The Golgi apparatus of spinal cord motor neurons in transgenic mice expressing mutant Cu,Zn superoxide dismutase becomes fragmented in early, preclinical stages of the disease

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ABSTRACT Dominant mutations of the SOD1 gene encoding Cu,Zn superoxide dismutase have been found in members of certain families with familial amyotrophic lateral sclerosis (ALS). To better understand the contribution of SOD1 mutations in the pathogenesis of familial ALS, we developed transgenic mice expressing one of the mutations found in familial ALS. These animals display clinical and pathological features closely resembling human ALS. Early changes observed in these animals were intra-axonal and dendritic vacuoles due to dilatation of the endoplasmic reticulum and vacuolar degeneration of mitochondria. We have reported that the Golgi apparatus of spinal cord motor neurons in patients with sporadic ALS is fragmented and atrophic. In this study we show that spinal cord motor neurons of transgenic mice for an SOD1 mutation display a lesion of the Golgi apparatus identical to that found in humans with sporadic ALS. In these mice, the stacks of the cisternae of the fragmented Golgi apparatus are shorter than in the normal organelle, and there is a reduction in Golgi-associated vesicles and adjacent cisternae of the rough endoplasmic reticulum. Furthermore, the fragmentation of the Golgi apparatus occurs in an early, presymptomatic stage and usually precedes the development of the vacuolar changes. Transgenic mice overexpressing the wild-type human superoxide dismutase are normal. In familial ALS, an early lesion of the Golgi apparatus of motor neurons may have adverse functional effects, because newly synthesized proteins destined for fast axoplasmic transport pass through the Golgi apparatus.

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease, which in the classical sporadic form is characterized by the involvement of both upper and lower motor neurons and pyramidal tract degeneration (1, 2). About 10% of cases of ALS are familial and these are characterized by additional pathological features that include involvement of sensory fibers in the posterior columns as well as cerebellar projections in the lateral columns (2, 3). Until recently there were no clues concerning the etiology and pathogenesis of the degeneration and death of motor neurons in ALS. Now, a percentage of patients with familial ALS has been identified as carrying one of several missense mutations in the gene for Cu,Zn superoxide dismutase (SOD) (4–6). Cu,Zn SOD is a metalloenzyme that catalyzes the dismutation of superoxide anion (O2·−) to hydrogen peroxide (H2O2) (7, 8). The gene is located on chromosome 21 and consists of five exons. No deletions of the Cu,Zn SOD gene have been identified in any of the families examined so far. This strongly suggests that the expression of the mutant enzyme is necessary for the pathogenesis of the disease.

To test this hypothesis, in 1994, Gurney et al. (9) generated transgenic mice expressing a mutant form of Cu,Zn SOD, which was identified in one of the families with familial ALS. The selected mutation was in exon 4 of the SOD gene and produced a Gly → Ala change at position 93 (G93A) of the enzyme. Multiple lines of transgenic mice carrying this mutation have now developed severe motor impairment. Clinical and pathological studies have shown a pattern of disease that, in many respects, and especially in its late phases, is very similar to that in familial ALS (9–12). In fact, whereas initial alterations in these mice are mainly characterized by vacuolar changes in axons, dendrites, and cell bodies of motor neurons, later the alterations consist of anterior horn neuronal loss (~50%), neuronal atrophy, axonal swellings with filamentous accumulations, and the presence of cytoplasmic inclusions, closely reminiscent of the Lewy-like bodies described in humans with familial ALS (3, 10, 11). Importantly, mice transgenic for the wild-type form of human SOD and expressing comparable levels of protein and enzyme activity to that seen in mice transgenic for the mutant form never developed clinical disease, and, pathologically, changes remained very subtle and were limited to mild cytoplasmic vacuolization, mainly in motor axons (11). The late pattern of disease, characterized by neuronal loss, never developed in mice overexpressing the wild-type human transgene. These data strongly support the hypothesis that pathological changes in this murine model of familial ALS are due to a new toxic function exerted by the mutant enzyme, rather than to loss of enzyme activity due to a dominant negative effect (9–12).

The cellular target or targets of the presumed toxic effect of the SOD mutations are not known, but significant insights have been gained from ultrastructural studies of degenerating neurons in these transgenic mice. Among the ultrastructural changes observed in mice expressing the mutant SOD transgene, the most readily notable were dilatation of the cisternae of the endoplasmic reticulum and peculiar alterations in mitochondria leading to swelling and linearization of their architecture to form long, curled membranous profiles (10, 11, 13). These changes accounted for most of the vacuolar changes that were observed in the first phase of the disease. In addition to these changes, however, there were also more subtle alterations of the Golgi cisternae, mainly consisting of their dilatation and apparent disorganization (11).

