factors for motor neurons and is comparable to glial cell line-derived neurotrophic factor (GDNF) [6]. Neurotrophic effects have been demonstrated *in vivo* on embryonic spinal motor neurons during development and on adult motor neurons after axotomy of the hypoglossal nerve [27, 28, 40]. In addition, overexpression of neuronal HGF has been shown to result in the attenuation of neuronal cell death and progression of disease in a familial ALS (FALS) transgenic mouse model [35]. Therefore, HGF and its receptor, c-Met [9], might be beneficial for motor neuron survival.

An essential histopathological feature of ALS is loss of the large anterior horn cells throughout the spinal cord, and the surviving motor neurons of the spinal cord often exhibit shrinkage. Among these residual large anterior horn cells, some appear to be normal. These surviving neurons in ALS patients are thought to possess some form of self-preservation mechanism. To gain new insight into the survival/trophic mechanism of these residual neurons, we focused on the HGF-c-Met system. To date, there have been no reports demonstrating the immunohistochemical expression of HGF and c-Met in motor neurons of the human ALS spinal cord. In the study presented here, we performed immunohistochemical analyses of the human spinal cord, not only from FALS patients with superoxide dismutase 1 (SOD1) gene mutations, but also from patients with sporadic ALS (SALS), and analyzed the expression of HGF and c-Met.

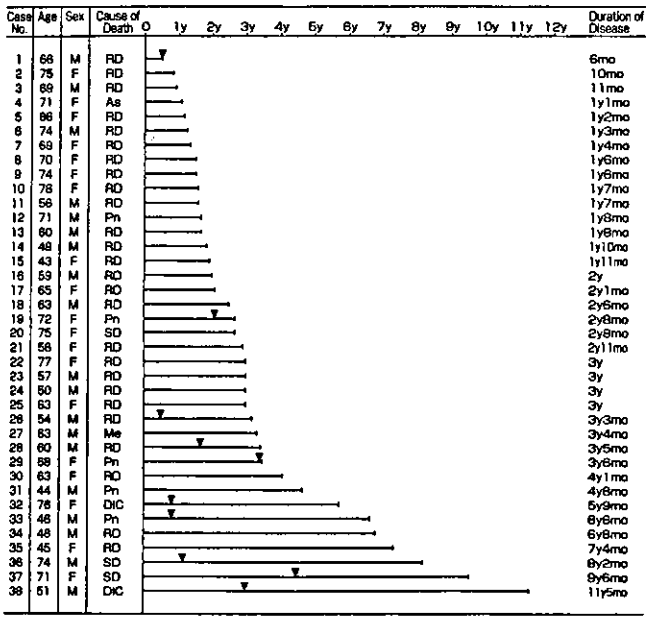

Fig. 1 Characteristics of 38 sporadic ALS cases. This figure includes each patient's age, sex, cause of death, and disease duration. The horizontal lines each show the duration of disease. Arrowheads indicate the time point at which the patients were placed on respirators. (*ALS* amyotrophic lateral sclerosis, *RD* respiratory distress, *As* asphyxia, *Pn* pneumonia, *SD* sudden death, *Me* melena, *DIC* disseminated intravascular coagulation, *y* years, *mo* months)

Materials and methods

Autopsy specimens

Immunohistochemical studies were performed on archival, buffered 10% formalin-fixed, paraffin-embedded spinal cord tissues obtained at autopsy from 38 SALS patients and 5 FALS patients who were members of two different families. The main clinical characteristics of the SALS patients are summarized in Fig. 1. The clinicopathological characteristics of the FALS patients are summarized in Table 1 and have been reported previously [14, 15, 18, 19, 26, 33, 36]. SOD1 analysis revealed that the members of the Japanese Oki family had a two-base pair deletion at codon 126 (frameshift 126 mutation) [14] and that the members of the American C family had an Ala to Val substitution at codon 4 (A4V) [33]. We also examined autopsy specimens of the spinal cord from 20 neurologically and neuropathologically normal individuals (11 males, 9 females; aged 37–75 years). This study was approved by the Ethics Committee of Tottori University (Permission No. 2001-150).

Histopathology and immunohistochemistry

After fixation in buffered 10% formalin, the specimens were paraffin-embedded, cut into 6-μm-thick sections, and examined by light microscopy. Spinal cord sections were subjected to routine staining with hematoxylin and eosin (H-E), Klüver-Barrera, Holzer, and Bielschowsky stains. Representative paraffin sections were used for immunohistochemical staining with the following primary antibodies: an affinity-purified rabbit antibody against human recombinant HGF purified from the culture medium of a Chinese hamster ovary cell that had been transfected with the human HGF expression vector (concentration: 5 μg/ml), and an affinity-purified rabbit antibody to human c-Met (C-12) [diluted 1:500 in 1% bovine serum albumin-containing phosphate-buffered saline (BSA-PBS), pH 7.4] (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were deparaffinized, and endogenous peroxidase activity was quenched by incubation for 30 min with 0.3% H_2O_2. The sections were then washed in PBS. Normal serum homologous with the secondary antibody was used as a blocking reagent. Tissue sections were incubated with the primary antibodies for 18 h at 4°C.

Table 1 Characteristics of five FALS cases (*FALS* familial amyotrophic lateral sclerosis, *SOD* superoxide dismutase, *LBHI* Lewy body-like hyaline inclusion, *mo* months, *y* years, *2-bp* two-base pair, *PCI* posterior column involvement type, + detected, *ND* not determined, *As* asphyxia, *IH* intraperitoneal hemorrhage, *RD* respiratory distress, *Pn* pneumonia)

Case	Age	Sex	Cause of death	FALS duration	SOD1 mutation	Subtype	Neuronal LBHI
Japanese Oki family							
1	46	F	As	18 mo	2-bp deletion (126)	PCI	+
2	65	M	IH	11 y	2-bp deletion (126)	PCI and degeneration of other systems	+
American C family							
3	39	M	RD	7 mo	A4V	PCI	+
4	46	M	Pn	8 mo	A4V	PCI	+
5	66	M	Pn	1 y	ND	PCI	+

PBS-exposed sections served as controls. For the preabsorption test, some sections were incubated with the anti-HGF antibody that had been preabsorbed with an excess amount of human recombinant HGF. Bound antibodies were visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using the appropriate Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako, Glostrup, Denmark) as the chromogen.

Western blot analysis

This analysis was carried out on three fresh autopsy specimens from spinal cord cervical segments: two SALS cases [2.5 years after onset (case 18 in Fig. 1, age 63 years) and 11 years 5 months after onset (case 38 in Fig. 1, age 51 years)], and one normal individual (age 68 years). In brief, specimens were homogenized in Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 2% sodium dodecyl sulfate (SDS), 25% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, and 62.5 mM TRIS-HCl (pH 6.8). The samples were heated at 100°C for 5 min. Soluble protein extracts (20 μg) from the samples were separated on SDS-polyacrylamide gels (4%–20% gradient, Bio-Rad) and transferred by electroblotting to Immobilon PVDF (Millipore, Bedford, MA). After blocking with 5% nonfat milk for 30 min at room temperature, the blots were incubated overnight at 4°C with the antibodies against human HGF and c-Met. Binding to HGF and c-Met was visualized with the Vectastain ABC Kit and DAB. Appropriate molecular weight markers (Bio-Rad) were included in each run.

Results

Histopathology

An essential histopathological finding of the spinal cord in ALS was loss of motor neurons throughout the course of the disease. In the specimens we examined, neuronal loss was most easily recognized in the cervical and lumbar enlargements. The surviving motor neurons showed shrinkage, and lipofuscin granule-filled neurons stood out. Among these residual motor neurons, some that were smaller in size appeared normal. The number of motor neurons decreased with ALS disease progression. Reactive astrocytosis and gliosis were also observed in the affected areas. In the affected antero-lateral columns that were most pronounced in the crossed and uncrossed corticospinal tracts, there was a loss of large myelinated fibers in association

