Reactive Astrogliosis in Neonatal Rat Spinal Cord after Exposure to Cerebrospinal Fluid from Patients with Amyotrophic Lateral Sclerosis

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Previous studies have proposed the presence of circulating toxic factor(s) in the cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (ALS). In the present study we show that there is an increased number of astrocytes intensely immunoreactive for glial fibrillary acidic protein (GFAP) in the gray matter of the spinal cords of neonatal rats exposed to ALS CSF. There is also increased expression of GFAP in the astrocytes of the white matter of neonatal rat spinal cords exposed to ALS CSF. Western blot analysis also confirmed the increased expression of GFAP. Accordingly, our study provides for the first time a clear evidence for the pathological response of glia to the circulating toxic factor(s) in the CSF of ALS patients.

Key Words: amyotrophic lateral sclerosis; reactive astrogliosis; glial fibrillary acidic protein; cerebrospinal fluid.

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder which mainly affects the motor neurons. The central nervous system responds to neural injuries with an increase in the number and size of astrocytes expressing glial fibrillary acidic protein (GFAP), a phenomenon generally referred to as "reactive astrogliosis" (6). GFAP is an intermediate filament cytoskeletal protein present selectively in astroglia (1) and represents the marker of astroglial activation (2, 4). Much work has been carried out regarding the astrogliosis in the cortex of ALS patients (10, 11, 14, 16). Reactive astrogliosis has also been observed in the spinal cords of autopsy specimens of ALS patients (20).

Our earlier studies have proposed circulating toxic factor(s) in the cerebrospinal fluid (CSF) of ALS patients which causes motor neuron degeneration (15, 19). We wanted to study the effect of these toxic factor(s) in the ALS CSF on the astrocytes in spinal cord. We have examined astrocytes labeled with GFAP in the spinal cords of neonatal rats which were exposed intrathecally to the CSF from patients suffering from ALS or other neurological (non-ALS) disorders. Spinal cords of neonatal rats (normal) which were unexposed to human CSF samples were also studied for the GFAP immunoreactivity.

CSF was collected from (a) 15 patients with ALS and (b) 11 age- and sex-matched non-ALS neurological patients suffering from one of the following diseases: lumbar disk prolapse (n = 4), noncompressive myelopathy (n = 2), compressive myelopathy (n = 2), syringomyelia (n = 2), and nutritional myelopathy (n = 1). All samples were collected for routine laboratory investigation and a part was used for this study after obtaining the informed consent from patients.

Wistar rat pups (3 days old) were anesthetized with ether. A 26 G (attached to a polyethylene tube and 5-µl Hamilton syringe) was inserted into the spinal subarachnoid space through the intervertebral space, 1 cm rostral to the base of the tail because the spinal cord ends 1 cm rostral to this point at this age. Membrane sterilized CSF (5 µl, 0.22-µm low protein binding filter, Millipore, Bedford, MA) from patients with ALS was injected into the spinal subarachnoid space at the rate of 1 µl/2.5 min using a microinjector. The needle was retained in place for 2–3 min. The same procedure was followed to inject the CSF from non-ALS patients in control rats (19).

After 48 h of CSF injection, rat pups were anesthetized with ether and perfused transcardially using 0.9% saline (3–5 ml) followed by 4% paraformaldehyde (20–25 ml). Spinal cords were removed and postfixed with 4% paraformaldehyde for 24 h. GFAP immunostaining was carried out on the 30-µm (Vibratome) sections from the lumbar region of the spinal cord using anti-GFAP antibodies (1:100, Boehringer Mannheim) and FITC-conjugated secondary antibodies (1:100, Cappel, U.S.A.)
as described previously (19). For Western blot analysis, lumbar regions of the spinal cords of untreated, non-ALS, or ALS CSF-exposed rats were washed in the homogenization buffer (50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 20 mM β-phosphoglycerate, 0.2 mM benzimidene, 0.2 mM AEBSF, leupeptin (1 µg/ml), 1 mM PMSF). Homogenization was carried out using a polytron homogenizer. Immunoblotting was done according to the method described by Towbin et al. (21). Protein concentration was determined by the method of Bradford (3) using bovine serum albumin as a standard. Thirty micrograms of total protein from these samples was electrophoresed on a 12% SDS–polyacrylamide gels (13). After electrophoresis, the samples were transferred to polyvinylidene fluoride membrane. The membranes were incubated overnight at 4°C with monoclonal antibodies against GFAP (1:5000, Boehringer Mannheim). The membranes were then incubated with the alkaline phosphatase-conjugated anti-mouse antibodies (1:5000, Kirkegaard and Perry Lab Inc.) for 1 h at room temperature. The bands were visualized using BCIP-NBT substrate chromogen.

