Intrathecal Upregulation of Granulocyte Colony Stimulating Factor and Its Neuroprotective Actions on Motor Neurons in Amyotrophic Lateral Sclerosis

Masahito Tanaka, MD, Hitoshi Kikuchi, MD, PhD, Takaaki Ishizu, MD, Motozumi Minohara, MD, PhD, Manabu Osoegawa, MD, PhD, Kyoko Motomura, MS, Takahisa Tateishi, MD, Yasumasa Ohyagi, MD, PhD, and Jun-ichi Kira, MD, PhD

Abstract
To investigate cytokine/chemokine changes in amyotrophic lateral sclerosis (ALS), we simultaneously measured 16 cytokine/chemokines (interleukin [IL]-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 [p70], IL-13, IL-17, interferon-γ, tumor necrosis factor-α, granulocyte colony stimulating factor [G-CSF], macrophage chemotactant protein-1 [MCP-1], and macrophage inflammatory protein-1β) in cerebrospinal fluid (CSF) and sera from 37 patients with sporadic ALS and 33 controls using a multiplexed fluorescent bead-based immunosassay. We also conducted immunohistochemical analyses from 8 autopsied ALS cases and 6 non-neurologic disease controls as well as cell culture analyses of relevant cytokines and their receptors. We found that concentrations of G-CSF and MCP-1 were significantly increased in ALS CSF compared with controls. In spinal cords, G-CSF was expressed in reactive astrocytes in ALS cases but not controls, whereas G-CSF receptor expression was significantly decreased in motor neurons of spinal cords from ALS cases. Biologically, G-CSF had a protective effect on the NSC34 cell line under conditions of both oxidative and nutritional stress. We suggested that G-CSF has potentially neuroprotective effects on motor neurons in ALS and that downregulation of its receptor might contribute to ALS pathogenesis. On the other hand, MCP-1 correlated with disease severity, which may aggravate motor neuron damage.

Key Words: Amyotrophic lateral sclerosis (ALS), Granulocyte colony stimulating factor (G-CSF), Granulocyte colony stimulating factor receptor (G-CSFR), Macrophage chemotactant protein-1 (MCP-1).

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that primarily affects upper and lower motor neurons in the spinal cord, brainstem, and motor cortex, culminating in respiratory failure. Several hypotheses for ALS pathogenesis have been advocated, including oxidative stress (1), mitochondrial dysfunction (2), excitotoxic damage (3), and abnormalities of protein folding (4). Recently, increasing evidence suggests that some cytokines or growth factors might also have an effect on ALS (5, 6). In the spinal cords of patients with ALS and animal models (mutant Cu/Zn superoxide dismutase [mSOD1] transgenic rodents), motor neuron loss and dramatic increases in activated microglia/macrophages, reactive astrocytes, and dendritic cells have been documented (7, 8). These glial cells possibly act on motor neurons destructively or protectively by secretion of cytokines/chemokines or growth factors.

Cytokines/chemokines and growth factors are key mediators of central nervous system (CNS) networks. Cytokines/chemokines are multifunctional proteins that have been shown to be involved not only in autoimmune diseases of the CNS such as multiple sclerosis (MS), but also in neurodegenerative disorders such as Alzheimer disease (9) and Parkinson disease (10). In ALS, several key mediators have been reported. Macrophage chemoattractant protein (MCP)-1 (7, 11, 12), interleukin (IL)-6 (13), tumor necrosis factor-α (TNF-α) (14), and transforming growth factor-β (TGF-β) (15) are all upregulated in either cerebrospinal fluid (CSF) or serum. Moreover, some cytokines/chemokines act on both immune cells and neurologic cells; for example, granulocyte colony stimulating factor (G-CSF) is not only a major growth factor in activation and differentiation of the granulocyte lineage, but also has a significant neuroprotective action on cerebral ischemia (16) and induces neurogenesis (17) like vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) in ALS animal models (18, 19).

