Abnormalities of Purkinje Cell Arborization in Brindled Mouse Cerebellum

A Golgi Study

TSUNEKAZU YAMANO, M.D., AND KINUKO SUZUKI, M.D.

Abstract. The cerebellum of the hemizygous brindled mouse (MO<sup>br</sup>/y), a murine model of Kinky hair disease (KHD) in human beings, was investigated immunohistochemically using the Golgi technique. In 15-day-old MO<sup>br</sup>/y, Purkinje cells showed considerable changes in their arborization such as perisomatic dendrite-like processes, numerous spine-like protrusions from somata and stem dendrites, focal swellings of stem and distal dendrites and generally poor development of dendritic trees. These changes closely resembled those of KHD. Similar changes except for the focal swellings of dendrites, could be found in control mice at day eight but never after day 12. In the MO<sup>br</sup>/y receiving intraperitoneal injections of cupric chloride (CuCl<sub>2</sub>) on postnatal (PN) days seven and ten, Purkinje cells appeared similar, if not identical, to those of controls at PN day 15. Focal swellings of dendrites transiently reappeared in treated animals after PN day 23 but spontaneously subsided by day 110. These results suggested that normal arborization of Purkinje cells in MO<sup>br</sup>/y is at least in part due to delayed maturation, which is correctable by cupric chloride (CuCl<sub>2</sub>) treatment. The "weeping willow" deformity, which characterizes Purkinje cells in KHD in humans were not observed in MO<sup>br</sup>/y. Because other neuronal populations, which are known to be deficient in KHD, appeared well preserved in the murine mutant, these dendritic deformities may be secondary to the loss of other neurons.

Key Words: Brindled mouse; Cerebellum; Dendrites; Dendritic arborization; Copper therapy; Golgi technique; Kinky hair disease.

INTRODUCTION

Menkes' Kinky hair disease (KHD) is an X-linked neurological disorder of infancy characterized by mental and growth retardation, seizures and peculiar hair (kinky or steely hair) (1, 2). Its metabolic defect is considered to be due to a failure in copper homeostasis in the body in which copper accumulates in certain organs but is deficient in others, including the brain (3–5). The principal neuropathological changes of KHD are widespread neuronal loss and gliosis in cerebrum and cerebellum with associated axonal degeneration in the white matter. In the cerebellum, abnormal dendritic arborization and perisomatic processes (somal sprouts) of Purkinje cells are unique features in most reported cases (1–3, 6–18).

To determine the nature and developing patterns of these structural abnormalities in Purkinje cells in KHD, we conducted a chronological morphological investigation of the cerebellum in the brindled mottled mouse, which is an allele of the X-linked mottled mutant and whose hemizygous male, MO<sup>br</sup>/y, has a homologous metabolic defect with KHD (19, 20).

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TABLE 1
Experimental Protocol

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* Each column indicates the number of animals used.

MATERIALS AND METHODS

Thirty seven male hemizygotes (MO<sup>w</sup>/y) and 33 normal littermate mice were used for the study. The day of birth was considered day 1. As has been reported previously, MO<sup>w</sup>/y gradually lose weight after PN days 10–12 and die before PN days 15–16 with severe cmaclation and extensive neuronal degeneration in the cerebrum (21, 22). A single intraperitoneal injection of cupric chloride (CuCl<sub>2</sub>) on day seven or ten gave some clinicopathological improvement in MO<sup>w</sup>/y, but injection on earlier or later days had little or no effect. Administration of CuCl<sub>2</sub> to MO<sup>w</sup>/y mice on PN days seven and ten effectively prevents neuronal degeneration in the cerebrum, and these animals survived without any clinical symptoms (23). However, transient mitochondrial changes were noted in Purkinje cells despite CuCl<sub>2</sub> treatment. Therefore we conducted a morphological investigation of the cerebellum using a Golgi impregnation technique in the following groups. The experimental schedules are summarized in Table 1.