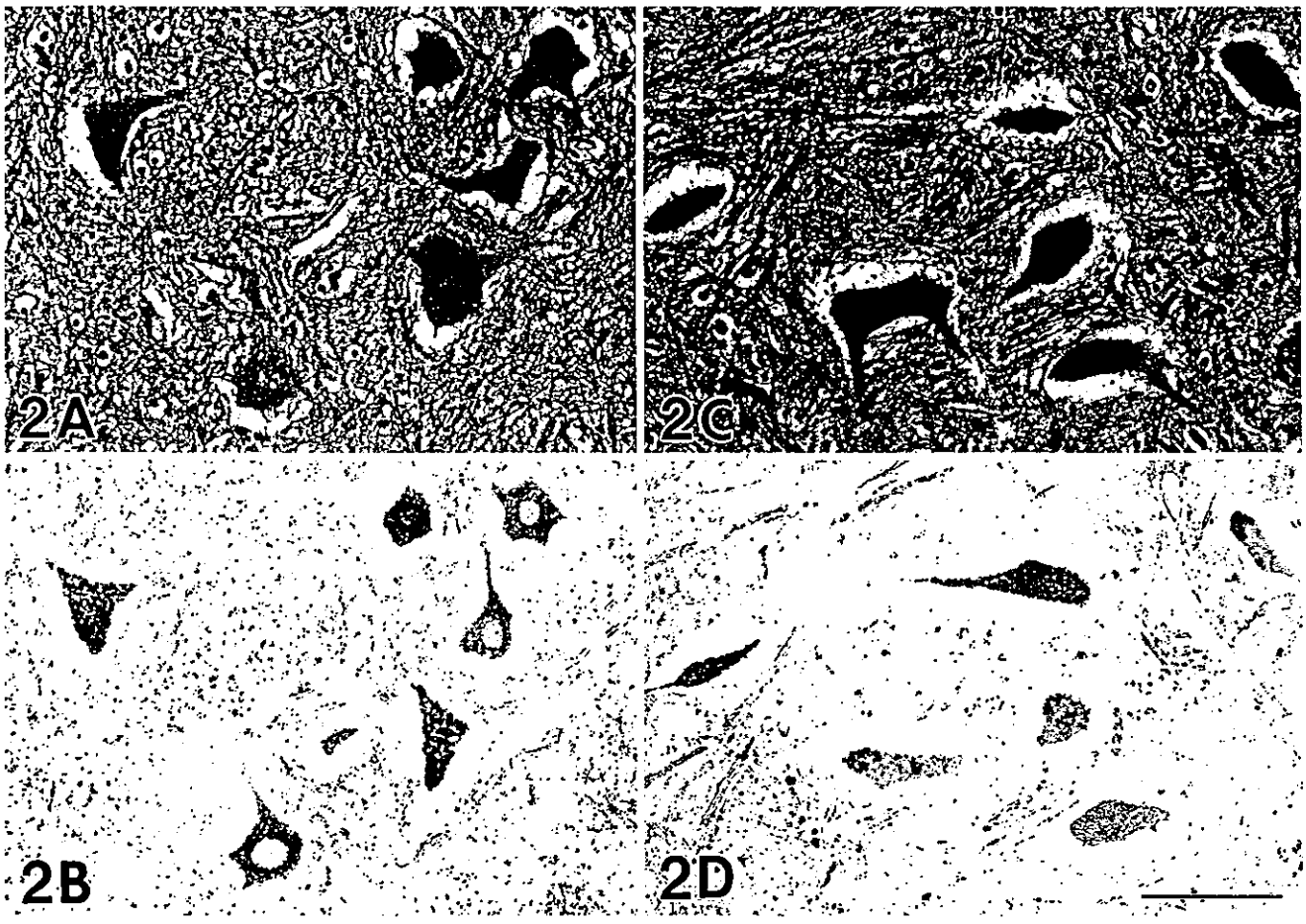

Fig. 2 Detection of HGF and its receptor, c-Met, in the normal anterior horn cells of the spinal cord. **A** Light microscopic preparation of the anterior horn cells stained with H-E. **B** Immunostaining with an antibody against HGF of the section consecutive to that shown in **A**. HGF immunoreactivity is identified in almost all of the motor neurons. Cytoplasmic staining is observed. No counterstaining. **C** Normal anterior horn cells stained with H-E. **D** Immunoreactivity for c-Met of the serial section of that shown in **C**. Almost all of the normal motor neurons are immunopositive for c-Met. Diffuse staining of the cell bodies and proximal dendrites is evident. No counterstaining. (*HGF* hepatocyte growth factor, *H-E* hematoxylin and eosin). *Bar* **A–D** 100 μm

with variable degrees of astrocytic gliosis. Fiber destruction was associated with the appearance of lipid-laden macrophages.

Analysis of the essential changes in the five cases of FALS revealed a subtype of FALS with posterior column involvement (PCI). This subtype is characterized by degeneration of the middle root zones of the posterior column, Clarke nuclei, and the posterior spinocerebellar tracts, in addition to spinal cord motor neuron lesions. A patient who had survived for a long period, with a clinical course of 11 years (case 2 in Table 1), showed multi-system degeneration in addition to the features of FALS with PCI. Neuronal Lewy body-like hyaline inclusions (LBHIs) were present in all five FALS cases.

The spinal cords of normal individuals did not exhibit any distinct histopathological alterations.

Immunohistochemistry

When control and representative paraffin sections were incubated with PBS alone (i.e., no primary antibody), no staining was detected. In addition, incubation of sections with anti-HGF antibody that had been pretreated with an excess of recombinant human HGF produced no staining in any of the sections. HGF immunoreactivity in normal spinal cords was identified in almost all of the motor neurons: cytoplasmic staining was observed with various staining intensities (Fig. 2A, B). A similar staining pattern was also observed for c-Met, the cell bodies and proximal dendrites being stained (Fig. 2C, D). Almost all of the motor neurons in normal spinal cords co-expressed both HGF and c-Met, although the staining intensity of positively stained neurons varied. No significant immunoreactivity for HGF and c-Met was seen in astrocytes and oligodendrocytes from normal subjects.

As for the anterior horn cells in ALS patients, some residual neurons expressed both HGF and c-Met strongly within about 2 years of disease onset (cases 1–15 in Fig. 1). Both antibodies produced a cytoplasmic staining pattern. Other neurons were either faintly stained by both antibodies or unstained. Around 2–3 years after disease onset in ALS patients (cases 16–25 in Fig. 1), the intensity of HGF and c-Met immunoreactivity peaked in some neurons that were positive for both proteins (Fig. 3). In ALS patients with a clinical course of over 3 years (cases 26–38 in Fig. 1), the number of residual neurons decreased strikingly, and respiratory assistance became essential for most patients; the residual neurons intensely expressing both HGF and c-Met decreased with disease progression, while the number of residual neurons negative for both proteins increased dramatically (Fig. 4). At 11 years 5 months after disease onset (case 38 in Fig. 1), although a small number of residual neurons were still evident, most of them were atrophic and immunonegative for both HGF and c-Met. However, even in this long-surviving patient, a few residual neurons expressing both HGF and c-Met were observed (Fig. 5). Thus, residual neurons that were positive for both HGF and c-Met were present throughout the disease course in every ALS patient. Expression of HGF and c-Met by only part of the neuronal cytoplasm and/or dendrites was observed more often as ALS-associated degeneration progressed (Figs. 4, 5). Observation of only the H-E-stained sections revealed no difference between the neurons positive for HGF and c-Met and those negative for both proteins (Figs. 4, 5).

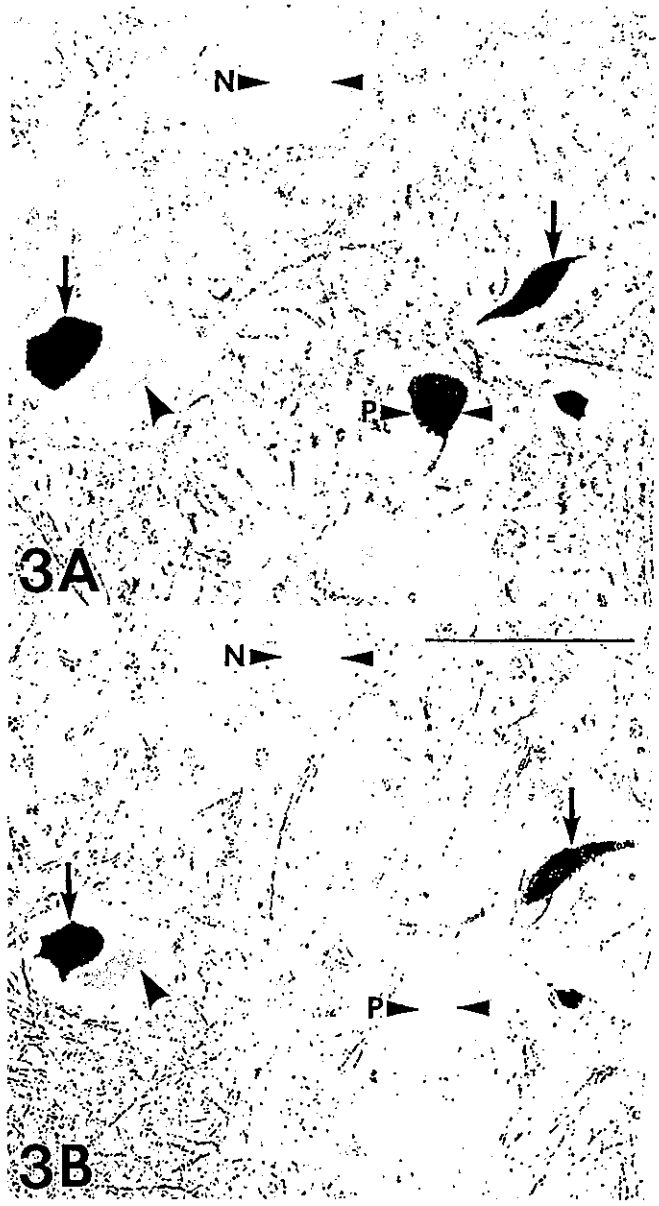

Fig. 3 Serial sections of spinal anterior horn cells in a patient with sporadic ALS after a clinical course of 2.5 years (case 18 in Fig. 1) immunostained with antibodies against HGF (**A**) and c-Met (**B**). Residual neurons intensely expressing both HGF and c-Met are evident (*arrows*). The staining pattern is diffuse in the cytoplasm. Other neurons are either faintly stained by both antibodies, or unstained (*big arrowhead*). However, neither the HGF-positive neuron (*small arrowheads* with *P*) nor the HGF-negative neuron (*small arrowheads* with *N*) in the HGF section shown in A appears in the section stained for c-Met in B. No counterstaining. *Bar* B (also for A) 100 μm

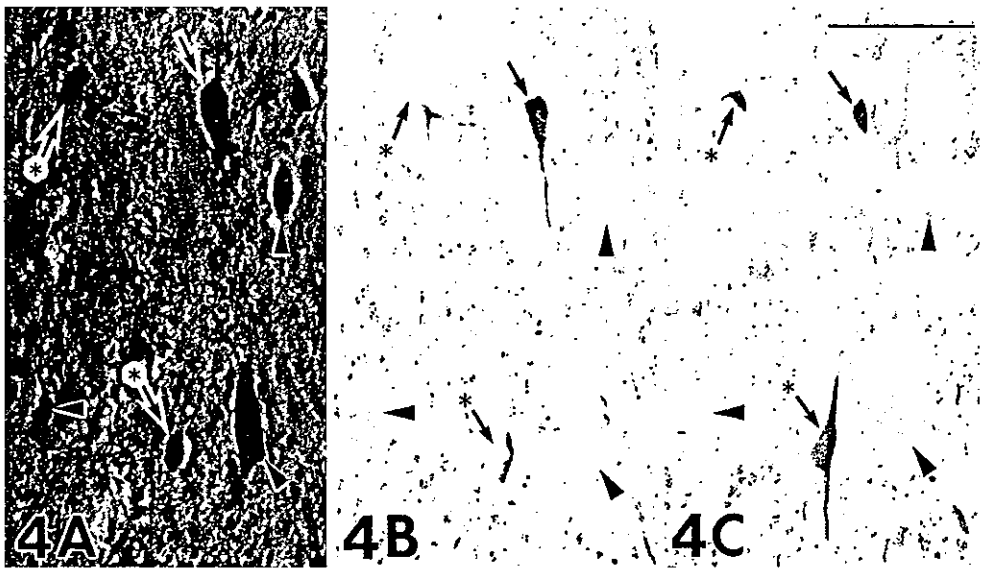

Fig. 4 Serial sections of the anterior horn cells in a patient with sporadic ALS after a clinical course of 4 years 8 months (case 31 in Fig. 1). **A** In the H-E preparation, residual motor neurons appear to be atrophic. There is no distinction among these atrophic neurons when observed in the H-E preparation alone. **B** Immunostaining with the antibody against HGF. The number of residual neurons intensely expressing HGF (*arrow*) is reduced in comparison with that in the ALS patient after a clinical course of 2.5 years (Fig. 3). Only part of the neuronal cell body and dendrite expresses HGF (*arrows* and *asterisks*). The number of HGF-negative neurons is increased (*arrowheads*). No counterstaining. **C** Immunostaining with the c-Met antibody. Similarly to the anti-HGF immunostaining, the number of c-Met-immunopositive neurons is diminished (*arrow*). In contrast, the number of c-Met-immunonegative neurons is increased (*arrowheads*). However, only part of the neuronal cytoplasm and dendrite is positive for c-Met (*arrows* and *asterisks*). No counterstaining. *Bar* **C** (also for **A**, **B**) 100 μm

