abolished the expression of TUNEL-positive cells on day 3 (Fig. 4).

Identification of early molecular events plays an important role in the determination of the mechanisms by which hrHGF protects the brain from ischemia-induced neuronal cell death. Thus, we primarily targeted early events after the start of reperfusion following transient ischemia. At first, we detected c-Met proteins using immunohistochemistry on the pyramidal cell layer in the hippocampal CA1 region and possibly in some astrocytes and interneurons out of the neuronal cell layer of naïve rats (Fig. 5A). The immunoreactivity and localization of c-Met of ischemic rats were comparable to those of naïve rats. Next, the amount of c-Met protein was determined in the hippocampal CA1 region after the reperfusion with or without hrHGF treatment. The amounts of c-Met protein in the CA1 region at 1, 6, and 24 h of reperfusion were comparable to those of the sham-operated control rats (Fig. 5B). We next examined the effects of in vivo treatment with hrHGF on the tyrosine phosphorylation of c-Met in the hippocampal CA1 region after

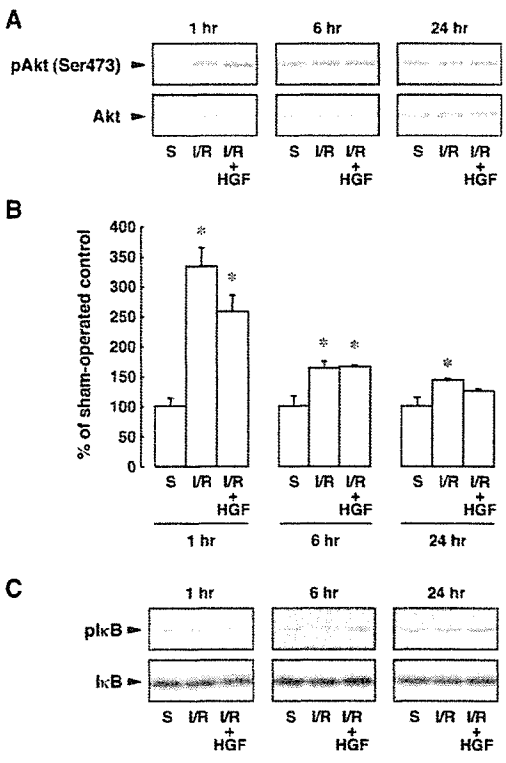

Fig. 6. Effects of hrHGF on phosphorylation of Akt and IκB in the hippocampal CA1 region after transient forebrain ischemia. (Panel A) Proteins (20 μg) from sham-operated (S) and 4-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with hrHGF (I/R+HGF) were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-Akt antibody [pAkt (Ser473)]. The blots were then stripped and re-probed with anti-Akt antibody (Akt). (Panel B) Bands corresponding to phospho-Akt (Ser473) were scanned, and the scanned bands were normalized by total Akt on the same blot. The results are expressed as the mean percentages of values for sham-operated group (S)±S.E.M. (n=3). * indicates a significant difference from sham-operated group (P<0.05). (Panel C) Proteins (50 μg) were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-IκB (pIκB) antibody. The blots were then stripped and re-probed with anti-IκB antibody.

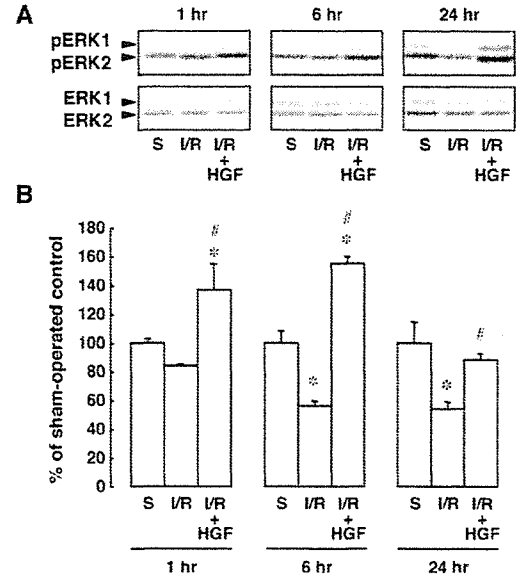

Fig. 7. Effects of hrHGF on phosphorylation of ERK1/2 in the hippocampal CA1 regions after transient forebrain ischemia. (Panel A) Proteins (20 μg) from sham-operated (S) and 4-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with hrHGF (I/R+HGF) were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-ERK1/2 antibody (pERK1/2). The blots were subsequently stripped and re-probed with anti-ERK antibody (ERK). (Panel B) Bands corresponding to phospho-ERK1/2 were scanned, and the scanned bands were normalized by total ERK on the same blot. The results are expressed as the mean percentages of values for sham-operated group (S)± S.E.M. (n=3). * indicates a significant difference from the sham-operated group (P<0.05). # indicates a significant difference from the hrHGF-untreated ischemic group (I/R) (P<0.05).

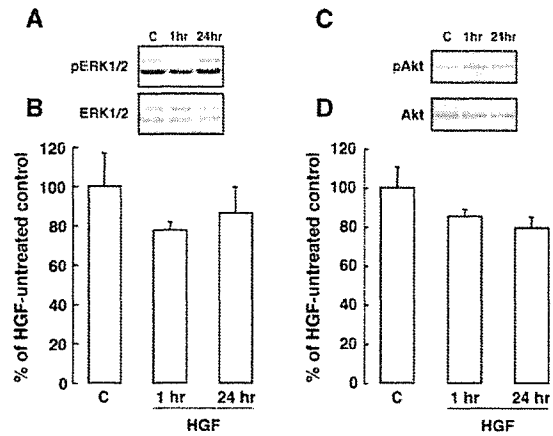

Fig. 8. Effects of hrHGF on phosphorylation of ERK1/2 in the hippocampal CA1 regions in the sham-operated rats. (Panels A and C) Proteins (20 μg) from non-operated naïve control (C) and sham-operated rats at 1 and 24 h after operation with hrHGF treatment were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-ERK1/2 antibody (pERK1/2 in A) and anti-phospho-Akt antibody [pAkt (Ser473) in B]. The blots were subsequently stripped and re-probed with anti-ERK antibody (ERK1/2 in A) and anti-Akt antibody (Akt in B). (Panels B and D) Bands corresponding to phospho-ERK1/2 and phospho-Akt were scanned, and the scanned bands were normalized by total ERK (B) and Akt (D) on the same blot. The results are expressed as the mean percentages of values for non-operated naïve control group (C)±S.E.M. (n=3).

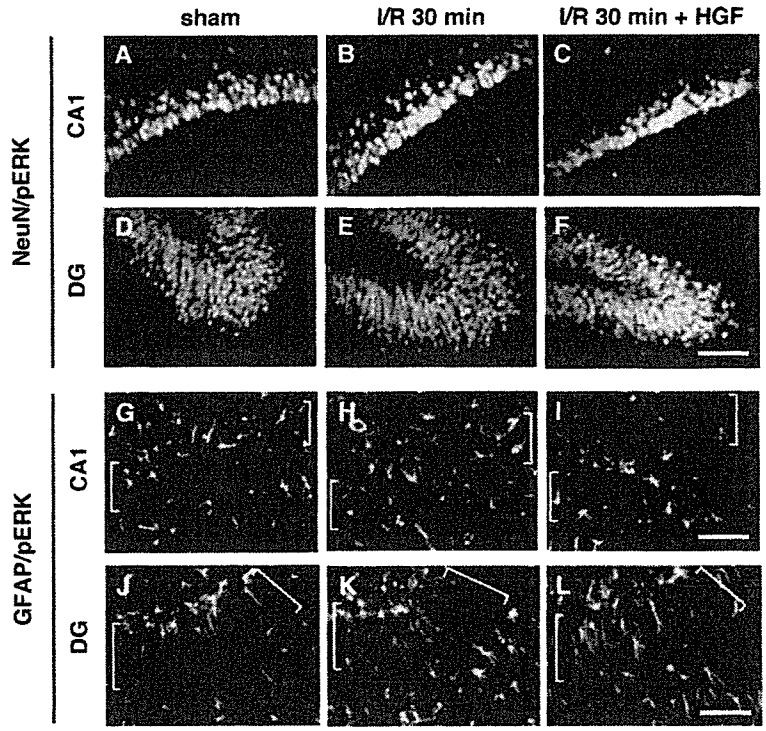

Fig. 9. Effects of hrHGF on localization of phosphorylated ERK1/2 in the hippocampal CA1 and dentate gyrus regions at the early period after transient forebrain ischemia. Representative photomicrographs of double staining with anti-phosphorylated ERK1/2 (red) antibody and anti-NeuN antibody as a marker for neuron (green) in the hippocampal CA1 region (A–C) and dentate gyrus (DG) region (D–F) of sham-operated rat (A and D), 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 30 min of reperfusion without hrHGF (B and E), and 4-vessel-occluded rat with hrHGF (C and F). Phosphorylated ERK1/2 was colocalized (yellow) with NeuN-positive neuron in the CA1 region of ischemic rat with hrHGF treatment (C) and in the dentate gyrus region of ischemic rat without (E) and with (F) hrHGF treatment. Representative photomicrographs of double staining with anti-phosphorylated ERK1/2 (red) antibody and anti-GFAP antibody as a marker for astrocytes (green) in the hippocampal CA1 region (G–I) and dentate gyrus (DG) region (J–L) of sham-operated rat (G and J), 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 30 min of reperfusion without hrHGF (H and K), and 4-vessel-occluded rat with hrHGF (I and L). Scale bar represents 50 μm. Pyramidal cell layer in CA1 region and granule cell layer in dentate gyrus region were marked with brackets.

the reperfusion. Immunoprecipitation with anti-c-Met antibody demonstrated that tyrosine phosphorylation of c-Met increased with hrHGF treatment compared with that of the sham-operated or hrHGF-untreated ischemic group (Fig. 5C). As the c-Met receptor is known to be phosphorylated on its Tyr1234 and Tyr1235 residues when the receptor is activated, we further examined the phosphorylation of these residues using a specific antibody that recognizes c-Met phosphorylated on Tyr1234 and Tyr1235. Although tyrosine phosphorylation of Tyr1234 and Tyr1235 residues of c-Met increased at 1 h of reperfusion relative to the level for sham-operated animals, hrHGF-induced tyrosine phosphorylation was larger than that of the hrHGF-untreated group (Fig. 5D and E). The increase in tyrosine phosphorylation of Tyr1234 and Tyr1235 in the hrHGF-treated ischemic groups was maintained at least for 24 h after the start of the reperfusion (Fig. 5D and E).