In patients with sporadic ALS, the Golgi apparatus of motor neurons, studied with an organelle-specific antiserum, is fragmented (14–18). This organelle-specific antiserum reacts with MG160, a conserved membrane sialoglycoprotein of the medial cisternae of the Golgi apparatus (19). MG160 has been cloned and sequenced, and the gene for MG160 has been

Abbreviations: ALS, amyotrophic lateral sclerosis; SOD, superoxide dismutase; RER, rough endoplasmic reticulum.

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assigned to the long arm of chromosome 16 (20, 21). Furthermore, developmental studies in chicken embryos have shown that MG160 is a primordial protein of the Golgi apparatus, appearing very early in development (22).

The fragmentation of the Golgi apparatus of motor neurons in ALS resembles a similar change of the organelle induced by drugs that disrupt microtubules (23, 24). Furthermore, all neurons containing ubiquitin-positive inclusions, thought to be pathognomonic for ALS (25), had fragmented Golgi apparatus (17). For these reasons it was proposed that a valid animal model for ALS should be associated with a fragmented Golgi apparatus of motor neurons. Such Golgi pathology may be important to explain, at least in part, the process leading to neuronal death in these patients, because the experimental fragmentation of the organelle has been associated with significant impairment of secretion (26–28). Furthermore, the Golgi apparatus is involved in numerous important functions such as the transport, processing, and targeting of virtually all proteins synthesized in the rough endoplasmic reticulum (RER) and is destined for the secretory pathways, the plasma membrane, or lysosomes (29, 30). Specifically in neurons, all newly synthesized proteins destined for fast axoplasmic transport pass through the Golgi apparatus (31).

In the present study we asked whether human SOD transgenic mice show an alteration of the Golgi apparatus similar to that seen in patients with sporadic ALS. In addition, we examined whether changes in the Golgi apparatus might occur early in the disease process, thus possibly being implicated in the pathogenesis of the neuronal dysfunction. Such questions cannot be answered from human studies, because examination of the central nervous system from ALS patients is generally done at the terminal stages of the disease. Finally, we asked the question whether alterations of the Golgi apparatus were dependent on the expression of the mutant enzyme or they could be produced by the overexpression of the wild-type form of human SOD.

**MATERIALS AND METHODS**

The production of transgenic mice, assessment of levels of SOD protein and enzyme activity, and clinical assessment of neurological disease have already been reported (9–12). Also reported have been both clinical and pathological changes in transgenic mice at different ages. Briefly, the first pathological alterations in the cytoplasm of motor neurons of mutant transgenic animals were seen at ~60 days, while the first clinical abnormalities were observed at ~80 days. In mice overexpressing the wild-type human transgene, clinical signs never developed, and only subtle alterations in the motor axons were observed at later time points.

Guided by those earlier findings, in this study, we selected mice expressing the mutant transgene and mice overexpressing the wild-type transgene, both at different ages from 31 days to 289 days. At least two animals were sacrificed per time point. For morphometric analysis, four mutant and four overexpressor transgenic animals were selected at ~100 days of age.

**Preparation of Tissues for Light Microscopy Immunohistochemistry.** Mice were perfused with phosphate-buffered physiologic saline (PBS), pH 7.35, at room temperature. Perfusion was continued with ~50 ml of freshly prepared 1% paraformaldehyde in PBS. The spinal cord was removed, cut into 2- to 3-mm cross sections, kept in the same fixative at room temperature for 6–8 hr, and processed as previously described (32).

**Morphometry and Statistical Methodology.** Morphometric studies were done with a CUE-2 image analyzer using the planomorphometry program according to a previously described protocol (Olympus, Lake Success, NY) (17). Mean surface area of neurons, their mean nuclear area, the area of the immunostained Golgi apparatus per neuron, the number of Golgi elements per neuron, and the percentage of cytoplasmic area occupied by the Golgi apparatus were measured. Ten motor neurons from each of four mutant SOD transgenic mice and 10 motor neurons from each of four overexpressors of wild-type SOD were analyzed. The statistical significance of values obtained were evaluated by Student's t test.

