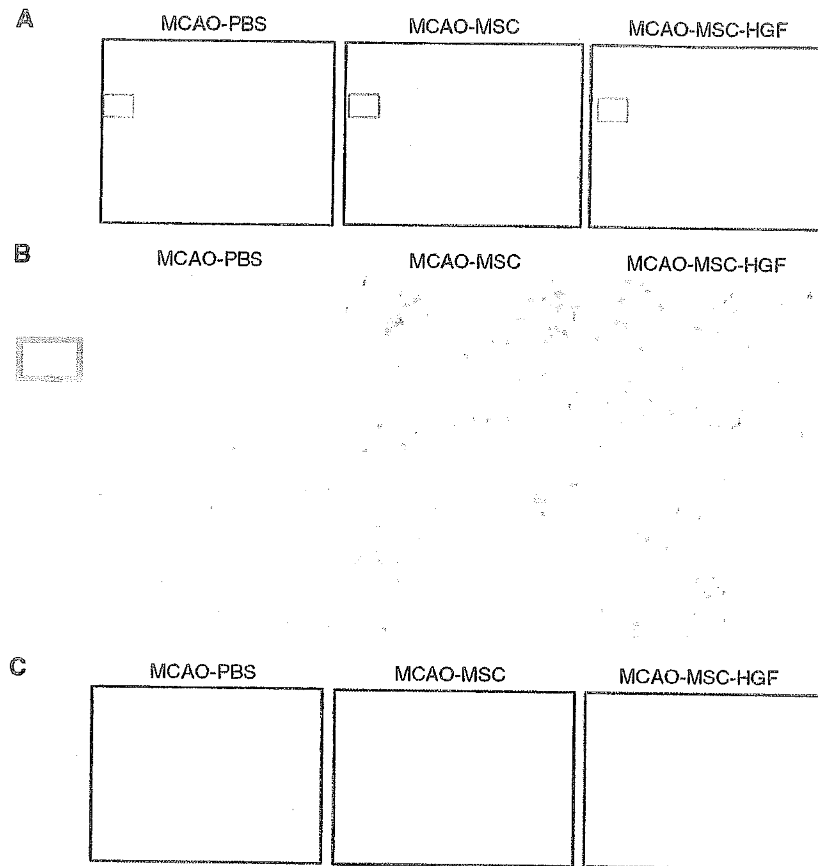

Figure 4 Immunohistochemistry for HGF expression *in vivo*. Mixed HGF expression (brown color) in the ischemic brains of groups 1, 2, and 4 detected by immunohistochemistry on days 2 (row A) and 14 (row C). Scale bar: 1.0 mm. The upper and lower rows identified in B (original magnification, ×400) show the cortex and basal ganglia, respectively, of the images shown in row A.

under the acute treatment. This was the same as the Chopp's group had reported, that transplanted MSCs to the transient MCAO model 24 h after ischemia occurred had improved neurological function recovery, but not significantly decreased infarction area (Chen *et al*, 2000, 2001; Li *et al*, 2000, 2001), while our data of the superacute treatment showed the contrast result. We thought that it might have been caused by the different therapeutic time window. Furthermore, we found significant neurological recovery of the rats treated with combined therapy on day 14, 24 h after MCAO occurred (acuter phase), than the rats of the MSC-only group treated even 2 h after MCAO occurred (superacute phase). It indicates that our combined therapeutic method may extend the therapeutic time window for treating brain ischemia at least until 24 h after the onset of MCAO, while compared with the MSC-only cell therapy. To treat transient ischemia, both the combined therapeutic method and superacute therapeutic time window might be important.

Mesenchymal stromal cell-only therapy also showed significant improvement of functional outcome and decrease of infarction volume when cells were administered 2 h after stroke. A more likely mediator of short-term benefit may reflect increased production of growth factors, including neurotrophins adjusted to the needs of the compromised tissue with an array of reducing host cells' apoptosis in the IBZ, including neurons, and promoting functional recovery of the remaining neurons (David and Thomas, 2002; Chopp and Li, 2002). After stroke, cerebral tissue reverts to an earlier stage of development and thus becomes highly responsive to stimulation by cytokines, trophins, and growth factors from the invading MSCs (Chopp and Li, 2002). The MSCs may simply provide the resources required by the ontogenous cerebral tissue to stimulate cerebral remodeling.

In the present study, the combined therapy group showed more therapeutic benefit than the MSC-only cell therapy. Hepatocyte growth factor gene-modified MSCs may also behave as small molecular factories, secrete an array of cytokines and trophic factors over an extended period and not in a single bolus dose, directly involved in promoting plasticity of the ischemic damaged neurons or in stimulating

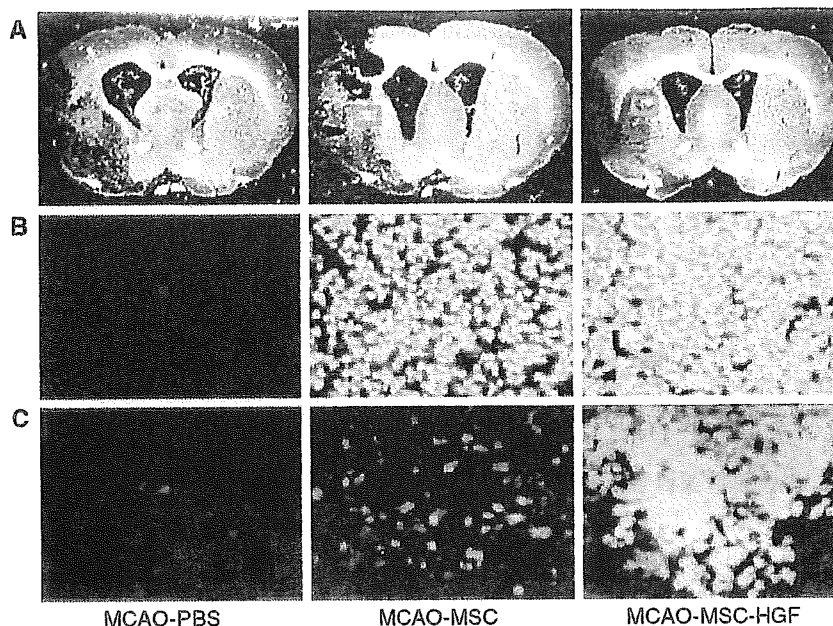

Figure 5 Expression of HGF and identification of transplanted MSCs. Photographs in row C show mixed HGF expression in the ipsilateral brain of groups 1, 2, and 4 with red fluorescence, and photographs in row B show transplanted donor MSCs of groups 2 and 4 with blue fluorescence, at 2 weeks after treatment. The microphotographs shown in rows B and C have the same size and higher power magnification than the blue squares in row A. Original magnification, $\times$200.

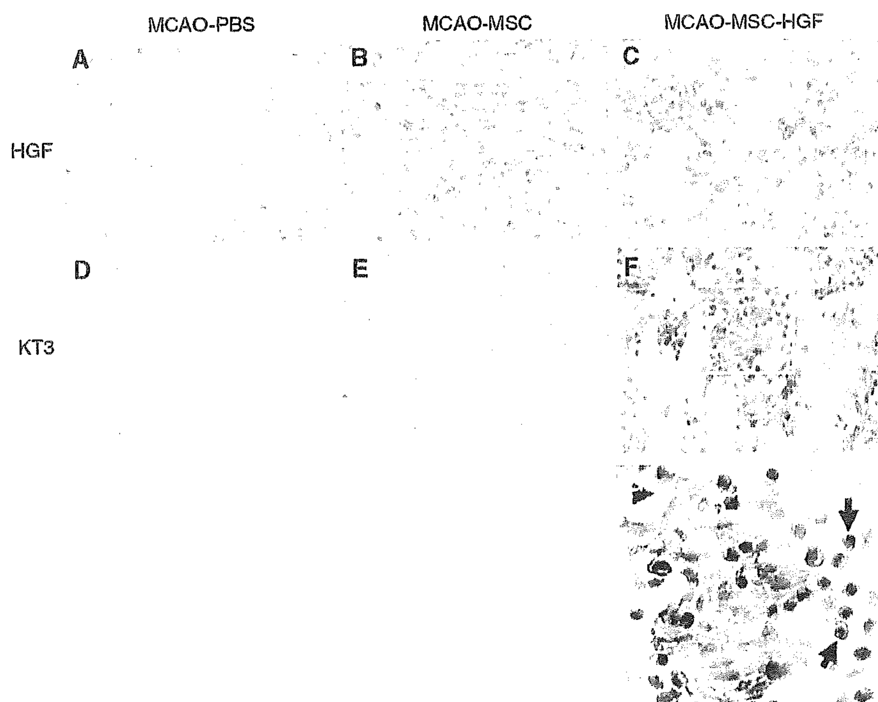

Figure 6 Immunohistochemistry for HSV-1 vector-transferred exogenous HGF and mixed HGF expression. The upper column (**A–C**) shows mixed HGF expression in groups 1, 2, and 4 with anti-rat HGF immunostaining, and the lower column (**D–F**) shows ex-HGF-2 expression with anti-ratHGFKT3 immunostaining at 2 weeks after treatment. Original magnification, $\times$200. (**G**) is the enlarged white square in (**F**), arrows mark HGF expression in the transplanted MSCs and an arrowhead marks HGF expression in the intracellular space.

