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Research Report

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

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ABSTRACT

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

1. Introduction

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

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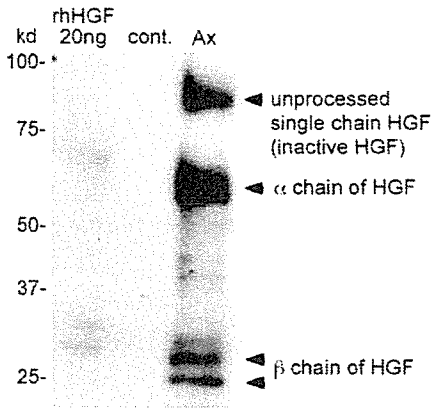

Fig. 1 – Western blot analysis of conditioned media (CMs) obtained from COS1 cells uninfected (cont.) or infected (Ax) with AxCAhHGF. The CMs harvested at 3 days after infection were concentrated by heparin beads, electrophoresed, blotted and immunolabeled for HGF as described in the text.

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

2. Results

2.1. *Bioassay of recombinant human HGF*

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

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

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

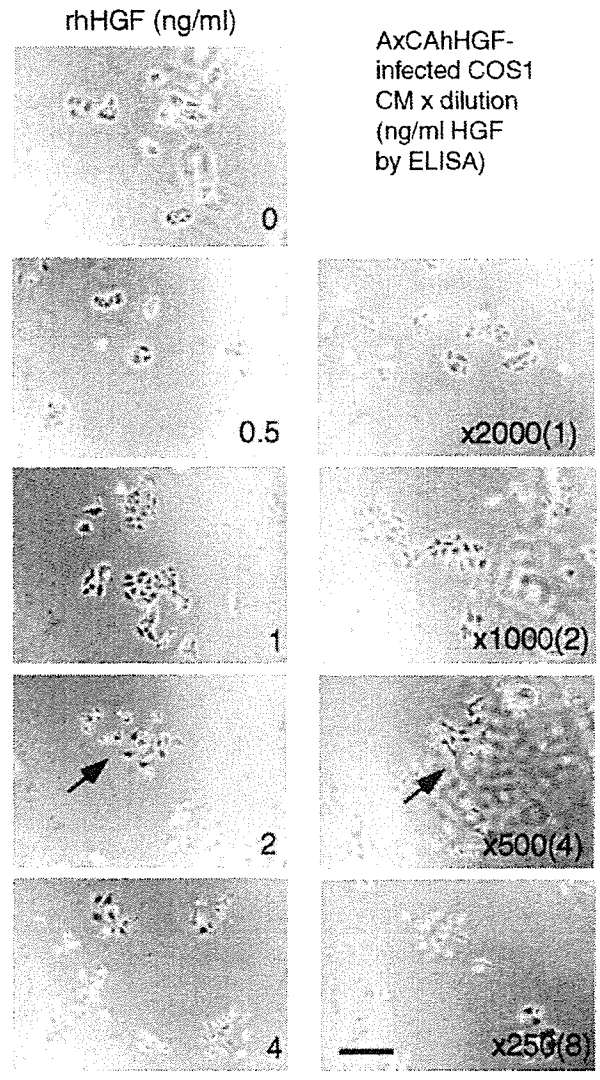

Fig. 2 – Madin-Darby canine kidney (MDCK) cell scatter assay for HGF bioactivity. MDCK cells were cultured in the presence or absence of AxCAhHGF-infected COS1 CM or rhHGF as described in the text. The activity of 1:500-diluted CM containing 4 ng/ml human HGF as measured by ELISA corresponds to that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