To explore the nature of intracellular signal transduction pathways for the in vivo protective effects of hrHGF treatment, we next examined the phosphorylation of Akt and ERK1/2 with or without hrHGF treatment. Transient ischemia increased Akt phosphorylation from 1 h after the reperfusion without changing the total level of Akt (Fig. 6A and B). Treatment with hrHGF did not affect the change in phosphorylation of Akt after transient ischemia (Fig. 6A and B). Activated Akt phosphorylates IκB kinase. In turn, the phosphorylated IκB kinase phosphorylates IκB, thereby resulting in degradation of IκB. Consequently, NFκB dissociated from IκB enters the nucleus to activate transcription of anti-apoptotic genes, including *bcl-x*$_L$. Immunoblotting showed that the phosphorylation of IκB was not altered by the ischemia regardless of treatment with or without hrHGF (Fig. 6C). In contrast to the increases in Akt

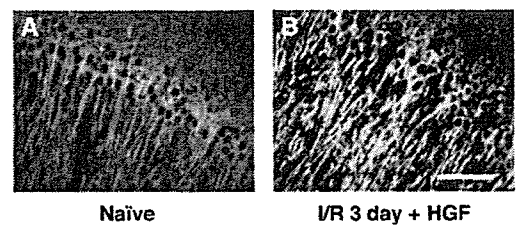

Fig. 10. Effects of hrHGF on localization of phosphorylated ERK1/2 and MAP-2 in the hippocampal CA1 on day 3 after transient forebrain ischemia. Representative photomicrographs of double staining with anti-phosphorylated ERK1/2 (red) antibody and anti-MAP2 antibody as a marker for neuron (green) in the hippocampal CA1 region of non-operated naïve rat (A) and 4-vessel-occluded rat after 15 min of transient forebrain ischemia followed by 72 h of reperfusion with hrHGF (I/R 3 day+HGF; B). Scale bar represents 50 μm.

phosphorylation, ERK phosphorylation decreased with time after the start of reperfusion (Fig. 7A and B). Treatment with hrHGF increased or recovered ERK phosphorylation without changing the total level of ERK (Fig. 7A and B). Thus, increased or recovered ERK phosphorylation with hrHGF treatment after the reperfusion was not due to a change in total amount of ERK1/2. There was no significant difference in phosphorylation of ERK and Akt in sham-operated animals at 1 and 24 h after treatment with hrHGF when compared with hrHGF-untreated naïve animals (Fig. 8).

We next examined cellular localization of ERK phosphorylation with or without hrHGF treatment after ischemia using double immunofluorescence histochemistry. NeuN-positive neuronal nuclei in the hippocampal CA1 region of sham-operated and ischemic rats at 30 min of the reperfusion were not stained with anti-phosphorylated ERK antibody (Fig. 9A and B), while a marked increase in immunoreactivity of phosphorylated ERK1/2 was evident in the dentate gyrus of ischemic rats at 30 min of the reperfusion (Fig. 9D and E). Treatment with hrHGF increased phosphorylation of ERK, which was colocalized with the NeuN-positive neuronal nuclei in the hippocampal CA1 region (Fig. 9C) and did not affect the phosphorylation of ERK in the dentate gyrus (Fig. 9F). GFAP-positive cells were colocalized with phosphorylated ERK in the CA1 (Fig. 9G) and dentate gyrus (Fig. 9J) in the sham-operated rats, while immunoreactivities of phosphorylated ERK, which were colocalized with GFAP, decreased in both CA1 (Fig. 9H) and dentate gyrus (Fig. 9K) in the ischemic rat. Although localization of phosphorylated ERK with GFAP in the CA1 (Fig. 9I) and dentate gyrus (Fig. 9L) after HGF treatment was comparable to that of ischemic rats, some GFAP-positive astrocytes in the CA1 regions were stained with phosphorylated ERK after treatment with hrHGF (Fig. 9I). Immunoreactivities of phosphorylated ERK in the neuronal nuclei in the hippocampal CA1 region were almost disappeared on day 3 after the reperfusion, although some neuronal nuclei were stained with phosphorylated ERK (Fig. 10).

As treatment with hrHGF decreased the number of TUNEL-positive cells in the hippocampal CA1 region on day 3 after transient forebrain ischemia, we next focused on the expression of anti-apoptotic protein Bcl-x_L with or without hrHGF treatment after ischemia. Treatment with hrHGF significantly increased the expression of Bcl-x_L proteins in the CA1 regions at 1 and 6 h of the reperfusion compared with that for hrHGF-untreated ischemic groups (Fig. 11A and B). Although no statistical difference was detected at 24 h, hrHGF tended to increase the expression of Bcl-x_L even at that time (Fig. 11B).

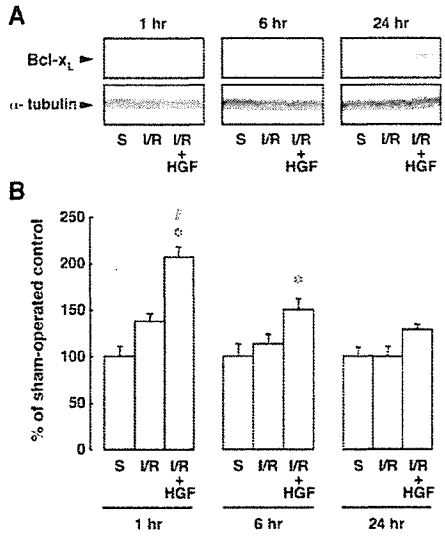

Fig. 11. Effects of hrHGF on expression of Bcl-x_L in the hippocampal CA1 regions after transient forebrain ischemia. (Panel A) Proteins (50 μg) from sham-operated (S) and 4-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with hrHGF (I/R+HGF) were separated by SDS-PAGE and subjected to immunoblotting with anti-Bcl-x_L antibody. The blots were subsequently stripped and re-probed with anti-α-tubulin antibody. (Panel B) Bands corresponding to Bcl-x_L were scanned, and the scanned bands were normalized by α-tubulin on the same blot. The results are expressed as the mean percentages of values for sham-operated group (S)±S.E. (n=3). * indicates a significant difference from the sham-operated group (P<0.05).

4. Discussion

The roles of HGF in various types of cells have been documented, whereas identification of HGF-induced intracellular signaling in the central nervous system and under pathophysiological conditions, including cerebral ischemia, remains to be a significant objective of research. The hippocampus, a region vulnerable to ischemic insults, plays a pivotal role in neuronal function, as it is associated with learning and memory function. Thus, it is worthwhile to clarify the protective effects of HGF against neuronal cell death and their mechanisms in the hippocampus.

In the present study, to determine the direct effect of hrHGF on the region vulnerable to ischemia, we infused hrHGF continuously for 3 days into the hippocampal CA1 region. The administration of hrHGF dose-dependently prevented the selective neuronal cell death in the CA1 region up to the concentration of 10 μg/3 days/animal. This protective effect against the pyramidal cell death in the CA1 region was comparable to the finding of Miyazawa et al. (1998), although they used a gerbil model of ischemia with injection of hrHGF into the striatum. Furthermore, we demonstrated that the expression of TUNEL-positive cells in the hippocampal CA1 region of ischemic rats was prevented by treatment with hrHGF. Our findings suggest that hrHGF is capable of directly protecting the hippocampal cells against ischemic injuries through anti-apoptotic pathways. As the total amount of c-Met was not affected for at least 24 h of reperfusion irrespective of treatment with or without hrHGF, the protective effects of hrHGF might not be due to changes in the amount of the HGF receptor, but due to the activation of c-Met. In accordance with this suggestion, tyrosine phosphorylation of c-Met was increased after the in vivo treatment with hrHGF, indicating the involvement of c-Met receptor activation. These results led us to determine events downstream of c-Met. HGF has been shown to protect cells from apoptosis via PI3-kinase/Akt pathways in several types of cells. For example, in the case of

cerebellar granule neurons, treatment with HGF prevented apoptosis that was induced by changing the medium containing serum with 25 mM K^+ to serum-free medium containing 5 mM K^+ (Zhang et al., 2000). HGF also has the ability to protect cerebellar granule neurons against NMDA- and quinolinic acid-induced excitotoxicity in a PI3-kinase/Akt-dependent and ERK-independent manner (Hossain et al., 2002). However, it remains to be determined whether Akt and ERK pathways are involved in protective effects of HGF in the hippocampus after in vivo ischemia. Therefore, we next focused on HGF-mediated changes in activation of Akt and ERK1/2 at early periods after transient forebrain ischemia, at which no obvious neuronal cell death in the hippocampal CA1 region was detected. Although transient ischemia significantly increased the phosphorylation of Akt at 1 h of reperfusion, the magnitude of Akt phosphorylation was comparable to that of ischemic rats regardless of treatment with or without hrHGF. Akt acts as an anti-apoptotic factor in various ways, including by phosphorylating IκB kinase, leading to IκB degradation following phosphorylation. As a result, NFκB dissociated from IκB sets a transcription of anti-apoptotic genes in motion. With respect to this suggestion, there were no obvious changes in phosphorylation of IκB after transient forebrain ischemia regardless of in vivo treatment with or without hrHGF. Therefore, Akt-dependent phosphorylation of IκB might not be the primary mechanism for the protective effects of in vivo treatment with hrHGF after transient forebrain ischemia. However, it remains to be determined whether IκB phosphorylation contributes to protective effects of hrHGF at the later stages after the start of the reperfusion. In addition to our results, previous studies by others have shown that phosphorylated Akt is transiently increased in the hippocampus after transient forebrain ischemia (Kawano et al., 2001; Yano et al., 2001). Although these findings suggest that Akt may play a role in neuronal survival in the hippocampus at an early period of reperfusion, changes in several other factors are capable of contributing to the selective neuronal cell death in the hippocampal CA1 region. As in the current study treatment with hrHGF did not affect Akt phosphorylation after ischemia, the role of phosphorylated Akt after transient forebrain ischemia remains controversial.

In contrast to the increase in the phosphorylation of Akt after transient forebrain ischemia, ERK1/2 phosphorylation in the hippocampal CA1 region was decreased with time after reperfusion. In vivo treatment with hrHGF transiently increased phosphorylation of ERK, which was colocalized with a neural nucleus marker NeuN at the early period of reperfusion, and attenuated the decrease in phosphorylation at 24 h of reperfusion without changing the total amount of ERK1/2. In contrast to the changes in the CA1 region, ERK1/2 in the dentate gyrus was highly activated in surviving neurons after transient forebrain ischemia regardless of treatment with or without hrHGF. The result of ischemia-induced marked increase in ERK phosphorylation in the dentate gyrus was in agreement with those of earlier studies (Hu et al., 2000; Li et al., 2001), suggesting that ERK pathway might act as neuroprotective signals after ischemia. We also demonstrated that treatment with HGF did not alter the levels of phosphorylated-ERK and Akt in the sham-operated rats compared to those of non-operated naïve control. Our results were consistent with findings that the action of exogenous HGF was specific to the injured organs (Tajima et al., 1992; Yanagita et al., 1993; Ohmichi et al., 1996). Therefore, phosphorylation of ERK in the HGF-treated ischemic rats might be the action in response to the protective effects of HGF against cell death. Although a number of studies using other types of neuronal cells have implicated ERK activation in cell survival (Yujiri et al., 1998), ERK does not seem to act as a factor for cell survival under all conditions of neuronal injury. For example, persistent activation of ERK in response to glutamate-induced oxidative stress is suggested to induce programmed cell death in primary cortical neurons (Stanciu et al., 2000). In this sense, we demonstrated that the immunoreactivity of ERK phosphorylation in the nucleus of ischemic rats with hrHGF treatment on day 3 was comparable to that of naïve control rats. In the present study, the immunostaining for c-Met proteins was detected in the pyramidal cell layer as well as in the GFAP-positive astrocyte that were comparable to those of earlier studies (Korhonen et al., 2000; Akimoto et al., 2004). In addition, we demonstrated that phosphorylation of ERK of hrHGF-treated animal was primarily seen in NeuN-positive neurons in the hippocampal CA1 region at the early period after the start of reperfusion. Therefore, transient activation of ERK1/2 through c-Met in neurons may contribute to the protective effects of hrHGF after transient forebrain ischemia.

