obviously reduced in the HGF group compared with the LacZ group (data not shown). These results suggested that HGF significantly reduced the levels of cleaved caspase-3 activation in neurons and oligodendrocytes after SCI, thereby promoting their survival.

HGF Enhances Angiogenesis After SCI

To examine the effect of HGF on vascular endothelial cells after SCI, immunostaining with anti-RECA-1 antibody was performed. In intact thoracic spinal cord, the vessels had delicate walls composed of homogeneously stained endothelial cells. Although most of the vessels disappeared at the epicenter at 1 week after SCI, several vessels were stained intensely and showed abnormally large lumina with thick walls (Fig. 6A, arrows), which were not observed in the intact spinal cord. Quantitative MCID analysis of the RECA-1-positive areas showed significant differences at the epicenter and 4 mm rostral to the epicenter between the two groups (Fig. 6B). In these areas, there were large amounts of fragmented RECA-1-IR resulting from the debris of dead endothelial cells. Thus, we focused on RECA-1-positive vessels with intact lumina for quantitative analysis. Accordingly, we found that significant differences in the number of RECA-1-positive vessels with lumina larger than 20 μm, representing newly formed vessels (Casella et al., 2002), between the two groups at the epicenter and at 4 mm rostral to the epicenter at 1 week after SCI (Fig. 6C).

HGF Promotes Regrowth of Serotonergic Fibers and Functional Recovery After SCI

To determine the effects of HGF on the axonal growth after SCI, axial sections of injured spinal cords were immunostained with anti-5HT antibody at 1 week and 6 weeks after SCI. 5HT-positive raphe-spinal serotonergic fibers were observed mainly in the gray matter in each group. Quantitative MCID analyses revealed that, whereas 5HT-positive fibers were almost undetectable in either group at 1 week after SCI, a significantly greater abundance of 5HT-positive fibers was detected, even in an area 4 mm caudal to the epicenter, in the HGF group compared with that in the LacZ group (Fig. 7A, arrows) at 6 weeks after SCI (Fig. 7A,B). Furthermore, at 1 week after SCI, the 5HT-positive fibers also showed c-Met-IR (Fig. 7C), and, at 6 weeks after SCI, they expressed GAP-43, which has been used as a marker of axonal regeneration (Kobayashi et al., 1997; Ramon-Cueto et al., 1998; Ikegami et al., 2005; Kaneko et al., 2006), even in a region 4mm caudal to the epicenter (Fig. 7D). Consistently with this, a greater abundance of GAP-43-positive fibers (Fig. 7E,F) and RT97-positive fibers (Fig. 7G,H) was observed, even in a region 4 mm caudal to the epicenter, in the HGF group compared with the LacZ group at 6 weeks after SCI; furthermore, c-Met-IR was also detected in these fibers (Fig. 7I,J). Most of the RT97-positive fibers were oriented longitudinally and parallel to each other (Fig. 7G), and these longitudinal fibers did not express GAP-43 (data not shown), indicating that they probably represented preserved axons after SCI.

The contusive SCI resulted in complete paralysis, followed by gradual recovery, reaching a plateau (BBB score 6.6 ± 1.1) at 6 weeks after SCI in the LacZ group. In the HGF group also, the animals suffered complete paralysis at 1 day after SCI, but these animals eventually showed better functional recovery than those in the LacZ group. Significant differences in the BBB scores were observed between the two groups from 7 days after SCI (Fig. 8). We believe that the difference between a BBB score of 8 (sweeping of hindlimbs) and

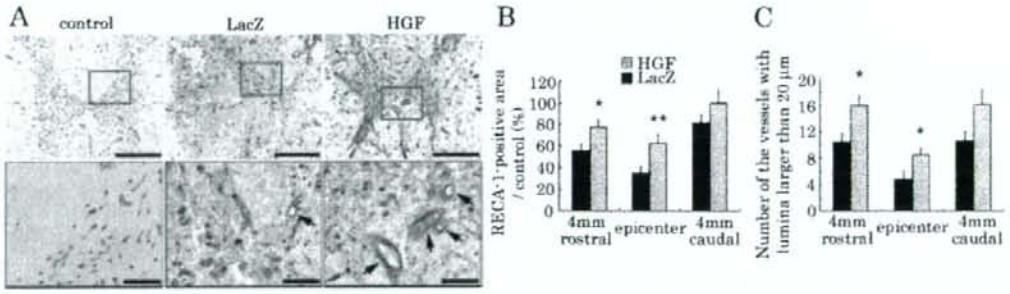

Fig. 6. Change in the microvasculature in the spinal cord after SCI. The axial sections of lesion epicenter at 1 week after SCI and intact thoracic spinal cord were immunostained with anti-RECA-1 antibody (**A**). Higher magnification views correspond to the boxed areas in the upper pictures. RECA-1-positive vessels with abnormally large lumina (arrows) emerged at 1 week after SCI in the two groups; these vessels were not observed in intact spinal cord and were considered to represent newly formed vessels after SCI. Quantitative analysis of the total area of RECA-1-positive endothelial cells (**B**) and the number of vessels with lumina larger than 20 μm (**C**) showed significant differences at the epicenter and at 4 mm rostral to the epicenter between the two groups. $*P < 0.05$, $**P < 0.01$; n = 5 each. Scale bars = 500 μm in A upper; 100 μm in A lower.

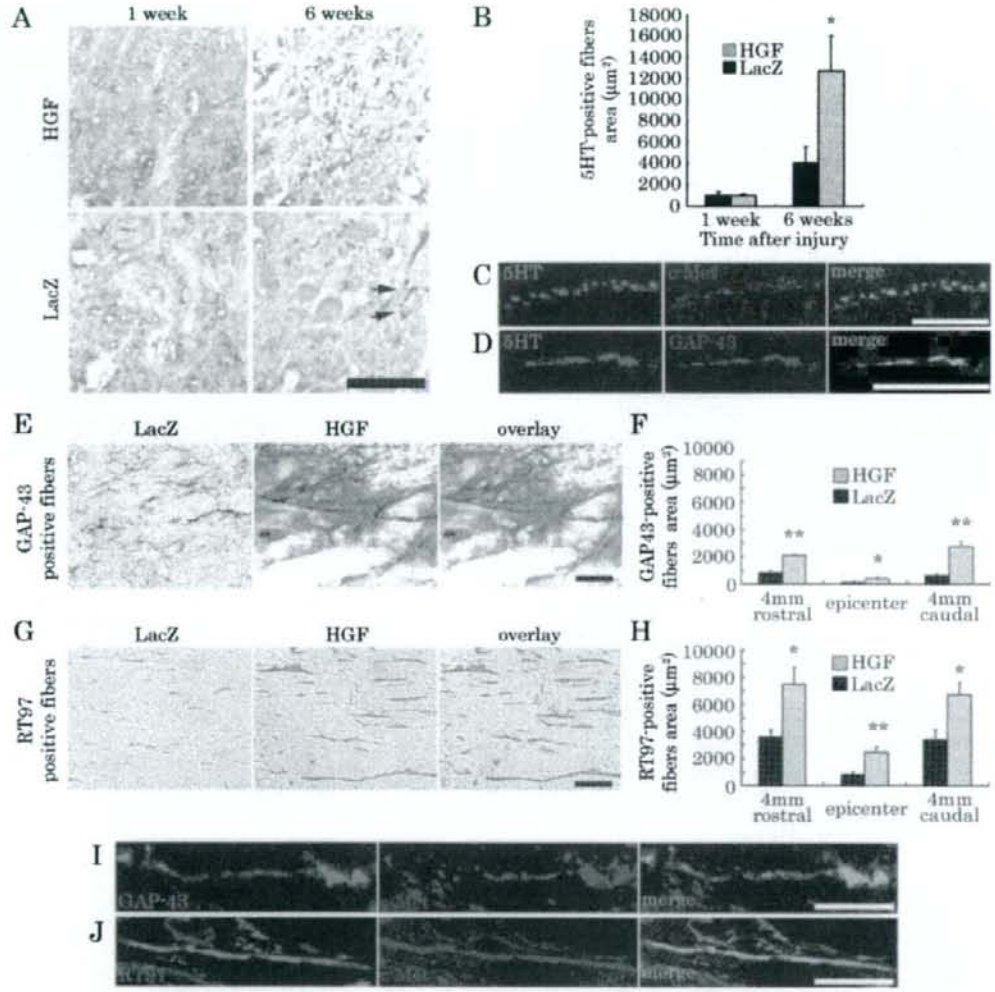

Fig. 7. Degeneration and regrowth of fibers after SCI. Immunostaining and quantification of 5-HT-positive fibers in axial sections at 4 mm caudal to the epicenter showed no significant difference between the two groups at 1 week after SCI but showed significant regrowth of the fibers in the HGF group compared with poor regrowth in the LacZ group (arrows) at 6 weeks after SCI (n = 5 each; **A,B**). 5HT-positive descending raphe-spinal fibers showed c-Met-IR (axial section) at 1 week after SCI (**C**) and expressed GAP-43 (sagittal section) at 4 mm caudal to the epicenter at 6 weeks after SCI (**D**). Representative images of midsagittal sections through an area 4 mm caudal to the epicenter showed a significantly greater abundance of GAP-43-positive fibers (**E**) and R97-positive fibers (**G**) in the HGF group compared with that in the LacZ group at 6 weeks after SCI. Note that significant differences in the immunopositive area were observed even in the region 4 mm caudal to the epicenter between the two groups (n = 5 each; **F,H**). Double immunostaining of midsagittal sections at 1 week after SCI showed c-Met-IR in the GAP-43-positive growth cones (**I**) and RT97-positive neurofilaments (**J**). $*P < 0.05$, $**P < 0.01$. Scale bars = 50 μm in A,E,G,J; 20 μm in C,D; 10 μm in I.

a BBB score of 9 (weight support on hindlimbs) is clinically substantial. From a clinical perspective, the recovery of the HGF group to weight-supported plantar steps (BBB score 10.1 ± 0.6) was noteworthy.