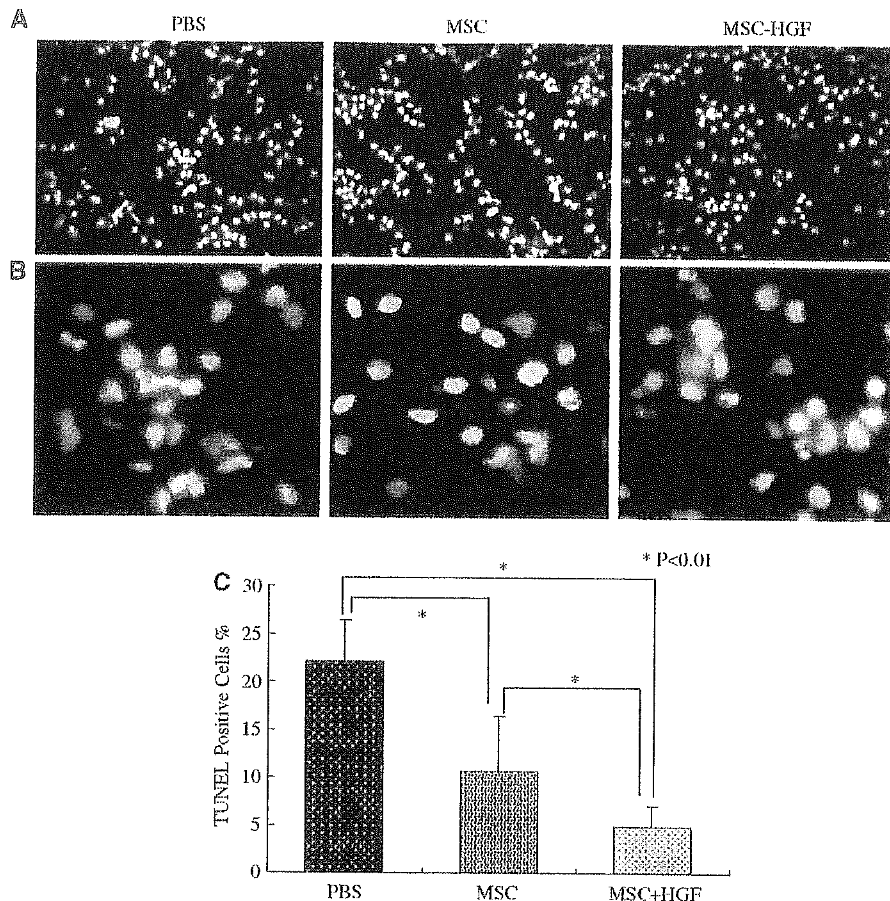

Figure 7 Apoptotic cells in the IBZ with TUNEL staining. (**A**) Column, fewer TUNEL-positive cells were detected in rats treated with MSC-HGF than others treated either with PBS or MSC-only (Rhodamine, red, TUNEL positive; Hoechst, blue, nuclear; original magnification with × 40 object). (**B**) Column, 4 times enlarged magnification of × 40 object. (**C**) The percentage of TUNEL-positive cells in the IBZ was significantly reduced in the MSC-HGF group compared with the other groups 7 days after treatment.

glial cells to secrete neurophins. Marrow stromal cells secrete many cytokines known to play a role in hematopoiesis (Dormady *et al*, 2001), and also supply autocrine, paracrine, and juxtacrine factors that influence the cells of the marrow microenvironment themselves (Haynesworth *et al*, 1996). The interaction of MSCs with the host brain may lead MSCs and parenchymal cells also to produce abundant trophic factors, which may contribute to recovery of function lost as a result of a lesion too (Williams *et al*, 1986).We speculate that *HGF* gene-modified MSCs also had carried out such ways not only to produce extended and abundant exogenous HGF, but also a variety of other cytokines and trophic factors, and interact with each other in an anatomically distributed, tissue-sensitive, and temporally ongoing way.

Other functions of HGF include reducing the BBB destruction without exacerbating cerebral edema, decreasing intracranial pressure, inducing angiogenesis, and interacting with other kinds of neurotrophic factors; cytokines that are secreted by MSCs themselves may also take part in improving the neurological recovery after stroke. We also speculate that the various cytokines secreted from MSCs or MSC-HGF activate the proliferation and differentiation of endogenous neural stem and progenitor cells in the subventricular zone, such as Chopp and Li (2002) had reported. Also, transplanted MSCs themselves might differentiate into some kinds of central never system cells (Woodbury *et al*, 2000). Actually, we also found some MSCs expressing glial phenotype 4 weeks after transplantation only in the combined therapy group (data not shown), which might suggest that *HGF* gene transduction could influence transplanted MSC differentiation. But tissue regeneration might be another part of the mechanisms that induced recovery after stroke mainly occurs in the chronic therapeutic time course.

Anyhow, our MSC-HGF combined therapy enhanced the therapeutic efficiency than the MSC-only

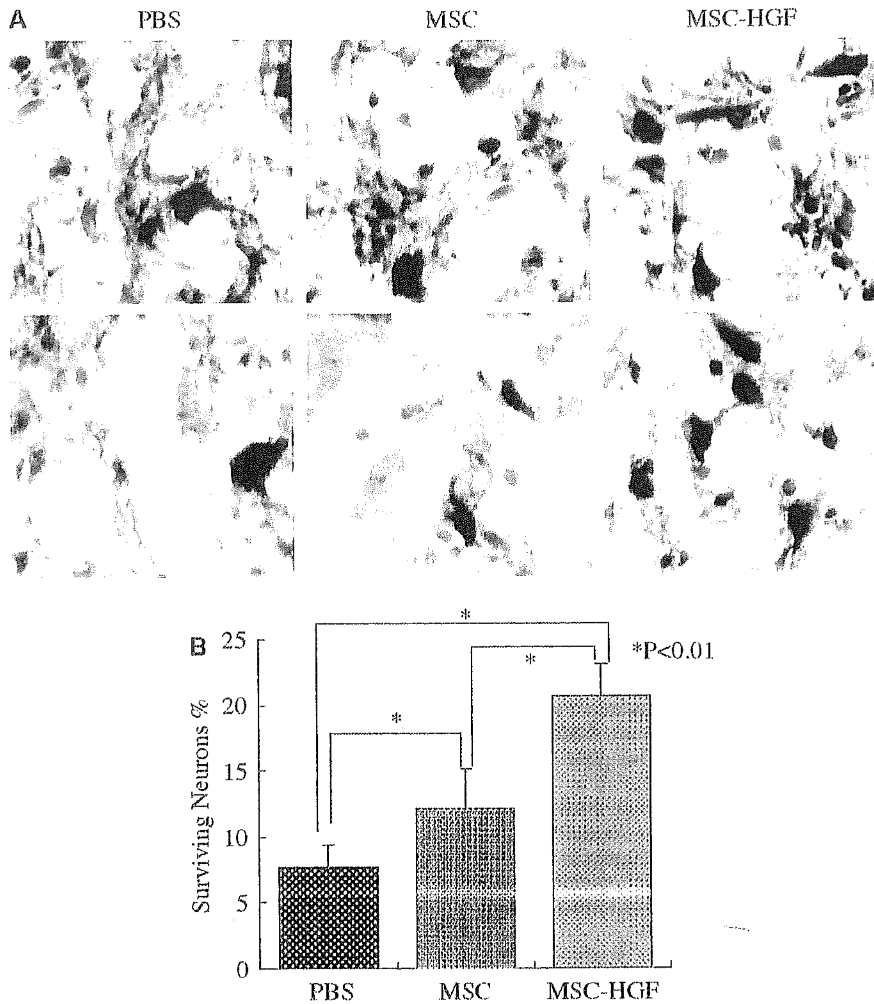

Figure 8 Remaining neurons in the cortex of IBZ with MAP-2 immunostaining. (**A**) Column, more neurons could be detected in rats treated with MSC-HGF than others treated either with PBS or MSC-only (MAP-2, dark brown, neurons; hematoxylin, blue, nuclear; original magnification, × 600). (**B**) The percentage of neurons in the cortex of IBZ was significantly increased in the MSC-HGF group compared with the other groups 7 days after treatment.

cell therapy for stroke in rats treated in both the superacute and acute phases. The target gene was successfully transferred to MSCs with the HSV-1 virus vector *in vitro*, and later the gene-modified MSCs served as both a therapeutic material and a vector platform that continuously carried the target gene into the brain and functioned *in vivo*. This method might be safer than direct gene transfer with viral vectors for *in vivo* treatments, more therapeutically efficient than MSC-only cell therapy, extend the therapeutic time window from superacute to at least 24 h after ischemia happened, and also could be used as a post-treatment method for stroke. Although the best therapeutic time schedule, the administration route of MSCs and best cytokine gene (or cocktail of the genes) should be explored for better clinical application. It may require a broad array of treatments to prevent neurological disorders in brain ischemia, which may offer a promise for human clinical treatment in future.

