Decreased Cystatin C Immunoreactivity in Spinal Motor Neurons and Astrocytes in Amyotrophic Lateral Sclerosis

Fumiaki Mori, PhD, Kunikazu Tanji, PhD, Yasuo Miki, MD, and Koichi Wakabayashi, MD

Abstract
Cystatin C (CC), a cysteine protease inhibitor involved in protein degradation, is a marker of Bunina bodies in lower motor neurons in amyotrophic lateral sclerosis (ALS). TAR-DNA binding protein-43 (TDP-43)–immunoreactive inclusions are also histological hallmarks of ALS but whether CC is found in motor neurons with or without TDP-43–positive inclusions in ALS is not known. To determine whether inclusion body formation affects cytoplasmic CC immunoreactivity, we examined spinal cords from 9 ALS patients and 12 control subjects by immunohistochemistry. Most anterior horn cells (AHCs) showed moderate to intense immunoreactivity in controls, whereas CC immunoreactivity was markedly decreased in AHCs in ALS cases. The proportion of CC-immunolabeled AHCs was significantly reduced regardless of whether they contained Bunina bodies. In contrast, the proportion of CC-immunolabeled AHCs was significantly reduced in those with TDP-43 inclusions. Cystatin C immunoreactivity of astrocytes in the spinal gray matter and white matter in ALS was significantly decreased compared with controls. These findings suggest that the formation of TDP-43 inclusions, but not of Bunina bodies, may be linked to the content of CC in spinal motor neurons and that perturbations in endogenous levels of CC in neuronal and glial cells may be part of the neurodegenerative processes in ALS.

Key Words: Amyotrophic lateral sclerosis, Bunina body, Cystatin C, Immunohistochemistry, Skeinlike inclusion, TDP-43.

INTRODUCTION
Cystatin C (CC) is a cysteine protease inhibitor that is involved in lysosomal and endosomal protein degradation (1, 2) and in cell-matrix interactions (3, 4). Previous immunohistochemical studies have demonstrated that antibodies against CC label the Bunina bodies (BBs) that are observed in amyotrophic lateral sclerosis (ALS), an adult-onset neurodegenerative disorder that affects both upper and lower motor neurons (2, 5). Bunina bodies are small eosinophilic intracytoplasmic inclusions 1 to 3 μm in diameter in residual lower motor neurons (6). Ultrastructural studies have shown that these inclusions consist of a homogeneous electron-dense granular matrix surrounded by vesicular and tubular structures (7). Ubiquitinated inclusions, including skeinlike and round inclusions, are also histological hallmarks of ALS (8). TAR-DNA binding protein-43 (TDP-43) is a major protein of ubiquitinated inclusions in cases of ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions with or without motor neuron disease (9, 10).

Recently, several investigators reported that the levels of TDP-43 protein in the cerebrospinal fluid from patients with ALS and frontotemporal lobar degeneration were higher than those in controls (11, 12). In contrast, concentrations of CC in the cerebrospinal fluid from patients with ALS are significantly lower than in normal controls, suggesting possible sequestration of CC in BBs (13, 14). The localization of CC in motor neurons with or without inclusions in ALS is, however, uncertain. We examined CC expression in spinal cord samples from patients with ALS and controls by immunohistochemistry and immunoelectron microscopy and found that CC immunoreactivity is markedly decreased in anterior horn cells (AHCs) and astrocytes in ALS compared with controls.

MATERIALS AND METHODS

Subjects
The spinal cords of 9 patients with sporadic ALS, 3 of whom had dementia (Cases 1–3), were studied (Table). The diagnosis of ALS was made on both clinical and pathological grounds. The ages at death ranged from 53 to 76 years, and the duration of illness ranged from 18 to 136 months. Bunina bodies were found in the AHCs in 6 of 9 cases and TDP-43–positive inclusions in all the cases. We also examined 6 neurologically normal individuals (normal control), aged between 51 and 84 years, and 6 patients with various neurological diseases affecting the spinal anterior horn with ages between 47 and 79 years (Table). For routine histological examination, the brain and spinal cord of each subject was fixed with 10% buffered formalin for 3 weeks and then embedded in paraffin; 4-μm-thick sections were cut and stained with hematoxylin and eosin (H&E) and by the Klüver-Barrera method.