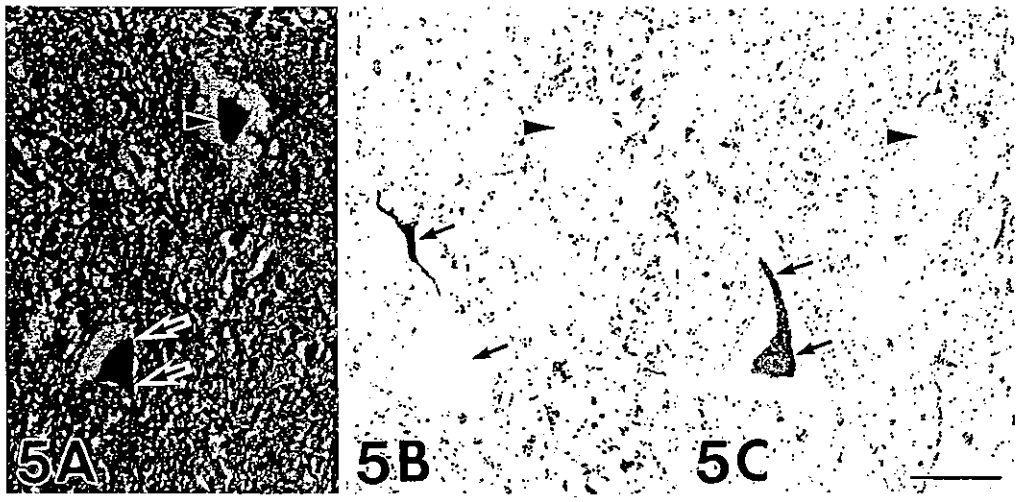

Fig. 5 Expression of HGF and c-Met detected by immunohistochemistry in a patient with sporadic ALS after a clinical course of 11 years 5 months (case 38 in Fig. 1). **A** Light microscopic preparation stained with H-E. Small and atrophic motor neurons are seen. **B** HGF immunoreactivity of the section consecutive to that shown in **A**. Although the residual neuron (*double arrows*) appears to be atrophic in the H-E preparation, this residual neuron expresses HGF: only a dendrite is HGF-positive (*double arrows*). No counterstaining. **C** c-Met immunoreactivity of the section consecutive to that shown in **B**. The residual neuron that appears to be atrophic in the H-E preparation is stained by the anti-c-Met antibody (*double arrows*). A neuron negative for both HGF and c-Met can be also observed (*arrowhead*). No counterstaining. Observation of the H-E-stained section in **A** reveals no difference between the atrophic neuron positive for HGF and c-Met (indicated by *double arrows*) and the atrophic neuron negative for both proteins (marked by an *arrowhead*). *Bar* **C** (also for **A**, **B**) 50 μm

With respect to the LBHI-bearing neurons in the anterior horns of FALS patients with SOD1 mutations, LBHIs in the anterior horn cells showed co-aggregation of both HGF and c-Met (Fig. 6). However, the intensity of cytoplasmic HGF and c-Met staining in LBHI-bearing neurons was either weak or negative. Except for the LBHI-bearing neurons, the stainability and intensity of HGF and c-Met staining in the residual neurons of SOD1-mutated FALS patients were identical to those of the SALS patients.

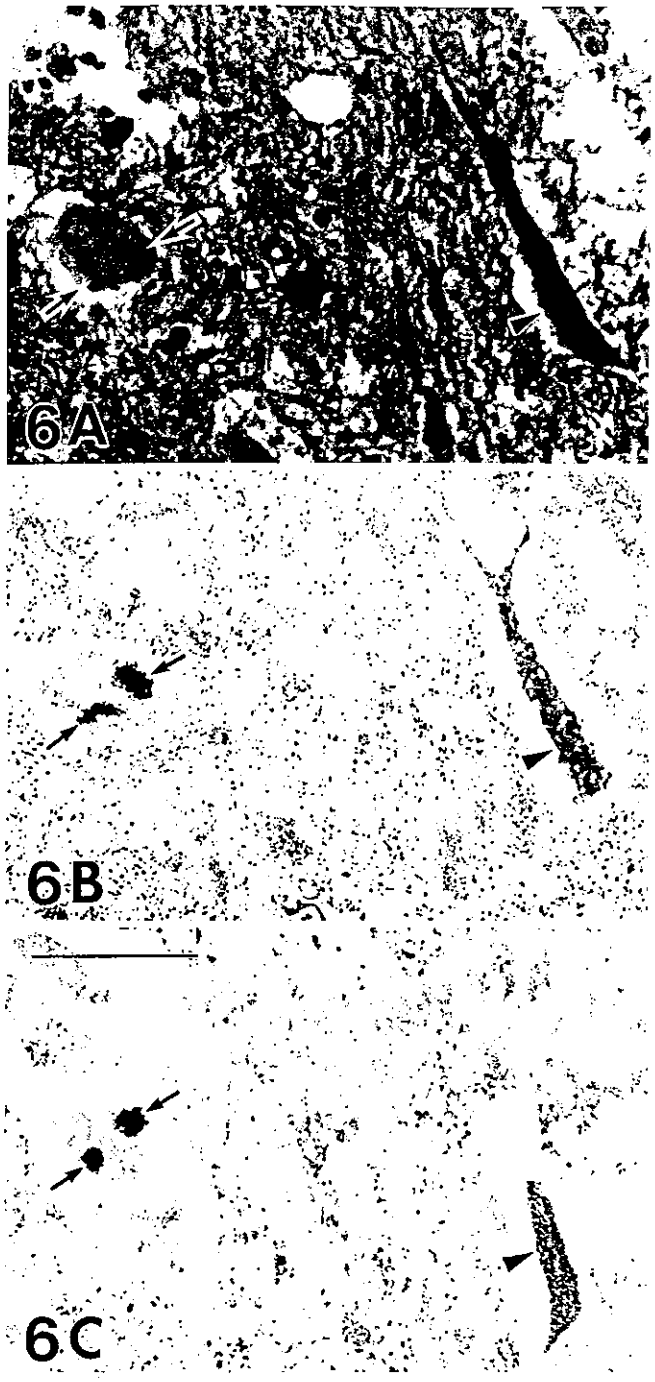

Fig. 6 Serial sections of neuronal LBHIs in a familial ALS patient with an SOD1 mutation (case 2 in Table 1). **A** In the H-E preparation, the neuronal LBHIs consist of eosinophilic cores with paler peripheral halos (*arrows*). An atrophic anterior horn cell without inclusions is also seen (*arrowhead*). **B** Immunostaining with the c-Met antibody is strongly positive for the neuronal LBHI core (*arrows*). However, the cytoplasmic staining intensity of the LBHI-bearing neuron is weak. By contrast, c-Met expression is evident even in the atrophic neuron (*arrowhead*). No counterstaining. **C** Immunostaining with the HGF antibody. The LBHIs show co-aggregation of both HGF and c-Met (*arrows*). Even though the cytoplasm of the shrunken neuron is positive for both HGF and c-Met (*arrowhead*), the cytoplasmic staining intensity of the LBHI-bearing neuron to HGF and c-Met is weak. No counterstaining. (*LBHI* Lewy body-like hyaline inclusion, *SOD1* superoxide dismutase 1). *Bar* **C** (also for **A**, **B**) 50 μm

Fig. 7 HGF and c-Met expression in reactive astrocytes in a patient with sporadic ALS after a clinical course of 4 years 1 month (case 30 in Fig. 1). **A** Although only part of the neuronal cytoplasm is HGF-positive (*arrow*), almost all of the neurons are either faintly stained by the anti-HGF antibody, or not at all (*arrowheads*). No significant HGF immunoreactivity is evident in astrocytes. No counterstaining. **B** c-Met immunoreactivity of the section consecutive to that shown in **A**. c-Met expression is observed in reactive astrocytes. In general, c-Met-positive reactive astrocytes are frequently present around the neurons negative for HGF and c-Met (*arrowheads*). An astrocyte in contact with a neuron negative for both proteins intensely expresses c-Met (*arrow* and *A*). Only part of the neuronal cytoplasm is immunopositive for HGF and c-Met (*arrow*). A c-Met-negative neuron (*arrowheads* and *N*) in the section stained for c-Met in **B** does not appear in the section stained for HGF in **A**. No counterstaining. *Bar* **B** (also for **A**) 100 μm

Although no significant HGF immunoreactivity was found in reactive astrocytes in any of the ALS patients throughout the disease course, c-Met was expressed in these cells. These c-Met-positive reactive astrocytes were frequently seen around neurons that were negative for HGF and c-Met (Fig. 7). Reactive astrocytes in contact with neurons negative for both proteins showed intense expression of c-Met. The number of reactive astrocytes expressing c-Met tended to reach a peak around 3–4 years after ALS onset (Fig. 7), and thereafter decreased along with disease progression after patients had begun to require respiratory assistance. Oligodendrocytes showed no significant expression of HGF and c-Met.

Western blot analysis

When the tissue homogenate of each fresh cervical segment of the spinal cord was examined by immunoblotting for HGF, double bands at positions corresponding to approximately 34 kDa and 69 kDa were observed, i.e., those with the same mobility as human HGF (Fig. 8A). Immunoblotting showed that the intensity of HGF immunoreactivity in the SALS patient with a clinical course of 2.5 years (case 18 in Fig. 1) appeared to be identical to, or slightly stronger than, that in a normal subject. In the SALS patient with a clinical course of 11 years 5 months (case 38 in Fig. 1), HGF expression was less than that in the normal subject. This observation supported the results of HGF immunohistochemistry.

Immunoblotting for c-Met revealed a single band corresponding to about 140 kDa in two SALS cases and a normal subject (Fig. 8B). This molecular mass was compatible with that of human c-Met. In the SALS case at 2.5 years after disease onset (case 18 in Fig. 1), c-Met was expressed more strongly than in the normal subject. However, the level of c-Met expression in the SALS case at 11 years 5 months after onset (case 38 in Fig. 1) decreased below that in the normal subject. This finding reflected the c-Met immunohistochemistry results.

