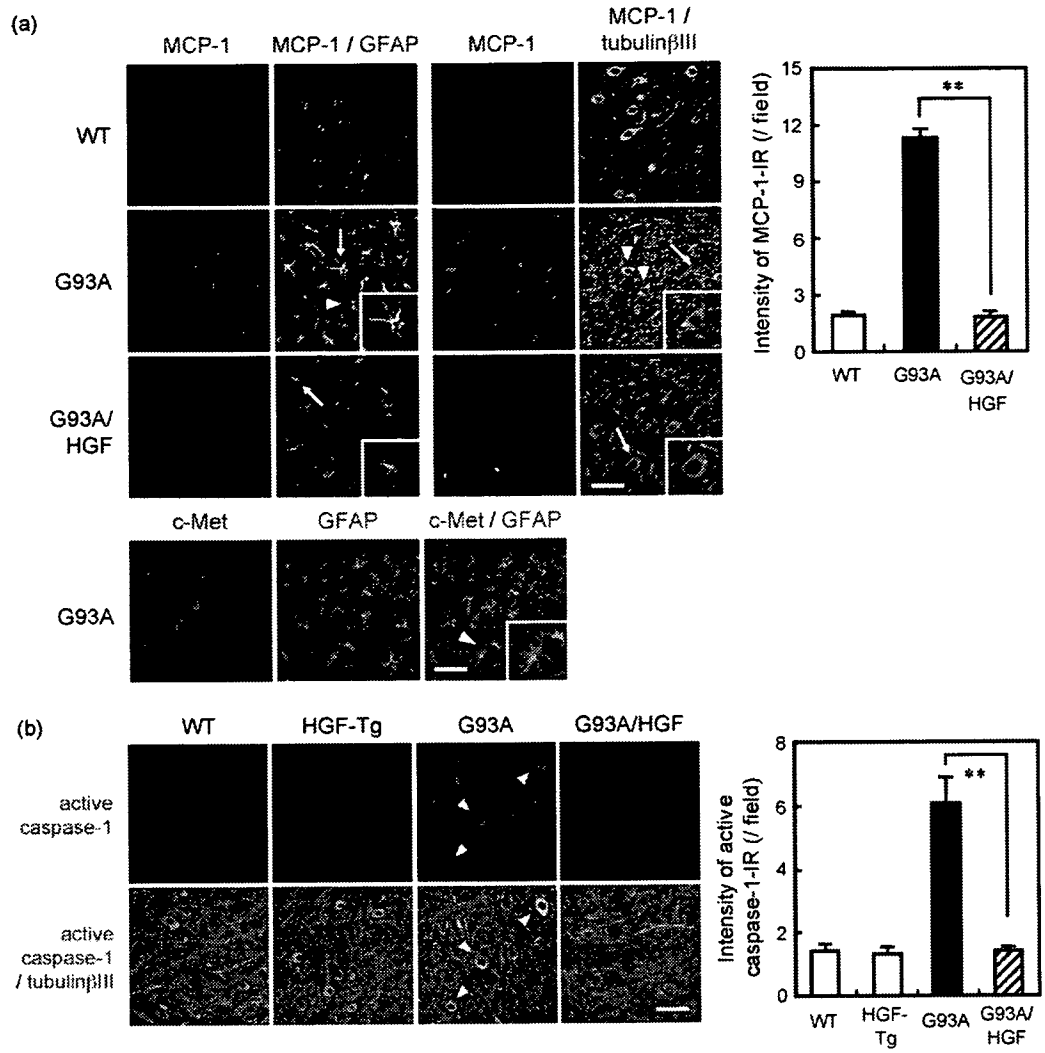

Fig. 4. Inhibitory mechanisms of HGF on gliosis in the facial nucleus of G93A mice. (a) Suppression of MCP-1 induction in G93A/HGF mice. Upper left panel, double immunofluorescence analysis of MCP-1 (red) and GFAP (green) or tubulinβIII (green) in the facial nucleus of WT, G93A and G93A/HGF mice at 8 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,

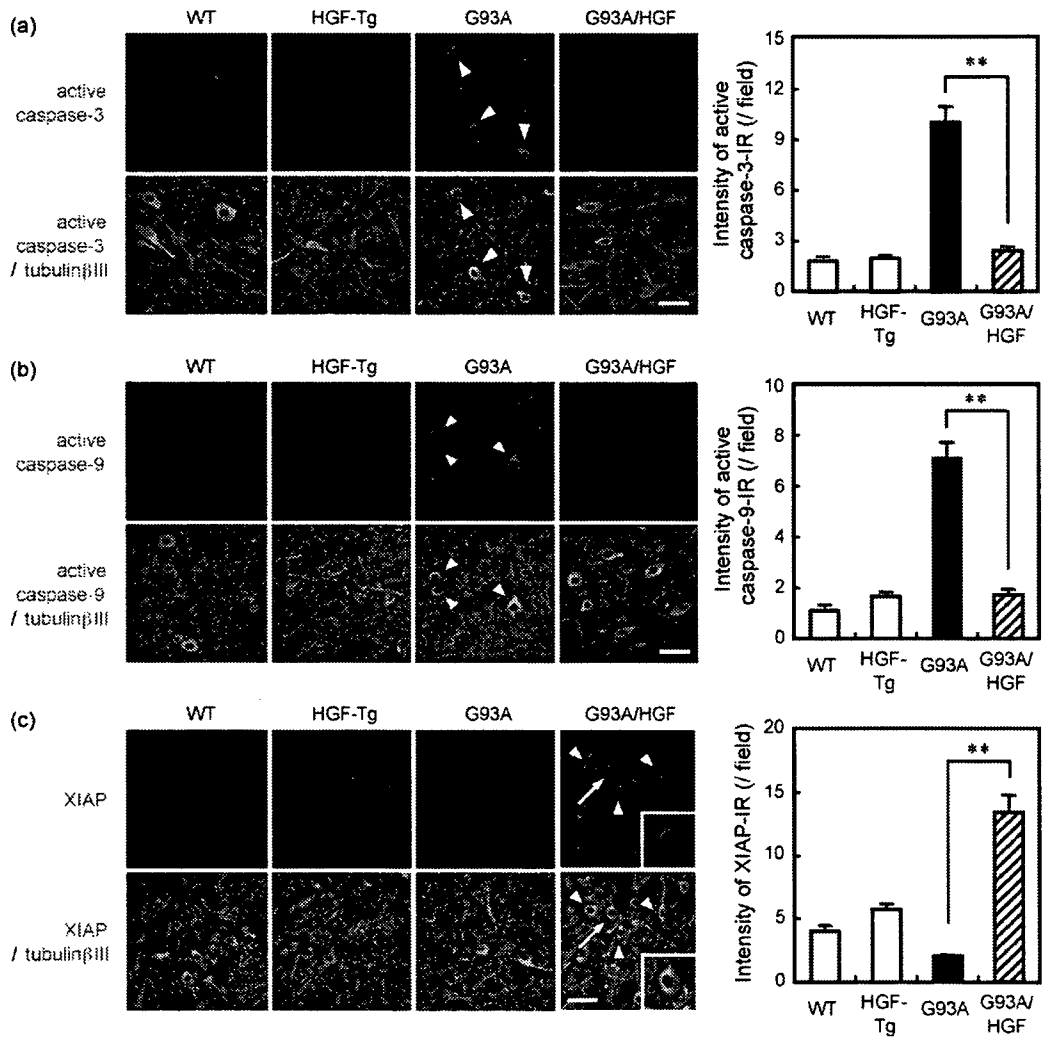

Fig. 5. Attenuation of pro-apoptotic protein activation in the facial motoneurons of G93A/HGF mice. (a) Left panel, double immunofluorescence analysis of active caspase-3 (red) and tubulinßIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. Active caspase-3-positive 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

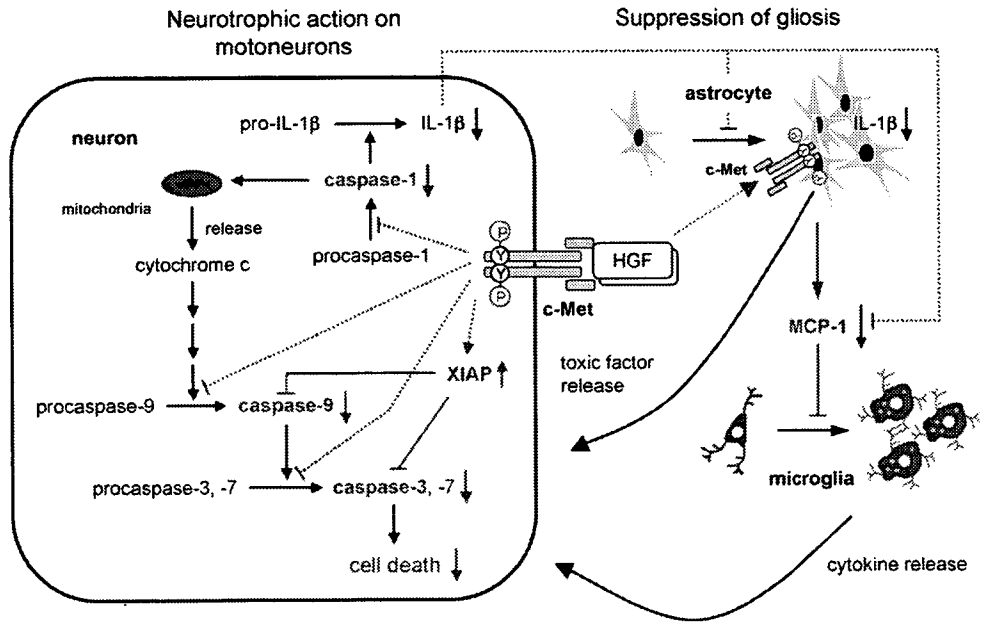

Fig. 6. Proposed working model of the molecular mechanisms of the neuroprotective effect of HGF on G93A mice are shown. In the motoneurons of G93A/HGF mice, HGF binds to c-Met on cell surface and induces autophosphorylation of the intracellular tyrosine residues of c-Met. Subsequently, HGF inhibits caspase-1 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 micro-array 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.

Acknowledgements

This work was supported by a Grant-in-Aid (T.N. and H.F.) from the Ministry of Education, Science, Culture, Sports and Technology, Japan, and by a Grant-in-Aid (H.F.) from the Ministry of Health, Labour, and Welfare, Japan. K.K. was supported by a grant for Young Research Residents from the Japan Foundation for Neuroscience and Mental Health, Tokyo.