Control: Untreated, normal littersmates.
Group I: Malnourished group. Normal littersmates fed only six hours daily for 12 days beginning with PN day five (21).
Group II: Normal littersmates which received intraperitoneal injections of CuCl<sub>2</sub>, 10 μg/g of body weight (BW) twice on PN days seven and ten.
Group III: Untreated male hemizygotes.
Group IV: Male hemizygotes which received intraperitoneal injections of CuCl<sub>2</sub>, 10 μg/g BW, twice on PN days seven and ten.
Group V: Male hemizygotes which received intraperitoneal injection of CuCl<sub>2</sub>, 10 μg/g BW, on PN day five only.
Group VI: Male hemizygotes which received intraperitoneal injection of CuCl<sub>2</sub>, 10 μg/g BW, on PN day seven only.
Group VII: Male hemizygotes which received intraperitoneal injection of CuCl<sub>2</sub>, 10 μg/g BW, on PN day ten only.

The mice were anesthetized with pentobarbital and were perfused with physiologic saline for three to five minutes on the day indicated in Table 1. The brains were quickly removed and were impregnated by the modified Golgi-Cox method according to Sholl (24). Mid-sagittal cerebellum was sectioned at 120 μm thick and examined with light microscopy. Golgi impregnated Purkinje cells were documented by photography and camera lucida reconstruction.

RESULTS

In the control group on PN day eight the morphological features of Purkinje cells were roughly divided into two types: cells with, or cells without, perisomatic dendrite-
like processes. The Purkinje cells with perisomatic processes (Fig. 1a) had several thick apical dendrites or a single thick stem dendrite with relatively short secondary and tertiary branches, mainly in the anterior lobe. Purkinje cells in the middle to posterior lobes on the other hand, lacked perisomatic processes and had well formed dendritic trees with a stem dendrite, four to six secondary and many tertiary dendrites (Fig. 1b).

After day 12, perisomatic dendrite-like processes were absent even in the anterior lobe and Purkinje cells in all lobes appeared morphologically mature. The dendritic trees expanded with long secondary dendrites and with increased numbers of tertiary dendrites (Fig. 1c). By day 23 the density of the dendrites had increased and the surfaces of the somata and stem dendrites were smooth (Fig. 1d). After day 30, the morphology of Purkinje cells remained unchanged up to day 110.
Fig. 2. Purkinje cells in MO−−/+ (Group III). a) Eight-day-old, anterior lobe, cerebellum. Golgi. ×1,100. b) Eight-day old, anterior lobe, cerebellum. Golgi. ×1,100. c) 12-day-old, anterior lobe, cerebellum. Golgi. ×930. Compare these Purkinje cells with those illustrated in Figure 1; the abnormal pattern of development is evident.

The dendritic arborization of Purkinje cells in Group I was similar to that of age-matched controls. However focal axonal swellings of Purkinje cells were occasionally noted in the inner granular layer. No significant changes were noted in Purkinje cells in Group II.

In the untreated hemizygous mice of Group III, most Purkinje cells in the anterior and middle lobes possessed perisomatic dendrite-like processes at day eight. These processes were more pronounced than those in age-matched controls and in some, a stem dendrite could not be identified with certainty (Fig. 2a, b). In these Purkinje cells each of the processes originating from the soma was similar in length and in caliber, and only on rare occasions were short secondary dendrites noted. Even in Purkinje cells with a stem dendrite, dendritic trees were far smaller than those in age matched controls. At day 12, Purkinje cells with perisomatic dendrite-like pro-
Fig. 3. Purkinje cells in MO<sup>sy</sup>/y (Group III) at day 15: a, b, c are Purkinje cells in anterior lobe of the cerebellum. There is irregular and poorly developed dendritic arborization. Perisomatic dendrite-like processes (arrows) are conspicuous. An arrowhead (b) indicates spine-like protrusions in the stem dendrite. Small arrows in c and d point to sites of focal dendritic swelling. A Purkinje cell in the posterior lobe of the cerebellum (d) reveals smooth somal contours and a well developed dendritic tree. However, focal swellings of the dendrites (small arrows) are present at branching points. Golgi. a) ×650, b) ×2,200, c) ×820, d) ×650.

processes and more than one thick apical dendrite could only be found in the anterior and middle lobes (Fig. 2c). Occasionally, very thick processes were studded with numerous spine-like protrusions (Fig. 2c). Purkinje cells with similar patterns of dendritic arborization as noted in Figure 2c were frequently observed in the hemizygotes on day 15 (Figs. 3, 4). However, on day 15, the trunk of stem dendrites and perisomatic dendrite-like processes were more voluminous (Fig. 3a–c). Numerous spine-like protrusions were also noted on the surface of dendrites and dendrite-like somatic processes (Fig. 3b). Secondary and tertiary branches were formed but the development of dendritic trees was far less than that of age-matched controls. Ad-
Fig. 4. Camera lucida drawings of Purkinje cells in Group II at day 15. Some are illustrated in Figure 3.

ditionally, focal swellings of stem dendrites and dendritic branches were commonly observed (Fig. 3c, d).

