Ultrastructural, Morphometric, and Immunocytochemical Study of Anterior Horn Cells in Mice with "Wasted" Mutation

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Abstract. Mice with the autosomal recessive gene "wasted" (wst/wst) manifest hind-limb paralysis and tremulousness, develop reduced secretory immune responses, and have abnormal DNA repair mechanisms. There is prominent vascular degeneration of neurons within anterior horns of the spinal cord and motor nuclei of the brainstem. A morphometric analysis of motor neurons in the spinal cord was performed on 2-hydroxyethyl methacrylate-embedded tissue from ten wst/wst mice, ten littermates (wst/+, +/+ ) without clinical deficits, and ten parental (+/+ ) control mice. Vacuolated neurons were present only in wst/wst mice (p = 0.0008). Fibrillar neurons were more numerous in the wst/wst mice than in littermates (p = 0.01) or controls (p = 0.007). The number of total or normal neurons did not differ significantly among the three groups. Volume measurements for normal, fibrillar, vacuolated, and total neurons were greater in wst/wst mice (p < 0.0008). Electron microscopic studies revealed vascular degeneration exclusively within neurons of wst/wst mice with the prominent accumulation of neurofilaments. Immunocytochemical staining of Araldite-embedded sections with monoclonal antibodies (MAb) to 68 kDa, 160 kDa, and 200 kDa neurofilament proteins showed prominent staining of vacuolated and fibrillar neurons in wst/wst mice exclusively with the MAb to 200 kDa neurofilaments. Dephosphorylation of tissue reduced the staining of 200 kDa neurofilaments in wst/wst mice. These studies suggest that phosphorylated neurofilaments may be important in events producing neuronal dysfunction. Therefore the "wasted" mutation may be an excellent model for the study of motor neuron disease.

Key Words: Amyotrophic lateral sclerosis; Anterior horn; Morphometry; Motor neurons; Mouse mutant; "wasted"; Neurofilaments.

INTRODUCTION

Mice bearing the autosomal recessive gene "wasted" (wst/wst) develop neurologic and immunologic abnormalities. In 1982, Shultz et al (1) reported the discovery of the spontaneous mutation, "wasted," in the inbred mouse HRS/J. Heterozygotes (wst/+) appeared normal whereas homozygotes (wst/wst) developed tremulousness, ataxia, weakness of hind limbs, and weight loss. Most affected mice did not survive beyond 30 days of age. Marked lymphoid hypoplasia (thymus dependent and independent), a high degree of spontaneous and gamma ray-induced chromosomal damage (2), and degeneration of Purkinje cells with focal demyelination in the cerebellar cortex were described in the homozygote. Thus, the wst/wst mouse was originally proposed by Shultz et al (1) as a murine model for ataxia telangiectasia. However, further immunologic and endocrinologic investigations did not find a relationship between this model and ataxia telangiectasia (3–8). Preliminary neuropathologic studies done in our laboratory challenged the conclusion as well (9).

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Studies revealed prominent vacuolar degeneration of neurons within anterior horns of the spinal cord and motor nuclei of the brainstem without abnormalities in Purkinje cells. The central nervous system abnormalities resembled human motor neuron disease rather than ataxia telangiectasia.

The present studies were designed to characterize further the neuropathology of the "wasted" mouse. Morphometry was used to assess anterior horn cells volumetrically and numerically. Electron microscopy and immunocytochemistry aided in further delineating neuronal lesions, particularly in elucidating abnormal neurofilament metabolism.

MATERIALS AND METHODS

Mice

The recessive mutation wst/wst first appeared spontaneously in the HRS/J colony at The Jackson Laboratory (Bar Harbor, ME). Because of the short lifespan of the homozygote, the mutation was transferred to a segregating background (C3HeB/FeJ × C57BL/6J) by ovary transplantation to increase viability through hybrid vigor. Normal littersmates (wst/+, +/+ ) from this first generation were mated. The original H-2 phenotype of these animals was attenuated by further ovary transplantation from a female wst/wst to a normal F1 female (C3HeB/FeJ × C57BL/6J) and subsequent breeding to a normal C57BL/6J male. This cycle was repeated 20 times (6).

Breeding pairs of mice bearing the wt mutation were obtained from The Jackson Laboratory. Our mice were bred in the immunogenetic facility at Mayo Clinic under the supervision of Dr. C. David. For all experiments, affected animals 28 days of age were used, whereas unaffected littermates (wst/+, +/+ ) and parental strain (C3HeB/FeJ × C57BL/6J) F1, served as controls.