DISCUSSION

Previous studies have shown that the HGF-c-Met system is involved in the mediation of inflammatory responses to tissue injury. In animal models in which the

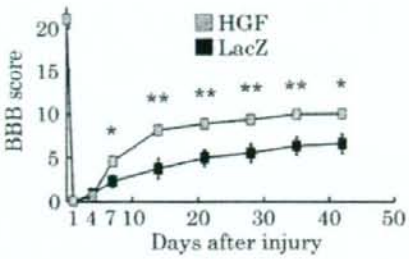

Fig. 8. BBB scores after SCI. A significant improvement in hindlimb motor function was observed in the HGF group compared with the LacZ group from 7 days after SCI (n = 14 each). $*P < 0.05$, $**P < 0.01$.

liver (Noji et al., 1990; Kono et al., 1992; Matsumoto and Nakamura, 1997), lung (Yanagita et al., 1993), or kidney (Kono et al., 1992; Igawa et al., 1993) tissues were experimentally damaged, the HGF mRNA expression and HGF activity were found to increase markedly in the damaged organs, peaking within 24 hr after the insult. Consistently with these increased expressions, the plasma HGF level also increased within 24 hr after the damage, suggesting that HGF could be delivered to these injured organs from other organs through the blood supply via an endocrine mechanism, in addition to being produced endogenously in these organs (Kono et al., 1992). In the CNS, on the other hand, it was reported that HGF mRNA was up-regulated exclusively in the periinfarct region at 14 days after cerebral ischemia (Nagayama et al., 2004). In the present study, we demonstrated for the first time that the HGF mRNA expression level gradually increased, peaking at 2 weeks after SCI, whereas the c-Met mRNA expression level increased markedly within 1 day of SCI. In addition, no increase in the plasma HGF levels was found after SCI. These findings suggest that the injured spinal cord cannot produce a sufficient amount of HGF by itself, compared with the remarkable increase in c-Met expression after SCI, nor can HGF be supplied through an endocrine mechanism, in contrast to the case following damage to other organs, as mentioned above. These results prompted us to perform an in vivo study to determine whether the application of exogenous HGF into the injured spinal cord might exert a beneficial effect and promote functional recovery in the spinal cord after SCI. We used the HSV-1 vector to introduce the exogenous HGF into the spinal cord, to compensate for the deficiency of endogenous HGF after SCI. The feasibility of using this vector for transgene expression in the nervous system in a safe and nontoxic manner has been examined in previous studies (Coffin et al., 1998; Palmer et al., 2000; Lilley et al., 2001).

We showed that the application of exogenous HGF into the injured spinal cord significantly attenuated caspase-3 activation in both neurons and oligodendrocytes, thereby reducing the area of demyelination and promoting the survival of cholinergic neurons. Previous studies have demonstrated the neurotrophic effects of HGF on a variety of neurons (Hamanoue et al., 1996; Maina and Klein, 1999; Caton et al., 2000), and, in one study, the application of HGF prevented the apoptosis of adult motoneurons after axonotomy of the hypoglossal nerve (Okura et al., 1999). In addition, HGF overexpression was reported to prevent delayed neuronal death and decrease the infarct volume after cerebral ischemia (Miyazawa et al., 1998; Hayashi et al., 2001; Shimamura et al., 2004; Niimura et al., 2006) by attenuating apoptosis. Consistently with these reports, in the present study, the neurotrophic and antiapoptotic effects of HGF on the neurons prevented neuronal loss after SCI, thereby reducing the size of the damaged area. Oligodendrocyte death, which is mediated by a pathway involving caspase-11 and caspase-3, leads to demyelination (Hisahara et al., 2001), and inhibition of the apoptosis of oligodendrocytes has been shown to reduce the area of demyelination and functional impairment after SCI (Tamura et al., 2005). These reports indicate that the induction of apoptosis in oligodendrocytes is directly correlated with demyelination and that inhibition of the apoptosis of oligodendrocytes could be potentially beneficial for recovery after SCI. In the present study, we demonstrated that HGF markedly attenuated the induction of caspase-3 in the oligodendrocytes after SCI, resulting in a significant reduction in the area of demyelination after SCI. Taken together, the antiapoptotic and neurotrophic effects of HGF on the neurons and oligodendrocytes contributed to a significant reduction of the area of parenchymal damage after SCI.

HGF is also well known as a potent angiogenic factor. HGF and c-Met are known to be expressed in endothelial cells and vascular smooth muscle cells (VSMCs; Nakamura et al., 1995, 1996), and a relationship between improved microcirculation and behavioral recovery after cerebral ischemia has been suggested (Shimamura et al., 2004, 2006). A change in the microvasculature of the spinal cord after contusion injury has been shown to be essential for the ability of the spinal cord to undergo self-repair (Loy et al., 2002; Hagg and Oudega, 2006). The cordons of vessels that form early at the lesion site may be the initial stage of the trabeculae described in the contusion injury model; these trabeculae have been reported to promote endogenous repair and support axonal outgrowth in the injured spinal cord (Beattie et al., 1997). Loy and colleagues demonstrated a biphasic angiogenic response after SCI, the first phase of which (3–7 days after injury; Casella et al., 2002), but not the second (28–60 days after injury), corresponded to the time course of functional recovery (Loy et al., 2002). Moreover, a relationship between the blood flow and functional recovery has been shown following strategic treatments to improve angiogenesis in the injured spinal cord during the acute phase of SCI (Glaser et al., 2004; Guizar-Sahagun et al., 2005). Thus, enhancing the formation of blood vessels, especially during the acute phase of SCI, may be a potential repair strategy, because nutritional and mechanical support by vessels is critical

for axonal regeneration. Interestingly, the number, length, and diameter of the vessels have been reported to reach their maximum within 1 week after SCI, and vessels with abnormally large lumina may represent newly formed vessels after SCI (Casella et al., 2002). In the present study, we showed that the introduction of HGF into the injured spinal cord increased the total area of RECA-1-positive endothelial cells and number of vessels with abnormally large lumina by 1 week after SCI, confirming that HGF also promoted angiogenesis during the acute phase of SCI. Because HGF simultaneously stimulates the migration of endothelial cells and VSMCs (Nakamura et al., 1995, 1996), blood vessels might mature in a well-coordinated way, without the release of inflammatory cells (Morishita et al., 2004). Consistently with this suggestion, HGF overexpression reduced cerebral ischemic injury, without causing cerebral edema, through angiogenic and neuroprotective actions (Shimamura et al., 2004). Taken together, our results suggest that HGF may promote angiogenesis without enhancing blood vessel permeability after SCI and contribute to a reduction in the area of damage and regeneration of the injured spinal cord.

Several researchers have reported that HGF plays a role as an axonal chemoattractant and enhances the axonal growth of motoneurons (Ebens et al., 1996; Wong et al., 1997; Caton et al., 2000) and cortical neurons (Yamagata et al., 1995). Furthermore, it has been reported that overexpression of HGF in the chronic stage of cerebral infarction enhances neurite extension and increases the number of synapses, leading to improvements in learning and memory (Shimamura et al., 2006). In the present study, we demonstrated that HGF significantly induced the regrowth of raphe-spinal 5HT-positive fibers, which are known to contribute to the locomotor functions after SCI in rats (Bregman, 1987; Saruhashi et al., 1996; Kim et al., 2004; Kaneko et al., 2006), and the fibers expressed GAP-43 at 6 weeks after SCI. Moreover, c-Met-IR was also detected in the 5HT-positive fibers, suggesting that HGF directly acted on these fibers as well as the neuronal bodies to promote axonal regrwoth and recovery of locomotor functions after SCI. On the other hand, most of the longitudinal RT97-positive fibers oriented parallel to each other (Fig. 7G) did not express GAP-43 but expressed c-Met (Fig. 7J). Because these RT97-positive fibers were observed more abundantly in the HGF group than in the LacZ group at 6 weeks after SCI (Fig. 7H), it is likely that HGF protected the axons from degeneration.

Overall, during the acute phase of SCI, HGF appears to exert significant neuroprotective and antiapoptotic effects, to promote the survival of neurons and oligodendrocytes, and also to enhance angiogenesis around the lesion epicenter after SCI. These effects significantly reduced the area of damage and provided a better scaffold for axonal regeneration. Furthermore, HGF directly acted on the 5HT-positive fibers to promote their regrowth, which likely contributed to the significantly better recovery of the motor functions during the chronic phase of SCI. In conclusion, we have demonstrated that HGF exerted multiple beneficial effects on the injured spinal cord and significantly enhanced endogenous repair after SCI.

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Hepatocyte growth factor (HGF) attenuates gliosis and motoneuronal degeneration in the brainstem motor nuclei of a transgenic mouse model of ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive loss of brainstem and spinal motoneurons. Although prevention of motoneuronal degeneration has been postulated as the primary target for a cure, accumulating evidence suggests that microglial accumulation contributes to disease progression. This study was designed to assess the ability of HGF to modulate microglial accumulation and motoneuronal degeneration in brainstem motor nuclei, using double transgenic mice overexpressing mutated $SOD1^{G93A}$ and HGF (G93A/HGF). Histological and immunohistochemical analyses of the tissues of G93A/HGF mice revealed a marked decrease in the number of microglia and reactive astrocytes and an attenuation of the loss of motoneurons in facial and hypoglossal nuclei compared with G93A mice. HGF overexpression attenuated monocyte chemoattractant protein-1 (MCP-1) induction, predominantly in astrocytes; suppressed activation of caspase-1, -3 and -9; and, increased X chromosome-linked inhibition of apoptosis protein (XIAP) in the motoneurons of G93A mice. The implication is that HGF reduces microglial accumulation by suppressing MCP-1 induction and prevents motoneuronal death through inhibition of pro-apoptotic protein activation. These findings suggest that, in addition to direct neurotrophic activity on motoneurons, HGF-suppression of gliosis may retard disease progression, making HGF a potential therapeutic agent for the treatment of ALS patients.

Keywords: Caspases; X chromosome-linked inhibitor of apoptosis protein (XIAP); Microglia; Monocyte chemoattractant protein-1 (MCP-1); c-Met

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration of motoneurons and their axons in the brainstem and spinal cord, leading to spasticity, hyperreflexia, generalized weakness of the limbs, muscle atrophy, and paralysis (Cleveland and Rothstein, 2001). Most cases (90%) are classified as sporadic ALS (SALS), as they are not associated with a documented family history. The remaining 10% are inherited and referred to as familial ALS (FALS). Gene mutations in copper/zinc superoxide dismutase 1 (SOD1) are responsible for 15–20% of FALS. In addition, mutations in the ALS2/alsin, senataxin (SETX), synaptobrevin/vesicle-associated membrane protein-associated protein B (VAPB) and dynactin genes recently have been associated with ALS (Pasinelli and Brown, 2006). Regardless of the type of gene mutations or whether the disease is familial or sporadic, motoneuronal degeneration is thought to constitute a common and primary event in ALS (Cleveland and Rothstein, 2001). Therefore, most efforts have been directed toward finding molecules that act directly on motoneurons in an attempt to reduce their degeneration, regardless of the effects of gliosis on surrounding motoneurons. However, treatment with minocycline, an antibiotic that inhibits microglial activation (Yrjanheikki et al., 1999; Van Den Bosch et al., 2002; Zhu et al., 2002), was found to slow disease progression in a transgenic mouse model of ALS that overexpresses mutated human $SOD1^{G93A}$ (G93A) (Kriz et al., 2002). Boillee et al. (2006) recently used the Cre-lox system to show that diminishing expression of mutated $SOD1^{G37R}$ in the microglia of a transgenic mouse model of ALS prolongs disease duration and survival. These lines of evidence demonstrate that

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microglia might contribute to disease progression that is caused by mutant SOD1 toxicity in a transgenic mouse model of ALS and raises the possibility that reducing the number of activated microglia could be incorporated as another strategy for ALS therapy.