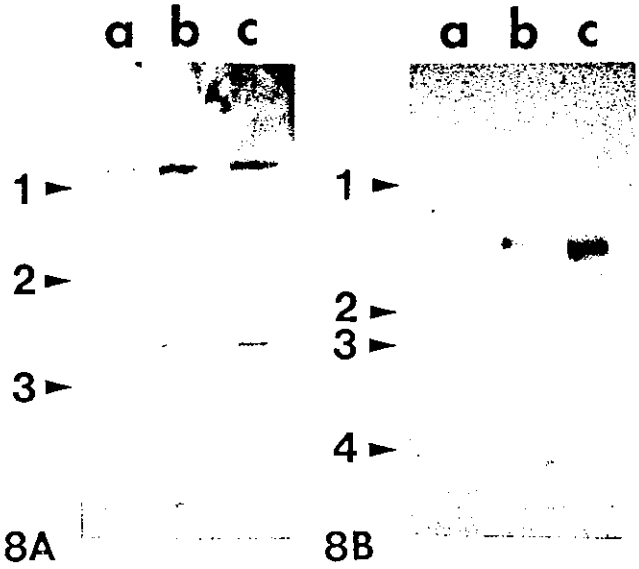

Fig. 8 Western blot analyses using the antibodies against HGF (A) and c-Met (B). A 20-μg amount of the soluble protein extract from each sample has been applied to each lane. A Molecular mass markers: *1* bovine serum albumin (66 kDa); *2* ovalbumin (45 kDa); *3* carbonic anhydrase (31 kDa). *Lane a* A sporadic ALS patient with a clinical course of 11 years 5 months (case 38 in Fig. 1), *lane b*: normal control, *lane c*: a sporadic ALS patient with a clinical course of 2.5 years (case 18 in Fig. 1). Double bands with molecular masses of approximately 69 kDa and 34 kDa are observed in all samples. The intensity of HGF immunoreactivity in *lane c* (ALS case 18) appears to be identical to, or slightly stronger than, that in *lane b* (normal). In contrast, HGF expression in *lane a* (ALS case 38) appears to be lower than that in *lane b* (normal). B Molecular mass markers: *1* myosin (200 kDa); *2* β-galactosidase (116 kDa); *3* phosphorylase B (97.4 kDa); *4* bovine serum albumin (66 kDa). A single band corresponding to about 140 kDa is detected in each sample. Expression of c-Met in *lane c* (case 18) is stronger than in *lane b* (normal). However, the level of c-Met expression in *lane a* (case 38) is decreased below that seen in *lane b* (normal)

Discussion

HGF is a heterodimeric protein composed of two polypeptide chains (69 kDa α chain and 34 kDa β chain) linked together by disulfide bonds [25]. HGF has four intrachain disulfide loops, called "kringle" domains, in its α chain [25]. In vitro, HGF is one of the most potent neurotrophic factors for motor neurons [6]. The HGF antibody used in the present study recognizes both the α and β chains. HGF acts via its receptor, c-Met, which is a transmembrane protein of 190 kDa, consisting of a 50-kDa extracellular α subunit and a 140-kDa β subunit with a tyrosine kinase domain [29]. According to the manufacturer (Santa Cruz), the c-Met antibody recognizes only the β subunit. This is consistent with the results of Western blot analyses, where use of a normal tissue homogenate yielded double bands of approximately 69 kDa and 34 kDa with the anti-HGF antibody, and a single band of about 140 kDa with the anti-c-Met antibody.

Under normal physiological conditions, HGF and c-Met immunoreactivity in the human spinal cord anterior horns was primarily identified in the neurons: cytoplasmic staining with antibodies against both proteins was observed in almost all of the anterior horn cells. Considering that HGF has extremely potent neurotrophic activity and acts through c-Met, our immunohistochemical findings indicate that even under normal physiological conditions, spinal motor neurons maintain themselves via an HGF-c-Met autocrine and/or paracrine system. No significant HGF and c-Met immunoreactions were seen in astrocytes and oligodendrocytes. Even if the astrocytes and oligodendrocytes produced only infinitesimal traces of HGF and c-Met, the protein production levels were below the limits of immunohistochemical detection.

A very striking feature was the presence of certain residual motoneurons intensely co-expressing both HGF and c-Met throughout the disease course of ALS. This suggests that the HGF-c-Met system may represent an endogenous survival mechanism that is activated by ALS

stress. Although some neurons co-expressed HGF-c-Met, while many were negative for both proteins among the residual motoneurons affected by ALS, there was no apparent difference among these residual ALS neurons when observed in H-E preparations alone. Those residual neurons showing high co-expression of HGF and c-Met would be less susceptible to ALS stress and protect themselves from ALS neuronal death. In addition, the residual neurons that are immunopositive for HGF and c-Met might maintain their viability via an autocrine and/or paracrine mechanism involving the HGF-c-Met system. This hypothesis is supported by the fact that HGF is one of the most potent survival-promoting factors for motor neurons, being comparable to GDNF in vitro [6]. Because of the neurite outgrowth-promoting property of HGF in vitro [7, 10, 22], residual neurons that are immunoreactive for HGF and c-Met might show enhanced neurite outgrowth to other neurons and regenerate the neuronal network to compensate for any functional deficiency and neuron loss. In other words, those motor neurons showing up-regulation of the HGF-c-Met cell-survival system, which is normally present in neurons, might show enhanced cell survival in the presence of ALS stress. In contrast, breakdown of the HGF-c-Met system in ALS motor neurons that are barely viable would result in cell death, and many residual neurons that are unable to express HGF and c-Met would be ultimately moribund. It remains to be determined whether this HGF-c-Met up-regulation is a direct or an indirect effect based on the pathogenesis of ALS itself, or whether HGF and c-Met play a primary or a secondary role in attenuating ALS-related neuronal death.

Focusing on FALS with SOD1 mutations, the SOD1 protein (probably the mutant form) aggregates in the anterior horn cells as neuronal LBHIs [14, 15, 33]. It is of great interest that these LBHIs corresponded to structures immunostained by both antibodies against HGF and c-Met. The cytoplasm of these LBHI-bearing neurons was only weakly immunopositive, or even immunonegative, for HGF and c-Met. Such sequestration into LBHIs has also been observed for normal constitutive proteins such as tubulin and tau protein [15, 16, 17], and this resulted in partial impairment of the maintenance of cell metabolism [15, 16, 17]. Although we cannot readily compare the sequestration of normal constitutive proteins with internalization of a cell-survival ligand-receptor system like HGF and c-Met, these findings lead us to speculate that internalization of HGF and c-Met into LBHIs in FALS patients may partly contribute to the breakdown of HGF autocrine and/or paracrine trophic support in these neurons and may be one of the endogenous mechanisms that accelerate neuronal death. This hypothesis would appear to be compatible with the aggregation toxicity theory.

Reactive astrocytosis and gliosis were observed in the anterior horns of patients with SALS and FALS. Based on observations of H-E preparations alone, these findings suggest that the reactive astrocytes merely fill the spaces created by neuronal loss. It is noteworthy that these reactive astrocytes in both types of ALS expressed c-Met. Thus, it appears that c-Met induction in reactive astrocytes is a common feature in ALS patients. It should be noted that astrocytes, in contact to neurons that were immunonegative for both proteins, intensely expressed c-Met. This may reflect c-Met regulation in astrocytes through an astrocyte-neuron relationship. Astrocytes play important roles in sustaining environments within the central nervous system, including neuronal maintenance [20, 32, 34, 37, 39]. Neuronal vulnerability to oxidative stress or toxicity is greater in neurons cultured alone than in neurons co-cultured with astrocytes [4, 5]. It has been shown that the activity of the glial-specific glutamate transporter (EAAT2/GLT1) in spinal cord astrocytes is selectively diminished in human SALS [30, 31] as well as in the SOD1-mutated ALS model transgenic animals of G85R [2], A4V [38], I113T [38], and G93A [1, 13]. Therefore, it can be postulated that HGF might protect c-Met-positive reactive astrocytes from down-regulation of the glial-specific glutamate transporter (EAAT2) via the HGF-c-Met system, and increase glutamate clearance, thereby leading to a reduction in glutamatergic neurotoxicity and resulting in promotion of neuronal survival [35]. However, 3–4 years after ALS onset, the number of c-Met-positiv reactive astrocytes decreased along with disease progression. These findings suggest that breakdown of the HGF-c-Met system is involved in the impairment of both astrocytic function itself and astrocyte-neuron relationship at the terminal stage of ALS. It remains to be determined whether this consideration applies to a degenerative condition such as ALS, in which affected astrocytes do not have a normal control mechanism. Taken together with the fact that overexpression of HGF has been reported to attenuate motor neuron death and prolong the life-span of FALS transgenic mouse model [35], our data in this report may lead to the development of a new HGF-based therapy for ALS, which for over 130 years has had an unknown etiology.

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治療戦略に有用な筋萎縮性側索硬化症（ALS）の動物モデルの開発*

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Key Words : amyotrophic lateral sclerosis, Cu/Zn superoxide dismutase, mutation, transgenic mice, transgenic rat

はじめに

筋萎縮性側索硬化症(amyotrophic lateral sclerosis : ALS) は上位および下位運動ニューロンを選択的かつ系統的に障害し，呼吸筋を含む全身の筋萎縮をきたす進行性疾患である．加えて現在までに有効な治療薬や治療法がほとんどないため，ALSは神経疾患のなかで最も過酷な疾患とされ，早期に病因の解明と有効な治療法の確立が求められている．遺伝学的解析法の進歩により，1993年に家族性ALSにおいてその一部の原因遺伝子がCu/Zn superoxide dismutase (Cu/Zn SOD) であることが明らかになり[1~3]，さらにはこのCu/Zn SOD遺伝子の突然変異をマウスに導入することにより，ヒトALSの病態を非常によく再現することに成功した[4~6]．ALSに対する治療法の開発には，① 臨床型および病理像を忠実に再現した動物モデルの作製とその病態機序の解明，および，② その動物モデルに対する治療法の開発の二つのステップが重要である．本稿では前半の①にあたる現在最も汎用されているトランスジェニック (Tg) マウス，および最近はじめて確立したTgラットによるALSモデル動物の開発を中心に述べる．

I．変異Cu/Zn SOD遺伝子導入によるALSマウスモデル

1. 変異Cu/Zn SODを導入したトランスジェニック (Tg) マウスによるALSモデル

Cu/Zn SOD遺伝子変異による家族性ALSの発症メカニズムはまだ十分には解明されていないが，変異によるSOD活性の低下が直接の原因ではなく，変異Cu/Zn SODが新たに獲得した"gain of toxic function"によるものと考えられている[7]．その最大の根拠は変異Cu/Zn SODを導入したTgマウスが，ヒトALSにきわめてよく似た表現型と病理所見を示す一方で[4~6]，Cu/Zn SOD遺伝子をノックアウトしてもALS症状は示さないことである[8]．これまでに数種類のヒト変異Cu/Zn SOD遺伝子を導入したマウスが報告されて[4~6]，その一部は米国Jacksonラボラトリーにより世界中に供給されている．わが国においても新潟大学の中野・菊川らがヒトALS患者の病理像が明らかとなっているI113T変異[9]を導入したTgマウスの作製に成功している．このマウスは変異蛋白の発現量が生理的なレベルに近く，しかも後肢の脱力という表現型が現れる時期が12ヵ

* Development of New Transgenic Models of Amyotrophic Lateral Sclerosis.

