to be decreased in aged persons, so we might have underestimated the decrease in rCBF in the ALS-D group.

The change observed was notable in the inferolateral premotor frontal cortex in 4 of the 5 ALS cases, this being consistent with the previous results on ^{123}I-IMP SPECT and visual inspection (4, 5). This result is also supported by a previous neuropathological study on ALS-D that showed involvement of the inferomedial premotor frontal cortex, which is known to play important roles in emotional control and intellectual function (10). Thus, the decreased rCBF in the bilateral frontal lobe may play a role in the cognitive dysfunction in this condition.

In some of our ALS patients, a subtle reduction of rCBF in some parts of the frontal lobes, but strangely not in the precentral motor cortex, was observed, but the region was relatively restricted and variable. Our results are contrary to the findings in some previous studies (2, 3, 5) that revealed bilateral frontal hypoperfusion in ALS on visual inspection. In this study, no rCBF reduction was seen in the bilateral motor cortices not only in ALS-D but also in ALS. This result might indicate that there is actually no rCBF reduction in ALS, or we also need to consider the limit of sensitivity of this method.

What should we think about the relation between ALS and ALS-D? Most ALS-D cases reported previously shared characteristic neuropathological findings such as motor neuronal degeneration and Bunina bodies with classic/sporadic ALS. From the standpoint that ALS is a disease with widespread involvement of not only the pyramidal tract but also other systems, ALS-D belongs to the same clinical entity as ALS. However, the pattern of rCBF reduction in the ALS-D group in this study is apparently different from that of ALS, the pattern of ALS-D resembling that of fronto-temporal dementia (FTD). The hyporperfusion in the ALS-D group in this study could lead us to think that ALS-D is one form of FTD. However, most ALS-D cases reported previously had characteristic neuropathological findings consistent with sporadic ALS, such as Bunina bodies. It is essential that ALS-D is defined by supporting neuropathological investigation. If dementia is ultimately superimposed on ALS, the same decreasing rCBF pattern as in ALS-D should be observed in ALS cases, especially in ones with a long history. Actually, the most prominent rCBF reduction was observed in the case with the longest duration of the disease (45 months) among the ALS-D cases (case 5 of ALS-D). However, none of the 16 ALS cases showed the same pattern as that in ALS-D.

Furthermore, it is interesting that the cortical hypoperfusion in the frontal lobe was observed in one ALS-D case before the development of dementia. Case 3 with ALS-D developed limb weakness as an initial symptom, and at 19 months after the onset, ^{123}I-IMP SPECT was performed. Although the reduction of rCBF in the frontal lobe was obvious, her intellectual impairment was negligible at that time and became markedly worse after the examination. This suggests that a reduction of frontal rCBF could precede clinically evident dementia.

Based on our 3D-SSP analysis, it is reasonable to suppose that the pathogenesis of ALS-D is different from that of ALS, and 3D-SSP analysis might have a high predictive value for the diagnosis of ALS-D even at the stage of cryptic dementia.

There is the possibility that ALS-D may be overlooked, because ALS patients with severe bulbar symptoms tend to have trouble in verbal communication. We should always consider possible dementia hidden behind ALS, and recommend a SPECT study for ALS patients with any subtle signs and symptoms suggesting dementia.

Conclusion

Using 3D-SSP, we have demonstrated that ALS-D patients have a significantly reduced rCBF in the bilateral premotor frontal lobes compared with controls and ALS patients. The present study indicated that SPECT with 3D-SSP can clearly and objectively distinguish ALS-D from ALS. This finding seems to be useful for the diagnosis of ALS-D even at an early stage of dementia. A decreased rCBF in the bilateral premotor frontal lobes may be associated with dementia in ALS-D patients and help us to recognize the pathogenesis of the disease.

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Hepatocyte growth factor promotes the number of PSD-95 clusters in young hippocampal neurons

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Abstract

Hepatocyte growth factor (HGF) and its receptor are expressed in various regions of the brain and have protective effects against excitotoxic injuries. However, their effects on synapse formation remain to be elucidated. To determine whether HGF has the ability to alter synaptic function during development, we investigated changes in the number of synapse detected by double immunostaining for NMDA receptor subunits and a presynaptic marker in cultured young hippocampal neurons. Whereas application of HGF increased the number of cluster of synapsin, a presynaptic protein, the clusters of NMDA receptor subunits NR1 and NR2B were not altered. Interestingly, colocalization of PSD-95, a scaffolding protein of the receptor, with synapsin was increased by HGF treatment without a change in the total amount of it. In addition, we investigated the expression of surface NMDA receptor, neuroligin, and neurexin, which were assessed by use of a cell-surface biotinylation assay. The application of HGF did not change the surface expression of these proteins. Furthermore, we determined the release of glutamate in response to depolarization. Treatment with HGF promoted depolarization-evoked release of glutamate. These results suggest that HGF modulates the expression of the scaffolding protein of the NMDA receptor at the synapse and promotes maturation of excitatory synapses in young hippocampal neurons.

Keywords: Hepatocyte growth factor; *N*-methyl-D-aspartate receptor; PSD-95

Introduction

The postsynaptic density (PSD), which underlies the postsynaptic membrane at excitatory synapses, links receptors of the neurotransmitter to intracellular signaling proteins and to the cytoskeleton (Kennedy, 1997; Kim and Sheng, 2004; Klauck and Scott, 1995; Ziff, 1997). The *N*-methyl-D-aspartate (NMDA) receptor, a glutamate-gated ion channel, is localized to the PSD at excitatory synapses in the brain and plays a pivotal role in the regulation of neuronal development and learning function. NMDA receptors are heteromeric complexes of NR1 and NR2A-NR2D or NR3 subunits (Das et al., 1998; Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991; Nakanishi, 1992; Nishi et al., 2001). Whereas NR1 is the principal subunit for the activity of the NMDA receptor, the NR2 subunits serve to modulate channel properties of these receptors (Hollmann and Heinemann, 1994). PSD-95, a major protein component of the PSD, interacts with NR2A and NR2B subunits and is thought to regulate channel activities, insertion, and internalization of the NMDA receptor (Lavezzari et al., 2004; Lin et al., 2004; Roche et al., 2001). As synaptic targeting and regulation of activities of NMDA receptors play an important role in synaptic plasticity, PSD-95 could be involved in the regulation of synaptic transmission. In this sense, neuroligin, a binding partner of PSD-95, connects the PSD to presynaptic terminals by binding between the extracellular domain of neuroligin and that of neurexin, a presynaptic binding partner (Dean and Dresbach, 2006). These findings suggest that pre- and

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postsynaptic protein complexes modulate structural and functional coordination at synapses in the brain. Neurotrophic factors are known to play an important role in the development of the central nervous system.