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Inhibition of apoptosis-inducing factor translocation is involved in protective effects of hepatocyte growth factor against excitotoxic cell death in cultured hippocampal neurons

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Abstract

Although hepatocyte growth factor (HGF) and its receptor are expressed in various regions of the brain, their effects and mechanism of action under pathological conditions remain to be determined. Over-activation of the *N*-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor, has been implicated in a variety of neurological and neurodegenerative disorders. We investigated the effects of HGF on the NMDA-induced cell death in cultured hippocampal neurons and sought to explore their mechanisms. NMDA-induced cell death and increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were prevented by HGF treatment. Although neither the total amounts nor the mitochondrial localization of Bax, Bcl-2 and Bcl-xL were affected, caspase 3 activity was increased after NMDA exposure. Treatment with HGF partially prevented this NMDA-induced activation of caspase 3. Although the amount of apoptosis-inducing factor (AIF) was not altered, translocation of AIF into the nucleus was detected after NMDA exposure. This NMDA-induced AIF translocation was reduced by treatment with HGF. In addition, increased poly(ADP-ribose) polymer formation after NMDA exposure was attenuated by treatment with HGF. These results suggest that the protective effects of HGF against NMDA-induced neurotoxicity are mediated via the partial prevention of caspase 3 activity and the inhibition of AIF translocation to the nucleus.

Keywords: apoptosis-inducing factor, hepatocyte growth factor, neuronal cell death, *N*-methyl-D-aspartate receptor.
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The hepatocyte growth factor (HGF), which was found to be a potent mitogen for hepatocytes (Nakamura *et al.* 1984, 1989), exerts its physiological activities as an organotrophic factor for regeneration and has protective effects in various organs (Zarnegar and Michalopoulos 1995; Matsumoto and Nakamura 1996; Balkovetz and Lipschutz 1999; Matsumoto and Nakamura 2001). In addition, motogenic, morphogenic, angiogenic and anti-apoptotic activities of HGF have been demonstrated in various types of cells (Nakamura *et al.* 1989; Zarnegar and Michalopoulos 1995; Matsumoto and Nakamura 1996; Thompson *et al.* 2004). These multipotent activities are thought to be mediated by the transmembrane tyrosine kinase receptor, c-Met (Bottaro *et al.* 1991; Higuchi and Nakamura 1991). HGF and c-Met receptor were found to be expressed in various regions of the brain and to function in a variety of ways in the central nervous system (Honda *et al.* 1995; Achim *et al.* 1997; Sun *et al.* 2002a,b). For example, HGF promoted the survival of tyrosine hydroxylase-positive midbrain neurons, as well as hippocampal and cortical neurons, during aging in culture (Honda *et al.* 1995; Hamanoue *et al.* 1996; Machide *et al.* 1998). HGF not only increased the number of calbindin D-expressing neurons in

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Abbreviations used: AIF, apoptosis-inducing factor; DIV, days *in vitro*; hrHGF, human recombinant hepatocyte growth factor; NMDA, *N*-methyl-D-aspartate; PFA, paraformaldehyde; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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postnatal rat hippocampal cultures (Korhonen *et al.* 2000), but also promoted dendritic growth and branching of layer 2 pyramidal neurons in cortical organotypic slice cultures from early postnatal mice (Gutierrez *et al.* 2004). In addition, the administration of human recombinant HGF (hrHGF) prevented neuronal death in the hippocampal CA1 region after transient global ischemia, and reduced the infarct size after transient focal cerebral ischemia and widespread cerebral embolism (Miyazawa *et al.* 1998; Tsuzuki *et al.* 2001; Date *et al.* 2004), thus suggesting that HGF has the ability to prevent ischemic brain injuries. Whereas it is conceivable that the angiogenic effect of HGF, at least in part, is involved in the rescue of the brain tissue in the *in vivo* ischemic brain, the questions remain as to whether HGF exerts protective effects by acting directly on neurons under pathological conditions, and how the effects are mediated by intracellular signalings.

The ionotropic glutamate receptors are ligand-gated ion channels that mediate the majority of excitatory neurotransmission in the central nervous system. The *N*-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor, is highly permeable to Ca^{2+} and Na^{+} (Dale and Roberts 1985). Although the intracellular calcium transient induced by the influx of Ca^{2+} through the NMDA receptor plays an important role in physiological activities such as learning and memory, an excessive stimulation of the NMDA receptor has been implicated in a variety of neurological and neurodegenerative disorders, including cerebral ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's chorea and amyotrophic lateral sclerosis (Dingledine *et al.* 1999). The hippocampus plays a pivotal role in neuronal function as it is associated with learning and memory function, but it is a region vulnerable to excitotoxicity. It has become an important objective to explore strategies to protect cells from NMDA-induced excitotoxicity and to determine the underlying mechanism of such protection. In the present study, to achieve further insight into the potency of HGF treatment and the mechanism of HGF-mediated neuronal protection, we examined the effect of hrHGF treatment on NMDA-induced neurotoxicity in cultured hippocampal neurons. Our findings demonstrate inhibition of NMDA-induced apoptosis-inducing factor (AIF) translocation into the nucleus as a possible mechanism for the protective effect of HGF against excitotoxicity in hippocampal neurons.

Experimental procedures

Recombinant HGF

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

Primary hippocampal cultures

Primary hippocampal cell cultures were prepared from gestational day 18 fetal rats as described previously (Huettner and Baughman 1986), with slight modifications. The hippocampi were dissected out and the cells were dissociated by incubation at 37°C for 30 min in Hank's balanced salt solution containing 15 U/mL papain, 210 U/mL deoxyribonuclease I, 1 mM L-cysteine and 0.5 mM EDTA. The dispersed cells were resuspended in Dulbecco's modified Eagle's medium containing 10% horse serum, then plated at a density of 40 000 cells/cm^2 on 12-well plates or in 35 mm dishes coated with poly-L-lysine. The medium 24 h after plating was changed to serum-free neurobasal medium containing 2% B27 supplements (Gibco-BRL, Rockville, MD, USA) and 0.5 mM glutamine. Cytosine arabinoside (1 μM) was added to inhibit the proliferation of non-neuronal cells. At 3 and 10 days *in vitro* (DIV), one half of the medium was replaced with fresh neurobasal medium containing the 2% B27 supplements and 0.5 mM glutamine. The cells were maintained at 37°C in a 5% CO_2 incubator and used for experiments at 14–16 DIV, at which time they contained 87.3 ± 4.5% NeuN-positive neurons and 12.8 ± 0.5% glial fibrillary acidic protein-positive astrocytes.

Cell viability assay

At 14–16 DIV, cells were washed twice with 10 mM HEPES buffer (washing buffer), pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl and 10 mM D-glucose. They were then incubated for 15 min at 37°C in a 5% CO_2 incubator with the desired concentration of NMDA in 10 mM HEPES buffer (stimulating buffer), pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM D-glucose and 10 μM glycine. Next, the cells were washed twice with the washing buffer and maintained in neurobasal medium containing 2% B27 supplements and 0.5 mM glutamine. After 24 h of incubation, the cells were incubated with 2 μg/mL propidium iodide (PI) for 20 min and then fixed in 4% parafomaldehyde to determine the total number of neurons by staining with anti-NeuN or anti-microtubule-associated protein 2ab (MAP2) antibody. Fluorescent images of cells were captured on a CCD camera (DP50, Olympus, Tokyo, Japan) mounted on an Olympus BX51 microscope equipped with a mercury arc lamp. The number of PI-, NeuN- or MAP2-positive cells was counted in 10 randomly chosen areas (245 × 320 μm) of each well. Results were obtained from 10 frames in four independent experiments. hrHGF was added at the desired concentrations 1 h before the addition of NMDA. For triple staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were detected using an *in situ* Apoptosis Detection Kit (MK500; Takara Bio Inc., Shiga, Japan). The cells were incubated with 2 μg/mL PI for 20 min and then stained with anti-MAP2 antibody to determine the total number of neurons. The microscopic observations were performed by an operator unaware of the study group.

Western immunoblotting

Cells were homogenized in ice-cold 0.32 M sucrose containing 0.2 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/mL each of antipain, aprotinin and leupeptin. Some cells were homogenized and their nuclei pelleted at 1 200 *g* for 10 min. The supernatant fluid was centrifuged at 10 000 *g* for 10 min to pellet the mitochondrial fraction. Samples were stored at −80°C until used and were thawed only once. Proteins that had been

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solubilized by heating at 100°C for 5 min in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol and 2% SDS, in 62.5 mM Tris-HCl, pH 6.8) were separated on 8.5, 10 or 15% 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). Immunoblots were scanned and quantified using computerized densitometry and an image analyzer (ATTO Co., Tokyo, Japan). Care was taken to ensure that bands to be semi-quantified were in the linear range of response. For removal of bound antibodies, the blots were incubated for 30 min at 65°C in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS and 0.1 M β-mercaptoethanol. The efficiency of the stripping procedure was confirmed by reacting the stripped blot with secondary antibody alone to ensure that there were no bound antibodies. Antibodies used for immunoblotting were anti-cleaved caspase 3 (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-c-Met, anti-Bax, anti-Bcl-x_L (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Bcl-2 (BD Transduction Laboratories, San Jose, CA, USA), anti-cytochrome *c* oxidase subunit IV (Molecular Probes, Inc., Eugene, OR, USA), anti-apoptosis-inducing factor (Chemicon, Temecula, CA, USA), anti-poly(ADP-ribose) (Biomol, Plymouth Meeting, PA, USA) and anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY, USA).