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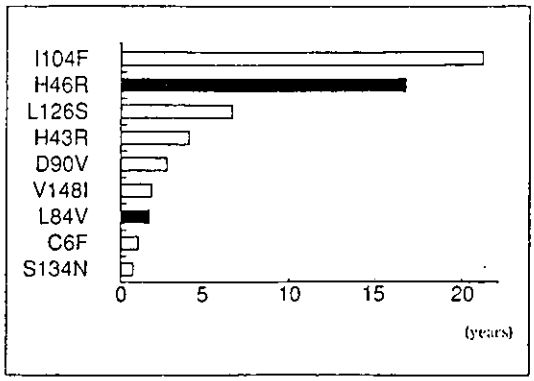

Fig. 1 Relationship between the duration of the disease and the mutations in the Cu/Zn SOD gene that have been reported form the Department of Neurology, Tohoku University School of Medicine. The mutations that we have introduced to Tg mice are indicated by the solid bars.

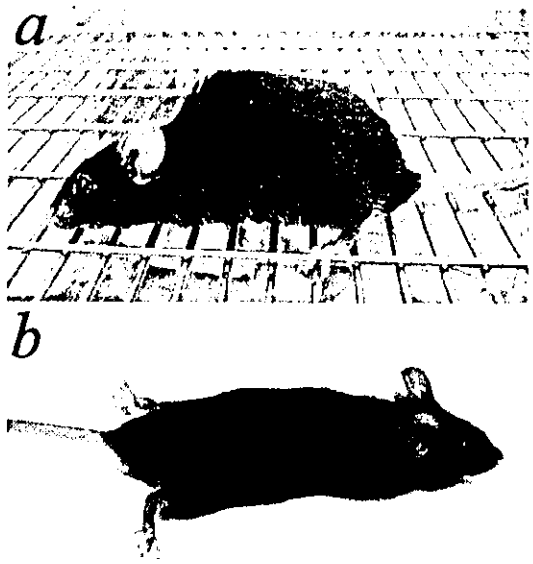

Fig. 2 a, An affected mouse from the L84V-*l*51 line mutant developed weakness in the forelimbs. b, An affected mouse from the H46R-*h*70 line developed weakness in the hind limbs. The mice in H46R-*h*70 line always develop weakness in their hindlimbs.

月と中年期以降の発症でかつ症状の進行も緩徐であるため，神経細胞の障害機構の解析や治療薬の薬効の評価に有用と考えられる．Cu/Zn SOD遺伝子変異に伴う家族性ALSおよび既存の変異Cu/Zn SOD遺伝子導入Tgマウスにおける病理像については加藤らの総説を参照されたい[10]．

2. 新しいTgマウスモデルの作製による臨床型の再現

東北大学神経内科ではこれまでに，日本人13家系の家族性ALSにおいて10種類のCu/Zn SOD遺伝子変異を報告してきた．これまでの臨床型の検討により，各点突然変異によりおよその罹病期間が決まっており，例えばH46R変異を伴う家系の患者は例外なく平均16.8±6.8年にわたるきわめて緩徐な進行を示した[1, 11]．すなわち同じ遺伝子に変異をもちながら臨床型に大きな違いがみられている（**Fig. 1**）[12~14]．そこでわれわれは各点突然変異による臨床型の違い，特に臨床経過の速さを決定する要因を検討するために，臨床経過が非常に緩徐なH46R変異および経過が急速なL84V変異[15]（Fig. 1）を導入したマウスを同一の遺伝的背景で作製することを開始した．このためにまずヒトゲノムライブラリーからプロモーター領域を含むヒトCu/Zn SOD遺伝子全長を単離し，site-directed mutagenesis法を用いて点突然変異（H46RおよびL84V）を導入しTgマウスの作製を行った．これまでにH46R変異を導入したTgマウスは2系統得られ，このうち導入した変異Cu/Zn SOD蛋白が多く発現したマウス（*h*70系統）は，生後約150日目に後肢の脱力で発症し，四肢麻痺へと進行して約30日の経過で死亡した．脊髄における総蛋白量あたりのCu/Zn SOD活性は低下していた．病理所見は，脊髄前角の運動ニューロンの変性・消失がみられ，ALS患者に認められるLewy body-like hyaline inclusionに類似した封入体が認められている[16]．一方，L84V変異を導入したTgマウスは数系統得られ，このうち3系統に発症がみられている．ここで興味深いことにはH46R変異を導入したマウスはすべて後肢からの発症であるのに対して，L84V変異を導入したマウスは一部ではあるが前肢からの発症が認められた（**Fig. 2**）[17]．このことはヒト家族性ALSにおいてH46R変異を伴う家系は全て下肢から発症が認められるのに対して，L84V変異を伴う家系では上肢から発症が認められることをよく再現している．さらには全生存期間が約180日とほぼ同等の系統（*l*51と*h*70

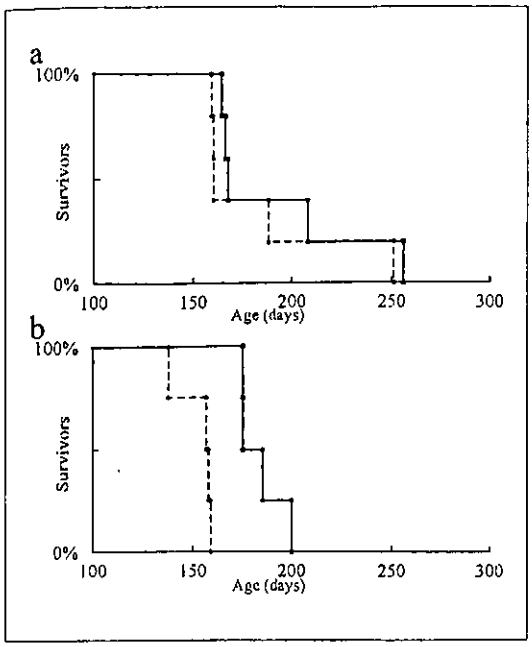

Fig. 3 a, Kaplan-Meier survival curves illustrating the age of onset (mean 184 d) and death (mean 189 d) for the L84V-*l*51 line. b, Kaplan-Meier survival curves illustrating the age of onset (mean 153 d) and death (mean 184 d) for the H46R-*h*70 line. The dashed lines with black dots designate the onset curves (percentage without weakness), whereas the solid lines with black square data points designate the survival curves (percentage surviving).

系統）でH46R変異とL84V変異を比較すると，発症から死亡までの経過はL84V変異（*l*51）が13.8日とH46R変異（*h*70）の25.7日と比較して急速であった（**Fig. 3**）．このことは各点突然変異によるヒトALSの罹病期間の違いもよく再現している[17]．

また今回作製したTgマウスはH46R，L84V両変異ともにSOD活性を上昇させず，組織学的にも空胞変性が少なかった．このことは従来のマウスに比較してこれらのマウスはよりヒトの病態に近いモデルであると考えられる[17]．今後はこれらのマウスを用いて，臨床型の違いを決定する因子を探索が可能であると同時に，治療法の開発の促進が期待される．

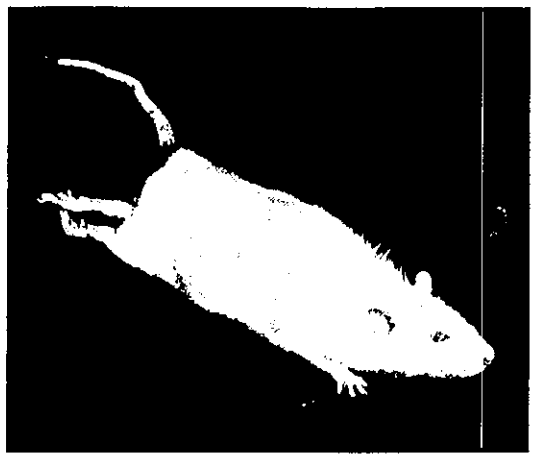

Fig. 4 An affected transgenic rat from H46R-4 demonstrates hindlimb weakness and abnormal posturing with segmental spasticity of the tail.

II. トランスジェニック（Tg）ラットによる新しいALSモデル

前述のように，ALSのモデル動物としては従来マウスが用いられてきたが，特に病態の首座である脊髄の解析には，その個体の大きさによる研究上のさまざまな制約があった．さらには脊髄の運動ニューロンに対して効率よく，しかも副作用を回避できる薬物の投与ルートとして髄腔内投与が注目されており，実際に米国ではALS患者への持続注入ポンプを用いた神経栄養因子の髄腔内投与が試みられている（日本でも岡山大学神経内科で治験が始まっている）．そこで東北大学の永井らは動物モデルにおける脊髄や脊髄腔に対する治療的なアプローチを可能とするために，世界にさきがけて変異Cu/Zn SOD導入TgラットによるALSモデルの作製に成功した（**Fig. 4**）[18]．

マウスと同様に臨床経過が非常に緩徐なH46R変異および経過が一般的でTgマウスが世界的に供給されているG93A変異[4]をもつCu/Zn SOD遺伝子をSDラット受精卵にマイクロインジェクションし，生まれたラットの尾からDNAを抽出し，遺伝子の導入を確認した．H46R変異を導入したTgラットは5系統，G93A変異を導入したTgラットは7系統得られた．導入したヒト変異Cu/Zn SOD蛋白の発現量は，H46R-4系統で最

Table 1 Lines of transgenic rats expressing human Cu/Zn SOD with H46R or G93A mutations[16]

	Relative Cu, Zn SOD protein level (human/rat)	Age of onset (days)	Duration (days)
H46R-4	6.0	144.7 ± 6.4	24.2 ± 2.9
H46R-13	3.0	−	−
G93A-39	2.5	118.6 ± 14.1	8.3 ± 0.7
G93A-24	0.8	−	−

も多く，内因性のラットCu/Zn SODとの比をとると約6倍であった（**Table 1**）．

H46R変異およびG93A変異をもつTgラットともに導入された変異ヒトCu/Zn SOD蛋白が多く発現した系統（H46R-4およびG93A-39）において運動ニューロン病の症状の発現が認められている（Table 1）．発症は，二つの変異を導入したTgラットとも後肢の筋力低下で始まり，対麻痺，四肢麻痺へと進行し死に至った（Fig. 4）．H46R-4は144.7日で発症し，24.2日の経過で死亡した．G93A-39は118.6日で発症し，8.3日の経過で死亡した．変異Cu/Zn SOD蛋白の発現量はG93A-39の方がH46R-4に比較して少ないのにもかかわらず，G93A-39はより早期に発症し，かつ非常に急速な進行を示している（Table 1）．これは前述のTgマウスと同様にラットにおいても導入した変異がG93A変異の家系は速い経過を示し，H46R変異が緩徐な経過を取ることをよく再現していた[16]．