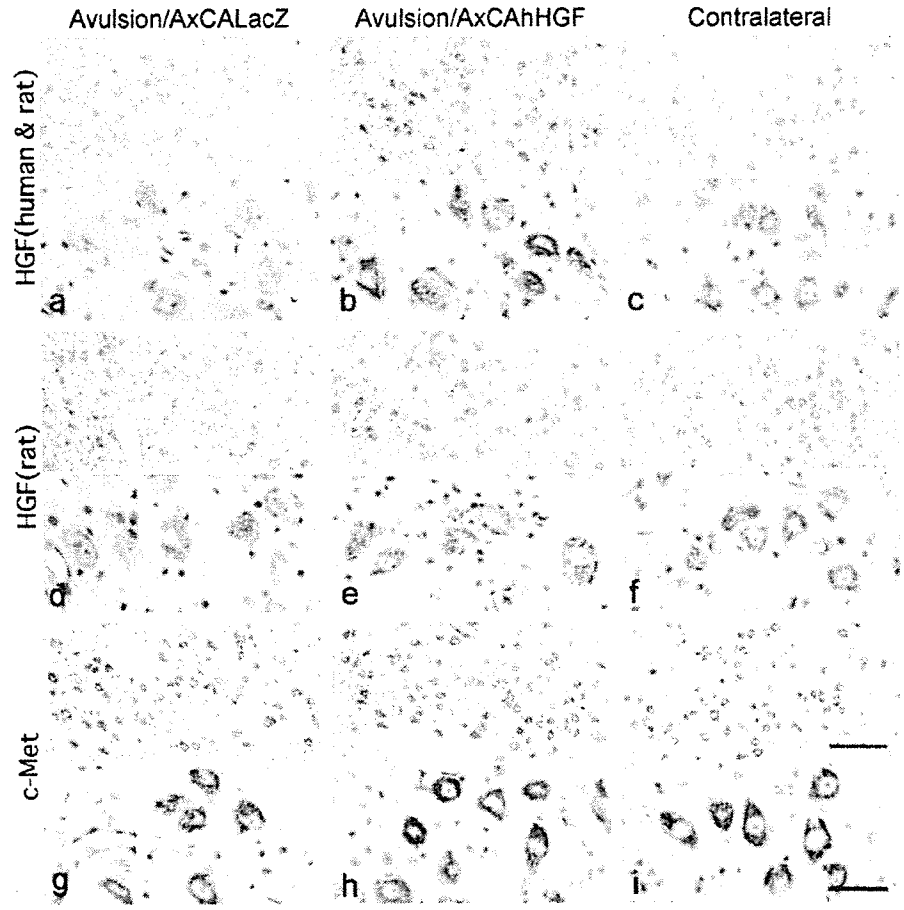

Fig. 3 – Low (top) and high (bottom)-magnified photomicrographs of immunohistochemistry of facial motoneurons at the ipsilateral (a, b, d, e, g, h) and contralateral (c, f, i) sides 7 days after facial nerve avulsion and the treatment with AxCALacZ (a, d, g) or AxCAhHGF (b, c, e, f, h, i) using antibodies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

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

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

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

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

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

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

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

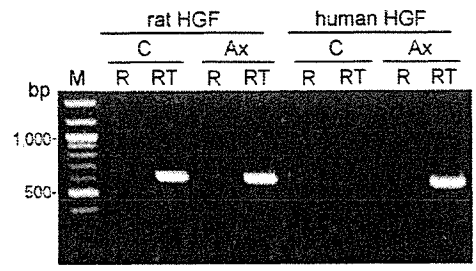

Fig. 4 – RT-PCR analysis of HGF mRNA transcripts in ipsilateral (Ax) and contralateral (C) sides of the brain stem tissue containing facial nuclei 7 days after facial nerve avulsion and AxCAhHGF treatment. The PCRs were performed on RNA without (R) or with (RT) reverse transcription. Primers that amplify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