Morphology

Animals were anesthetized with pentobarbital given intraperitoneally and killed by intracardiac perfusion with 4% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The spinal cord and brain of each mouse were examined. Brains were cut into six coronal sections and spinal cords were sectioned coronally into 15 to 20 blocks, 1 to 2 mm thick. For morphometric analysis, the tissues were embedded in 2-hydroxyethyl methacrylate by using the JB4 system (Polysciences, Warrington, PA) and cut into 1 μm semithin sections. Spinal cord and brain sections were stained with a modified hematoxylin and eosin (H&E) method. Spinal cords from additional animals were embedded in Araldite (Polysciences). Osmicated tissue was processed for electron microscopy and nonosmicated tissue was used for immunocytochemical studies.

Morphometry

Ten "wasted" mice (wst/wst), ten normal littermates (wst/+, +/+ ), and ten parental mice (+/+ ) were examined. A Zeiss image analysis system with video camera was used.

The right-sided anterior horn of each section was examined and comprised an area lying anteriorly and laterally from the central canal. A microscope eyepiece grid, each square corresponding to 34 ± 2 μm of tissue, was used to select fields. The first field analyzed was a random multiple, zero through five, of each 34 μm square, counting laterally from the central canal. Thereafter, fields every six squares (approximately 204 μm) were analyzed, minimizing gaps and obviating overlap when coupled to the 220 × 220 μm video frame of the computer. A ×40 Zeiss objective lens was used for analysis.

An average of 300 neurons was counted per animal and provided a relative standard error of less than 5% (10). A total of 8,752 neurons was analyzed. Neurons were delineated manually and categorized into three groups: normal, fibrillar, and vacuolated. Vacuolated neurons had readily apparent "bubbles" of cytoplasm. Fibrillary neurons contained distinct "thread-like"
bands interweaving to form a meshwork. These neurons were not vacuolated but had a less defined nucleus and nucleolus. Neurons on the edge of the video frame were not counted if less than half of their area was inside the frame; their areas, however, were calculated. Gray matter area was determined by manual elimination of white matter and automatic editing of vessel walls and lumens from area determinations. In addition, one random field in the right posterior horn of four animals, each bordered by white matter, was analyzed (a total of 1,186 neurons) to document the absence of pathologic features in the posterior horn.

Neuronal volumetric (Vv) and numerical densities (Nv) were calculated (10) for each of the three groups. Vv = total area of the neuron of interest/total area gray matter analyzed. Nv = K/β[(Na)²/Vv]², where Na = number neurons/gran gray matter area (μm²), K is a size distribution factor selected by an arbitrary constant between 1 and 1.1 (chosen here to be 1), and β is a shape coefficient selected from published graphs to be 1.5, assuming an approximate neuron length : width ratio of 1.5 (11). Statistical analysis was performed with an unpaired t-test.

Immunocytochemistry

Immunocytochemical testing was performed on Araldite-embedded spinal cord sections of wst/wst mice, “wasted” littermates, and parental control mice. Slides were etched for 15 minutes (min) with a 1:1 solution of aged sodium ethoxide in absolute alcohol, followed by rehydration with graded concentrations of alcohol. Dephosphorylation of sections was achieved with a solution of 43 μl bovine alkaline phosphatase (type VII, Sigma, St. Louis, MO), in 1 ml Tris-HCl (pH 9) at 32°C for 2.5 hours (h), as per Grundke-Iqbal et al (12). Neurofilament staining was obtained by using monoclonal antibodies (MAb) to 68 kDa, 160 kDa, and 200 kDa neurofilaments (Boehringer Mannheim, West Germany) diluted 1:4 with phosphate-buffered saline (PBS) buffer, in conjunction with the ABC immunohistochemistry kit (Vector Laboratories, Burlingame, CA). Additional sections were performed with MAb SM 131, 32, and 33 to neurofilaments from Sternberger-Meyer Immunocytochemicals, Inc. (Jarrettsville, MA) (13). Staining was achieved with Hanks-Yates reagent (Polysciences) in Tris buffer solution in the presence of hydrogen peroxide after 15 min. Controls included substituting (PBS) for the primary antibody.