今回作製されたTgラットでは従来のマウスに比較して約20倍の大きさをもつために，脳脊髄液（髄液）の採取および解析ならびに薬剤や遺伝子治療用のベクターの髄腔内投与がきわめて容易である．将来的な遺伝子治療を含めた新しい治療法開発のために非常に有用なモデルとなることが期待される．実際われわれはこの新しいTgラットに対し，ポンプを利用して脳由来神経栄養因子BDNFやわが国で開発された新規の栄養因子である肝細胞増殖因子HGFなどの神経栄養因子の髄腔内投与を持続的に行いその治療効果を判定している．将来的には神経幹細胞の髄腔内投与や脊髄への移植も可能であり，今後の研究の発展に役立つことが期待される．

おわりに

1993年のALSにおけるCu/Zn SOD遺伝子変異の発見は家族性のALSとはいえきわめて意義が大きい．この遺伝子異常を導入したモデル動物の作製が進んでいるが，SODの異常によりなぜ選択的に運動ニューロンが障害を受けるのかの病態機序は依然として不明であり，これからの課題である．ALSの病態の解明あるいは新しい治療法の開発のためにはモデル動物の利用およびその解析は必須と思われる．また，2001年には東海大学の秦野・池田らにより新規ALS原因遺伝子ALS2の同定が行われ，このALS2遺伝子はグアニンヌクレオチド交換因子をコードすることが推定されている[19]．さらにはDNAマイクロアレイなどを利用した発現遺伝子プロファイリング解析に基づくALS病態関連遺伝子の同定の試みも進行しつつあり[20]，新たな知見に基づく遺伝子改変動物の作製も必要と考えられる．

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Development of New Transgenic Models of Amyotrophic Lateral Sclerosis

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Mutations in Cu/Zn superoxide dismutase (SOD1) have been linked to some familial cases of amyotrophic lateral sclerosis (ALS). In order to reproduce the different degree of toxicity to the mutant protein by mutations, we generated new transgenic mice with two mutations from which the progression of the disease in human family is rapid (L84V) or extremely slow (H46R). By comparing the two transgenic mice with different SOD1 mutations, we demonstrate that the time course and the first symptoms in these mice were likely to human SOD1-mediated familial ALS. Similarly to the previous studies of SOD1 mutant familial ALS patients and mutant SOD1-expressing transgenic mice, Lewy body-like hyaline inclusions in the motor neurons in spinal cords are seen in the L84V and H46R transgenic mice. These transgenic mice do not have elevated dismutation activity. It is probable the reason why these mice has little vacuolar pathology. Because the vacuoles are usually undetectable in human ALS spinal cord, we think that L84V and H46R ALS transgenic mice precisely demonstrate pathological changes that occur in human ALS.

In addition, we report here that rats that express a human SOD1 transgene with two different ALS-associated mutations (G93A and H46R) develop striking motor neuron degeneration and paralysis. As in the human disease and transgenic ALS mice, pathological analysis demonstrates selective loss of motor neurons in the spinal cords of these transgenic rats. The larger size of this rat model as compared with the ALS mice will facilitate studies involving manipulations of spinal fluid (implantation of intrathecal catheters for chronic therapeutic studies ; CSF sampling) and spinal cord (e.g., direct administration of viral- and cell-mediated therapies).

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Transplantation of neural stem cells into the spinal cord after injury

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Abstract

Thanks to advances in the stem cell biology of the central nervous system (CNS), the previously inconceivable regeneration of the damaged CNS is approaching reality. The availability of signals to induce the appropriate differentiation of the transplanted and/or endogenous neural stem cells (NSCs) as well as the timing of the transplantation are important for successful functional recovery of the damaged CNS. Because the immediately post-traumatic microenvironment of the spinal cord is in an acute inflammatory stage, it is not favorable for the survival and differentiation of NSC transplants. On the other hand, in the chronic stage after injury, glial scars form in the injured site that inhibit the regeneration of neuronal axons. Thus, we believe that the optimal timing of transplantation is 1–2 weeks after injury.

Keywords: Neural stem cell; Central nervous system; Regeneration; Myelin; Nogo; Spinal cord injury; Transplantation

1. Introduction

Ever since the famous neuroanatomist Ramon y Cajal wrote in the early 20th century that the central nervous system (CNS, i.e. the brain and spinal cord) does not regenerate once it is injured [1], this theory has been popularly accepted. The lack of regenerative properties of the mammalian CNS, especially in the spinal cord, could be attributable to a combination of factors, including the inhibitory character of CNS myelin and injury-induced glial scars, the apparent inability of endogenous adult neural stem cells (NSCs) in the spinal cord to induce de novo neurogenesis upon injury [2], and the lack of sufficient trophic support [3]. However, in the 1980s, studies were reported on the transplantation of peripheral nerves [4] and fetal spinal cord [5] for spinal cord injuries. These studies indicated that the introduction of an appropriate environment into the injured site can cause injured axons to regenerate. In addition, reports described spinal cord regeneration, including the promotion of the regeneration of injured axons by neurotrophic factors [6], and the identification of axonal growth inhibitors [7]. These studies indicated that the regeneration of the injured spinal cord might really be possible. Although researchers first focused on the effectiveness of fetal spinal cord transplantation for spinal cord injuries [8–10], donor shortage and ethical problems precluded the practical clinical application of this approach. As a result of remarkable advances in neuroscience in recent years, NSCs have stepped into the limelight as a new transplant material. This paper outlines the present state and future prospects of basic studies on NSC transplantation for the damaged CNS, including spinal cord injuries.

1.1. Rationale behind cell transplantation for spinal cord injury and damaged CNS

Traumatic spinal cord injury affects many people, including young people, and can result in severe damage, leading to paraplegia, tetraplegia, or worse. Many strategies, including surgical, pharmacological, neurophysiological, and technological approaches, have been used in attempts to develop new therapies that will allow patients to regain the use of their paralyzed limbs. One such strategy is cell transplantation into the damaged spinal cord. The rationales for this approach can be summarized as follows: (a) to promote the functional reconstruction of neuronal circuits, i.e. the production of new inputs to a de-afferented region that form new synaptic connections or new interconnections, the replacement of damaged interneurons within a structure, the formation of an interconnecting bridge that receives inputs from a healthy brain region and provides modulated inputs to a damaged part of the brain, the formation of a barrier to

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abnormal collateral growth of axons, or the production of a substrate that facilitates the growth of axons; (b) the trophic effects, i.e. to produce neurochemically active substances such as neurotransmitters, growth factors, antibodies, or growth substrates; and (c) to promote the remyelination of axons [11]. Experiments on neural transplantation for spinal cord injuries started in 1981 with peripheral nerve transplantations performed by Aguayo and coworkers [4,12]. The advantage of this strategy is that the CNS myelin-derived inhibitor for axonal regeneration is absent in the peripheral nervous system (PNS) (this issue on CNS myelin-derived inhibitor is discussed later). In 1993, Bregman and coworkers reported the treatment of immature and adult rats in which the thoracic spinal cord had been partially transected, with transplanted fetal spinal cord, which does not yet express the CNS myelin component. The rats receiving the transplant showed elongation of the injured axons with functional recovery, and this result was more pronounced in immature rats [5]. Such transplants survive and integrate with the host tissue, and may be associated with functional improvement. In fact, the transplantation of fetal CNS tissue has already been performed in human patients with Parkinson's disease, resulting in some clinical improvement [13]. For spinal cord injury, however, such treatment has not yet been established. One underlying reason for the lack of research is that a large number of fetuses are required to obtain enough tissue to treat even one patient (for Parkinson's disease, 4–8 fetuses are required), a requirement that generates both practical and ethical problems. On the other hand, recent progress in the biology of NSCs has made it possible to routinely expand neural progenitor cells obtained from a small amount of fetal CNS tissue in vitro, as floating cell aggregates called neurospheres [14]. The expansion of neural progenitor cells in vitro may overcome the practical and ethical problems associated with fetal tissue transplantation and provide a potential source of the graft material for clinical efforts to regenerate injured spinal cord.

1.2. Neural stem cells

NSCs are undifferentiated nervous system cells that are capable of proliferation, repeated subculture (self-replicating capacity), and differentiation into the three types of cells composing the central nervous system, that is, neurons, astrocytes, and oligodendrocytes (multipotency). Studies are in progress throughout the world in two major areas of research to develop therapeutic strategies for CNS injuries and diseases using NSCs: (i) the activation of endogenous NSCs and (ii) the transplantation of NSCs.

Stem cell biologists, such as those studying hematopoiesis, include the ability to repair post-traumatic tissue in the stem cell definition. Stem cells fitting this definition were not thought to exist in the CNS until evidence appeared that endogenous NSCs contribute to the recovery of the damaged CNS [15–17]. Owing to the development of a selective culture technique for NSCs (the neurosphere technique) [14,18,19], enormous progress has been made in elucidating the biological properties of neural stem cells and their location in the body. In this culture technique, cells collected from the CNS are cultured in a non-adhesive culture dish containing serum-free medium supplemented with a high concentration of either epidermal growth factor (EGF) or fibroblast growth factor-2 (FGF-2), or both. A small number of NSCs present among the cells respond to the growth factor(s) and selectively proliferate in suspension to form balls of cells (neurospheres). If these balls are separated and each individual cell is cultured under the same conditions, neurospheres form again, and, with repeated subculture, continue to form (self-replicating capacity). If these cells are plated in adhesive culture dishes and cultured in growth factor-free medium supplemented with serum, they can differentiate into neurons, oligodendrocytes, and astrocytes (multipotency). Thus, once the desired CNS tissue is obtained, this culture technique allows the acquisition of a large volume of NSCs, resolving the donor procurement problem associated with fetal tissue transplantation.

Nevertheless, previous reports suggested that endogenous NSCs existing in the adult rat spinal cord proliferate and differentiate exclusively into astrocytes, but not into neurons, upon injury [2,20,21]. Furthermore, although recent results showed that forebrain damage due to ischemia could be recovered by activating endogenous NSCs to induce de novo neurogenesis [16,17], such a strategy has not been successful in the injured spinal cord. This observed inability of endogenous NSCs in the adult spinal cord to execute de novo neurogenesis could not be solely attributed to their intrinsic properties, since adult spinal cord derived NSCs are able to make new neurons when transplanted into an adult neurogenic site (i.e. the hippocampal dentate gyrus) [22]. Dr. Masato Nakafuku and his colleagues have suggested that the status of the Notch signal pathway contributes to the apparent restriction of de novo neurogenesis in the adult spinal cord [23]. In the adult spinal cord, neural progenitors and/or NSCs are thought to be surrounded by many mature cells (including both neurons and glia) that express Notch ligands and can inhibit the differentiation of neurons from endogenous progenitors. Considering this situation, the simple question is: How can we induce neurogenesis by transplanting exogenous NSCs or neural progenitor cells in the apparently non-neurogenic adult spinal cord? Is it really possible? In fact, previous studies have reported that neural progenitors or NSCs cannot differentiate into neurons when transplanted back into the spinal cord [21,24]. However, in our recent work we showed that by using in vitro expansion and transplanting the cells at the appropriate time point (which is very important!), neural progenitor cells derived from rat fetal spinal cord can divide and differentiate into neurons in vivo and integrate into the host tissue in the injured spinal cord [25] (Fig. 1). Furthermore, functional recovery was achieved by this NSC-transplantation procedure. (This experiment will be described in details later.)

