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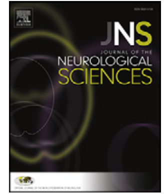

Loss of calretinin- and parvalbumin-immunoreactive axons in anterolateral columns beyond the corticospinal tracts of amyotrophic lateral sclerosis spinal cords

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ABSTRACT

In amyotrophic lateral sclerosis (ALS) spinal cords, diffuse myelin pallor (dMP) in the anterolateral columns (ALCs) beyond the corticospinal tracts has been frequently observed; however, its origin still remains to be elucidated. To address this issue, we focused on calretinin (CR) and parvalbumin (PV), since these buffer calcium-binding proteins (CaBP) are predominantly expressed in axons in the ALCs of neurologically normal human spinal white matter. Immunohistochemical methods revealed that numbers of both CR-immunoreactive (ir) and PV-ir axons were significantly lower in ALS patients' spinal cords with dMP compared to those in controls. In ALS patients' spinal cords without dMP, there were also significant reductions in the number of these CaBP-ir axons compared to controls. In contrast, the number of CR-ir neurons in the spinal gray matter did not differ significantly among ALS patients and controls. These findings suggest that a loss of CaBP-ir axons may precede the development of dMP in ALS patients' spinal cords, and the dying back mechanism would underlie this phenomenon.
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1. Introduction

Since Charcot's description [1], the pathognomonic significance of corticospinal tract degeneration [1–3] has been established in the spinal cords of amyotrophic lateral sclerosis (ALS) patients. Diffuse myelin pallor (dMP) [1,4–7] in the anterolateral columns (ALCs) outside the corticospinal tracts has also been observed frequently in ALS patients, and this finding suggests the presence of another tract degeneration in the area. Some authors have proposed possible mechanisms of dMP; however, the origins of the degenerating fibers have not been clarified [8,9]. We previously documented that many macrophage/microglial infiltrations in the ALCs of ALS patients and the distributions corresponded well with those of dMP, and immunoelectron microscopy revealed that these infiltrating cells surrounded and phagocytosed myelin degradations [6]. These results suggest that dMP would be derived from degenerating fiber tracts lying widely in the ALCs outside the corticospinal tracts and not runnning in the posterior columns. Several candidates for such fiber tracts are reticulospinal-, tectospinal-, vestibulospinal-, and rubrospinal-tracts, and medial longitudinal fasciculus. We considered it unlikely, however, since, in the ALS brainstems, macrophage/microglial infiltrations were mostly restricted within the corticospinal tracts, and those in other areas were similar to controls, indicating that the dMP in the ALCs beyond the corticospinal tracts of ALS patients would be derived from intrinsic spinal cord lesions, and not degenerating nerve fibers connecting with the brainstems [6]. During immunohistochemical examinations of the human spinal cords, we found that calretinin (CR)- and parvalbumin (PV)-immunoreactive (ir) axons were widely distributed in the ALCs outside the corticospinal tracts and not observed in the posterior columns. Therefore, we quantitatively examined the relationship between degrees of dMP and numbers of CR- and PV-ir axons in the spinal white matter of ALS patients.

2. Materials and methods

2.1. Histological examinations

We examined the spinal cords of 15 sporadic ALS patients (56.3 ± 10.5 years) and 5 subjects without neurological complications (controls, 64.4 ± 18.9 years). Clinical profiles of the ALS patients in this study were reported previously [7]. Ten-percent buffered formalin-fixed, paraffin-embedded, 5-μm-thick transverse sections were examined by Klüver–Barrera (KB) staining and immunohistochemical methods. We used a goat polyclonal calretinin (CR) antibody (Chemicon, Temecula, CA), a mouse monoclonal parvalbumin (PV) antibody (Sigma, Saint Louis, MO), a rabbit polyclonal PV antibody (Novus Biologicals Inc., Littleton, CO), and mouse monoclonal

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SMI-31 (Covance, Berkeley, CA) as primary antibodies. After being deparaffinized, the specimens were quenched with 0.3% H_2O_2, and treated with nonimmune serum as the blocking agent. All sections were autoclaved for 10 min in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval and then incubated for 48 h at 4 °C with the primary antibodies. Antibody binding was visualized using the avidin–biotin immunoperoxidase complex method employing an Elite ABC kit (Vector Laboratories, Burlingame, CA). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was the final chromogen. For the immunofluorescent study, Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (Molecular Probes-Invitrogen, Eugene, OR), Alexa Fluor 488 donkey anti-goat IgG (H + L) antibodies (Molecular Probes-Invitrogen), and Alexa Fluor 568 donkey anti-mouse IgG (H + L) antibodies (Molecular Probes-Invitrogen) were used as secondary antibodies. To avoid autofluorescence signals, sections were treated with Sudan Black B for 10 min and rinsed in 70% ethanol. Specimens were mounted with Vectashield (Vector Laboratories) and examined under a microscope equipped with a confocal system (FV1000; Olympus). The images obtained were processed further using Adobe Photoshop CS2 version 9 (Adobe, San Jose, CA).