Hepatocyte growth factor (HGF), which was originally identified and cloned as a mitogen for hepatocytes (Nakamura et al., 1984, 1989), elicits diverse cellular responses such as mitogenic, motogenic, and morphogenic activities in various types of cells (Matsumoto and Nakamura, 1996; Zarnegar and Michalopoulos, 1995). Although HGF has physiological roles as acting as an organotropic for regeneration and protection of a variety of organs (Balkovetz and Lipschutz, 1999; Matsumoto and Nakamura, 1996, 2001; Zarnegar and Michalopoulos, 1995), exogenous HGF attenuates ischemia-induced injuries under pathophysiological conditions, including cardiac ischemia and reperfusion (Nakamura et al., 2000) and hind limb ischemia (Morishita et al., 1999; Van Belle et al., 1998). HGF and its receptor c-Met were found to be expressed in various regions of the brain and to function in a variety of ways in the central nervous system (Achim et al., 1997; Honda et al., 1995; Sun et al., 2002a,b). Therefore, HGF might have the ability to prevent brain injuries under pathophysiological conditions. Indeed, we previously demonstrated that administration of HGF prevented ischemic brain injuries and also improved learning and memory dysfunction of ischemic rats (Date et al., 2004; Niimura et al., 2006). Although overactivation of the NMDA receptor is associated with degenerative diseases (Dingledine et al., 1999), this receptor plays a key role in the regulation of neuronal development and learning function. Whereas HGF is likely to play an important role during brain development (Giacobini et al., 2007; Ohya et al., 2007), it is still not fully clarified whether HGF affects the development of excitatory synapses, including those bearing NMDA receptors. To determine the role of HGF in the regulation of synaptogenesis, we investigated the effect of HGF on synaptic clustering of NMDA receptor subunits and PSD-95 in cultured hippocampal young neurons. The results obtained show that HGF increased the number of PSD-95 clusters without changing the number of clusters and surface expression of NMDA receptor subunits. Furthermore, HGF promoted depolarization-evoked glutamate release.

Methods

Recombinant HGF

Human recombinant HGF was purified from conditioned medium of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA as described earlier (Nakamura et al., 1989). The purity of the human recombinant HGF was >98%, as determined by SDS–PAGE.

Primary hippocampal cultures

Primary hippocampal cell cultures were prepared from fetal rats at gestational day 18 as described previously (Huettner and Baughman, 1986), with slight modifications (Ishihara et al., 2005). The pooled hippocampi were dissociated by incubation at 37 °C for 30 min in Hank's balanced salt solution containing 15 U/mL papain, 210 U/mL deoxyribonuclease I, 1 mM L-cysteine, and 0.5 mM EDTA. The dispersed cells were resuspended in Dulbecco's Modified Eagle's Medium containing 10% horse serum and were plated at a density of 40,000 cells/cm^2 in 35-mm dishes coated with poly-L-lysine. At 24 h after plating, the medium was replaced with serum-free neurobasal medium containing 2% B27 supplements (Gibco-BRL, Rockville, MD, USA) and 0.5 mM glutamine. To inhibit proliferation of non-neuronal cells, we added cytosine arabinoside (1 μM) to each dish. At 3 days *in vitro* (DIV), one-half of the medium was replaced with fresh Neurobasal medium having the 2% B27 supplements and 0.5 mM glutamine. Cultures were maintained at 37 °C in a 5% CO_2 incubator. HGF (30 ng/mL) was added to cultured hippocampal cells at 4 DIV, and then the cultures were maintained for the next 3 days. The dose of used in the present study, 30 ng/mL, was based on the data of Korhonen et al. (2000), Ueda et al. (2001), and those obtained in our preliminary study: treatment with 10 ng/mL HGF increased tyrosine phosphorylation of c-Met protein at 4 DIV to a lesser degree than that with 30 ng/mL HGF.

For experiment on high K^+-evoked glutamate release, hippocampal cells were depolarized for 1 min at 37 °C with 10 mM HEPES buffer, pH 7.4, containing 60 mM KCl, 67 mM NaCl, 2 mM $CaCl_2$–$2H_2O$, 10 mM D-glucose. The collected samples were derivatized with *o*-phthalaldehyde and then injected into a high-performance liquid chromatograph (EICOM, Kyoto, Japan).

Immunohistochemistry

After cells had been fixed in −20 °C methanol, they were then incubated for 1 h at 37 °C or overnight at 4 °C with a primary antibody, and then with the secondary antibody for 1 h. They were then incubated with another primary antibody for 1 h at 37 °C and subsequently with the corresponding secondary antibody for 1 h at 37 °C. The primary antibodies used were mouse anti-NR1 (BD Biosciences), mouse anti-NR2B (BD Biosciences), mouse anti-PSD-95 (Affinity BioReagents), and rabbit anti-synapsin (Affinity BioReagents) antibodies. The secondary antibody used for anti-NR1, anti-NR2B, and anti-PSD-95 antibodies was Cy3-conjugated anti-mouse IgG (Amersham) antibody; and that for anti-synapsin was biotinylated anti-rabbit IgG (Vector Laboratories), which was visualized with Streptavidin FITC (Amersham). Images of cells were captured by a CCD camera (DP50) mounted on an Olympus BX52 microscope equipped with a mercury arc lamp. Images were processed by using Adobe Photoshop (Adobe Systems, Mountain View, CA). To count the number of clusters, we evaluated at least 1 dendrite (50-μm length) in each of 5 randomly selected cells in each of 5 separate cultures. The number of NR1-, NR2B-, PSD-95-, or synapsin I-positive clusters and synapsin I-positive clusters, which were colocalized with NR1, NR2B, or PSD-95, on basal dendritic segments were counted manually. The microscopic observations were performed by a person unaware of the study group.

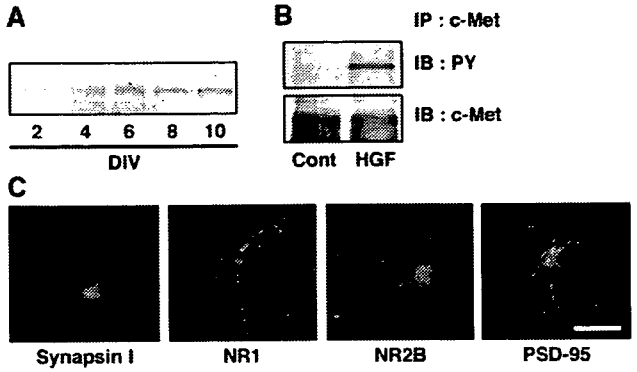

Fig. 1. (A) Proteins from hippocampal cells cultured for 2, 4, 6, 8 or 10 days *in vitro* were analyzed by immunoblotting with anti-c-Met antibody. (B) Proteins from cultured hippocampal cells at 0 (Cont) and 10 min after treatment with 30 ng/mL HGF were immunoprecipitated (IP) with anti-c-Met antibody, and the precipitates were then analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody (PY). The blots were stripped and then re-probed with antibody against c-Met. (C) Cultured hippocampal cells were fixed at 4 days *in vitro* and immunostained with anti-synapsin I, anti-NR1, anti-NR2B, or anti-PSD-95 antibodies. Scale bar represents 25 μm.