Immunohistochemistry
Serial 4-μm-thick paraffin-embedded sections were cut from the seventh cervical, eighth thoracic, and fourth lumbar...
### TABLE. Clinical and Pathological Features of Patients With ALS and Controls

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Age at Death, Years</th>
<th>Sex</th>
<th>Disease Duration, Months</th>
<th>Pathology of Anterior Horn Cells</th>
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<tr>
<td>1</td>
<td>ALS</td>
<td>53</td>
<td>F</td>
<td>18</td>
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<td>2</td>
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<td>67</td>
<td>F</td>
<td>18</td>
<td>2 Bunina Body (+), TDP-43 Positive (+)</td>
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<td>ALS</td>
<td>59</td>
<td>M</td>
<td>24</td>
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<tr>
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<td>M</td>
<td>24</td>
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<tr>
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<td>M</td>
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<tr>
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<td>F</td>
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<td>M</td>
<td>108</td>
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<td>M</td>
<td>108</td>
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<tr>
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<td>−</td>
<td>0 Neuronal Loss (−), Bunina Body (−)</td>
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<tr>
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<td>16</td>
<td>Multiple-system atrophy</td>
<td>79</td>
<td>M</td>
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<tr>
<td>17</td>
<td>Multiple-system atrophy</td>
<td>66</td>
<td>M</td>
<td>108</td>
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<tr>
<td>18</td>
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<tr>
<td>21</td>
<td>Metastatic carcinoma</td>
<td>55</td>
<td>M</td>
<td>13</td>
<td>1 Neuronal Loss (−), Bunina Body (−)</td>
</tr>
</tbody>
</table>

Pathological scores: 0, none; 1, mild; 2, moderate; 3, severe; +, present; −, absent.

ALS, amyotrophic lateral sclerosis; F, female; M, male; TDP-43, TAR-DNA binding protein-43.

### FIGURE 1. Cystatin C immunoreactivity in the spinal cord in control (A–C) and amyotrophic lateral sclerosis (ALS) (D–F) cases. (A) Anterior horn cells (AHCs) show strong immunoreactivity. (B) Higher magnification of the area indicated by an asterisk in (A) shows diffuse granular cytoplasmic staining. (C) Medium- to small-sized neurons in Clarke column show weak to moderate immunoreactivity. (D) Anterior horn cells in ALS show weak immunoreactivity. (E) Higher magnification of the area indicated by asterisk in (D) shows no or only weak immunoreactivity. (F) An AHC containing Bunina bodies (arrowheads) was stained with H&E (left) and subsequently immunostained with anti-cystatin C antibody (right). Scale bars = (A–E) 50 μm; (F) 10 μm.
spinal cord segments; 3 sections 40 μm apart were subjected to immunohistochemical investigations using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were pretreated with heat retrieval using an autoclave for 10 minutes at 121°C in 10 mmol/L citrate buffer (pH 6.0) and were then immunostained with a polyclonal antibody against CC (A0541; DakoCytomation, Glostrup, Denmark; 1:5000), a polyclonal antibody against TDP-43 (10782-1-AP; ProteinTech Group, Inc, Chicago, IL; 1:4000), and a monoclonal antibody against phosphorylated TDP-43 (phospho Ser409/410; Cosmo Bio Co, Ltd, Tokyo, Japan; 1:1000). Diaminobenzidine was used as the chromogen. The sections were counterstained with hematoxylin.

Sections from patients with ALS were also double immunolabeled with a polyclonal antibody against CC (1:5000) and a monoclonal antibody against phosphorylated TDP-43 (1:1000). The immunoproducts of the monoclonal antibody were detected by the ABC method with diaminobenzidine as the chromogen. The immunoproducts for the polyclonal antibody were detected by the ABC method with streptavidin-alkaline phosphatase as the tertiary reagent and Vector Blue (Vector Laboratories) as the chromogen for alkaline phosphatase.