Hepatocyte growth factor (HGF) was initially identified and molecularly cloned as a potent mitogen for mature hepatocytes (Nakamura et al., 1984, 1989). Subsequent studies revealed that HGF exerts multiple biological effects, including mitogenic, motogenic, morphogenic, and anti-apoptotic activities in a wide variety of cells, including neurons, by binding to the c-Met receptor tyrosine kinase (c-Met) (Funakoshi and Nakamura, 2003). HGF is one of the most potent *in vitro* and *in vivo* survival-promoting factors for neurons. For example, neurotrophic effects of HGF have been demonstrated in cultured hippocampal neurons (Honda et al., 1995) and in cultured embryonic spinal motoneurons (Ebens et al., 1996; Yamamoto et al., 1997; Novak et al., 2000), and its anti-apoptotic activity in motoneurons is comparable to that of glial cell line-derived neurotrophic factor (GDNF) (Ebens et al., 1996). Indeed, reflecting the *in vitro* neurotrophic activity of HGF on motoneurons and the expression of c-Met in motoneurons of G93A mice, the transgenic overexpression of rat HGF in the nervous system attenuates spinal motoneuronal death and axonal degeneration, delays onset of the disease and prolongs the lifespan of G93A mice (Sun et al., 2002). However, many studies have suggested that some neurotrophic factors show survival-promoting effects only on certain subtypes of motoneurons in the spinal cord and brainstem (Sakamoto et al., 2003; Guillot et al., 2004). Therefore, the effects of HGF on the degeneration of ALS brainstem motoneurons in G93A mice remain unclear. Furthermore, the role of HGF on microglial accumulation, another research target in the search for an ALS cure, is poorly understood. In the present study, the effects of HGF on microglial accumulation and motoneuronal degeneration in brainstem (facial and hypoglossal) motor nuclei of G93A mice were examined using double transgenic mice overexpressing mutated human $SOD1^{G93A}$ and HGF. The molecular mechanisms by which HGF functions in suppressing microglial accumulation and attenuating motoneuronal degeneration in G93A mice were also examined.

2. Materials and methods

2.1. Animals

Neuron-specific enolase promoter-driven HGF transgenic (HGF-Tg) mice were generated as previously described (Sun et al., 2002). Transgenic mice overexpressing mutated (glycine to alanine in position 93) human SOD1 (G93A) [B6SJL-TgN (SOD1-G93A)^{1}Gur1] (Gurney et al., 1994) were purchased from the Jackson Laboratory (Bar Harbor, ME). This mouse strain has a low copy number of $SOD1^{G93A}$ and shows a delayed onset of ALS, slower disease progression, and a longer lifespan compared with mice carrying a high copy number of the transgene (G1H). This strain resembles the slow progressing phenotype of patients with ALS and is useful for the accurate evaluation of the molecular mechanisms involved in the action of HGF during disease progression. HGF-Tg mice were crossed with G93A transgenic mice to generate G93A/HGF-double transgenic animals. The HGF and G93A transgenic heterozygous mice were maintained by mating transgenic males with C57/BL6 females. Mouse genotypes were determined by polymerase chain reaction (PCR) and Southern blot analysis as previously reported (Gurney et al., 1994; Sun et al., 2002). Non-transgenic littermates served as controls. Experimental protocols were approved by the Animal Experimentation Ethics Committee of Osaka University Graduate School of Medicine. All efforts were made to minimize animal discomfort and the number of animals used.

2.2. Tissue preparation

Wild-type (WT), HGF-Tg, G93A, and G93A/HGF mice at 6 and 8 months of age were sacrificed with an overdose injection of pentobarbital sodium salt. Tissues were fixed by treating them in increasing concentrations of ethanol. After dehydration, the tissues were treated with xylene and embedded in paraffin. Serial tissue sections were cut on a microtome to a thickness of 7 μm, deparaffinized, and used for either Nissl staining or for immunohistochemistry.

2.3. Motoneuron survival

The neuroprotective effect of HGF on facial and hypoglossal motoneurons was evaluated by counting the number of motoneurons (i.e., motoneuronal survival). The number of motoneurons in the facial and hypoglossal nuclei of WT, HGF-Tg, G93A, and G93A/HGF mice were counted in every fifth section of 16 Nissl-stained 7-μm-thick paraffin sections ($n = 4$). Densely stained motoneurons with a clear nucleolus and in a defined area of facial and hypoglossal nuclei were counted using Win ROOF analysis software (Mitani Corp., Fukui, Japan) as previously described (Sun et al., 2002).

2.4. HGF ELISA

HGF protein levels in the brainstem of WT and HGF-Tg mice were measured by ELISA using an anti-rat HGF polyclonal antibody (Tokushu Meneki, Tokyo, Japan) as previously described (Sun et al., 2002).

2.5. Immunohistochemistry

The sections were stained with the following antibodies: (i) c-Met polyclonal antibody (1:50; Santa Cruz Biotech, Santa Cruz, CA); (ii) tubulinßIII monoclonal antibody (1:500; Berkeley Antibody Co. Inc., Richmond, CA); (iii) caspase-1 (p10) polyclonal antibody (1:100; Santa Cruz Biotech); (iv) active caspase-3 polyclonal antibody (1:200; Promega, Madison, WI) (v) cleaved caspase-9 polyclonal antibody (1:50; Cell Signaling Technology, Beverly, MA); (vi) X chromosome-linked inhibitor of apoptosis protein (XIAP) monoclonal antibody (1:50; BD Pharmingen, San Diego, CA); (vii) glial fibrillary acidic protein (GFAP) monoclonal antibody (1:500; Chemicon International, Temecula, CA); (viii) Iba1 polyclonal antibody (1:2000; Wako Pure Chemical, Osaka, Japan); and (ix) monocyte chemoattractant protein (MCP)-1 polyclonal antibody (1:100; Abcam, Cambridge, UK). These antibodies were applied to the sections for 1 h at room temperature (RT) or overnight at 4 °C after blocking with phosphate-buffered saline (PBS) containing 5% goat serum and 0.1% Triton X-100 at RT for 1 h. After washing the sections with PBS, immunoreactivity (IR) was visualized by incubating them for 20 min at RT with secondary antibodies conjugated with Alexa Fluor 488 (green) or Alexa Fluor 546 (red) diluted 1:500 (Invitrogen, Carlsbad, CA). When double-immunostaining for XIAP and tubulinßIII was performed, antibodies against XIAP and tubulinßIII were labeled with Alexa Fluor 546 and 488, respectively, using a Zenon labeling kit according to the manufacturer's instructions (Invitrogen). These fluorescence-immunostained sections were observed under a LSM 5 PASCAL confocal microscope (Carl Zeiss, Germany). The staining specificity of the antibodies was also assessed by the absence with first antibody or by pre-incubation with an excess amount of immunized peptide. The intensities of immunoreactivity against GFAP, Iba-1, MCP-1, active caspase-1, -3, -9 and XIAP were determined as previously described (Sun et al., 2002).

2.6. Statistical analysis

Results were expressed as the mean ± S.E. Statistically significant differences among the four groups of mice were assessed by ANOVA, followed by

Scheffe's post hoc test. Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

3. Results

3.1. c-Met is expressed in facial and hypoglossal motoneurons in a transgenic mouse model of ALS

The effect of HGF on gliosis and motoneuronal degeneration was investigated using a mouse model of ALS (G93A) in which mutated human $SOD1^{G93A}$ is overexpressed (Gurney et al., 1994). Expression of the c-Met/HGF receptor (c-Met) was examined in the facial and hypoglossal nuclei of wild-type (WT) and G93A mice. Immunofluorescence analysis with antibodies against c-Met and tubulinßIII (a neuronal marker) showed that c-Met immunoreactivity (IR) was detectable in the facial and hypoglossal nuclei, and that it was localized in the large-size neurons of WT and G93A mice at both 6 (data not shown) and 8 months (Fig. 1a) of age. These results demonstrate that c-Met is present in facial and hypoglossal motoneurons of WT and G93A mice at the ages that correspond to the middle and end stages of the disease.

3.2. Introduction of HGF into the brainstem of transgenic mice overexpressing HGF (HGF-Tg)

The role of HGF was examined using transgenic mice overexpressing rat HGF (HGF-Tg) (Sun et al., 2002). In HGF-Tg mice, exogenous rat HGF mRNA is located specifically in the brain and spinal cord, as evidenced by an RNase protection assay (Sun et al., 2002). An HGF ELISA, which specifically recognizes rodent HGF, revealed that the levels of HGF in the brainstem of HGF-Tg mice were 2-fold higher than in WT mice (Fig. 1b) with no effect on serum HGF levels (data not shown), demonstrating the successful introduction of HGF into the brainstem of HGF-Tg mice.

3.3. Neuroprotective effect of HGF on facial and hypoglossal motoneurons in G93A mice

The effect of HGF on facial and hypoglossal nuclei against ALS was evaluated by generating double transgenic mice (G93A × HGF-Tg) that overexpressed a mutated form of human $SOD1^{G93A}$ and rat HGF (G93A/HGF). This mating resulted in the generation of four groups of mice: (1) WT, (2) HGF-Tg, (3) G93A and (4) G93A/HGF. Nissl staining showed that the facial motoneurons of G93A mice at 8 months of age were atrophic and were present in lower numbers compared with WT and HGF-Tg mice (Fig. 2a), i.e., 55% of WT (Fig. 2b). In contrast, the facial motoneurons of G93A/HGF mice exhibited a healthier morphology (i.e., less atrophic) than those of G93A mice (Fig. 2a). The mean number of facial motoneurons in G93A/HGF mice was significantly larger than that of the G93A mice, and was almost the same as that of WT mice (Fig. 2b).