This study aimed to characterize multiple cytokine profiles in ALS CSF using multiplexed fluorescent bead-based immunoassay (20, 21), a recently developed powerful technology, and to clarify the biologic significance of relevant cytokines through immunohistochemical analyses of...
autopsied ALS spinal cords and cell culture assays. We found significant upregulation of G-CSF in the CSF and spinal cord of patients with ALS and proved protective effects of G-CSF in motor neuron cell lines.

MATERIALS AND METHODS

Patients

A total of 37 patients with sporadic ALS (19 males and 18 females; mean age ± standard deviation [SD] at examination, 59.5 ± 12.9 years) (Table 1) were examined. All patients with ALS were subjected to a thorough neurologic examination and diagnosed as clinically definite or probable cases of ALS based on the El Escorial diagnostic criteria (22) at the Department of Neurology, Kyushu University Hospital, from 1997 to 2004. The mean duration of ALS was 19.6 ± 20.1 months at the time of examination. The disability level associated with the development and progression of ALS was determined using the revised ALS functional rating scale (ALSFRS-R) (23). The mean ALSFRS-R score was 38.2 ± 6.2. The disease progression rate was defined as 48 (ALSFRS-R full score)—ALSFRS-R score/disease duration expressed in months. In addition, 33 control patients with other noninflammatory neurologic diseases (OND) examined during the same period (23 males and 10 females; age at examination, 56.0 ± 13.2 years) were also enrolled. The patients with OND did not have malignancies and they had not undergone immunologic treatment. They comprised 5 patients with sporadic spinocerebellar atrophy, 4 with late cortical cerebellar atrophy, 4 with olivopontocerebellar atrophy, 3 with cervical spondylosis, 2 with metabolic neuropathy, 2 with lumbar herniation, 2 with spastic spinal paraplegia, and one each with striatoni gral degeneration, Shy-Drager syndrome, myopathy, facioscapulohumeral muscular dystrophy, thoracic outlet syndrome, cervical plexopathy, ossification of the posterior longitudinal ligament, idiopathic dystonia, essential myoclonus, alcoholic ataxia, and hysteria.

Cerebrospinal Fluid and Serum Sample Collection

CSF samples were obtained from all patients (37 patients with ALS and 33 patients with OND) and immediately centrifuged at 800 rpm at 4°C for 5 minutes. Supernatants were stored at −80°C until the cytokine assay. The mean CSF cell count and total protein level were also measured for all patients (Table 1). Serum samples were simultaneously obtained from most patients (28 patients with ALS and 26 patients with OND) and also stored at −80°C until the cytokine assay. None of the patients had systemic inflammation at the time that blood and CSF were drawn.

Tissue Sources

Spinal cord specimens were obtained at autopsy from 8 ALS cases and 6 nonneurologic disease controls (Table 2). The nonneurologic controls did not receive either chemotherapy

| TABLE 1. Demographic Features of Patients With Sporadic Amyotrophic Lateral Sclerosis (ALS) and Those With Other Noninflammatory Neurologic Diseases (OND) |
|-----------------|-----------------|-----------------|
| ALS             | OND             |
| Number of patients | 37              | 33              |
| Sex (male/female) | 19/18           | 23/10           |
| Age at examination (mean ± SD, months) | 59.5 ± 12.9     | 56.0 ± 13.2     |
| Disease duration (mean ± SD, months) | 19.6 ± 20.1     | NA              |
| Immunologic treatment (for the past year) | —               | —               |
| ALSFRS-R score (mean ± SD) | 38.2 ± 6.2      | NA              |
| Cell count (mean ± SD, per µL) | 0.79 ± 0.83     | 1.36 ± 1.61     |
| Total protein amount (mean ± SD, mg/dL) | 35.7 ± 15.0     | 34.3 ± 10.9     |
| SD, standard deviation; ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; CSF, cerebrospinal fluid; NA, not applicable. |