Cell-surface biotinylation assay

Cell-surface biotinylation assay of hippocampal neurons was performed as described previously (Shen et al., 2000) with minor modifications. Cultured hippocampal neurons were washed 3 times with ice-cold PBS, pH 7.4, containing 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$. Surface proteins were then biotinylated with 1.0 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) for 20 min in PBS at 4 °C. To remove unreacted sulfo-NHS-SS-biotin, we washed the cells 3 times with ice-cold 50 mM Tris/PBS, pH 7.4, containing 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 1.0 mM $CaCl_2$, and 0.5 mM $MgCl_2$. The cells were then lysed with ice-cold lysis buffer (10 mM sodium phosphate, pH 7.4, containing 100 mM NaCl, 0.2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μM PMSF, 5 μg/ml each of antipain, aprotinin, and leupeptin). For isolation of biotinylated proteins, we used UltraLink-immobilized neutravidin beads (Pierce). After incubation, the beads were washed and bound proteins were eluted with SDS sample buffer.

Western immunoblotting

Hippocampal cells were homogenized in ice-cold 0.32 M sucrose containing 0.2 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of antipain, aprotinin, and leupeptin. Samples were stored at −80 °C until used and were thawed only once. Proteins were solubilized by heating at 100 °C for 5 min in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, and 2% SDS in 62.5 mM Tris–HCl, pH 6.8) and were separated on polyacrylamide gels. Protein blots were reacted with the appropriate antibodies, and the bound antibody was detected by the enhanced chemiluminescence method (Amersham Biosciences Inc., Piscataway, NJ, USA). Quantification of the immunoreactive bands was performed by using an image analyzer (ATTO Co., Tokyo, Japan). Care was taken to ensure that bands to be semiquantified were in the linear range of response.

Antibodies used for immunoblotting were anti-c-Met (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-phosphotyrosine (clone 4G10, Upstate Biotechnology, Inc., Lake Placid, NY, USA), anti-NR1 (BD Biosciences, San Jose, CA), anti-NR2B (BD Biosciences), anti-PSD-95 (Affinity BioReagents), anti-GluR1 (Upstate Biotechnology), anti-synapsin (Affinity BioReagents), anti-neuroligin 1 (Synaptic Systems, Göttingen, Germany), and rabbit anti-neurexin (Calbiochem) antibodies.

Statistics

The results were expressed as the means±SEM. Statistical comparison between 2 groups was evaluated by using Student's *t*-test. Differences with a probability of 5% or less were considered to be significant ($p<0.05$).

Results

First, we examined the protein expression of HGF receptor c-Met in cultured hippocampal cells at 2, 4, 6, 8, and 10 DIV. In agreement with the results of previous studies (Hossain et al., 2002; Machide et al., 1998), c-Met protein (140 kDa) was expressed in the cultured hippocampal cells throughout the culture period (Fig. 1A). To determine whether c-Met proteins in the young hippocampal cells could be activated in response to the application of HGF, we examined the tyrosine phosphorylation of c-Met after HGF treatment. The tyrosine phosphorylation of c-Met was elevated relative to the initial amount at 10 min after the application of HGF without any change in the amount of total c-Met protein (Fig. 1B). By immunohistochemical analysis, we detected the expression of NMDA receptor subunits NR1 and NR2B, PSD-95, and the presynaptic marker synapsin I at 4 DIV (Fig. 1C). Attempts to examine clustering of NR2A subunits by using several different commercially available NR2A specific antibodies were not successful.

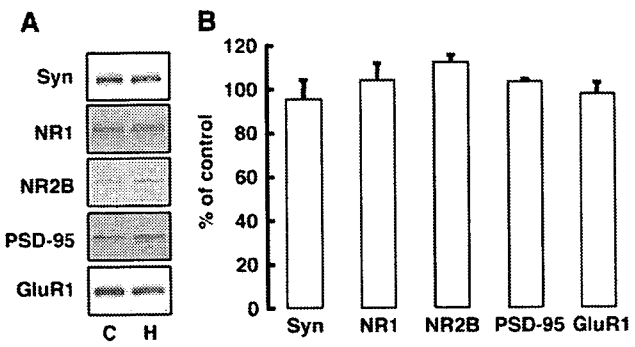

Fig. 2. (A) After 4 days in culture, hippocampal cells were incubated for 3 days with (H) or without (C) 30 ng/mL HGF. Total proteins were analyzed by immunoblotting with anti-synapsin I (Syn), anti-NR1, anti-NR2B, anti-PSD-95, or anti-GluR1 antibodies. (B) Bands corresponding to synapsin I, NR1, NR2B, PSD-95, and GluR1 on immunoblots were scanned. Comparison between HGF-treated (H) and -untreated (C) groups was done using scanned optical densities ($n=4$ each). Results are expressed as the average percentages of the HGF-untreated group±SEM of 4 separate cultures.

Next, we determined the effects of HGF on total amounts of synapsin I, NMDA receptor subunits, and PSD-95 at 7 DIV. As shown Fig. 2, total amounts of these proteins after the application of HGF were comparable to those under normal conditions. Total amount of GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) was also comparable to that under normal condition (Fig. 2).

Then, we examined the effects of HGF on clustering and synaptic localization of the NMDA receptor subunits and PSD-95 at 7 DIV. Whereas the number of clusters of presynaptic marker synapsin I was increased at 7 DIV ($F=1.017$, $p=0.036$, Fig. 3; $F=0.227$, $p=0.047$, Fig. 4; $F=0.893$, $p<0.0001$, Fig. 5), there was no change in the number of NR1 (Fig. 3) or NR2B (Fig. 4) subunit clusters. The number of double-positive immunofluorescent puncta containing NR1 (Fig. 3) or NR2B (Fig. 4) subunit and synapsin I after HGF treatment was comparable to that under normal conditions. In contrast, HGF increased the number of PSD-95 clusters ($F=0.617$, $p=0.002$, Fig. 5) and that of PSD-95 clusters colocalized with synapsin I (merge) at 7 DIV ($F=0.915$, $p<0.0001$, Fig. 5). We also examined the effect of HGF on clustering of GluR1 subunit of the AMPA receptor. GluR1 staining showed the diffuse pattern during the first week in culture. The finding was consistent with previous results (Rao et al., 1998). We could not show the data for GluR1 in the present study, as it was difficult to demonstrate the effect of HGF on distinct GluR1 clusters along the dendrites.

Furthermore, to examine the surface expression of the NMDA receptor, we performed a cell-surface biotinylation assay at 7 DIV. Treatment with HGF did not affect the surface expression of

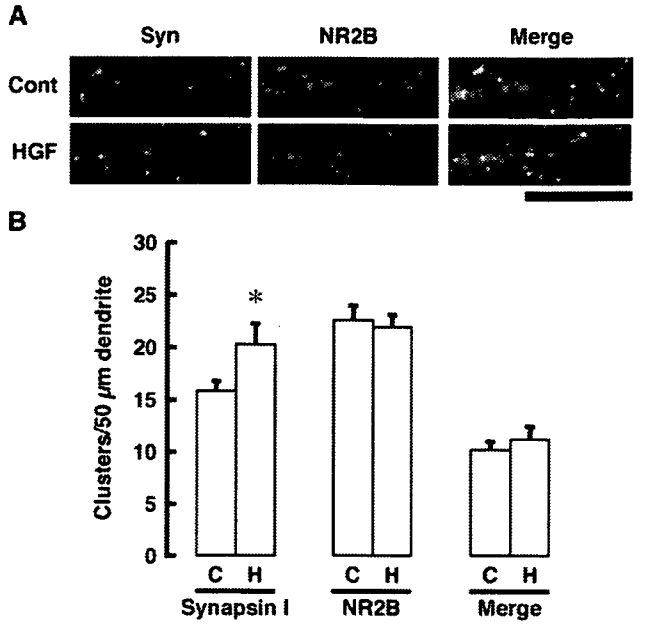

Fig. 4. (A) Hippocampal cells were cultured for 4 days and then treated (H) or not (C) with 30 ng/mL HGF for the following 3 days. The cells were fixed and double-immunostained with anti-synapsin I (for Syn) and anti-NR2B (for NR2B) antibodies. Images show magnified regions of dendritic segments. Scale bar represents 25 μm. (B) The number of clusters of synapsin I or NR2B and that double-positive for synapsin I/NR2B in a 50-μm dendrite length was counted. Results are expressed as the means±SEM of 5 separate cultures. *Significant difference from the control group ($p<0.05$).