The hypoglossal motoneurons of G93A mice also were atrophic, and reduced in number compared with WT and HGF-Tg mice (Fig. 2c), with a decrease in mean number to 57% of WT mice (Fig. 2d). Similar to the facial motoneurons of G93A/HGF mice, the hypoglossal motoneurons of G93A/HGF mice also exhibited a healthier morphology than did those of G93A mice (Fig. 2c). The mean number of hypoglossal motoneurons of G93A/HGF mice was significantly larger than that found in G93A mice (Fig. 2d). These results demonstrate that HGF exerts a neuroprotective effect on brainstem motoneurons against ALS-associated neurotoxicity.

3.4. HGF suppresses gliosis in facial and hypoglossal nuclei of G93A mice

The effect of HGF on gliosis in facial and hypoglossal nuclei of G93A mice was examined using antibodies against Iba1

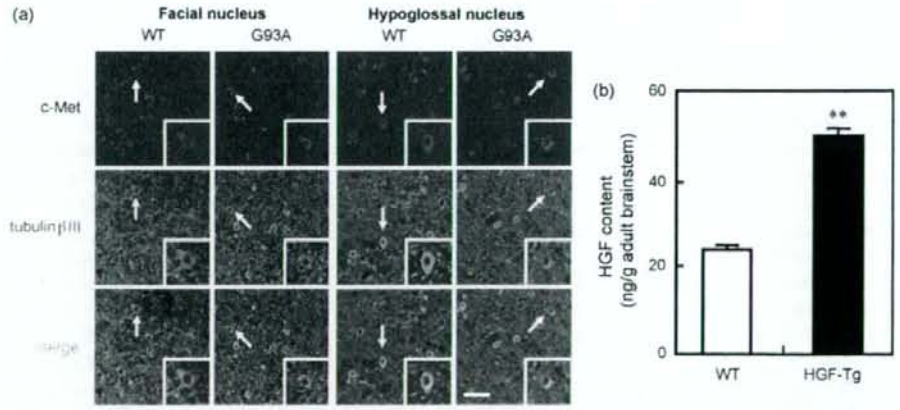

Fig. 1. Expression of c-Met and HGF in the brainstem. (a) Double-immunofluorescence analysis for c-Met (red) and tubulinßIII (green; a marker for neurons) in the facial and hypoglossal nuclei of WT and G93A mice at 8 months of age. A high-magnification view of the area indicated by the arrow is also boxed in each photo. c-Met immunoreactivity was detectable in large-sized neurons of the facial and hypoglossal nuclei. Scale bar = 50 μm. (b) Protein levels of HGF in the brainstems of 6-month-old wild-type (WT) and HGF transgenic (HGF-Tg) mice were analyzed by ELISA ($n = 6$ in each group). Data represent the mean ± S.E. $^{**}P < 0.01$ compared with WT mice.

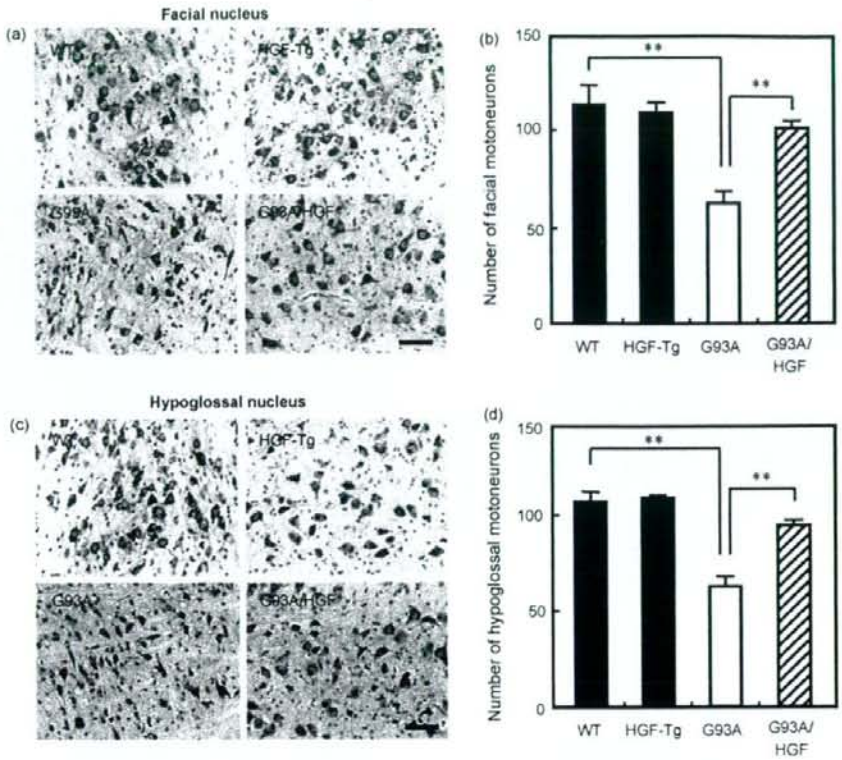

Fig. 2. Neuroprotective effect of HGF on facial (a and b) and hypoglossal (c and d) motoneurons in G93A mice. (a and c) Photomicrographs of representative Nissl-stained sections of the facial (a) and hypoglossal (c) nuclei of WT, HGF-Tg, G93A, and G93A/HGF mice at 8 months of age are shown. Scale bars = 50 μm. (b and d) Quantification of the mean numbers of surviving Nissl-stained neuronal cells with a clear nucleolus in the facial (b) and hypoglossal (d) nuclei of 8-month-old mice of each group. Motoneuron survival is expressed as the neuronal cell count on every fifth section of 16 sections. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ compared with WT and G93A/HGF mice.

(a microglia marker; Imai et al., 1996) and GFAP (an astrocyte marker). As shown in Fig. 3a, microglia densely accumulated in the facial nuclei of G93A mice at 8 months of age, while the number of microglia was lower in the facial nuclei of WT and HGF-Tg mice. The mean intensity of Iba1-IR in the facial nuclei of G93A mice increased 3.7-fold over the WT signal. In contrast, the mean intensity of Iba1-IR in the facial nuclei of G93A/HGF mice decreased to 59% of that found in G93A mice. Similar results were obtained for the hypoglossal nuclei (Fig. 3a).

Immunofluorescence analyses showed that a large number of hypertrophic astrocytes (i.e., exhibiting astrocytosis) were evident in the facial and hypoglossal nuclei of the G93A mice, while the number of astrocytes was low in the nuclei of both WT and HGF-Tg mice. Consistent with the reduction of Iba1-IR and GFAP-IR, the numbers of Iba1-positive and GFAP-positive cells were lower in G93A/HGF mice compared with G93A mice (data not shown). These results demonstrate that HGF is capable of suppressing both microgliosis (accumulation of activated microglia) and astrocytosis in the facial and hypoglossal nuclei of G93A mice.

3.5. HGF suppresses active caspase-1 and monocyte chemoattractant protein (MCP)-1 in facial and hypoglossal nuclei of G93A mice

The mechanisms by which HGF suppresses gliosis in the facial and hypoglossal nuclei of G93A mice were examined. The mRNA expression of monocyte chemoattractant protein (MCP)-1 is critical for recruitment of inflammatory cells of the monocytic lineage after inflammation or injury to the central nervous system (Berman et al., 1996). Compared with non-neurological disease controls, MCP-1 is markedly increased in the spinal cords of ALS patients and in transgenic mice overexpressing $SOD1^{G37R}$ (G37R mice, a model of ALS) (Henkel et al., 2004, 2006; Baron et al., 2005). Intrathecal treatment with cyclosporin, which is thought to reduce MCP-1 levels, prolongs survival of late stage G93A mice (Keep et al., 2001). Therefore, HGF modulation of MCP-1 levels in the facial and hypoglossal nuclei of 8-month-old G93A mice was examined using immunohistochemistry. MCP-1-IR primarily localized to astrocyte-like cells in the facial nuclei of G93A mice. Double-fluorescence immunostaining revealed that MCP-1-IR with strong immunoreactivity co-localized well

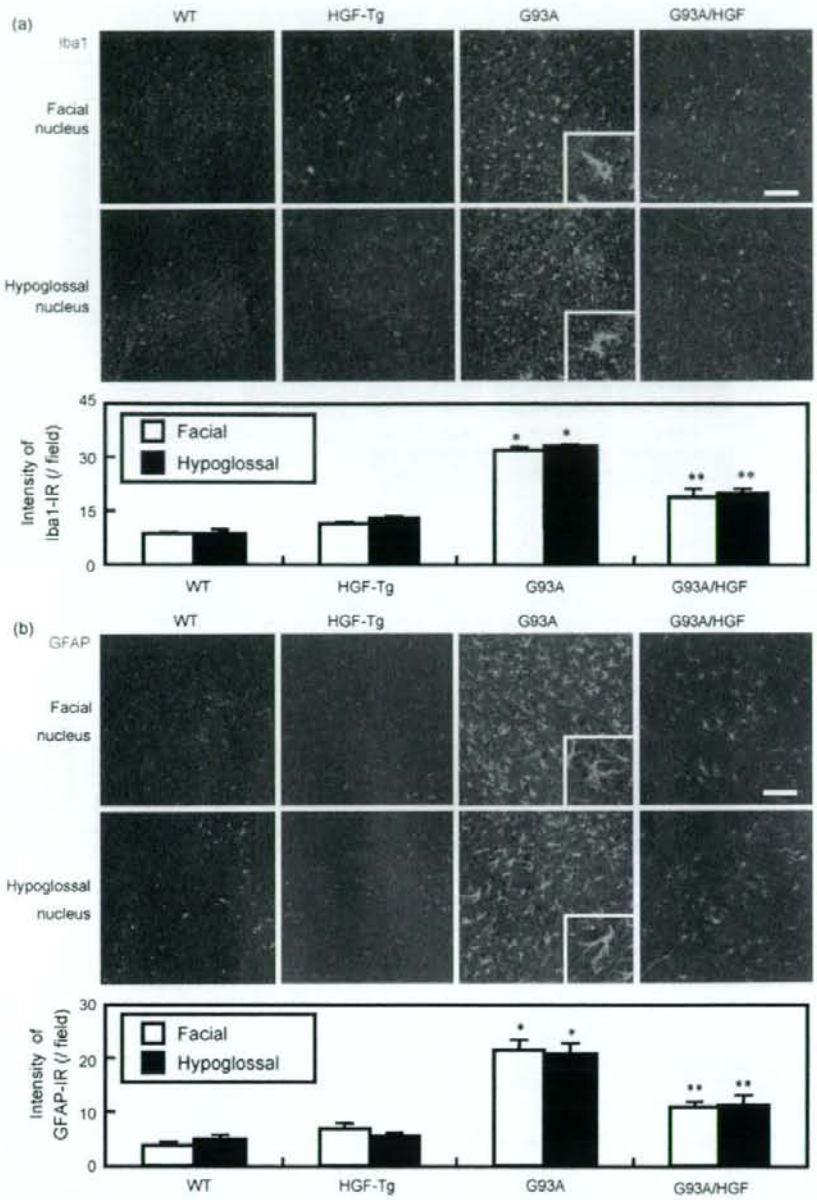

Fig. 3. Suppression of gliosis in the facial and hypoglossal nuclei of G93A/HGF mice. (a) Suppression of microglial accumulation in G93A/HGF mice. Immunofluorescence analysis for Iba1 (a marker for microglia) in the facial and hypoglossal nuclei of WT, HGF-Tg, G93A and G93A/HGF mice at 8 months of age. Scale bar = 100 μm. The intensity of Iba1-IR is shown in the facial (open column) and hypoglossal (closed column) nuclei. Data represent the mean ± S.E. ($n = 4$ for each group). *$P < 0.05$ vs. WT mice and **$P < 0.05$ vs. G93A mice. (b) Suppression of astrocytosis in G93A/HGF mice. Immunofluorescence analysis for GFAP (a marker for astrocyte) in the facial and hypoglossal nuclei of WT, HGF-Tg, G93A and G93A/HGF mice at 8 months of age. Scale bar = 100 μm. The intensity of GFAP-IR is shown in the facial (open column) and hypoglossal (closed column) nuclei. Data represent the mean ± S.E. ($n = 4$ for each group). *$P < 0.05$ vs. WT mice and **$P < 0.05$ vs. G93A mice.

with GFAP, indicating that MCP-1-IR is predominantly induced in reactive astrocytes (Fig. 4a, upper left panel). While double-fluorescence immunostaining of MCP-1 and tubulinßIII showed that weakly immunostained MCP-1-IR cells in G93A mice were motoneurons, the signal was below the detection limit in the facial nuclei of WT mice (Fig. 4a, upper middle panel). In contrast with G93A mice, G93A/HGF mice showed much lower levels of MCP-1-IR in the facial nuclei

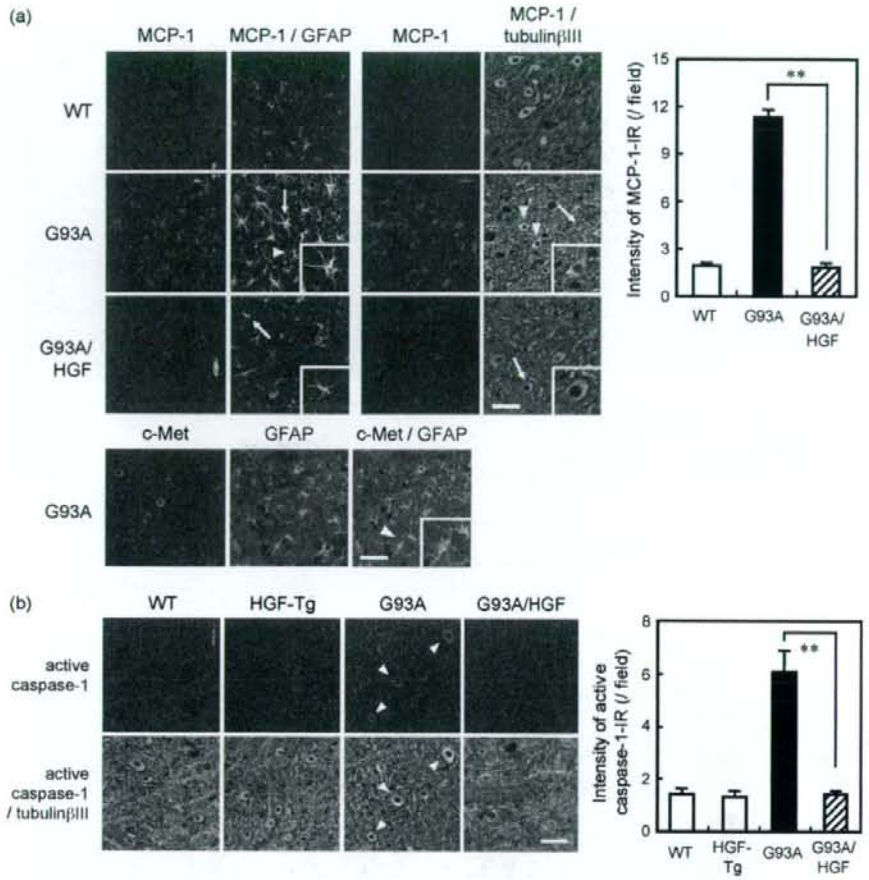

Fig. 4. Inhibitory mechanisms of HGF on gliosis in the facial nucleus of G93A mice. (a) Suppression of MCP-1 induction in G93A/HGF mice. Upper left panel, double immunofluorescence analysis of MCP-1 (red) and GFAP (green) or tubulinβIII (green) in the facial nucleus of WT, G93A and G93A/HGF mice at 8 months of age. Scale bars = 50 μm. Arrowheads indicate MCP-1-positive cells lacking GFAP-IR or double labeled with tubulinβIII-IR. A high-magnification view of the area indicated by the arrow is also shown. Lower left panel, double immunofluorescence analysis of c-Met (red) and GFAP (green) in the facial nucleus of G93A mice. A high-magnification view of the area indicated by the arrows is also boxed in each photograph. c-Met immunoreactivity was undetectable in reactive astrocytes of the facial nucleus of G93A mice. Upper right panel, the intensity of MCP-1-IR is shown in the facial nucleus of WT, G93A and G93A/HGF mice. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ vs. G93A mice. (b) Suppression of caspase-1 activation in the facial nucleus of G93A/HGF mice. Left panel, double-immunofluorescence analysis of active caspase-1 (red) and tubulinβIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. Active caspase-1-positive neurons are indicated by arrowheads. Right panel, the intensity of active caspase-1-IR is shown in the facial nucleus of WT, G93A and G93A/HGF mice. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ vs. G93A mice.

(Fig. 4a, upper left and middle panels). The mean intensity of MCP-1-IR in the facial nuclei of G93A mice increased 5.8-fold relative to WT mice. Meanwhile, the mean intensity of MCP-1-IR in the facial nucleus of G93A/HGF mice was decreased, and was similar to the level observed in WT mice (Fig. 4a, upper right panel). Similar results were obtained for the hypoglossal nuclei of WT mice, G93A and G93A/HGF mice (data not shown).

How could MCP-1 induction in G93A mice be attenuated by HGF? Interleukin (IL)-1ß has been postulated to play a role in the induction of MCP-1 and astrocytosis *in vivo* (Giulian et al., 1988; Herx and Yong, 2001) and *in vitro* (John et al., 2004). IL-1ß is generated by proteolytic cleavage of pro-IL-1ß by IL-1ß-converting enzyme (ICE)/caspase-1 activation (Thornberry et al., 1992). Therefore, the effect of HGF on caspase-1 activation, which is abundant long before neuronal death and/or phenotypic onset (Pasinelli et al., 2000), mediation of disease processes from the early stage of the disease, was examined. Active caspase-1-IR was detectable in the facial motoneurons of G93A mice at 6 months of age (during the middle stage of the disease when motoneuronal death is not evident), but the immunofluorescent signal was undetectable in WT and HGF-Tg mice (Fig. 4b). G93A/HGF mice showed much lower levels of active caspase-1-IR in the facial motoneurons (Fig. 4b, left panel). The mean intensity of active caspase-1-IR in the facial nucleus of G93A mice increased significantly to 4.2-fold higher than in WT mice. Meanwhile, the mean intensity of caspase-1-IR in the facial nucleus of G93A/HGF mice was decreased, and was at almost the same level as in WT mice (Fig. 4b, right

panel). Similar results were obtained for the hypoglossal motoneurons (data not shown). Suppression of active caspase-1 induction by HGF might help reduce IL-1ß levels in motoneurons which, in turn, suppresses MCP-1 induction. This scenario explains the suppressive effect of HGF on gliosis, despite the observation that c-Met-IR was below the detection limit in astrocytes (Fig. 4a, lower panel) and microglia (data not shown) at the developmental stage examined.

3.6. HGF induces XIAP and attenuates pro-apoptotic protein activation in facial and hypoglossal motoneurons of G93A mice

The mechanism of the HGF neuroprotective effect on facial and hypoglossal motoneurons was examined using immunohistochemistry. Previous studies have demonstrated that caspases are activated in spinal motoneurons of a transgenic mouse model of ALS at various stages throughout the clinical course, and that caspase-mediated apoptosis is a mechanism of motoneuronal degeneration in ALS (Pasinelli et al., 2000; Li et al., 2000; Guegan et al., 2001; Inoue et al., 2003). Therefore, the effect of HGF on the activation of caspases-3 and -9 was examined. Active caspase-3-IR and caspase-9-IR were induced in facial motoneurons of G93A mice at 6 months of age, while the signal was not detected in the nuclei of WT or HGF-Tg mice (Fig. 5a and b). However, G93A/HGF mice showed much lower levels of active caspase-3-IR and caspase-9-IR in facial motoneurons (Fig. 5a and b, left panel). The mean intensities of active caspase-3-IR and caspase-9-IR in the facial nuclei of G93A mice increased significantly (5.6- and 6.4-fold,

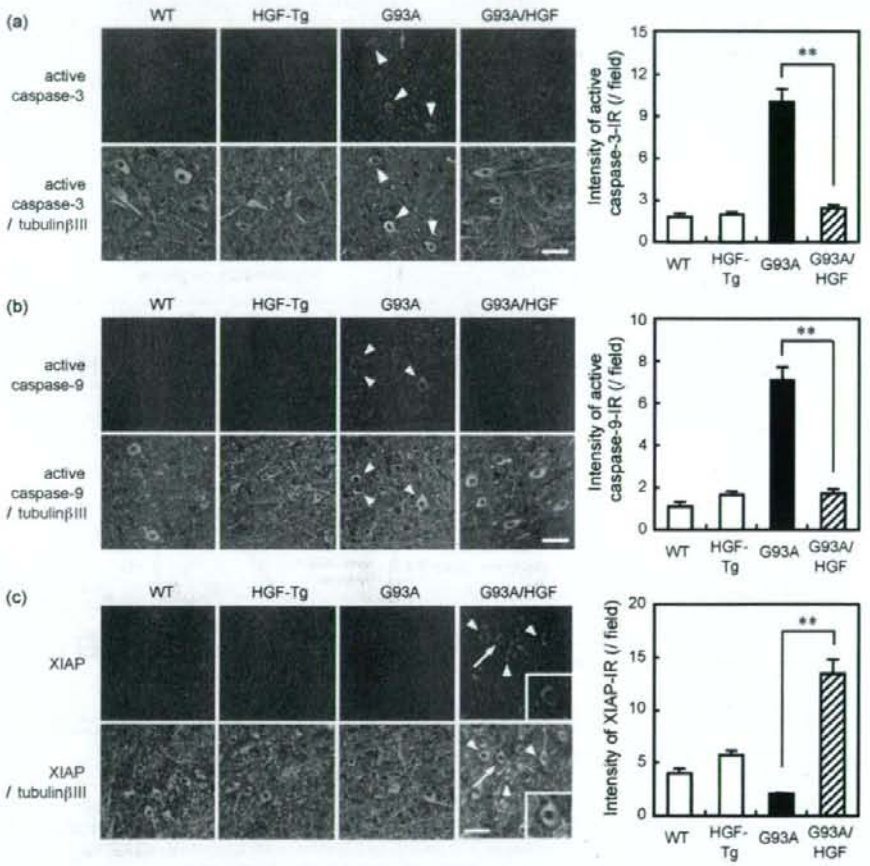

Fig. 5. Attenuation of pro-apoptotic protein activation in the facial motoneurons of G93A/HGF mice. (a) Left panel, double immunofluorescence analysis of active caspase-3 (red) and tubulinßIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. Active caspase-3-positive neurons, indicated by arrowheads, are evident. Right panel, the intensity of active caspase-3-IR is shown in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ vs. G93A mice. (b) Left panel, double immunofluorescence analysis of active caspase-9 (red) and tubulinßIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. Active caspase-9-positive neurons are indicated by arrowheads. Right panel, the intensity of active caspase-9-IR is shown in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ vs. G93A mice. (c) Left panel, double immunofluorescence analysis for XIAP (red) and tubulinßIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. XIAP-positive neurons are indicated by arrowheads. A high-magnification view of the area indicated by the arrow is also shown for G93A/HGF mice. Scale bars = 50 μm. Right panel, the intensity of XIAP-IR is shown in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice. Data represent the mean ± S.E. ($n = 4$ for each group). $^{**}P < 0.01$ vs. G93A mice.

respectively) relative to WT mice. Meanwhile, the mean intensities of active caspase-3-IR and caspase-9-IR in the facial nuclei of G93A/HGF mice were decreased, and were at almost the same level as in WT mice (Fig. 5a and b, right panel). Similar results were obtained for the hypoglossal motoneurons (data not shown). These results suggest that HGF-dependent prevention of facial and hypoglossal motoneuron degeneration in G93A mice was mediated, at least in part, by inhibition of caspase-dependent neuronal cell death.

X chromosome-linked inhibitor of apoptosis protein (XIAP) is a member of a family of protein inhibitors of apoptosis. The protein antagonizes the caspase cascade through direct inhibition of the activation of caspases-3, -7 and -9 (Deveraux et al., 1997). Therefore, the ability of HGF to modify expression of XIAP in facial and hypoglossal nuclei was examined. Immunofluorescence analysis revealed that XIAP-IR was markedly induced in facial motoneurons of G93A/HGF mice at 6 months of age, while the signal was low in WT and HGF-Tg mice, and below the detection limit in G93A mice (Fig. 5c, left panel). The mean intensity of XIAP-IR in the facial nuclei of G93A/HGF mice increased significantly to 6.4-fold over G93A mice. Similar results were obtained for the hypoglossal motoneurons (data not shown). These results suggest that, in addition to attenuation of caspase-1 activation, HGF induced XIAP expression in the presence of ALS-toxicity.

4. Discussion

4.1. HGF suppresses gliosis in facial and hypoglossal nuclei of a transgenic mouse model of ALS

ALS is characterized by a selective degeneration of motoneurons, regardless of the type of causal mutation or whether the disease is familial or sporadic. Most efforts have been directed toward the prevention of motoneuronal degeneration. However, several studies have suggested that gliosis in the vicinity of degenerating motoneurons may contribute to ALS disease progression, raising the possibility that gliosis might be a good target for curative efforts. In this regard, a single factor with neurotrophic and gliosis-suppressing activities may be beneficial for curing ALS. This study provides the first evidence that introduction of HGF into the nervous system suppresses induction of microglial accumulation in the facial and hypoglossal nuclei of G93A mice at 8 months of age, in addition to its suppressive activity on astrocytosis, using double transgenic mice overexpressing $SOD1^{G93A}$ and HGF. It was recently reported that using the

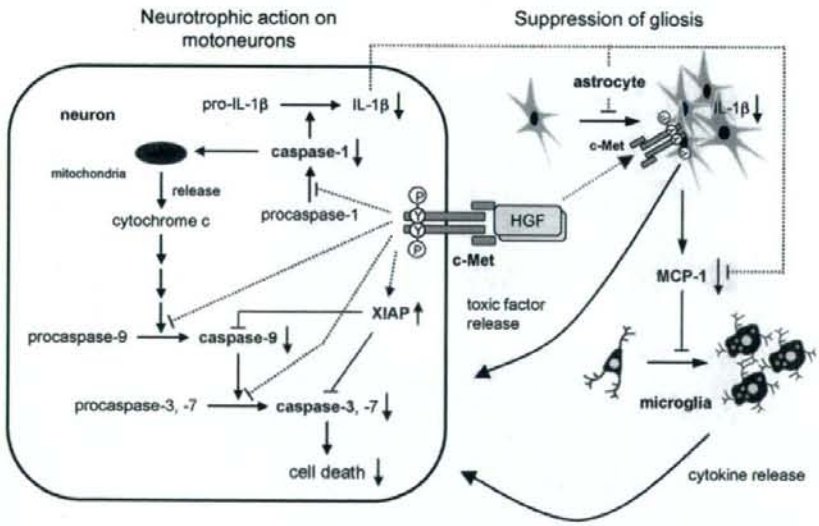

Fig. 6. Proposed working model of the molecular mechanisms of the neuroprotective effect of HGF on G93A mice are shown. In the motoneurons of G93A/HGF mice, HGF binds to c-Met on cell surface and induces autophosphorylation of the intracellular tyrosine residues of c-Met. Subsequently, HGF inhibits caspase-1 activation, induces XIAP and inhibits its downstream caspases, caspase-3, -7 and -9, thereby effectively dampening caspase-dependent cascades. Therefore, the neurotrophic action of HGF on motoneurons is, at least in part, promoted by preventing caspase-mediated cell death signals, which are commonly activated in patients with SALS and FALS, and in G93A mice. In addition to the neuroprotective effect of HGF on motoneurons, HGF also suppresses microglial accumulation, which is contributed in the progression of motoneuronal degeneration by producing cytotoxic cytokines, in G93A mice. By inhibition of caspase-1 activation in motoneurons and, presumably, the subsequent reduction of IL-1ß levels, HGF also suppresses MCP-1 induction in motoneurons and reactive astrocytes, and suppresses microgliosis as well as astrocytosis. HGF-induced attenuation of MCP-1 induction, which is thought to be responsible for the recruitment of monocytic lineage cells including microglia (Meeuwsen et al., 2003), might aid in the lessening of microglial accumulation in the brainstem, thereby presumably leading to a reduction of cytokine release from accumulated microglia (Weydt et al., 2004). The direct function of HGF on astrocytes may also play an important role, since c-Met is induced in reactive spinal cord astrocytes of G93A mice. Furthermore, recent study has reported that astrocytes contribute to motoneuronal degeneration by releasing toxic factors selectively to motoneurons (Nagai et al., 2007; Di Giorgio et al., 2007), suggesting that suppressing effects of HGF on astrocytosis is also advantageous for the treatment of brainstem and spinal motoneurons of ALS patients. In addition, other mechanisms may be involved in the neurotrophic activity and gliosis suppressing activity of HGF.

Cre-lox system to decrease expression of the mutant $SOD1^{G37R}$ transgene by only 25% in microglia of a transgenic mouse model of ALS significantly prolongs disease duration (Boillee et al., 2006). In microglia/motoneuron co-cultures, microglia that express mutant $SOD1^{G93A}$ induce more motoneuron death and decrease neurite numbers and length compared with wild-type microglia (Xiao et al., 2007). Therefore, even a small reduction in the number of disease progressing cells (microglia expressing mutant $SOD1^{G93A}$) (i.e., a small reduction of microglial accumulation) may be beneficial for retarding progression of the disease. It can be postulated that the attenuation of microglial accumulation to 60% in the facial and hypoglossal nuclei of G93A/HGF mice compared with those of G93A mice (Fig. 3a) might be sufficient to affect the disease. This raises the possibility that HGF application would be valuable for ALS therapy with respect to the reduction of microglial accumulation and subsequent disease progression.

The precise mechanism by which HGF attenuates microglial accumulation has not been elucidated. However, the present findings suggest two possibilities. The first possible mechanism is inhibition of caspase-1 activation in motoneurons, which in turn inhibits proteolytic cleavage of pro-IL-1ß to IL-1ß through active caspase-1 (Thornberry et al., 1992). The subsequent suppression of IL-1ß-dependent induction of MCP-1 mRNA is thought to be crucial for recruiting monocytic cells, such as microglia (Meeuwsen et al., 2003) (Fig. 6). In the present study, caspase-1 activation in motoneurons and MCP-1 induction in reactive astrocytes of the facial and hypoglossal nuclei of G93A mice were markedly suppressed in G93A/HGF mice (Fig. 4). Thus, it seems likely that HGF suppresses microglial accumulation by decreasing IL-1ß levels through inhibition of caspase-1 activation in motoneurons and reduction of MCP-1 levels in reactive astrocytes of the facial and hypoglossal nuclei of G93A mice, thereby preventing synergism between microglial accumulation and astrocytosis (Fig. 6). In addition to its effect through motoneurons, it seems likely that HGF directly acts on c-Met expressed in astrocytes to reduce IL-1β and MCP-1 levels, in turn ameliorating astrocytosis and microgliosis. Support for this supposition is evidenced by elevated c-Met levels in spinal cord astrocytes of G93A mice at the end stage of the disease (Sun et al., 2002; data not shown), and by HGF suppression of MCP-1 in the tubular epithelial cells (TEC) of the kidney via an NF-kB-mediated process (Gong et al., 2004). Indeed, upregulation of IL-1β in both spinal motoneurons and reactive astrocytes of G93A mice at 7 months of age is largely attenuated in G93A/HGF mice (Ohya and Funakoshi, unpublished data). Therefore, HGF may suppress gliosis via direct and indirect activities on glial cells. In addition to the above mechanisms, other mechanisms may be involved in the HGF-dependent suppression of microglial accumulation, and such possibilities are under the investigation.

Recent *in vitro* studies provided evidence that astrocytes expressing mutant SOD1 contribute to motoneuronal degeneration mediated by the release of soluble factors that are toxic to degenerate primary motoneurons or motoneurons that are derived from ES cells of mutant SOD1 mice (Nagai et al., 2007; Di Giorgio et al., 2007). In addition to suppressing microglial accumulation, HGF also suppresses astrocytosis in the facial and hypoglossal nuclei of G93A mice. These results raise the possibility that one of the molecular mechanisms by which HGF prevents motoneuronal degeneration is mediated by suppressing both microglial accumulation and astrocytosis.

4.2. Molecular mechanism of HGF neuroprotective effect on facial and hypoglossal motoneurons against ALS-toxicity

Caspases are activated in the spinal motoneurons of G93A mice, and a dominant negative inhibitor of the IL-1ß-converting enzyme (ICE)/caspase-1, anti-apoptotic protein Bcl-2 and a broad caspase inhibitor, zVAD-fmk, significantly slow the onset of ALS in a transgenic mouse model (Friedlander et al., 1997; Kostic et al., 1997; Pasinelli et al., 2000; Li et al., 2000; Guegan et al., 2001; Inoue et al., 2003). The results of the present study provide evidence that caspase-1, -3 and -9 are activated in facial and hypoglossal motoneurons of G93A mice, while their activation is suppressed in G93A/HGF mice (Figs. 4b, 5 and 6). These results suggest that the effects of HGF on caspase-dependent apoptosis in motoneurons may retard the early disease process.

Independent of its caspase-1 inhibition function, the upregulation of XIAP in brainstem motoneurons by HGF may also be beneficial in retarding the disease. XIAP functions as a ubiquitin ligase toward mature caspase-9 and second mitochondria-derived caspase activator (Smac), which is also known as direct IAP binding protein with low PI (DIABLO) and promotes caspase activation in the caspase-9 pathway by binding IAPs and preventing them from inhibiting caspases (Shi, 2004), to inhibit apoptosis (Morizane et al., 2005). Inoue et al. (2003) reported that gene transfer of XIAP attenuates disease progression without delaying onset through inhibition of caspase-9 activation in G93A mice, suggesting that caspase-9 contributes to the duration of the disease. Collectively, the actions of HGF cause not only caspase-1 inhibition, but also upregulation of XIAP and inhibition of its downstream caspases in brainstem motoneurons. These actions of HGF may be, at least in part, involved in the mechanisms associated with retarding disease onset and duration, and prolonging the lifespan in the familial ALS (FALS) mouse model. We previously reported that HGF delays onset and prolongs lifespan, but does not extend duration in G93A mice due to insufficient delivery of HGF in the late stages (Sun et al., 2002). Thus, improved delivery of HGF may further enhance its effect at later stages of ALS.

Immunocytochemical, Western blotting and DNA microarray analyses have shown that caspase expression is upregulated in patients with sporadic ALS (SALS) and/or FALS compared with non-ALS controls (Ilzecka et al., 2001; Inoue et al., 2003; Calingasan et al., 2005; Jiang et al., 2005). These results suggest that activation of these caspases may be a common pathway of disease progression for both FALS and SALS. Furthermore, in both SALS and FALS patients, HGF and c-Met are regulated in a manner similar to that seen in

FALS mice (Kato et al., 2003). Therefore, HGF delays onset and may prolong disease duration through inhibition of a common caspase-dependent pathway in ALS. Post-diagnostic HGF therapy could be considered not only for mutant SOD1-related FALS, but also for SALS.

4.3. HGF may be an effective agent for ALS therapy

Since motoneuronal death is the major and common characteristic of both FALS and SALS (Cleveland and Rothstein, 2001), neurotrophic factors have been proposed as highly potent therapeutic agents for motoneuronal degeneration (Sendtner et al., 1992; Funakoshi et al., 1995, 1998; Wang et al., 2002; Sun et al., 2002; Kaspar et al., 2003; Azzouz et al., 2004). Some neurotrophic factors, including HGF, glial cell-line derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF), confer neuroprotective properties to spinal motoneurons in a transgenic mouse model of ALS (present study; Sun et al., 2002; Wang et al., 2002; Kaspar et al., 2003; Azzouz et al., 2004), raising the possibility of their use as therapeutics. However, some neurotrophic factors may not prevent the death of subpopulations of spinal cord and brainstem motoneurons under degenerative conditions, including ALS-toxicity (Sakamoto et al., 2003; Guillot et al., 2004). The effects of these factors on brainstem motoneurons in the transgenic mouse model of ALS are not well understood. The finding that HGF is capable of attenuating motoneuronal death in both brainstem (present study) and spinal motoneurons (Sun et al., 2002), might be useful in future therapeutic applications of HGF in ALS patients. The potential of HGF to decrease gliosis, including microglial accumulation, in addition to its direct neurotrophic activity on motoneurons might be of further benefit.

In summary, this study provides the first evidence that HGF exerts a neuroprotective effect on facial and hypoglossal motoneurons against ALS-toxicity by preventing motoneuronal death via suppression of pro-apoptotic protein activation and by reducing gliosis via inhibition of MCP-1 induction. Although development of a delivery method for the HGF protein and gene may be required before clinical application, these findings suggest that HGF may be an effective therapeutic agent for the treatment of brainstem and spinal motoneurons in ALS patients.

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Research Report

Hepatocyte growth factor (HGF) promotes oligodendrocyte progenitor cell proliferation and inhibits its differentiation during postnatal development in the rat

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ABSTRACT

Hepatocyte growth factor (HGF) was initially cloned as a mitogen for hepatocytes and has been identified as a neurotrophic factor for a variety of neurons. However, few attempts have assessed the role of HGF in cells of oligodendrocyte lineage. The purpose of this study was to elucidate the role of HGF in such cells during development. Double immunostaining for either c-Met/HGF receptor or phospho-c-Met with either NG2 or RIP in rat striatum at postnatal day 3 (P3), P7, and P14 revealed that c-Met was phosphorylated on tyrosine residues and thereby activated in NG2$^+$ oligodendrocyte progenitor cells (OPCs) at P3–P14 and in RIP$^+$ oligodendrocytes at P14. Intrastriatal injections of recombinant human HGF at both P7 and P10 revealed that the relative ratio of BrdU$^+$/NG2$^+$ cells per total number of NG2$^+$ cells increased, while BrdU$^+$/MBP$^+$ oligodendrocyte numbers decreased. Western blot analysis showed a down-regulation of myelin basic protein (MBP) after HGF injection. Electron microscopy revealed that the numbers of myelinated nerve fibers decreased after HGF treatment. Furthermore, administration of anti-HGF IgG into the striatum increased the number of BrdU$^+$/MBP$^+$ oligodendrocytes. These findings demonstrated that HGF increases proliferation of OPCs and attenuates their differentiation into myelinating oligodendrocytes, presumably by favoring neurite outgrowth that may be inhibited by the myelin inhibitory molecules on oligodendrocytes. Down-regulation of HGF mRNA in the striatum from P7 to P14, as revealed by quantitative real-time RT-PCR, may be favorable for OPC differentiation into myelinating oligodendrocytes. Our findings suggest that c-Met signaling, together with HGF regulation, plays an important role in developmental oligodendrogenesis.

1. Introduction

Proliferation, differentiation, and maturation of oligodendrocyte progenitor cells (OPCs) and oligodendrocytes, together with their mutual communication with neurons, are essential for the functional development of the nervous system. These events are tightly and precisely regulated by the communication of developing oligodendrocyte lineage cells with surround-

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ing cells, such as astrocytes and neuronal axons, especially during postnatal development. It has been shown that two different notch signaling pathways play a critical role in the switching of OPCs from a proliferation state to a differentiation state at postnatal day 6 (P6) in rodents (Hu et al., 2003; Hu et al., 2006). Prior to P6, the astrocyte-expressed Jagged1 protein transactivates OPCs to induce intracellular notch signaling via Hairy Enhancer of Split-1 (HES-1), which contributes to the maintenance of OPCs in an undifferentiated state (Wang et al., 1998). However, Jagged1 expression sharply decreases at P6, a time point concurrent with the onset of myelination, and its inhibitory activity toward OPC differentiation becomes weak. On the other hand, after P6, levels of the axon-derived notch ligand "F3/contactin" increase, which in turn transactivate OPCs, inducing a second intracellular notch signaling molecule "Deltex1", which contributes to OPC differentiation into mature oligodendrocytes. Therefore, the switch from a proliferation state to a differentiation state is dependent upon the two different notch pathways, "Jagged1/notch/HES-1" and "contactin/notch/Deltex1" (Hu et al., 2003). In addition, other factors, such as platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF2), have been found to be partially involved in these processes during either development or adulthood (Bogler et al., 1990; Butt and Dinsdale, 2005b; McKinnon et al., 1990). While FGF2 is known to be involved in the induction of demyelination in adult CNS (Butt and Dinsdale, 2005a), the molecular mechanisms by which OPCs proliferate, differentiate, and mature during the course of development are still not fully understood.