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J Neuropathol Exp Neurol

Vol. 66, No. 11
November 2007
pp. 1037–1044

ORIGINAL ARTICLE

Intrathecal Delivery of Hepatocyte Growth Factor From Amyotrophic Lateral Sclerosis Onset Suppresses Disease Progression in Rat Amyotrophic Lateral Sclerosis Model

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Abstract

Hepatocyte growth factor (HGF) is one of the most potent survival-promoting factors for motor neurons. We showed that introduction of the HGF gene into neurons of G93A transgenic mice attenuates motor neuron degeneration and increases the lifespan of these mice. Currently, treatment regimens using recombinant protein are closer to clinical application than gene therapy. To examine its protective effect on motor neurons and therapeutic potential we administered human recombinant HGF (hrHGF) by continuous intrathecal delivery to G93A transgenic rats at doses of 40 or 200 μg and 200 μg at 100 days of age (the age at which pathologic changes of the spinal cord appear, but animals show no clinical weakness) and at 115 days (onset of paralysis), respectively, for 4 weeks each. Intrathecal administration of hrHGF attenuates motor neuron degeneration and prolonged the duration of the disease by 63%, even with administration from the onset of paralysis. Our results indicated the therapeutic efficacy of continuous intrathecal administration of hrHGF in transgenic rats and should lead to the consideration for further clinical trials in amyotrophic lateral sclerosis using continuous intrathecal administration of hrHGF.

Key Words: Amyotrophic lateral sclerosis, Continuous intrathecal delivery, Hepatocyte growth factor, Neurodegeneration, Superoxide dismutase-1 (SOD1), Transgenic rat.

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This work was supported by a grant from the Ministry of Health, Labor, and Welfare, Japan (YI, MA, SK, HF). Research funding was also provided by the Haruki ALS Research Foundation (YI, MA, HW) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MA, SK, HF).

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective motor neuron death (1). Approximately 10% of cases of ALS are inherited, usually as an autosomal dominant trait (2). In ~25% of familial cases, the disease is caused by mutations in the gene encoding cytosolic copper-zinc superoxide dismutase (SOD1) (3–5). The cause of ALS is still unclear, and clinical trials have as yet failed to identify any truly effective therapeutic regimens for ALS, with only riluzole providing a modest improvement in survival. Various substances have been shown to have therapeutic effects in a murine model of ALS. However, there have been a few reports of prolongation of survival with treatment starting around the time of disease onset (6–12).

We (13) and another group (14) developed a rat model of ALS expressing a human SOD1 transgene with 2 ALS-associated mutations: glycine to alanine at position 93 (G93A) and histidine to arginine at position 46 (H46R) (3, 5). Similar to its murine counterpart, this rat transgenic (Tg) ALS model reproduces the major phenotypic features of human ALS. Some experimental manipulations are difficult in Tg mice because of size limitations; however, this Tg rat model allows routine implantation of infusion pumps for intrathecal drug delivery. Intrathecal drug application is a well-established method for therapy and has been used in clinical trials in patients with ALS (15). This route of administration bypasses the blood-brain barrier, allowing rapid access to potential binding sites for the test compound in the spinal cord (16).

Hepatocyte growth factor (HGF) was first identified as a potent mitogen for mature hepatocytes and was first cloned in 1989 (17). Detailed studies indicated that HGF is expressed in the CNS (18) and is a novel neurotrophic factor (19, 20). HGF is one of the most potent survival-promoting factors for motor neurons, comparable to glial cell line-derived neurotrophic factor in vitro (21). Sun et al (22) reported that introduction of the HGF gene into neurons of G93A Tg mice attenuates motor neuron degeneration and increases the lifespan of these mice. Thus, HGF is a good candidate agent for treatment of ALS. Currently, treatment using recombinant protein is closer to clinical application than gene therapy. However, HGF has a very

short half-life (23–25) and shows poor penetration into the CNS. Therefore, we examined the effects of continuous intrathecal delivery of human recombinant HGF (hrHGF) into Tg rats using implanted infusion pumps for selective and less invasive supply of HGF to the spinal cord.

MATERIALS AND METHODS

Animal Preparation and Clinical Evaluation

G93A Tg rats were genotyped by polymerase chain reaction (PCR) assay using DNA obtained from the tail as described (13). To examine the dose and effects of hrHGF on disease onset, we began administration of 40 or 200 μg of hrHGF (provided by H. Funakoshi and T. Nakamura, Osaka University, Osaka, Japan) or vehicle (0.1 M sulfoxide PBS) for 4 weeks to groups of eight 100-day-old Tg rats, when the pathologic changes of the spinal cord appeared, but the animals did not show weakness. All animals were killed at 130 days by deep anesthesia, and the spinal cords were examined. Because treatment of patients with ALS patients is initiated only after diagnosis based on clinical signs and symptoms, we tested the effects of hrHGF on survival with administration beginning at around the age of onset of paralysis. We administered 200 μg of hrHGF or vehicle alone to groups of eight 115-day-old G93A Tg rats for 4 weeks, and the animals were observed until their death. To analyze the mechanism of action of hrHGF administration beginning at onset of paralysis we treated groups of six 115-day-old G93A Tg rats with 100 μg of hrHGF or with vehicle alone for 2 weeks (a dose comparable to 200 μg for 4 weeks). All rats were killed 2 weeks after commencement of administration of hrHGF, and their lumbar spinal cords were examined. Further groups of 3 G93A Tg rats and 3 non-Tg rats at 70, 100, and 130 days were used to measure the levels of rat HGF and c-Met. All rats were handled according to approved animal protocols of our institution and had free access to food and water throughout the experimental period and before and after pump implantation.

The onset of ALS was scored as the first observation of abnormal gait, evidence of limb weakness, or loss of extension of the hindlimbs when picked up at the base of the tail. We defined the appearance of paralysis as disease onset, although this is not a sensitive indicator and appears later than the decrease in activity (10). However, the appearance of paralysis is a suitable marker of disease onset because it is closer to the state at which patients will be diagnosed with the disease.

Footprints were collected every 3 days by letting the rats walk on a straight path after dipping their hind paws in black ink. We measured 3 strides within the area showing regular gait and calculated the means. Footprint measurements were made for rats that began treatment at 115 days. Examiners were blinded to which group each of the rats belonged in.

Preparation of the Osmotic Pumps and Transplant Surgery

Osmotic pumps (model number 2004 or 2002; Durect Corporation, Cupertino, CA) were incubated in sterile saline at 37°C for 40 hours to attain a constant flow rate before use. Pumps were filled to capacity with hrHGF solution or vehicle using a filling needle. An infusion tube was made by connecting a 1-cm length of polyethylene tubing (PE 60; Becton Dickinson, Franklin Lakes, NJ) to a small caliber tube 9 cm in length (PE 10; Becton Dickinson) using an adhesive (ARON ALPHA; Konishi Co., Osaka, Japan). The end of the infusion tube was connected to the shorter end of the flow moderator, the longer end of which was inserted into the pump.

Surgery for placement of the pump and intrathecal administration was performed as follows. Tg rats were anesthetized using diethyl ether and 1% halothane in a mixture of 30% oxygen and 70% nitrous oxide. The skin over the third to fifth lumbar spinal process was incised and the paravertebral muscles were separated from the vertebral lamina with scissors. The fifth lumbar vertebra was laminectomized, and the dura mater was exposed for insertion of the infusion tube. Particular care was taken not

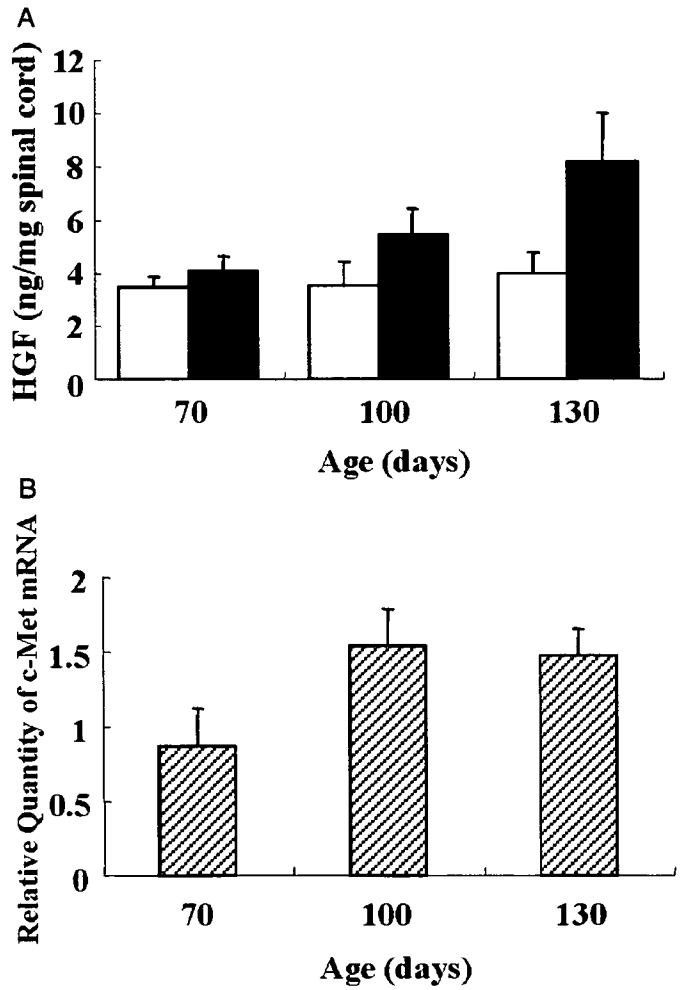

FIGURE 1. Increased levels of rat hepatocyte growth factor (HGF) and c-Met expression in the spinal cords of G93A transgenic (Tg) rats (n = 3) and non-Tg rats (n = 3). **(A)** Levels of endogenous rat HGF expression. Open bars, non-Tg rats; closed bars, G93A Tg rats. **(B)** Levels of c-Met mRNA of G93A Tg rats compared with non-Tg rats.

to injure the dura mater during laminectomy. A small hole was bored through the dura mater with a 24-gauge needle, and a polyethylene tube (PE 10, Becton Dickinson) was inserted into the subarachnoid space approximately 3 cm rostrally. A subcutaneous pocket was made into which the osmotic pump and pump side tube were implanted. The infusion tube was attached to the fascia over the paravertebral muscles at the incision margin with silk string. A drop of adhesive (ARON ALPHA) was applied, and the incision was closed by suturing the muscles and skin.

