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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References

Achim C. L., Katyal S., Wiley C. A., Shiratori M., Wang G., Oshika E., Petersen B. E., Li J. M. and Michalopoulos G. K. (1997) Expression of HGF and cMet in the developing and adult brain. *Brain Res. Dev. Brain Res.* **102**, 299–303.

Balkovetz D. F. and Lipschutz J. H. (1999) Hepatocyte growth factor and the kidney: it is not just for the liver. *Int. Rev. Cytol.* **186**, 225–260.

Bottaro D. P., Rubin J. S., Faletto D. L., Chan A. M., Kmiecik T. E., Vande Woude G. F. and Aaronson S. A. (1991) Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251**, 802–804.

Budd S. L., Tenneti L., Lishnak T. and Lipton S. A. (2000) Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *Proc. Natl Acad. Sci. USA* **97**, 6161–6166.

Cande C., Cecconi F., Dessen P. and Kroemer G. (2002) Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? *J. Cell Sci.* **115**, 4727–4734.

Cheung E. C., Melanson-Drapeau L., Cregan S. P., Vanderluit J. L., Ferguson K. L., McIntosh W. C., Park D. S., Bennett S. A. and Slack R. S. (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. *J. Neurosci.* **25**, 1324–1334.

Cregan S. P., Dawson V. L. and Slack R. S. (2004) Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* **23**, 2785–2796.

Dale N. and Roberts A. (1985) Dual-component amino-acid-mediated synaptic potentials: excitatory drive for swimming in *Xenopus* embryos. *J. Physiol.* **363**, 35–59.

Danial N. N. and Korsmeyer S. J. (2004) Cell death: critical control points. *Cell* **116**, 205–219.

Date I., Takagi N., Takagi K., Kago T., Matsumoto K., Nakamura T. and Takeo S. (2004) Hepatocyte growth factor improved learning and memory dysfunction of microsphere-embolized rats. *J. Neurosci. Res.* **78**, 442–453.

Deveraux Q. L., Leo E., Stennicke H. R., Welsh K., Salvesen G. S. and Reed J. C. (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J.* **18**, 5242–5251.

Dingledine R., Borges K., Bowie D. and Traynelis S. F. (1999) The glutamate receptor ion channels. *Pharmacol. Rev.* **51**, 7–61.

Gutierrez H., Dolcet X., Tolcos M. and Davies A. (2004) HGF regulates the development of cortical pyramidal dendrites. *Development* **131**, 3717–3726.

Hamanoue M., Takemoto N., Matsumoto K., Nakamura T., Nakajima K. and Kohsaka S. (1996) Neurotrophic effect of hepatocyte growth factor on central nervous system neurons *in vitro*. *J. Neurosci. Res.* **43**, 554–564.

Hayashi K., Morishita R., Nakagami H., Yoshimura S., Hara A., Matsumoto K., Nakamura T., Ogihara T., Kaneda Y. and Sakai N. (2001) Gene therapy for preventing neuronal death using hepatocyte growth factor: *in vivo* gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Ther.* **8**, 1167–1173.

Higuchi O. and Nakamura T. (1991) Identification and change in the receptor for hepatocyte growth factor in rat liver after partial hepatectomy or induced hepatitis. *Biochem. Biophys. Res. Commun.* **176**, 599–607.

Honda S., Kagoshima M., Wanaka A., Tohyama M., Matsumoto K. and Nakamura T. (1995) Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. *Brain Res. Mol. Brain Res.* **32**, 197–210.

Hossain M. A., Russell J. C., Gomez R., Laterra J. and Gomes R. (2002) Neuroprotection by scatter factor/hepatocyte growth factor and FGF-1 in cerebellar granule neurons is phosphatidylinositol 3-kinase/akt-dependent and MAPK/CREB-independent. *J. Neurochem.* **81**, 365–378.