**Immunoelectron Microscopy**

Vibratome sections of the spinal cord from 2 ALS patients (Cases 3 and 5) and 2 control subjects (Cases 11 and 15) were blocked with normal goat serum and incubated with a polyclonal antibody against CC (1:5000) and a monoclonal antibody against phosphorylated TDP-43 (1:1000). The immunoproducts of the monoclonal antibody were detected by the ABC method with diaminobenzidine as the chromogen. The immunoproducts for the polyclonal antibody were detected by the ABC method with streptavidin-alkaline phosphatase as the tertiary reagent and Vector Blue (Vector Laboratories) as the chromogen for alkaline phosphatase.

**Semiquantitative Analysis**

The numbers of CC-immunoreactive neurons in controls and ALS patients were assessed using a semiquantitative rating scale as follows: −, unstained; +, weakly stained; ++, moderately or intensely stained. The staining intensity was first graded as − or +, and the intensity that was neither − nor ++ was designated as +. In each case, the numbers of neurons were counted in Rexed laminae VIII and IX of lumbar spinal cord. In ALS cases, the presence or absence of BBs was confirmed in each neuron on sections stained with H&E. After photographing BB-containing neurons, the sections were immunostained with anti-CC antibody. The numbers of CC-immunoreactive neurons were assessed using the previously mentioned scale. The number of CC-immunoreactive neurons with or without TDP-43-positive inclusions was counted on sections double immunolabeled with anti-CC and anti–TDP-43 antibodies.

**FIGURE 2.** Cystatin C immunoreactivity of anterior horn cells in normal and diseased controls and amyotrophic lateral sclerosis. The proportions of neurons with intense/moderate (+++, black column), weak (+, gray column), or no immunostaining for cystatin C (−, white column) relative to the total number of neurons in the anterior horn are indicated.

**FIGURE 3.** (A) Cystatin C immunoreactivity of anterior horn cells with (+) and without (−) Bunina bodies in amyotrophic lateral sclerosis (ALS) cases. The proportions of neurons with intense or moderate (+++, black column), weak (+, gray column), or no immunostaining for cystatin C (−, white column) relative to the total number of neurons in the anterior horn are shown. (B) Cystatin C immunoreactivity of anterior horn cells with (+) and without (−) TAR-DNA binding protein-43 (TDP-43)–positive inclusions in ALS cases. The proportions of neurons with intense or moderate (+++, black column), weak (+, gray column), or no immunostaining for cystatin C (−, white column) relative to the total number of neurons in the anterior horn are shown.
Counting was performed at 200× original magnification using an eyepiece graticule and parallel sweeps of the microscope stage.

The intensity of CC immunoreactivity of astrocytes in the anterolateral and posterior funiculi of the spinal cord in each case was classified on an arbitrary scale (0, none; 1, weak; 2, moderate; 3, intense) in CC-immunostained sections.

**Statistical Analysis**
Calculations were performed using Statcel software (OMS Publishing, Tokorozawa, Japan). Statistical comparison was performed with repeated-measures analysis of variance and Student or Welch t-test. Values were expressed as mean ± SEM. Probability values at p < 0.05 were considered significant.

**RESULTS**

**CC Immunoreactivity in Spinal Gray Matter**

In normal and diseased controls, anti-CC antibody strongly immunolabeled the cytoplasm of AHCs in a diffuse granular pattern (Figs. 1A, B). The cytoplasm in medium- and small-sized neurons in the intermediolateral nucleus, Clarke column, and posterior horn showed weak to moderate immunoreactivity (Fig. 1C). In ALS cases, CC immunoreactivity was decreased in most AHCs (Fig. 1D), although BBs were intensely immunolabeled (Fig. 1E). Sequential staining of the same sections with H&E and anti-CC antibody revealed that BBs were clearly immunolabeled with anti-CC antibody (Fig. 1F). The outer portion of relatively large BBs was more intensely immunostained. Cystatin C immunoreactivity in the intermediolateral nucleus, Clarke column, and posterior horn was preserved in ALS cases.