Measurement of Rat and Human HGF in the Lumbar Spinal Cord

Slices of the fifth lumbar cord from 3 G93A Tg rats and 3 non-Tg rats at 70, 100, and 130 days as well as from 130-day-old G93A Tg rats treated with 40 or 200 μg of hrHGF or vehicle alone for 4 weeks starting at 100 days were homogenized in buffer (20 mM Tris-HCl, pH 7.5, 0.1% Tween-80, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA) and centrifuged at 15,000 rpm for 30 minutes. Supernatants were separated and the concentrations of rat endogenous HGF were measured using an enzyme-linked immunosorbent assay (ELISA) kit, which is specific for rat HGF without detecting human HGF (22) (Institute of Immunology, Tokyo, Japan). For measurement of human HGF in the treated rats we used a human HGF-specific ELISA kit (IMMUNIS, Institute of Immunology), which is not reactive with rat HGF (26, 27).

Measurement of c-Met mRNA in the Lumbar Spinal Cord of Tg Rats

Aliquots of 1 μg of total RNA from the lumbar cords of rats were used as templates for synthesis of double-stranded cDNA. Real-time quantitative PCR was performed for c-Met and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [GAPDH forward primer, 5′-CCATCACTGCCACTCAGAAGAC-3′; GAPDH reverse primer, 5′-TCATACTTGGCAGGTTTCTCCA-3′; GAPDH TaqMan probe, 5′(FAM)-ACCACGAGCACTGTTTCAATAGGACCC-(TAMRA)3′; c-MET forward primer, 5′-GTACGGTGTCTCCAGCATTTTT-3′; c-Met reverse primer, 5′-AGAGCACCACCTGCATGAAG-3′; TaqMan probe, 5′(FAM)-CGTGTTCCTACCCCCAATGTATCCGT- (TAMRA)3′]. An ABI Prism 7700 Sequence Detection System (Applied Biosystems Perkin-Elmer, Foster City, CA) was used to monitor emission intensities using the above primer pairs and TaqMan fluorogenic probes. The c-Met mRNA level of G93A Tg rats relative to non-Tg rats was calculated using the Comparative C_T Method (Applied Biosystems).

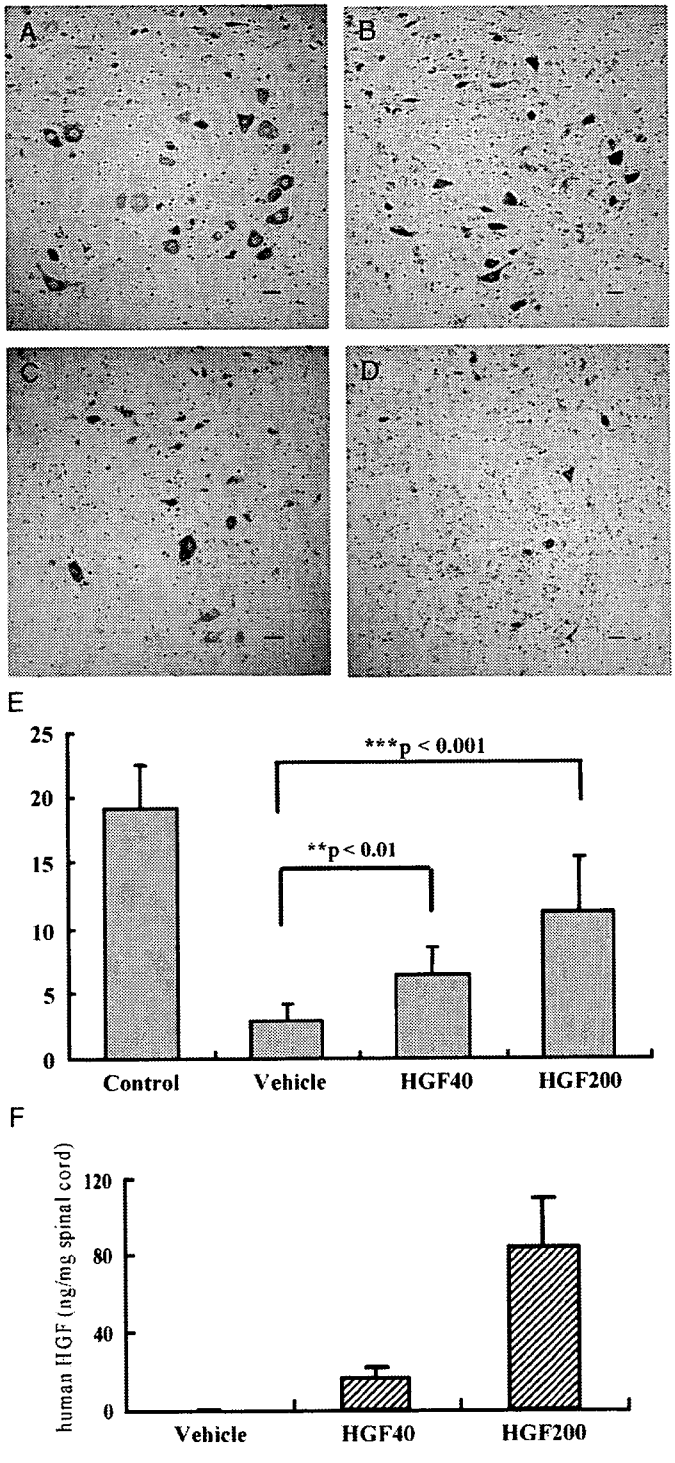

FIGURE 2. Intrathecal administration of hepatocyte growth factor (HGF) to G93A transgenic (Tg) rats at 100 days showed a protective effect against motor neuron death. **(A–D)** Histologic evaluation of the anterior horn with Nissl staining at 130 days: **(A)** lumbar cord of non-Tg rats; **(B)** 200 μg of human recombinant HGF (hrHGF)-treated; **(C)** 40 μg of hrHGF-treated; and **(D)** vehicle-treated G93A Tg rats. Scale bar = 40 μm. **(E)** Quantitative morphometric evaluation of surviving motor neurons of the fifth lumbar anterior horn at 130 days. We counted neurons that were >40 μm in diameter. Significantly larger numbers of motor neurons survived in hrHGF-treated G93A Tg rats ($p < 0.01$ and $p < 0.001$, 40 and 200 μg of hrHGF, respectively), compared with vehicle-treated G93A Tg rats (n = 8 in each group). **(F)** Levels of human HGF concentration in lumbar spinal cords of G93A Tg rats treated with 200 μg of hrHGF, 40 μg of hrHGF, and vehicle.

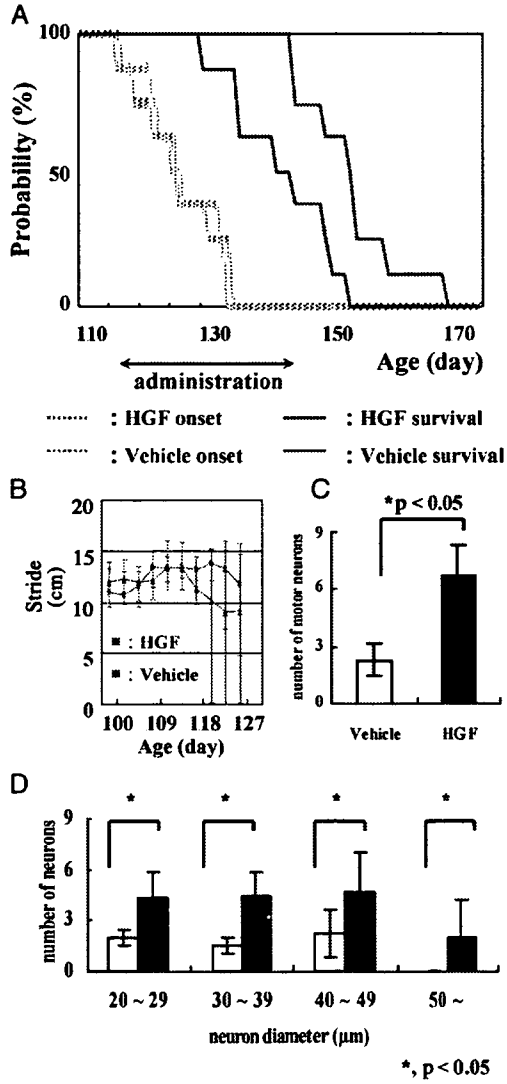

FIGURE 3. Intrathecal administration of hepatocyte growth factor (HGF) from 115 days (just before disease onset) retarded disease progression. **(A)** Survival periods were 143.25 ± 17.0 days in the vehicle-treated group (solid blue line) and 154.3 ± 16.4 days in the 200 μg of human recombinant HGF (hrHGF)-treated group (solid red line). Survival of hrHGF-treated animals was extended significantly ($p = 0.0135$), although there were no significant differences in onset (dotted lines, $n = 8$ in each group, $p = 0.6346$). **(B)** Footprint analysis demonstrated a delay in decline of stride length in G93A transgenic (Tg) rats treated with 200 μg of hrHGF relative to vehicle-treated G93A Tg rats (error bars, ± SD). **(C, D)** Quantitative morphometric evaluation of surviving motor neurons that were >40 μm in diameter **(C)** and neuron size distribution **(D)** in the fifth lumbar anterior horn of G93A Tg rats 2 weeks after administration from 115 days. Significantly larger number of motor neurons survived in the hrHGF-treated G93A Tg rats compared with vehicle-treated G93A Tg rats (6.7 ± 1.6 vs 2.3 ± 0.9; $p = 0.002$, $n = 6$ in each group) **(C)**.