Immunoprecipitation

For immunoprecipitation of c-Met, cells were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 2 mM sodium orthovanadate, 0.1 mM PMSF, and 5 μg/mL each of antipain, aprotinin and leupeptin. The lysates were pre-incubated for 1 h with protein G-agarose beads to remove any proteins that had bound non-specifically to the protein G-agarose beads. The supernatant fluid was then incubated with anti-c-Met antibody for 2 h or overnight at 4°C. Protein G-agarose beads were added and the incubation was continued at 4°C for 2 h. The immune complexes isolated by centrifugation were washed, and the bound proteins were eluted by heating at 100°C in SDS sample buffer.

Immunohistochemistry

After having been fixed with ice-cold methanol and blocked, the cells were incubated overnight at 4°C with anti-AIF antibody, and then incubated for 1 h with Cy3-conjugated anti-rabbit IgG antibody at room temperature (25 ± 2°C). Thereafter, they were incubated with anti-NeuN antibody for 2 h and then with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody for 1 h. Fluorescent images of cells were captured on a CCD camera (DP50) mounted on an Olympus BX51 microscope equipped with a mercury arc lamp.

Statistics

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

Results

First, we confirmed the presence of the HGF receptor c-Met protein in cultured hippocampal neurons. In agreement with the results obtained from studies using cultured cortical (Machide *et al.* 1998) and cerebellar granule (Hossain *et al.* 2002) neurons, immunoblotting analysis showed that c-Met protein (140 kDa) was expressed in the cultured hippocampal neurons as well as in the adult hippocampal tissue (Fig. 1a). Also, by immunohistochemical analysis, we confirmed the expression of c-Met protein in dendrites of the cultured hippocampal neurons (Fig. 1b). To determine whether c-Met proteins in the cultured hippocampal neurons were activated in response to the application of hrHGF, we next examined the effect of hrHGF on the tyrosine phosphorylation of c-Met by performing immunoprecipitation with anti-c-Met antibody, followed by immunoblotting with anti-phosphotyrosine antibody. The tyrosine phosphorylation of c-Met was elevated relative to the initial amount as early as 10 min after the addition of 30 ng/mL hrHGF (Fig. 1c) and this increase was maintained for at least 60 min, although the amount of phosphorylated c-Met decreased gradually (Fig. 1c).

Next, hippocampal neurons were exposed to various concentrations of NMDA (10–300 μM) for 15 min. After

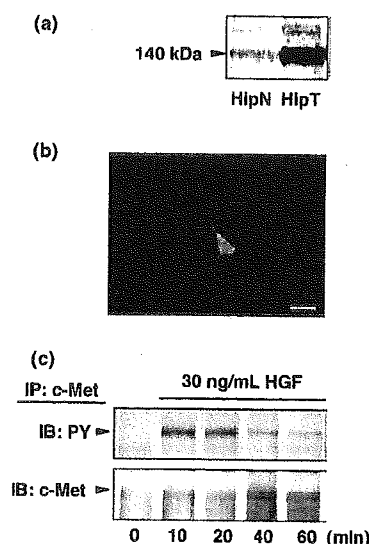

Fig. 1 Effects of hrHGF on tyrosine phosphorylation of c-Met in cultured hippocampal neurons. (a) Proteins (50 μg) from cultured hippocampal neurons at 14 DIV (HipN) and hippocampal tissue from an adult rat (HipT) were analyzed by immunoblotting with anti-c-Met antibody. c-Met protein migrated upon electrophoresis with an apparent molecular mass of 140 kDa. (b) Hippocampal neurons were fixed at 14 DIV and immunostained with anti-c-Met antibody. Scale bar represents 10 μm. (c) Proteins (200 μg) from cultured hippocampal neurons at 0, 10, 20, 40 and 60 min after treatment with 30 ng/mL hrHGF were immunoprecipitated (IP) with anti-c-Met antibody, and the precipitates were then analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody (PY). The blots were then stripped and re-probed with antibody against c-Met.

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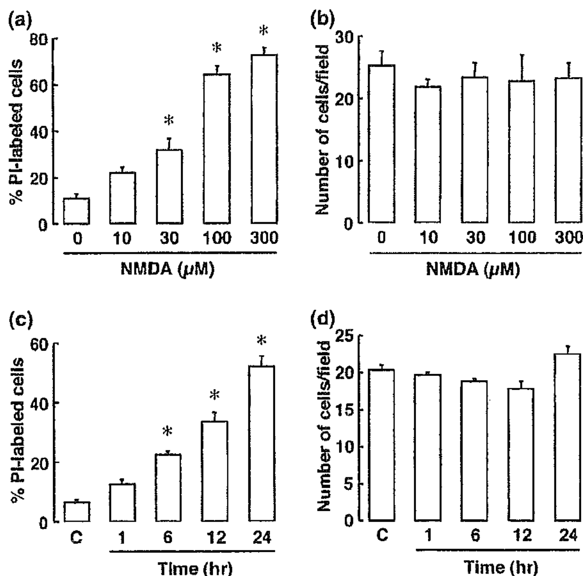

Fig. 2 Concentration-dependent profile and time course of changes in NMDA-induced excitotoxic damage in cultured hippocampal cells. (a) Cultured hippocampal cells were exposed to 10, 100 or 300 μM NMDA for 15 min. After 24 h of incubation, the cells were double-stained with propidium iodide (PI) for injured cells and anti-NeuN antibody for total neurons. *Indicates a significant difference from the NMDA-untreated group ($p < 0.05$). (b) Total number of NeuN-positive neurons counted for each condition in (a) in randomly chosen areas (245 × 320 μm). (c) Time course of the change in the number of PI-labeled cells. The percentage of PI-labeled cells among the total number of cells within the same field was determined. C indicates incubation under normal culture conditions for 24 h without pre-treatment with NMDA. *Indicates a significant difference from the NMDA-untreated group ($p < 0.05$). (d) Total number of NeuN-positive neurons counted for each condition in (c) in randomly chosen areas (245 × 320 μm). Results are the means ± SE from 10 frames in four wells in four independent experiments.

24 h of incubation under normal culture conditions, the number of PI-labeled neurons was increased (Fig. 2a) without changing the total number of neurons (Fig. 2b). The average percentages of PI-labeled cells among the total neurons (NeuN-labeled cells) treated with 0, 10, 30, 100 and 300 μM NMDA were 10.7 ± 2.2, 21.7 ± 2.6, 31.6 ± 4.9, 64.0 ± 3.7 and 72.3 ± 3.2%, respectively (Fig. 2a), and these increases were significant at the concentrations of 30, 100 and 300 μM NMDA (Fig. 2a). We next examined the time course of changes in the number of PI-labeled cells after the application of 100 μM NMDA for 15 min. The application of NMDA time-dependently increased the number of PI-labeled cells (Fig. 2c) without changing the total number of neurons (Fig. 2d). The average percentages of PI-labeled cells among the total NeuN-labeled neurons at 1, 6, 12 and 24 h after the incubation under normal conditions were 12.3 ± 1.5, 22.2 ± 1.2, 33.4 ± 3.1 and 51.9 ± 3.5%, respectively (Fig. 2c). The increase was significant from 6 h after the application of NMDA (Fig. 2c).

To determine the effects of hrHGF on NMDA-induced neuronal cell death, we added various concentrations of hrHGF to the medium containing hippocampal neurons. Treatment with hrHGF dose-dependently attenuated the increase in the number of PI-labeled cells, with the decrease being significant at 10 ng/mL hrHGF and above (Figs 3a and b). The total number of neurons counted for each condition was not changed (Fig. 3c). To further elucidate the protective effects of hrHGF against this neuronal cell death, we next investigated DNA fragmentation by TUNEL staining. The application of NMDA significantly increased the percentages of TUNEL-positive and TUNEL-PI-positive neurons (Figs 3d and e) without changing the number of neurons (Fig. 3f). Treatment with hrHGF attenuated the increase in the number of TUNEL-positive and TUNEL-PI-positive neurons (Figs 3d and e). We next examined the amounts of pro-apoptotic protein, Bax, and anti-apoptotic proteins, Bcl-2 and Bcl-xL. Not only the total amounts, but also the mitochondrial localization, of Bax, Bcl-2 and Bcl-xL, which were normalized with respect to actin or COX IV, were unaffected by the application of NMDA (Figs 4a and b). Total amounts of these proteins after the application of NMDA with hrHGF treatment were comparable with those of the non-treated control group (Fig. 4c). The effects of hrHGF on the activation of caspase 3 after the application of NMDA for 15 min were then examined. Activation of caspase 3 was assessed by immunoblotting with an antibody that recognizes the activated form of caspase 3. The application of NMDA time-dependently increased the amount of activated caspase 3 (Figs 5a and b). The average level of activated caspase 3 at 1, 12 and 24 h after incubation under normal conditions was 136.0 ± 17.0, 180.2 ± 12.8 and 221.2 ± 20.7%, respectively, of the level of the non-treated control group (Fig. 5b). The increase in activation was significant from 12 h after the incubation under normal culture conditions (Figs 5a and b). Treatment with hrHGF partially attenuated the increase in amount of activated caspase 3 at 24 h after incubation under normal culture conditions (Figs 5a and b). The average percentage of activated caspase 3 level at 24 h after incubation under normal culture conditions with hrHGF treatment was 170.6 ± 14.7% (Fig. 5b).