In ischemic brain, we cannot fully rule out the possibility of contribution of astrocytes to the protective effect of HGF (Sun et al., 2002), as astrocytes also have the HGF receptor c-Met (Machide et al., 2000) and we demonstrated in the current study that the phosphorylated ERK was detected in some astrocytes in the hippocampal CA1 region of hrHGF-treated animal after ischemia. The difference between pathological activation and HGF-induced activation of ERK may exert diverse intracellular outcomes. In this sense, a study showed the ability of HGF to protect cardiac muscle cells against apoptosis by ERK-dependent phosphorylation of GATA-4 (Kitta et al., 2003). This HGF-induced GATA-4 phosphorylation played a pivotal role in the expression of Bcl-x_L (Kitta et al., 2003). Although phosphorylation of GATA family members by ERK in the hippocampal CA1 region remains unclear, the activation of transcription factors through c-Met/ERK pathway appears to be involved in the expression of anti-apoptotic proteins and in protection of cells against ischemic injury. With respect to this suggestion, the amount of Bcl-x_L protein in the hippocampal CA1 region after transient forebrain ischemia was increased by in vivo treatment with hrHGF. It is well known that Bcl-x_L has the anti-apoptotic effects in various types of cells. Therefore, the increase in the expression of Bcl-x_L protein at the early period of reperfusion may be one of the mechanisms for hrHGF-mediated protective effects. Recently, HGF was shown to activate Bcl-2 expression and to inhibit high D-glucose-induced translocation of Bax protein to the mitochondrial membrane in endothelial cells (Nakagami et al., 2002). Therefore, we cannot fully exclude the possibility that the protective effects of in vivo treatment with hrHGF may also rely on increased expression of

other anti-apoptotic proteins and/or inhibition of pro-apoptotic proteins, including prevention of Bax translocation to the mitochondria.

For clinical use, HGF might be injected to the subarachnoid space, as circulating proteins cannot be penetrated blood–brain barrier. In addition, HGF gene transfer might be superior due to the short half-life of recombinant HGF (Uematsu et al., 1999, Liu et al., 1992). In this sense, recent studies demonstrated that injection of HGF gene into the subarachnoid space prevented cell death in the hippocampal CA1 region after transient forebrain ischemia in gerbils (Hayashi et al., 2001) and reduced ischemic injury in a rat permanent middle cerebral artery occlusion model (Shimamura et al., 2004), suggesting that HGF has a potent cerebroprotective effect and thus is a prospective agent for therapy against ischemic brain injuries. However, HGF-mediated intracellular signaling in the ischemic brain was not fully understood. It is thus an important objective to elucidate mechanisms for protective effects of HGF under pathologic conditions in the central nervous system. Especially, the contribution of ERK and/or Akt phosphorylation, which are major events downstream of c-Met, to the protective effect of HGF in the hippocampus after ischemia remained to be determined. Our results suggest that treatment with hrHGF is capable of protecting against neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia in rats, which may be related to an ERK-dependent pathway.

Acknowledgments

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Hepatocyte growth factor attenuates cerebral ischemia-induced increase in permeability of the blood–brain barrier and decreases in expression of tight junctional proteins in cerebral vessels

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Abstract

Hepatocyte growth factor (HGF) exerts its physiological activities as that of an organotropic factor for regeneration and can prevent ischemia-induced injuries; however, its effect and mechanism of action under *in vivo* pathophysiological conditions remains to be determined. Recently, we demonstrated that treatment with human recombinant HGF (hrHGF) attenuated the disruption of the blood–brain barrier (BBB) observed after microsphere embolism-induced sustained cerebral ischemia. To see if tight junctional proteins were involved in this attenuation, in the present study, we investigated the effects of HGF on the levels of occludin and zonula occludens (ZO)-1 in cerebrovascular endothelial cells after microsphere embolism. Sustained cerebral ischemia was induced by the injection of 700 microspheres (48 μm diameter) into the right internal carotid artery of rats. hrHGF was injected into the right ventricle of the brain by using an osmotic pump at a dose of 30 μg/7 days per animal. The levels of tight junctional proteins in the endothelial cells were examined by immunohistochemical analysis. Treatment with hrHGF attenuated the decrease in the expression of occludin and ZO-1 proteins in the endothelial cells that occurred after sustained cerebral ischemia. Furthermore, treatment with hrHGF resulted in retention of these tight junctional proteins in fluorescein isothiocyanate (FITC)-albumin-perfused cerebral vessels, which did not leak FITC–albumin in the ipsilateral cortex. These results suggest that HGF-mediated maintenance of the tight junctional proteins in the endothelial cells may be a possible mechanism for the protective effect of HGF against the disruption of the BBB after cerebral ischemia.

Keywords: Cerebral ischemia; Microsphere embolism; Blood–brain barrier; Tight junctions; Occludin; ZO-1; Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a cytokine that evokes diverse cellular responses, including mitogenic, motogenic, morphogenic, angiogenic, and anti-apoptotic activities in various types of cells [13,18,22,25]. Whereas HGF exerts its physiological activities as an organotrophic factor for regeneration, HGF can also prevent ischemia-induced injuries, including hind limb ischemia [16,23] and cardiac ischemia/reperfusion [19], via its anti-apoptotic and angiogenic activities. We recently demonstrated that treatment with human recombinant HGF (hrHGF) attenuated cerebral ischemia-induced learning dysfunction [3]. Furthermore, in that study, we showed that the disruption of the blood–brain barrier, which was determined by FITC–albumin leakage, and the decrease in the viable area of the ipsilateral hemisphere were lessened by the hrHGF treatment. Such effects might have been due to HGF-mediated protection against cerebral ischemia-induced impairment of the tight junctional barrier [11], which is formed by the interaction of membrane-associated accessory proteins, including claudin and occludin [14], on the plasma membranes of adjacent cells [5,6]. Zonula occludens (ZO)-1, ZO-3, and cingulin are considered to be cytoplasmic tight junctional accessory proteins, which connect tight junctions to the actin cytoskeleton [7,10].

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Although HGF can exert multipotent activities under pathophysiological conditions, the effects of HGF on the tight junctional proteins, which play an important role in the maintenance of tight junctional barrier in the endothelial cells, after severe cerebral ischemia have not yet been clarified. In the present study, we examined immunohistochemically the effect of hrHGF on the levels of the tight junctional proteins occludin and ZO-1 after cerebral ischemia. Our findings demonstrate that treatment with hrHGF attenuated the ischemia-induced decrease in the levels of tight junctional proteins in the endothelial cells. The results suggest that the maintenance by HGF of these proteins in the endothelial cells may be a possible mechanism for the protective effect of HGF against disruption of the blood–brain barrier (BBB) after cerebral ischemia.

Human recombinant HGF (hrHGF) was purified from the culture media of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA, as described previously [18,21]. The purity of hrHGF was >98%, as determined by SDS-PAGE.

Male Wistar rats weighing 180–220 g (Charles River Japan Inc., Atsugi, Japan) had free access to food and water according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Guideline for Experimental Animal Care issued by the Prime Minister's Office of Japan. All efforts were made to minimize the animals' suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. The study protocol was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Science.

Microsphere-induced cerebral embolism was performed by the method described previously [15]. In brief, after the right external carotid and pterygo-palatine arteries had been temporarily occluded with strings, a needle connected to a polyethylene catheter (3French, Atom Co., Tokyo) was inserted into the right common carotid artery. Seven-hundred microspheres (47.5 ± 0.5 μm in diameter, PerkinElmer Life Science), suspended in 20% dextran solution, were injected into the right internal carotid artery through the cannula. After the injection, the puncture wound was repaired with surgical glue. Then, the strings occluding the right external carotid and pterygo-palatine arteries were released. Fifteen hours after the operation, the behavior of the operated rats was scored on the basis of paucity of movement, truncal curvature, and forced circling during locomotion, which are considered to be typical symptoms of stroke in rats [15]. Each item was rated from 3 to 0 (3: very severe; 2: severe; 1: moderate; 0: little or none). Rats with a total score of 7–9 points were used in the present study. The rats that underwent a sham operation received the same volume of vehicle without microspheres.

An osmotic pump (Alzet model 2001 Palo Alto, CA, USA) containing human recombinant HGF (30 μg) or a physiologic saline solution was implanted at 10 min after the microsphere embolism. A cannula device connected to the subcutaneously implanted osmotic pump was inserted into the right ventricle of the brain at a point located 1.5 mm lateral and 0.8 mm posterior to the bregma, and at a depth of 4 mm from cortical surface. The hrHGF was infused at a flow rate of 1.0 μl/h and a dose of 30 μg/7 days per animal.

Albumin fluorescein isothiocyanate (FITC–albumin, Sigma, Tokyo, Japan) was injected into both carotid arteries for morphological observation of cerebral blood vessels. Injection of FITC–albumin was conducted by the method of Cavaglia et al. [1]. FITC–albumin solution (10 mg/ml 0.1 mol/l phosphate-buffered saline, pH 7.4) was perfused through bilateral carotid arteries at a rate of 1 ml/min (10 ml/kg) by using a syringe pump (CFV-2100, NIHON KOHDEN, Japan). In order to avoid elevation of systemic blood pressure, the same amount of blood was withdrawn from the inferior vena cava. Immediately following perfusion, the rats were decapitated and their brains were removed. Brains were fixed for 24 h in 4% paraformaldehyde, then cryoprotected by sequential immersion in 10, 20, and 30% sucrose solutions for 4 h for each concentration. The brains were embedded in O.C.T. compound (SAKURA, Tokyo, Japan). Coronal brain sections (20 μm thick) were cut on a cryostat, thaw-mounted on poly-L-lysine-coating microslide glasses (Muto Pure Chemicals, Japan), and then dried over 2 h. Fluorescence was detected by using an Olympus fluorescence microscope (BX-52, Olympus, Tokyo, Japan) equipped with a mercury arc lamp. Images were incorporated into a personal computer by using Viewfinder Lite and Studio Lite software (Pixcera, Los Gatos, CA, USA).

For immunohistochemical detection of tight junctional protein-positive endothelial cells, coronal sections were incubated overnight with polyclonal rabbit anti-occludin or ZO-1 antibodies (Zymed, San Francisco, CA, USA) at room temperature and then incubated with biotinylated anti-rabbit IgG (Vector, Burlingame, CA, USA). The sections were incubated with streptavidin–fluorescein (Amersham, Buckinghamshire, UK). After having been washed, they were next incubated overnight at room temperature with mouse anti-rat endothelial

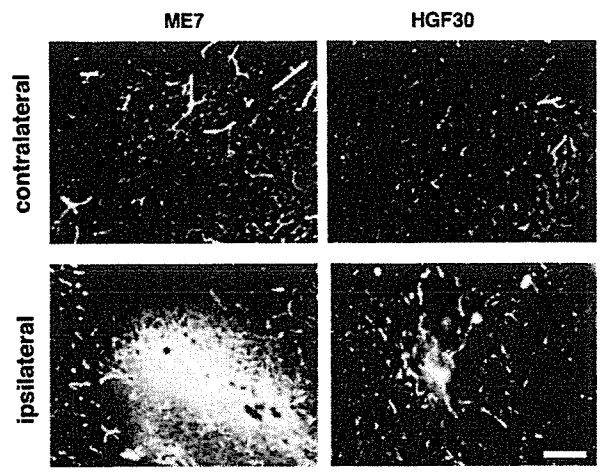

Fig. 1. Images of fluorescein isothiocyanate-conjugated albumin (FITC–albumin)-perfused vessels in the contralateral and ipsilateral temporal cortices of hrHGF-untreated (ME7) and 30 μg/7 days per animal hrHGF-treated ME (HGF30) rats on day 7 after the operation. Bar: 200 μm.