Histopathologic and Immunohistochemical Analyses

To examine the dose and effects of hrHGF against disease onset, we began administration of 40 or 200 μg of hrHGF or vehicle alone to groups of eight 100-day-old Tg rats each for 4 weeks. At 130 days, G93A Tg rats were administered hrHGF or vehicle, and non-Tg rats were deeply anesthetized with diethyl ether and killed for histopathologic evaluation. To examine the effects of hrHGF administration beginning at onset of paralysis, 100 μg of HGF or vehicle alone was administered to groups of six 115-day-old Tg rats for 2 weeks. These animals were killed by deep anesthesia with diethyl ether 2 weeks after the operation. Under deep anesthesia these animals were perfused via the aorta with physiologic saline at 37°C and their lumbar spinal cords were removed. The fifth lumbar spinal cord tissue was embedded in OCT compound (Sakura Finetek Japan Co., Tokyo, Japan), frozen in an acetone/dry ice bath after fixation with 4% paraformaldehyde, and supplemented with 0.1 M cacodylate buffer (pH 7.3) containing 30% sucrose. Other spinal cord tissue specimens were frozen in dry ice and cut into frozen sections (12-μm-thick) and then washed with PBS. To evaluate the effects of HGF on motor neuron loss we compared the numbers of lumbar motor neurons in each group by counting as mentioned below. To evaluate the effects of HGF on apoptosis and to determine whether HGF receptors were activated, we compared the results of immunohistochemical staining of the lumbar cords for activated caspase-3, activated caspase-9 (Cell Signaling Technology, Inc., Beverly, MA), and phosphorylated c-Met (activated HGF receptor) (BioSource International, Camerillo, CA). The staining specificity of the antibodies was assessed by preabsorption of the primary antibody with excess peptide, omission of the primary antibody, or replacement of the primary antibody with normal rabbit IgG (22). We examined every seventh section from 42 serial sections of the fifth lumbar spinal cord. We counted neurons that had a clear nucleolus and were multipolar with neuronal morphology (13, 22), >40 μm in diameter, and located in a defined area of the anterior horn of the spinal cord. Cell counts were performed using ImageJ software (National Institutes of Health, Bethesda, MD) on images captured electronically (28).

Western Blotting

Lysates from the lumbar spinal cord of each rat were prepared in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris, pH 8.0). Equal amounts of proteins from the lysates (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene diflouride membranes, and immunoblotted. The primary antibodies used were anti-caspase-3 (Sigma-Aldrich, St. Louis, MO), anti-caspase-9 (Stressgen Biotechnologies Corporation, Victoria, BC, Canada), anti-X-linked inhibitor of apoptosis protein (XIAP) (Cell Signaling Technology, Inc.), and anti-excitatory amino acid transporter 2 (EAAT2) antibodies (Chemicon International, Temecula, CA). After incubation of membranes with HRP-coupled

secondary antibodies, proteins were visualized using ECL or ECL Plus Western Blotting Detection Reagents (Amersham Biosciences Inc., Piscataway, NJ) and a Fluorochem image analyzer (LAS-3000 mini; Fuji Photo Film Co., Tokyo, Japan).

Statistical Analysis

The Kaplan-Meier and log-rank test were used for statistical analyses of differences in onset and survival between groups. For statistical analyses of differences in body weight, footprint, motor neuron cell count, and Western blotting we used analysis of variance and post hoc tests. The data are reported as means ± SD.

RESULTS

Measurement of the Levels of Rat HGF and c-Met Expression in Untreated Animals

Groups of 3 G93A Tg rats and non-Tg rats at 70, 100, and 130 days were used to measure the levels of rat HGF without any treatment. In the lumbar cords of untreated G93A Tg rats, the HGF concentrations increased with disease progression (Fig. 1A). At 70 days the level of rat HGF in the lumbar cords of G93A Tg rats was 4.05 ± 0.6 ng/mg and was the same as that of non-Tg rats. Increases of 35% and 107% were observed in the rat HGF level at 100 and 130 days, respectively, compared with non-Tg rats.

In addition, we measured the levels of c-Met mRNA in the lumbar spinal cords of Tg rats relative to non-Tg rats by real-time quantitative PCR. In the lumbar cords of G93A Tg rats the level of c-Met mRNA expression was the same as that in non-Tg rats at 70 days. However, a 55% increase in the level of c-Met mRNA expression compared with that of non-Tg rats was observed at 100 days and the higher level of expression was retained at 130 days (Fig. 1B).

Administration of hrHGF to 100-Day-Old G93A Tg Rats for 4 Weeks

To examine the efficacy of hrHGF on motor neurons in the spinal cords of Tg rats against onset of disease we administered 40 and 200 μg of hrHGF or vehicle alone to 100-day-old G93A Tg rats for 4 weeks (n = 8 in each group). Animals were killed at 130 days, and their lumbar spinal cords were examined. Because administration of hrHGF for more than 30 days may induce antibodies against hrHGF, we did not treat rats for longer than this period. We confirmed elevation of human HGF concentrations in the lumbar cords of hrHGF-treated rats using a specific sandwich immunoassay. The mean human HGF concentrations were 83.9 ± 25.1, 15.6 ± 5.4, and 0 ng/mg for rats treated with 200 μg of hrHGF, 40 μg of hrHGF, and vehicle, respectively (Fig. 2F). The endogenous rat HGF concentration is 4 to 5 ng/mg at this age (Fig. 1A). The human HGF concentration in the spinal cord of G93A Tg rats treated with 200 μg of hrHGF

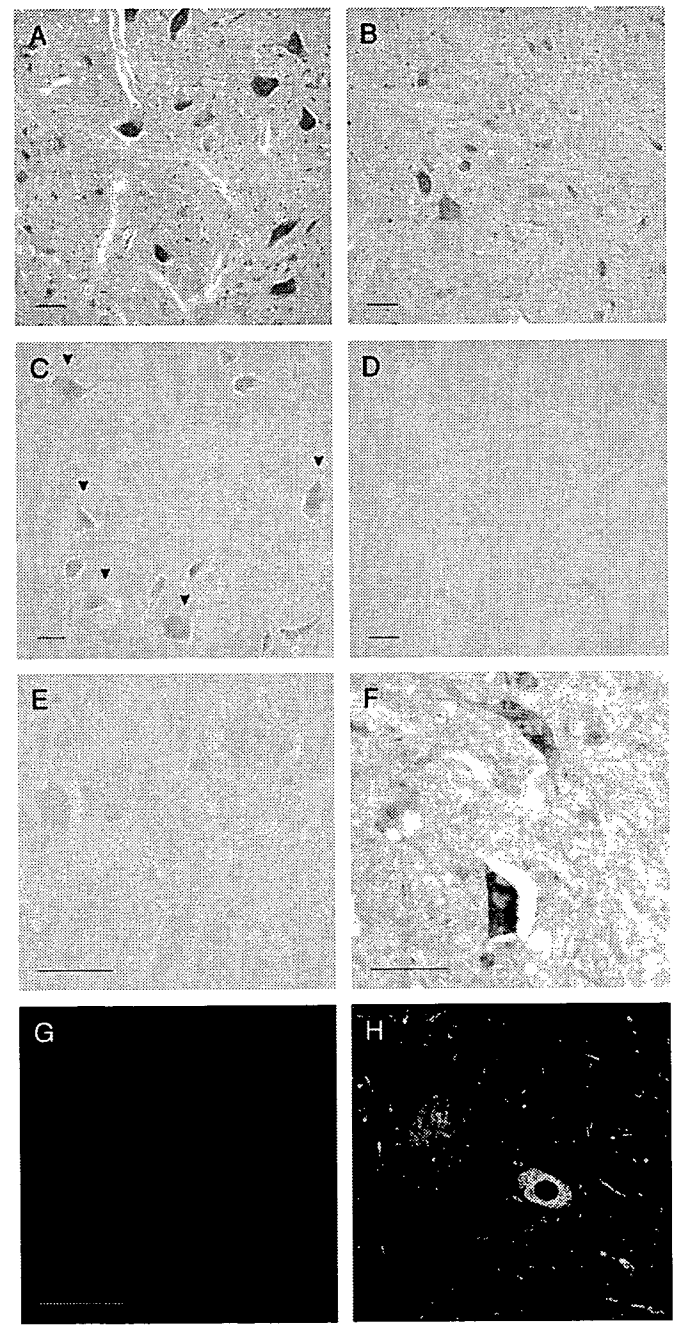

FIGURE 4. Sections of the fifth lumbar anterior horn from G93A transgenic (Tg) rats treated with human recombinant hepatocyte growth factor (hrHGF) **(A, C, E, G)** or vehicle **(B, D, F, H)** for 2 weeks starting at 115 days were stained with hematoxylin and eosin **(A, B)** and antibodies to phosphorylated c-Met **(C, D)**, activated caspase-3 **(E, F)**, and activated caspase-9 **(G, H)**. Scale bar = 50 μm. There were larger numbers of remaining large motor neurons in hrHGF-treated G93A Tg rats (6.7 ± 1.6) **(A)** than in vehicle-treated G93A Tg rats (2.3 ± 0.9) **(B)**. Phosphorylated c-Met staining was more distinct in hrHGF-treated G93A Tg rats **(C)** than in vehicle-treated G93A Tg rats **(D)**. In contrast, activated caspase-3 staining was stronger in vehicle-treated G93A Tg rats **(F)** than in hrHGF-treated G93A Tg rats **(E)**. Activated caspase-9 staining was detectable in vehicle-treated G93A Tg rats **(H)** compared with little reactivity in hrHGF-treated G93A Tg rats **(G)**.

was increased by approximately 20-fold relative to the endogenous rat HGF. All vehicle-treated G93A Tg rats developed weakness in the hindlimbs with a mean onset of 118.8 ± 4.3 days. Seven of 8 G93A Tg rats treated with 40 μg of rhHGF developed the disease before 130 days. In contrast, only 3 of 8 animals treated with 200 μg of rhHGF developed paralysis before this stage. At 130 days the average numbers of motor neurons in the ventral horn were as follows: non-Tg rats, 19.2 ± 3.3; vehicle only, 2.9 ± 1.3; 40 μg of hrHGF, 6.3 ± 2.1; and 200 μg of hrHGF, 11.2 ± 4.2. Significantly more motor neurons survived in hrHGF-treated (40 μg, $p < 0.01$; 200 μg, $p < 0.001$) than in vehicle-treated G93A Tg rats (Fig. 2A–E). hrHGF prevented motor neuron death in G93A Tg rats in a dose-dependent manner.

Administration of hrHGF to 115-Day-Old G93A Tg Rats for 4 Weeks

We next examined the therapeutic potential of HGF when administration was started at around the age of onset of paralysis. We administered 200 μg of hrHGF or vehicle alone to 115-day-old G93A Tg rats for 4 weeks. There were no statistically significant differences ($p = 0.6346$) in onset between the groups (200 μg of hrHGF, 126.8 ± 13.1 days; vehicle, 126.3 ± 13.8 days) (Fig. 3A, dotted lines). In contrast, 200 μg of hrHGF extended mean survival by 11 days compared with vehicle-treated G93A Tg rats ($p = 0.0135$) (Fig. 3A, solid lines), although G93A Tg rats showed very rapid disease progression and died within 20 days of disease onset. The average periods from the onset to death were 16.9 ± 8.17 and 27.5 ± 11.1 days in vehicle ($n = 8$) and hrHGF ($n = 8$) groups, respectively. The latter represented an increase of 62.7% relative to vehicle-treated controls. Footprint analysis of stride length in 200 μg of hrHGF-treated G93A Tg rats showed significant improvement compared with vehicle-treated G93A Tg rats at 118 days ($p = 0.0424$) (Fig. 3B). Thus, despite the very rapid disease progression in this model and short treatment period of 4 weeks, hrHGF treatment improved motor performance and prolonged survival even with treatment beginning around the onset of paralysis.