| TABLE 2. Patient Data for the Neuropathologic Study |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient | Age | Sex | Location of Onset | Clinical Course (years) | Mechanical Ventilation | Cause of Death |
| A1 | 60 | F | Upper limbs | 7 | - | Respiratory failure |
| A2 | 54 | M | Bulbar | 3 | - | Respiratory failure |
| A3 | 68 | M | Upper limbs | 6 | - | Respiratory failure |
| A4 | 69 | F | Lower limbs | 6 | - | Respiratory failure |
| A5 | 45 | M | Upper limbs | 5 | + | Pneumonia |
| A6 | 51 | M | Upper limbs | 2.4 | - | Respiratory failure |
| A7 | 60 | F | Bulbar | 1 | - | Respiratory failure |
| A8 | 67 | F | Lower limbs | 1.6 | - | Respiratory failure |
| Control C1 | 86 | F | NA | NA | NA | Respiratory failure |
| C2 | 69 | F | NA | NA | NA | Lungs cancer |
| C3 | 61 | M | NA | NA | NA | Gastric cancer |
| C4 | 62 | M | NA | NA | NA | Lung cancer |
| C5 | 62 | F | NA | NA | NA | Hepatocellular carcinoma* |
| C6 | 61 | M | NA | NA | NA | Chronic renal failure* |

* Patients C4 and C5 had a complication of bronchopneumonia. ALS, amyotrophic lateral sclerosis; NA, not applicable.
or colony stimulating factor medication. The postmortem interval ranged from 2.5 to 10 hours (mean, 5.5 hours). For immunostaining of G-CSF and G-CSFR, autopsied specimens (8 ALS cases, A1–8; 6 controls, C1–6) were fixed for several days in 10% buffered formalin, embedded in paraffin, and then sliced into 6-μm-thick sections.

Multiplexed Fluorescent Bead-Based Immunoassay of Cerebrospinal Fluid and Serum Samples

CSF supernatant and serum samples were simultaneously analyzed for 16 different cytokines and chemokines, namely IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, interferon (IFN)-γ, TNF-α, G-CSF, MCP-1, and macrophage inflammatory protein (MIP)-1β, using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA) as described previously (20, 21) according to the manufacturer’s instructions (24, 25). Briefly, 50 μL of each CSF supernatant and various concentrations of each cytokine standard (Bio-Rad) were added to 50 μL of antibody-conjugated beads (Bio-Rad) in a 96-well filter plate (Millipore, Billerica, MA). Cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800, and 3,200 pg/mL) assayed in the same manner as the CSF samples. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay System was used throughout the experiments, and the inter- and intraassay variabilities were reportedly less than 10% according to the manufacturer (20, 21, 24, 25). The detection limit for each cytokine was determined by recovery of the corresponding cytokine standard, and the lowest values with more than 70% recovery were set as the lower detection limits. The lower detection limits were as follows: 0.2 pg/mL for IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, and IL-17; 0.78 pg/mL for IL-4, IL-12 (p70), IL-13, IFN-γ, TNF-α, and MIP-1β; and 3.13 pg/mL for G-CSF and MCP-1. All samples were analyzed undiluted in duplicate.

Antibodies for Immunohistochemistry

The following primary antibodies were used: anti-G-CSF rabbit polyclonal and mouse monoclonal antibodies (1:500; Chugai Seiyaku, Tokyo, Japan); anti-G-CSF mouse monoclonal antibody (1:500; s-1284; Abcam, Cambridge, U.K.); antiligial fibrillary acid protein (GFAP) rabbit polyclonal antibody (1:1000; DAKO, Copenhagen, Denmark); and anti-CD68 mouse monoclonal antibody (1:200; clone Kp-1; DAKO). The secondary antibodies were horseradish peroxidase–conjugated anti-rabbit (PI-1000) and anti-mouse (PI-2000) (all from Vector Laboratories, Burlingame, CA), and fluorescein isothiocyanate (FITC)-mouse Ig antibody and Texas-red rabbit Ig antibody (Amersham, London, U.K.).