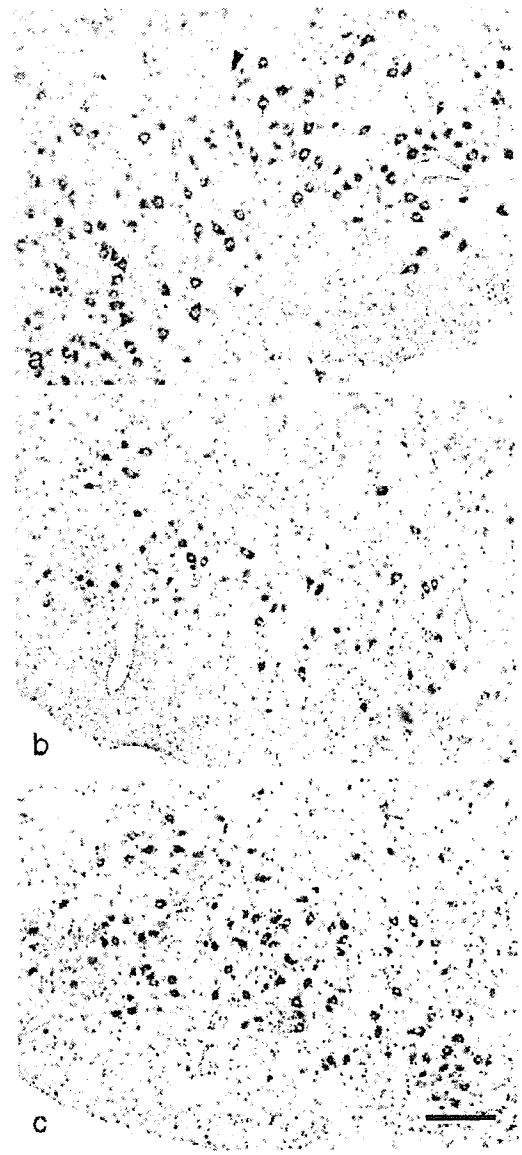

Fig. 5 – Photomicrographs of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=200 μm.

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

2.3. *Neuroprotective effects of HGF gene transfer*

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

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

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

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

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

3. Discussion

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

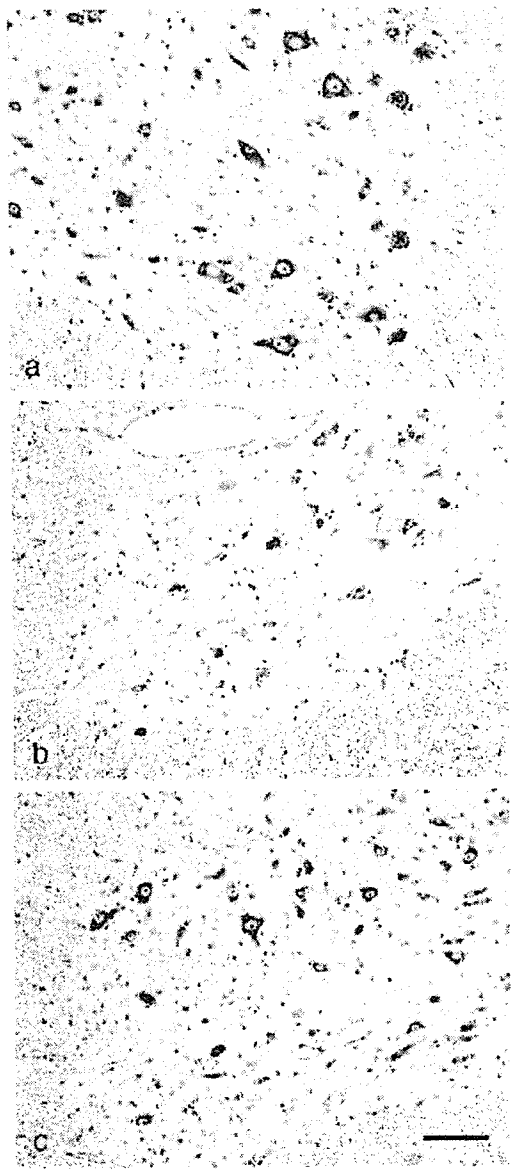

Fig. 6 – Photomicrographs of C7 spinal motoneurons at the contralateral (a) and ipsilateral (b, c) side 4 weeks after the right C7 spinal nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Nissl stain. Scale bar=100 μm.