RESULTS

Clinical Findings

Animals began to manifest a stereotypic neurologic abnormality by 14 to 18 days of age: tremulousness, incoordination, and decreased interest in the surrounding environment. This was followed by hind-limb paralysis and death by 28 to 31 days of age. The tremor appeared to involve all four limbs and was of high frequency but low amplitude. The animals had difficulty negotiating a 60° inclined plane. Most of the animals failed to gain weight.

Neuropathologic Findings

Light Microscopy: The most striking abnormality was extensive vacuolar degeneration of anterior horn cells of the spinal cord (Fig. 1A, B). Less severe abnormalities were present in various motor nuclei of the brainstem. Occasional neurons showed prominent axonal swelling in association with an accumulation of neurofilaments. Degeneration of certain anterior horn cells was contrasted with adjacent neuronal cells which were entirely normal, as were all cells examined in the posterior horn. Review of the cerebellum and its white matter tracts revealed normal anatomical structure (Fig. 2). Other areas of the nervous system such as the basal ganglia, hypothalamus, thalamus, and cortex failed to show neuronal degeneration. No inflammatory or reactive changes were seen accompanying neuronal degeneration. Myelin was well preserved. No abnormalities were noted in astrocytes or oligoden-droglia.
Fig. 1. A. Spinal cord section from wt/wt mouse shows prominent vacuolar degeneration of an anterior horn cell. ×450. B. Fibrillar changes in cytoplasm of motor neurons. Cells in bottom of micrograph have undergone vacuolar changes. Axonal swelling is shown by arrows. Glycol methacrylate-embedded section stained with modified H&E. ×450.

Electron Microscopy: Ultrastructural studies revealed prominent microvacuolation and macrovacuolation of neurons within the anterior horns of the spinal cord (Fig. 3). The earliest change appeared to be swelling of mitochondria and presumed dilation of the endoplasmic reticulum (Figs. 3, 4A). This was associated with accumulation of electron dense intracytoplasmic and intra-axonal inclusions (Fig. 4B). Widened proximal axonal segments were present, enclosing aggregates of neurofila-
ments. There was evidence of nerve fiber degeneration with accumulation of dense bodies, filaments, and tubules within the degenerating axons (Fig. 4B) while myelin sheaths remained intact. Macrophages were conversely absent. No ultrastructural abnormalities were noted in the processes of astrocytes or within myelin-producing cells. The final consequence of the degenerative process in neurons was accumulation of electron-lucent vacuoles outlined by a thin membrane containing loosely organized membrane remnants.

Morphometry: All wst/wst animals showed neuronal vacuolar degeneration of anterior horn cells (approximately 4% of all neurons counted), whereas no littermates (wst/+ or +/+ ) exhibited these abnormalities (p < 0.0008) (Table 1). Fibrillary neurons were more numerous in the wst/wst (approximately 8% of all neurons) than in normal littermates (approximately 0.5% of all neurons) (p = 0.01) or in parental mice (0.2%) (p = 0.007). Normal littermates and parental mice did not differ significantly from each other in number of fibrillary neurons. None of the groups of mice differed significantly from the others in the number of normal neurons or total neurons.

Volume measurements for normal, fibrillary, vacuolated, and total neurons were greater in wst/wst than in normal littermates or parental mice (p < 0.0001). There was no difference in volume measurement between littermates and parental mice.

Immunocytochemical Staining for Neurofilaments: Prominent staining of vacuolated (Fig. 5A) and fibrillary (Fig. 5B) neurons was found only in wst/wst mice by MAb to 200 kDa neurofilament protein (Table 2). Most vacuolated neurons viewed
Fig. 3. Multiple small vacuoles in cytoplasm of greatly enlarged neuron from “wasted” mouse. Most microvacuoles originate from widening of cisternae of endoplasmic reticulum or degenerative changes in mitochondria (arrowheads). In center of this abnormal neuron there is accumulation of neurofilaments (nf). Arrows show surface of neuron. Note the absence of morphologic changes in myelin sheaths. × 5,500.
Fig. 4. A. Higher magnification of one of the areas in abnormal neuron shown in Figure 3. Vacuoles are lined by membrane and are surrounded by numerous neurofilaments. × 50,000. B. Electron dense inclusions within axonal swelling of "wasted" mouse consist primarily of degenerating mitochondria. × 22,500.
<table>
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<th>Animals</th>
<th>Normal Mean</th>
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<th>Fibrillary Mean</th>
<th>p value</th>
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p = statistical significance by unpaired Student t-test.
stained with the MAb against 200 kDa protein (Fig. 6A), suggesting that these two pathologic phenomena may be related. Rarely, labeling occurred with the MAb to the 160 kDa neurofilament; none occurred with MAb to the 68 kDa neurofilament. No staining of neurons occurred in either the littermates or parental mice with any of the MAbs. Axons stained uniformly in all mice, but more vividly with the MAb.
### TABLE 2