We next focused on caspase-independent pathways. To assess these, we determined the time course of change in the amount of AIF after the application of NMDA. The amount of AIF was not affected by NMDA application (Figs 6a and b). The intracellular localization of AIF was then investigated using double immunofluorescence histochemistry for it and the neural nuclear marker, NeuN. AIF was localized in the cytosol in control hippocampal neurons (Fig. 6c). After the application of NMDA, AIF was translocated to the nucleus at 12 (not shown) and 24 h (Fig. 6c). The application of NMDA significantly increased the percentages of AIF-

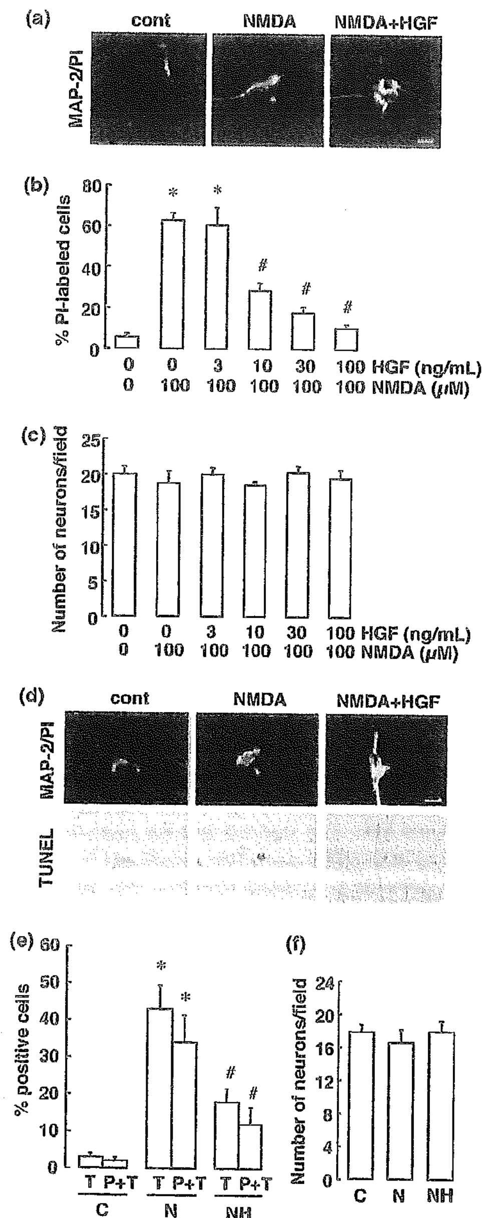

Fig. 3 Effects of hrHGF on NMDA-induced increase in the number of PI-labeled, TUNEL-positive and PI-stained/TUNEL-positive hippocampal cells. (a) Representative photomicrographs of hippocampal cells double-stained with PI (red) for injured cells and MAP2 (green) as a marker of neurons in cultures of non-treated control cells (cont) and 100 μM NMDA-treated cells without (NMDA) or with (NMDA + HGF) 30 ng/mL hrHGF. Scale bar represents 10 μm. (b) hrHGF was added at the indicated concentrations (3, 10, 30 and 100 ng/mL) 1 h before the addition of NMDA. After 24 h of incubation, the cells were double-stained with propidium iodide (PI) for injured cells and anti-MAP2 antibody for total number of neurons. (c) Total number of MAP2-positive neurons counted for each condition in (b) in randomly chosen areas (245 × 320 μm). Results are the means ± SE from 10 frames in four wells in four independent experiments. (d) Representative photomicrographs of hippocampal cells triple-stained with PI (red) for injured cells, MAP2 (green) as a marker of neuron and TUNEL for DNA-damaged cells in cultures of non-treated control cells (cont) and 100 μM NMDA-treated cells without (NMDA) or with (NMDA + HGF) 30 ng/mL hrHGF. Scale bar represents 10 μm. (e) Changes in the number of TUNEL-positive (T) and PI-stained/TUNEL-positive (P + T) cells in cultures of non-treated control cells (C) and 100 μM NMDA-treated cells without (N) or with (NH) 30 ng/mL hrHGF. The percentage of MAP2-positive cells among the total number of cells within the same field was calculated. (f) Total number of MAP2-positive neurons counted for each condition in (e) in randomly chosen areas (245 × 320 μm). Results are the means ± SE from 10 frames in four wells in four independent experiments. *Indicates a significant difference from the NMDA- and hrHGF-untreated group ($p < 0.05$). #Indicates a significant difference from the NMDA-treated and hrHGF-untreated group ($p < 0.05$).

NeuN-positive neurons (Fig. 6d) without changing the total number of neurons counted (Fig. 6e). Treatment with hrHGF attenuated the translocation of AIF to the nucleus at 24 h after the application of NMDA (Figs 6c and d) without changing the total number of neurons (Fig. 6e). We further examined poly(ADP-ribose) polymerase activity at 24 h, which was estimated by immunoblotting analysis using an anti-poly(ADP-ribose) antibody to detect poly(ADP-ribose) polymer formation. The application of NMDA significantly increased the level of poly(ADP-ribosyl)ated proteins, which were detected as diffuse bands of several molecular weights (Figs 7a and b). Treatment with hrHGF prevented poly(ADP-ribose) polymer formation (Figs 7a and b).

Discussion

We have demonstrated that treatment with hrHGF not only dose-dependently attenuates NMDA receptor-mediated neuronal cell injury, which was determined by PI uptake, but also decreases the number of TUNEL-positive and TUNEL-PI-positive hippocampal neurons, suggesting that hrHGF protects hippocampal neurons from NMDA-induced apoptotic cell death. In a recent study using mature sympathetic neurons of the superior cervical ganglion, HGF was shown to exert an anti-apoptotic effect through phosphatidylinositol-3 kinase/Akt and ERK 1/2 signaling pathways (Thompson *et al.* 2004). In addition, it was reported that HGF prevented hypoxia/reoxygenation-induced apoptosis in murine lung endothelial cells by blocking Bax translocation to the mitochondria, the blocking being mediated by the p38 MAPK pathway, and by stabilizing Bcl-xL protein levels (Wang *et al.* 2004b). However, the intracellular signaling pathways involved in protection against neuronal injuries have not been extensively studied. We found that neither the total amount nor the mitochondrial localization of Bax

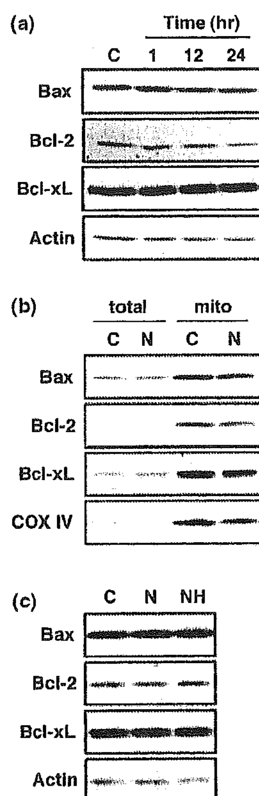

Fig. 4 Expression of apoptotic signaling proteins in cultured hippocampal neurons after the application of NMDA. (a) Cells were treated with 100 μM NMDA for 15 min, followed by incubation under normal culture conditions for 1, 12 or 24 h. Total proteins (10 μg) were analyzed by immunoblotting with anti-Bax, anti-Bcl-2, anti-Bcl-xL and anti-actin antibodies. There were no changes in the total amounts of these proteins after the application of NMDA. (b) The mitochondrial fraction was isolated 24 h after the application of NMDA, and the mitochondrial localization (mito) of Bax, Bcl-2 and Bcl-xL proteins in non-treated control (C) and 100 μM NMDA-treated (N) cells was analyzed by immunoblotting. Additional immunoblotting analysis confirmed the presence of cytochrome *c* oxidase subunit IV (COX IV) in mitochondrial fractions. (c) Effects of hrHGF on total amounts of Bax, Bcl-2 and Bcl-xL 24 h after the application of NMDA. Total proteins (10 μg) were analyzed by immunoblotting with anti-Bax, anti-Bcl-2, anti-Bcl-xL and anti-actin antibodies. Total amounts of these proteins with hrHGF treatment (NH) were comparable with those of NMDA-treated cells without hrHGF (N) and non-treated control cells (C).

