Cerebellar Involvement in Murine Sphingomyelinosis: A New Model of Niemann-Pick Disease

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Abstract. Mice with sphingomyelinosis (spm) with a C57BL/KsJ inbred background showed hepatosplenomegaly as early as four weeks (wk) of age and cerebellar signs around seven wk. Almost all animals died by 14 wk. Sudanophilic lipid accumulated in the liver, spleen, and lymph nodes as well as in the brain. The striking neuropathological change was a marked atrophy of the cerebellum, where Purkinje cells were predominantly involved. Loss of Purkinje cells started at the age of six wk before the cerebellar signs had become evident clinically. The cell loss appeared to be more marked in the vermis than in the hemispheres. Cytoplasmic inclusions in most cells consisted of myelin figures composed of concentric membranous lamellae. These inclusions were found mainly in the Purkinje cells at an early stage; thereafter, they were widely distributed in the granule cells, Golgi cells, some glial cells, macrophages and endothelial cells. The neuronal inclusions were frequently located in the vicinity of the Golgi apparatus; there were no unusual mitochondrial configurations. The clinicopathological characteristics of the mutant mice are similar to those of the human Niemann-Pick disease type C.

Key Words: Cytoplasmic inclusions; Mutant mouse; Niemann-Pick disease; Purkinje cell; Sphingomyelinosis.

INTRODUCTION

Niemann-Pick disease is a disorder of lipid metabolism characterized by the accumulation of sphingomyelin in reticulo-endothelial cells and in the central nervous system. The patients have a genetic deficiency of sphingomyelinase. Animal models of the human disease have been found in Siamese cats (1, 2), a poodle dog (3) and mice (4, 5). Murine sphingomyelinosis, an autosomal recessive mutation in an inbred strain of the C57BL/KsJ, was described in 1982 (6). The affected mice developed hepatosplenomegaly and abnormalities in motor coordination with tremor of the body and extremities, prominently manifested with progressive cerebellar signs. Increased levels of sphingomyelin and a deficiency of sphingomyelinase were demonstrated in the liver, spleen and brain (7, 8).

We describe here the neuropathological findings of the mutant mice in which the cerebellar cortex is predominantly involved. Ultrastructurally, affected nerve cells and swollen axons are filled with cytoplasmic inclusions composed of concentric membranous lamellae that are distinctly similar to those observed in human Niemann-Pick disease. Loss of Purkinje cells, which probably accounts for the clinical

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signs of cerebellar impairment, was statistically evaluated in the vermis and hemispheres.

MATERIALS AND METHODS

The spheromyelinosis (gene symbol: *spm*) mouse was discovered as a new mutant in the C57BL/KsJ inbred strain in 1975; it has since been maintained on this genetic background in the Research Laboratories, Nippon Shinyaku Co., Ltd. Reproduction of the mutants was carried out by inbreeding heterozygous males (+/*spm*) and normal females which were recipients of transplanted ovaries from homozygotes (*spm/*spm*). Thirty affected mice of the C57BL/KsJ at the 37–45th inbred generations, aged 4 to 12 wk, and 20 of their age-matched normal siblings were used in this study. Affected animals had hepatosplenomegaly, detectable by laparotomy, as early as four wk of age. Around six wk, hepatosplenomegaly was maximal. Around seven wk, loss of body weight, behavioral abnormalities and gait disturbances appeared. Beyond eight wk, tremor of the body and extremities was evident and ataxic movements progressed to inability to walk. By 12 wk, the mice had difficulty in taking food and water. Almost all of the mutant mice died by 14 wk.

Anesthetized animals were perfused through the aorta with a fixative solution of buffered 10% formalin for light microscopy, or with a mixture of 4% paraformaldehyde, 2.5 to 3.6% glutaraldehyde in 0.2 M cacodylate buffer pH 7.3 for electron microscopy. The histologic sections were routinely stained with hematoxylin and eosin (H&E), Klüver-Barrera (luxol-fast blue) and Bodian (silver protargol) methods, lipid stains (Sudan III and black B), periodic acid-Schiff (PAS), and immunohistochemical stains for glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). The blocks for electron microscopy were postfixed with 1% osmium tetroxide, dehydrated and embedded in Epon 812.