In untreated rats or rats exposed to non-ALS CSF, GFAP immunoreactivity was observed in fibrous astrocytic processes of the white matter in spinal cord sections (Fig. 1A). The astrocytic processes were long and radially arranged. In contrast, the gray matter remained poorly labeled with GFAP antibodies.

In spinal cord sections of rats exposed to ALS CSF (Fig. 1B), astrocytes that are intensely immunoreactive for GFAP were observed in the entire gray matter. These reactive astrocytes looked like protoplasmic astrocytes. There was also an increased GFAP immunoreactivity in the astrocytes of the white matter of spinal cords of ALS CSF-treated rats compared to normal or non-ALS CSF-exposed rats. The predominantly radial nature of astrocytes in the white matter was not altered by ALS CSF treatment. Western blotting analysis showed that the GFAP content in spinal cords of the ALS CSF-exposed rats was more compared to un-

**FIG. 1.** Representative photomicrographs of a spinal cord section from lumbar region from 6-day-old rat exposed to CSF from non-ALS (A) and ALS (B) patients. Note an increased number of astrocytes, intensely immunoreactive for GFAP in the gray matter (GM), and increased immunoreactivity in the white matter (WM) in (B) compared to (A). Scale bar, 30 µm.
treated or non-ALS CSF-treated rats (Fig. 2), thus corroborating the immunocytochemical findings.

In the present study 11 control (non-ALS) CSF samples were used. Of the 11, 1 control CSF sample (from patient with intrinsic spinal cord lesion) gave a positive result. CSF samples from 15 ALS patients were used for the injections. Increased immunoreactivity for GFAP in the gray and white matter of the spinal cords was produced by 11 of 15 ALS CSF samples. In our earlier studies also, we have shown that not all the CSF samples from ALS patients cause the same amount of degeneration in terms of aberrant phosphorylation of neurofilaments in the neuronal soma (15, 19).

An attempt was made to correlate the findings of enhanced GFAP expression and the clinical features. All 15 patients had sporadic form of ALS. However, no correlation with the age, sex, severity, duration, and bulbar involvement was observed with the intensity of GFAP staining. It is of interest to note that CSF samples from two of the subjects who had monomelic amyotrophy, a benign form of motor neuron disease, with the involvement restricted to one upper limb (8) showed positive result while four others with classical features of ALS with bulbar involvement were negative.

Similar results in terms of increased levels of GFAP with massive hypertrophy of astrocytes in the spinal cord and brain stem of the Wobbler mouse have been observed which has been used as an animal model of motor neuron disease (5, 7, 12). There was a progressive increase in the glial reactivity from the first month onward, coinciding with the onset of motor neuron degeneration (18).

To explain the nature of Wobbler neuropathy, it has been suggested that the primary defect is the loss of motor neurons, and glial reactivity represents a secondary defect. The second possibility is that the principal event is an initial astrocyte defect which leads to motor neuron degeneration. It was observed that the GFAP-positive astrocytes with numerous short processes appear very early in the gray matter of the spinal cord of Wobbler mice and then in the white matter, while GFAP-reactive astrocytes, with long unbranched processes, were seen throughout the white matter in normal animals (9). The increase in astrocyte reactivity was associated with modifications in the arrangement of astrocytic processes which may be an important primary event in the course of the Wobbler disease.

A recent study has also shown that the normal close association between astrocytic processes and motor neuron soma was decreased in the ALS cases (17). There was also a reduction or absence of superoxide dismutase1 immunoreactivity in astrocytes of the spinal cords of ALS patients compared to normal (17). These results again suggest the disease mechanism in ALS may involve alterations in spinal cord astrocytes.

Studies in our laboratory have shown aberrant phosphorylation of neurofilaments (NF) in the soma of the cultured chick spinal neurons (15) and the ventral horn neurons of neonatal rat spinal cord (19) following exposure to ALS CSF. These studies have proposed the presence of circulating toxic factor(s) in the ALS CSF which induced the degeneration of motor neurons. Our present results clearly provide an evidence for the pathological response of astrocytes to the circulating toxic factor(s) in the CSF of ALS patients. In our previous study aberrant phosphorylation of NF in motorneurons was observed 48 h after the injection of ALS CSF. Even after 24 h of ALS CSF injection aberrant phosphorylation of NF is neuronal soma was observed, to a lesser extent compared to 48 h (unpublished observation). We have also observed an increase in the GFAP expression after 48 h of exposure to ALS CSF, even though there was a slight increase in the GFAP expression even after 24 h. Hence, the response of astrocytes was seen during the same period when the aberrant phosphorylation of NF in motor neurons takes place (19). Accordingly, the possibility that a primary defect in astrocytes, leading to the degeneration of motor neurons in ALS cannot be discounted.

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REFERENCES


