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The primary locus of motor neuron death in an ALS–PDC mouse model

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Abstract

A mouse model of amyotrophic lateral sclerosis–parkinsonism–dementia complex based on the consumption of cycad seed flour was used to determine whether the observed pathology of motor neuron loss begins in the distal axons or the spinal cord. Assessments of neuromuscular junction integrity and motor neurons were performed at multiple time points. Mice fed cycad pellets performed worse on the wire hang than controls. Microglial activation in cycad-fed mice was observed with motor neuron degeneration at 12 weeks, but reactive astrocyte proliferation was not observed. After 33 weeks of cycad feeding, motor neuron loss had stabilized, with no evidence of neuromuscular junction endplate denervation. These data suggest that neuronal pathology begins at the soma and proceeds distally in a ‘dying forward’ pattern.

Keywords

amyotrophic lateral sclerosis–parkinsonism–dementia complex; axonopathy; cycad; neuromuscular junction

Introduction

The collection of neurological diseases now termed amyotrophic lateral sclerosis–parkinsonism–dementia complex (ALS–PDC) was first described by Mulder *et al.* [1] who identified an unusually high incidence of what at first appeared to be a nearly classical form of ALS among the Chamorro population of Guam in the years immediately after World War II. Later, Mulder and Kurland [2] also identified extremely high incidence levels of a parkinsonism coupled with dementia as a second neurological disorder. Approximately 7% of affected individuals showed features of both disorders. Whiting [3] established a firm link between ALS–PDC and the consumption of the seeds of *Cycas micronesica*, the indigenous cycad palm of Guam and nearby islands of the Western Pacific. These early observations linking cycad to the disease have recently been confirmed by Borenstein *et al.* [4].

In-vivo feeding of washed cycad seed flour to adult male CD-1 or C57/Bl-6 mice showed that many of the behavioural and pathological features of ALS–PDC could be reproduced. Daily

dietary exposure to washed cycad seed flour was shown to induce progressive motor and cognitive deficits in adult CD-1 mice, as well as neurodegeneration in neocortex, various hippocampal regions, substantia nigra, olfactory bulb and spinal cord as measured using TUNEL and caspase-3 labelling [5]. Our studies have also shown that specific molecules found in high concentrations in cycad, variant steryl glucosides, are neurotoxic to motor neurons. Mice treated with these neurotoxins showed progressive motor neuron loss at both the lumbar and thoracic levels continuing beyond the 15-week toxin-feeding period. Increases in reactive astrocyte proliferation and micro-glial activation coupled with caspase-3-positive motor neurons were also found in the lumbar segment of the ventral horn [6].

Transgenic mouse models have been widely used to study the familial forms of ALS, Parkinson's disease, and Alzheimer's disease. Mice expressing human SOD1 mutations are currently the most widely used ALS model. Fischer *et al.* [7], investigating the origin of motor neuron pathology in SOD1G93A mice reported early neuromuscular junction denervation before the onset of motor neuron loss. These findings implied that motor neuron pathology begins at the distal axon and travels proximally towards the motor neuron before neuronal degeneration begins.

We have earlier examined the similarities and dissimilarities between the cycad mouse model of ALS and mSOD mice [8] and noted a number of similarities as well as significant differences. In general, both models showed significant decrease in total brain volume and volumetric reductions in specific brain areas including substantia nigra, striatum and hippocampus. Cycad-fed mice and mSOD mice displayed significant reductions in total spinal grey matter specifically confined to the ventral horn volume. The primary difference between the two models was that, while both models showed reduced grey matter volumes, only the mSOD mouse model showed a corresponding reduction in spinal white matter volume.

To further compare the cycad model with mutant SOD1 pathology, we have now examined the timeline of pathological events leading to motor neuron degeneration. Our results show a loss of motor neurons in the absence of significant neuromuscular denervation, suggesting a ‘dying forward’ pathophysiology.