Immunohistochemical Analysis of Autopsied Spinal Cord

Immunohistochemistry was performed using an indirect immunoperoxidase method as previously described (26, 27). The deparaffinized sections (for anti-G-CSF, anti-G-CSFR, anti-CD68, and anti-GFAP) were hydrated in ethanol and then incubated with 0.3% hydrogen peroxide in absolute methanol for 30 minutes at room temperature to inhibit endogenous peroxidase. After rinsing in tap water, the sections were completely immersed in distilled water and then heated in 0.01 M citrate buffer (pH 6.0) in a microwave for 10 minutes for antigen retrieval. After this pretreatment, the sections were incubated with a primary antibody diluted in 5% nonfat milk in 20 mM Tris-HCl (pH 7.6) containing 0.5 M NaCl, 0.05% Na3 and 0.05% Tween 20 (TBST) at 4°C overnight, and then with a 1: 200 dilution of the appropriate secondary antibody for 1 hour at room temperature. The colored reaction product was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) solution (0.02% DAB, 0.003% H2O2, 50 mM Tris-HCl, pH 7.6). We also checked the localization of G-CSF using fluorescent immunohistochemistry. For double-fluorescence immunohistochemistry of G-CSF, astrocytes and microglia, anti-G-CSF antibody, anti-GFAP, and anti-CD68 antibodies were used, respectively. FITC-mouse antibody and Texas-red rabbit antibody were used as secondary antibodies. After washing with secondary antibody, sections were incubated with Sudan black III in 70% ethanol for 10 minutes to attenuate autofluorescence (28) and then mounted in Fluoromount and observed on a FluoView FV300 fluorescent microscope (Olympus, Tokyo, Japan). To investigate the frequency of G-CSF-positive astrocytes in GFAP-positive astrocytes, we counted the numbers of GFAP-positive and both GFAP- and G-CSF-positive astrocytes in the unilateral anterior horn of one section of L3 spinal cord from each patient with ALS.

Spinal cord specimens of ALS and control cases were processed simultaneously in the same reaction trays to obtain comparable staining intensity. To determine G-CSFR and G-CSF immunoreactivity, we evaluated all anterior horn cells in one section at the L3 level of spinal cord from each patient with ALS and control. In 8 patients with ALS, 95 (average 12 per patient) G-CSFR immunoreactive anterior horn cells were counted and in 6 controls, there were 198 (average, 33); whereas G-CSF immunoreactive anterior horn cells counted in the 8 patients with ALS totaled 119 (average, 15 per patient) and those in the 6 controls were 208 (average, 35). Images of the anterior horn cells were scanned by a DP12 (Olympus) using Adobe Photoshop software version 6.0 (Adobe Systems Inc., San Jose, CA). Densitometric analysis was performed using Scion Image software version 4.02 (Scion Corp., Frederick, MD). The quantity of G-CSFR- or G-CSF-immunoreactive products in the anterior horn cells was expressed as the optical density (OD) (pixels/μm2/cytoplasm). The relative amount of G-CSFR or G-CSF was calculated as (mean OD of the entire cytoplasm of anterior horn cells) - (mean OD of posterior columns) in accord with a previous report (26).

