Neuropathological Changes in a Mouse Model of Progressive Myoclonus Epilepsy: Cystatin B Deficiency and Unverricht-Lundborg Disease

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Abstract. Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is a recessively inherited neurodegenerative disease caused by loss-of-function mutations in the gene encoding cystatin B, a cysteine protease inhibitor. Mice with disruptions in this gene display myoclonic seizures, progressive ataxia, and cerebellar pathology closely paralleling EPM1 in humans. To provide further insight into our understanding of EPM1, we report pathological findings in brains from cystatin B-deficient mice. In addition to confirming the loss of cerebellar granular cell neurons by apoptosis, we identified additional neuronal apoptosis in young mutant mice (3–4 months old) within the hippocampal formation and entorhinal cortex. In older mutant mice (16–18 months old), there was also gliosis most marked in the presubiculum and parasubiculum of the hippocampal formation, as well as the entorhinal cortex, neocortex, and striatum. Furthermore, widespread white matter gliosis was also noted, which may be a secondary phenomenon. Within the cerebral cortex, cellular atrophy was a prominent finding in the superficial neurons of the prosubiculum. Finally, we show that mutant mice in either a “seizure-prone” or “seizure-resistant” genetic background display similar neuropathological changes. These findings indicate that neuronal atrophy is an important consequence of cystatin-B deficiency independent of seizure events, suggesting a physiological role for this protein in the maintenance of normal neuronal structure.

Key Words: Cystatin B; EPM1; Neuropathology; Progressive myoclonus epilepsy; Protease inhibitor; Unverricht-Lundborg disease.

INTRODUCTION

Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1, or Baltic myoclonus) is a familial neurodegenerative disease characterized by childhood onset, easily provoked myoclonus, tonic-clonic seizures, progressive ataxia, and late intellectual decline (1–3). Histopathological studies of patients with EPM1 show loss of cerebellar Purkinje cells, as well as loss and degeneration of neurons in the cerebral cortex, striatum, thalamus, brainstem nuclei, and spinal motor neurons (2, 4). The EPM1 phenotype is due to loss-of-function mutations in the gene encoding cystatin B (5–8), a cysteine protease inhibitor that is known to block the actions of lysosomal proteases such as cathepsins B, H, L, and S (9–13). The fact that mice lacking cystatin B display apoptosis in cerebellar granule cells indicates a necessary role for cystatin B in preventing this type of programmed cell death (14). Moreover, the protein is widely distributed in the central nervous system and other tissues, and increased cystatin B gene expression is present in the kindling model of epilepsy (5, 15, 16). Mice homozygous for a disruption in the cystatin B gene develop a phenotype similar to EPM1 in humans, with myoclonic seizures, progressive ataxia, and neuronal loss (14). Initial studies of the central nervous system in these mice clearly demonstrated apoptotic loss of cerebellar granule cells, but detailed examination of the cerebral pathology in these mice was not performed (14).

Clinical investigations of EPM1 suggest that abnormal control of cortical neuronal activity is important in the pathogenesis of myoclonic epilepsy (17, 18). However, the anatomical lesions giving rise to myoclonic epilepsy in EPM1 are unclear. The interpretation of histopathology in human tissue is confounded by the toxic effect of antiepileptic drugs and postmortem specimens obtained at late stages of the disease (2, 4, 19, 20). Cystatin B-deficient mice provide an excellent animal model for exploring the anatomical and physiological bases for EPM1 (14). In this study, we examined the cerebral morphology in cystatin B-deficient mice by using a combination of classical histological techniques, image analysis, and immunohistochemistry to fully characterize the anatomical sites of pathology in EPM1 disease.