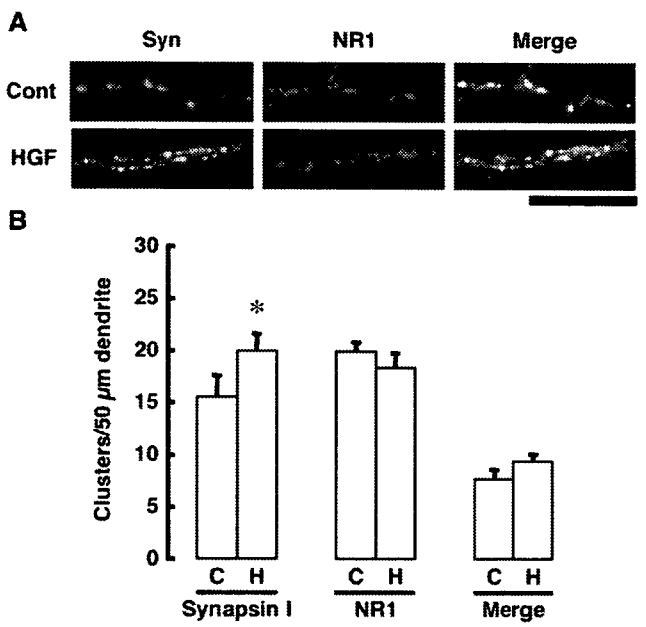

Fig. 3. (A) Hippocampal cells were cultured for 4 days and then treated (H) or not (C) with 30 ng/mL HGF for the following 3 days. The cells were fixed and double-immunostained with anti-synapsin I (for Syn) and anti-NR1 (for NR1) antibodies. Images show magnified regions of dendritic segments. Scale bar represents 25 μm. (B) The number of clusters of synapsin I or NR1 and that double-positive for synapsin I/NR1 in a 50-μm dendrite length was counted. Results are expressed as the means±SEM of 5 separate cultures. *Significant difference from the control group ($p<0.05$).

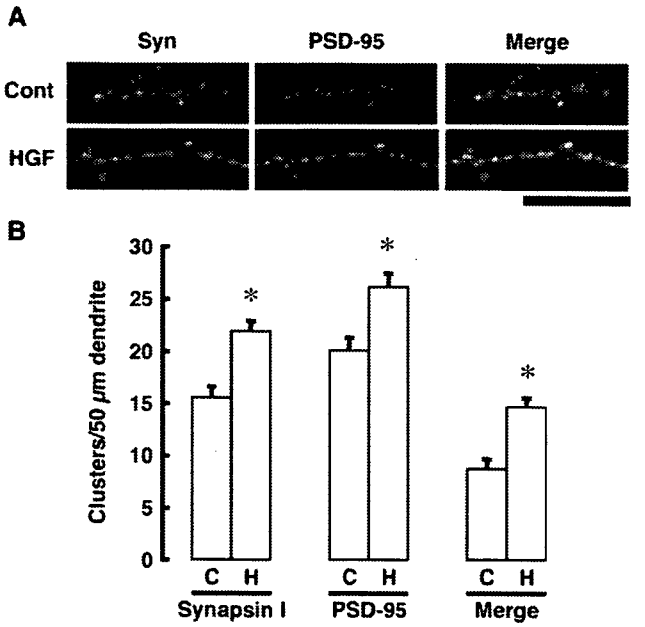

Fig. 5. (A) Hippocampal cells were cultured for 4 days and then treated (H) or not (C) with 30 ng/mL HGF for the following 3 days. The cells were fixed and double-immunostained with anti-synapsin I (for Syn) and anti-PSD-95 (for PSD-95) antibodies. Images show magnified regions of dendritic segments. Scale bar represents 25 μm. (B) The number of clusters of synapsin I or PSD-95 and that double-positive for synapsin I/PSD-95 in a 50-μm dendrite length was counted. Results are expressed as the means±SEM of 5 separate cultures. *Significant difference from the control group ($p<0.05$).

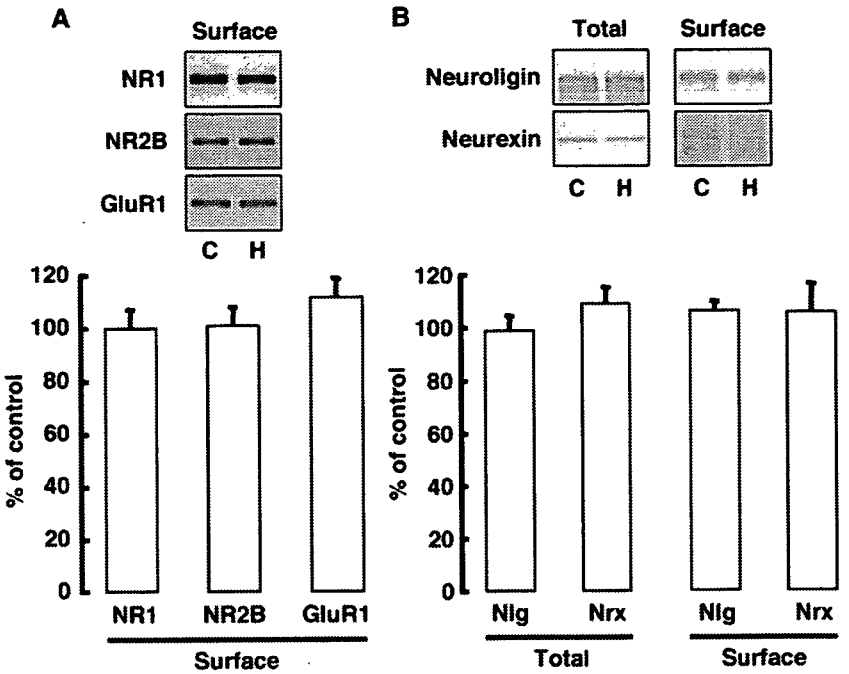

Fig. 6. Four-day hippocampal cultures were incubated in the presence (H) or absence (C) of 30 ng/mL HGF for 3 days. Cell-surface proteins were then labeled with membrane impermeable sulfo-NHS-SS-biotin. Biotinylated proteins were precipitated with avidin beads and analyzed by immunoblotting with anti-NR1, anti-NR2B, and anti-GluR1 antibodies (A) or with anti-neuroligin and anti-neurexin antibodies (B). Total proteins were analyzed by immunoblotting with anti-neuroligin and anti-neurexin antibodies (B). Bands corresponding to NR1, NR2B, and GluR1 (A) or neuroligin and neurexin (B) on immunoblots were scanned. Results are expressed as the average percentages of the control ± SEM of 4 separate cultures.