Methods

Adult male CD-1 mice were maintained in individual housing at a 12-h light/dark cycle. All procedures were carried out in accordance with the regulations and guidelines of the Canadian Council on Animal Care and the National Institutes of Health. Coordination and activity testing commenced at 6 months of age and performed in the following groups during three periods of the study: during the first period from the start of cycad feeding to time point 1 (17 weeks), $N=10$ control and $N=18$ cycad-exposed mice; between time point 1 and time point 2 (26 weeks), $N=6$ control and $N=13$ cycad-exposed mice; between time point 2 and time point 3 (43 weeks), $N=3$ control and $N=9$ cycad-exposed mice. Mice were fed daily a 0.5 g pellet consisting of ground cycad seed chips (previously washed for 7 days) starting at week 4 of the study. Control animals received commercial white flour pellets.

Behavioural tests were performed as described earlier [9]. Loss of hind limb extension reflex during tail suspension is a sign of motor neuron dysfunction [10]. A reflex score was assigned to each animal twice weekly and the scores from each trial were averaged [6]. Every week as a test of motor strength [11] mice were placed on a wire lid approximately 60 cm above a padded surface, turned upside down, and the time to fall was recorded. Rotarod performance was observed for quantitative analysis of motor coordination [12]. Mice were placed on a rod (diameter 3.6 cm; length 15 cm) rotating at 16 rpm and their latency time to fall was measured. Each mouse had two trials weekly with a 1-day intertrial interval. Paw print analysis was

performed to detect alterations in stride length and stance width [13]. A strip of paper was placed on the floor of a walkway (length 100 cm, width 6 cm, walls 10 cm high). Hind paws were dipped in paint before walking down the strip of paper. Measurements from two consecutive strides were averaged for each mouse. To measure exploratory activity and anxiety, mice were placed in an open field chamber (diameter 2 m, walls 0.5m high) every second week and allowed to roam for 5 min, while monitored by overhead video camera. Activity was assessed with a circular grid overlaying the screen during playback and the number of grid crossings was counted. Statistics were performed by unpaired two-tailed Student's *t*-tests and repeated-measures two-way analysis of variance.

At each chosen time point of euthanization, mice were perfused with phosphate-buffered saline followed by 4% paraformaldehyde. The spinal cord and gastrocnemius muscles were dissected, transferred to a sucrose gradient, frozen in OCT compound, and sectioned transversely on a cryostat (20, 40 μ m, respectively).

Primary antibodies used were anti-choline acetyltransferase (ChAT) goat polyclonal (1:100, Chemicon International, Temecula, California, USA), anti-ionized calcium-binding adapter molecule 1 (Iba1) rabbit polyclonal (1:1000, Wako Chemicals, Richmond, Virginia, USA), and antiglial fibrillary acidic protein (GFAP) mouse monoclonal (1:100, Cell Signaling, New England Biolabs Ltd., Pickering, Ontario, Canada). Secondary antibodies (1:200) used were FITC anti-goat (Jackson Immuno Research Laboratories Inc., West Grove, Pennsylvania, USA), Alexa 546 anti-rabbit (Molecular Probes, Burlington, Ontario, Canada), and FITC anti-mouse (Jackson). For the analysis of muscle, we followed the protocol described by Fischer *et al.* [7]. In brief, muscle sections were incubated in 5 μ g/ml Alexa 594 α -bungarotoxin (Invitrogen, Burlington, Ontario, Canada) followed by anti-neurofilament 160 mouse monoclonal (1:300, Chemicon) and anti-synaptic vesicle protein 2 (SV2; 1:30; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA).