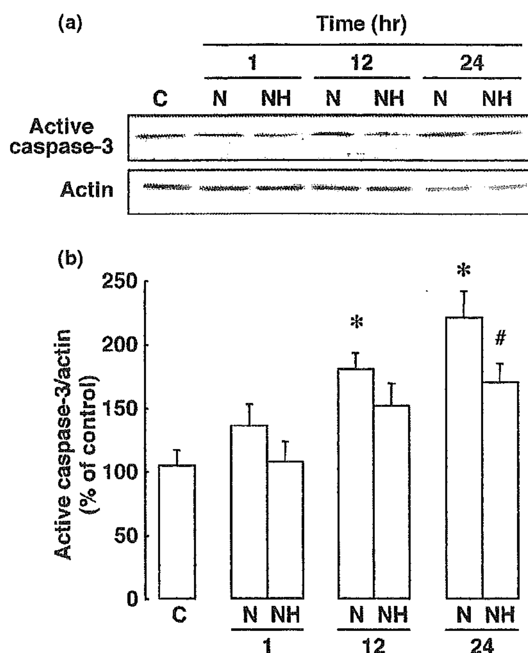

Fig. 5 Effects of hrHGF on NMDA-induced activation of caspase 3 in cultured hippocampal cells. (a) Cells were treated with 100 μM NMDA for 15 min, followed by incubation under normal culture conditions for 1, 12 and 24 h. Typical blots for cleaved caspase 3 and actin from non-treated control cells (C) and from NMDA-treated cells without (N) or with (NH) 30 ng/mL hrHGF. (b) Bands corresponding to cleaved caspase 3 and actin were scanned, and the scanned bands were normalized by actin on the same blot. Results are the means ± SE ($n = 4–7$). *Indicates a significant difference from the NMDA- and hrHGF-untreated group ($p < 0.05$). #Indicates a significant difference from the corresponding NMDA-treated group ($p < 0.05$).

protein was altered after the application of NMDA. The levels of Bcl-2 and Bcl-xL proteins were also unaffected, regardless of treatment with or without hrHGF. These findings suggest that the anti-apoptotic effect of HGF on hippocampal neurons was not likely to be due to the inhibition of Bax mitochondrial translocation, or increase in the expression of Bcl-2 and Bcl-xL proteins. In contrast to our findings, application of NMDA at higher concentrations, and for longer periods, to primary cultures of forebrain increased the expression of Bax relative to that of Bcl-xL (McInnis *et al.* 2002). Therefore, we cannot rule out the possibility that HGF might have the ability to protect hippocampal neurons against NMDA-induced neurotoxicity via regulation of the expression of pro- and anti-apoptotic Bcl-2 family proteins under severer pathological conditions. In this sense, it was demonstrated that HGF rapidly induced Bcl-xL expression in cardiomyocytes cultured under the condition of serum starvation (Nakamura *et al.* 2000).

Mitochondria have been shown to play a pivotal role in caspase-dependent and -independent apoptotic pathways. The caspase-dependent pathway is initiated by the release of cytochrome *c* from mitochondria, and the released cytochrome *c* associates with apoptotic protease-activating factor APAF1 to activate caspases (Danial and Korsmeyer 2004). It has been documented that the NMDA-induced apoptotic cascade consists of calcium overload of mitochondria, production of reactive oxygen species, opening of the mitochondrial permeability transition pore, release of cyto-

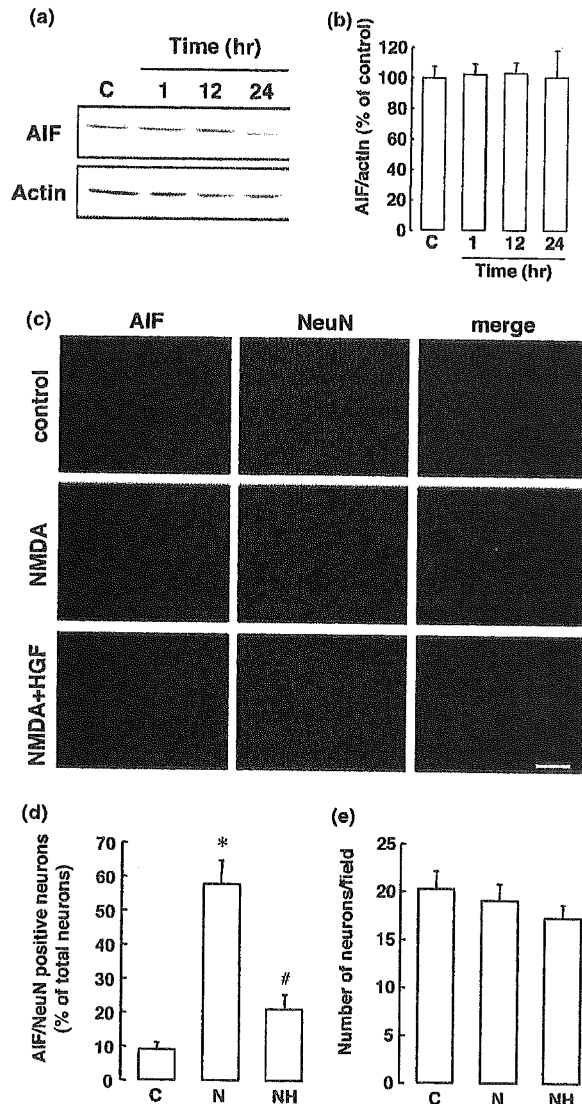

Fig. 6 Effects of hrHGF on NMDA-induced change in localization of AIF in cultured hippocampal cells. (a) Cells were treated with 100 μM NMDA for 15 min and then incubated under normal culture conditions for 1, 12 or 24 h. Total proteins (10 μg) were analyzed by immunoblotting with anti-AIF and anti-actin antibodies. (b) Bands corresponding to AIF and actin were scanned, and the scanned bands were normalized by actin on the same blot. Results are the means ± SE (n = 4–7). There were no changes in total amounts of AIF after the application of NMDA. (c) Representative photomicrographs of hippocampal cells double-stained for AIF (red) and NeuN (green), the latter being a nuclear marker of neurons in cultures of non-treated control cells (control), and 100 μM NMDA-treated cells without (NMDA) or with (NMDA + HGF) 30 ng/mL hrHGF. Scale bar represents 10 μm. (d) Changes in the number of AIF/NeuN-positive cells in cultures of non-treated control cells (C) and 100 μM NMDA-treated cells without (N) or with (NH) 30 ng/mL hrHGF. The percentage of NeuN-positive cells among the total number of cells within the same field was calculated. (e) Total number of NeuN-positive neurons counted for each condition in (d) in randomly chosen areas (245 × 320 μm). Results are the means ± SE from 10 frames in four wells in four independent experiments. *Indicates a significant difference from the NMDA- and hrHGF-untreated group ($p < 0.05$). #Indicates a significant difference from the NMDA-treated and hrHGF-untreated group ($p < 0.05$).

chrome *c* and, ultimately, activation of caspase 3 (Budd *et al.* 2000; Tenneti and Lipton 2000). In the present study, the NMDA-induced activation of caspase 3, which was comparable with the findings on cerebrocortical neurons (Budd *et al.* 2000; Tenneti and Lipton 2000; Okamoto *et al.* 2002), was partially attenuated by hrHGF treatment. Therefore, the protective effect of hrHGF, at least in part, involves inhibition of caspase-dependent pathways, although further study will be required to clarify targets of hrHGF in the caspase-dependent apoptotic cascade. It was reported that overexpression of the X chromosome-linked inhibitor of apoptosis protein, XIAP, a member of the inhibitor of apoptosis protein (IAP) family, attenuated both the activation of caspase 3 and neuronal cell death in the hippocampal CA1 region after transient forebrain ischemia (Xu *et al.* 1999). It is likely that the second baculoviral inhibitory repeat domain of XIAP is sufficient to inhibit caspases 3 and 7 (Deveraux *et al.* 1999), and that XIAP is the most potent inhibitor of caspase-dependent apoptosis (Deveraux *et al.* 1999) among the IAP family, including cIAP1, cIAP2, XIAP, NAIP, Livin/KIAP, BRUCE/Apollon and Survivin (Keane *et al.* 2001; Silke and Vaux 2001). An effective level of XIAP protein maintained by HGF might play a role in the inhibition of

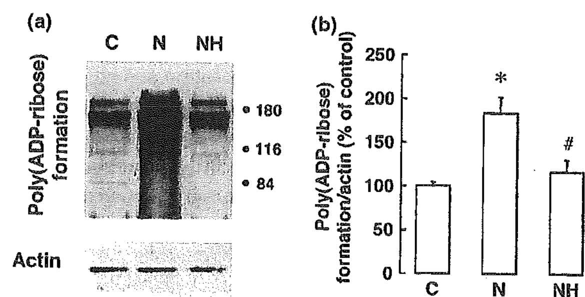

Fig. 7 Effects of hrHGF on poly(ADP-ribose) formation in cultured hippocampal cells. (a) Cells were treated with 100 μM NMDA for 15 min and then incubated under normal culture conditions for 24 h. Typical blots for poly(ADP-ribose) formation and actin from non-treated control cells (C) and 100 μM NMDA-treated cells without (N) or with (NH) 30 ng/mL hrHGF. (b) Bands corresponding to poly(ADP-ribose) formation and actin were scanned, and scanned bands were normalized by actin on the same blot. Results are the mean ± SE (n = 4–5). *Indicates a significant difference from the NMDA- and hrHGF-untreated group ($p < 0.05$). #Indicates a significant difference from the NMDA-treated and hrHGF-untreated group ($p < 0.05$).

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caspase 3 activity and could be involved in the protective effects of hrHGF against NMDA-induced excitotoxicity. However, we cannot fully rule out the possibility that HGF might be associated with other targets to protect hippocampal neurons as hrHGF only partially inhibited caspase 3 activity.