Histologic evaluation of the lumbar spinal cord indicated that hrHGF treatment prevented the pathologic changes typical of Tg rats. Two weeks after commencement of administration at 129 days, vehicle-treated rats showed substantial loss of motor neurons (2.3 ± 0.9) compared with hrHGF-treated rats (6.6 ± 1.6) (Figs. 3C, 4A, B). A significantly larger number of motor neurons survived in hrHGF-treated G93A Tg rats than in vehicle-treated G93A Tg rats ($p = 0.002$). Histologic evaluation of the lumbar spinal cord revealed much greater numbers of phosphorylated c-Met-positive cells (which were presumed to be motor neurons because of their large size, multipolar form, and localization in the anterior horn of the spinal cord) in hrHGF-treated G93A Tg rats compared with vehicle-treated G93A Tg rats at 2 weeks after the start of administration at 129 days (Fig. 4C, D). These observations indicated that the administered hrHGF was used in the spinal cord in G93A Tg rats. Consistent with the observation that apoptosis is involved in the pathogenesis of ALS (29–32), immunohistochemical analyses indicated large numbers of cells positive for activated caspase-3 and caspase-9 in vehicle-treated rats (Fig. 4F, H), compared with little or no reactivity in hrHGF-treated rats (Fig. 4E, G). To assess the mechanisms of suppression of caspase-3 and caspase-9 activation in hrHGF-treated rats, we next examined the level of XIAP by Western blotting, as XIAP inhibits activation of these pro-caspases and its levels are decreased in ALS mice (31). Western blotting analysis revealed increased XIAP expression

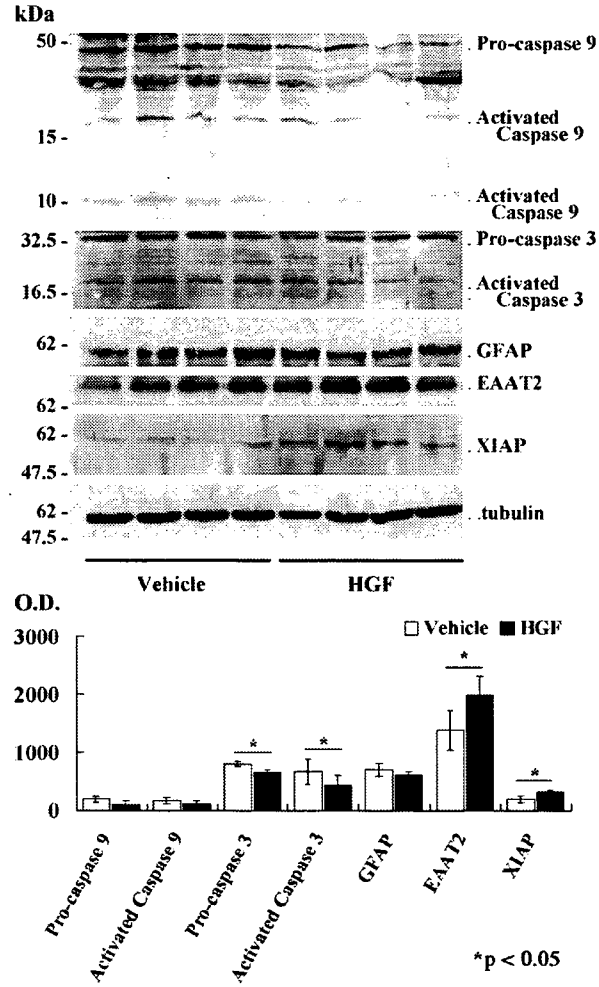

FIGURE 5. Caspase-3 and -9, glial fibrillary acidic protein (GFAP), excitatory amino acid transporter 2 (EAAT2), X-linked inhibitor of apoptosis protein (XIAP), and β-tubulin expression in the lumbar spinal cord. Western blotting of lumbar spinal cord lysates from G93A transgenic (Tg) rats treated with 100 μg of human recombinant hepatocyte growth factor (hrHGF) or vehicle for 2 weeks from 115 days. Western blotting analysis revealed increased levels of EAAT2 and XIAP expression in the spinal cords of hrHGF-treated G93A Tg rats compared with vehicle-treated G93A Tg rats (XIAP, $p = 0.0099$; EAAT2, $p = 0.0417$; $n = 4$). On the other hand, activated caspase-3 and -9 expression levels were decreased in hrHGF-treated G93A Tg rats. There were significant differences in caspase-3 expression between hrHGF- and vehicle-treated G93A Tg rats (pro-caspase-3, $p = 0.0031$; activated caspase-3, 0.0154; $n = 4$). GFAP expression was equivalent in both groups.

in the spinal cord of G93A Tg rats, and the increase in hrHGF-treated rats was only 60% of that in vehicle-treated G93A Tg rats. On the other hand, activated caspase-3 and 9 levels were decreased in hrHGF-treated G93A Tg rats ($p = 0.0154$ and $p = 0.2364$, 75% and 69% of vehicle-treated G93A Tg rats, respectively). These were all considered to be effects of HGF on motor neurons. Finally, we examined whether HGF improves the function of other cell types, such as astrocytes. There was a 60% increase in glial-specific glutamate transporter (EAAT2) in hrHGF-treated rats compared with vehicle-treated controls, although there was little difference in GFAP expression levels between the 2 groups (Fig. 5).

DISCUSSION

In this study, we demonstrated dose-dependent effects of hrHGF on motor neurons in the G93A Tg rat model of ALS, with administration starting at 100 days. Furthermore, we showed that hrHGF retards disease progression in this animal model treated from 115 days at the time of disease onset. There have been many studies of possible treatments in a mouse model of ALS (33, 34), but few agents have been shown to prolong survival with administration starting around disease onset (6–12). In this study, recombinant hrHGF retarded disease development even with administration beginning around the age onset of paralysis. Here, we showed the therapeutic effects of intrathecal delivery of a neurotrophic factor as a protein, rather than a transgene, on ALS beginning at the onset of paralysis. The average survival period of hrHGF-treated rats was 62.7% longer than that of vehicle-treated controls, comparable with the improved survival obtained by viral delivery of insulin-like growth factor-1 (6). We defined the appearance of paralysis as disease onset, although this is not a sensitive indicator and appears later than the decrease in activity (10). However, the appearance of paralysis is a clinically relevant marker of disease onset because it is closer to the state at which patients will be diagnosed with the disease.

We confirmed elevation of the human HGF concentration in the lumbar cords of hrHGF-treated G93A Tg rats using a specific sandwich immunoassay. Histologic evaluation of the lumbar spinal cord revealed greater numbers of phosphorylated c-Met-positive motor neurons in hrHGF-treated G93A Tg rats. This finding suggested that HGF receptors of motor neurons were activated well by administered hrHGF (35). These observations indicated that the administered hrHGF penetrated into the spinal cord and was utilized in the motor neurons of spinal cord. Previous studies demonstrated that many trophic factors have protective effects on motor neurons. In human trials of neurotrophic factors, such as brain-derived neurotrophic factors, glial cell line-derived neurotrophic factor, and insulin-like growth factor-1, the delivery (accessibility) of the protein to the motor neurons and glia in the spinal cord has been argued to be essential. Our results confirmed that chronic intrathecal administration with implanted infusion pumps supplied appropriate therapeutic doses to spinal cord motor neurons.

The HGF concentrations in cerebrospinal fluid are increased in many neurologic disorders, including ALS (26). In G93A Tg rats, the level of endogenous HGF in the spinal cord showed significantly greater elevation when the pathologic changes began in the spinal cord and increased with progression of the disease compared with the level of endogenous HGF in the spinal cord of non-Tg rats. After onset, the level of endogenous HGF almost doubled relative to that in non-Tg rats (Fig. 1A). These results were compatible to observations in patients with sporadic as well as familial ALS (36, 37). The level of c-met RNA expression in the lumbar cord of G93A rats increased to 155% of the normal level from before onset, and this elevated expression was retained after onset of disease (Fig. 1B). Kato et al (36) demonstrated that autocrine and paracrine trophic support of the HGF-c-met system contributes to attenuation of the degeneration of residual spinal cord motor neurons in ALS, whereas disruption of the HGF-c-met system at an advanced stage of disease accelerates cellular degeneration (37). Administration of hrHGF delayed the pathologic changes in G93A Tg rats. This effect of HGF may be due to replenishment of the relative insufficiency of HGF in G93A Tg rats in the present study.

Consistent with the findings that apoptosis is involved in ALS (29–31), large numbers of cells immunopositive for activated caspase-3 and -9 were observed in vehicle-treated animals in contrast to little or no reactivity in hrHGF-treated rats. This result was verified by quantitative Western blotting analysis, which indicated that HGF could block caspase activation of apoptosis. Caspase-3 and -9 are the main factors involved in execution of the caspase cascade. The survival-prolonging effect of HGF may be explained by suppression of induction and activation of caspase-9, as this enzyme is involved in determining disease duration (31). These observations suggest that the mechanism of the therapeutic effect of HGF in G93A Tg rats includes inhibition of the caspase cascade or of the cell death mechanism preceding the caspase cascade. In addition, EAAT2 and XIAP expression levels were increased in the hrHGF-treated group compared with vehicle-treated controls, indicating that HGF affected not only motor neurons via inhibition of the caspase cascade but also other cell types, such as astrocytes, which support motor neurons by maintaining or reinforcing internal cell protective functions, such as EAAT2 and XIAP.

Our results demonstrate pathologic improvements and retarded progression of ALS in G93A Tg rats by intrathecal administration of hrHGF from around the time of disease onset. Because HGF and c-Met are thought to be regulated in cases of not only familial but also sporadic ALS in a manner similar to the Tg mouse model of ALS (36), our findings suggest the possibility of clinical use of HGF in both familial and sporadic ALS. The results indicating the efficiency of hrHGF administration even from the onset of paralysis should prompt further clinical trials in ALS.

ACKNOWLEDGMENT

We thank Rieko Kamii for technical assistance.