**Preparation of Tissues for Ultrastructural Immunohistochemistry.** Spinal cords were fixed by perfusion and immersion in 4% paraformaldehyde and 0.05% glutaraldehyde in PBS, pH 7.35. Frozen sections were permeabilized for 30 min in 0.05% saponin, incubated overnight in a polyclonal antiserum against MG160, prepared as described in ref. 32, and incubated 2 hr each in biotinylated anti-rabbit IgG and ABC Elite (Vector Laboratories), all in PBS with 2% fish gelatin. Sections were post-fixed for 30 min in 1.5% glutaraldehyde, stained with 0.05% 3,3'-diaminobenzidine tetrahydrochloride with 0.03% hydrogen peroxide with 10 mM imidazole and 7.5% sucrose in 50 mM Tris buffer, pH 7.4, post-fixed for 1 hr on ice in 1% osmium tetroxide with 1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer, pH 7.4, and embedded in Araldite (33).

**RESULTS**

**Light Microscopy.** All animals expressing the mutant transgene for Cu,Zn SOD showed a severe fragmentation of the Golgi apparatus, which was identical to that described in ALS patients (Fig. 1). It is important to note that the fragmentation of the Golgi was present as early as 31 days of age, the earliest time point examined, which is ~2 months before the first observable signs of clinical dysfunction. At that time point (31 days), the severity of the fragmentation of the Golgi apparatus in any affected motor neuron was comparable to that observed at later time points. In contrast, the neurons of mice transgenic for the wild-type human SOD did not show fragmentation of the Golgi apparatus, even as late as 289 days of age.

**FIG. 1.** Mutant Cu,Zn SOD transgenic animal at 104 days of age. Section of mouse spinal cord immunostained with polyclonal antiserum against MG-160, a Golgi-specific membrane protein, is shown. Arrow points to a neuron with fragmented or dispersed Golgi apparatus. For comparison note the immediately adjacent neuron, which contains a normal network of immunostained elements of the Golgi apparatus. All neurons in mice overexpressing the wild-type human enzyme had a similar normal appearance. Arrowhead points to the axon of the neuron with the dispersed Golgi apparatus; the initial thin segment of the axon is followed by the enlarged segment that probably corresponds to the described spheroids in these mice and in ALS. (x750.)
As previously reported, the first changes observed in motor neurons of transgenic mice at both light and ultrastructural levels consist of vacuolization of motor axons, followed by the neuronal cytoplasm and dendrites (10, 11). Vacuoles derive from swellings of both the cisternae of the endoplasmic reticulum and, especially, mitochondria, which eventually transform into linear or curved membranous arrays.

In paraffin sections from all animals examined in this study, we did not observe vacuolization of the neuronal cytoplasm without fragmentation of the Golgi apparatus, whereas fragmentation of this organelle was frequently observed in neurons without apparent cytoplasmic vacuolization. As shown in Table 1, in symptomatic animals of ~100 days of age, there was variation in the percentage of motor neurons with fragmented Golgi. In animals with greater Golgi fragmentation (8290 and 8291), there was greater vacuolization, and only 10% of neurons with Golgi fragmentation were not vacuolated. In animals with lesser Golgi fragmentation (9042 and 9043), vacuolization was also less severe, and 70–80% of motor neurons with fragmented Golgi were not vacuolated. However, by electron microscopy we detected a few neurons with a few cytoplasmic vacuoles and an apparently intact Golgi apparatus (data not shown), suggesting that fragmentation of the Golgi apparatus and cytoplasmic vacuolization occur independently, although the Golgi fragmentation probably occurs earlier. As more neurons become affected, more neurons will present both Golgi changes and vacuolar pathology. In neurons where vacuoles and altered Golgi apparatus coexist, vacuoles appear to be surrounded by dispersed round elements of the immunostained fragmented Golgi apparatus (Fig. 2).

Additional morphometric analyses were done in the above mutant transgenic mice and in mice overexpressing wild-type human SOD, at comparable ages (Table 2). Significant differences were observed between the motor neurons of the mutant transgenic mice with fragmented Golgi apparatus and the motor neurons of the overexpressors with normal Golgi. The mutant mice showed smaller motor neurons with smaller nuclei and more numerous Golgi elements, indicative of fragmentation, but they showed less total "amount" of Golgi apparatus. These findings are similar to previous observations in human ALS.