We further investigated the role of a caspase-independent pathway in the protective effect of hrHGF against NMDA-induced excitotoxicity. An important protein in the caspase-independent mechanism is thought to be the apoptosis-inducing factor, AIF (Cregan *et al.* 2004), which is usually located in the mitochondrial intermembranous space (Susin *et al.* 1999) and likely protects against oxidative stress in normal cells (Klein *et al.* 2002). Once cell death signaling is set in motion, AIF is translocated to the nucleus to mediate chromatin condensation and large-scale (50 kbp) DNA fragmentation (Lorenzo *et al.* 1999; Susin *et al.* 1999; Cande *et al.* 2002; Ye *et al.* 2002). Excessive calcium influx through the NMDA receptor leads to the activation of neuronal nitric oxide synthase and the production of nitric oxide (NO). Subsequently, the interaction of NO and superoxide generates peroxynitrite ($ONOO^-$), which is capable of damaging DNA. This damage leads to poly(ADP-ribose) polymerase-1 (PARP-1) activation. Overactivation of PARP-1 triggers a poly(ADP-ribosyl)ation-dependent mechanism that mediates relocation of AIF from mitochondria to the nucleus. Therefore, it is likely that NMDA receptor-mediated excitotoxicity is involved in the damage caused to DNA by oxidative stress, and in the activation of the DNA damage-sensing enzyme PARP-1 (Mandir *et al.* 2000). In this sense, NMDA-induced translocation of AIF to the nucleus and neuronal death were abolished in the cortical neurons from PARP-1 knockout mice (Yu *et al.* 2002; Wang *et al.* 2004a). We have demonstrated in this study that AIF was translocated to the nucleus of the hippocampal neurons after the application of NMDA. This phenomenon was recently reported to occur in cortical neurons (Wang *et al.* 2004a; Cheung *et al.* 2005). Treatment with hrHGF prevented this AIF translocation and poly(ADP-ribose) formation. It has also been reported that HGF exhibits a protective effect on cardiomyocytes subjected to H_2O_2-induced oxidative stress (Ueda *et al.* 2001). Therefore, HGF may inhibit poly(ADP-ribose) formation and the translocation of AIF into the nucleus through an attenuation of NMDA receptor-mediated oxidative stress. Although the administration of hrHGF or the gene of HGF prevents neuronal cell death after cerebral ischemia *in vivo* (Miyazawa *et al.* 1998; Hayashi *et al.* 2001; Tsuzuki *et al.* 2001; Date *et al.* 2004; Shimamura *et al.* 2004), the question remains as to how the protective effects against neuronal injuries are mediated by intracellular signaling. Our findings first demonstrated inhibition of AIF translocation into the nucleus as a possible mechanism for the protective effect of HGF against NMDA-induced excitotoxicity in hippocampal neurons.

Earlier findings implicated NMDA receptors in a variety of neurological and neurodegenerative disorders that include brain ischemia, epilepsy, Parkinson's and Alzheimer's diseases, Huntington's chorea and amyotrophic lateral sclerosis. Thus, it is important to determine the effect of HGF and to explore the nature of intracellular signal transduction pathways via c-Met under various NMDA-mediated pathophysiological conditions to develop appropriate therapeutic strategies for these diseases. Our results suggest that treatment with hrHGF is capable of protecting hippocampal neurons against NMDA-induced excitotoxicity via the partial prevention of caspase 3 activity and the inhibition of AIF translocation to the nucleus.

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Anxiolytic Effect of Hepatocyte Growth Factor Infused into Rat Brain

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Key Words
Anxiety · Hepatocyte growth factor · Rat

Abstract
Background: Hepatocyte growth factor (HGF) has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination. HGF may have a role in the mediation of anxiety. Very little is known about other central behavioral effects of HGF. ***Objective:*** Our aim was to determine what effect HGF has on anxiety in rats. ***Methods:*** HGF was infused at a constant rate into cerebral lateral ventricles and its effect on anxiety in rats was monitored. ***Results:*** In the elevated plus maze test and the black and white box test, HGF administration caused all indicators of anxiety to increase. No significant effect on general locomotor activity was seen. ***Conclusion:*** HGF infusion into the brain produces an anxiolytic effect.

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Introduction

Hepatocyte growth factor (HGF) is a potent angiogenic growth factor [1–3]. Recently, it has been reported that HGF is induced in neurons during ischemia [4] and that HGF is neuroprotective against postischemic delayed neuronal death in the hippocampus [5, 6].

In the brain, HGF is expressed by specific classes of neurons in addition to nonneuronal cells in the ependyma and choroid plexus [7]. In contrast to HGF, c-Met transcripts have been predominantly localized in neurons of the cerebral cortex, hippocampus and septum [8–10]. HGF elevated the proto-oncogene c-*fos* mRNA in cultured septal neurons, showing a functional interaction between c-Met and its ligand [10]. This result, together with the presence of c-Met in the developing brain, raised the possibility that HGF may have a neurotrophic activity on central neurons. In keeping with this hypothesis, Hamanoue et al. [11] showed that HGF promoted the survival of cultured mesencephalic tyrosine hydroxylase-positive neurons. HGF acts on calbindin-D-containing hippocampal neurons and increases their neurite outgrowth, suggesting that HGF plays an important role in the maturation and function of hippocampal neurons [12]. Transfection of HGF gene into the subarachnoid space prevent-

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ed delayed neuronal death, accompanied by a significant increase in HGF in the cerebrospinal fluid (CSF). Prevention of delayed neuronal death by HGF is due to the inhibition of apoptosis through the blockade of bax translocation from the cytoplasm to the nucleus. HGF gene transfer into the subarachnoid space may provide a new therapeutic strategy for cerebrovascular disease [13].

HGF has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination [14]. Mice with a targeted mutation of the gene encoding urokinase plasminogen activator receptor (uPAR), a key component in HGF/scatter factor (SF) activation and function, have decreased levels of HGF/SF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages. Mice of the uPAR -/- strain survive until adulthood, and behavior testing demonstrates that they have an increased anxiety state [14]. HGF may have a role in the mediation of anxiety.

This is the first report to determine what effect HGF infused into cerebral lateral ventricles has on anxiety in rats.

Materials and Methods

Animals

Five-week-old male Wistar rats (Seack Yoshitomi Co., Fukuoka, Japan) were used for the present study. The number of rats was each 10 rats for experimental and control groups. The rats were housed in pairs for 3 weeks prior to the start of behavioral experiments in a sound-proof room at 24 ± 0.5°C, 50 ± 5% relative humidity, with controlled 12-hour light-dark cycles (light from 18:00 to 6:00), and were allowed free access to food and water. The room was cleaned at random in a dim, red light. All testing was performed in July during the dark phase using a dim, red light. Animal care was in accordance with the guidelines for animal experimentation of Oita Medical University.

Surgical Procedures

Each rat was anesthetized with chloral hydrate (400 mg/kg, i.p.), a brain infusion cannula (brain infusion kit, model 1007D, Alzet Corp., Palo Alto, Calif., USA) was stereotactically implanted into the lateral cerebral ventricle (0.92 mm caudal and 1.6 mm lateral to the bregma and 3.5 mm deep), and a mini-osmotic pump (micro-osmotic pump, model 1003D; Alzet Corp.) was placed into subcutaneous tissue of the back. After the operation, rats were injected with ceftriaxone sodium (20 mg/kg, i.p.). Either HGF (30 μg) in the experimental group or a vehicle in the control group (Ringer's solution, pH = 7.4) was infused at a constant rate into the lateral ventricle of the rat via the micro-osmotic pump over a 3-day period. Tsuzuki et al. [15] reported that continuous intraventricular administration of the human recombinant HGF by using an osmotic mini-pump reduced the infarct volumes in the brain lesion and prevented apoptotic neuronal cell death.

Materials

A vehicle (Ringer's isotonic solution, pH 7.4) was used as a control. HGF was synthesized in the Division of Biochemistry, Department of Oncology, Biomedical Research Center, Osaka University Medical School.

Behavioral Testing

The first day of testing was concerned with measuring anxiety. All rats were subjected to the 'elevated plus maze', followed on the same day by the 'black and white box' test. Ethological measures in elevated plus maze comprised frequency scores for supported head dipping (exploratory movement of head/shoulders over the side of the maze), and stretched attend posture (exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion). At the end of the day, rats received inescapable electric foot shocks to condition fear. On the second day, rats performed the conditioned fear test. Conditioned response models of fear and anxiety are based on classical procedures of fear conditioning [16]. On day 1 of fear conditioning, each rat was individually subjected to 5 min of inescapable electric foot shock (10 shocks of 1 s duration and 2 mA intensity, each shock separated by an interval of 40 s) in a chamber with a grid floor (31 × 30 × 25 cm). Twenty-four hours after the foot shock, the rats were again placed in the shock chamber and observed for 5 min without shocks. During the 5-min observation period, freezing behavior was recorded using a video camera. Every 10 s, the behavior was classified as either freezing or active. Freezing was defined as the absence of any observable movement of the body and/or vibrissae, aside from the movement necessitated by respiration. We also investigated general locomotor activity.

Elevated Plus Maze

The elevated plus maze consisted of two opposite open arms (50 × 10 cm) without side walls and two opposite enclosed arms (50 × 8 × 40 cm), and was elevated 50 cm above the floor. The rats were placed in the middle of the maze facing one of the open arms, and immediately left alone in the test room. They were observed and their responses were recorded for 300 s via a video camera. Five parameters were measured during 5 min: (1) time spent in the open arms, (2) total number of entries into the open arms, (3) number of stretched attend postures, and (4) number of head dips over the edge of the platform.

Black and White Box

The wall of the test box was 27 cm high, the size of each compartment was 23 × 27 cm, and the two compartments were connected by a 10-cm high semicircular hole. Both white and red light sources were 40 W, and the light sources were located 17 cm above the floor of the two compartments. The rats were placed in the center of the white compartment and the number of entries and time spent in the black and white compartments during 5 min were recorded. An entry into another compartment was scored whenever a rat placed all four paws in that compartment.