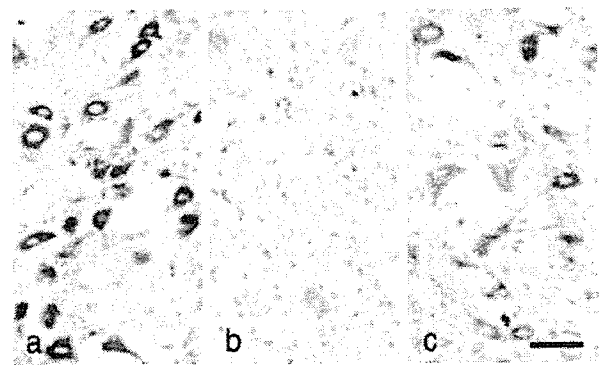

Fig. 7 – Photomicrographs of ChAT immunohistochemistry (a–c) of facial motoneurons at the contralateral (a) and ipsilateral (b, c) side 7 days after the facial nerve avulsion and the treatment of AxCALacZ (b) or AxCAhHGF (c). Pictures (a) and (c) were taken from the same section. Scale bar=50 μm.

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

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

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

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

4. Experimental procedures

4.1. *Adenovirus preparation*

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

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

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

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

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

4.4. *Animals and surgical procedures*

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

4.4.1. *Facial nerve avulsion*

Adult Fischer 344 male rats (12–14 weeks old, 200–250 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg). Under a dissecting microscope, the right facial nerve was exposed at its exit from the stylomastoid foramen. Using microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve as described previously (Sakamoto et al., 2000). Immediately following the avulsion, microsyringe was inserted into the stylomastoid foramen and 20 μl solution of AxCAhHGF (1×10^8 pfu), AxCALacZ (1×10^8 pfu) or PBS was injected through the facial canal. The wounds were covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ) and suture closed, and the animals were sacrificed at 1 and 4 weeks postoperation as described below.

4.4.2. *Spinal root avulsion*

Anesthetized animals were placed in a supine position. Under a dissecting microscope, the right seventh cervical segment (C7) nerve was exposed by separating the surrounding cervical muscles and connective tissue until the point where the vertebral foramen was identified. Using microhemostat forceps, the C7 ventral and dorsal roots and dorsal root ganglia (DRG) were avulsed and removed from the peripheral nerve as described previously (Watabe et al., 2000). Immediately following avulsion, a small piece of Gelfoam presoaked with 10 μl solution of AxCAhHGF (1×10^8 pfu), AxCALacZ (1×10^8 pfu) or PBS was placed in contact with the lesioned vertebral foramen. The wounds were suture closed and animals were sacrificed at 4 weeks postoperation as described below.

4.5. *HGF ELISA of brain stem tissue containing facial nucleus*

One week after facial nerve avulsion and the treatment with AxCALacZ or AxCAhHGF, the animals ($n=3$) were euthanized with a lethal dose of pentobarbital sodium and the brain stem tissue containing the facial nucleus (11–14 mg wet weight) was collected. ELISA for rat HGF and human HGF was performed as described (Sun et al., 2002; Funakoshi and Nakamura, 2003).