MATERIALS AND METHODS

The production of cystatin B-deficient mice has been previously described (14). Ten 16- to 18-month-old mice (hereafter called “adult” mice) homozygous for the loss of cystatin B and 10 age-matched littermates were killed and dissected (aged 12–19 months, mean = 15.3). Whole brains were collected and immersion-fixed in 10% phosphate-buffered formalin. In addition, four 3- to 6-month-old mice (hereafter called “young”
mice) homozygous for the loss of the cystatin B gene and 4 age-matched control mice were similarly harvested. Of the adult mice, 5 were from litters in which cystatin B-deficient animals showed little or no evidence of seizure activity (“adult seizure-resistant mutant mice”), and 5 were from litters in which the cystatin B-deficient animals show seizure activity (“adult seizure-prone mutant mice”). This difference is accounted for by slight differences in the genetic background of the 2 groups, as previously described (14). Mutant mice in a mixed 129SvJ and C57Bl/6 background do not display seizures, while mutant mice in an isogenic 129SvJ background display seizures. Brains were sectioned in the coronal plane at 2-mm intervals and embedded in paraffin according to standard protocols. Histological sections were prepared at 0.1-mm intervals and stained with cresyl violet, hematoxylin and eosin (H&E), or Luxol fast blue and H&E. Selected sections were immuno-stained for glial fibrillary acidic protein (GFAP, Dako, Glostrup, Denmark, 1:500), calbindin D (Novocastra, Newcastle upon Tyne, UK, 1:100) neurofilament (neurofilament cocktail, Dako, 1:100), and by the TUNEL assay with diaminobenzidine as a chromogen to detect nick-damaged DNA (Boehringer Mannheim [Roche], Mannheim, Germany). An MCID 5+ image-analysis system linked to a Coolsnap digital camera was used to gather images and measure neuronal size and quantify the number of GFAP-positive cells. In the striatum, 10 contiguous high-power (×400) fields were analyzed bilaterally: the number of all neuronal somata completely contained by the microscopic field was measured. A t-test for paired samples was used to compare differences between genotypes. To assess the degree of gliosis, the proportion of 10 high-power fields occupied by GFAP-positive processes was measured in the area of interest. Within the prosubiculum and parasubiculum, on 1 hemisphere in each brain, the somal area of all supercificial granular neurons in the prosubiculum and parasubiculum was measured. Data were analyzed by using SPSS for Windows.

RESULTS

We examined mice from 2 age groups (“young” mice, 3–6 months of age, and “adult” mice, 16–20 months of age) that lack cystatin B (“mutants”) for pathological features compared to control mice. We found a severe reduction in the brain and body weights of mutant mice compared to controls. However, the ratio of brain to body weight was unaltered between mutant mice and controls, suggesting that the effects of cystatin B deficiency in the mouse are not specific to the CNS (Table). Upon microscopic examination, seizure-prone and seizure-resistant adult mutant mice showed the same spectrum of abnormalities. In all adult mutant mice, we identified striking cerebellar atrophy with a predominant loss of cerebellar granular cells, similar to the pathology that we previously reported (14) (Fig. 1A). In addition, we found a patchy loss of Purkinje cells accompanied by Bergmann gliosis, with some Purkinje cells showing a limited amount of ballooning and clearing of the cytoplasm (Fig. 1C). These alterations were predominantly present in the cerebellar vermis, where 5% to 20% (mean = 7%) of Purkinje cells displayed this alteration. In the lateral cerebellum, 1% to 5% of Purkinje cells showed similar changes (mean = 1.7%). In 5 of 10 adult mutant mice, we identified swollen axons in the granular cell layer and white matter (Fig. 1E, F). These swollen axons did not stain with antibodies to calbindin D, and neither swollen axons nor vacuolated Purkinje cells were present in the control mice or younger mutant mice. Immunohistochemical staining of all mutant mice did not identify abnormal accumulation of neurofilament in any morphologically identifiable subpopulations. Neurons of the deep cerebellar nuclei and inferior olivary nuclear complex from cystatin B-deficient mice did not show significant alterations.

We performed immunostaining for GFAP and found widespread fibrillary gliosis of white matter, particularly in the cerebral hemispheres, in all adult mutant mice. Myelin staining revealed no abnormalities (data not shown), suggesting that the white matter gliosis might be secondary to neuronal loss or atrophy. We further identified mild widespread gliosis in the cerebral cortex. One striking feature was the relative lack of gliosis in Ammon’s horn, the dentate fascia, and the first segment of the subiculum. However, gliosis abruptly increased and was maximal in the presubiculum and parasubiculum and was also prominent in the entorhinal cortex and neocortex (Fig. 2A). Within the subcortical grey matter, we found prominent gliosis in the striatum but none in the adjacent septal nuclei and basal forebrain (Fig. 2B). In all mutant mice, there was also atrophy and gliosis of the optic tracts (data not shown).