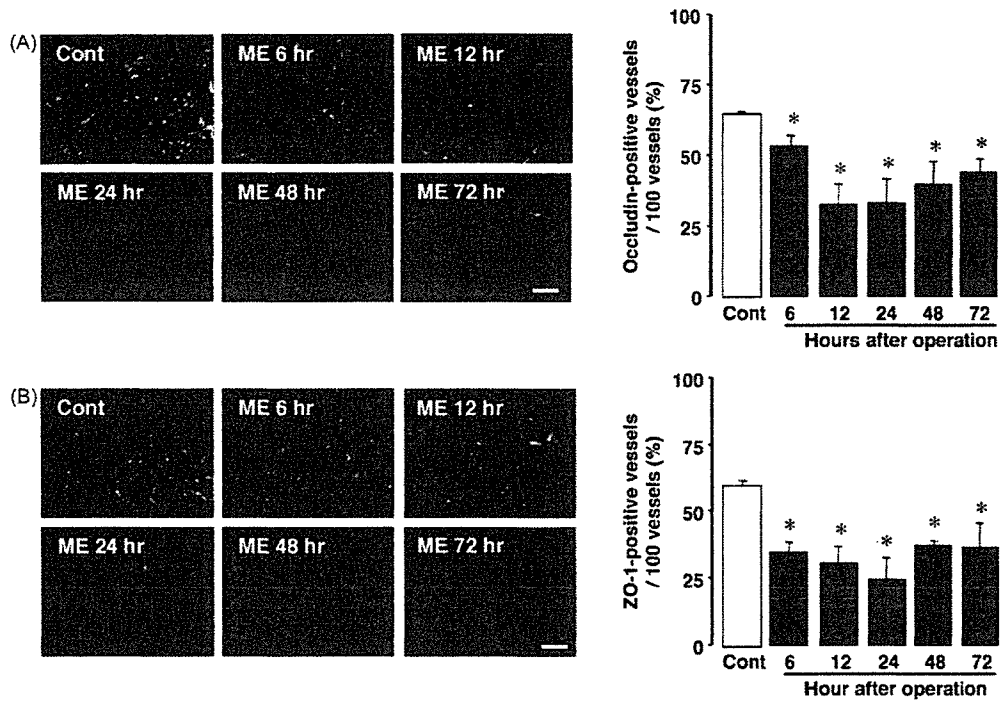

Fig. 2. Images showing double staining for occludin (A, green) or ZO-1 (B, green), and RECA (A and B, red) in the non-operated naïve control (Cont) and hrHGF-untreated ME rats at 6, 12, 24, 48, and 72 h after the operation. Time course of changes in the percent of occludin- or ZO-1-positive endothelial cells is shown in the right panels. Each value represents the mean ± S.E.M., $n = 4$ each. Bar: 100 μm. *Significant difference from non-operated naïve control rats ($P < 0.05$).

cell antigen (RECA) antibody (Serotec, Oxford, UK) as a marker of cerebral microvessels. Subsequently, the sections were incubated with Cy-3-labeled goat anti-mouse IgG (Amersham). By use of image analysis software (Image-Pro Express; Media Cybernetics), the number of occludin- or ZO-1-positive endothelial cells in the temporal cortex was counted in four sections per animal, as the temporal cortex was significantly affected by the microsphere embolism. The percentage of occludin- or ZO-1-positive endothelial cells per 100 blood vessels in the temporal cortex was then calculated. The microscopic observations were carried out by a person unaware of the study group.

The results were expressed as the means ± S.E.M. Statistical comparison among multiple groups was evaluated by analysis of variance followed by Fisher's protected least significant difference test. Differences with a probability of 5% or less were considered significant ($P < 0.05$).

In the present study, microspheres were injected into 104 rats. Seventy-seven of the surviving rats (74.0%) showed stroke-like symptoms with a total score of 7–9 points. Seventeen (16.3%) and four animals (3.8%) showed the stroke-like symptoms with a score of 4–6 and 1–3 points, respectively. Six rats (5.8%) died before all examinations could be completed.

At first we examined BBB leakage after the microsphere embolism (ME) by injecting FITC–albumin into the carotid arteries. Fig. 1 shows the effect of treatment with hrHGF on FITC–albumin leakage in the ipsilateral temporal cortex on day 7 after the operation. FITC–albumin leakage in the ipsilateral temporal cortex of ME rats (ME7) was inhibited by hrHGF treatment (HGF30). There was no FITC–albumin leakage in the contralateral temporal cortex of either group. These results were similar to those of our previous studies [3,11].

Tight junctional proteins play a pivotal role in the physical barrier function. Therefore, next we examined changes in the number of tight junctional protein-positive endothelial cells in the temporal cortex after the microsphere embolism. The number of occludin- and ZO-1-positive endothelial cells started to decrease at 6 h and remained significantly lower than the control for up to day 3 after the microsphere embolism (Fig. 2A and B). The lowest number of occludin- or ZO-1-positive endothelial cells in the ipsilateral temporal cortex was evident at 24 h after the embolism (Fig. 2A and B).

On the basis of results from the time-course study, we next examined the effect of hrHGF on the number of tight junctional protein-positive endothelial cells in the temporal cortex at 24 h after the microsphere embolism by using double-immunofluorescence staining with occludin or ZO-1, and RECA as a marker of cerebral microvessels. Treatment with hrHGF attenuated the decrease in the number of occludin- (Fig. 3A) or ZO-1-positive endothelial cells (Fig. 3B).

Finally, we used anti-tight junctional proteins antibodies to stain coronal sections of brain from rats that had been perfused with FITC–albumin. As shown in Fig. 4, occludin and ZO-1

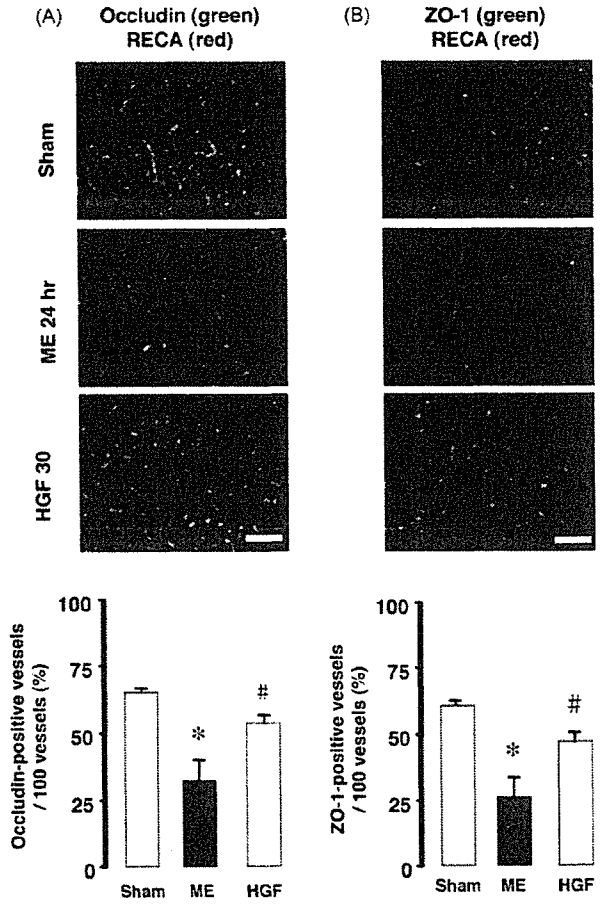

Fig. 3. Images of double staining with occludin (green, A) or ZO-1 (green, B) and RECA (red) as a marker of endothelial cells in the sham-operated (Sham), hrHGF-untreated ME (ME 24 h) and hrHGF-treated ME (HGF30) rats at 24 h after the operation. Scale bar: 100 μm. Quantified data are shown in the lower panels. Each value represents the mean ± S.E.M., $n = 4$ each. *Significant difference from sham-operated rats ($P < 0.05$). #Significant difference from hrHGF-untreated ME rats ($P < 0.05$).

proteins were decreased in amount or completely lost in the temporal cortex where FITC–albumin leakage was detected, whereas these tight junctional proteins were present in the FITC–albumin-perfused cerebral vessels in the retrosplenial granular cortex (non-infarct area), where BBB integrity was undamaged. Treatment with hrHGF (HGF 30) prevented both the loss of occludin and ZO-1 proteins in the FITC–albumin-perfused cerebral vessels in the temporal cortex and the leakage of FITC–albumin there.

Microsphere embolism-induced, sustained cerebral ischemia was earlier reported to cause disruption of the BBB [3,11], and this disruption was suggested to lead to the development of brain injuries after ischemia. Indeed, it has been proposed that the majority of stroke-induced brain injuries are related to disruption of the BBB, resulting in secondary damage to neurons [17,20]. Therefore, it has become an important objective to maintain the integrity of the BBB to protect brain from ischemic injuries, including tissue degeneration and dysfunction of learning and memory.

We demonstrated in a previous study that treatment with hrHGF improved the acquisition of spatial learning and memory impaired by microsphere embolism. Furthermore, FITC–albumin leakage and tissue degeneration were attenuated by hrHGF treatment [3]. These results suggested that HGF acts on the endothelial cells and prevents endothelial cell death, consequently maintaining BBB integrity after severe cerebral ischemia due to microsphere embolism. However, the mechanism underlying protection against disruption of the BBB has not been extensively examined. To achieve further insight into the effects of HGF on cerebrovascular endothelial cells after the embolism, we focused on the *in situ* levels of tight junctional proteins in the endothelial cells. We demonstrated that the expression of occludin and ZO-1 in the endothelial cells was decreased at the early period after the embolism and that the decreased level remained at least until 72 h. The increase in FITC–albumin leakage roughly paralleled the decrease in the number of occludin- or ZO-1-positive vessels.

Peripheral inflammatory pain was reported to decrease the expression of occludin in microvasculature samples of brain and to lead to increased BBB permeability [9]. Furthermore, the markedly increased BBB permeability during human immunodeficiency virus-elicited encephalitis was associated with significant disruption of tight junctions, as demonstrated by the absence of immunoreactivity for occludin and ZO-1 within the vessels [2]. Therefore, the decreased expression of these tight junctional proteins in the endothelial cells may be related to the increased BBB permeability seen under *in vivo* pathophysiological conditions such as cerebral ischemia. Findings by others and us led us to examine the effects of hrHGF on the level of tight junctional proteins in the endothelial cells after the embolism. We obtained data showing that treatment with hrHGF prevented the decrease in the expression of occludin and ZO-1 in the endothelial cells. Thus, it is possible that HGF maintained the expression of tight junctional proteins in the endothelial cells, thereby resulting in prevention of BBB leakage.

The question arises as to the mechanism by which the expression of tight junctional proteins occludin and ZO-1 was retained in the endothelial cells after the embolism followed by HGF treatment. It is noteworthy that vascular endothelial growth factor (VEGF), which increases vascular permeability in the brain *in vivo*, causes a loss of occludin and ZO-1 from the endothelial cell junctions [24]. Furthermore, angiopoietin-1 (Ang-1), which is an important anti-permeability factor [4], appears to induce the expression of tight junctional proteins in brain capillary endothelial cells [8,12]. In contrast to the action of Ang-1, that of Ang-2 may inhibit ZO-1 expression [26]. In addition to the protective effect of HGF against cerebral ischemia-induced apoptotic death of endothelial cells [3], these findings suggest that HGF may promote the expression of occludin and ZO-1 indirectly via regulating the opposing actions of VEGF and Ang-1 proteins on the endothelial cells, thereby resulting in attenuation of BBB leakage in the ischemic brain.