2.2. Quantification of axons immunoreactive for CR and PV in the spinal white matter of controls and ALS patients

Intensities of dMP in the ALCs outside the corticospinal tracts of ALS spinal cords were classified into 3 categories by KB staining (none: MP−, moderate: MP+, and severe: MP++), as previously reported [7]. Briefly, if the intensity of KB staining in the ALCs was similar to that of posterior columns, it was designated as MP−, when the intensity was markedly decreased like that of severely degenerated corticospinal tracts, it was designated as MP++, and if the intensity was intermediate between MP− and MP++, it was designated as MP+. The data on the numbers of axons immunoreactive for CR and PV were obtained from controls and ALS patients who were MP− ($n = 5$), MP+ ($n = 5$), and MP++ ($n = 5$). The ALCs were divided anteriorly into three equal parts from the medial to lateral margins of the anterior horn [6]. Photographs from the lateral, mid-lateral, and mid-medial portions (Fig. 2) were taken at 600-fold magnification, we extended them to 90 × 40 mm (corresponding to 0.15×0.067 mm $= 0.01$ mm^2) on paper, and the numbers of CR-ir and PV-ir axons were counted. The total examined area of ALCs in one slice was 0.03 mm^2 (0.01 mm^2 × 3) in each patient.

2.3. Quantification of neurons immunoreactive for CR in the spinal gray matter

The spinal gray matter was divided into three distinct areas (Fig. 2) based on a slight modification of the method which we previously employed [7]. The anterior part (AP), intermediate part (IMP), and posterior part (PP) were bilaterally examined to quantify the numbers of CR-ir neurons with visible nuclei, and we compared those in ALS spinal gray matter to controls or those among the 3 ALS categories.

2.4. Statistical analyses

Statistical evaluations were performed using ANOVA with Newman–Keuls post hoc comparison, and $p < 0.05$ was considered significant. The results are presented as the mean ± standard deviation.

3. Results

3.1. Double immunohistochemistry using fluorescent antibodies

Confocal microscopy revealed that all immunoreactivities of CR (Fig. 1a) and PV (picture not shown) in the axons showed colocalization with those of the axonal marker (Fig. 1b) in the ALCs outside the corticospinal tracts of control spinal cords (Fig. 1c).

3.2. Light-microscopic observations

CR-ir axons were present in two regions with a different density, neither of which could be assigned with certainty to anatomically defined fiber tracts. There were CR-ir axons at a high density along the anterior and lateral white matters lying close to the gray/white border. Outside these areas, there was a group of CR-ir axons scattered at a low density. In the anterior and lateral corticospinal tracts, CR-ir axons were scarcely observed and, in the posterior columns, there were no axons immunopositive for CR. PV-ir axons (Fig. 2) were present at a high density around the ventral and lateral aspects of the white matter abutting the gray matter. Outside these areas, PV-ir axons were occasionally seen at a low density in the ALCs and no immunoreactivities were detcted in the posterior columns. CR-ir axons were more widely distributed in the ALCs of spinal cords than PV-ir axons. Among the neurons in the gray matter, CR immunoreactivities were observed mainly in small-sized neurons in Rexed's laminae I, II, VII, and VIII and no immunoreactivities of PV were seen, which is consistent with a previous study [10]. Compared to controls (Fig. 3a), the loss of CR-ir (Fig. 3b) or PV-ir axons (data not shown) in the ALCs was observed in ALS spinal cords. On the other hand, the numbers of neurons immunopositive for CR (Fig. 3b, inset) did not show any distinct alterations compared to controls (Fig. 3a, inset) on the same slides.

3.3. Quantitative analyses of CR-ir axons

In controls, the average number of CR-ir axons in the ALCs of the spinal cords was 1686 ± 354/0.03 mm^2. Compared to data we previously reported [6], the number of CR-ir axons corresponds to 52.7% of that of phosphorylated neurofilament (SMI-31)-positive axons in the spinal ALCs of controls. In ALS spinal cords with MP+ and MP++, the numbers of CR-ir axons were significantly lower compared to those of controls (controls vs. MP+: $p < 0.001$, controls vs. MP++: $p < 0.001$). In ALS spinal cords without MP, the numbers of CR-ir axons were also decreased compared to those of controls ($p < 0.001$). In comparison among ALS cases, the numbers of CR-ir axons were significantly decreased depending on the degree of dMP (MP− vs. MP+: $p < 0.05$, MP− vs. MP++: $p < 0.01$, MP+ vs. MP++: $p < 0.05$) (Fig. 4).

3.4. Quantitative analyses of PV-ir axons

In controls, the average number of PV-ir axons in the ALCs of the spinal cords was 911 ± 175/0.03 mm^2. Compared with the data we previously reported [6], it corresponds to 28.5% of that of phosphorylated neurofilament-positive axons. We found that, in ALS spinal cords with MP++, there were no PV-ir axons in the ALCs; therefore, statistical comparisons were performed among controls and ALS patients with MP− and MP+. The numbers of PV-ir axons in the ALS spinal cords with MP+ were significantly reduced compared to controls ($p < 0.001$). In the ALS spinal cords without dMP, the numbers of PV-ir axons were also significantly lower compared to those of controls ($p < 0.001$). In comparisons among ALS cases, the numbers of PV-ir axons showed significant reductions depending on the degree of dMP (MP− vs. MP+: $p < 0.001$) (Fig. 4).

3.5. Quantitative analyses of CR-ir neurons in the spinal gray matter

In contrast to the decrease in the numbers of CR-ir axons in the spinal white matter of ALS cases, there were no significant quantitative differences of CR-ir neurons in the spinal gray matter between ALS patients and controls, or among the 3 ALS categories (Fig. 5).

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