**Electron Microscopy.** The immunoperoxidase localization of the anti-MG160 antiserum appeared predominantly in one to two medial cisternae of the Golgi stacks, flanked by 1–3 unstained cisternae (Figs. 3–5). The Golgi apparatus from normal-appearing spinal cord motor neurons was found in the proximity of the RER, and consisted of the usual stacks of 5–7 cisternae, which were surrounded by numerous unstained smooth vesicles (Figs. 3 and 4). The elements of the fragmented Golgi apparatus were detected at the margins of cytoplasmic vacuoles. The stacks of the cisternae were shorter than in the normal-appearing Golgi, and there was a reduction of Golgi-associated unstained vesicles, while neighboring RER was scanty or absent (Fig. 5). The adjacent cytoplasmic vacuoles were partially enclosed by remnants of membranes, the origins of which could not be determined (Fig. 5).

**DISCUSSION**

We have suggested that fragmentation of the Golgi apparatus is an early response of motor neurons to injury during the

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**Table 1.** Fragmentation of the Golgi apparatus of spinal cord motor neurons in mutant SOD transgenic mice

<table>
<thead>
<tr>
<th>Mutant SOD transgenic mouse</th>
<th>Age, days</th>
<th>Total percentage of neurons with fragmented Golgi apparatus (with or without vacuolization)</th>
<th>Percentage of total neurons with fragmented Golgi apparatus but no vacuolization</th>
<th>Total number of neurons counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>8290</td>
<td>Cervical</td>
<td>20</td>
<td>2.0</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Lumbar</td>
<td>25</td>
<td>2.5</td>
<td>99</td>
</tr>
<tr>
<td>8291</td>
<td>Cervical</td>
<td>27</td>
<td>2.7</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Lumbar</td>
<td>29</td>
<td>2.9</td>
<td>102</td>
</tr>
<tr>
<td>9042</td>
<td>Cervical</td>
<td>13</td>
<td>9.1</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Lumbar</td>
<td>15</td>
<td>10.5</td>
<td>153</td>
</tr>
<tr>
<td>9043</td>
<td>Cervical</td>
<td>8</td>
<td>6.4</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Lumbar</td>
<td>11</td>
<td>8.8</td>
<td>162</td>
</tr>
</tbody>
</table>
development of ALS and in certain motor neuropathies (14–18). However, it was difficult to discern in those human studies whether such Golgi alterations represented a terminal event in a dying neuron or earlier changes contributing significantly to the process of neuronal degeneration. Time-course studies to explore whether changes of the Golgi apparatus are indeed early events in ALS and related diseases are, obviously, not possible in humans, although it would be possible in an appropriate animal model. Our transgenic model with expression of a mutant form of Cu,Zn SOD, identified in one of the families with familial ALS, gave us the perfect opportunity to look into this important question.

Cu,Zn SOD is an important enzyme in the cascade of events leading to the neutralization of injurious oxygen radicals in tissues. Its main function is to scavenge superoxide anion (\( \text{O}_2^- \)) into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), which is then decomposed to water by glutathione peroxidase. How mutant human Cu,Zn SOD damages motor neurons is unclear, but most hypotheses propose that damage is mediated by free radicals. Hypotheses include the generation of hydroxyl radical (34), the catalysis of peroxynitrite-mediated nitration of proteins (35), and the release of free copper, which might then catalyze oxidative damage to lipids and proteins. For example, mutation may alter the capacity of the enzyme to catalyze free radical formation or may promote nitrating reactions. Superoxide reacts with nitric oxide (NO) to form peroxynitrite anion (ONOO\(^-\)). The reaction of mutant Cu,Zn SOD with ONOO\(^-\) may promote formation of toxic nitronium intermediates, which would produce unwanted nitration of proteins (35, 36). Thus, either lipid peroxidation promoted by free radical species or protein nitration may initiate the pathogenesis of ALS.

This study shows fragmentation of the Golgi apparatus of spinal cord neurons in mutant transgenic mice with an identical pattern to that observed in patients with ALS. In addition, this study strongly suggests that the Golgi apparatus may be one of the earliest targets of the SOD mutation. Two features support this contention. First, the fragmentation of the Golgi apparatus was seen in asymptomatic animals. Second, while virtually all neurons with vascular changes contained fragmented Golgi apparatus, many neurons with fragmented Golgi had no vascular changes. These data strongly suggest that the alterations of the Golgi apparatus usually precede the vascular changes in motor neurons of transgenic animals. The discovery of lesions in the Golgi apparatus at very early times in the transgenic studies, before the development of any other pathological or clinical evidence of disease, is potentially important. The Golgi apparatus is a crucial component of the cell, because it is involved in the transport and processing of polypeptides as well as in their targeting to different destinations, such as plasma membranes and lysosomes, and to their secretion (29, 30). Previous studies with ALS patients had suggested that fragmentation of a structure with such crucial roles in the cellular handling of proteins was likely to be associated with significant impairment of function. The new immunouultrastructural data of this study support this hypothesis. Specifically, the paucity of vesicles in the vicinity of the shortened Golgi cisternae (Fig. 5) imply an impairment of molecular traffic between Golgi cisternae and from the Golgi apparatus to the periphery—i.e., along dendrites, axons, and presynaptic terminals. It is quite possible that membranes of the Golgi apparatus, or key proteins mediating the anchorage of membrane-bound vesicles, may be a common feature of many neurodegenerative diseases.