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BRAIN RESEARCH 1111 (2006) 187–195

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Research Report

Adenoviral gene transfer of hepatocyte growth factor prevents death of injured adult motoneurons after peripheral nerve avulsion

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ARTICLE INFO

Article history:
Accepted 27 June 2006
Available online 1 August 2006

Keywords:
Adult rat
Avulsion
Facial nerve
Hepatocyte growth factor
Motoneuron
Spinal nerve

ABSTRACT

Hepatocyte growth factor (HGF) exhibits strong neurotrophic activities on motoneurons both in vitro and in vivo. We examined survival-promoting effects of an adenoviral vector encoding human HGF (AxCAhHGF) on injured adult rat motoneurons after peripheral nerve avulsion. The production of HGF in COS1 cells infected with AxCAhHGF and its bioactivity were confirmed by ELISA, Western blot and Madin-Darby canine kidney (MDCK) cell scatter assay. The facial nerve or the seventh cervical segment (C7) ventral and dorsal roots of 3-month-old Fischer 344 male rats were then avulsed and removed from the stylomastoid or vertebral foramen, respectively, and AxCAhHGF, AxCALacZ (adenovirus encoding β-galactosidase gene) or phosphate-buffered saline (PBS) was inoculated in the lesioned foramen. Treatment with AxCAhHGF after avulsion significantly prevented the loss of injured facial and C7 ventral motoneurons as compared to AxCALacZ or PBS treatment and ameliorated choline acetyltransferase immunoreactivity in these neurons. These results indicate that HGF may prevent the degeneration of motoneurons in adult humans with motoneuron injury and motor neuron diseases.

1. Introduction

Hepatocyte growth factor (HGF) was initially identified and purified as a potent mitogen of primary cultured hepatocytes (Nakamura et al., 1984, 1989). HGF is a heterodimeric protein composed of α and β chains and induces proliferation, migration, differentiation of target cells as well as organogenesis and neovascularization (Funakoshi and Nakamura, 2003). In the nervous system, HGF exhibits strong neurotrophic activities for motoneurons both in vitro and in vivo (Caton et al., 2000; Ebens et al., 1996; Funakoshi and Nakamura, 2003; Honda et al., 1995; Koyama et al., 2003, Maina and Klein, 1999; Naeem et al., 2002; Novak et al., 2000; Okura et al., 1999; Sun et al., 2002; Wong et al., 1997; Yamamoto et al., 1997). There have

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doi:10.1016/j.brainres.2006.06.104

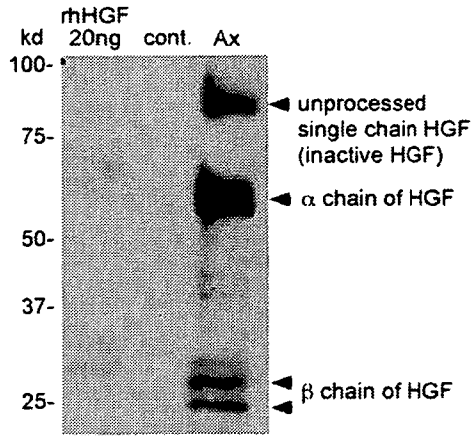

Fig. 1 – Western blot analysis of conditioned media (CMs) obtained from COS1 cells uninfected (cont.) or infected (Ax) with AxCAhHGF. The CMs harvested at 3 days after infection were concentrated by heparin beads, electrophoresed, blotted and immunolabeled for HGF as described in the text.

been no reports, however, concerning the neurotrophic effects of HGF on adult motoneuron death after proximal nerve injury. In animal models of adult motoneuron injury, avulsion of cranial and spinal nerves causes marked motoneuron degeneration in adult rats (Koliatsos et al., 1994; Moran and Graeber, 2004; Ruan et al., 1995; Sakamoto et al., 2000, 2003a, 2003b; Søreide, 1981; Watabe et al., 2000, 2005; Wu, 1993), so that these animal models can be useful for therapeutic evaluation of neurotrophic factors or neuroprotective molecules against adult motoneuron death (Ikeda et al., 2003; Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000, 2005). We have recently shown that adenoviral gene transfer of glial-cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), transforming growth factor-β2 (TGFβ2) and growth inhibitory factor (GIF)/metallothionein-III (MT-III) prevented the death of adult rat facial and spinal motoneurons after facial nerve and cervical spinal root avulsion (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). In the present study, we investigated whether HGF protects injured motoneurons after facial nerve or spinal root avulsion by using a recombinant adenoviral vector encoding human HGF.

2. Results

2.1. Bioassay of recombinant human HGF

In this study, we constructed a recombinant adenoviral vector encoding human HGF (AxCAhHGF). To test the ability of AxCAhHGF to induce human HGF expression in vitro, COS1 cells were infected with AxCAhHGF and the conditioned media (CMs) were harvested at 3 days postinfection. The levels of human HGF in uninfected and infected CMs analyzed by enzyme-linked immunosorbent assay (ELISA) were 1.9± 0.4 ng/ml and 2004.8±160 ng/ml, respectively ($n=3$). Western blot analysis of the CM harvested at 3 days postinfection showed immunoreactive bands of α-chain, β-chain and pro-HGF (inactive, unprocessed single chain precursor form) (Fig. 1). The CM obtained from uninfected COS1 cells did not show any immunoreactive bands. The Madin-Darby canine kidney (MDCK) cell scatter assay showed definite bioactivitiy of AxCAhHGF-infected COS1 CM; i.e., the activity of 1:500-diluted CM containing 4 ng/ml HGF as measured by ELISA corresponded to that of 2 ng/ml recombinant human HGF (rhHGF) that induced scattering of MDCK cells (Fig. 2).

2.2. Adenoviral-vector-mediated HGF gene expression in facial nuclei

We then examined the expression of adenovirus-mediated HGF in injured motoneurons after avulsion. We have pre-

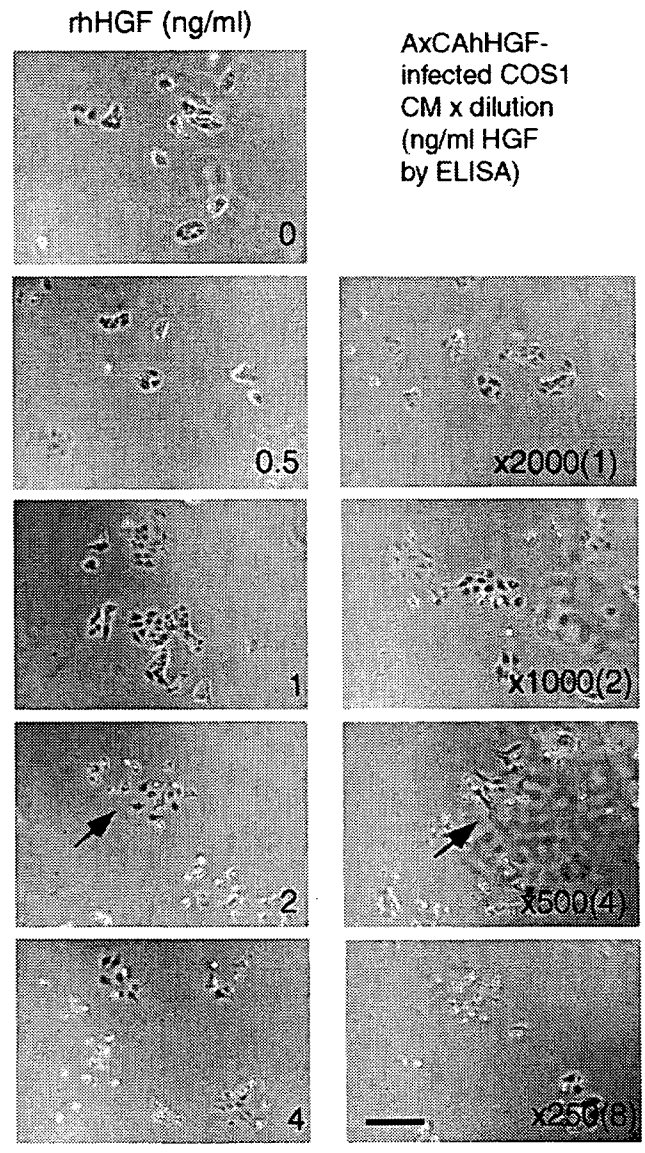

Fig. 2 – Madin-Darby canine kidney (MDCK) cell scatter assay for HGF bioactivity. MDCK cells were cultured in the presence or absence of AxCAhHGF-infected COS1 CM or rhHGF as described in the text. The activity of 1:500-diluted CM containing 4 ng/ml human HGF as measured by ELISA corresponds to that of 2 ng/ml recombinant human HGF that induced scattering of MDCK cells (arrows). Scale bar=50 μm.