Cell Culture and Cell Viability Assays

NSC34 cells (a gift from Dr. Tabira, National Institute for Longevity Sciences, Obu, Japan), hybrid cell lines created by fusing neuroblastoma cells with motor neuron-enriched spinal cord cell preparations, were used for evaluation of the effects of G-CSF on motor neurons (2, 29, 30). The culture medium was Dulbecco’s modified Eagle’s medium (DMEM)
plus 10% (vol/vol) fetal bovine serum (FBS). Cells were grown at 37°C under 5% CO₂ humidified atmosphere. To assay the effect of stress conditions on ALS, Lee et al investigated apoptosis using hydrogen peroxide (H₂O₂) and serum deprivation in an ALS culture model (31). Therefore, to determine the adequate condition for NSC34 cell death, we used H₂O₂ as well as serum deprivation. We cultured NSC34 cells with H₂O₂ at concentrations of 0, 0.05, 0.1, and 0.5 mM, respectively. Sufficient NSC34 cell death was observed with 0.5 mM H₂O₂ (data not shown), which was similar to a previous report (32). We therefore exposed NSC34 cells to 0.5 mM H₂O₂ in the absence or presence of various doses of G-CSF (Chugai-seiyaku). Cell viability was assayed using the WST-8 test (33) with 4-[3-(2-methoxy- nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt, which is reduced by NADH to formazan. The amount of formazan generated in the cells is proportional to the number of living cells. NSC34 cells were seeded in 96-well plates at 2,000 cells/well and preincubated for 24 hours in DMEM plus 10% FBS before assay. After preincubation, the medium was removed and replaced with fresh DMEM plus 10% FBS for 2 hours. Small aliquots of H₂O₂ were then added to give final concentrations of 0.5 mM, and cells were treated with various doses of G-CSF. After that, WST-8 solution (DOJINDO, Kumamoto, Japan) was added to each well of the plate and incubated for 2 hours in the CO₂ incubator. Finally, we measured the absorbance at 450 nm, which was absorption spectrum of WST-8 formazan.

**FIGURE 1.** Cytokine/chemokine levels in cerebrospinal fluid (CSF) supernatants from patients with amyotrophic lateral sclerosis (ALS) (n = 37) and patients with other noninflammatory neurologic diseases (OND) (n = 33) measured using a multiplexed fluorescent bead-based immunoassay. Bars indicate the mean concentration of each group.
using a microplate reader. Similarly, in the serum deprivation experiments, NSC34 cells were seeded in 96-well plates at 1,000 cells/well and preincubated for 24 hours in DMEM plus 10% FBS. After preincubation, the medium was removed and replaced with fresh DMEM without FBS, and cells were treated with various doses of G-CSF. After 48 hours, cell viability was assayed. Cytotoxicity assays were performed using 8 replicate wells.

**Immunostaining of the NSC34 Cell Line**

For immunostaining, cells were plated in 24-well plates with cell disks precoated with poly-L-lysine and cultured in DMEM plus 10% FBS. Cells were fixed in acetone for 5 minutes at room temperature and then immersed in 1% NP-40 in PBS for 10 minutes. After this pretreatment, the cell disks were incubated with a primary antibody (anti-G-CSF or anti-G-CSFR) diluted in 5% normal goat serum in PBS at 4°C overnight and then with the appropriate secondary antibody (FITC-mouse antibody or Texas-red rabbit antibody) for 1 hour at room temperature. The cell disks were then washed and mounted under coverslips.

**Reverse Transcriptase–Polymerase Chain Reaction for the Granulocyte Colony Stimulating Factor and Granulocyte Colony Stimulating Factor Receptor**

Total RNA from NSC34 cells was isolated by RNeasy Mini Kit (QIAGEN, Valencia, CA) and cDNA was synthesized using oligo(dT), dNTP mixture, transcriptor reverse transcriptase (Roche, Penzberg, Germany). Polymerase chain reaction (PCR) was carried out using the following primers: a mG-CSF forward primer (5’-TCCATGACATCTGGCAGG-3’) and a mG-CSF reverse primer (5’-CGGCCTCTCCTGACCATAGT-3’) with an expected product length of 1.4 kbp, and a m-G-CSFR forward primer (5’-AGTCCACAGCGAGGTTAGT-3’) and a m-G-CSFR reverse primer (5’-GTAGGCCTAGTTCATACCTG-3’) with an expected product length of 279 bp. PCR amplification steps consisted of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes (for G-CSF) or 45 seconds (for G-CSFR) using the Gene Amp PCR system 2400 (Perkin Elmer, Wellesley, MA). PCR products were electrophoresed on 1.5% or 2.5% agarose gels and visualized with ethidium bromide staining. PCR products were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and automatic DNA sequencing was conducted on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

**Statistical Analysis**

The nonparametric Mann-Whitney U test was used to compare CSF and serum cytokine/chemokine levels between the ALS and OND patient groups. Spearman’s rank correlation test was used for statistical analysis of the correlations between individual CSF cytokine and chemokine levels, between CSF and serum cytokine/chemokine levels, and between cytokine/chemokine levels and either the ALSFRS-R score or disease progression rate. Statistical analysis of differences among corrected OD values obtained from the immunohistochemical studies of all motor neurons between the ALS and control groups was performed using the unpaired t test. The effects of G-CSF treatment were evaluated using analysis of variance with StatView (version 5; Abacus Concepts, Berkeley, CA). Statistical significance was set at p < 0.05.