NMDA receptor subunits NR1 and NR2B (Fig. 6A). The surface expression of AMPA receptor subunit GluR1 was not affected by treatment with HGF (Fig. 6A). At 7 DIV, we further examined the effects of HGF on the expression of neuroligin, which is a binding partner of PSD-95 and connects the PSD to the presynaptic release machinery, and on that of neurexin, which is a presynaptic binding partner of neuroligin. There were no changes in total amounts of neuroligin and neurexin irrespective of HGF treatment (Fig. 6B). The surface expression of these proteins was also unaffected regardless of treatment or not with HGF (Fig. 6B).

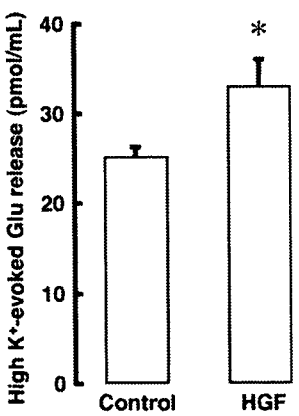

Fig. 7. Hippocampal cells were cultured in the presence (H) or absence (C) of 30 ng/mL HGF for 3 days and then stimulated by high K^+ (60 mM)-evoked depolarization. The amount of released glutamate was quantified by HPLC. Values represent the means ± SEM of 6 separate cultures. *Significant difference from the control group ($p<0.05$).

Although the total amount of synapsin I was not altered irrespective of the HGF treatment, the number of presynaptic clusters of synapsin I was increased. These findings raise the possibility that HGF would affect synaptic transmission. Next, to examine the effect of HGF on depolarization-evoked release of glutamate, we determined the amount of glutamate released into the medium following high-K^+ (60 mM) stimulation at 7 DIV. HGF treatment increased the amount of depolarization-evoked glutamate release relative to the amount released under normal conditions ($F=0.144$, $p=0.045$, Fig. 7).

Discussion

In the present study, we examined the effect of HGF on synapse formation in young hippocampal neurons by focussing excitatory synapses containing the NMDA receptor and PSD-95, which is a receptor-anchoring protein. First, the HGF receptor c-Met protein in cultured hippocampal neurons was detected at 2 DIV and was clearly identified from 4 DIV. Therefore, the treatment with HGF was started at 4 days, and the effect of HGF on synapse formation was examined 3 days later. We found that tyrosine phosphorylation of c-Met was induced by application of HGF, indicating that c-Met protein in cultured hippocampal neurons at 4 DIV could reveal the functional significance of HGF. The findings are consistent with the observations that the c-Met is expressed in developing hippocampus and plays a pivotal role for the maturation of neurons in the hippocampus (Achim et al., 1997; Honda et al., 1995; Jung et al., 1994; Korhonen et al., 2000; Thewke and Seeds, 1999).

Earlier it was demonstrated that brain-derived neurotrophic factor (BDNF) increased the expression level of presynaptic vesicle-associated proteins and promoted GABAergic maturation (Yamada et al., 2002). Other neurotrophic factors may also play a pivotal role in synapse formation during development. However, the effects of HGF on synaptogenesis in young neurons were still not fully understood. We demonstrated that treatment with HGF increased the number of clusters of the presynaptic marker protein synapsin I without causing a change in the total amount of this protein. In contrast, HGF had no effect on the protein expression levels of NMDA receptor subunits NR1 and NR2B or PSD-95. We demonstrated that the number of double-positive immunofluorescent puncta containing synapsin and NR1 or NR2B was not altered by HGF treatment. Furthermore, we demonstrated that the surface expression of NMDA receptor subunits was unaffected irrespective of HGF treatment. These results suggest that HGF had no effect on the number of synapses containing the NMDA receptor in young hippocampal neurons. Interestingly, we found that treatment with HGF increased the number of double-positive immunofluorescent puncta containing synapsin and PSD-95. The finding that the total amount of PSD-95 after the application of HGF was not altered suggests that PSD-95 was translocated to synaptic sites by HGF treatment. The binding of PSD-95 to NR2 subunits has been implicated in not only the localization and anchoring of receptors but also the regulation of ion channel function, synaptic activity, and intracellular signaling (Kim and Sheng, 2004; Migaud et al., 1998; Sattler et al., 1999; Yamada et al., 1999). Therefore, HGF-mediated recruitment of PSD-95 may be involved in structural and functional maturation of synapses containing the NMDA receptor during development.

As it is known that PSD-95 enhances neuroligin-1 clustering and maturation of excitatory synapses at the expense of inhibitory contacts (Prange et al., 2004), we next examined the cell-surface expression of neuroligin and that of neurexin, which is a presynaptic protein that interacts trans-synaptically with postsynaptic neuroligin. We demonstrated that HGF did not alter the surface expression of either molecule. Postsynaptic differentiation is likely to occur prior to formation of a functional active zone at the presynapse in young hippocampal neurons (Gerrow et al., 2006). Furthermore, overexpression of postsynaptic PSD-95 in hippocampal neurons causes maturation of glutamatergic synapses (El-Husseini et al., 2000). Therefore, HGF might promote the distribution of PSD-95 to the postsynaptic membrane for neuronal development, although further studies will be required to determine whether HGF is involved in the development of excitatory synapses. HGF enhances endothelial junctional integrity by increasing the availability of β-catenin (Liu et al., 2002). Interestingly, a synaptic scaffolding molecule is localized at synapses by the action of β-catenin and recruits neuroligin and PSD-95 (Iida et al., 2004).

In addition, a postsynaptic complex of scaffolding protein PSD-95 and neuroligin is likely to modulate the presynaptic release probability of transmitter vesicles (Futai et al., 2007). In the present study, HGF increased the number of clusters of the presynaptic marker synapsin I. Therefore, HGF may promote functional synaptogenesis by recruiting PSD-95 to the postsynaptic membrane, although the number of double-positive immunofluorescent puncta containing synapsin I and NMDA receptor subunit and the expression of surface neuroligin and neurexin were not affected. We demonstrated that depolarization-evoked release of glutamate was enhanced by HGF treatment. Interestingly, the glutamate concentration at the synaptic cleft is likely to be higher when PSD-95 is postsynaptically over-expressed (Futai et al., 2007). The reason for such a higher glutamate concentration is suggested to be the release of more vesicles at synapses; although other possibilities, such as a decrease in the exclusion of released glutamate from the extracellular spaces, cannot be ruled out. Under pathophysiological conditions, such as cerebral ischemia, excessive glutamate release was occurred and induced neuronal injuries. In this sense, HGF exerts protective effects against excitotoxic injuries *in vitro* and *in vivo*. In contrast, glutamate plays an important role in neuronal development (McDonald and Johnston, 1990). Therefore, depolarization-evoked glutamate release promoted by HGF treatment in young hippocampal neurons may be involved in the regulation of neuronal development, but not in the process of excitotoxicity.