Although abnormalities of Purkinje cells were particularly prominent in the anterior and middle lobes, a certain degree of dendritic change was also observed in the posterior lobe (Fig. 3d). Axons of some Purkinje cells displayed focal swellings in the inner granular layer at the anterior and middle lobes on days 12 and 15.

In the CuCl₂-treated hemizygous mice of Group IV, perisomatic dendrite-like processes, or more than one thick apical dendrite, were observed at day 12 in some Purkinje cells in anterior and middle lobes. However, they were far fewer than those in age-matched untreated hemizygous mice in Group III. The dendritic trees of some Purkinje cells were well developed and comparable to age-matched controls. No focal swellings of dendrites were observed in these Purkinje cells (Fig. 5a). Purkinje cells with perisomatic dendrite-like processes were very few in this group on day 15, even in the anterior and middle lobes. Two stem dendrites were rarely observed; spine-like protrusions were not recognized on the surface of stem dendrites or cell somata. On day 23, only rare perisomatic processes were encountered in the anterior lobe. However, segmental swellings in dendrites, which were not present at day 15 in this group, became a conspicuous feature after day 23 (Figs. 5, 6). The swellings were more prominent in the secondary and tertiary dendrites of the Purkinje cells (Fig. 5) in the anterior and middle lobes. Except for these segmental swellings, the
Fig. 5. a) A Purkinje cell in the anterior lobe of the cerebellum of MO₉/₁₀ in Group IV at day 15 shows a well developed dendritic tree and is similar to the normal Purkinje cell in age-matched controls. Although the somata show normal smooth contours and the dendritic trees are expanded, these focal swellings of dendrites (arrows), in particular at the branching points, are conspicuous features in the Purkinje cells at days b) 23, c) 25, and d) 30. The swellings are found in both proximal and distal dendrites (b); at times predominantly in the distal dendrites (c) or at times predominantly in proximal dendrites (d). Golgi. a) ×920, b) ×920, c) ×1,530, d) ×630.
dendritic trees were well-developed. Purkinje cells in the posterior lobe did not show any abnormalities.

Although the segmental swellings in dendrites were still observed at day 60, they decreased in size (Fig. 7). Perisomatic dendrite-like processes were never observed at this age. A few focal or segmental swellings were still present in some Purkinje cell dendrites at day 94, but no swellings were noted at day 110 and the dendritic trees of Purkinje cells were identical to those of age-matched controls.

The morphological alteration of Purkinje cell arborization in Group V and VI on days 12 and 15 was the same as that in Group IV. However, in Group VII, which received CuCl₂ injection once at day 10, the morphological changes of Purkinje cells at days 12 and 15 were very similar, if not identical, to those of untreated hemizygous mice in Group III.

DISCUSSION

The abnormal dendritic arborization of Purkinje cells in the cerebellum of patients with KHD are remarkable and well documented in the literature (6–8, 16, 17). These changes consist of perisomatic dendrite-like processes (somal sprouts), spine-like protrusions on the surface of stem and secondary dendrites, abnormal swellings in stem dendrites, focal, fusiform enlargements within secondary and tertiary dendrites, axonal swellings (torpedoes), the "weeping willow" deformity in dendritic trees and a reduction in the size of dendritic trees. These abnormalities, with an exception of...
the "weeping willow" deformity in dendritic trees, were observed in the cerebella of hemizygous brindled mice. Marked emaciation occurs in brindled mice before death (21). However, the absence of similar morphological changes in severely malnourished mice (Group I) suggests that these alterations are primary changes in KHD and brindled mice. It is unsettled whether perisomatic dendrite-like processes in the Purkinje cells in KHD represent a persistence of perisomatic processes of immature Purkinje cells (25–27) or newly formed processes due to a metabolic derangement of Purkinje cells (7, 16). Our study with brindled mottled mice provides evidence that these abnormal arborizations are consistent with delayed maturation in Purkinje cells and can be corrected by CuCl₂ treatment.