### RESULTS

**Concentrations of Each Cytokine/Chemokine in Cerebrospinal Fluid Supernatants and Sera**

Among the cytokines/chemokines measured, G-CSF, MCP-1, and IL-5 levels were significantly higher in patients with ALS than in patients with OND (G-CSF: 19.3 ± 17.5 versus 9.8 ± 6.9 pg/mL, p = 0.0043; MCP-1: 251.4 ± 79.4 versus 214.9 ± 82.8 pg/mL, p = 0.0368; IL-5: 1.2 ± 0.97 versus 0.97 ± 0.22 pg/mL, p < 0.0001), whereas IL-10 levels were significantly lower in patients with ALS than in patients with ALSFRS-R score or disease progression rate.

**FIGURE 2. Correlations between the concentrations of individual cytokines/chemokines in cerebrospinal fluid and between the concentration of each cytokine/chemokine and various clinical parameters.**

(A) Relationship between the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R) score and macrophage chemoattractant protein-1 (MCP-1) concentration in cerebrospinal fluid (n = 37). (B) Relationship between the disease progression rate and MCP-1 concentration in cerebrospinal fluid (n = 37).
OND (0.40 ± 0.09 versus 0.48 ± 0.11 pg/mL, p = 0.0018) (Fig. 1). The other cytokine/chemokine levels did not differ significantly between the 2 groups. In CSF, even when the cutoff level was set at 1 pg/mL, patients with ALS still showed significantly higher concentrations of G-CSF, MCP-1, and IL-5 compared with controls (p = 0.0056, p = 0.0368, and p < 0.0001, respectively). In sera, there were no significant differences between the 2 groups (data not shown). The serum concentrations of G-CSF and MCP-1 were 5.06 ± 5.5 pg/mL and 63.4 ± 24.5 pg/mL, respectively.

**Correlations Between Individual Cytokine/Chemokine Levels and Between Each Cytokine/Chemokine Level and Various Clinical Parameters**

In CSF, the concentration of MCP-1 was negatively correlated with the ALSFRS-R score (r = −0.538, p = 0.0013) (Fig. 2A) and positively correlated with the disease progression rate (r = 0.342, p = 0.0403) (Fig. 2B). None of the other cytokines/chemokines in the CSF showed any correlations among themselves or with any clinical parameter. In the sera, no correlations were found for either individual cytokine/chemokine levels or for each cytokine/chemokine level and clinical parameters. No significant correlations were found between individual CSF and serum cytokine/chemokine levels in each group. In addition, MCP-1 showed a significant positive correlation with the CSF protein level and IL-8 (r = 0.359, p = 0.0425 and r = 0.432, p = 0.0095, respectively).

**Immunohistochemical Changes in Cytokines/Chemokines and Their Receptors in Amyotrophic Lateral Sclerosis Spinal Cords**