### Table 2. Morphometric studies in spinal cord motor neurons in SOD transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Mean neuron area</th>
<th>Mean nuclear area</th>
<th>Golgi area/neuron</th>
<th>Golgi elements/neuron</th>
<th>% Golgi/neuron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal SOD transgenic mice (overexpressors)</td>
<td>1138 ± 492.8</td>
<td>222.8 ± 72.6</td>
<td>213.2 ± 62.5</td>
<td>28.1 ± 7.1</td>
<td>18.7 ± 3</td>
</tr>
<tr>
<td>Mutant SOD transgenic mice</td>
<td>899.9 ± 248.5</td>
<td>122.4 ± 39.1</td>
<td>76.6 ± 29.3</td>
<td>48.5 ± 13.8</td>
<td>8.5 ± 2.3</td>
</tr>
</tbody>
</table>

Areas are in square micrometers. *P* values are based on Student's *t*-test. Forty motor neurons from each category were analyzed; 10 motor neurons from each of four overexpressors (mice 8730, 8731, 8986, and 8987) and 10 motor neurons from each of four SOD mutants (mice 8290, 8291, 9042, and 9043). Five-micron sections of cells containing nuclei were analyzed. To avoid analyzing a cell twice, in the serial sections, only every 10th section was included in the study. Statistics were based on the number of animals with 7 degrees of freedom.

![Fig. 3](image1.png) Normal-appearing neuron. Three groups of the Golgi apparatus show one immunostained medial cisterna in a stack of unstained 5 to 7 cisternae. Note that the stacks of Golgi cisternae are surrounded by unstained vesicles. A few cisternae of the RER are seen at the lower left of the electron micrograph. (×16,000.)

![Fig. 4](image2.png) One stack of Golgi cisternae surrounded by numerous clear vesicles from a normal-appearing motor neuron. Note immunostained medial cisternae of the organelle and abundance of the RER in the proximity of the Golgi apparatus. (×16,000.)
It is unlikely that the fragmentation of the Golgi apparatus is due to a Brefeldin A-like effect of the mutant proteins (44, 45). In Brefeldin A-treated cells, the Golgi stacks are disrupted, while they are present here (Fig. 5); furthermore, in Brefeldin A-treated cells, proteins of the Golgi apparatus are redistributed in the RER including the nuclear envelope, which was never observed in sporadic ALS or in these transgenic mice (14–18, 40, 46).

 Whereas vacuoles in the cytoplasm of motor neurons and processes have been found in human SOD transgenic mice, these changes have not been reported in human ALS, although they have been observed in motor neurons in tetanus, in which oxidative stress has been postulated as a causative factor (47). However, vacuoles are predominantly a feature of the early phase of disease in the G1 line of mice which have relatively high levels of expression of the human transgene. Animals with lower expression of the transgene, such as the G20 and G5 lines of animals, only show minimal vacuolar changes. These animals—i.e., the G20 (9–11) and G5 lines (unpublished data)—survive long enough to develop a pattern of disease which is characterized by neuronal loss, atrophy of anterior horns, and filamentous inclusions. These changes are essentially identical to those in human ALS, particularly the familial form (48, 49).

 Therefore, it is quite possible that in humans with ALS, if there is indeed an early phase of vacuolar changes, these are modest and more similar to those in transgenic mice with intermediate levels of expression of the mutant transgene. Furthermore, it is likely that the vacuolar phase would go undetected because, in ALS patients, the examination of the central nervous system is generally performed at the end stage of the disease process.

 Another significant result of this study is that mice overexpressing the wild-type human SOD fail to develop fragmentation of the Golgi apparatus as late as 289 days after birth. This observation is consistent with the conclusion that the fragmentation of the Golgi apparatus in SOD transgenic mice is dependent on the presence of the mutant enzyme. This supports the gain of function hypothesis for the pathogenesis of mutant SOD-induced cellular injury in this animal model of ALS.

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