<table>
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<th>Monoclonal antibodies</th>
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<tr>
<td>814342*</td>
<td>200</td>
<td>−</td>
<td>−/−</td>
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<td>68</td>
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<tr>
<td>SM1 33‡</td>
<td>Nonphosphorylated neuronal marker</td>
<td>−</td>
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</table>

* Antibodies from Boehringer Mannheim.
† Each + equals an average of one labeled neuron per high-power field (×40), per approximately 70 neurons.
‡ Antibodies from Sternberger-Meyer Immunocytochemicals, Inc.

to the 200 kDa neurofilament than the 160 kDa neurofilament and less to the 68 kDa protein. No difference was seen in the degree of axonal staining between "wasted" mice and control mice.

Dephosphorylation of tissue reduced the number of 200 kDa labeled neurons and the intensity of the brown reaction product (Fig. 6B). Dephosphorylation prior to labeling with MAbs to the 160 kDa or 68 kDa neurofilament produced no discernible change in neuronal staining from non-enzymatically treated tissue. Axons labeled with MAb to the 200 kDa neurofilament showed slightly diminished intensity of staining after dephosphorylation; the change was not discerned in axons labeled to the 160 kDa and the 68 kDa neurofilaments. In order to document further the conclusion that "wasted" neurons contain phosphorylated neurofilament of 200 kDa, sections were stained with neurofilament antibodies from Sternberger-Meyer Immunocytochemicals, Inc. Prominent labeling was seen with MAb SM1 31, which reacts primarily with a phosphorylated epitope of 200 kDa protein. No staining was seen with MAb SM1 32, which reacts with nonphosphorylated neurofilaments.

**DISCUSSION**

These studies were designed to quantitate neuronal abnormalities in "wasted" mice and to test the hypothesis that abnormal neurofilament metabolism occurs in diseased neurons. Our results indicate that the "wasted" (wst/wst) mouse is different from its littermates (wst/+ and +/+ ) in exhibiting vacuolar degeneration of anterior horn cells, more fibrillary neurons, and greater neuronal volume measurements. However, there is no significant neuronal loss in "wasted" mice. "Wasted" mice do show prominent perikaryal neuronal staining with MAb to 200 kDa neurofilament protein. The perikaryal neurofilaments appear to be phosphorylated, because treatment with alkaline phosphatase diminishes the intensity of staining. In addition, these abnormal neurons exhibit staining with a MAb that reacts specifically with

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phosphorylated epitopes of the 200 kDa neurofilament protein. The specificity of the neuropathologic abnormalities for anterior horn cells in the "wasted" mouse suggests that this may be an important animal model of human motor neuron disease.

Unlike our results in the "wasted" model, morphometric studies performed on spinal cords of patients with amyotrophic lateral sclerosis (ALS) suggest neuronal loss. In morphometric quantification performed on limb motor cells of the sixth
cervical region, Tsukagoshi et al (14) found the total number of larger motor neurons to be decreased significantly in 11 of 12 cases of ALS. However, in that study ballooned anterior horn cells were discounted. Using computer-aided analysis, Swash et al (15) described focal loss of motor neurons in the eighth cervical segments in ALS, without any constant or columnar pattern of disease distribution. Hanyu et al (16) showed a decrease in the number of large fibers of the ventral root in 14 of 17 cases of ALS, whereas the number of small fibers increased in three of five cases. Also at variance with our findings in the “wasted” mouse is a morphometric analysis of motor neuron volume in the lumbar swelling in which there was no significant difference between controls and ALS (17). However, distention of axons has been found qualitatively to be more severe in patients with ALS of shorter duration (18–20). The presence of neuronal and axonal swellings in the “wasted” mouse suggests that the mouse may be a model for the earliest neuropathologic events in human motor neuron disease.