Huettner J. E. and Baughman R. W. (1986) Primary culture of identified neurons from the visual cortex of postnatal rats. *J. Neurosci.* **6**, 3044–3060.

Keane R. W., Kraydieh S., Lotocki G., Alonso O. F., Aldana P. and Dietrich W. D. (2001) Apoptotic and antiapoptotic mechanisms after traumatic brain injury. *J. Cereb. Blood Flow Metab.* **21**, 1189–1198.

Klein J. A., Longo-Guess C. M., Rossmann M. P., Seburn K. L., Hurd R. E., Frankel W. N., Bronson R. T. and Ackerman S. L. (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature* **419**, 367–374.

Korhonen L., Sjoholm U., Takei N., Kern M. A., Schirmacher P., Castren E. and Lindholm D. (2000) Expression of c-Met in developing rat hippocampus: evidence for HGF as a neurotrophic factor for calbindin D-expressing neurons. *Eur. J. Neurosci.* **12**, 3453–3461.

Lorenzo H. K., Susin S. A., Penninger J. and Kroemer G. (1999) Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. *Cell Death Differ.* **6**, 516–524.

Machide M., Kamitori K., Nakamura Y. and Kohsaka S. (1998) Selective activation of phospholipase C gamma1 and distinct protein kinase C subspecies in intracellular signaling by hepatocyte growth factor/scatter factor in primary cultured rat neocortical cells. *J. Neurochem.* **71**, 592–602.

Mandir A. S., Poitras M. F., Berliner A. R., Herring W. J., Guastella D. B., Feldman A., Poirier G. G., Wang Z. Q., Dawson T. M. and Dawson V. L. (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly (ADP-ribose) polymerase. *J. Neurosci.* **20**, 8005–8011.

Matsumoto K. and Nakamura T. (1996) Emerging multipotent aspects of hepatocyte growth factor. *J. Biochem.* **119**, 591–600.

Matsumoto K. and Nakamura T. (2001) Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. *Kidney Int.* **59**, 2023–2038.

McInnis J., Wang C., Anastasio N., Hultman M., Ye Y., Salvemini D. and Johnson K. M. (2002) The role of superoxide and nuclear factor-kappaB signaling in *N*-methyl-D-aspartate-induced necrosis and apoptosis. *J. Pharmacol. Exp. Ther.* **301**, 478–487.

Miyazawa T., Matsumoto K., Ohmichi H., Katoh H., Yamashima T. and Nakamura T. (1998) Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: a novel neurotrophic factor. *J. Cereb. Blood Flow Metab.* **18**, 345–348.

Nakamura T., Nawa K. and Ichihara A. (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **122**, 1450–1459.

Nakamura T., Nishizawa T., Hagiya M., Seki T., Shimonishi M., Sugimura A., Tashiro K. and Shimizu S. (1989) Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342**, 440–443.

Nakamura T., Mizuno S., Matsumoto K., Sawa Y., Matsuda H. and Nakamura T. (2000) Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J. Clin. Invest.* **106**, 1511–1519.

Okamoto S., Li Z., Ju C., Scholzke M. N., Mathews E., Cui J., Salvesen G. S., Bossy-Wetzel E. and Lipton S. A. (2002) Dominant-interfering forms of MEF2 generated by caspase cleavage contribute to NMDA-induced neuronal apoptosis. *Proc. Natl Acad. Sci. USA* **99**, 3974–3979.

Silke J. and Vaux D. L. (2001) Two kinds of BIR-containing protein — inhibitors of apoptosis, or required for mitosis. *J. Cell Sci.* **114**, 1821–1827.

Shimamura M., Sato N., Oshima K., Aoki M., Kurinami H., Waguri S., Uchiyama Y., Ogihara T., Kaneda Y. and Morishita R. (2004) Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *Circulation* **109**, 424–431.

Sun W., Funakoshi H. and Nakamura T. (2002a) Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. *Mol. Brain Res.* **103**, 36–48.