In order to estimate the time course and magnitude of cell loss in the cerebellum, 5-μm-thick sections were used; these samples included the vermis and hemispheres. The number of Purkinje cells, within a 1-mm-long sector of cortex, were counted in at least five different regions. Their numbers were expressed as a percentage of the counts in age-matched controls. All the Purkinje cells in the lamina were counted at a magnification of ×400.

RESULTS

In the mutant mice at the age of five wk, before any neurological signs had appeared, the liver and spleen were grossly enlarged and pale, their average weights being approximately 2.5 times and 10 times as large as those of the controls, respectively (Fig. 1a). The lymph nodes were also swollen. However, there were no gross abnormalities in the brains of the mutants. Swollen macrophages laden with lipid material were present in the spleen (Fig. 1b). The material was histochemically positive with the Sudan III and black B stains, faintly positive with PAS and negative with luxol-fast blue. Ultrastructurally, macrophages were filled with cytoplasmic “myelin figures” composed of concentric membranous lamellae (Fig. 1c, d). Histologic sections of the cerebellar cortex at the age of five wk revealed minimal changes, and a few swollen Purkinje cells were encountered (Fig. 2a). Electron microscopically, swollen Purkinje cells contained cytoplasmic inclusions in the vicinity of the Golgi apparatus (Fig. 2b) and in some of the dendritic processes in the molecular layer. The inclusions in the Purkinje cell consisted of “myelin figures” similar to those seen in macrophages in splenic sinuses and, occasionally, of multivesicular bodies (Fig. 2c). These inclusions did not demonstrate any morphologic continuity with mitochondria; the latter displayed no unusual features in their size or configuration. A few inclusions were found in the stellate cells. The perikaryon of granule cells contained “myelin figures” and “zebra bodies” near the Golgi apparatus (Fig. 2d). Several myelinated fibers filled with inclusions were observed among the granule cells. Golgi cells also contained “myelin figures” near the Golgi apparatus and in
Fig. 1. a. Hepatosplenomegaly of sphingomyelinosis mouse (A9) at age of five wk. The liver weighing 3.8 g is 2.4 times and the spleen weighing 1.7 g is 10 times as large as the control (C5), weighing 1.6 g and 0.17 g, respectively. b. Clusters of lipid-laden cells in sinuses of the spleen. H&E. ×250. c. A cell in the splenic sinus is filled with cytoplasmic inclusion bodies. ×5,000. d. The inclusions show "myelin figures" composed of concentric membranous lamellae. ×35,000.

their dendrites. Swollen neurites filled with inclusions were occasionally encountered in the mossy fiber rosette. A few small inclusions were present within the perikaryon of astrocytes and oligodendrocytes. Macrophages filled with inclusions were observed in the areas with glomeruli. Some swollen axons, probably climbing fibers, were noted in the white matter (Fig. 2e). Many inclusions were found in the endothelial cells of blood vessels, but none in pericytes.

In the mutant mice aged six through ten wk there was a rapid decrease in number of Purkinje cells in both vermis and hemispheres. The remaining nerve cells in the cerebral and cerebellar cortices were swollen and filled with sudanophilic lipid within the cytoplasm (Fig. 3a, b). Swollen axons and dendrites were frequently encountered. Astrocytic proliferation in the Purkinje layer was demonstrated by GFAP immunostain (Fig. 3c). Preservation of myelin sheaths was obtained in the white matter by MBP immunostain (Fig. 3d).

At age 12 wk the brains of the mutant mice were obviously smaller in size, a mean weight being about two thirds that of the controls, and the degree of brain atrophy was more remarkable in the cerebellum than in the cerebrum (Fig. 4a). In the cerebellar cortex a diffuse and marked loss of Purkinje cells was noted in the mutants.
Fig. 2. a. Cerebellar cortex at five wk shows minimal changes. Rarely a swollen Purkinje cell (arrowhead) is seen. H&E. ×250. b. The Purkinje cell contains inclusions in the vicinity of the Golgi apparatus. No unusual configurations are seen in mitochondria. ×12,000. c. At a higher magnification inclusions in the Purkinje cell are a myelin figure and a multivesicular body. ×15,000. d. A granule cell contains “myelin figures” and a “zebra body” near the Golgi apparatus. ×12,000. e. A myelinated axon, probably a climbing fiber, in the white matter is swollen by accumulation of inclusions. ×6,500.