A potential source of error in our study is in identification of the three classes of neurons: normal, fibrillar, and vacuolated. Anterior horn cells lacking a distinct nucleus or vacuoles present the greatest chance of misclassification. The glial population could also be confused with small, especially abnormal, anterior horn cells. Other sources of error include inaccuracies in the manual tracing of neurons and gray matter, deficiencies in the computer’s ability to discriminate between vessels and gray matter, and volume extrapolations from two-dimensional areas.

Neurofilaments, a class of intermediate filaments, share with other cytoskeletal elements the function of regulating the size and configuration of neurons (21, 22). The neurofilament of 160 kDa and especially the neurofilament of 200 kDa are extensively phosphorylated (23, 24). Generally, phosphorylation-dependent epitopes can be demonstrated in axons but not in perikarya or dendrites; dephosphorylation-dependent epitopes, conversely, are present in perikarya and in large primary dendrites but not in axonal processes (13).

Neurofilaments accumulate in neuronal perikarya and processes in various disorders, many of which show abnormal distributions of phosphorylated epitopes of the neurofilament proteins. Between one and 21 days after crush of the proximal sciatic nerve in the rat, phosphorylated neurofilaments usually not found in neuronal perikarya are detected there (25). In chronic aluminum intoxication in rabbits, neurofibrillary swelling in proximal axons of motor neurons is associated with neurofilamentous accumulation in the perikarya and dendrites, much of which is phosphorylated (26). Neuronal vacuolization and neurofilament proliferation have been described in the wobbler mouse paralytic disorder (27), although no studies analyzing the distribution of neurofilaments have been performed. Motor neurons from Brittanay spaniels with a rapidly progressive form of hereditary canine spinal muscular atrophy may show perikaryal neurofilament accumulation as well (28).

Abnormal distributions of neurofilament immunoreactivity also have been described in neurons of several human diseases. In Alzheimer’s disease, phosphorylated epitopes of neurofilaments are associated with neurofibrillary tangles (12). In Parkinson’s disease, the perikarya of dopaminergic neurons in the substantia nigra show Lewy bodies that contain phosphorylated neurofilaments (29). In ALS, neurofilament epitopes in the perikarya of motor neurons have been detected with an antibody recognizing the phosphorylated 200 kDa protein subunit as in our experiments (30). Neuronal accumulation of phosphorylated neurofilaments has also been described in an infantile neurodegenerative disease. In this condition there is also staining with antibody directed against the 200 kDa neurofilament protein and loss of labeling on
dephosphorylation (31). Anterior horn cells, the neurons most severely involved, are exceedingly swollen in this disorder and, by nonmorphometric analysis, are present in near-normal numbers. Thus, our findings have a striking resemblance to several human neurodegenerative conditions.

Abnormal phosphorylation has been found in association with the diminished transport of neurofilaments from the perikaryon into the axon (32–34) and is consistent with several mechanisms. Delayed transport may lead directly to pathologic accumulation of neurofilaments. Conversely, slowed axonal transport may prolong the presence of neurofilaments in the vicinity of active kinase systems. An increase in neuronal volume may be expected as a result of diminished transport of filaments out of the cell body. In our model, perikaryal collections of phosphorylated neurofilaments are found simultaneously with increased neuronal volume. Although descriptions of swollen proximal axons in aluminum intoxication (26) and human ALS (18–20) exist in the literature, increases in neuronal volumes have not been found (17). Other explanations exist for the presence of perikaryal phosphorylated neurofilament epitopes and their relationship with neurologic disease. They may represent a reactive response of neurons to injury, as illustrated by perikaryal phosphorylation following axotomy of the rat sciatic nerve (25). They may reflect the interference of toxic agents with protein phosphorylation. Positive labeling with MAbs to phosphorylated neurofilament epitopes may also represent cross-reactivity among cytoskeletal proteins, especially phosphorylated ones. The relationship of mitochondrial degeneration and neurofilament accumulation may be enlightening in the “wasted” model, and it may prove relevant to human mitochondrial disorders.

A search for retroviruses in the “wasted” mouse may provide a possible explanation for the initiation of aberrant cell processes. Woloschak (personal communication) has demonstrated altered expression of retrovirus sequences in the spleen, brain, and thymus of “wasted” mice. An endogenous murine leukemia virus is expressed in the “wasted” mouse, correlated with the presence of the wt allele (35). In view of the immune deficiency in the “wasted” mouse, abnormal expression of an endogenous retrovirus genome may be associated with this disorder of anterior horn cells.

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