Pre- and postsynaptic functions mediated by PSD-95 and related proteins may play a key role in neural development and synaptic plasticity. In this sense, the expression of PSD-95 and neuroligin is increased during the development of the hippocampus (Petralia et al., 2005; Song et al., 1999). Furthermore, PSD-95 is acutely translocated to the postsynaptic contact in response to neuronal activity in the visual cortex (Song et al., 1999).

Earlier it was shown that HGF enhanced synaptic long-term potentiation in the hippocampal CA1 region and also augmented NMDA receptor-mediated currents (Akimoto et al., 2004). Therefore, HGF may modulate not only the release of neurotransmitters but also postsynaptic functions via activation of certain enzymes during the development in young hippocampal neurons.

It was suggested that, in addition to PSD-95, a preformed complex containing guanylate kinase-associated protein (GKAP), SH3, and an ankyrin repeat-containing protein (Shank) might recruit a number of proteins required for maturation of excitatory synapse (Gerrow et al., 2006). Therefore, we cannot rule out the possibility that postsynaptic proteins other than PSD-95 were involved in the synaptogenesis induced by HGF treatment.

Although the mechanisms for synaptic recruitment of PSD-95 after the treatment with HGF are still unknown, the results in the present study suggest that HGF initially regulates the localization of PSD-95, subsequently promotes depolarization-evoked excitatory neurotransmission during development. Because PSD-95 appears to be important in controlling synaptic plasticity and learning (Migaud et al., 1998), HGF may modulate the ability for synaptic maturation and function.

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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)^{dl}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 preincubation 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

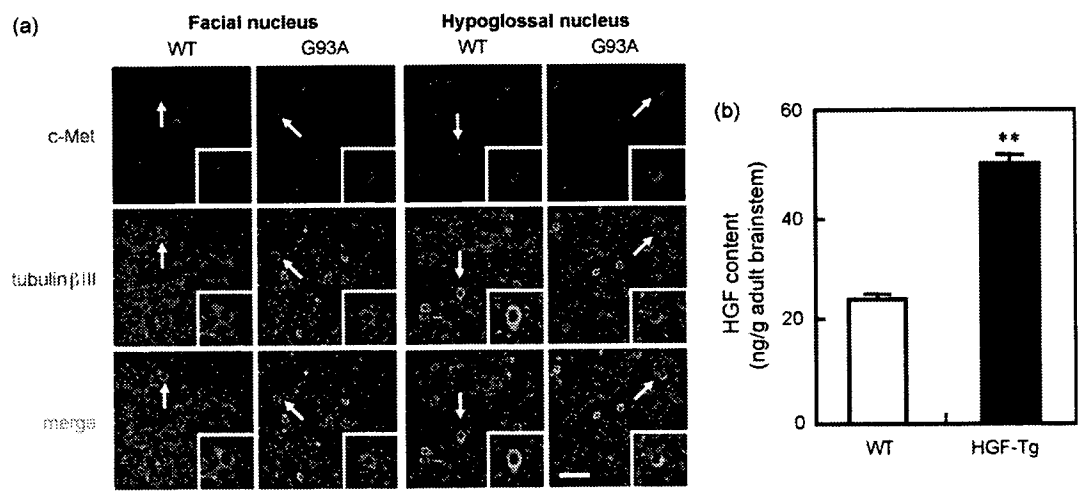

Fig. 1. Expression of c-Met and HGF in the brainstem. (a) Double-immunofluorescence analysis for c-Met (red) and tubulinßIII (green; a marker for neurons) in the facial and hypoglossal nuclei of WT and G93A mice at 8 months of age. A high-magnification view of the area indicated by the arrow is also 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.

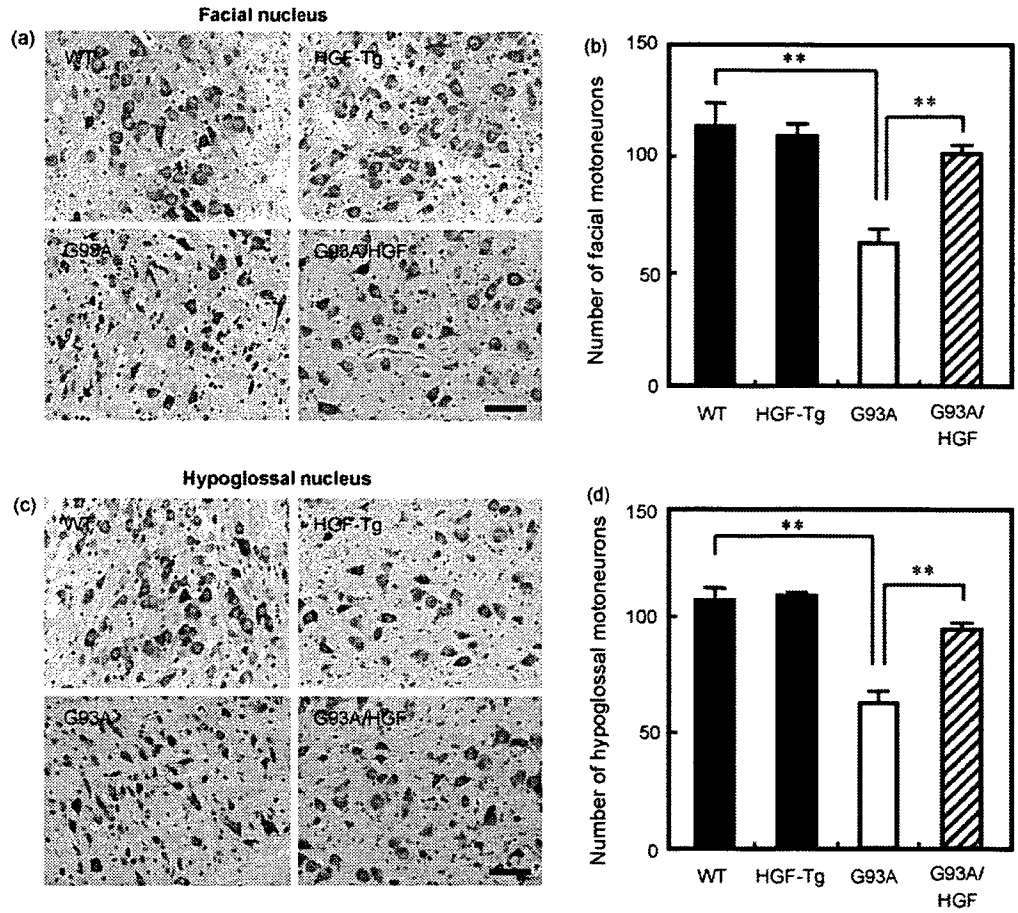

Fig. 2. Neuroprotective effect of HGF on facial (a and b) and hypoglossal (c and d) motoneurons in G93A mice. (a and c) Photomicrographs of representative Nissl-stained sections of the facial (a) and hypoglossal (c) nuclei of WT, HGF-Tg, G93A, and G93A/HGF mice at 8 months of age are shown. Scale bars = 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