The loss of the Purkinje cells seemed to be more conspicuous in the vermis than in the hemispheres. In some areas of the vermis, a severe loss of Purkinje cells and a swelling of Golgi cells were encountered (Fig. 4b). Occasional swollen axons were also seen in the granular layer (Fig. 4c, d).

The number of Purkinje cells per 1 mm of the lamina in mutant and control mice, 4 to 12 wk of age, is shown in Table 1. Until the age of five wk there were no
significant differences in the number of Purkinje cells compared to the controls. A sharp decline in the number of Purkinje cells started at the age of six wk before signs of cerebellar disease had appeared; this cellular loss reached its maximum extent at ten wk. After the tenth wk, during the terminal period of the mutant mouse's life span, only a few Purkinje cells survived. The magnitude of the Purkinje cell loss in the mutants over this time course is more clearly demonstrated in Figure 5, where cell counts are indicated as percentages of those of age-matched controls.

DISCUSSION

Neuropathological investigation of sphingomyelinosis (spm) mice has shown them to be an authentic model of Niemann-Pick disease in humans. Another model of Niemann-Pick disease in mice, transmitted by an autosomal recessive gene (fm; foam-cell reticulosis), was described by Lyon et al (4). The spm gene may not be identical to the fm gene, since there are several differences observed in these two models, and differences in genetic background should be considered. The onset of the disorder of spm mice is earlier than that of foam-cell reticulosis mice, which was reported to be inactive at three months of age or later. Neurological signs such as tremor have not been reported in fm mice. The liver and spleen of spm mice were variably enlarged with the C57BL/KsJ genetic background, while, in fm mice of the
CBS strain these organs were not enlarged and did not accumulate lipids (4). Sphingomyelin and cholesterol have been reported to accumulate in the liver of *spm* mice of three different genetic backgrounds examined (C57BL/KsJ, C57BL/6J and DBA/2J), although hepatosplenomegaly was not pronounced in *spm* mice of C57BL/6J and DAB/2J backgrounds (8). The survival time of *spm* mice is relatively shorter than that of *fm* mice, which have survived for 100 to 180 days. Moreover, there is no evidence of sphingomyelinase deficiency in *fm* mice (9). One other mutant mouse with a lipid storage was described as having deficiencies of sphingomyelinase and glucocerebrosidase (5).

The ultrastructure of the neuronal storage material in *spm* mice has a distinct similarity to that observed in human Niemann-Pick disease. The membrane-bound cytoplasmic bodies contain loosely packed lamellae which are concentric in some planes of section (10). Sometimes the limiting membrane contains two or three collections of lamellae and some have fused to produce more complex bodies (11). These inclusions are usually located in lysosomes or near the Golgi apparatus. Dense osmiophilic inclusions are often found. The occurrence of swollen axons filled with inclusions might represent some neuroaxonal dystrophic feature.
### TABLE 1
Numbers of Purkinje Cells in Sphingomyelinosis Mice as a Function of Age