4.6. *Reverse transcription followed by polymerase chain reaction (RT-PCR)*

One week after facial nerve avulsion and the treatment with AxCAhHGF, the brain stem tissue containing the facial nucleus ($n=3$) was collected as described above. Total RNA was isolated from the tissue using RNA isolation reagent (TRIZOL, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with RNase-free DNase (Roche, Penzberg, Germany) in transcription buffer for 30 min. First strand cDNA was synthesized from 250 ng of total RNA using random primer and Superscript II reverse transcriptase (Invitrogen) for one PCR analysis. The PCR reactions were carried out in PCR buffer containing cDNA template, 200 μM dNTPs, 2 mM $MgCl_2$, 0.2 μM of each primer and 25 unit/ml of ExTaq DNA polymerase (TaKaRa, Osaka, Japan). Specific oligonucleotide primers for PCR were designed to amplify rat HGF cDNA (Tashiro et al., 1990; GenBank Accession no. NM_017017; forward, 5′-GCCAAAACAAAA-CAACTG-3′; reverse, 5′-GACACCAAGAACCATTCTCA-3′) that yield 615 bp amplified products, and human HGF cDNA (Seki et al., 1990; M60718; forward, 5′-AAACATATCTGCGGAGGATC-3′; reverse, 5′-ACGATTTGGAATGGCACATC-3′) that give 561 bp amplified products. The PCR amplification program consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min for 40 cycles. For negative control reactions, non-reverse transcribed RNA samples were processed for PCR to exclude the possibility of the contamination of genomic or adenoviral DNA as a source of amplified products. The PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. To confirm the sequence identity of the amplified products, the PCR fragments were subcloned into pCRII (Invitrogen) and sequenced by a model 373A sequencer and the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

4.7. *Histological analysis*

Rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% paraformaldehyde in 0.1 M PB. The brain stem tissue containing facial nuclei and their intramedullary nerve tracts after facial nerve avulsion or the C7 spinal cord tissue after spinal root avulsion was dissected and immersion fixed in the same fixative for 2 h. As for facial nerve avulsion, we routinely checked the absence of extra-axial portion of facial nerve on the avulsed side under a dissecting microscope and confirmed the absence of any peripheral nerve tissues at the level of facial nerve outlet from the brain stem in microscopic sections prepared from every animal as described below. As for C7 spinal root avulsion, the absence of C7 ventral and dorsal roots as well as DRG on the lesioned side was confirmed under the dissecting microscope. A small longitudinal incision was made in the anterolateral white matter through the level of C7 ventral root outlets on the contralateral side in aid of identifying the level of C7 spinal ventral horn in histological sections.

For immunohistochemistry, the brain stem tissues were either embedded in paraffin or cryoprotected in 30% sucrose in 0.1 M PB and serial paraffin or cryostat sections were made. For immunostaining for HGF, deparaffinized sections were pretreated with 0.3% H_2O_2 in methanol, incubated with 0.05% trypsin for 15 min at 37 °C and preincubated with 3% heat-inactivated goat serum in 0.1% Triton-X100 in phosphate-buffered saline (T-PBS). Sections were then incubated overnight at 4 °C with rabbit anti-human HGFα antibody (H55; recognizes human and rat HGFα; IBL, Fujioka, Japan) or rat HGFα antibody (H56; recognizes rat, but not human, HGFα; IBL) diluted 1:200 in T-PBS followed by the incubation with biotinylated goat anti-rabbit IgG at a dilution of 1:200 and with ABC reagent (Vector). Immunostaining for ChAT on cryostat sections was performed using rabbit polyclonal antibody to ChAT (1:1000; Chemicon, Temecula, CA) and ABC method as described previously (Watabe et al., 2000). Sections were visualized by 3,3′-diaminobenzidine tetrahydrocholoride (DAB)–H_2O_2 solution and counterstained with hematoxylin.

For negative controls, the primary antibodies were omitted or replaced by non-immunized animal sera.

For motoneuron cell counting, serial paraffin-embedded brain stem or C7 spinal cord sections were made. Every fifth section (6 μm thickness; 24 μm interval) was picked up, deparaffinized and stained with cresyl violet (Nissl staining). Facial motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei were counted in 25 sections. For spinal motoneuron cell counting, ventral horn motoneurons located in Rexed's lamina IX having nuclei greater than 15 μm in diameter with distinct nucleoli on both sides of the C7 spinal cord were counted in 35 sections. The data were then expressed as the mean ± SD from 4 to 8 animals, and statistical significance was assessed by Mann–Whitney *U* test.