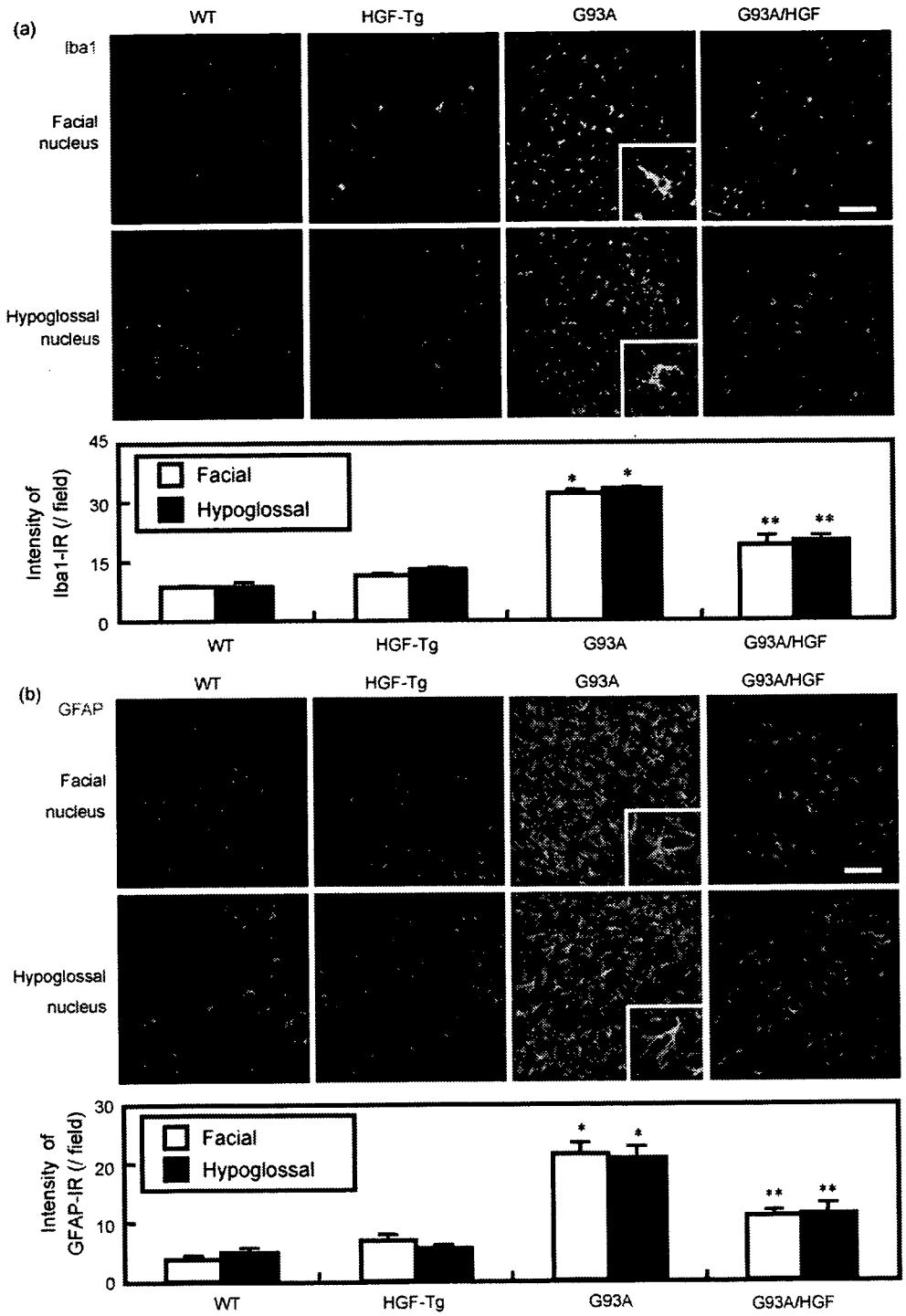

Fig. 3. Suppression of gliosis in the facial and hypoglossal nuclei of G93A/HGF mice. (a) Suppression of microglial accumulation in G93A/HGF mice. Immunofluorescence analysis for Iba1 (a marker for microglia) in the facial and hypoglossal nuclei of WT, HGF-Tg, G93A and G93A/HGF mice at 8 months of age. 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

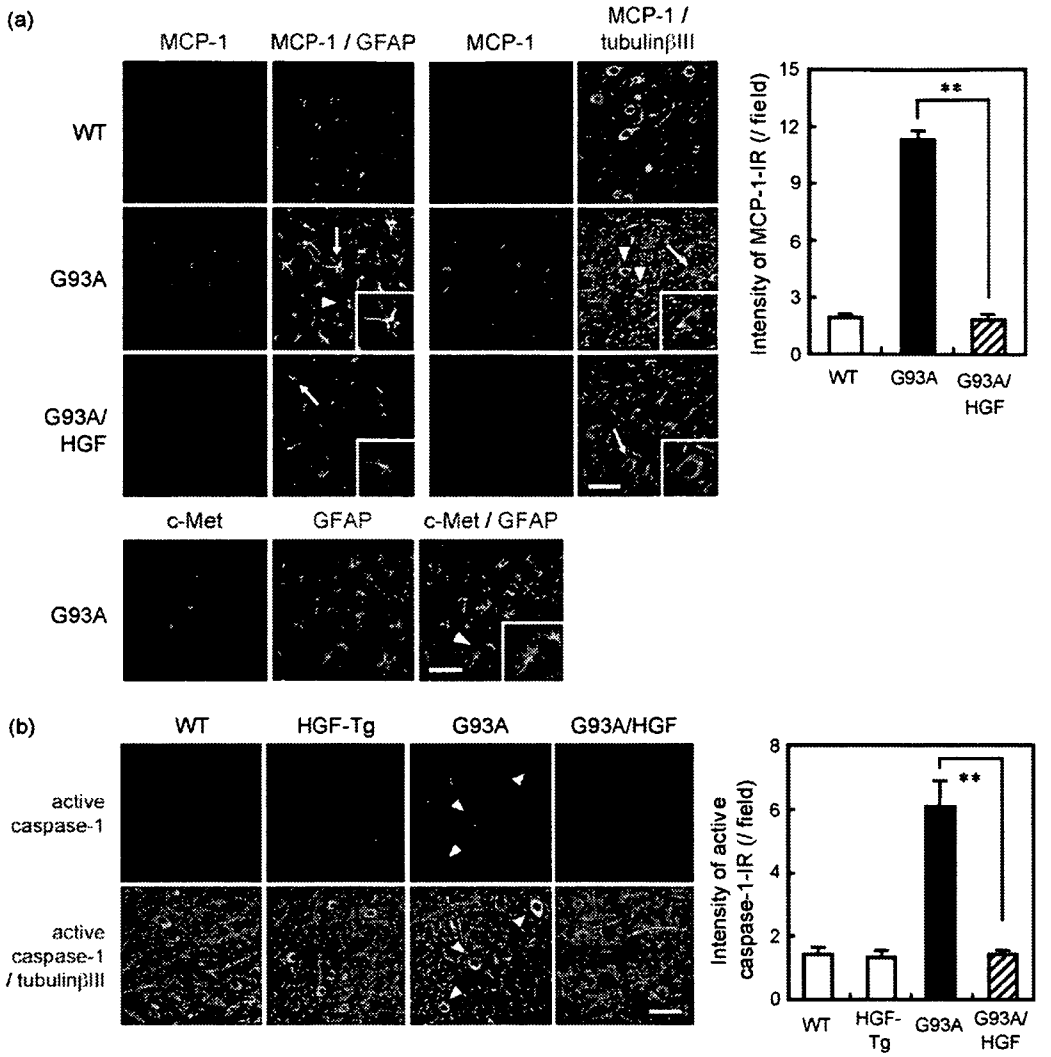

Fig. 4. Inhibitory mechanisms of HGF on gliosis in the facial nucleus of G93A mice. (a) Suppression of MCP-1 induction in G93A/HGF mice. Upper left panel, double immunofluorescence analysis of MCP-1 (red) and GFAP (green) or tubulinβIII (green) in the facial nucleus of WT, G93A and G93A/HGF mice at 8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