Many of the clinical signs and degenerative changes in the brains of patients with KHD are considered to be the result of copper deficiency (3, 4). However, there have been no convincing reports suggesting that copper supplementation is efficacious in the treatment of KHD (28–30).

In contrast, trace amounts of copper effectively prevented not only the clinical signs and symptoms (31, 32), but also the degeneration of neurons in the cerebrum.
of brindled mice, murine models of KHD when the cupric salts were administered at a critical period of brain development. Copper concentration in the cerebrum increases rapidly after PN day four in normal mice but in hemizygous brindled mice (MO<sup>br</sup>/y) this rapid raise in copper concentration could not be detected. Following CuCl<sub>2</sub> treatment at days seven and ten, brain copper concentration increases transiently in MO<sup>br</sup>/y but returns to subnormal levels later (33). Despite low copper concentration in adult MO<sup>br</sup>/y neuronal degeneration does not occur in the cerebrum but in the cerebellum, where transient but pronounced mitochondrial changes occur in Purkinje cells (23). This Golgi study clearly shows that CuCl<sub>2</sub> administration is associated with maturation of Purkinje cells in Groups IV, V and VI. Perisomatic dendrite-like processes regressed to a considerable degree, and the development of dendritic trees was promoted at day 15. However, in the mice in Group VII, very little effect was noted. These results indicate that for maturation of Purkinje cells, copper must be given before day seven.

Focal swellings in dendrites observed in hemizygous males on day 15 disappeared following administration of CuCl<sub>2</sub>, but recurred after day 23 in Group IV. As described in our other paper (34), the Purkinje cells with such dendritic swellings contained abnormally enlarged mitochondria in their somata and dendrites and the regression of these swellings coincided with the disappearance of abnormal mitochondria. Abnormal mitochondria have been well documented in Purkinje cells with abnormal arborization in patients with KHD (8, 18) and hemizygous brindled mice at day 15 (35). Thus, there appears to be some causal relationship between mitochondrial abnormalities and the focal swellings in dendrites.

Dendritic abnormalities may also result from disturbed cytoskeletal components in Purkinje cells, since disarray of the microtubules occurs in these swollen portions of dendrites (34). Strong immuno-reactivity with anti-actin serum on the irregularly swollen Purkinje cell dendrites, which contain 5–6 nm filaments, indicates an accumulation of actin in these dendrites (36).

Focal axonal swellings (torpedoes) of Purkinje cells as observed in KHD were also observed in the brindled mutant. However, they were also observed in severely malnourished normal littermates (Group I). Similar focal swellings of Purkinje cell axons have also been well documented in other neurological mutant mice (37, 38). Therefore these abnormalities appeared to be nonspecific degenerative changes in axons. However, spontaneous regression of torpedoes appeared to be taking place in hemizygous mice since only a few were recognized after day 94.

One prominent feature of Purkinje cells, which was pronounced in KHD but absent in brindled hemizygous mice, was the "weeping willow" deformity of dendritic trees. Such abnormalities have been reported in experimental animals following destruction of the cerebellar interneurons, especially stellate and granule cells (39, 40). Reduction of these neurons has been well documented in KHD (7, 8, 16, 17) but the degree of severity varied considerably among cases. In hemizygous brindled mice, only Purkinje cells are affected and other cerebellar neurons appeared well preserved (21, 23). Therefore absence of this "weeping willow" deformity in the dendritic trees of the brindled mouse mutant offers good supporting evidence for the view that such changes are secondary to the reduction of interneurons in KHD (16, 17).

The dendritic abnormalities in MO<sup>br</sup>/y are similar to those of KHD. With CuCl<sub>2</sub> administration, these abnormalities which result from delayed maturation could be corrected in MO<sup>br</sup>/y. Although mitochondrial changes in the somata and dendrites and focal swellings of dendrites in Purkinje cells recurred, they are transient phe-

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nomena and disappeared spontaneously. These observations indicate that at least in the mouse mutant MOw/y, CuCl₂ administration at days seven and ten, a period which probably corresponds to the third trimester of gestation in human beings, is effective treatment for this degenerative change.

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