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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).

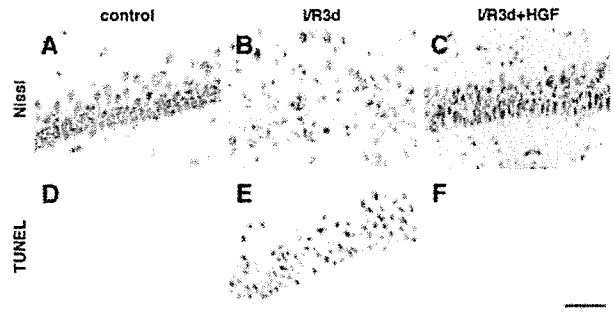

Figure 1 Effect of hepatocytes growth factor (HGF) on neuronal cell death in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A–C**) Photomicrographs of cresyl violet-stained hippocampal CA1 region of nonoperated naïve rats (**A**), four-vessel-occluded 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.

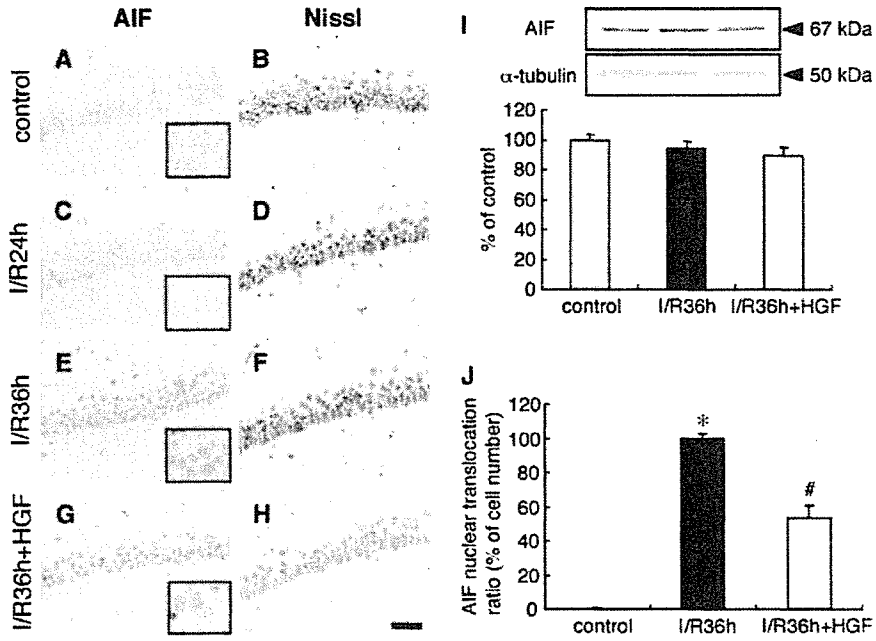

Figure 2 Effect of hepatocyte growth factor (HGF) on the expression of apoptosis-inducing factor (AIF) in the nucleus of the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A** and **B**) Photomicrographs of staining with anti-AIF antibody (**A**) and Nissl 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).