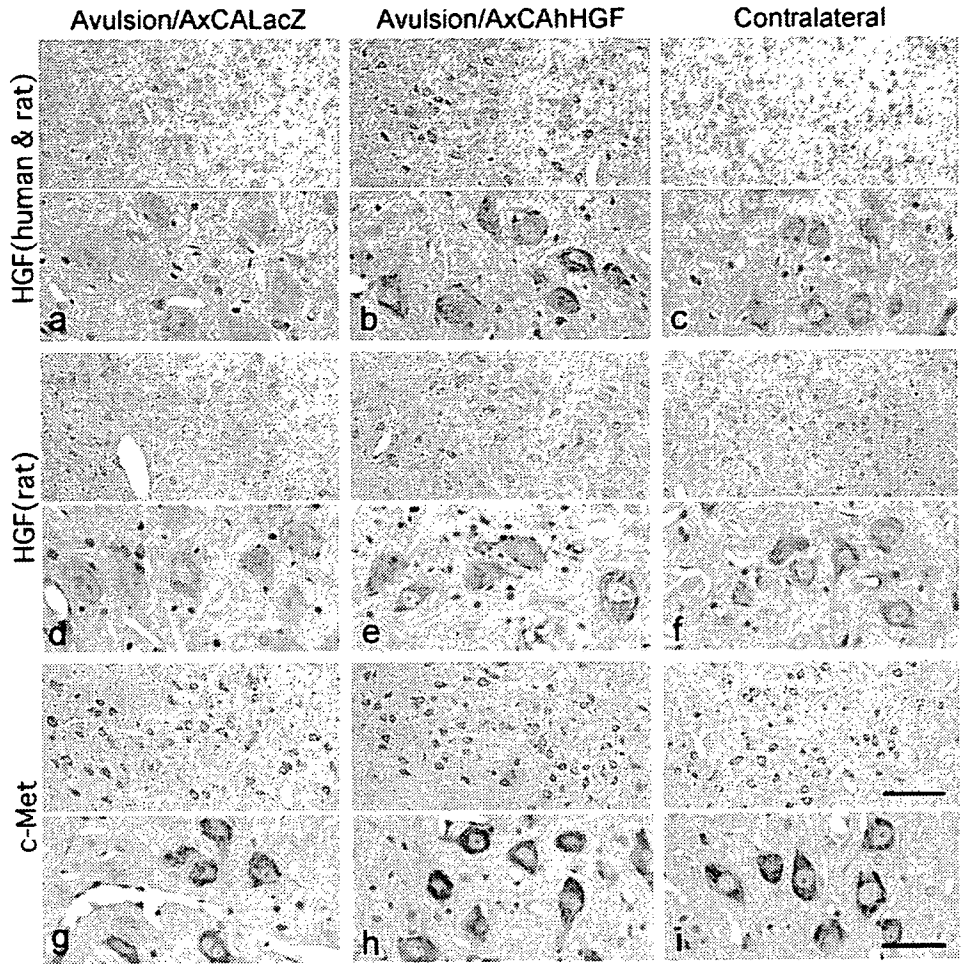

Fig. 3 – Low (top) and high (bottom)-magnified photomicrographs of immunohistochemistry of facial motoneurons at the ipsilateral (a, b, d, e, g, h) and contralateral (c, f, i) sides 7 days after facial nerve avulsion and the treatment with AxCALacZ (a, d, g) or AxCAhHGF (b, c, e, f, h, i) using antibodies against human and rat HGF (a–c), rat HGF (b–f) and c-Met (g–i). Counterstained with hematoxylin. Injured facial motoneurons after avulsion and AxCAhHGF treatment are more intensely immunolabeled by anti-human and rat HGF antibody (b) compared with injured motoneurons with AxCALacZ treatment (a) or contralateral intact motoneurons (c). Immunoreactivity of injured motoneurons treated with AxCALacZ (d) or AxCAhHGF (e) is comparable to that of contralateral intact motoneurons (f) when anti-rat HGF antibody was used. Immunoreactivity for c-Met is consistently demonstrated in both injured and contralateral motoneurons (g–i). Scale bars=200 μm (top), 50 μm (bottom).

viously demonstrated that injured motoneurons and their axons were labeled with X-Gal after facial or seventh cervical segment (C7) avulsion and inoculation of an adenovirus encoding bacterial β-galactosidase gene as a reporter (AxCA-LacZ) into lesioned stylomastoid or vertebral foramen, respectively (Sakamoto et al., 2000; Watabe et al., 2000). This indicates the diffusion of the virus through the facial canal or intervertebral foramen, its adsorption to injured axons, retrograde transport of the virus via intramedullary facial or spinal nerve tracts to soma of the motoneurons and

Table 1 – HGF protein levels in brain stem tissue containing facial nuclei after facial nerve avulsion and treatment with adenoviral vectors

Treatment (n=animal number)	Human HGF (ng/g)		Rat HGF (ng/g)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
AxCALacZ (n=3)	u.d.	u.d.	20.3±3.8	17.2±2.3
AxCAhHGF (n=3)	61.8±36.1	13.1±11.4	21.2±3.1	22.8±2.8

Seven days after facial nerve avulsion and the treatment with AxCALacZ or AxCAhHGF, the brain stem tissues containing facial nuclei (10–14 mg wet weight) were examined by human- and rat-specific HGF ELISA. u.d.=under the detection limit (<2.4 ng/g tissue).

successful induction of the virus-induced foreign gene in these neurons (Sakamoto et al., 2000, 2003a,b; Watabe et al., 2000). In the present study, 1 week after avulsion and treatment with AxCAhHGF, injured facial motoneurons were more intensely immunolabeled by an antibody that recognizes both human and rat HGF (Fig. 3b), compared with injured motoneurons treated with AxCALacZ (Fig. 3a) or uninjured motoneurons on the contralateral side (Fig. 3c). Immunoreactivity of injured motoneurons treated with AxCAhHGF (Fig. 3e), AxCALacZ (Fig. 3d) or phosphate-buffered saline (PBS) (not shown) was comparable to that of contralateral intact motoneurons (Fig. 3f) when an antibody that recognizes only rat HGF was used. These immunohistochemical results suggest that endogenous rat HGF was preserved in injured motoneurons after avulsion, while adenovirus-induced exogenous human HGF was successfully expressed in these neurons. Immunoreactivity for HGF receptor c-Met was consistently demonstrated in both ipsilateral and contralateral motoneurons after avulsion and AxCAhHGF or AxCALacZ treatment (Figs. 3g–i). No significant immunoreactivity for HGF and c-Met was observed in astrocytes, oligodendrocytes or microglia.

We further examined the expression of exogenous human HGF and endogenous rat HGF in brain stem tissue containing facial nuclei after facial nerve avulsion and adenovirus treatment by human-specific (Funakoshi and Nakamura, 2003) or rat-specific (Sun et al., 2002) ELISA (Table 1). Rat HGF levels measured by ELISA showed no significant difference between injured and contralateral sides. Human HGF levels were more than twofold compared with endogenous rat HGF levels after AxCAhHGF infection. Human HGF was also detectable in the tissues at the contralateral side after AxCAhHGF infection, which was considered to originate from injured and infected motoneurons at the ipsilateral side (Table 1).

One week after facial nerve avulsion and the treatment with AxCAhHGF, RT-PCR analysis showed that virus-induced human HGF mRNA transcripts were expressed in the brainstem tissue containing the facial nucleus on the ipsilateral, but not the contralateral side, whereas endogenous rat HGF

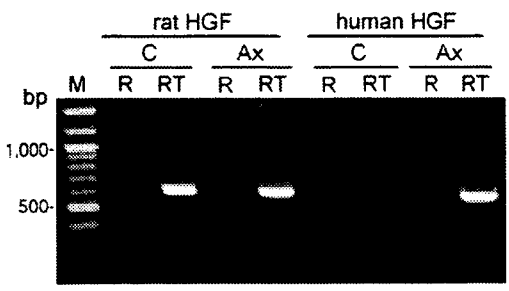

Fig. 4 – RT-PCR analysis of HGF mRNA transcripts in ipsilateral (Ax) and contralateral (C) sides of the brain stem tissue containing facial nuclei 7 days after facial nerve avulsion and AxCAhHGF treatment. The PCRs were performed on RNA without (R) or with (RT) reverse transcription. Primers that amplify rat or human HGF mRNA transcripts were used as described in the text. M=DNA size marker.

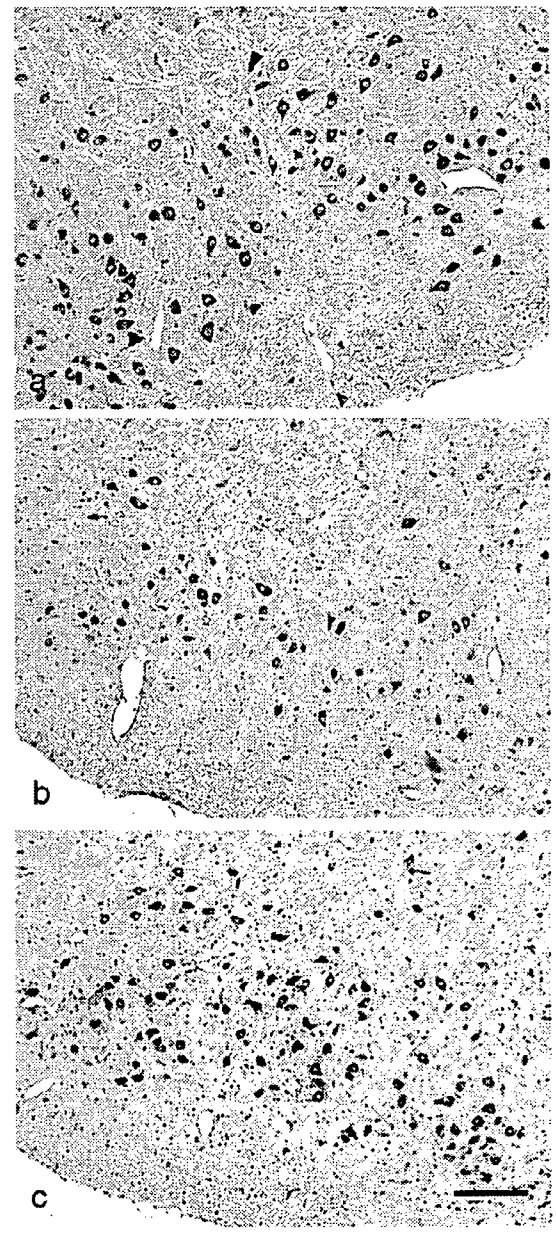

Fig. 5 – Photomicrographs of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=200 μm.

mRNA was consistently detected in the tissues on both ipsilateral and contralateral sides after avulsion (Fig. 4).

2.3. *Neuroprotective effects of HGF gene transfer*

Four weeks after facial nerve or C7 spinal root avulsion and treatment with phosphate-buffered saline (PBS) or AxCALacZ, the number of surviving facial or spinal motoneurons declined to 30–50% of that on the contralateral side as described previously (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). The treatment with AxCAhHGF prevented the loss of facial (58.8±5.9% survival) and spinal (75.4±4.4% survival)

motoneurons after avulsion compared with the treatment with PBS (30.2±6.7% survival of facial motoneurons; 44.6±9.3% survival of C7 motoneurons) or AxCALacZ (32.4±4.3% survival of facial motoneurons; 46.0±5.3% survival of C7 motoneurons) (Sakamoto et al., 2000) (Figs. 5, 6; Table 2). The treatment with AxCAhHGF after avulsion attenuated the decrease of choline acetyltransferase (ChAT) immunoreactivity in injured facial motoneurons compared with the treatment with PBS or AxCALacZ (Fig. 7). We found no perivascular or intrathecal lymphocytic/mononuclear cell infiltration in the facial nuclei and the spinal cord tissues that would be histologically defined and identified in case of immunogenic reaction against adenovirus infection (Figs. 5, 6).