Hepatocyte growth factor (HGF) was initially identified and cloned as a mitogen for primary hepatocytes (Nakamura et al., 1984, 1989) and was later found to be a novel neurotrophic factor for various types of neurons in both the CNS and PNS (Funakoshi and Nakamura, 2003; Maina et al., 1998), such as hippocampus (Honda et al., 1995), midbrain dopaminergic neurons (Hamanoue et al., 1996), cerebral cortical neurons (Sun et al., 2002a), sensory neurons (Funakoshi and Nakamura, 2001; Maina et al., 1997), motor neurons (Ebens et al., 1996; Yamamoto et al., 1997), cerebellar granular cells (Zhang et al., 2000), and cortical interneurons (Powell et al., 2001) in vitro. HGF was also found to be a neurotrophic factor in vivo in rodent models of brain ischemia (Ishihara et al., 2005; Miyazawa et al., 1998) and motor nerve injuries (Hayashi et al., 2006; Okura et al., 1999). Anxiolytic effects of HGF are also evident (Isogawa et al., 2005). In addition, we found that HGF gene transfer in the neurons of a transgenic mouse model of amyotrophic lateral sclerosis (ALS) overexpressing $SOD1^{G93A}$ attenuates the degeneration of both spinal and brainstem motor neurons, retards the progression of functional motor impairment, and improves the life span of animals with ALS (Sun et al., 2002b) (Kadoyama, Funakoshi et al., unpublished data). Taken together, both the presence and regulation of the HGF-c-Met/HGF receptor system in familial as well as sporadic patients with ALS suggest a physiological role for HGF in retarding the progression of the disease in such patients (Kato et al., 2003). In contrast to its activities on neurons, little information is available on the role of HGF in oligodendrocyte lineage cells, except for in vitro evidence that c-Met is present in both cultured OPCs and oligodendrocytes, and that HGF is capable of accelerating the proliferation of OPCs in culture (Yan and Rivkees, 2002).

As null mutations of both HGF and c-met show embryonic lethality (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995), making the analysis of the role of HGF in postnatal development difficult, we took advantage of two approaches in the present study to assess the role of HGF in oligodendrogenesis during postnatal development in vivo. First, we used antibodies specific for c-Met as well as antibodies specific for phospho-c-Met, which recognize the intracellular, phosphorylated c-Met tyrosine residues (phospho-$Tyr^{1230/1234/1235}$) for detection of its activation in vivo. Second, we treated animals with recombinant human HGF (rhHGF) or anti-HGF IgG by stereotaxic injection into the striatum and examined the in vivo role of HGF in oligodendrogenesis by immunohistochemistry, Western blotting, and electron microscopy. The results demonstrated that HGF plays an important role in the promotion of both OPC proliferation and attenuation of its differentiation into myelinating oligodendrocytes.

2. Results

2.1. *c-Met/HGF receptor is present in OPCs and oligodendrocytes during early postnatal development in vivo*

In rat striatum, MBP^+ oligodendrocytes became prominent as dense fiber bundles at P14 compared with P7 (Fig. 1a). $NG2^+$ OPCs were evident from P3 to P14 (Fig. 1b), demonstrating the importance of this early postnatal period in both OPC and oligodendrocyte development in the striatum. Therefore, to examine the biological role of HGF in oligodendrocyte development, we first assessed whether c-Met was expressed in oligodendrocyte lineage cells from P3 to P14 by immunostaining using a specific antibody against c-Met. Double fluorescence immunostaining of c-Met and NG2, a marker for OPC, revealed that $c\text{-}Met^+/NG2^+$ immunoreactivity (IR) (green/red) was evident in addition to the large numbers of c-Met single-positive cells at P3 (Fig. 1b, upper panel). At both P7 and P14, NG2 IR overlapped c-Met IR, demonstrating the presence of c-Met IR in OPCs during all postnatal periods examined. It has been reported that NG2 staining overlaps with that of another OPC marker, PDGFRα, in the striatum at P3, and thus NG2 and PDGFRα can serve as reliable markers for the identification of O2A progenitor cells; however, there is a small population of $NG2^+$ cells that do not overlap with $PDGFR\alpha^+$ cells at late stages of postnatal development (Nishiyama et al., 1996). To further confirm the expression of c-Met in OPCs from the early postnatal striatum, the population of $NG2^+$ cells was compared with $PDGFR\alpha^+$ cells from P3 to P14. Double immunostaining revealed that $NG2^+$ cells overlapped $PDGFR\alpha^+$ cells from P3 to P11; $NG2^+$ endothelial cells were easily distinguishable from OPCs (Fig. 1c). On the other hand, it should be noted that there is a small population of $NG2^+$ cells that does not overlap with $PDGFR\alpha^+$ cells in the striatum at P14 (data not shown). The presence of $c\text{-}Met^+/PDGFR\alpha^+$ cells at P14 demonstrated that c-Met is present in OPCs during all developmental stages examined. Double immunostaining for c-Met and RIP, a marker for oligodendrocytes, revealed that c-Met IR was detected in RIP^+ oligodendrocytes (red) at P14 (Fig. 1b, lower panel). These findings suggest that both OPCs and oligodendrocytes are potential target cells for HGF in the striatum during early postnatal development.

2.2. *Numbers of OPCs and oligodendrocytes were reciprocally regulated from P7 to P14*

To assess the regulation of oligodendrogenesis from P3 to P14, we examined the number of both PDGFRα$^+$ (Fig. 2a) and RIP$^+$ (Fig. 2b) cells since at P14 there is a population of NG2$^+$ cells that do not overlap with PDGFRα. The number of PDGFRα$^+$ OPCs increased from P3 to P7 and decreased from P7 to P14 (Fig. 2a). In contrast, the number of RIP$^+$ oligodendrocytes increased from P7 to P14 (Fig. 2b). Double immunostaining of the oligodendrocytic markers and c-Met (Figs. 2c, d) revealed that c-Met was expressed in both PDGFRα$^+$ OPCs and RIP$^+$ oligodendrocytes at all developmental stages (from P3 to P14). Therefore, the numbers of OPCs and oligodendrocytes were reciprocally regulated from P7 to P14. Additionally, c-Met is expressed in most populations of OPCs and oligodendrocytes, suggesting that the HGF-c-Met system plays an important role in oligodendrogenesis during early postnatal development.

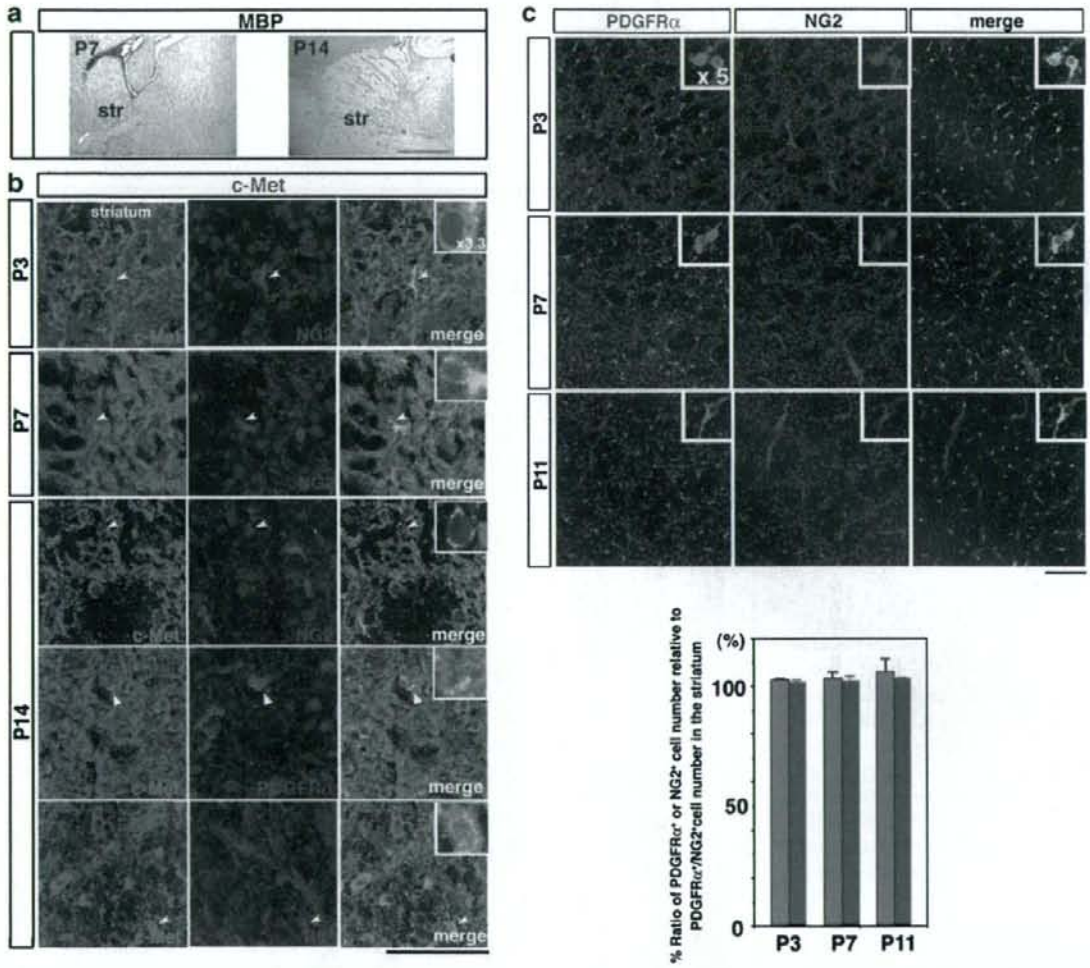

Fig. 1 – c-Met IR is present in NG2$^+$ OPCs from P3 to P14 and in RIP$^+$ oligodendrocytes at P14. (a) Immunostaining for anti-MBP antibody in the sagittal sections of P7 and P14 rat brains. Slides were counterstained with hematoxylin. Only weak MBP IR is detected in the striatum at P7, while strong MBP IR with a cingulated pattern is detected in the striatum at P14. Str, striatum. Scale bar = 1 mm. (b) Double fluorescence immunostaining for c-Met (green) and NG2 (red), for c-Met (green) and PDGFRα (red), or for c-Met (green) and RIP (red) (bottom panel) and counterstained with TOPRO-3 iodide (blue) for nuclear staining in P3 (upper panel), P7 (middle panel), and P14 (lower panel) rat striatum. Arrowheads indicate double-immunostained cells with NG2/c-Met, PDGFRα/c-Met, or RIP/c-Met. The insets designate higher (×3.3) magnification views, indicated by arrowheads. Scale bar = 100 μm. (c) Double fluorescence immunostaining for PDGFRα (green) and NG2 (red) in the striatum during development (upper panel). Lower panel shows the quantification of the ratio of PDGFRα single-positive cells (green) or NG2 single-positive (red) cells per double-positive cells at P3, P7, and P11. The insets designate higher (×5) magnification views. Scale bar = 100 μm.