neuronal depletion often seen in KSS (13). The degeneration of myelin tracts, astrocitosis and selective neuronal degeneration are also observed in MERRF neuropathology (13).

The clinical features and MRI abnormalities broadly correlate with the severe cerebellar, midbrain, and dorsal column degeneration. The cerebellar, inferior olivary nuclei, and dorsal column degeneration explains the profound ataxia and cerebellar speech disorder. The dysphagia may have been related to primary esophageal dysmotility or neurogenic, associated with degeneration of the brainstem nuclei, or a combination of the 2. The fatigue, external ophthalmoplegia, and ptosis were probably myopathy related. However, despite generalized cortical and hippocampal astrocitosis and microgliosis, and hippocampal CA 1 to 4 neuronal depletion, there was no obvious clinical cognitive impairment, although it was believed that academically he had underachieved.

This mosaic pattern of COX-deficient/SDH-positive neurons in a case of a multiple mtDNA deletion disorder is not previously described. Kaido et al (14) found COX-deficient neurons in an autopsy case with mitochondrial encephalopathy with a point mutation at tRNA^{Ile} (nt4269) A to G, although they did not relate this histochemical finding to the neuropathology. As shown in Figure 2, the percentage of COX-deficient/SDH-positive neurons in the corpus striatum, pons, medulla, and frontal cortex resembles or even exceeds the percentage of COX-deficient/SDH-positive muscle fibers seen in quadriceps muscle. Previous observations indicate that both the proportions of mutant mtDNA and the quantity of different size deletions increase chronologically in patients with multiple deletion disorders (15). The phenotypic expression of disease may depend on populations of neurons that reach a threshold of heteroplasmy resulting in neuronal dysfunction or death.

We found an intriguing paradox such that the percentage of COX-deficient/SDH-positive neurons did not always correlate directly with the degree of neuropathological damage. For example, in the cerebellum there is a predilection for Purkinje cell loss and white matter changes but the percentage of COX-deficient neurons was only 8.4%, one of the lowest levels observed. The hippocampus also exhibited marked neuronal depletion yet percentages of COX-deficient neurons were again relatively low (14.4%). In contrast, there was a remarkably high prevalence of COX-deficient neurons (up to 64%) in several CNS regions, including the nucleus ambiguus, pontine nuclei, and corpus striatum, which were clinically but not pathologically affected. Whether or not these neurons retain their functional ability is unknown, but if their performance was impaired it would help to explain the symptoms of dysphagia, profound ataxia, and the extrapyramidal movement disorder manifested in this case.

The low levels of COX-deficient/SDH-positive neurons observed in the cerebellum and hippocampus is probably due to the high levels of neuronal loss observed in these areas. Why certain cell populations should be selectively vulnerable is unclear but it is interesting to note that certain neuronal populations are affected in genetically and phenotypically distinct mitochondrial disorders. In this case, and in several cases with KSS, MERRF, and MELAS, there is neuronal degeneration and depletion in the cerebellar cortex, dentate nucleus, substantia nigra, red nucleus, spinal cord, and myelin loss in the cerebellum and dorsal columns of the spinal cord (13). The neurons in these regions could be more susceptible to metabolic damage at a lower threshold level of mtDNA mutations for a number of reasons: (a) a higher dependence on oxidative phosphorylation, (b) higher levels of local toxic factors such as excitatory amino acids, (c) a lower threshold for apoptosis in which mitochondria appear to play a pivotal role (16).

The observation that loss of COX activity occurs in individual neuronal cells as well as in muscle fibers is highly relevant to the pathophysiology of neuronal loss and dysfunction in patients with mitochondrial disease. Multiple mtDNA deletions are also seen in neurodegenerative conditions and normal brain aging, not dissimilar but at a much lower level to those in our patient. Our observation of COX-deficient neurons may also have important implications in the neuropathology of these conditions. Indeed recent studies from our laboratory have highlighted that COX-deficient neurons are seen in spinal cord sections from patients with amyotrophic lateral sclerosis (4). Preliminary studies (see Results section) suggest that COX-deficient neurons are also present, although at lower levels, in brains from aged individuals.

ACKNOWLEDGMENTS

We thank Gill Borthwick, Chris McDermott, Geoff Taylor, Theresa Wardell, and Jean Dawes for their help and advice.

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Received February 17, 2000
Revision received April 10, 2000
Accepted April 14, 2000