Next, we focused on the cytokines/chemokines that were significantly upregulated in ALS CSF. Immunoreactivity for G-CSF was observed in the cytoplasm of anterior horn cells in all 8 ALS cases and 6 controls (Fig. 3A, B). G-CSF immunoreactivity was strongly enhanced in the cytoplasm of several reactive astrocytes in the anterior horn of ALS cases.
relative to controls (inset in Fig. 3B), and G-CSF was similarly immunolabeled in the cytoplasm of anterior horn cells in ALS cases and controls. Double immunostaining also revealed G-CSF expression in GFAP-positive astrocytes (Fig. 3E–G) but not CD68-positive cells (Fig. 3H–J). Among 151 GFAP-positive astrocytes counted in 8 patients with ALS (average, 19 per patient), 25 (16.6%) of the cells also expressed G-CSF. G-CSFR immunoreactivity was also detected in the cytoplasm of anterior horn cells in both ALS cases and controls (Fig. 3C, D). The corrected OD values for G-CSFR immunoreactivity in the anterior horn cells were significantly less in ALS cases than controls (p < 0.0001) (Fig. 3K). On the other hand, there was no significant difference of G-CSF immunoreactivity in the anterior horn cells between both groups (Fig. 3L). Several ALS cases had enhanced immunoreactivity for G-CSF in reactive astrocytes in the lesions (inset in Fig. 3D).

Neuroprotective Effect on the NSC34 Cell Line

We next examined the expression of G-CSFR in NSC34, a mouse motor neuron cell line. Immunoreactivity of G-CSFR was revealed in the cytoplasm of NSC34 cells under steady-state conditions, and the expression of G-CSFR mRNA was also detected by reverse transcriptase–polymerase chain reaction analysis. In (B), a, b, and c indicate positive control, sample and negative control, respectively. Viability of NSC34 cells that were exposed to hydrogen peroxide (H₂O₂) (0.5 mM) (C) or 10% fetal bovine serum (FBS) deprivation conditions (D). G-CSF treatment (at concentrations of 0.5, 1, or 2 µg/mL) had a significant dose-dependent survival effect on NSC34 cells exposed to 0.5 mM hydrogen peroxide. G-CSF treatment (at concentrations of 0.5, 1, or 2 µg/mL) also had a significant survival effect on NSC34 cells under FBS deprivation conditions. Scale bar in (A) = 20 µm. Data are the mean ± standard deviation of optical density (OD), which was proportional to the number of living cells.

DISCUSSION

Multiplexed fluorescent bead-based immunoassay is a novel technology that can sensitively and simultaneously measure multiple cytokines/chemokines in just 50 µL of body fluid and is thus useful especially for application to
CSF, which is only available in very small volumes (20, 21). The main new findings presented here using this technology combined with immunohistochemistry and cell culture techniques are as follows: 1) G-CSF was significantly upregulated intrathecally together with MCP-1 in sporadic ALS; 2) in ALS cases, G-CSF was overproduced in reactive astrocytes, whereas G-CSFR was downregulated in motor neurons relative to controls; 3) G-CSF had a protective effect on a motor neuron cell line with respect to oxidative and nutritional stress through G-CSFR expression in the cells; and 4) intrathecal MCP-1 levels were negatively correlated with the ALSFRS-R score and positively correlated with the disease progression rate, suggesting an association with advancing disease.

G-CSF is well known as a growth factor for granulocytes, but its role in ALS is yet to be determined. Here, we found significant increases in the concentration of G-CSF in ALS CSF, and the level was greater than in sera, suggesting an intrathecal origin. In vitro, stimulated astrocytes reportedly produce a high level of G-CSF (34, 35), although production in vivo has not been proven. We demonstrated G-CSF production in neurons as well as glial cells in the anterior horn of patients with ALS and noneurologic disease controls. Our results are consistent with the findings of Schneider et al (17), who showed G-CSF expression in the neurons of rodent cortex, hippocampus CA3 field, and cerebellar and brainstem nuclei in protein and mRNA levels. However, the production of G-CSF was strongly enhanced in reactive astrocytes in the anterior horn of ALS cases compared with controls, suggesting a glial origin for the increased G-CSF in ALS CSF. Moreover, its autocrine secretion by neurons is also possible.