In conclusion, we demonstrated for the first time that HGF has the ability to attenuate the disruption of the BBB seen after

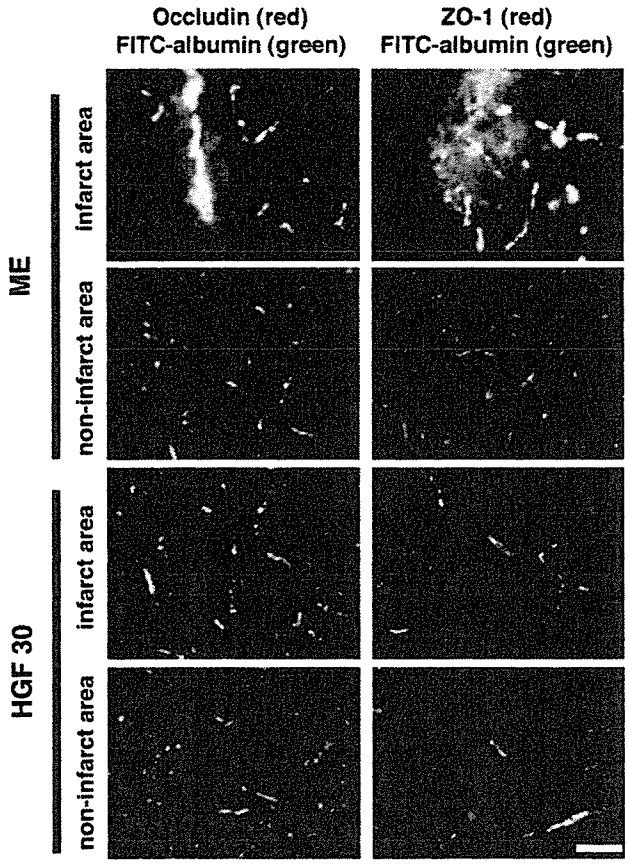

Fig. 4. Images of double staining with occludin (red) or ZO-1 (red) and FITC–albumin-perfused vessels (green) in the right retrosplenial granular cortex (as a non-infarct area) and in the right temporal cortex (as an infarct area) of hrHGF-untreated ME (ME) and hrHGF-treated ME (HGF 30) rats at 24 h after the operation. Scale bar: 50 μm.

sustained cerebral ischemia mediated by preventing the decrease in expression of tight junctional proteins in the endothelial cells.

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The protective effect of hepatocyte growth factor against cell death in the hippocampus after transient forebrain ischemia is related to the improvement of apurinic/apyrimidinic endonuclease/redox factor-1 level and inhibition of NADPH oxidase activity

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Abstract

Early oxidative DNA damage is regarded to be an initiator of neuronal apoptotic cell death after cerebral ischemia. Although evidence suggests that HGF has the ability to protect cells from oxidative stress, it remains unclear as to how HGF suppresses oxidative DNA damage after cerebral ischemia. Apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) is a multifunctional protein in the DNA base repair pathway that is responsible for repairing apurinic/apyrimidinic sites in DNA after oxidation. We demonstrated that both the immunoreactivity and the number of APE/Ref-1-positive cells in the hippocampal CA1 region were decreased after transient forebrain ischemia and that treatment with HGF suppressed this reduction. The expression of Cu/ZnSOD and MnSOD in the hippocampal CA1 region did not change after ischemia, regardless of treatment with or not with HGF. The activity of NADPH oxidase was increased mainly in glia-like cells in the hippocampal CA1 region after ischemia, and this increase was attenuated by HGF treatment. These results suggest that the protective effects of HGF against cerebral ischemia-induced cell death in the hippocampal CA1 region are related to the improvement of neuronal APE/Ref-1 expression and the inhibition of NADPH oxidase activity in glia-like cells.

Keywords: Apurinic/apyrimidinic endonuclease/redox factor-1; Cerebral ischemia; Hepatocyte growth factor; Hippocampus; NADPH oxidase

Hepatocyte growth factor (HGF), which was originally identified and purified as a multifunctional protein in hepatocytes [17,18], exerts its activities such as mitogenic, motogenic, morphogenic, angiogenic, and anti-apoptotic activities in various types of cells [14,17,19]. HGF and its receptor c-Met were found to be expressed in the central nervous system [1,8,9] and to prevent cell death induced by transient global or focal ischemia or by widespread cerebral embolism in rats [4,7,15,21]. Although the protective effect of HGF against ischemia-induced injuries, at least in part, are related to its angiogenic and anti-apoptotic activities [4,7,21], the precise mechanisms underlying the protective effect of HGF remain to be determined in the *in vivo* ischemic brain.

Early oxidative DNA damage is regarded to be an initiator of neuronal apoptotic cell death after cerebral ischemia [13]. Recent studies suggest that HGF has a protective activity against oxidative stress-induced cell death. For example, HGF protected cardiac myocytes and mesangial cells from H_2O_2-induced oxidative stress [12,20,25]. Furthermore, HGF prevented apoptotic cell death after hypoxia/reoxygenation-induced oxidative stress in hepatocytes [24]. In addition, we recently showed that HGF attenuated the primary oxidative DNA damage after transient forebrain ischemia [21]. Although these findings suggest that HGF has the ability to protect cells from ischemia-induced oxidative stress, it remains unclear as to how HGF suppresses oxidative DNA damage in the brain.

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Apurinic/apyrimidinic endonuclease/redox factor-1 (APE/Ref-1) is a multifunctional protein in the DNA base repair pathway that is responsible for repairing apurinic/apyrimidinic sites in DNA after oxidation. In the course of the repair of oxidative DNA damage or of 5-phosphoglycolate residues resulting from ionizing radiation-induced strand breakage, APE/Ref-1 functions as a 3-phosphodiesterase. In the processing of DNA alkylation damage, APE/Ref-1 acts as 5′-endonuclease [5]. It is noteworthy that the expression of APE/Ref-1 was decreased in the early period after transient forebrain ischemia [11] or transient focal cerebral ischemia [6]. Therefore, APE/Ref-1 might function in the protective effect of HGF against the oxidative DNA damage after cerebral ischemia.

In the present study, we examined the mechanism responsible for the protective effect of HGF against apoptotic cell death after transient forebrain ischemia by focusing on oxidative DNA damage, which is elicited at the early period of apoptosis. We demonstrated that HGF attenuated the decrease in the level of the DNA repair enzyme APE/Ref-1, as well as blocked the activity of NADPH oxidase after transient forebrain ischemia.

Human recombinant HGF (HGF) was purified from the culture medium of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA, as described earlier [19]. The purity of HGF was >98%, as determined by SDS-PAGE.

Male Wistar rats (200–250 g, Charles River Japan Inc., Atsugi, Japan) had free access to food and water according to the Guideline of Experimental Animal Care issued by the Prime Minister's Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Science. Transient (15 min) forebrain ischemia was produced by the four-vessel occlusion procedure for rats described previously [29]. Only rats that showed a completely flat electroencephalogram and a loss of consciousness during the duration of the carotid artery occlusion were chosen for use in the present study.

HGF was infused into the right hippocampal CA1 region by using an osmotic pump (Alzet model 1003D; DURECT Corp., Cupertino, CA, USA) attached to a 30-gauge needle implanted 3.5 mm posterior and 2.5 mm lateral to the bregma, and at a depth of 2.4 mm from the cortical surface. The infusion of HGF was begun 10 min after the start of reperfusion, at a flow rate of 1.0 μl/h and a concentration of 100 μg/ml (10 μg per 3 days per animal).

At various times after the start of reperfusion, animals were sacrificed by decapitation. The hippocampal CA1 and dentate gyrus regions were dissected on ice in ice-cold 125 mM Tris–HCl, pH 7.4, containing 320 mM sucrose, 2 mM sodium orthovanadate, 20 mM sodium diphosphate decahydrate, 20 mM DL-α-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of antipain, aprotinin, and leupeptin (homogenization buffer) described previously [21,22]. The dissected hippocampal region was then homogenized in the ice-cold homogenization buffer. The samples were stored at −80 °C until used and were thawed only once.

Hippocampal homogenates that had been 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) were separated on 12% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane as described [29]. Protein blots were incubated with the appropriate antibodies, and the bound antibody was detected by the enhanced chemiluminescence method (Amersham Biosciences Inc., Piscataway, NJ, USA). Quantification was performed by using computerized densitometry and an image analyzer (ATTO Co., Tokyo, Japan). Antibodies used were anti-Cu/ZnSOD antibody (SOD-101, StressGen, Victoria, Canada) and anti-MnSOD antibody (SOD-111, StressGen).

For APE/Ref-1 staining, the coronal sections (5 μm) were incubated overnight with rabbit anti-APE/Ref-1 antibody (Novus Biologicals, Littleton, CO, USA) at 4 °C. After washing, the sections were incubated with biotinylated anti-rabbit IgG antibody (Dako, Carpinteria, CA, USA) for 2 h and then with ABC complex solution (Vector Laboratories, Burlingame, CA, USA) for 2 h. Color development was performed by incubating the sections with 3,3′-diaminobenzidine and hydrogen peroxide (Vector Laboratories).

NADPH oxidase activity staining was performed according to the method described by Murphy et al. [16]. The sections were preincubated in buffer (2.5 mM Tris–HCl, pH 7.4 containing 0.08% Triton X-100 and 2 mg/ml BSA) for 30 min in the presence or absence of 10 μM dicumarol (Sigma-Aldrich, St. Louis, MO, USA). The preincubation solution was replaced with buffer containing 100 μM nitro blue tetrazolium (NBT, Sigma-Aldrich) and 100 μM LY83583 (Calbiochem, La Jolla, CA, USA) with or without dicumarol. The staining reaction was initiated by addition of 1 mM NADPH in buffer (Boehringer Ingelheim, Indianapolis, IN, USA), and the reaction mixture was incubated at 37 °C for up to 30 min.

The results were expressed as the means ± S.E. Statistical comparison among multiple groups was evaluated by ANOVA following by Fisher's protected least significant difference test.

We previously demonstrated that the oxidative DNA damage, which was estimated by staining with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody, occurred at the early period of apoptosis [21]. In addition, we found that HGF attenuated oxidative DNA damage at 6 h after the start of reperfusion [21]. Thus, we focused in the present study on the expression of the DNA repair enzyme APE/Ref-1 in the hippocampus at that time point. The number of APE/Ref-1-positive cells in the hippocampal CA1 region significantly decreased without changing the total number of cells after ischemia (Fig. 1B and M), and the HGF treatment prevented this decrease (Fig. 1C and M). The decrease in the level of immunoreactivity in the APE/Ref-1-positive cells after ischemia was also reversed by the HGF treatment (Fig. 1C). TUNEL-positive cells in the hippocampal CA1 region were not evident at 6 h after the reperfusion and appeared from 48 h after the reperfusion [22]. On the other hand, neither the number of APE/Ref-1-positive cells nor the immunoreactivity of APE/Ref-1 in the hippocampal dentate gyrus was altered after the ischemia, regardless of treatment or not with HGF (Fig. 1G–I and N).