Sun W., Funakoshi H. and Nakamura T. (2002b) Overexpression of HGF retards disease progression and prolongs life span in a transgenic mouse model of ALS. *J. Neurosci.* **22**, 6537–6548.

Susin S. A., Lorenzo H. K., Zamzami N. *et al.* (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446.

Tenneti L. and Lipton S. A. (2000) Involvement of activated caspase-3-like proteases in *N*-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. *J. Neurochem.* **74**, 134–142.

Thompson J., Dolcet X., Hilton M., Tolcos M. and Davies A. M. (2004) HGF promotes survival and growth of maturing sympathetic neurons by PI-3 kinase- and MAP kinase-dependent mechanisms. *Mol. Cell Neurosci.* **27**, 441–452.

Tsuzuki N., Miyazawa T., Matsumoto K., Nakamura T. and Shima K. (2001) Hepatocyte growth factor reduces the infarct volume after transient focal cerebral ischemia in rats. *Neurol. Res.* **23**, 417–424.

Ueda H., Nakamura T., Matsumoto K., Sawa Y., Matsuda H. and Nakamura T. (2001) A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. *Cardiovasc. Res.* **51**, 41–50.

Wang H., Yu S. W., Koh D. W., Lew J., Coombs C., Bowers W., Federoff H. J., Poirier G. G., Dawson T. M. and Dawson V. L. (2004a) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. *J. Neurosci.* **24**, 10 963–10 973.

Wang X., Zhou Y., Kim H. P., Song R., Zarnegar R., Ryter S. W. and Choi A. M. (2004b) Hepatocyte growth factor protects against hypoxia/reoxygenation-induced apoptosis in endothelial cells. *J. Biol. Chem.* **279**, 5237–5243.

Xu D., Bureau Y., McIntyre D. C., Nicholson D. W., Liston P., Zhu Y., Fong W. G., Crocker S. J., Korneluk R. G. and Robertson G. S. (1999) Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *J. Neurosci.* **19**, 5026–5033.

Ye H., Cande C., Stephanou N. C., Jiang S., Gurbuxani S., Larochette N., Daugas E., Garrido C., Kroemer G. and Wu H. (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat. Struct. Biol.* **9**, 680–684.

Yu S. W., Wang H., Poitras M. F., Coombs C., Bowers W. J., Federoff H. J., Poirier G. G., Dawson T. M. and Dawson V. L. (2002) Mediation of poly (ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**, 259–263.

Zarnegar R. and Michalopoulos G. K. (1995) The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J. Cell Biol.* **129**, 1177–1180.

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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.

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.

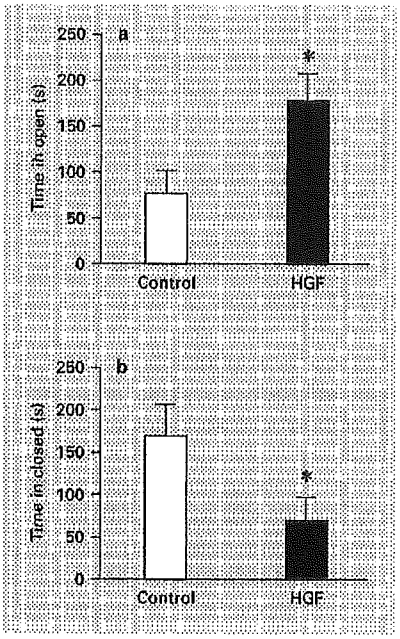

Fig. 1. **a** Time spent in the open arm of the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. **b** Time spent in the closed arm of the elevated plus maze was significantly decreased in the HGF-infused group compared to the control group. * $p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