<table>
<thead>
<tr>
<th>Age</th>
<th>4 wk</th>
<th>5 wk</th>
<th>6 wk</th>
<th>7 wk</th>
<th>8 wk</th>
<th>10 wk</th>
<th>12 wk</th>
</tr>
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<tbody>
<tr>
<td>A. n = 19</td>
<td></td>
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<tr>
<td>Vermis</td>
<td>51.3 ± 6.0</td>
<td>46.3 ± 5.4</td>
<td>30.4 ± 4.9*</td>
<td>17.1 ± 4.1*</td>
<td>5.8 ± 1.2*</td>
<td>1.2 ± 0.7*</td>
<td>1.0 ± 1.7*</td>
</tr>
<tr>
<td>Hemisphere</td>
<td>46.9 ± 5.5</td>
<td>45.3 ± 4.7</td>
<td>36.4 ± 3.2*</td>
<td>24.7 ± 6.0*</td>
<td>7.4 ± 2.2*</td>
<td>2.0 ± 0.9*</td>
<td>1.2 ± 1.9*</td>
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<tr>
<td>C. n = 10</td>
<td></td>
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<tr>
<td>Vermis</td>
<td>51.5 ± 6.5</td>
<td>52.3 ± 5.1</td>
<td>50.6 ± 4.9</td>
<td>46.5 ± 3.4</td>
<td>42.3 ± 1.8</td>
<td>40.4 ± 2.1</td>
<td>38.5 ± 2.4</td>
</tr>
<tr>
<td>Hemisphere</td>
<td>46.0 ± 5.2</td>
<td>48.8 ± 5.7</td>
<td>47.7 ± 5.0</td>
<td>46.7 ± 3.1</td>
<td>45.7 ± 1.2</td>
<td>41.4 ± 2.1</td>
<td>37.1 ± 5.4</td>
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<tr>
<td>Ratio (A/C)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Vermis</td>
<td>99.6%</td>
<td>88.5%</td>
<td>60.1%</td>
<td>36.8%</td>
<td>13.7%</td>
<td>3.0%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Hemisphere</td>
<td>101.9%</td>
<td>92.9%</td>
<td>76.3%</td>
<td>52.8%</td>
<td>16.2%</td>
<td>4.8%</td>
<td>3.2%</td>
</tr>
</tbody>
</table>

Average number of Purkinje cells per 1-mm-long sector (5 μm thick) of the layer counted at least in five different regions of the cerebellar vermis and hemispheres at various ages in weeks (wk). A = sphingomyelinosis mice, C = age-matched controls, n = number of mice examined.

*p < 0.01 by Student's t-test.
Loss of Purkinje cells is often reported in mutant animals with neurological signs, such as nervous (nr), described by Landis et al (12), Purkinje cell degeneration (pcd) by Mullen et al (13), leaner (tg\(^{+}\)) by Meier et al (14) in mice and jaundice (\(j\)) in rats by Schutta et al (15). In nr mice, mitochondrial abnormalities with a spherical configuration are observed in all Purkinje cells, while in spm mice, no abnormalities are seen in the mitochondria of Purkinje cells which are filled with inclusions. In pcd mice, many Purkinje cells retain the abnormal basal accumulation of polysomes and unusual configurations of endoplasmic reticulum (16). Spm mutants show a loss of Purkinje cells which occurs later than in nr and pcd mutants. Thus, the loss of Purkinje cells might be, at least in part, the cause of neurological signs in spm mice. However, the extremely severe tremor could not be explained by this, since \(j\) rats show only slight behavioral changes in spite of almost complete loss of Purkinje cells.

Statistically, the number of Purkinje cells in spm mice seems to remain almost normal until 5 weeks of age. It was at 6 weeks that depletion of Purkinje cells became apparent. At 7 weeks, cerebellar signs became manifest and the number of Purkinje cells fell to 36.8% of the control value in the vermis and to 52.8% in the hemispheres. The percentage of surviving Purkinje cells fell to 2.6% and 3.2% in the vermis and hemispheres, respectively, by 12 weeks. The cerebellar involvement appeared to be more severe in the vermis. It was surprising to find the loss of Purkinje cells more extensive in pcd mice than in spm because the gait ataxia described in pcd is only moderate (13). Purkinje cells in the vermis are probably more concerned with stability of the trunk and with gait than those in the hemispheres. It remains uncertain how many Purkinje cells are needed to retain cerebellar functions. The possibility that persistence of 10 to 15% of Purkinje cells can sustain relatively good motor coordination has been considered (17).
Human Niemann-Pick disease consists of at least six clinically distinct disorders. All show an autosomal recessive mode of inheritance and are characterized by the accumulation of sphingomyelin in visceral and nervous tissues (18). Type C, the juvenile form, is characterized by CNS involvement beginning after the first year of life, with less severe hepatosplenomegaly than is found in type A of an infantile onset; there is often a predominance of cerebellar symptoms. Neuropathological changes in this type include marked atrophy of the cerebellar cortex, particularly in the vermis, where Purkinje cells degenerate (19). The high incidence of neuroaxonal dystrophy may be an interesting aspect pointing to a special feature in this type (20, 21). The mutant strain of spm mice, in which clinicopathological features are very similar to those of human Niemann-Pick disease type C, may be an excellent model for elucidating the pathogenesis of lysosomal storage disorders and, to assess therapeutic trials (22).

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