We next examined the amounts of the antioxidant enzymes Cu/ZnSOD and MnSOD. The expression of Cu/ZnSOD in either

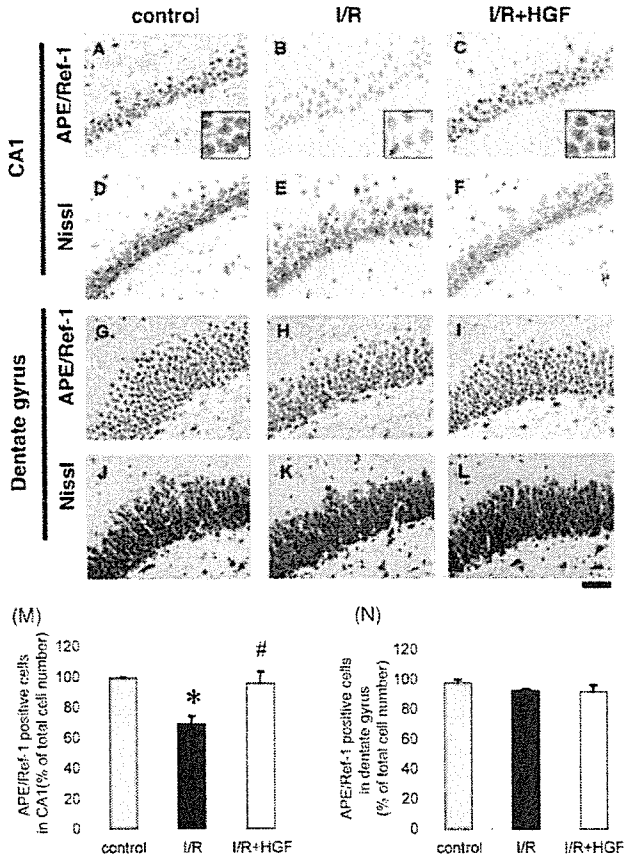

Fig. 1. Effect of HGF on the expression of APF/Ref-1 in the hippocampal CA1 region and dentate gyrus after transient forebrain ischemia. Representative photomicrographs of cells stained with anti-APE/Ref-1 antibody in the hippocampal CA1 (A–C) and dentate gyrus (G–I) region of non-operated naïve rats (A and G), ischemic rats at 6 h of reperfusion without (B and H) or with (C and I) HGF treatment, with enlargements in the boxes in A–C. Representative photomicrographs of Nissl-stained cells in the hippocampal CA1 region of non-operated naïve rats (D and J), ischemic rats at 6 h of reperfusion without (E and K) or with (F and L) HGF treatment. Scale bar represents 50 μm and 10 μm in enlargements. Effect of HGF on the number of APE/Ref-1-positive cells in the hippocampal CA1 region (M) and dentate gyrus (N) of ischemic rats at 6 h of reperfusion without (I/R) or with (I/R + HGF) HGF treatment. The percentage of APE/Ref-1 positive cells among the total number of cells, which was estimated by Nissl staining of the same field of an adjoining section, was calculated. Results are given as the mean percentages of the non-operated naïve control ± S.E. $n = 3$. $^{*}p < 0.05$ vs. non-operated naïve control, $^{\#}p < 0.05$ vs. I/R6 h.

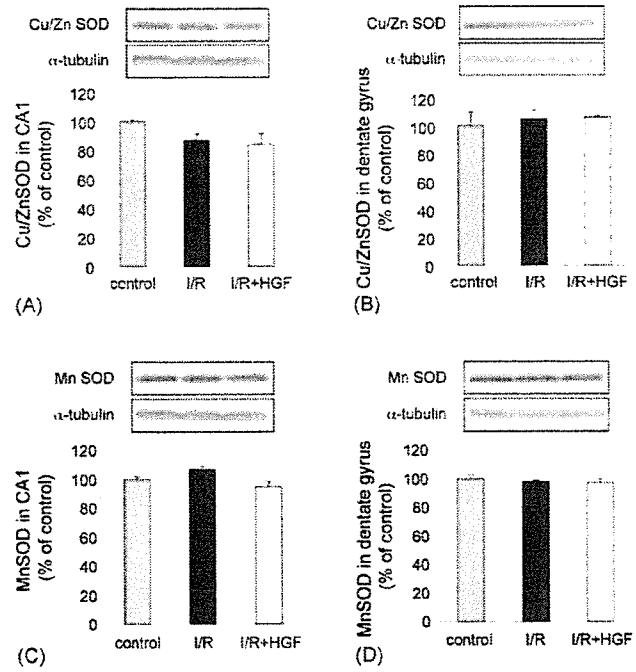

Fig. 2. Effect of HGF on the amounts of Cu/ZnSOD and MnSOD in the hippocampal CA1 region and dentate gyrus after transient forebrain ischemia. Proteins (50 μg) from non-operated naïve control and ischemic rats at 6 h of reperfusion in the hippocampal CA1 region (A) and dentate gyrus (B) without (I/R) or with (I/R + HGF) HGF treatment were separated by SDS-PAGE and subjected to immunoblotting with anti-Cu/ZnSOD antibody. The blots were reprobed with anti-α-tubulin antibody. The scanned bands of Cu/ZnSOD were normalized by α-tubulin on the same blots. (C–D), Same as "A–B," except anti-MnSOD antibody was used instead of anti-Cu/ZnSOD. Results are the mean percentages of the non-operated naïve control ± S.E. $n = 3$.

the hippocampal CA1 region (Fig. 2A) or dentate gyrus (Fig. 2B) did not change after ischemia with or without the HGF treatment. As to MnSOD, its expression in the hippocampal CA1 region or dentate gyrus was not altered after ischemia with or without the HGF treatment (Fig. 2C and D).

NADPH oxidase is known to produce reactive oxygen species (ROS) after ischemia and reperfusion. Therefore, next we stained sections for NADPH oxidase activity. This activity was detected mainly in glia-like cells and vascular smooth muscle cells (Fig. 3A–C). The number of active NADPH oxidase-positive glia-like cells was markedly elevated in CA1 region after ischemia (Fig. 3B, E, and M), and was significantly decreased by the HGF treatment (Fig. 3C, F, and M). The number of active NADPH oxidase-positive cells did not change in the dentate gyrus after ischemia, regardless of treatment or not with HGF (Fig. 3J–K and N). We confirmed that NADPH oxidase activity in CA1 region was not detectable in the presence of dicumarol, a NADPH oxidase inhibitor (Fig. 3G–I).

We previously demonstrated that HGF attenuated apoptotic cell death in the hippocampal CA1 region after transient forebrain ischemia [21,22]. Although HGF may have done so by preventing oxidative DNA damage [22], the mechanism for its effect remains obscure in the *in vivo* ischemic brain. So we focused on the molecules involved in DNA oxidation to determine a possible mechanism for the protective effect of HGF against cell death after transient forebrain ischemia.

APE/Ref-1 is a multifunctional protein in the DNA base repair pathway. A decrease in the level of Ref-1 protein was implicated in oxidative stress associated with apoptotic neuronal cell death after transient forebrain ischemia [11]. Also the overexpression of APE/Ref-1 protein in Drosophila reduced the somatic mutation and recombination frequency caused by oxidative DNA damage [28]. We previously demonstrated that transient forebrain ischemia increased expression of 8-OHdG, as an indicator of oxidative DNA damage, at 6 h after the start of reperfusion [21]. Therefore, decreased level of immunoreactivity and number of APE/Ref-1-positive cells may be involved in elevated

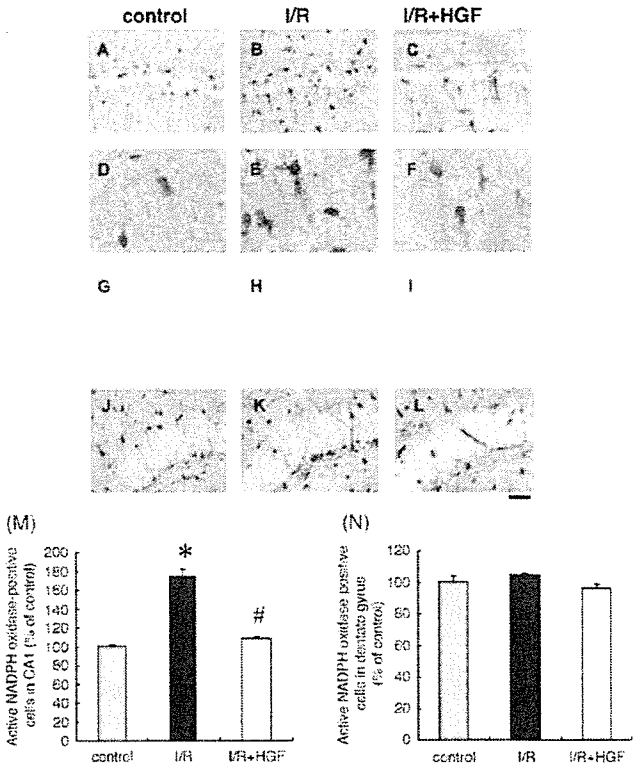

Fig. 3. Effect of HGF on the number of active NADPH oxidase-positive cells in the hippocampal CA1 region and dentate gyrus after transient forebrain ischemia. Representative photomicrographs of activity staining for NADPH oxidase in the absence of 10 μM dicumarol in the hippocampal CA1 region of non-operated naïve rats (A, D), ischemic rats at 6 h of reperfusion without (B, E) or with (C, F) HGF treatment. "D–F" show enlarged areas of "A–C," respectively. Representative photomicrographs showing lack of NADPH oxidase activity in the presence of 10 μM dicumarol in the hippocampal CA1 region of non-operated naïve rats (G), ischemic rats at 6 h of reperfusion without (H) or with (I) HGF treatment. Scale bar represents 50 μm (A–C, G–I) and 15 μm (D–F). Representative photomicrographs of activity staining for NADPH oxidase in the absence of 10 μM dicumarol in the hippocampal dentate gyrus of non-operated naïve rats (J), ischemic rats at 6 h of reperfusion without (K) or with (L) HGF treatment. Effect of HGF on the number of active NADPH oxidase-positive cells in the hippocampal CA1 region (M) and dentate gyrus (N) of ischemic rats at 6 h of reperfusion. The percentage of active NADPH oxidase-positive cells within a field was calculated. Results are the mean percentages of the non-operated naïve control ±S.E. $n=3$. $^{*}p<0.05$ vs. non-operated naïve control, $^{\#}p<0.05$ vs. I/R6 h.

expression of 8-OHdG at the early period after the start of reperfusion. Our results suggest that HGF inhibited expression of 8-OHdG in the nucleus by preventing APE/Ref-1-psotive cells after ischemia. This effect of HGF is implicated in the inhibition of subsequent apoptosis-inducing factor (AIF) translocation to the nucleus [21]. Since the expression of APE/Ref-1 was down-regulated by oxidative stress after ischemia, we next examined the levels of ROS-eliminating enzymes, i.e., cytosolic superoxide dismutase (Cu/ZnSOD) and mitochondrial SOD (MnSOD). The levels of Cu/ZnSOD and MnSOD were not altered in the CA1 and dentate gyrus regions after ischemia compared with those of non-operated animals, irrespective of treatment with HGF after the ischemia. As for MnSOD, our results are consistent with those of the previous study in which the expression of MnSOD was not altered by HGF treatment [2]. Therefore, the prevention of the drop in the APE/Ref-1 level rather than changes in the expression of SODs at the early stage of the reperfusion may be involved in the protective effect of HGF.

We further examined the activity of NADPH oxidase, an enzyme that plays an important role in the production of superoxide in cells [30]. NADPH oxidase is localized at the plasma membrane in various types of cells, including hippocampal neurons and glial cells [16,26]. The activity of NADPH oxidase was increased after transient forebrain ischemia [3], and the inhibition of it reduced the infarct volume after transient cerebral ischemia [10]. Furthermore, neuronal injury after transient cerebral ischemia was reduced in NADPH oxidase KO mice [30]. In addition, it was demonstrated that hypoxia/reoxygenation induced the production of ROS in microglial cells [27] and that glucose deprivation induced the rapid accumulation of ROS in cultured astrocytes [23]. These findings indicate that the activation of NADPH oxidase in glial cells may contribute to oxidative cell damage after ischemia. In the present study, the activity of NADPH oxidase was increased in glia-like cells in the hippocampal CA1 region after ischemia, and this increase was attenuated by HGF treatment. However, we cannot fully exclude the possibility that the activity of NADPH oxidase in neurons may also contribute to cell damage after ischemia. The activity of NADPH oxidase in the dentate gyrus was neither altered at all after ischemia nor influenced by treatment with HGF. Our results thus suggest that the protective effects of HGF are related to the attenuation of the reduction in the neuronal APE/Ref-1 level and the inhibition of ROS production mediated by NADPH oxidase activation mainly in glia-like cells. It is possible that the improvement of APE/Ref-1 by HGF treatment is independent of the inhibition of NADPH oxidase activity. Therefore, further studies will be required to clarify whether the inhibition of NADPH oxidase activity by HGF leads to the improvement of APE/Ref-1 in the hippocampal CA1 region after transient forebrain ischemia.

There still remains a question as to whether ROS produced in glia-like cells can penetrate the plasma membrane or not. It is known that NADPH oxidase produces superoxide, which is changed to H_2O_2 by SODs [31]. Since H_2O_2 is a membrane-permeable ROS, it is possible that H_2O_2 produced in glia-like cells diffuses to neurons, enters them, and is subsequently changed into the hydroxyl radical, which would cause oxidative damage in neurons after the ischemia. However, we cannot fully rule out the possibility that ROS is produced in the neurons themselves and that HGF attenuates it.

Since HGF has the protective effect against neuronal cell death after ischemia, it may regard as a prospective agent available for the treatment of other neurological and neurodegenerative diseases. Thus, clarification of the mechanism underlying this protective effect of HGF is an important goal, and may lead to remarkable progress for the therapy against various neurological and neurodegenerative diseases. In the present study, we provided the first data demonstrating that treatment with HGF prevented the ischemia/reperfusion-induced reduction in neuronal APE/Ref-1 expression and concomitantly halted the increase in NADPH oxidase activity at the early period after the

start of reperfusion in the hippocampal CA1 region. Although the interaction between neurons and glial cells with respect to the protective effects of HGF is still not fully clear, the improvement of the neuronal APE/Ref-1 level and reduction in NADPH oxidase activity in glia-like cells may be related to the HGF-induced attenuation of ischemia-induced AIF translocation to the nucleus and subsequent neuronal cell death.

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Intrathecal Injection of Epidermal Growth Factor and Fibroblast Growth Factor 2 Promotes Proliferation of Neural Precursor Cells in the Spinal Cords of Mice With Mutant Human SOD1 Gene

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We investigated three steps of neural precursor cell activation—proliferation, migration, and differentiation—in amyotrophic lateral sclerosis spinal cord treated with intrathecal infusion of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) into the lumbar spinal cord region of normal and symptomatic transgenic (Tg) mice with a mutant human Cu/Zn superoxide dismutase (SOD1) gene. We observed that 5-bromodeoxyuridine (BrdU) + nestin double-labeled neural precursor cells increased in the spinal cords of Tg mice compared with non-Tg mice, with a much greater increase produced by EGF and FGF2 treatment. The number of BrdU + nestin double-labeled cells was larger than that of BrdU + ionized calcium-binding adapter molecule-1 (Iba1), BrdU + glial fibrillary acidic protein (GFAP), or BrdU + highly polysialylated neural cell adhesion molecule (PSA-NCAM) double-labeled cells, but none expressed neuronal nuclear antigen (NeuN). On further analysis of the gray matter of Tg mice, the number of BrdU + nestin and BrdU + PSA-NCAM double-labeled cells increased more in the ventral horns than the dorsal horns, which was again greatly enhanced by EGF and FGF2 treatment. Because neural precursor cells reside close to the ependyma of central canal, the present study suggests that proliferation and migration of neural precursor cells to the ventral horns is greatly activated in symptomatic Tg mice and is further enhanced by EGF and FGF2 treatment and, furthermore, that the neural precursor cells preferentially differentiate into neuronal precursor cells instead of astrocytes in Tg mice with EGF and FGF2 treatment.

Key words: neural precursor cells; epidermal growth factor; fibroblast growth factor 2; spinal cord; amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disease that is characterized by selective loss of central and peripheral motor neurons. There are many hypotheses about the cause of this disease, for example, glutamate toxicity (Bruijn et al., 2004; Ganel et al., 2006), axonal transport deficiency (Farah et al., 2003; Rao and Nixon, 2003; Kieran et al., 2005), lack of trophic factor (Murakami et al., 2003; Bruijn et al., 2004; Narai et al., 2005), and mitochondrial dysfunction (Menzies et al., 2002; Kirkinezos et al., 2005; Manfredi and Xu, 2005). In approximately 15–20% of familial ALS cases, a variety of dominant missense mutations or small deletions in the Cu/Zn superoxide dismutase (SOD1) gene has been identified (Deng et al., 1993; Rosen et al., 1993; Brown and Robberecht, 2001). Several lines of transgenic (Tg) mice have been established that express a mutant human SOD1 gene and thus act as valuable models of human ALS (Gurney et al., 1994; Wong et al., 1995). These Tg mice have proved to be the best model to date for ALS studies.

Recent studies have shown that the adult brain has the ability to produce new neurons (Doetsch et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002; Nakatomi et al., 2002). There are endogenous neural precursor cells in the subventricular zone and subgranular zone of the dentate gyrus. Division of these cells is enhanced by traumatic and ischemic brain injury, and differentiation of these cells into neurons is activated (Yagita et al., 2001; Iwai et al., 2002, 2003; Nakatomi et al., 2002;

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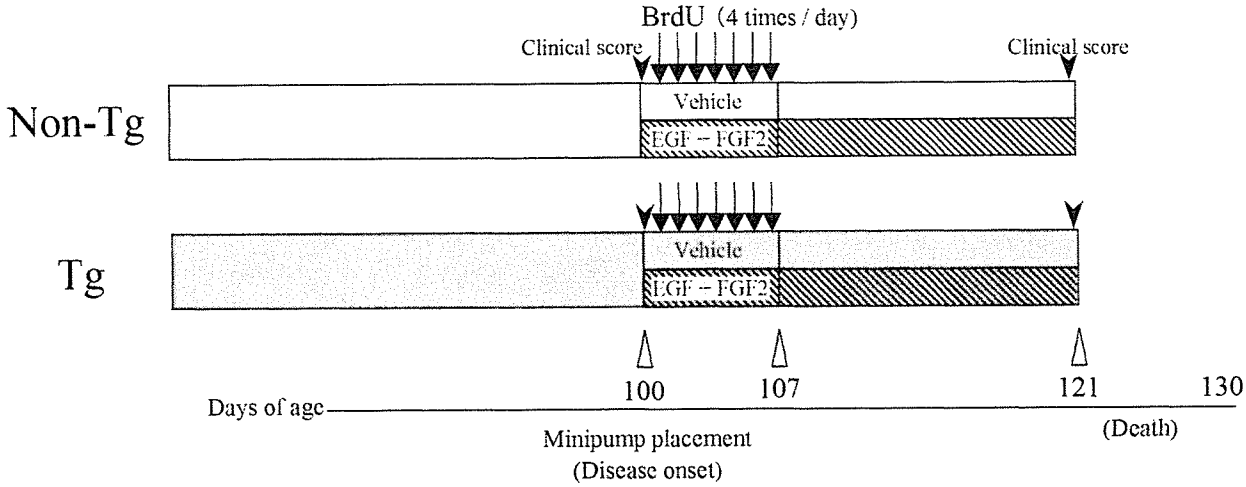

Fig. 1. Experimental paradigm for EGF and FGF2 treatment, BrdU injection, and sacrifice of mice. EGF and FGF2 or vehicle was first intrathecally infused in both non-Tg and Tg mice using osmotic minipumps at 100 days of age (average disease onset day) and lasting for 7 days. After minipump placement, clinical scores for all the mice were evaluated for the first time. BrdU was intraperitoneally injected four times per day for these 7 days in all groups. At 21 days after minipump placement, clinical scores were evaluated for the second time, and the mice were sacrificed.

Rice et al., 2003; Jin et al., 2003). In normal spinal cord, endogenous neural precursor cells reside close to the ependyma of the central canal (CC; Johansson et al., 1999; Martens et al., 2002), and these differentiate mainly into glial cells (Horner et al., 2000). In injured spinal cord, neural and glial precursor cells are activated to proliferate (Kojima and Tator, 2002; Lang et al., 2004; Mothe and Tator, 2005; Zai and Wrathall, 2005). However, most of these neural precursor cells differentiate into glial cells, and rarely into neurons. Proliferation of neural precursor cells in ALS spinal cord has not been reported in detail.

Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) have both neuroprotective and cell proliferative effects, but the combination of EGF and FGF2 has much stronger cell proliferative effects than either EGF or FGF2 alone in normal and injured brain and spinal cord (Alonso, 1999; Kojima and Tator, 2002; Martens et al., 2002; Nakatomi et al., 2002). However, a comprehensive study of stem cell activation in ALS spinal cord using EGF and FGF2 has not been reported. In the present study, therefore, we intrathecally infused EGF and FGF2 into the lumbar spinal cord region of normal mice and symptomatic Tg mice with a mutant human SOD1 gene and examined the proliferation, migration, and differentiation of intrinsic neural precursor cells in the lumbar spinal cord.

MATERIALS AND METHODS

Animal Model

All experimental procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine and Dentistry of Okayama University. A Tg mouse line with the G93A human SOD1 mutation (G1H/+) was obtained from Jackson Laboratories (Bar Harbor, ME; Gurney et al., 1994). G93A Tg mice experience disease onset at about 100 days of age and die approximately 20–30 days later. One-hundred-day-old G93A Tg male mice (Tg) and age-matched non-Tg wild-type B6SJL male littermates (non-Tg) were examined simultaneously. There were four experimental groups: non-Tg mice treated with vehicle (n = 6), non-Tg mice treated with recombinant human EGF (catalog No. 1376454; Roche Pharmaceutical, Tokyo, Japan) and recombinant human FGF2 (catalog No. 1120417; Roche; n = 6), Tg mice treated with vehicle (n = 8), and Tg mice treated with EGF and FGF2 (n = 8).

EGF and FGF2 Infusion

EGF and FGF2 were dissolved in phosphate-buffered saline (PBS) at a concentration of 20 μg/ml. For continuous intrathecal infusion into the lumbar spinal cord region of each animal at 100 days of age, the solution was loaded into an osmotic minipump (Alzet minipump, model 2001; Alza Corporation, Palo Alto, CA), which was designed for a nominal infusion rate of 1 μl/hr for 7 days and was attached to sterile polyethylene tubing (Becton Dickinson, Sparks, MD). Under anesthesia achieved by an intraperitoneal injection of ketamine hydrochloride (50 mg/kg; Sankyo Pharmaceuticals, Tokyo, Japan) and xylazine (12 mg/kg; Bayer AG, Leverkusen, Germany), the minipump was placed subcutaneousuly at a lumbar site. A rostrally directed cannula was threaded through the muscle close to the exposed region of the spinal column, and the tip of the cannula was placed in the spinal subarachnoid space at the level of L6 to S1. Infusion of EGF and FGF2 or vehicle (PBS) was continued for 7 days (Fig. 1).

5-Bromodeoxyuridine Labeling

A cell proliferation marker, 5-bromodeoxyuridine (BrdU; Sigma, St. Louis, MO), was dissolved in saline. After placement

of the minipump, intraperitoneal injections of BrdU began. Injections contained a dose of 50 mg/kg BrdU and were given every 6 hr (four times per day) for 7 days (total of 28 injections). By using this method, we were able to label all the cells that initiated DNA synthesis during days 1–7 (Fig. 1).

Clinical Scores

The clinical scores of the mice were evaluated twice: once following minipump placement and once 21 days after minipump placement (Fig. 1). Body weight was measured, and a rotarod test was administered to assess behavior. Based on our previous rotarod testing method (Abe et al., 1997; Klivenyi et al., 1999; Manabe et al., 2002), initially mice were placed on a rod that was rotated at 1 rpm. The speed was then increased by 1 rpm every 10 sec until the mouse fell off. The number of rotations before the mouse fell off was recorded as an indicator of each mouse's grasping power.

Motor Neuron Count

After the second clinical score evaluation, animals were deeply anesthetized with an intraperitoneal infusion of sodium pentobarbital (10 mg; Abbott Laboratories, Abbott Park, IL) and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The region of the spinal cord spanning L4–L5 was removed and further fixed by immersion in the same fixative for 4 hr, then frozen after cryoprotection with a series of phosphate-buffered sucrose solutions of increasing concentration (10%, 15%, and 20%).

Transverse sections of 10 μm thickness were cut through the middle of the L4 segment with a cryostat, and the motor neurons in L4 were counted in five transverse sections from each lumbar cord after staining with cresyl violet (Nissl stain). All cells in both ventral horns (VH) below a lateral line across the spinal cord from the central canal were microscopically video-captured, and only cells with a diameter greater than 20 μm that showed clear nucleoli were counted by investigators, who were blinded to the treatment conditions. In addition, five sections from each mouse were immunostained for choline acetyltransferase (ChAT goat antiserum; Chemicon, Temecula, CA) using a Vector ABC kit (Vector Laboratories, Burlingame, CA) to confirm the results of the motor neuron counting in the Nissl-stained sections.

Immunofluorescence Analysis

To determine whether the BrdU-labeled cells are neural or neuronal precursor cells, or are microglial cells, or differentiate into neuronal or astroglial cells, we performed double-immunofluorescence studies using BrdU plus nestin, highly polysialylated neural cell adhesion molecule (PSA-NCAM), ionized calcium-binding adapter molecule-1 (Iba1), neuronal nuclear antigen (NeuN), or glial fibrillary acidic protein (GFAP). To undertake detailed analysis for the BrdU + nestin and BrdU + GFAP double-labeled cells, we performed triple-immunofluorescence studies with BrdU, nestin, and GFAP. Nestin, PSA-NCAM, Iba1, NeuN, and GFAP are markers of neural precursor, neuronal precursor, microglial, differentiating neuronal, and astroglial cells, respectively.

The sections were first incubated in 2 N HCl at 25°C for 20 min to denature the DNA, then rinsed in 0.1 M boric acid (pH 8.5) at 25°C for 10 min. The sections were next incubated with rat monoclonal anti-BrdU (1:100; Oxford Biotechnology, Oxfordshire, United Kingdom) and antibody for each cellular marker, that is, mouse monoclonal anti-nestin (1:100; Chemicon), mouse monoclonal anti-PSA-NCAM (1:200; Chemicon), rabbit polyclonal anti-Iba1 (1:200; Wako, Osaka, Japan), mouse monoclonal anti-NeuN (1:400; Chemicon), or goat polyclonal anti-GFAP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) for double-immunofluorescence studies or rabbit polyclonal anti-GFAP (1:1,000; Dako, Copenhagen, Denmark) for triple-immunofluorescence studies overnight at 4°C. After being washed in PBS, the sections were incubated simultaneously with fluorescein isothiocyanate-labeled donkey anti-rat IgG (1:100; Chemicon) and Texas red-labeled horse anti-mouse IgG (1:100; Vector Laboratories), tetramethylrhodamine isothyocyanate (TRITC)-labeled goat anti-rabbit IgG (1:100; Vector Laboratories), TRITC-labeled rabbit anti-goat IgG (1:100; Vector Laboratories), or Alexa Fluor 405-labeled goat anti-rabbit IgG (1:100; Molecular Probes, Invitrogen, Eugene, OR) for 1 hr at room temperature. To confirm the specificity of the primary antibody, a set of sections was stained in a similar way without the anti-BrdU, anti-nestin, anti-PSA-NCAM, anti-Iba1, anti-NeuN, or anti-GFAP antibodies.

The sections for double-immunofluorescence studies were scanned by using a confocal microscope equipped with an argon and HeNe1 laser (LSM-510; Zeiss, Jena, Germany), and those for triple-immunofluorescence studies were scanned using a confocal microscope equipped with LD405, argon, and HeNe1 laser (FV300; Olympus, Tokyo, Japan). Sets of fluorescent images for double-immunofluorescence studies were acquired sequentially for the green and red channels to prevent crossover of signals from the green to the red or from the red to the green channels, and those for triple-immunofluorescence studies were acquired in the same manner. For quantification of the number of BrdU-labeled cells, the positively stained cells were counted in 1,024 pixels from a 450-μm × 450-μm area from five transverse sections of each lumbar cord per animal. We noted the total number and the regional differences among the gray matter (GM), white matter (WM), and CC and carried out closer analysis of the GM between the VH and both dorsal horns (DH), which are above a lateral line across the spinal cord from the central canal. In the same manner, the double-labeled cells were also counted for BrdU + nestin, BrdU + PSA-NCAM, BrdU + Iba1, BrdU + NeuN, and BrdU + GFAP.

Statistical Analysis

Results are expressed as mean ± SD. Statistical differences between groups with respect to body weight and rotarod test score were evaluated by using Welch's *t*-test. Differences between groups in the motor neuron count were evaluated by using Student's *t*-test. For the immunofluorescence studies, the cell count in each class was evaluated by using Mann-

TABLE I. Clinical Score at 121 Days of Age[†]

	Non-Tg		Tg	
	Vehicle	EGF + FGF2	Vehicle	EGF + FGF2
Body weight (g)	22.5 ± 2.8	20.3 ± 1.5	22.5 ± 1.9	23.1 ± 1.6
Rotarod score (rotation number)	371.6 ± 155.1	437.4 ± 104.9	63.3 ± 53.8*	62.4 ± 77.4**

[†]EGF and FGF2 or vehicle were intrathecally infused into lumber spinal cord region of non-Tg and Tg mice at 100 days of age using osmotic minipumps for 7 days. Clinical scores of the mice were evaluated at 121 days of age (21 days after minipump placement). Data are presented as mean ± SD of body weight (g) and rotation number in rotarod test.

*$P < 0.01$ compared with non-Tg mice with vehicle.

**$P < 0.01$ compared with non-Tg mice with EGF and FGF2.

Whitney's U test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Clinical Scores

After minipump placement, no significant differences were observed in either body weight or rotarod score between non-Tg and Tg mice or those with or without EGF and FGF2 (data not shown). After 100 days of age, Tg mice with and without EGF and FGF2 treatment began to show motor symptoms, such as dragging and atrophy of the hindlimbs, but they remained alive until they were sacrificed 21 days after minipump placement. No significant differences were observed between the groups with respect to body weight at this time (Table I). However, the Tg mice had significantly reduced rotarod scores ($P < 0.01$) in the groups with and without EGF and FGF2 compared with both groups of non-Tg mice. The rotarod scores were not significantly different in the Tg groups with and without EGF and FGF2.

Histological Observations

Histological evaluation of the L4 segment revealed substantial motor neuron loss in the VH of Tg mice treated with vehicle (230.5 ± 91.0, $P < 0.01$) compared with non-Tg mice treated with vehicle (561.4 ± 107.0) and in the VH of Tg mice treated with EGF and FGF2 (266.0 ± 80.4, $P < 0.01$) compared with non-Tg mice treated with EGF and FGF2 (563.5 ± 111.1; Fig. 2). However, treatment with EGF and FGF2 (266.0 ± 80.4) did not ameliorate motor neuron loss in Tg mice compared with Tg mice treated with vehicle (230.5 ± 91.0). Immunostaining for ChAT supported these results (data not shown).

Changes in BrdU-Labeled Cell

Among the two vehicle-treated groups, the total number of BrdU-labeled cells increased 2.0-fold in the lumbar spinal cords of Tg mice compared with the non-Tg mice at 21 days after minipump placement (Fig. 3, Table II). Upon regional analysis, most BrdU-labeled cells were found to be located in the WM, with some in the GM and CC (Fig. 3, Table II). This pattern was consistent in non-Tg and Tg mice. Upon closer analysis of the GM, the numbers of BrdU-labeled cells were found to be increased more in the VH than in the DH in Tg mice, although the number of BrdU-labeled cells was greater in the DH than the VH in non-Tg mice (Fig. 3, Table II).

With EGF and FGF2 treatment, the total number of BrdU-labeled cells in the lumbar spinal cord of both non-Tg and Tg mice increased 2.3- and 2.1-fold compared with the vehicle groups, respectively, and the total number of BrdU-labeled cells in the Tg mice was 1.9-fold that of the non-Tg group (Fig. 3, Table II). Upon regional analysis, the numbers of BrdU-labeled cells were found to be increased in all regions (WM, GM, and CC) in mice treated with EGF and FGF2 compared with vehicle treatment in both non-Tg and Tg mice (Fig. 3, Table II). Although most BrdU-labeled cells were located in the WM, with some in the GM and CC, the increase in the number of BrdU-labeled cells in the WM (1.0%) was smaller than that in the GM (1.2%) and CC (1.9%) in Tg mice treated with EGF and FGF2. Upon closer analysis of the GM, the number of BrdU-labeled cells increased more in both the VH and the DH relative to numbers in vehicle-treated mice in both non-Tg and Tg groups, and the number of BrdU-labeled cells increased more in the VH (39.9 ± 14.0) than in the DH (30.9 ± 13.3, $P < 0.01$) in Tg mice, although the increase was greater in the DH in non-Tg mice (Fig. 3, Table II).

Double-Immunofluorescence Analysis

Double labelling with BrdU and nestin, PSA-NCAM, Iba1, NeuN, or GFAP showed that many BrdU-labeled cells expressed nestin and also (but to a lesser extent) Iba1, GFAP, and PSA-NCAM, but none expressed NeuN in the lumbar spinal cords of either non-Tg or Tg mice with or without EGF and FGF2 treatment (Fig. 3, Table II). With EGF and FGF2 treatment, the increases in the rates of BrdU + nestin, PSA-NCAM, Iba1, or GFAP double-labeled cells in Tg mice were 1.3, 1.6, 1.2, and 0.5, respectively, so the increase in the rate of BrdU + nestin and BrdU + PSA-NCAM double-labeled cells was greater than that of BrdU + GFAP. Upon regional analysis of the GM of Tg mice treated with EGF and FGF2, the number of BrdU + nestin double-labeled cells was