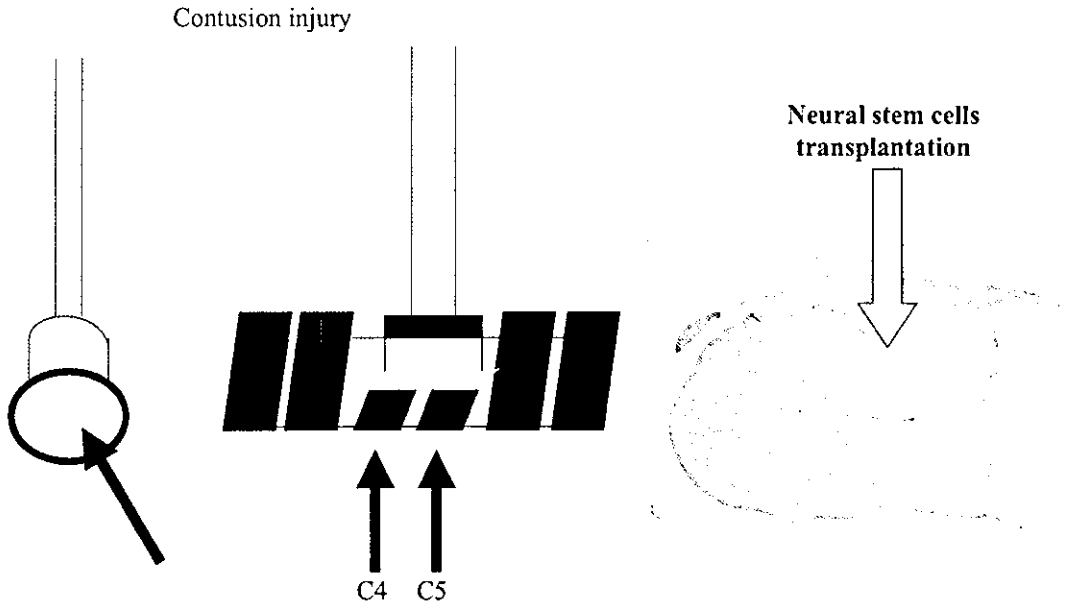


Fig. 1. Transplantation of in vitro-expanded NSCs derived from fetal rat spinal cord into the rat contusion injury model at the C4–5 level. Transplantation was performed 9 days after the spinal corsd injury (SCI + TP, $n = 15$). For transplantation, spheres that had been passaged two to five times were resuspended in the medium without FGF-2 at 5–10 × 10^6 cells/ml as described previously [25].

Relevant studies have recently been published. In one, a rat spinal cord injury model was treated by the transplantation of neural progenitor cells that had been induced to differentiate from mouse ES cells by retinoic acid treatment [26]. Despite some positive outcome, there are some important limitations to this method. For instance, it is known that retinoic acid treatment of ES cells induces a variety of different cell types in addition to neural progenitor cells. Thus, it is possible that a small number of poorly differentiated cells in the grafted cell suspension could result in tumorigenesis from the transplant or induce the formation of non-neural tissue. Furthermore, there may be difficulties in developing this application for human ES cells to treat human spinal cord injury, because human ES cells are not currently readily available for therapeutic purposes in many countries. Moreover, the conditions required to induce the differentiation of neural cells from ES cells in vitro and to select them once they have differentiated have not been optimized. In another study, Vacanti et al. transplanted gels packed with adult rat-derived NSCs into rat models of thoracic spinal cord transection, with similar results [27].

1.3. Cytokines involved in the induction of NSC differentiation: consideration of the optimal timing of NSC transplantation

To establish efficient NSC transplantation into the injured spinal cord, it is essential to elucidate the regulatory mechanism of NSC differentiation. Previous studies reported on the cytokines were involved in this process. Weiss and coworkers reported that brain-derived neurotrophic factor (BDNF) promoted the differentiation of fetal mouse striate body-derived neural stem cells into neurons [28]. Ghosh and Greenberg reported that neurotrophin-3 (NT-3) promoted the differentiation of fetal rat cerebral cortex-derived NSCs into neurons [29]. Dr. Ronald McKay and his colleagues reported that platelet-derived neurotrophic factor (PDNF), ciliary neurotrophic factor (CNTF), and thyroid hormone (T3) instructively induced fetal rat hippocampus-derived NSCs into neurons, astrocytes, and oligodendrocytes, respectively [30]. More recently, Dr. Tetsuya Taga and his colleagues reported that leukemia inhibitory factor (LIF) and bone morphogenic protein-2 (BMP-2) promoted the differentiation of fetal mouse neuroepithelium-derived NSCs into astrocytes [31]. The results of these studies have in common that members of the so-called interleukin-6 (IL-6) superfamily, such as CNTF and LIF, induce NSCs to differentiate into astrocytes, indicating that gp130-mediated-signaling plays a role in this process. However, their results differ in the conditions for differentiation into neurons and oligodendrocytes, presumably reflecting differences in the timing of cell collection, tissue of origin, and method of culture.

Consistent with these findings indicating that various cytokines affect the cell fates of NSCs in a context-dependent manner, it is well known that the host microenvironment influences the survival and differentiation of NSC transplants [32]. Dr. Lars Olson and his colleagues have reported relevant results indicating that NGF, BDNF, and CNTF

increased moderately upon spinal cord injury, but they unfortunately did not reach levels sufficient for spontaneous axonal regeneration [3]. Recent studies have shown that neural stem and progenitor cells also exist in the normal adult rat spinal cord, proliferate after injury, migrate to the injured site, and differentiate, mostly into astrocytes [2,20,33]. In light of the previously reported in vitro results, we believe that the post-traumatic increase in CNTF expression in the spinal cord is one of the factors inducing endogenous NSCs to differentiate into astrocytes, and that the low expression of NT-3 and BDNF, which promote the induction of endogenous NSC differentiation into neurons and oligodendrocytes, creates a microenvironment that is unfavorable for NSC differentiation into neurons and oligodendrocytes.

To achieve success using NSC transplantation, not only is the induction of the differentiation of transplanted cells an important problem, but so is the improvement of transplant survival rates. Within the injured spinal cord, the levels of various inflammatory cytokines (TNFα, IL-1α, IL-1β, and IL-6) peak 6–12 h after injury and remain elevated until the fourth day. Because these inflammatory cytokines are known to have biphasic actions, neurotoxic and neurotrophic, their actions within the injured spinal cord require careful interpretation. We believe that the highly increased expression of these cytokines within 7 days after injury is neurotoxic, resulting in a microenvironment unfit for the survival of NSC transplants. In fact, when we performed NSC transplantation 24 h after the injury, almost none of the grafted cells survived, or, in some cases, a small number of cells survived that formed a small mass. On the other hand, the expression of the anti-inflammatory cytokine TGFβ does not increase immediately after injury, but gradually increases later, peaking on the fourth day after injury. Thus, it appears that TGFβ acts to relieve the inflammatory situation [34].

To summarize the above discussion on the survival and differentiation of NSC transplants, we believe that the optimal time to transplant NSCs is not immediately after injury. At this stage, IL-1β and IL-6 levels are rapidly increased within the injured spinal cord; these cytokines would induce Jak/Stat-signaling, which is likely to direct the endogenous NSCs within the adult spinal cord exclusively into astrocytic fates [35], and they would not have a chance to become neurons. This hypothesis may explain why the injured spinal cord is non-neurogenic. However, this acute inflammatory phase only lasts up to 1 week after injury, which indicates that this period can be avoided for NSC transplantation. However, if too much time passes after the injury, a glial scar forms around the injured site and inhibits the regeneration of axons; therefore, we currently consider the optimal time to transplant NSCs to be 7–14 days after injury. In fact, our recent reports demonstrated that the transplantation of in vitro-expanded NSCs results in mitogenic neurogenesis when the transplantation into the injured adult rat spinal cord is performed 9 days after injury, but not when the transplantation is done within a few days of the injury [25,34,36] (Fig. 2). In addition to the neurogenesis from the transplanted NSCs, the benefits of NSC transplantation at this time point could also result from microvascular regen-

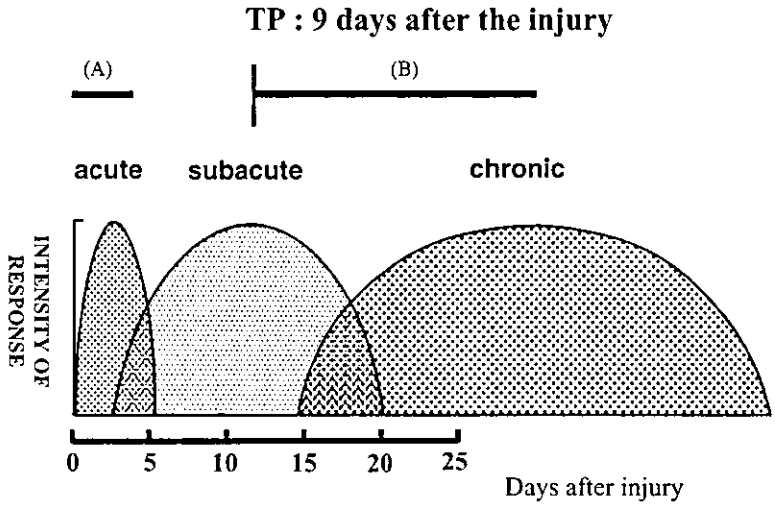


Fig. 2. Changes in the microenvironment after spinal cord injury. Recently, it was reported that endogenous neural stem cells, which are present in the adult rat spinal cord, proliferate and differentiate exclusively into astrocytes, but not into neurons, upon injury. In our recent study [25], however, transplanted neural progenitor cells differentiated into neurons in vivo. This apparent difference could be explained as follows. First, because we transplanted a large number of immature neural progenitor cells, enough autocrine and/or paracrine factors may have been supplied by the donor cells to facilitate their own survival and neuronal differentiation. Second, because we transplanted neural progenitor cells 9 days after the injury, the microenvironment around the injury site may no longer have been in the acute phase indicated (A) in this figure. The proliferation of the endogenous neural stem cells after the injury is actively induced in this acute inflammation phase. Their differentiation into astrocytes may be due to the microenvironment in this phase that favors the generation of astrocytes to prevent the extension of inflammation to the surrounding area. By 9 days after the injury, the microenvironment around the injured site may have changed to be suitable for grafted cells to survive and differentiate into neurons and oligodendrocytes. However, delaying transplantation until the chronic phase is also unlikely to lead to functional recovery, due to the enlarged cystic cavity at the injury site and glial scar formation.

eration in the host, considering previous findings from fetal neural tissues transplanted into the cerebral cortex [37,38]. Correspondingly, a recent report indicates that the formation of new vessels occurs most actively 7–14 days after a contusion injury to the rat spinal cord [39].