Results

Behavioural assessments

Cycad feeding of the experimental mice began after 5 weeks of restraining on the behavioural test series. The first euthanization was after 17 weeks of cycad feeding (T1). During this period, we noted that the ability of the cycad-treated mice to remain on the wire hang began to decline after 13 weeks of cycad feeding, but did not reach statistical significance [$F(1,16)=0.27$, Fig. 1a]. Cycad-fed animals showed an increased stride length during locomotion starting at week 9, but these measures did not reach significance [$F(1,16)=0.28$, Fig. 1c]. Cycad-fed animals consistently had a greater average body weight [$F(1,15)=3.06$, $P<0.001$] and spent less time in the outer perimeter of the open field [$F(1,8)=5.45$, $P<0.0001$] than their control-fed counterparts (Fig. 1b).

During the period between time point 1 and time point 2 (26 weeks), behavioural motor functions continued to decline in cycad-fed mice. Cycad animals continued to perform worse than the control group between weeks 18 and 26 (time point 2, $P<0.01$) as the performance of cycad-fed animals continued to decline ($N=6$ control, $N=13$ cycad). The remaining animals continued their diet of cycad until the final time point (T3) at 43 weeks. Performance on the wire hang was remarkably worse for cycad-exposed animals ($P<0.001$). During the latter half of the study, body weight did not differ between controls or treated mice at any of the time points ($P=0.2$, Fig. 1a and b). Cycad-exposed mice showed a significantly longer stride length in comparison with control mice ($P<0.05$) throughout the length of the study (Fig. 1c). This effect on gait did not become more pronounced at later measurements.

In a 5-min open field trial to measure exploratory activity, cycad-fed mice were significantly hypoactive compared with controls ($P<0.01$) in measures of total grid crossings around the perimeter of the arena (Fig. 1d). This hypoactivity was specific to the perimeter. There was no difference between cycad-fed mice and controls in the proportion of time spent in the perimeter versus the central areas of the arena (data not shown).

Mice exposed to cycad showed no significant deficits in the leg extension reflex when suspended by the tail. In addition, latency time to fall from a rotarod was similar for both groups (data not shown).

Histological assessment of spinal cord

In comparison with control mice, cycad-fed mice showed significant motor neuron loss [$F(1,17)=14.19$, $P<0.05$] and increased microglial activation [$F(1,19)=5.015$, $P<0.05$, Fig. 2 and Fig. 3, respectively]. Compared with control mice, early signs of chromatolysis in motor neurons were observed in the anterior horns at the earliest time point, whereas surviving motor neurons were indistinguishable from controls (Fig. 3e and g). At the final time point, remaining motor neurons in the anterior horns of cycad-fed mice were often chromatolytic (Fig. 3f and h). Immunostaining for GFAP showed no changes in reactive astroglial proliferation at any time during the study [$F(1,18)=0.057$, $P>0.05$, data not shown].

Muscle endplate innervation

Motor endplates remained innervated throughout the early phases of exposure to cycad during the period in which motor neuron loss and microglial activation were detectable. After 33 weeks of cycad exposure, a majority of endplates were still completely innervated in both groups of mice (Fig. 4): $79.38\pm5.98\%$ for control animals and $84.22\pm14.46\%$ for cycad-fed animals. Denervated endplates made up $15.41\pm5.72\%$ for control animals and $13.45\pm13.03\%$ for cycad-fed animals. The differences between cycad-fed and control groups did not achieve statistical significance at any time point.

Discussion

Although the mechanism(s) of cycad toxin-induced neurodegeneration remains uncertain, the link between our earlier [5,11] and current data and the epidemiological studies of ALS-PDC in relation to cycad seed consumption seems well founded. This study showed that cycad-fed animals have increased microglial activation and decreased motor neuron numbers in the ventral horn of the lumbar spinal cord. The first significant signs of behavioural disorder are recognizable at about week 19. Quantitative analyses of the integrity at the neuromuscular junction showed unremarkable changes throughout the study even with the presence of significant early motor neuron loss.

These results suggest that motor neuron pathology in this model of ALS-PDC begins at the cell body and proceeds distally in a 'dying forward' pattern. The apparent correspondence between motor neuron loss and microglial activation supports a role for these cells in motor neuron degeneration. In contrast, Fischer *et al.* [7] using the G93A mSOD model reported evidence of early neuromuscular junction denervation before the onset of motor neuron loss.