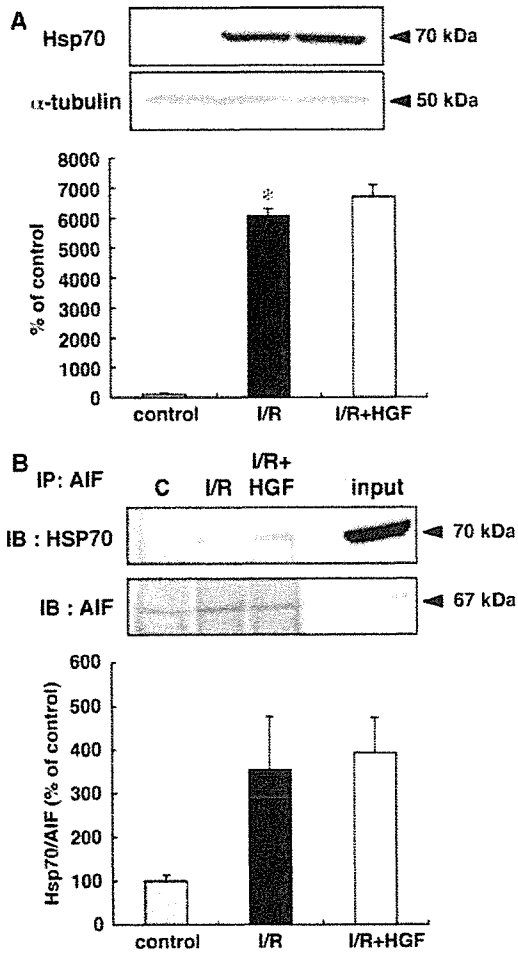

Figure 3 Effect of hepatocyte growth factor (HGF) on the expression of heat-shock protein 70 (Hsp70) and the interaction of Hsp70 with apoptosis-inducing factor (AIF) in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) Proteins (20 μg) from nonoperated 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.

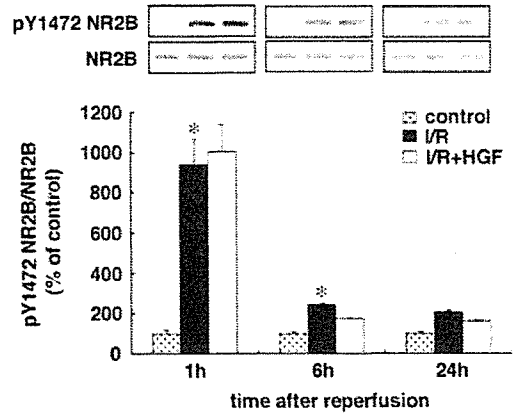

Figure 4 Effect of hepatocyte growth factor (HGF) on phosphorylation of the *N*-methyl-D-aspartate (NMDA) receptor in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. Proteins (50 μg) from nonoperated naïve rats and four-vessel-occluded rats at 1, 6, and 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.

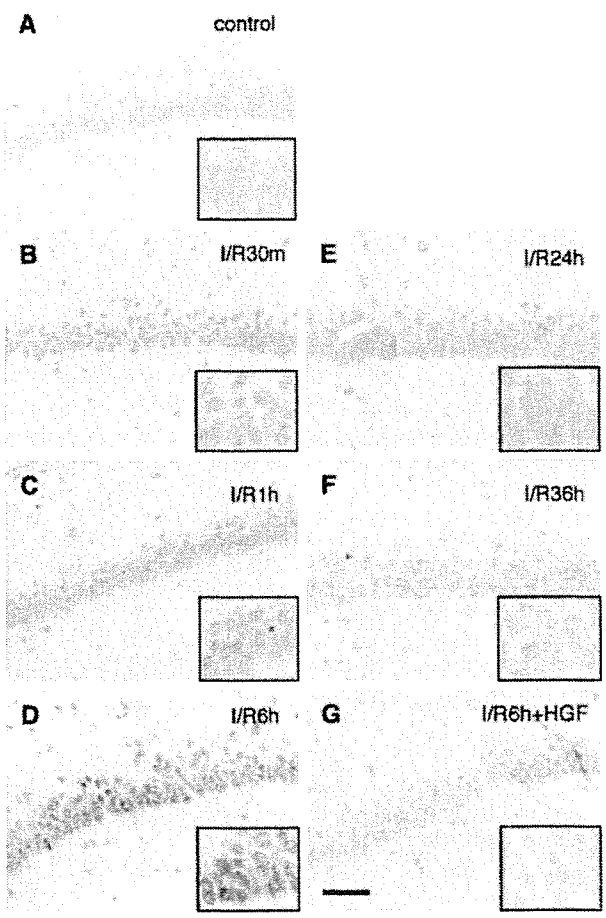

Figure 5 Effect of hepatocyte growth factor (HGF) on oxidative DNA damage in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-8-OHdG (8-hydroxy-2′-deoxyguanosine) antibody in the hippocampal CA1 region of nonoperated 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).