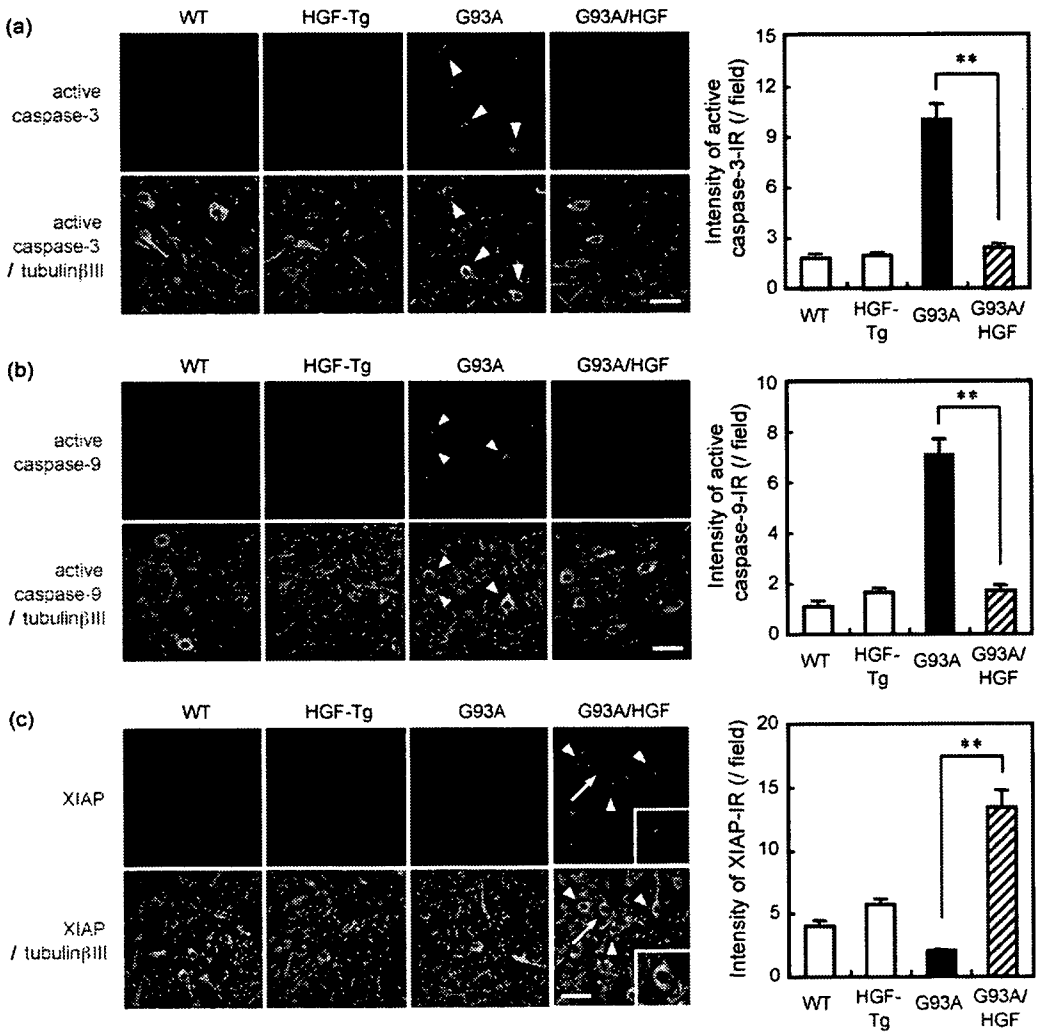

Fig. 5. Attenuation of pro-apoptotic protein activation in the facial motoneurons of G93A/HGF mice. (a) Left panel, double immunofluorescence analysis of active caspase-3 (red) and tubulinßIII (green) in the facial nucleus of WT, HGF-Tg, G93A and G93A/HGF mice at 6 months of age. Active caspase-3-positive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

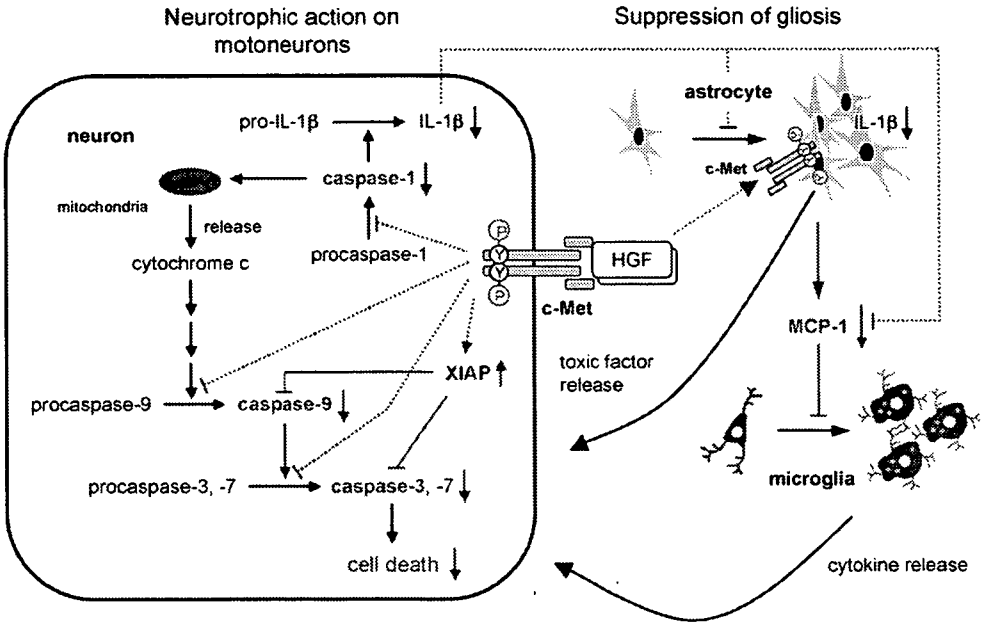

Fig. 6. Proposed working model of the molecular mechanisms of the neuroprotective effect of HGF on G93A mice are shown. In the motoneurons of G93A/HGF mice, HGF binds to c-Met on cell surface and induces autophosphorylation of the intracellular tyrosine residues of c-Met. Subsequently, HGF inhibits caspase-1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

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