The observed differences between the ALS-PDC and the mSOD models might arise because of fundamental differences in pathological processes. At present, we cannot exclude the possibility that in cycad-fed mice sprouting of surviving motor neurons occurs to replace the axons of those cells destroyed by cycad neurotoxins potentially masking early denervation of muscle endplates.

Conclusion

Our data show profound differences in the staging of motor neuron loss between the mSOD and cycad models of ALS, with results indicative of differential axonal pathologies. However, similarities at various levels of the central nervous system, including the observation that microglial activation in both models occurs, suggests a common inflammatory response at some phase of the disease process.

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Figure 1.

Abnormalities in grip strength, gait, and open field activity in cycad-fed mice. (a) Wire hang performance from pre-diet to time point 3 of cycad-fed animals and their control counterparts. Cycad-fed mice performed significantly worse than controls with differences between groups becoming more pronounced towards later time points ($P<0.01$). (b) Cycad-fed mice have a greater body weight at the start of the study. Both groups show a trend of increasing body weight throughout the course of the study. (c) Gait analysis during linear locomotion. Cycad-fed mice showed a significantly longer stride length compared with control animals ($P<0.05$). (d) Open field assessment of free locomotion. Cycad-fed mice were significantly less active at the outer perimeter of the arena ($P<0.01$; at T1, $N=10$ control, $N=18$ cycad; at T2, $N=6$ control, $N=13$ cycad; at T3, $N=3$ control, $N=9$ cycad).

Figure 2.

Motor neuron loss and chromatolysis in the lumbar spinal cord of cycad-fed mice after exposure to cycad. ChAT and Nissl staining of mouse spinal cord showed motor neuron loss and increased chromatolysis in cycad-fed mice (b and f) compared with the more numerous healthy motor neurons of control mice (a and e) at 12 weeks of cycad exposure. After 33 weeks of cycad exposure, motor neuron loss and chromatolysis was observed only in cycad-fed mice (d and h) compared with controls (c and g). Quantification of motor neurons in lumbar cord shows lower counts in cycad-fed mice at all time points (i) ($P < 0.05$). Mean counts were compared by analysis of variance. Bars = 40 μ m (a–d); 20 μ m (e–g). The ‘*’ mark the motor neuron that is

enlarged in the inset; and the insets show an enlarged $100\times$ magnification of selected motor neurons.

Figure 3.

Microglial activation in the spinal cords of cycad-fed mice. (a and b) Immunostaining for microglia with Iba1 at 12 weeks of cycad exposure. (c and d) Microglial activation at 33 weeks of exposure. At both time points of testing, microglial activation was significantly greater in cycad-fed animals than control animals. The inset shows a higher power view of a labelled cell. (e) Quantification of microglia in lumbar cord shows higher counts in cycad-fed mice at all time points ($P<0.5$). Mean counts were compared by analysis of variance. Bars = 40 μ m.

Figure 4.

Motor endplate innervations in cycad-fed mice. Quantification of motor endplate innervation status at 12 and 33 weeks shows no difference of endplate innervation between groups. Data are shown as percent of total endplates classified as innervated, intermediate or denervated per group. Numbers in parentheses indicate the total number of endplates quantified per group. Endplates were depicted as fully innervated (a) when there is complete overlap of the axon terminal with the endplate, where yellow (merge) indicates overlap (inset). Neuromuscular junctions associated with a preterminal axon only, or showing partial overlap of the axon terminal with the endplate were depicted as having intermediate innervation (b). Complete denervation is evident when the endplate was not associated with an axon (arrow in c). Immunostaining for axons with neurofilament/ SV2 (green) together with AChR staining with α-bungarotoxin (red) shows neuromuscular junction integrity. Bars=50 μm.