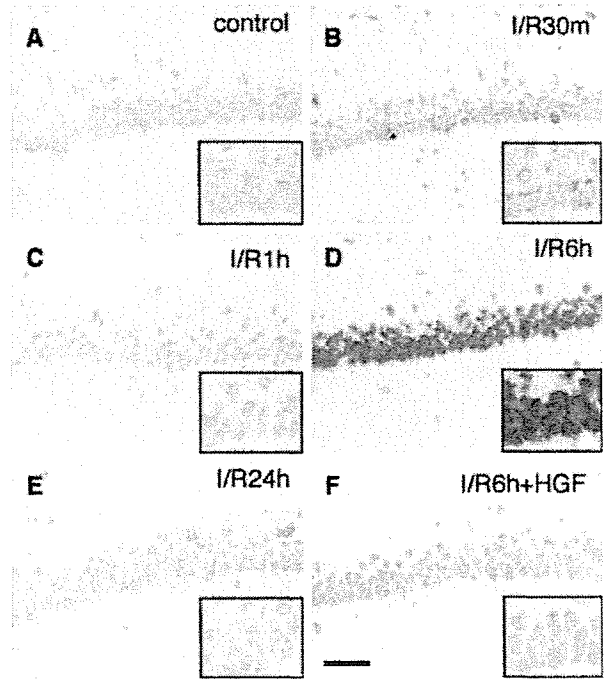

Figure 6 Effect of hepatocyte growth factor (HGF) on poly(ADP-ribose) polymer formation in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-poly(ADP-ribose) (PAR) antibody in the hippocampal CA1 region of nonoperated 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

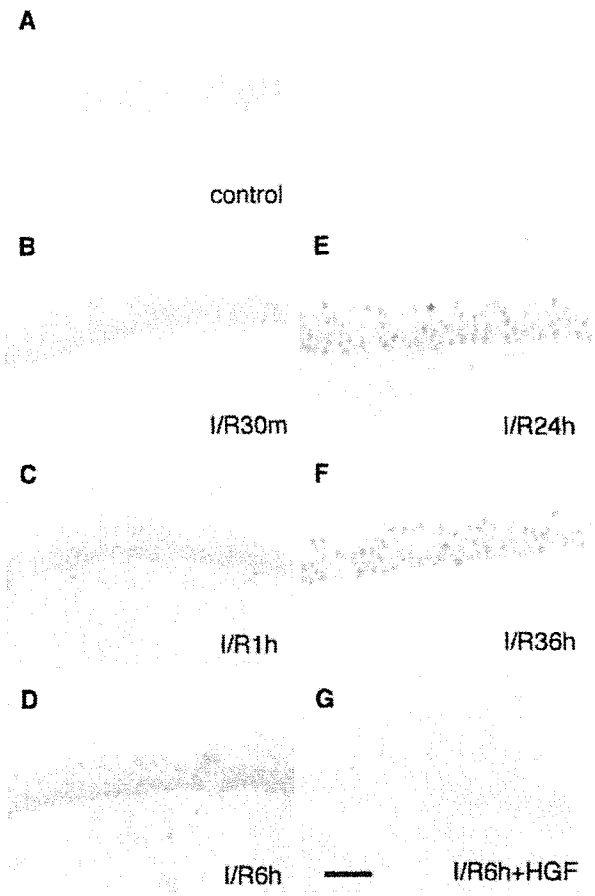

Figure 7 Effect of hepatocyte growth factor (HGF) on the expression of p53 in the hippocampal cornu ammonis (CA) subregion 1 (CA1) region after transient forebrain ischemia. (**A**) A photomicrograph of staining with anti-p53 antibody in the hippocampal CA1 region of nonoperated naïve rats. (**B–F**) 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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