Semiquantitative analysis showed that 89.9% of AHCs were moderately or intensely immunolabeled and 10.1% were weakly immunolabeled in normal controls (Fig. 2). Similarly, 95.0% of AHCs were moderately or intensely immunolabeled and 5.0% were weakly immunolabeled in diseased controls (Fig. 2). Staining intensity differences between normal and diseased controls were not statistically significant. In ALS, a small proportion of AHCs (18.9%) showed moderate or intense immunoreactivity, whereas more than half of AHCs (55.3%) showed weak immunoreactivity and a quarter (25.8%) were unstained (Fig. 2). Staining intensity differences between normal control and ALS cases and between diseased control and ALS cases were significant.

**Relationship Between Decrease of CC Immunoreactivity and Inclusion Body Formation in AHCs**

More than half of BB-containing AHCs (56.9%) showed weak CC immunoreactivity; the rest (43.1%) were unstained (Fig. 3A). Cystatin C immunoreactivity was also decreased in neurons without BBs; 42.8% of AHCs without BBs showed weak immunoreactivity and 32.3% were unstained (Fig. 3A).

![FIGURE 4. Cystatin C immunoreactivity in astrocytes in the spinal cord in control (A–C) and amyotrophic lateral sclerosis cases (D–F). (A, B) Astrocytes in the anterior horn (A) and in the white matter (B) show strong immunoreactivity. (C) Higher magnification of the area indicated by an asterisk in (B) shows diffuse cytoplasmic immunoreactivity. (D, E) Reactive astrocytes in the anterior horn (D) and in the white matter (E) show faint immunoreactivity. (F) Higher magnification of the area indicated by an asterisk in (E) demonstrates weak cytoplasmic immunoreactivity. Scale bars = 25 μm.](image-url)
There was no significant difference in the staining intensity between AHCs with and without BBs.

Cystatin C immunoreactivity was decreased in most neurons with TDP-43-positive inclusions; 46.8% of AHCs with TDP-43-positive inclusions showed weak immunoreactivity and 52.1% were unstained (Fig. 3B). Cystatin C immunoreactivity was also decreased in neurons without TDP-43-positive inclusions; 61.4% of AHCs without TDP-43-positive inclusions showed weak immunoreactivity and 18.3% were unstained (Fig. 3B). Cystatin C immunoreactivity was weaker in the AHCs with TDP-43-positive inclusions than those without.

**CC Immunoreactivity in the Spinal White Matter**

In normal and diseased controls, the cytoplasm and processes of astrocytes both in the gray matter and white matter showed diffuse or finely granular immunoreactivity (Figs. 4A–C). Oligodendroglial cells were not stained. In ALS, reactive astrocytes in the anterior horn contained a small amount of CC-immunoreactive fine granules (Fig. 4D). Cystatin C immunoreactivity in astrocytes in the white matter was markedly decreased in 4 cases and mildly decreased in 3 cases of ALS compared with controls (Figs. 4E, F). Semi-quantitative analysis showed that CC immunoreactivity of astrocytes in the anterolateral and posterior funiculi in ALS was significantly decreased compared with normal and diseased controls (Fig. 5).