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<td>Brain Weight, Body Weight, and Brain/Body Weight Ratios in Control and Cystatin B-Deficient Mice*</td>
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* Four of 5 cystatin B-deficient mice originated from seizure-prone strains and 1 mouse from a seizure-resistant strain. Although the brain weights are significantly smaller in the cystatin B-deficient mice compared to controls, the brain to body weight ratios are similar. P values are derived from t-tests for paired samples.
The distribution of gliosis led us to hypothesize that neuronal loss in the areas of gliosis might contribute to the neurological abnormalities in cystatin B-deficient mice. Previous studies revealed TUNEL-positive staining and ultrastructural changes of cerebellar granule cell neurons in mutant mice, which are indications of apoptotic cell loss. In this study, we found apoptotic bodies in the cerebral hemispheres only after extensive, careful searching. On rare occasions, we noted TUNEL-positive cells in the cerebrum of 2 of the 10 adult cystatin B-deficient mice (both were seizure-prone, isogenic 129SvJ-background mutant mice). However, in all 4 of the younger “seizure-prone” mutant mice we examined, we observed apoptotic neurons in the dentate fascia of the hippocampal formation, and in 2 of these 4 mice, within Ammon’s horn and entorhinal cortex (Fig. 2C). We observed no apoptotic bodies in either younger controls (Fig. 2D) or in the striatum of younger mutant mice. Quantitative studies in the striatum revealed a decrease in neuronal density of approximately 20% per high-power field in adult mutant mice compared to age-matched controls (mean number of neurons per high-power field: mutants = 94, controls = 76, df = 16, t = 3.01, p < 0.01), suggesting that, in this particular area, there is cell loss despite the difficulty in detecting apoptosis. We noted no difference in either the presence or absence of gliosis nor in the neuronal density of the striatum between adult isogenic 129SvJ (seizure-prone) mutant mice and adult mixed background (seizure-resistant) mutant mice (p > 0.05).

In addition to neuronal cell death by apoptosis, we noted an apparent decrease in mean neuronal cross-sectional area within the presubiculum and prosubiculum within the hippocampal formation (Fig. 3). To differentiate between selective loss of large neurons and cellular atrophy, we studied an anatomically identifiable subpopulation of neurons: the superficial granular neurons of the prosubiculum and parasubiculum. The mean somal area of these neurons was significantly larger in adult control littersmates (Fig. 3, solid circles) than in either adult mixed background (seizure-resistant) mutant mice or in adult isogenic 129SvJ (seizure-prone) mutant mice. Furthermore, the adult cystatin B-deficient mice from both backgrounds had smaller mean somal areas than either younger mutant mice or younger controls, indicating that the reduction in neuronal somal area is likely to be due to neuronal atrophy.

**DISCUSSION**

The histopathology of Unverricht-Lundborg disease in humans has been described in only a small number of cases. The prominent histological features that have been described are cerebellar Purkinje cell loss, cerebellar atrophy, gliosis, and neuronal degeneration in the medial and reticular nuclei of the thalamus, as well as neuronal degenerative changes in the cerebral cortex, striatum, mammillary bodies, anterior and lateral thalamic nuclei, pons, medulla, the ventral grey matter of the spinal cord, brainstem, and multiple brainstem nuclei (2, 4, 19). However, interpretation of the histopathology in humans with the disease is confounded by the effects of phenytoin therapy in some patients, who show a marked sensitivity to the neurotoxic effects of this drug (20, 21). Our examination of cystatin B-deficient mice reveals many histological features in common with human subjects. Although the cerebellar Purkinje cell population is relatively preserved in mice lacking cystatin B, there is Bergmann cell gliosis and ballooning of Purkinje cells. The swollen axons in the mouse model may be afferent axons, which lack calbindin D reactivity, a feature that would be expected if the axons originated from Purkinje cells. The relative preservation of this cell population in the mice may be due to the fact that they have not been treated with drugs, as were all published human cases. Nevertheless, our results show that the lack of cystatin B is sufficient to cause Purkinje cell abnormalities independent of the use of phenytoin. The minor alterations that we observed in Purkinje cells might also be secondary to granular cell loss and deafferentation. The basis of the relatively selective loss of the cerebellar granular cells in these mice is still not understood. Determining the molecular triggers of the apoptotic cascade in this model may aid in identifying the biochemical mechanism of this phenotype.