In the present study, we demonstrated for the first time that G-CSF has protective effects on NSC34, a cell line with motor neuron characteristics. Both hydrogen peroxide-causing oxidative stress and serum deprivation-inducing apoptotic cell death were used for in vitro motor neuron damage, because oxidative stress and apoptosis are thought to operate in ALS. G-CSF is known to extend the lifespan of neurophilys by delaying apoptotic cell death (36) and has also been shown to play as an antiapoptotic role after myocardial infarction by inducing antiapoptotic proteins such as Bcl-2 and Bcl-XL (37). In terms of neuronal cells, Schäbitz et al noted in vivo neuroprotective effects of G-CSF in focal cerebral ischemia (16). Schneider et al also reported recently that G-CSF and G-CSFR are widely expressed in CNS neurons and that their expression is induced by ischemia (17). They showed that G-CSF markedly improved long-term outcomes after cerebral ischemia in rats and that G-CSF not only had antiapoptotic activity, but also stimulated neurogenesis. Taking all of these data into consideration, enhanced G-CSF production by glial cells, and possibly neurons, is likely to be a secondary host defense mechanism in ALS and might promote motor neuron survival in a paracrine or autocrine manner.

However, as shown densitometrically in the present immunohistochemical study, G-CSF expression was reduced in motor neurons of the autopsied ALS spinal cords. In respect to growth factor receptors, it has been reported that ciliary neurotrophic factor receptor (38), IGF-1 receptor (39), and p75 nerve growth factor receptor (40) are upregulated in ALS spinal cord motor neurons, whereas VEGF receptor-3 expression in neuropil is reduced in the ALS spinal cord (41). Moreover, hepatocyte growth factor receptor-negative neurons are increased in the ALS spinal cord (42), and glial cell line-derived neurotrophic factor receptor persists in ALS spinal motor neurons (43). Thus, changes in growth factor receptor expression with disease progression are not uniform in ALS. The decreased expression of G-CSFR in motor neurons might potentially cancel out the neuroprotective effects of G-CSF in the advanced stage of the disease, yet the mechanism of G-CSFR downregulation requires further studies.

The lack of any correlation between G-CSF concentration and clinical parameters of ALS in the present study might be partly explained by downregulation of G-CSFR in motor neurons and by its effects being masked by an enhanced proinflammatory cytokine such as MCP-1. Because G-CSF administered peripherally can pass through the intact blood–brain barrier (17), it might be potentially beneficial, at least in the early stages of ALS.

It is notable that only the CSF MCP-1 concentration had a significant correlation with disease severity, increasing with not only advancing disease severity, but also faster progression. An increase in CSF MCP-1 concentration in ALS has also been detected in several previous studies, but no indication has been given of any correlation with disease severity (7, 12, 44). The discrepancy between our results and previous studies might be because of the larger number of patients included in the present study as well as the different methodologies used. The observations that the MCP-1 level was higher in CSF than sera and that the levels in the 2 compartments were not correlated suggest that there is differential regulation of MCP-1 production and selective activation of intrathecal MCP-1 synthesis in ALS. The higher level of MCP-1 in CSF than sera in ALS was in accordance with previous findings (12, 44). According to the findings of a previous study in which an immunohistochemical technique was used to show glial production of MCP-1 as well as upregulation of MCP-1 mRNA in sporadic ALS spinal cord tissues, the main source of MCP-1 is likely to be glial cells (7). All these observations strongly support the hypothesis that MCP-1 produced intrathecally by glial cells aggravates motor neuron degeneration in ALS.

MCP-1 activates microglia, which then produce abundant proinflammatory cytokines, including IL-5 and IL-8. The low but significant increase in CSF IL-5 and positive correlation between MCP-1 and IL-8 might reflect a proinflammatory cytokine cascade after microglial activation. Moreover, activated microglia express inducible nitric oxide synthase (iNOS), leading to the production of NO (45), which is neurotoxic to motor neurons (46). Enhanced expression of iNOS has also repeatedly been shown in ALS spinal cord (47–49). Therefore, it is reasonable to suggest that MCP-1 mostly acts as a disease-aggravating factor in ALS, probably through glial inflammation.

In summary, we found simultaneous upregulation of neuroprotective (G-CSF) and glial inflammatory cytokines intrathecally in ALS. In addition to glial inflammation, partial disruption of the G-CSF/G-CSFR system might contribute to the acceleration of neuronal degeneration in ALS.
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