General Locomotor Activity

We investigated general locomotor activity of the rats by means of infrared photobeam breaks, since locomotion influences exploratory activity. The apparatus was 36 cm in height and the floor size was 30 × 30 cm. We measured the locomotor activity by photobeam breaks for 2 h.

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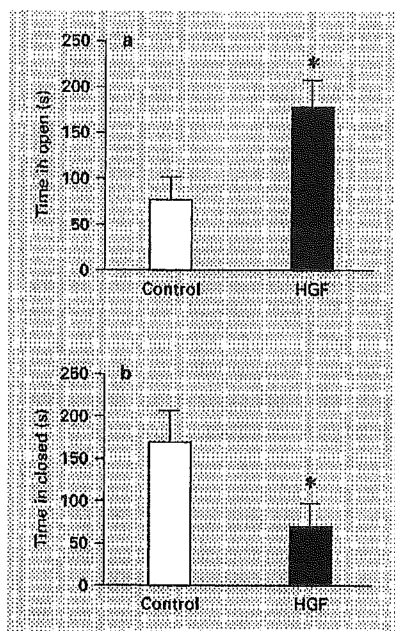

Fig. 1. a Time spent in the open arm of the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. **b** Time spent in the closed arm of the elevated plus maze was significantly decreased in the HGF-infused group compared to the control group. * $p < 0.05$ vs. vehicle- and HGF-infused group.

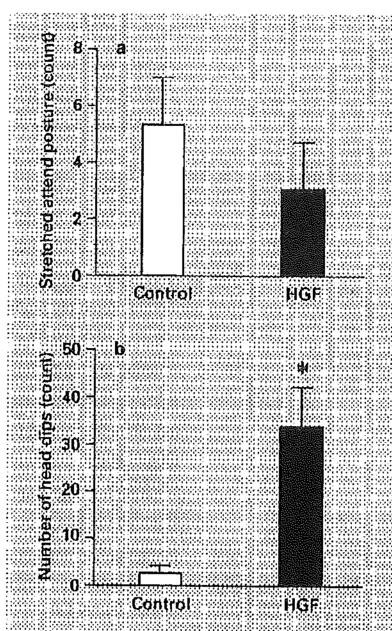

Fig. 2. a No effect of HGF or the control vehicle on the number of stretched attend postures in the elevated plus maze was seen. **b** The number of head dips in the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. * $p < 0.05$ vs. vehicle- and HGF-infused group.

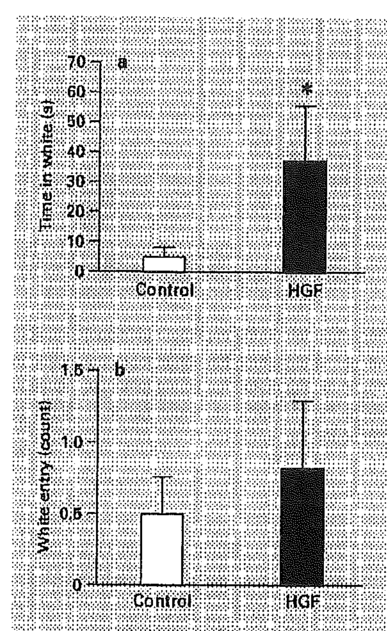

Fig. 3. In the black and white box test, the time spent in the white chamber was significantly increased in the HGF-infused group, compared to the controls (**a**). **b** No significant effect of HGF on the number of white chamber entries was seen. * $p < 0.05$ vs. vehicle- and HGF-infused group.

Statistical Analysis

The data were presented as means ± SE of the individual values from each group. Behavioral data (except for general locomotor activity) were analyzed using the Student t test for independent samples. The data of general locomotor activity were subjected to a two-way ANOVA. Statistical significance was accepted for $p < 0.05$.

Results

Time spent in the open arm of the elevated plus maze was significantly increased in the HGF-infused group, compared to the vehicle-treated group [t(18) = 2.43, $p < 0.031$; fig. 1a]. Time spent in the closed arm of the elevated plus maze was significantly decreased in the HGF-infused group compared to the control group [t(18) = 2.23, $p < 0.045$; fig. 1b]. No effect of HGF or the control vehicle on the number of stretched attend postures in the elevated plus maze was seen [t(18) = 1.01; fig. 2a]. The number of head dips in the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. The number of head dips was significantly increased in the HGF-infused group compared to the vehicle-treated group [t(18) = 2.61, $p < 0.023$; fig. 2b]. In the black and white box test, the time spent in the white chamber was significantly decreased in the HGF-infused group, compared to the controls [t(18) = 2.25, $p < 0.048$; fig. 3a]. No significant effect of HGF on the number of white chamber entries was seen [t(18) = 0.65; fig. 3b]. The amount of conditioned fear stress-induced freezing behavior was significantly decreased in the HGF-infused group compared to the vehicle-treated group [t(18) = 2.38, $p < 0.036$; fig. 4]. No significant differences between the two groups were seen in general locomotor activity ($F_{2, 35} = 1.30$; fig. 5).

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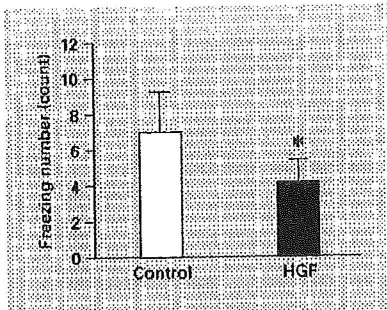

Fig. 4. Level of freezing induced by conditioned fear was significantly decreased in the HGF-infused group, compared to the controls. * $p < 0.05$ vs. vehicle- and HGF-infused group.

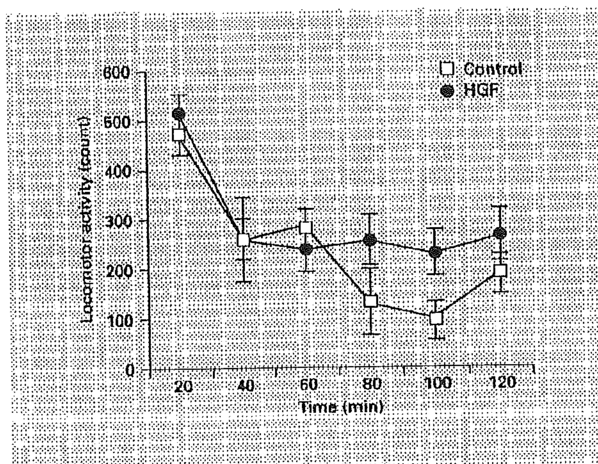

Fig. 5. No significant effect of HGF on general locomotor activity (number of photobeam breaks) was seen. Data are means ± SEM.

Discussion

This study provides the first evidence that HGF has an anxiolytic effect on the rat. HGF infusion into a lateral ventricle decreased anxiety as measured in the elevated plus maze and black and white box tests.

HGF was originally known as a cell mitogen and motogen, and has since been found to be a multifunctional growth factor with a variety of biological activities in numerous types of cells [17, 18]. The variety of biological functions attributed to HGF results from its interaction with its only known high-affinity transmembrane receptor, c-Met tyrosine kinase, present on target cells including central neurons [10, 19]. Coexpression of c-Met and HGF is oncogenic, and has been implicated in the progression of certain malignancies, in part, by decreasing tumor cell death and apoptosis [20, 21]. HGF and c-Met have been found to be present in specific subtypes of hippocampal neurons, cortex, septum, and cerebellum of both developing and adult mammalian brains [10, 12], but few reports exist concerning the biological activity of HGF in the CNS. A HGF-activating protease, HGF activator (HGFA), has recently been identified as a key enzyme that regulates the activity of HGF in vivo. HGFA appears to be associated with the cell surface. The HGFA antibody stained only astrocytes in the white matter in all the brain tissues. Expression of the mRNAs of HGF and HGFA was also seen in white matter astrocytes [22]. Recent studies have recognized effects of HGF on motor neuron survival, development and maturation, and on the function of cortical and hippocampal neurons in the developing brain [11, 12]. Tsuboi et al. [23] reported that consistent with the immunohistochemical data, a significantly higher concentration of HGF in Alzheimer's disease (AD) CSF was found as compared with controls. A significant correlation was also seen between CSF HGF levels and white matter high-signal foci determined on brain magnetic resonance imaging in AD patients. CSF HGF levels correspond with the white matter damage in AD brain [23].

Treatment with HGF induced an anxiolytic effect. But the mechanism of action of HGF has not been elucidated. The c-Met receptor has a heterodimeric protein which contains intracellular tyrosine kinase domains. Binding of HGF to c-Met might induce the anxiolytic effect [24]. HGF has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination. Mice with a targeted mutation of the gene encoding uPAR, a key component in HGF/SF activation and function, have decreased levels of HGF/SF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages. Mice of the uPAR -/- strain survive until adulthood, and behavior testing demonstrates that they have an increased anxiety state [14]. HGF may have a role in the mediation of anxiety.

In summary, this study reports that HGF infusion into the brain produced an anxiolytic effect in rats, as evaluated using the elevated plus maze, black and white box tests and conditioned fear test.

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