**Immunoelectron Microscopy**

Two types of BBs were observed in the AHCs in patients with ALS. One type consisted of moderately electron-dense amorphous material surrounded by many vesicular structures, as has been considered to be an early stage of BB formation (15). The other consisted of highly electron-dense granular material surrounded by a few vesicular structures, sometimes with a clear central area containing filaments approximately 10 nm in diameter and cell organelles such as mitochondria, corresponding to an advanced stage of BB formation (15, 16). Cystatin C labeling of BBs was localized to the vesicular structures but not to the electron-dense material. In the early-stage BBs, gold particles were seen in the vesicular structures within and around BBs.
DISCUSSION

Previous immunohistochemical studies have shown that CC immunoreactivity is widely distributed in brain neurons, astrocytes, and choroid plexus epithelium (17); normal human motor neurons in the brainstem and spinal cord show diffuse granular cytoplasmic immunoreactivity (18). Consistent with previous studies, we found CC immunoreactivity in AHCs to be more intense than that of small- and medium-sized neurons in the normal human spinal cord. In addition, astrocytes in the spinal gray matter and white matter were also intensely immunolabeled with anti-CC antibody.

Cystatin C has been reported to be upregulated in response to various brain injuries. In Alzheimer disease, CC immunostaining was increased in pyramidal neurons of the temporal cortex (19); and in temporal lobe epilepsy, CC immunoreactivity was increased in astrocytes in the molecular layer of the dentate gyrus (20, 21). Cystatin C expression is also increased in neurons and reactive astrocytes in the murine hippocampus after transient forebrain ischemia (22, 23) and perforant pathway transection (24), and the CC mRNA and protein levels are increased in neurons, astrocytes, and microglia in the striatum after dopamine depletion (25). These results indicate that CC may play an important role in neuroprotection by inhibition of cysteine proteases (26).

By contrast, we found that CC immunoreactivity was markedly decreased in AHCs in ALS, suggesting that there could be some differences in the roles of CC between ALS and other neurological disorders. Because CC is a cysteine protease inhibitor, decreased CC may imply increased proteolysis via upregulation of AHC cysteine proteases in ALS. This is supported by the finding that the immunoreactivity of cathepsin B, one of the cysteine proteases inhibited by CC, was decreased in the cytoplasm of AHCs in ALS (27).

In the present study, the presence of BBs was not correlated with a decrease of CC immunoreactivity, whereas the presence of TDP-43–positive inclusions was associated with decreased proportions of AHCs with CC immunoreactivity. Van Welselm et al (18) reported the relationship between the severity of neuronal loss and the proportion of skeinlike inclusion-containing neurons in the spinal cord, although no relationship was found with BBs. Our findings suggest that the formation of TDP-43–positive inclusions but not of BB may reduce CC content in spinal motor neurons or that reduction of CC in neurons may accelerate the formation of TDP-43–positive inclusions, but not of BB.

Our immunoelectron microscopic results clearly demonstrate that CC is localized to the vesicular membranous structures, but not to the electron-dense material, of BBs. It is noteworthy that CC immunoreactivity of BBs is decreased with the maturation of BBs. Moreover, the presence of BBs had no influence on the decrease of CC immunoreactivity of AHCs in ALS. Thus, it is likely that the decrease of CC immunoreactivity of AHCs is not simply caused by an increased sequestration of CC into BBs.

To our knowledge, this is the first demonstration of reduced cytoplasmic CC immunoreactivity in astrocytes both in the gray matter and white matter in ALS. Moreover, in situ hybridization analysis indicated that CC is synthesized and released by glial cells and choroid plexus epithelium (26). These findings suggest that the decrease of CC production in astrocytes might be responsible for the reduction of CC concentration reported in the cerebrospinal fluid in ALS cases (13, 14).

TAR-DNA binding protein 43–immunoreactive inclusions occur both in oligodendrocytes and astrocytes in ALS but are more frequent in the former (28, 29). Accumulation of TDP-43 might cause decreased CC in astrocytes, or decreased CC in astrocytes may cause TDP-43 to accumulate as insoluble protein aggregates.

In conclusion, we have demonstrated that the decreased CC immunoreactivity in AHCs is closely associated with the presence of TDP-43–positive inclusions in patients with ALS and that CC immunoreactivity in astrocytes in the spinal cord is also decreased in ALS. These perturbations in endogenous levels of CC in neuronal and glial cells may be part of the neurodegenerative processes in ALS.

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REFERENCES


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