To investigate the properties of new neurons derived from donors in more detail, we took advantage of the fact that the 1.1-kb promoter element of the Tα-1 tubulin gene is only active in cells of the neuronal lineage (including neuronal progenitors and post-mitotic neurons), and not those of the glial lineage [40–43]. Here, we used rats that had been treated with transplanted neurospheres derived from the fetal spinal cords (E14.5) of Tα-1-EYFP transgenic rats. By injecting BrdU intraperitoneally, we could label cells that had divided after the BrdU injection. The presence of post-mitotic neurons that were double positive for BrdU-labeling and EYFP expression demonstrated that donor-derived progenitor cells underwent mitotic neurogenesis within the host spinal cord.

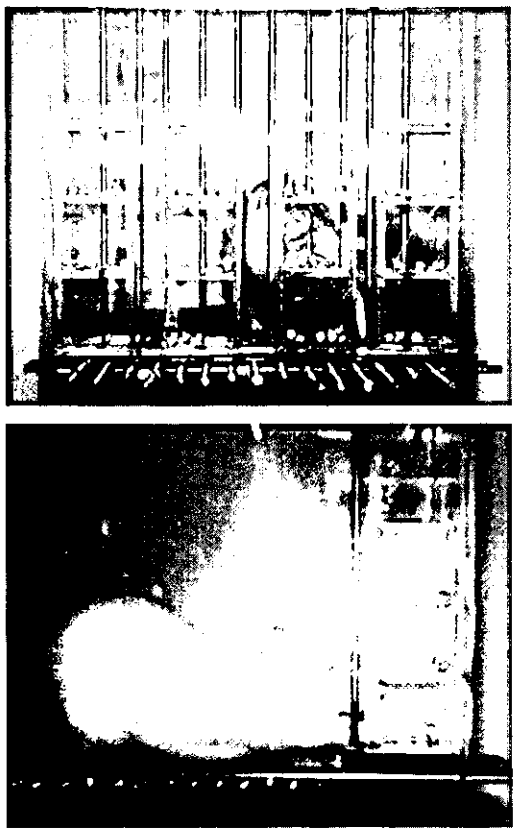
1.4. Reconstruction of neuronal circuits and functional recovery

Our studies showed that transplantation of NSCs at the appropriate time after the injury is an important factor for inducing their neuronal differentiation within the injured host spinal cord [25]. However, the functional recovery cannot result from neurogenesis alone. The ensuing synapse formation, myelination, and various other sequential events would be required for this. Thus, we investigated whether donor NSC-derived neurons became integrated into host neuronal circuits by making synapses. Five weeks after transplanting neurospheres derived from the fetal spinal cords of Tα-1-EYFP transgenic rats, donor-derived EYFP-positive neurons extended their axons within host spinal cord. We observed EYFP-positive pre-synaptic structures with pre-synaptic vesicles that were connected with EYFP-negative post-synaptic structures with post-synaptic densities. We also found EYFP-negative pre-synaptic structures that were connected with EYFP-positive post-synaptic structures. Interestingly, we found some cases in which EYFP-positive neurons had formed a synapse with host motor neurons at the injury site [25].

One of the factors contributing to the failure of axons to regenerate in the CNS, unlike in the peripheral nervous system, is the presence of factors that inhibit axon regeneration. Regardless of what excellent transplantation material the NSCs are, an effective method of NSC transplantation cannot be established without resolving the problem of axon regeneration inhibitors in the CNS. The axon elongation inhibitors in the CNS that have been discovered to date are broadly classified into myelin-related proteins (i.e. Nogo and myelin-associated glycoprotein (MAG)), semaphorin, and chondroitin sulfate, which are derived from glial scar tissue formed in the injured site. These inhibitors may account for the lack of axonal regeneration in the CNS after trauma in adult mammals. Dr. Bregman and her colleagues have already reported that the concomitant use of the IN-1 monoclonal antibody, which recognizes Nogo-A, in fetal spinal cord transplantation for spinal cord injuries resulted in excellent regeneration of injured axons and motor function recovery [44]. Recently, Dr. Stephen Strittmatter and his colleagues demonstrated that intrathecal administration of the peptide antagonist NEP1-40, which blocks the binding of the extra-cellular domain of Nogo (Nogo-66) to its receptor (NogoR), to rats with a mid-thoracic spinal cord hemisection resulted in significant axon growth in the corticospinal tract, and improved functional recovery [45]. Furthermore, NogoR was shown to play a major role in the inhibition of axonal outgrowth by CNS myelin and in limiting axonal regeneration after CNS injury, based on the following findings. First, NEP1-40 blocks Nogo-66 or CNS myelin inhibition of axonal outgrowth. Second, MAG, another CNS myelin-derived inhibitor for axonal regeneration, is a functional ligand for the Nogo-66 receptor, indicating that MAG and Nogo-66 activate NogoR independently and serve as redundant NogoR ligands that may limit axonal regeneration after CNS injury [46].

The next question is whether functional recovery is actually achieved by NSC transplantation. In our study of adult rats with spinal cord contusion injury [25], we observed behavioral improvement in skilled forelimb movement in rats that had received transplanted neural progenitor cells compared with control rats. In the behavioral test, known as the "pellet retrieval test," which examines the skilled forelimb movement by measuring the ability of animals to retrieve food pellets, a significantly favorable effect of NSC transplantation was demonstrated (Fig. 3). Because a deficit of upper limb skilled movement is an important symptom for patients with spinal cord injury, this finding indicates a benefit to patients of the future therapeutic application of neural progenitor cells. Collectively, our results indicate that if NSCs are transplanted in the subacute phase, and neither in the acute phase after spinal cord injury nor in the chronic phase characterized by marked glial scarring, they engraft and contribute to some degree of functional recovery.

The possible effects of NSC grafts for functional recovery would be similar to the effects of transplanting fetal neural tissues [9], which have been discussed above. In terms of neuronal circuits, previous studies indicate that the ascending sensory fiber components of the dorsal columns may play an important role in mediating the performance of skilled forelimb reaching movements, such as pellet retrieval. Namely, the damage to the ascending fibers within the dorsal column could be responsible for the severe reaching hypometria due to the rat cervical spinal cord contusion injury we observed in our study. Thus, one possible explanation for the behavioral improvement in the rats that received transplanted NSCs is that the neurons derived from the grafted cells "relayed" signals from the disrupted fibers in the host, including ascending fibers that existed in the dorsal column (Fig. 4a).



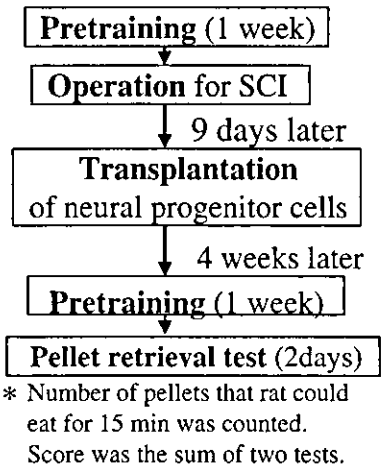


Fig. 3. Functional recovery of the contusion spinal cord injury revealed by pellet retrieval tests. Left: pellet retrieval test. Rats could obtain pellets only with their forelimbs. Right: time-table for the pellet retrieval tests. The pellet retrieval test was a modification of Diener and Bregman's method [10]. Before testing, rats were trained on each task for 1 week before the spinal cord injury, and were re-trained for an additional 1 week before the test was administered. Tests were performed 2 days in succession, and the final number was tallied as the sum of each day's number. The examiner performing the pellet retrieval tests was unaware of the experimental group for each rat. As a result, significant recovery of the skilled forelimb movement was achieved by the transplantation of in vitro-expanded neurosphere cells. As a result, significant recovery of the skilled forelimb movement was achieved by the transplantation of in vitro-expanded neurosphere cells [25].

Another possible explanation is that glial cells derived from grafted cells contributed to the behavioral improvement. Oligodendrocytes derived from grafted cells might have remyelinated fibers that had been demyelinated as a result of injury and restored the salutatory conduction along the neuronal axons of long projection neurons (Fig. 4b). In addition to the oligodendrocytes, astrocytes derived from donor neural progenitor cells might have played active roles in the generation of neuronal cells [25], axonal regeneration of host neuronal axons, enhancement of axonal extension of donor-derived neurons, synapse formation, and/or physiological maturation of neuronal cells. Astrocytes derived from the transplanted fetal spinal cord NSCs are likely to have similar functions as those derived from fetal brain, which regulate the precise growth of neuronal axons [47] and promote the maturation of neuronal cells physiologically [48]. Also, such functions of fetal spinal cord NSC-derived astrocytes could be distinct from those of the reactive astrocytes that were induced after the spinal cord injury. Dr. Fred Gage's group reported that astrocytes in an adult neurogenic site (the hippocampus) play active roles in inducing neurogenesis [49]. However, notably, astrocytes from adult spinal cord do not have these activities. Attractive future experiments will be to characterize such astrocytes-derived neurogenic inducing activities in more detail and to examine whether fetal spinal cord NSC-derived astrocytes have such activities.

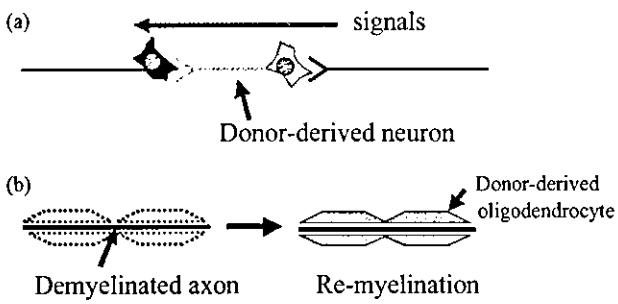


Fig. 4. Possible mechanisms for functional recovery. (a) Neurons derived from transplanted cells relay ascending signals that had been disrupted by the injury. (b) Demyelinated axons are re-myelinated by transplanted cells. In addition to these possibilities, the trophic effects of the transplanted neural stem/progenitor cells and their progenies might contribute to the functional recovery.