Other histological features shared between the human disease and the mouse model include striatal and corticomedial gliosis accompanied by atrophy of cortical neurons. The consistency of this finding suggests that cystatin B is important in maintaining normal neuronal architecture and size. We also observed indistinguishable differences in neuronal loss throughout the brain of adult mutant mice that lack seizures compared that adult mutant mice that display seizures, which supports the theory that neuronal loss alone is not responsible for the establishment or propagation of the epileptic phenotype. Furthermore, this indicates that seizure events do not cause the neuropathology found in this mouse model. In contrast, the gliosis and atrophy of the optic tracts may be related to the ophthalmic complications seen in this model (14), although further studies of the retina would be needed to establish this. The brains of adult cystatin B-deficient mice are smaller than controls, and this size difference is paralleled by low body weight. In humans, advanced neurodegenerative syndromes are often accompanied by severe wasting (22, 23), a finding that correlates with both the degree and type of neurological impairment. We cannot be certain that the decrease in brain size and neuronal atrophy are not due to malnutrition, yet the presence of neuronal death indicates that factors intrinsic to the central nervous system do play a role.
Fig. 1. A: Section of cerebellum at level of mid pons in a 17-month-old cystatin B-deficient mouse: the folia are markedly atrophic in comparison to those in (B), an age-matched control mouse. Luxol fast blue/H&E staining; magnification, ×25. C: A ballooned Purkinje cell, with drop-out of adjacent Purkinje cells in a cystatin B-deficient mouse compared with (D) normal Purkinje cells in an age-matched control. E: Axonal swelling in the depleted granular cell layer of a cystatin B-deficient mouse (depicted with an arrow). Luxol fast blue/H&E staining; magnification, ×1,000). F: Immunohistochemistry of cystatin B-deficient cerebellum section for detection of neurofilament highlights axonal swellings, which appear to originate in afferent axons. Magnification, ×630.
A wide variety of neurodegenerative conditions (e.g., Alzheimer disease and amyotrophic lateral sclerosis) give rise to atrophic neurons (24, 25), and although TUNEL-positive neurons are reported in many diseases, such as Alzheimer disease and AIDS-related dementia (24, 26–28), they are rare in others, such as ALS (29, 30). However, some cells that stain with TUNEL are not undergoing apoptosis (31). Although cell death is clearly an important phenomenon in EPM1, the paucity of apoptotic bodies in adult mice and the evidence for neuronal atrophy suggests that cellular dysfunction may also be important in producing the phenotype. The greater neuronal density of the cerebellar granular cell layer, as well as an increased susceptibility of granule cells to undergo apoptosis when they lack cystatin B, might account for the relative ease with which apoptosis can be identified in the cerebellum as opposed to the cerebral hemispheres in our mutant mice. However, even within the cerebral
hemispheres, gliosis and apoptotic activity is distributed unevenly, suggesting that there is differential neuronal sensitivity to cystatin B deficiency. The role of cystatin B in the maintenance of different neuronal populations remains to be explored.

It has been suggested that the brain area responsible for generation of the myoclonic jerk component of the EPM1 phenotype is the cortex (17, 18). One hypothesis about how this might occur involves excessive cortical firing resulting from a primary increase in neuronal hyperexcitability, loss of inhibitory neurons, and/or excessive excitatory inputs. The striking cerebellar granular layer cell loss reported in this study is expected to contribute to excessive excitatory inputs to the thalamic nuclei based on the following neuroanatomical pathways. Cerebellar outflow to the cortex proceeds mainly through excitatory output from the dentate nucleus. Cerebellar pathology resulting in excessive output by dentate neurons is associated with cortical myoclonus (32). In contrast, loss of dentate neurons results in reticular myoclonus (33, 34). Cerebellar granule cells are excitatory to Purkinje cells, which in turn are inhibitory to dentate neurons. Granule cell loss, along with Purkinje cell loss, without deep nuclear atrophy, as was demonstrated in the cystatin B deficient brains of this study, might lead to or exacerbate the cortical myoclonus of EPM1. Other evidence supporting the notion that granule cell loss can participate in the generation of myoclonus includes the case of mercury toxicity, which produces myoclonus, in part due to damage to granule cells (35, 36). Neurophysiologic studies on cystatin B-deficient mice are now needed to complement these histopathologic findings to test the role of cerebellar output in the cortical myoclonus of EPM1.

Furthermore, neurochemical and cytoarchitectural studies on this physiological model of myoclonic epilepsy should reveal the populations of neurons most susceptible to the lack of cystatin B. We observed a number of pathological changes that mirrored what is found in humans with EPM1, as well as some previously unknown changes. Another result of this study is that, among mice lacking cystatin B, those with frequent myoclonic seizures did not have lesions markedly different from those lacking myoclonic seizures. Further studies are required to elucidate the basis of the difference in behavioral phenotype. The presence of neuronal atrophy in all of the adult mutant mice suggests that therapeutic intervention might be possible to rescue affected neurons before they undergo extensive atrophy.

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Authors Patrick Shannon, MD and Len A. Pennacchio, PhD contributed equally to this study.

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