Table 2 – Survival of motoneurons after facial nerve and spinal root avulsion and treatment with adenoviral vectors

Treatment (n=animal number)	Ipsilateral motoneuron number	Contralateral motoneuron number	% Survival
Facial nerve avulsion			
PBS (n=8)	213±41	712±38	30.2±6.7
AxCALacZ (n=4)	239±29	741±73	32.4±4.3
AxCAhHGF (n=7)	441±87*	745±38	58.8±5.9*
Spinal root avulsion			
PBS (n=4)	66±22	144±20	44.6±9.3
AxCALacZ (n=4)	69±9	150±12	46.0±5.3
AxCAhHGF (n=4)	108±15**	143±14	75.4±4.4**

Numbers of facial motoneurons and the percent survival at the ipsilateral (lesion) side relative to the contralateral (control) side 4 weeks after avulsion and treatment with phosphate-buffered saline (PBS), AxCALacZ and AxCAhHGF. Results are presented as the mean±SD. Statistical comparison was done by Mann–Whitney U test. *P<0.01 vs. PBS- and AxCALacZ-treated groups. **P<0.05 vs. PBS- and AxCALacZ-treated groups.

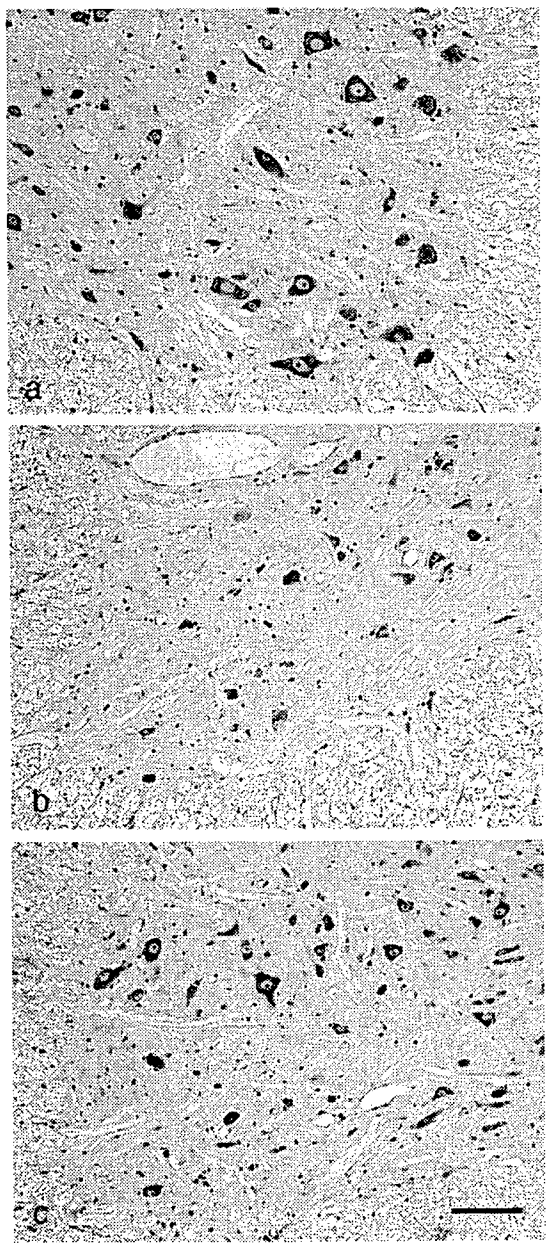

Fig. 6 – Photomicrographs of C7 spinal motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right C7 spinal nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=100 μm.

3. Discussion

HGF binds to tyrosine kinase receptor c-Met and triggers diverse biological responses that include cell motility, proliferation, morphogenesis, neurite extension and anti-apoptotic activities in a variety of cells (Funakoshi and Nakamura, 2003; Maina and Klein, 1999). Although the function of HGF in the nervous system has not been fully elucidated, it has recently been shown that HGF plays a strong neuroprotective

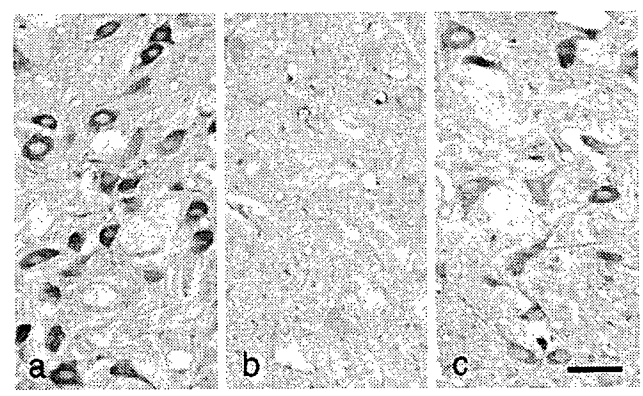

Fig. 7 – Photomicrographs of ChAT immunohistochemistry (a–c) of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 7 days after the facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Scale bar=50 μm.

role for motoneurons both in vitro and in vivo (Caton et al., 2000; Ebens et al., 1996; Honda et al., 1995; Koyama et al., 2003; Naeem et al., 2002; Novak et al., 2000; Okura et al., 1999; Sun et al., 2002; Wong et al., 1997; Yamamoto et al., 1997). It has been demonstrated that HGF-c-Met receptor coupling leads anti-apoptotic activities via MAP kinase (Hamanoue et al., 1996) and phosphatidylinositol-3 kinase/Akt (Hossain et al., 2002; Zhang et al., 2000) pathways and prevents caspase-1 and inducible nitric oxide synthase induction in motoneurons (Sun et al., 2002). In addition, HGF up-regulates the expression of excitatory amino acid transporter 2/glutamate transporter 1 (EAAT2/GLT1) in primary cultured astrocytes, which may improve glutamate clearance and reduce glutamate-mediated neurotoxicity (Sun et al., 2002).

In the present study, we investigated whether the treatment of AxCAhHGF can prevent the degeneration of motoneurons in adult rats after facial nerve and spinal root avulsion. We produced AxCAhHGF that induced bioactive HGF protein in infected COS1 cells in vitro as demonstrated by ELISA, Western blot analysis and MDCK scatter assay. Immunohistochemistry and RT-PCR results indicated that AxCAhHGF successfully infected injured motoneurons after facial nerve avulsion, suggesting the autocrine and paracrine neurotrophic effects of exogenous HGF on injured motoneurons after avulsion. Subsequently, we demonstrated that the treatment of AxCAhHGF delayed the loss of injured facial and spinal motoneurons. In addition, peripheral nerve avulsion as well as axotomy induces rapid decrease of ChAT immunoreactivity in injured motoneurons (Sakamoto et al., 2000; Watabe et al., 2000). In the present study, AxCAhHGF treatment after facial nerve avulsion improved ChAT immunoreactivity in injured motoneurons. We have previously shown that the treatments of recombinant adenoviral vectors encoding GDNF, BDNF, TGFβ2 and GIF promote the survival of motoneurons and attenuated ChAT immunoreactivity in the same avulsion model (Sakamoto et al., 2000, 2003a, 2003b; Watabe et al., 2000). Similarly, the present results clearly indicate that HGF have neuroprotective effects on injured adult motoneurons.

It has been reported that HGF mRNA is up-regulated in the spinal cord of human sporadic amyotrophic lateral sclerosis (ALS) (Jiang et al., 2005), and certain residual anterior horn cells in the spinal cord of ALS patients co-express both HGF and c-Met with the same or even stronger intensity compared with those of normal subjects (Kato et al., 2003). Transgenic mice expressing human mutant Cu/Zn superoxide dismutase (G93A mice) overexpressing HGF exhibited significant prolongation in survival and decreased motoneuron death compared with G93A mice with normal HGF expression (Sun et al., 2002). These reports indicate that HGF may have protective effects on motoneuron degeneration in ALS. Together with the present data, it is therefore conceivable that HGF may prevent the degeneration of motoneurons in adult patients with motoneuron injury and motor neuron diseases such as ALS.

In conclusion, we examined neuroprotective effects of HGF on injured adult motoneurons. The treatment of an adenoviral vector encoding HGF after facial nerve and spinal root avulsion significantly improved the survival of injured facial and spinal motoneurons and ameliorated ChAT immunoreactivity in these neurons. These results indicate that HGF may be a potential neuroprotective agent against motoneuron injury and motor neuron diseases in adult humans.

4. Experimental procedures

4.1. *Adenovirus preparation*

The human HGF cDNA was excised from pBS-hHGF with deletion of 15 base pairs (Seki et al., 1990) and subsequently cloned into *Swa*I cloning site of a cassette cosmid pAxCAwt (TaKaRa, Osaka, Japan) carrying an adenovirus type-5 genome lacking the E3, E1A and E1B regions to prevent the virus replication. The cosmid pAxCAwt contains the CAG (cytomegalovirus-enhancer-chicken β-actin hybrid) promoter on the 5′ end and a rabbit globin poly (A) sequence on the 3′ end. The cosmid was then cotransfected to 293 cells with the adenovirus genome lacking the E3 region (Miyake et al., 1996). A recombinant adenoviral vector encoding HGF (AxCAhHGF) was propagated and isolated from 293 cells and purified by two rounds of CsCl centrifugation. Generation of recombinant adenovirus containing bacterial β-galactosidase gene (AxCA-LacZ) has been described elsewhere (Kanegae et al., 1996).

4.2. *Analysis of HGF expression in COS1 cells infected with AxCAhHGF*

COS1 cells were infected with AxCAhHGF at a multiplicity of infection (moi) of 100 in serum-free Dulbecco's minimum essential medium (DMEM) (Invitrogen, Carlsbad, CA) for 1 h and incubated with serum-free DMEM in 5% CO_2 at 37 °C. The conditioned media (CMs) were harvested at 3 days postinfection for ELISA and Western blot analysis. The ELISA was performed as described (Sun et al., 2002; Funakoshi and Nakamura, 2003). For Western blot analysis, CM was treated with heparin beads to concentrate HGF and the CM or rhHGF (Nakamura et al., 1989; Seki et al., 1990) was electrophoresed on 4–20% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gels under reduced condition and transferred to PVDF membrane (Atto, Tokyo, Japan). The blotted membrane was then blocked with 3% skim milk and incubated overnight with rabbit anti-HGF (1:500; Tokusyu Meneki, Tokyo, Japan) followed by incubation with goat anti-rabbit IgG–HRP conjugate (1:1,000; DAKO, Glostrup, Denmark). Reactions were visualized by enhanced chemiluminescence detection using an ECL Western blotting detection kit (Amersham, Piscataway, NJ).

4.3. *Bioassay of adenoviral HGF; MDCK scatter assay*

MDCK cells cultured in DMEM with 10% fetal bovine serum (FBS) were trypsinized, seeded on 24-well plate (5000 cells/well) in the presence or absence of AxCAhHGF-infected COS1 CMs or rhHGF in DMEM with 5% FBS and incubated for 24 h at 37 °C. The cell scattering was viewed under a phase contrast microscope.

4.4. *Animals and surgical procedures*

The experimental protocols were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience.