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Prevention of apoptosis-inducing factor translocation is a possible mechanism for protective effects of hepatocyte growth factor against neuronal cell death in the hippocampus after transient forebrain ischemia

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Hepatocyte growth factor (HGF) is one of the prospective agents for therapy against a variety of neurologic and neurodegenerative disorders, although the precise mechanisms for the effect of HGF remain to be elucidated. We showed that treatment with HGF protected hippocampal cornu ammonis (CA) subregion 1 neurons from apoptotic cell death after transient forebrain ischemia. Accumulating evidence indicates that ischemia-induced neuronal damage occurs via caspase-independent pathways. In the present study, we focused on the localization of apoptosis-inducing factor (AIF), which is an important protein in the signal-transduction system through caspase-independent pathways, to investigate the possible mechanism for the protective effect of HGF after transient forebrain ischemia. Hepatocyte growth factor attenuated the increase in the expression of AIF protein in the nucleus after transient forebrain ischemia. We further explored the upstream components of AIF translocation. Primary DNA damage induced by Ca^{2+} influx and subsequent NO formation are thought to be the initial events for AIF translocation, which results in the subsequent DNA damage by AIF. Hepatocyte growth factor prevented the primary oxidative DNA damage, as was estimated by using anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) antibody. Oxidative DNA damage after ischemia is known to lead to the activation of poly(ADP-ribose) polymerase (PARP) and p53, resulting in AIF translocation. Marked increases in the PAR polymer formation and the expression of p53 protein after ischemia were effectively prevented by HGF treatment. In the present study, we first showed that HGF was capable of preventing neuronal cell death by inhibiting the primary oxidative DNA damage and then preventing the activation of the PARP/p53/AIF pathway.
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Introduction

Transient forebrain ischemia leads to the degeneration of vulnerable neurons in the brain, including pyramidal neurons in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region. As the degeneration of neurons induced by cerebral ischemia results ultimately in dysfunction of the central nervous system (CNS), it is an important objective to explore strategies for protecting cells from cerebral ischemia-induced death. In this context, treatment with several neurotrophic factors have been attempted to prevent ischemic brain injury and to restore normal neuronal function.

Hepatocyte growth factor (HGF) is a multifunctional cytokine originally identified and purified as a potent mitogen for hepatocytes (Nakamura *et al*,

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1984, 1987). Hepatocyte growth factor is known to evoke diverse cellular responses, including mitogenic, motogenic, morphogenic, angiogenic, and antiapoptotic ones in various types of cells (Nakamura *et al*, 1984, 1989; Matsumoto and Nakamura, 1996). Hepatocyte growth factor and its receptor c-Met were recently found to be expressed in the CNS (Honda *et al*, 1995; Achim *et al*, 1997), and to promote the survival of hippocampal and cortical neurons during the aging of cells in culture (Honda *et al*, 1995; Hamanoue *et al*, 1996). In addition, exogenous HGF prevented neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia in gerbils and attenuated the development of cerebral infarction after transient focal ischemia and widespread cerebral embolism in rats (Tsuzuki *et al*, 2001; Miyazawa *et al*, 1998; Hayashi *et al*, 2001; Date *et al*, 2004). These findings suggest that HGF has the ability to prevent cell injuries and to improve function in the CNS. Although such protective effects might be mediated by multipotent activities of HGF, including its antiapoptotic activity, their precise mechanisms remain unclear.

Although it is well known that caspase-dependent pathways play a role in apoptotic cell death after cerebral ischemia (Le *et al*, 2002; Niwa *et al*, 2001; Davoli *et al*, 2002; Wick *et al*, 2004), caspase inhibitors are likely to reduce ischemic injury after transient focal ischemia but not after a moderately long global ischemia (Li *et al*, 2000). The result indicates that caspase-independent pathways can contribute to cell death after transient forebrain ischemia. In this sense, accumulating evidence indicate that caspase-independent pathways are also involved in ischemia-induced neuronal damages (Zhang *et al*, 2005; Plesnila *et al*, 2004; Cao *et al*, 2003). An important protein in this pathway is thought to be the apoptosis-inducing factor (AIF), which is usually located in mitochondria in normal cells and acts as a mitochondrial oxidoreductase (Susin *et al*, 1999; Daugas *et al*, 2000). Once AIF is released from lesioned mitochondria, it produces reactive oxygen species (ROS) in the cytoplasm and also leads to large scale ($\sim$50 kbp) DNA fragmentation in the nucleus. This AIF-related apoptotic pathway is not affected by caspase inhibitors (Susin *et al*, 1999, 2000; Daugas *et al*, 2000; Cande *et al*, 2002). Cerebral ischemia appears to cause translocation of AIF from the mitochondria to the nucleus (Culmsee *et al*, 2005; Zhu *et al*, 2003; Plesnila *et al*, 2004; Zhao *et al*, 2004; Cao *et al*, 2003), suggesting that AIF plays a role in ischemia-induced neuronal cell death. Questions remain as to whether the inhibition of this caspase-independent pathway is involved in the protective effect of HGF in the ischemic brain, and if so, at what point in the process does HGF act. In the present study, we focused on the effect of HGF on the expression of AIF protein in the nucleus in the hippocampal CA1 region after transient forebrain ischemia and also explored the upstream components of AIF translocation. We first showed that HGF decreased nuclear translocation of AIF triggered by ischemia and reperfusion, which might be mediated by the prevention of primary oxidative DNA damage and the attenuation of subsequent activation of poly(ADP-ribose) polymerase (PARP) and p53.

Materials and methods

Recombinant Hepatocyte Growth Factor

Human recombinant HGF was purified from culture medium conditioned by Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA, as described earlier (Nakamura *et al*, 1989). The purity of HGF was >98%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Animal Model

Male Wistar rats weighing 200 to 250 g (Charles River Japan Inc., Atsugi, Japan) were used in the present study. The animals were housed in a cage and maintained on a 12-h light/12-h dark cycle at a temperature of 23°C$\pm$1°C with a humidity of 55%$\pm$5% throughout the experiment. The animals had free access to food and water according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guideline of Experimental Animal Care issued by the Prime Minister Office of Japan. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* techniques, if available. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Science. Transient (15 mins) forebrain ischemia was produced by the four-vessel occlusion procedure for rats described previously (Takagi *et al*, 2003). In brief, rats were anesthetized intraperitoneally with 40 mg/kg sodium pentobarbital. The right and left second cervical vertebras were exposed, and both visible vertebral arteries were permanently electrocauterized. Two silk threads were placed around both common carotid arteries without interrupting the blood flow. Twenty-four hour after electrocauterization, anesthesia was induced with 3% enflurane and maintained with 1.5% enflurane in a mixture of oxygen/nitrous oxide (25%/75%). Bitemporal subdermal electroencephalogram (EEG) needle electrodes were placed in reference to a frontal subdermal electrode. After a baseline EEG level had been established, both common carotid arteries were exposed and occluded with aneurysm clips for 15 mins. Then, the clips were removed, and the rat was allowed to recover. Rectal temperature was continuously monitored during ischemia and was maintained at 37.0°C to 37.5°C with a heating pad. Only rats that showed a completely flat EEG and a loss of consciousness during the occlusion were chosen for use in the present study. Sham-operated animals received exactly the same surgical procedure, but without the arterial occlusion. Each set of animals received the same

degree of surgical preparation and the same recovery paradigms to minimize variations that might result from surgical procedures.

In vivo Hepatocyte Growth Factor Treatment

Hepatocyte growth factor was diluted in physiologic saline and infused into the right hippocampal CA1 region by using an osmotic pump (Alzet model 1003D; DURECT Corp., Cupertino, CA, USA) attached to a 30-G 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. Before the start of infusion just after needle implantation, each osmotic pump was preincubated in physiologic saline at 37°C according to the instructions for use of the Alzet. The infusion of HGF was begun at 10 mins after the start of reperfusion at a flow rate of 1.0 μL/h and a concentration of 100 μg/mL (10 μg/3 days/animal). As a control, physiologic saline was used for the infusion.

Tissue Preparation

At various times after the start of reperfusion, animals were killed by decapitation, and their heads were quickly near-frozen in liquid nitrogen. The hippocampi were removed on ice, and hippocampal slices (730 μm) were prepared with a McIlewain tissue chopper (Brinkmann, Mickle Laboratory Engineering Co., Ltd, Gomshall, Surrey, UK). The hippocampal CA1 regions were dissected on ice in ice-cold 125 mmol/L Tris-HCl, pH 7.4, containing 320 mmol/L sucrose, 2 mmol/L sodium orthovanadate, 20 mmol/L sodium diphosphate decahydrate, 20 mmol/L DL-α-glycerophosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL each of antipain, aprotinin, and leupeptin (homogenization buffer). The dissected CA1 region was homogenized in the ice-cold homogenization buffer. The samples were stored at −80°C until used and were thawed only once.

Western Immunoblotting

Hippocampal CA1 homogenates that had been solubilized by heating at 100°C for 5 mins in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, and 2% SDS in 62.5 mmol/L Tris-HCl, pH 6.8) were separated on 10% or 12% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. 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) as described by the manufacturer. Quantification was performed by using computerized densitometry and an image analyzer (ATTO Co., Tokyo, Japan). Care was taken to ensure that bands to be semiquantified were in the linear range of response. For removal of bound antibodies, immunoblots were heated for 30 mins at 65°C in 62.5 mmol/L Tris-HCl buffer, pH 6.8, containing 2% SDS and 0.1 mol/L β-mercaptoethanol. The efficacy of the stripping procedure was confirmed by reacting the stripped blot with secondary antibody alone to ensure that no bound antibodies had remained. Antibodies used were antiphospho-NR2B (Tyr 1472) (Chemicon, Temecula, CA, USA), anti-NR2B (clone 13; Transduction Laboratories, Lexington, KY, USA), anti-AIF (Chemicon, Temecula, CA, USA), anti-heat-shock protein (Hsp) 70 (Calbiochem, La Jolla, CA, USA), and anti-α-tubulin (Sigma-Aldrich, St Louis, MO) antibody.

Immunoprecipitation

For immunoprecipitation of AIF, hippocampal CA1 tissues were lysed in a buffer containing 10 mmol/L Tris-HCl, pH 7.5, 0.5% Triton X-100, 150 mmol/L NaCl, 2 mmol/L sodium orthovanadate, 0.1 mmol/L phenylmethylsulfonyl, 5 μg/mL each of antipain, aprotinin, and leupeptin. The lysates were preincubated for 1 h with protein G–agarose beads and then centrifuged to remove any proteins that adhered nonspecifically to the protein G–agarose beads. The supernatant was then incubated at 4°C with anti-AIF antibody overnight. Next, protein G–agarose beads were added, and the incubation was continued at 4°C for 2 h. The immune complexes were isolated by centrifugation and washed, and the bound proteins were eluted by heating at 100°C in SDS sample buffer.

Histological Analysis

Animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (pH 7.4, phosphate buffer (PB)) under deep anesthesia. Their brains were quickly removed, cut into approximately 5-mm-thick coronal slabs, and postfixed overnight with 4% PFA in 0.1 mol/L PB. The slabs were embedded in paraffin and cut serially at 5 μm with a microtome. The coronal sections were then stained with cresyl violet acetate to assess neuronal damage. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were detected by using an *in situ* Apoptosis Detection Kit (MK500; Takara Bio Inc., Shiga, Japan). Surviving pyramidal cells and TUNEL-positive cells in the hippocampal CA1 region were counted under ×400 magnification (Olympus BX-52) in five to seven sections per animal. Results were expressed as the average number of cells per mm^2 in the areas comprising the hippocampal CA1 pyramidal cell layer. For immunostaining, sections were incubated with 100 mmol/L Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 30 mins, and then treated with 3% hydrogen peroxide for 5 mins to quench endogenous peroxidase. After blocking, the sections were incubated overnight at 4°C with mouse anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) (QED Bioscience, San Diego, CA, USA), mouse anti-poly(ADP-ribose) (PAR; Biomol, Plymouth Meeting, PA, USA), rabbit anti-AIF (Chemicon) or rabbit anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. After having been washed, the sections were incubated with biotinylated anti-rabbit immunoglobulin G (IgG) antibody (DAKO, Carpinteria, CA, USA) for AIF and p53 or with

biotinylated anti-mouse IgG antibody (DAKO) for 8-OHdG and PAR for 2 h and then with avidin: biotinylated enzyme complex solution (Vector) for 2 h. Color development was performed by incubating with 3,3′-diaminobenzidine and hydrogen peroxide (Vector). For 8-OHdG detection, sections were treated with RNase A (50 μg/mL in phosphate-buffered saline) at 37°C for 30 mins before blocking. Images were obtained by using an Olympus microscope (BX-52) or a Bio-Rad MRC 1024 confocal imaging system equipped with a krypton–argon laser and Nikon Diapot microscope, and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). The microscopic observations were performed by a person unaware of the study group.

Statistics

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

Results

Effect of Hepatocyte Growth Factor on Neuronal Cell Death after Transient Forebrain Ischemia

At first, we examined the effect of HGF on neuronal cell death in the hippocampal CA1 region of the four-vessel-occluded rats on day 3. Neuronal cell death in the hippocampal CA1 region after ischemia was significantly prevented by treatment with HGF at 10 μg/3 days/animal (Figures 1A–1C, Table 1). The dose used in the present study was based on the data obtained in our preliminary study, which showed that treatment at 10 μg/3 days/animal exerted the maximum protective effect. We next examined the effect of HGF on the number of TUNEL-positive cells in the hippocampal CA1 region after ischemia. The increase in the number of TUNEL-positive cells in the hippocampal CA1 region after ischemia was almost completely suppressed by HGF treatment (Figures 1D–1F, Table 1).

Effect of Hepatocyte Growth Factor on the Expression of Apoptosis-Inducing Factor in the Nucleus after Transient Forebrain Ischemia

To elucidate the mechanism for the antiapoptotic effect of HGF on the hippocampal CA1 neurons, we focused on the expression of AIF protein in the nucleus, which is one of the important proteins in the caspase-independent pathway. Immunoblotting showed that the total amount of AIF protein after ischemia was not altered regardless of treatment or not with HGF compared with that of nonoperated naïve control rats (Figure 2I). Apoptosis-inducing factor in nonoperated naïve rats was expressed in the neuronal cytoplasm (Figure 2A). The expression of AIF in the nucleus was evident at 24 h after the start of reperfusion (Figure 2C), and it became intensive at 36 h (Figure 2E). The number of AIF-positive nuclei increased after ischemia (Figures 2E and 2J), and this increase was attenuated by the HGF treatment (Figures 2G and 2J).

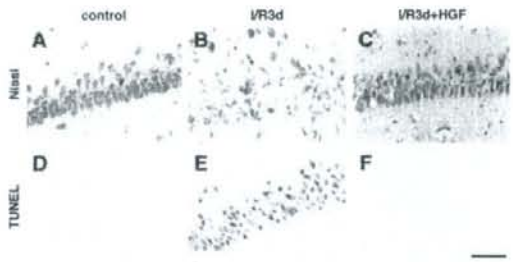

Figure 1 Effect of hepatocytes growth factor (HGF) on neuronal cell death in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A–C**) Photomicrographs of cresyl violet-stained hippocampal CA1 region of nonoperated naïve rats (**A**), four-vessel-occluded rats not treated with (**B**), or treated (**C**) with HGF at 10 μg/3 days/animal. (**D–F**) Photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-stained hippocampal CA1 region from nonoperated naïve rats (**D**), four-vessel-occluded rats not treated with (**E**), or treated (**F**) with HGF at 10 μg/3 days/animal. Scale bar represents 50 μm.

Table 1 Effect of HGF on the number of viable neurons in the hippocampal CA1 region after transient forebrain ischemia followed by 3-day reperfusion (I/R3day)

Cell number (cells/mm²)	*Control*	*I/R3day*	*I/R3day+HGF*
Cresyl violet-stained cells	77.03 ± 1.69	3.80 ± 0.95*	51.06 ± 17.23*
TUNEL-stained cells	0	77.02 ± 10.28*	2.57 ± 2.11*

CA1 = cornu ammonis (CA) subregion 1; HGF = hepatocyte growth factor; I/R = ischemia/reperfusion; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.
The number of cresyl violet- and TUNEL-stained cells were counted. Values represented the means ± s.e. $n = 3$–6. *$P < 0.05$ versus nonoperated naïve control; #$P < 0.05$ versus I/R3day, ANOVA with *post hoc* Scheffe.

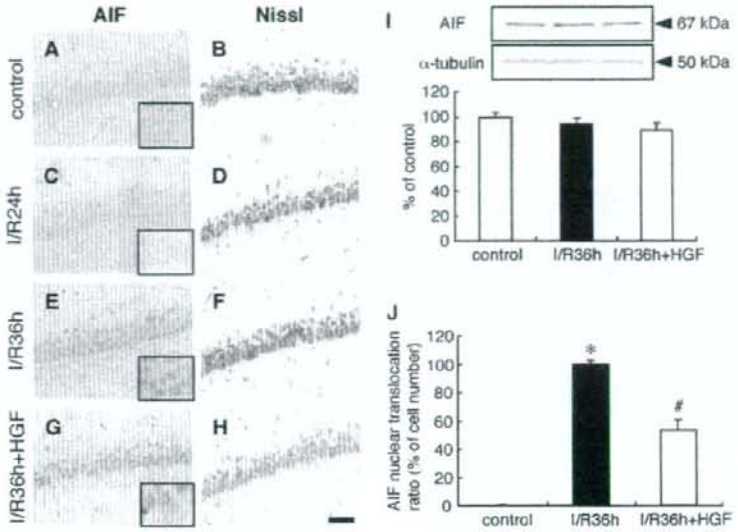

Figure 2 Effect of hepatocyte growth factor (HGF) on the expression of apoptosis-inducing factor (AIF) in the nucleus of the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A** and **B**) Photomicrographs of staining with anti-AIF antibody (**A**) and Nissl staining (**B**) in the hippocampal CA1 region of nonoperated naïve rats and its enlargement in the box in (**A**). (**C–F**) Photomicrographs of staining with anti-AIF antibody (**C**, **E**) and Nissl staining (**D**, **F**) in the hippocampal CA1 region of four-vessel-occluded rats at 24 h (**C**, **D**) and 36 h (**E**, **F**) of reperfusion, with enlargements in the boxes in (**C** and **E**). (**G** and **H**) Photomicrographs of staining with anti-AIF antibody (**G**) and Nissl staining (**H**) in the hippocampal CA1 region of four-vessel-occluded rats at 36 h of reperfusion with HGF treatment and its enlargement in the box in (**G**). Scale bar represents 50 and 10 μm (in enlargements). (**I**) Proteins (20 μg) from nonoperated naïve rats and four-vessel-occluded rats at 36 h of reperfusion without (I/R) or with HGF (I/R + HGF) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting with anti-AIF antibody. The blots were then stripped and reprobed with anti-α-tubulin antibody. Bands corresponding to AIF or α-tubulin were scanned, and the scanned bands of AIF were normalized by α-tubulin on the same blot. (**J**) Effect of HGF on the number of AIF-positive nuclei in the hippocampal CA1 region of four-vessel-occluded rats at 36 h of reperfusion. The percentage of AIF-positive cells among the total number of cells, which was estimated by Nissl staining within the same field of an adjoining section, was calculated. Results are the mean percentages of nonoperated naïve control $\pm$s.e. $n = 3$. $^*P < 0.05$ versus nonoperated naïve control, $^\#P < 0.05$ versus I/R36 h, analysis of variance (ANOVA) with *post hoc* Fischer's protected least significant difference.

Effect of Hepatocyte Growth Factor on the Interaction of Heat-Shock Protein 70 with Apoptosis-Inducing Factor after Transient Forebrain Ischemia

We next investigated the expression of Hsp70 protein after the start of reperfusion with or without HGF treatment, as Hsp70 is known to be an endogenous inhibitor of AIF. In nonoperated naïve rats, Hsp70 protein was barely expressed in the hippocampal CA1 region (Figure 3A). The amount of Hsp70 protein was significantly increased after transient ischemia, and the level of Hsp70 was not influenced by the HGF treatment (Figure 3A). Furthermore, we examined changes in the interaction of Hsp70 with AIF after ischemia with or without HGF treatment. Although the interaction of Hsp70 with AIF was elevated to 353.4% $\pm$ 124.1% of the control value after ischemia, it was not influenced by HGF treatment (393.9% $\pm$ 81.6%) (Figure 3B).

Effect of Hepatocyte Growth Factor on the Activity of the *N*-Methyl-D-Aspartate Receptor after Transient Forebrain Ischemia

The activation of the *N*-methyl-D-aspartate (NMDA) receptors after ischemia leads to a marked increase in Ca^{2+} influx, which causes activation of nitric oxide synthase (NOS) and subsequent production of nitric oxide (NO). Using antityrosine phosphorylated NR2B antibody, we examined tyrosine phosphorylation of the NR2B subunit at the src site, Y1472, in the NMDA receptor after ischemia with or without HGF treatment as an indicator of its activity. The tyrosine phosphorylation of the subunit at 1 h of reperfusion was 10 times larger than that of control rats, and it returned to the control level by 24 h of reperfusion (Figure 4A). Treatment with HGF did not affect the ischemia-induced tyrosine phosphorylation of the NR2B subunit at any of the all time points investigated (Figure 4A).

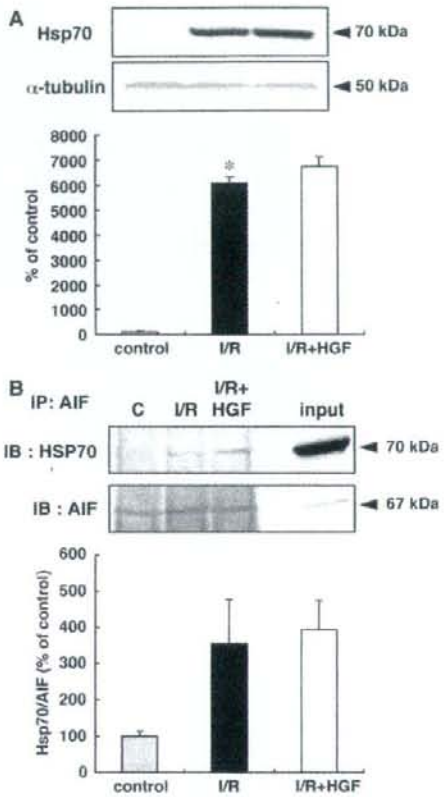

Figure 3 Effect of hepatocyte growth factor (HGF) on the expression of heat-shock protein 70 (Hsp70) and the interaction of Hsp70 with apoptosis-inducing factor (AIF) in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) Proteins (20 μg) from nonoperated naïve rats and four-vessel-occluded rats at 36 h of reperfusion without (I/R) or with HGF (I/R + HGF) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting with anti-Hsp70 antibody. The blots were then stripped and reprobed with anti-α-tubulin antibody. Bands corresponding to Hsp70 or α-tubulin were scanned, and the scanned bands of Hsp70 were normalized by α-tubulin on the same blot. Results are the mean percentages of the nonoperated naïve control ± s.e. (**B**) Proteins (500 μg) from nonoperated naïve rats and four-vessel-occluded rats at 36 h of reperfusion without (I/R) or with HGF (I/R + HGF) were immunoprecipitated (IP) with anti-AIF antibody, and the precipitates were then separated by SDS-PAGE and subjected to immunoblotting (IB) with anti-Hsp70 antibodies. The blots were subsequently stripped and reprobed with anti-AIF antibodies. Bands corresponding to AIF or Hsp70 were scanned, and the scanned bands of Hsp70 were normalized by precipitated AIF on the same blot. Results are the mean percentages of the nonoperated naïve control ± s.e. $n = 3$. $^*P < 0.05$ versus nonoperated naïve control, analysis of variance (ANOVA) with *post hoc* Fischer's protected least significant difference.

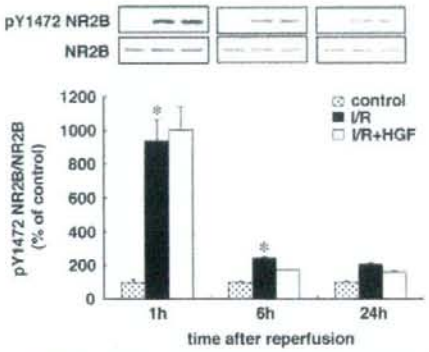

Figure 4 Effect of hepatocyte growth factor (HGF) on phosphorylation of the *N*-methyl-D-aspartate (NMDA) receptor in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. Proteins (50 μg) from nonoperated naïve rats and four-vessel-occluded rats at 1, 6, and 24 h of reperfusion without (I/R) or with HGF (I/R + HGF) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting with anti-phospho-NR2B antibody [pY1472 NR2B]. The blots were then stripped and reprobed with anti-NR2B antibody [NR2B]. Bands corresponding to pY1472 NR2B or NR2B were scanned, and the scanned bands of pY1472 NR2B were normalized by NR2B on the same blot. Results are the mean percentages of the nonoperated naïve control ± s.e. $n = 3$. $^*P < 0.05$ versus nonoperated naïve control, analysis of variance (ANOVA) with *post hoc* Fischer's protected least significant difference.

Effect of Hepatocyte Growth Factor on Oxidative DNA Damage Induced by Transient Forebrain Ischemia

Next, we examined the immunoreactivity of 8-OHdG, which is often used as a marker of oxidative DNA damage. We at first examined the time course of changes in its immunoreactivity after transient forebrain ischemia. The immunoreactivity of 8-OHdG was very faint in nonoperated naïve rats (Figure 5A), whereas it increased at 30 mins of reperfusion (Figure 5B). The maximum increase in the immunoreactivity was detected at 6 h of reperfusion (Figure 5D), and the immunoreactivity gradually disappeared thereafter (Figures 5E and 5F). The prominent increase in 8-OHdG expression in the nucleus at 6 h of reperfusion was almost completely suppressed by the HGF treatment (Figure 5G).

Effect of Hepatocyte Growth Factor on Poly(ADP-Ribose) Polymer Formation after Transient Forebrain Ischemia

Poly(ADP-ribose) polymerase is activated in response to DNA damage. We next examined poly(ADP-ribose) (PAR) polymer formation as a marker of PARP activity by using anti-PAR antibody.

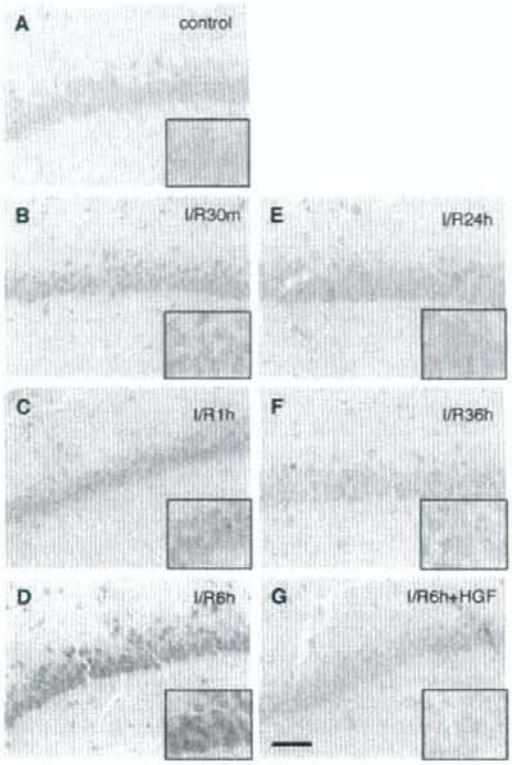

Figure 5 Effect of hepatocyte growth factor (HGF) on oxidative DNA damage in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) antibody in the hippocampal CA1 region of nonoperated naïve rats and its enlargement in the box. (**B–F**) Photomicrographs of staining with anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) antibody in the hippocampal CA1 region of four-vessel-occluded rats at 30 mins (**B**), 1 h (**C**), 6 h (**D**), 24 h (**E**), and 36 h (**F**) of reperfusion and an enlarged area in each box. (**G**) A photomicrograph of staining with anti-8-OHdG antibody in the hippocampal CA1 region of four-vessel-occluded rats at 6 h of reperfusion with HGF treatment and its enlargement in the box. $n = 3$. Scale bar represents 50 and 10 μm (in enlargements).

PAR polymer formation in naïve control was barely detected in the cytoplasm, whereas this formation was seen neither in the dendrites nor in the nuclei (Figure 6A). The immunoreactivity gradually rose, and the maximum increase in the nuclei was seen at 6 h of reperfusion (Figure 6D), which was comparable to the changes in the expression of 8-OHdG. Treatment with HGF suppressed ischemia-induced PAR polymer formation in the nuclei at 6 h of reperfusion (Figure 6F).

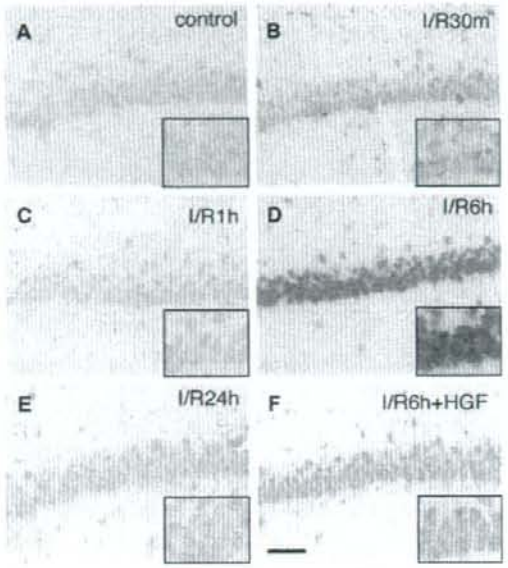

Figure 6 Effect of hepatocyte growth factor (HGF) on poly(ADP-ribose) polymer formation in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-poly(ADP-ribose) (PAR) antibody in the hippocampal CA1 region of nonoperated naïve rats and its enlargement in the box. (**B–E**) Photomicrographs of staining with anti-PAR antibody in the hippocampal CA1 region of four-vessel-occluded rats at 30 mins (**B**), 1 h (**C**), 6 h (**D**), and 24 h (**E**) of reperfusion and an enlarged area in each box. (**F**) A photomicrograph of staining with anti-PAR antibody in the hippocampal CA1 region of four-vessel-occluded rats at 6 h of reperfusion with HGF treatment and its enlargement in the box. $n = 3$. Scale bar represents 50 and 10 μm (in enlargements).

Effect of Hepatocyte Growth Factor on the Expression of p53 Protein after Transient Forebrain Ischemia

Oxidative DNA damage is known to induce p53 activation, leading to AIF translocation to the nucleus. So finally, we examined the expression of p53 protein after ischemia with or without HGF treatment. Faint cytoplasmic expression of p53 protein was found in the naïve control (Figure 7A), whereas the immunoreactivity gradually increased up to 6 h of reperfusion (Figures 7B–7D). The increased immunoreactivity remained at 36 h of reperfusion (Figures 7E–7F). The notable increase in the expression of p53 at 6 h of reperfusion was prevented by HGF treatment, making the expression comparable to that for the nonoperated naïve control (Figure 7G).

Discussion

We showed that treatment with HGF protected hippocampal CA1 neurons from apoptotic cell death

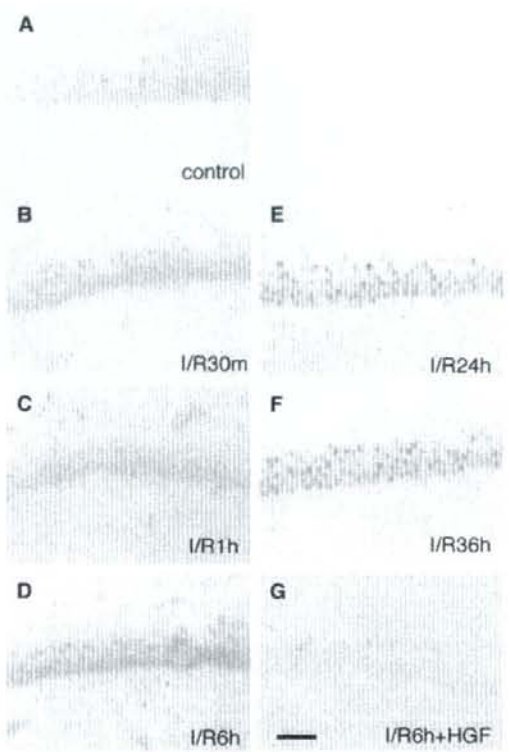

Figure 7 Effect of hepatocyte growth factor (HGF) on the expression of p53 in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-p53 antibody in the hippocampal CA1 region of nonoperated naïve rats. (**B–F**) Photomicrographs of staining with anti-p53 antibody in the hippocampal CA1 region of four-vessel-occluded rats at 30 mins (**B**), 1 h (**C**), 6 h (**D**), 24 h (**E**), and 36 h (**F**) of reperfusion. (**G**) A photomicrograph of staining with anti-p53 antibody in the hippocampal CA1 region of four-vessel-occluded rats at 6 h of reperfusion with HGF treatment. $n = 3$. Scale bar represents 50 μm.

after transient forebrain ischemia. Although caspases have been identified as major molecules in the mechanism responsible for apoptotic cell death, accumulating evidence recently indicates that caspase-independent mechanisms may also play an important role in cell death (Rideout and Stefanis, 2001; Johnson *et al*, 1999; Lankiewicz *et al*, 2000; Zhan *et al*, 2001; Miller *et al*, 1997; D'Mello *et al*, 2000; Keramaris *et al*, 2000; Selznick *et al*, 2000). In the caspase-independent pathway, AIF is characterized as one of the crucial proteins (Cregan *et al*, 2004). Apoptosis-inducing factor is located in the mitochondrial intermembrane space in intact cells, but it is translocated to the nucleus after cerebral ischemia, and thereby causes DNA fragmentation in a caspase-independent manner (Culmsee *et al*, 2005; Zhu *et al*, 2003; Plesnila *et al*, 2004; Zhao *et al*, 2004; Cao *et al*, 2003). In the present study, we focused on the localization of AIF in cells to investigate the possible mechanism of the protective effect of HGF after transient forebrain ischemia. Our findings show that HGF attenuated the increase in the expression of AIF in the nucleus after ischemia without a change in the total amount of AIF, suggesting that the inhibition of AIF translocation to the nucleus contributes to the protective effect of HGF on apoptotic cell death.

To further investigate the mechanism for inhibiting AIF translocation after HGF treatment, we examined the expression of Hsp70 and its interaction with AIF. Only Hsp70 among Hsp family proteins, including Hsp10, 27, 60, 70, and 90, is regarded to be an endogenous inhibitor of AIF, as it directly binds to AIF and inhibits the import of AIF into the nucleus (Ravagnan *et al*, 2001; Gurbuxani *et al*, 2003). In this sense, it was recently reported that overexpression of Hsp70 proteins induced an increase in the interaction of Hsp70 with AIF, and reduced neonatal hypoxic/ischemic brain injury (Matsumori *et al*, 2005). We showed that although both the expression of Hsp70 and interaction with AIF were increased after transient cerebral ischemia, they were not influenced by HGF treatment. Therefore, it is conceivable that neither the amount of Hsp70 protein nor the interaction of Hsp70 with AIF contributes to the neuroprotective mechanism of HGF after transient forebrain ischemia.

Primary DNA damage induced by neuronal NOS (nNOS) activation and NO formation is thought to be an initial event leading to AIF release from mitochondria, which release results in subsequent secondary DNA damage (DNA fragmentation) by AIF (Yu *et al*, 2003). It is widely accepted that excessive Ca^{2+} influx through the activated NMDA receptor, which activation can be estimated by tyrosine phosphorylation of the NR2 subunit of the NMDA receptor (Wang and Salter, 1994; Kohr and Seeburg, 1996; Zheng *et al*, 1998; Chen and Leonard, 1996), leads to the activation of nNOS and subsequent production of NO (Castilho *et al*, 1998; Dawson *et al*, 1991). To determine whether protective effect of HGF required the phosphorylation-dependent activities of the NMDA receptor, we examined tyrosine phosphorylation of NR2B subunit of the NMDA receptor. Although tyrosine phosphorylation of NR2B subunit was significantly increased after the start of reperfusion compared with that of the naïve control, it was not influenced by HGF treatment. These results suggest that HGF treatment altered neither the intracellular Ca^{2+} concentration regulated by tyrosine phosphorylation of the NR2B subunit nor the production of NO after transient forebrain ischemia.

Calcium influx through the activated NMDA receptor elicits production of not only NO but also

mitochondrial ROS, leading to formation of peroxynitrite ($ONOO^-$) and subsequent hydroxyl radical, which eventually results in oxidative DNA damage (Yu *et al*, 2003). To assess oxidative DNA damage after ischemia with or without HGF treatment, we measured 8-OHdG as an indicator of oxidative DNA damage, as its expression is elevated after oxidative DNA damage (Pastoriza Gallego and Sarasin, 2003; Toyokuni *et al*, 1997). In fact, the expression of 8-OHdG was shown earlier to be increased after transient forebrain ischemia (Won *et al*, 2001; Hwang *et al*, 2004; Baek *et al*, 2000). In agreement with these findings, we showed that the expression of 8-OHdG was elevated at the early period after the start of reperfusion. It is noteworthy that this elevated 8-OHdG expression was almost completely suppressed by HGF treatment. Our results suggest that HGF inhibited AIF translocation to the nucleus by preventing the primary oxidative DNA damage after ischemia.

Oxidative DNA damage is also known to lead to the activation of PARP and p53 after ischemia (Komjati *et al*, 2004; Koh *et al*, 2004; Nagayama *et al*, 2000; Banasiak and Haddad, 1998; McGahan *et al*, 1998; Huang *et al*, 1995; Renolleau *et al*, 1997; Tomasevic *et al*, 1999). Poly(ADP-ribose) is a DNA repair enzyme that reveals its activity by utilizing nicotinamide adenine dinucleotide$^+$ as a substrate. Therefore, excessive DNA damage induces a marked activation of PARP to repair DNA, and thereby depletes energy, which results in the release of cytochrome *c*, endonuclease G, and AIF (Yu *et al*, 2003; Meli *et al*, 2003). In addition, activation of p53 induced by DNA damage elicits ROS production and subsequent mitochondrial membrane disruption, which are associated with cytochrome *c*-independent apoptosis (Li *et al*, 1999). It has also been shown that AIF translocation is involved in p53-mediated neuronal injury (Cregan *et al*, 2004). Taking these findings into consideration, PARP and p53 activation after ischemia might be one of the steps in the AIF-dependent apoptotic pathway. Therefore, we further investigated the activity of PARP, which was assessed by the immunohistochemistry using an anti-PAR antibody to detect PAR polymer formation, and the expression of p53 protein after ischemia with or without HGF treatment. Marked increases in the PAR polymer formation and the expression of p53 protein after ischemia were effectively prevented by HGF treatment, suggesting that HGF reduced AIF translocation after ischemia by inhibiting PARP and p53 activation.

Questions remain as to how HGF can suppress primary oxidative DNA damage. It has been shown that HGF protected cardiomyocytes from H_2O_2-stimulated apoptosis by increasing Bcl-X_L expression (Nakamura *et al*, 2000) and by activating the MAP kinase kinase-mitogen-activated protein kinase pathway (Kitta *et al*, 2001). Furthermore, extracellular signal regulated kinase activation downregulated p53 in cancer cells, thereby reducing ROS production and subsequent depolarization of the mitochondrial membrane (Ostrakhovitch and Cherian, 2005). Recently, activation of the phosphatidylinositol 3′-kinase-Akt pathway induced by HGF protected hepatocytes from hypoxia-reoxygenation-induced oxidative stress and apoptosis by inhibiting the activation of rac1 small GTPase (Ozaki *et al*, 2003). Hepatocyte growth factor itself is unlikely to exert a direct effect on the redox state (Ozaki *et al*, 2003). Whereas HGF prevented ceramide-induced apoptosis by increasing catalase expression, the signaling cascade via c-Met to induce the expression of catalase remains unclear (Kannan *et al*, 2004). Therefore, further studies will be required to determine signal-transduction pathways via c-Met, which may inhibit the primary oxidative DNA damage that occurs in the ischemic brain *in vivo*.

We recently showed that HGF protected cultured hippocampal neurons against NMDA-induced excitotoxicity via the partial prevention of caspase-3 activity and the inhibition of AIF translocation to the nucleus (Ishihara *et al*, 2005). Therefore, although the inhibition of AIF-dependent pathway contributes to the protective effects of HGF, we cannot fully rule out the possibility that HGF can prevent cell death through the inhibition of caspase-dependent pathway in the ischemic brain. Alternatively, our results suggest that the potent protective effects of HGF on apoptotic cell death after transient forebrain ischemia might be mediated by the inhibition of AIF translocation in addition to a prevention of caspase-dependent pathway.

Although we suggest that suppression of the primary oxidative damage at the early stage after transient forebrain ischemia is, at least, involved in the protective effects of HGF, whether HGF inhibits the translocation of AIF by attenuating oxidative DNA damage remains to determine. It was recently showed that Bcl-2 transfection in the peri-infarct region blocked AIF translocation to the nucleus and prolonged cortical neuron survival (Zhao *et al*, 2004). Therefore, it is possible that HGF inhibits translocation of AIF after transient forebrain ischemia mediated by an expression of Bcl-2 family proteins, such as Bcl-2.

Although HGF has the ability to prevent ischemic brain injuries and is a prospective agent for therapy against a variety of neurologic and neurodegenerative disorders, the intracellular signaling associated with its protective effects is not fully understood. It is thus an important objective to elucidate the molecular basis of the protective effects of HGF under pathologic conditions. In the present study, we showed that HGF was capable of preventing *in vivo* ischemia-induced neuronal cell death by inhibiting the primary oxidative DNA damage and then preventing activation of the PARP/p53/AIF pathway.

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ALSに対する新しい治療薬としての肝細胞増殖因子(HGF)の研究

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筋萎縮性側索硬化症（ALS）と診断された方のうち、ごく一部の方が家族性（遺伝性）ALSに属し原因遺伝子の同定が行われていますが、残りの多くの方は原因不明の孤発性ALSに分類されています。それでは、原因が不明であれば治療法を諦めなければならないのでしょうか？　私達はそうは考えていません。

ALSは、運動神経細胞が特異的に変性・脱落することにより運動麻痺をきたす点では共通しているのです。逆に言うと、運動神経細胞変性の予防・進行を軽減できたら、ALSに対して大きな治療効果があがると期待されているのです。肝細胞増殖因子（以下、HGF）こそ、私達はALSの運動神経細胞変性を抑制できる薬として期待しているのです。ここでは、HGFのもつALSモデル動物に対する治療効果について記述します。そしてヒトALS治療法開発へ向けた最近の研究の進歩について解説します。

1. 肝細胞増殖因子(HGF)とは？

肝細胞増殖因子（Hepatocyte growth factor: HGF）と聞いてALSと何が関係しているか不思議に思う方もおられるかもしれません。HGFは名前の通り、肝臓の再生を担う因子として世界に先駆け日本で大阪大学中村敏一らにより発見・精製・クローニングされた物質です。HGFは、はじめ肝臓の再生因子としての機能に注目し研究を進めましたが、その後の研究の発展により生体内の種々の臓器の細胞保護・再生を担う分子としての機能が、多彩な動物疾患モデルで明らかとなってきています。例えば、急性肝障害や肝硬変モデル、急性腎炎や慢性腎炎モデル、心筋虚血や肺繊維症モデル等に対する治療効果の報告があります。

それでは神経系ではどうでしょうか？　HGFは、様々な神経細胞に対して細胞保護効果を示す新規神経栄養因子として機能します。ALSで問題となる運動神経細胞に対する細胞保護効果は、中でも強力であることが明らかとなっています。HGFが運動神経系の発生過程でも必須の因子であることは、HGFの受け手側であるc-Met受容体の一部の機能を欠失したマウス（ノックインマウス）の解析でも明らかにされています。

すなわち、上記マウスではHGFの機能がc-Metを介して十分行えないために、筋肉の発達と神経の筋肉への伸長がうまくいかなくなります。この結果は、HGF がALSで特異的に変性する運動神経系の発達に必須の因子であることを示しています。これらHGFの機能を背景に、私達はHGFがALSに起こる運動神経系の変性を抑制・治療できないかALSモデル動物を用いて解析を進めています。

2. HGFのALSモデル動物に対する治療効果

1) ALSモデル動物

ALSに対するHGFの治療効果の解析には、まずALSモデル動物が必要です。私達はALSモデル動物として1994年にGurneyらにより作出された動物を使用し研究をすすめました。この動物は家族性ALSの原因遺伝子であるsuperoxide dismutase-1 (SOD1)の93番目のアミノ酸がGからAに変異したヒトSOD1G93Aを強制的に発現させたマウスで、ヒトのALSをよく反映したモデル動物です（以下ALSマウスと呼びます）。この動物は、生後約7ヵ月半で運

動麻痺を発症し、その後約2週間で死亡します。

2) HGFはALSモデルマウスの運動神経細胞の変性を抑制し、運動機能を改善し、寿命を延長する

ALSマウスの運動神経細胞にHGFを効率よく供給することでHGFの治療効果を解析するために、私達は以下の方法をとりました。まず、神経細胞でHGFを大量に産生するマウスを作出します（以下HGFマウスと呼びます）。ALSマウスとHGFマウスとを交配しますと、ALSマウスの神経細胞にHGFを大量に供給することが可能になります（このマウスを以下ALS/HGFマウスと呼びます）。そうすると、ALSマウスに比べてALS/HGFマウスでは、ほぼすべての広範囲の運動神経細胞死が減少し、寿命が約1ヵ月延びることが明らかとなりました。

この結果は、ヒトに単純換算すると寿命を6年間延ばしたことに相当します。ALS/HGFマウスでは運動機能も大幅に改善することから、HGF治療では、動物レベルでは運動機能を維持しながら延命効果をもつと言えます。HGFの特徴は、運動神経細胞に直接作用して神経細胞保護作用を示すこと、もうひとつの特徴はグリア細胞（非神経細胞）にも働いて間接的に運動神経細胞保護作用を示すことです。いいかえると、HGFは神経細胞とグリア細胞に別々に機能し、その二重効果により強力に運動神経細胞保護作用を示す治療分子といえます（図1）。現時点でこのような二重効果がはっきり示されている因子はHGFだけといえます。

3. HGFとその受容体はヒトでもALSモデル動物と同様の発現制御を受けている

さて、それではHGFのALSマウスの治療効果はヒトALSでも同等と考えていいのでしょうか？　上記結果はあくまで家族性ALS（FALS）の動物モデルの結果ですので、孤発性ALSでは結果が異なる可能性もないとはいえません。

実際ヒトに投与していない段階で孤発性ALSに対するHGF治療効果を推察することは難しいですが、ヒトALS患者の病理組織像および生化学解析で以下のような結果を得ています。

まず、HGFとその受容体c-Metの発現は、家族性

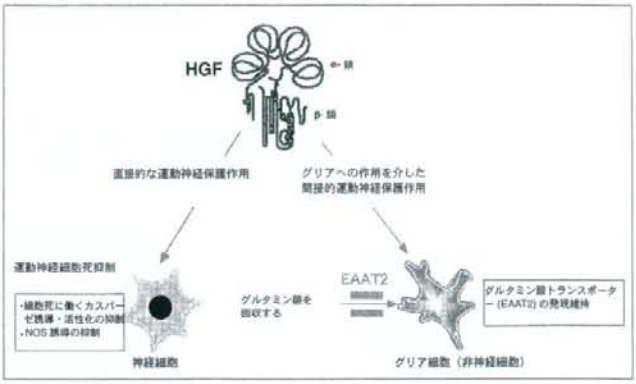

図1．HGFの構造とHGFのALS進行抑制・治療効果の作用機序．HGFはα-鎖とβ-鎖から構成されている（Nakamura T他 Nature, 1989）。HGFは、運動神経細胞に直接作用して運動神経細胞を細胞死ストレスから保護する。HGFはグリア細胞にも働いて、グルタミン酸をグリア細胞に回収する効率をあげる。このことで間接的に運動神経細胞をグリタミン酸毒性（ALSで問題となる）から保護する。HGFは、運動神経細胞をALSストレスから保護することで運動機能を維持し、寿命を延長します。

ALSでも孤発性ALSでも、私達がALSマウスで解析した結果とほとんど同じような発現をしていました。このことは孤発性ALSにおいてもHGF-c-Metが家族性ALSマウスと同様、ALS進行を抑制するように発現制御をうけていると期待される結果でした。これらの結果から、ALSマウスの時と同様にHGFの神経への供給が効率よくできたら家族性ALSに加えて孤発性ALSの進行が遅延し、治療効果が得られることが期待されています。

4. HGFの臨床適用への道

私達はALSマウスへHGFを遺伝子の形で供給して治療効果があることを示してきました。それではヒトの治療を考えた際は同じ方法が可能でしょうか？私達はヒトのALS治療にはHGF蛋白質を使用する方が有利と考えています。それは、遺伝子治療に比べて多くの病院で治療を受けやすいからです。

私達はそのためにいくつかの大学と共同でヒトALS治療に向けたHGF蛋白質の供給方法の検討を行っています。これまでに東北大学との共同研究で、実際HGF蛋白質投与でALSラット（東北大学神経内科青木先生、糸山教授の教室で開発されたALSモデル動物）で治療効果があることが明らかとなっています。現在は、さらにサルを用いたHGFの運動神経細胞保護作用の確認を行っている段階です。私達は実際のALS治療法開発に向けて今後さらに研究・努力していきたいと考えています。そして、少しでも研究成果がALSへの福音となることが私達の心からの夢なのです。

（資料 2.）

平成 19 年度　総括研究報告書

研究成果に関する一覧表およびその刊行物

厚生労働科学研究費補助金（こころの健康科学研究事業）
研究報告書

筋萎縮性側索硬化症に対する肝細胞増殖因子を用いた画期的治療法の開発

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研究要旨：本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（HGF）を用いた画期的治療法を開発することとそれに関わる基盤研究を進めることにある。ALS の病因研究および治療研究には変異 Cu/Zn superoxide dismutase（SOD1）遺伝子導入 ALS ラットが重要な役割を果している。私共はこの ALS ラットを用いて運動ニューロンに対し神経栄養因子作用を有する recombinant human HGF（rhHGF）の髄腔内持続投与で ALS に対する有効性を示してきた。多くの神経栄養因子のなかでもこの様に変異 SOD1 トランスジェニック動物による ALS モデルに対して明確な治療効果を示したものは少なく、この有効性を ALS 患者に臨床応用する意義と必要性がある。しかも、臨床応用の最も可能性の高いルートとしての髄腔内投与での効果が ALS ラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験および容量設定を開始した。マーモセットによる ALS モデルは確立されていないので、rhHGF の安全試験および臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いる。ALS ラットおよび脊髄損傷ラットで効果が確認された容量（400μg/4weeks）の rhHGF をマーモセット頸髄圧挫損傷モデルに対して損傷後よりくも膜下腔に持続投与したところ、著明な損傷範囲の縮小および良好な運動機能回復が得られた。霊長類脊髄損傷に対しても rhHGF を用いた治療法が有効であることが確認され、同時に安全性も確認中である。

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A. 研究目的

筋萎縮性側索硬化症（ALS）は運動ニューロンの選択的な細胞死が惹起されて、全身の筋萎縮と脱力が進行する原因不明の難治性神経筋疾患である。しかも 2～3 年の経過で呼吸筋の麻痺をきたす極めて予後不良な疾患であるが、現状では有効な治療法がない。ALS の病因と病態の解明を行ない、それを基盤にした新規治療法の開発が世界的に切望されている。

わが国で発見された神経栄養因子である肝細胞増殖因子（Hepatocyte Growth Factor、以下 HGF）は、運動ニューロンに対する強力な保護作用が知られており、私たちは遺伝子工学的に ALS マウスにおける HGF の運動ニューロン死に対する抑制効果を確認している。さらには ALS の臨床応用を目指し、私たちが開発した大型 ALS 動物モデルである変異 Cu/Zn superoxide dismutase（SOD1）導入 ALS ラットに対して HGF 蛋白の髄腔内投与実験を行い、その有効性も確認している。

今年度はげっ歯類脊髄損傷モデルに対する HGF の有効性およびその作用メカニズムを検証した上で、霊長類サル脊髄損傷モデルに対する recombinant human HGF (rhHGF)を用いた治療法の有効性・安全性を証明し、その結果を用いてヒト ALS 患者に対する画期的治療法を確立することが本研究の目的である。

B. 研究方法

1）成体ラットの脊髄損傷モデルに対する HGF の投与

我々は成体ラットの胸髄損傷後急性期には、HGF 受容体である c-Met の発現上昇に比べ HGF の発現上昇が脊髄内で不足していることを発見した。そこで herpes simplex virus 1 (HSV-1) vector を損傷 3 日前に直接脊髄に注入することで、exogenous HGF が脊髄に供給された理想的な状態をつくり（対照群； LacZ 発現）、同部に圧挫損傷を作成し HGF の有効性を検討した。損傷後急性期にはニューロンおよびオリゴデンドロサイトのアポトーシスを抑制し、血管新生を促進することで損傷範囲を縮小し、慢性期にかけてセロトニン線維の再生を促進することで良好な後肢運動機能回復が得られることを報告した。

そこで rhHGF を用いた臨床応用へ向け、ラットモデルにおける容量設定を行うために、既に ALS ラットで有効性が確認され容量にて下記の rhHGF の投与を行った。まず、成体 SD ラットの第 10 胸髄レベルに圧挫損傷を作成し、直後に第 12 胸髄レベルからくも膜下腔に髄腔内カテーテルを挿入する。先端を損傷直上部へ誘導し浸透圧ミニポンプを用いて rhHGF を 2 週間持続投与（200μg/2weeks）する。この容量は ALS ラットで有効であった容量である。対照群には PBS を投与する。損傷後 6 週目まで BBB scoring を用いて運動機能回復を評価し、組織学的検討を加えることで rhHGF の有効性を評価する。

2）コモンマーモセットの脊髄損傷モデルに対する rfHGF の髄腔内投与

次に、霊長類コモンマーモセットの第 5 頸髄レベルに圧挫損傷を作成し、直後より第 7 頸髄レベルから同様に髄腔内カテーテルを挿入する。rhHGF（対照群；PBS）をくも膜下腔に 4 週間持続投与（400μg/4weeks）し、損傷後 12 週目まで Bar grip test および open field scoring を用いてマーモセットの運動機能回復の評価および異常行動の有無を確認する。また、損傷後 1 週目・3 週目・12 週目に頸髄 MRI を撮影し経時的な損傷範囲の評価および腫瘍形成の有無を確認する。組織学的検討を加えることで、rhHGF を用いた本治療法の霊長類脊髄損傷に対する有効性および安全性を確立する。

3）治験に関するプロトコールの作成

ALS 患者を対象として臨床試験としては医師主導治験を計画しており、東北大学トランスレーショナルリサーチセンターと共にプロトコールの検討を行う。

（倫理面への配慮）

すべての遺伝子操作は本学 DNA 組換え実験指針に従い、また動物実験は同動物実験指針に従った上で動物愛護面に配慮しかつ利用動物数を極力減らすように務めた。

C 及び D.　研究結果及び考察

1）成体ラットの脊髄損傷モデルに対する HGF の投与

成体 SD ラットの第 10 胸髄圧座損傷モデルに損傷直後より ALS ラットで有効性が確認され容量にて rhHGF を 2 週間にわたり持続投与したところ、human HGF の脊髄損傷部への高率な導入および内在性 rat HGF の発現上昇が ELISA 法にて確認され、有意に良好な下肢運動機能回復を認めた。また rhHGF 群では損傷中心部においても白質有髄線維が著明に保たれており、H.E. 染色より空洞形成も著明に抑制されていることが明らかとなった。即ち、損傷後より rhHGF をくも膜下腔に持続投与することによっても、これまでの HSV-1 vector を用いた脊髄内への HGF 導入法と同様の効果が得られることが明らかとなった。

2）コモンマーモセットの脊髄損傷モデルに対する rfHGF の髄腔内投与

コモンマーモセット第 5 頸髄レベルに圧挫損傷を作製し、直後より同様に ALS および脊髄損傷ラットモデルでの有効容量から換算した rhHGF を 4 週間持続投与したところ、Bar grip test および open field scoring 共に有意に良好な運動機能回復を認めた。さらには損傷後 12 週目の MRI 像（T2WI）より、空洞形成ならびに異常高信号領域が左右および頭尾側、腹側背側いずれの方向にも rhHGF 群で著明に縮小していることが明らかとなった。また損傷後 12 週までの間、異常行動ならびに MRI 像における腫瘍形成は一切認められなかった。これらの結果より、rhHGF を損傷後にくも膜下腔に持続投与する治療法が、霊長類脊髄損傷に対しても有効であることが明らかとなった。

3）治験に関するプロトコールの作成

東北大学トランスレーショナルリサーチセンターと共にプロトコールの検討に入っている。シーズ開発戦略会議などを通じて、現在の問題点としては以下が指摘されている。

- ポンプを利用した薬剤の連続投与という形態が、安全性の確保に対する障害として懸念される。
- ポンプメーカー（メドトロニクス社）との共同研究という形がとれるかの交渉が大切である。
- 髄腔内投与の場合はまず薬剤の動態を要求されるので、これにどのように対応するか。
- 企業治験に持ち込むのが良策であるが企業の体力に依存するため、フェーズ 1・フェーズ 2A を探索的臨床試験として実施する方向性も検討すべきか。

E. 結論

本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（HGF）を用いた画期的治療法を開発することとそれに関わる基盤研究を進めることにある。臨床応用の最も可能性の高いルートとしての髄腔内投与での効果がALSラットで確認されたので、霊長類（マーモセット）に対する髄腔内投与での安全試験および容量設定を開始した。マーモセットによるALSモデルは確立されていないので、HGFの安全試験および臨床用量決定には慶応大学の岡野らが確立したマーモセットによる脊髄損傷モデルを用いた。

マーモセット脊髄損傷モデルに対して損傷後よりrhHGFをくも膜下腔に持続投与し、損傷範囲の著明な縮小ならびに有意に良好な運動機能の回復を認めた。霊長類脊髄損傷に対してもラットと同じ体重比の容量で有効性が確認され、また腫瘍形成や異常行動が認められなかったことから、本治療法がヒトALSに対し有効かつ安全な治療法となり得る可能性が大きく示唆された。

F. 健康危険情報

特になし

G. 研究発表

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