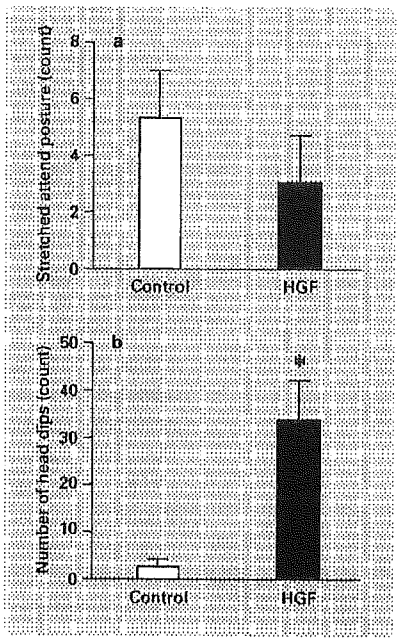

Fig. 2. **a** No effect of HGF or the control vehicle on the number of stretched attend postures in the elevated plus maze was seen. **b** The number of head dips in the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. * $p < 0.05$ vs. vehicle- and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

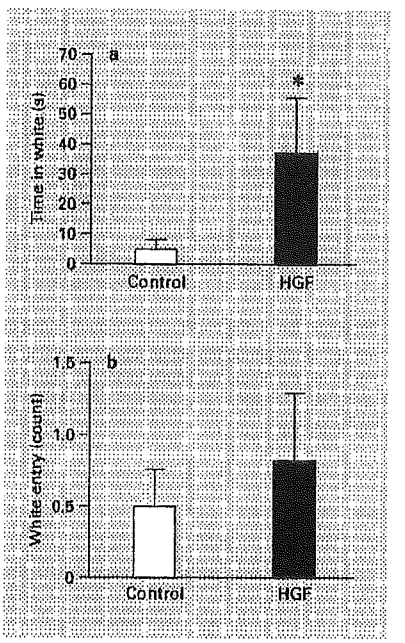

Fig. 3. In the black and white box test, the time spent in the white chamber was significantly increased in the HGF-infused group, compared to the controls (**a**). **b** No significant effect of HGF on the number of white chamber entries was seen. * $p < 0.05$ vs. vehicle- and HGF-infused group.

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).

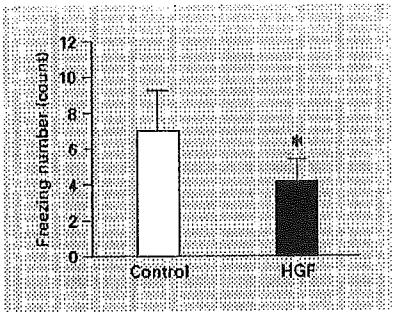

Fig. 4. Level of freezing induced by conditioned fear was significantly decreased in the HGF-infused group, compared to the controls. * $p < 0.05$ vs. vehicle- and HGF-infused group.

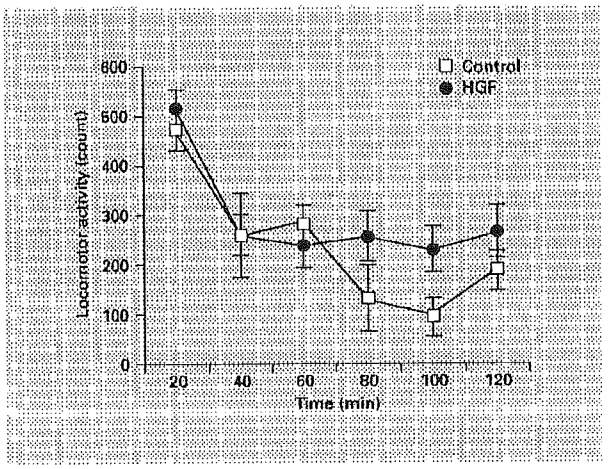

Fig. 5. No significant effect of HGF on general locomotor activity (number of photobeam breaks) was seen. Data are means ± SEM.

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.

References

1 Aoki M, Morishita R, Taniyama Y, Kida I, Moriguchi A, Matsumoto K, Nakamura T, Kaneda Y, Higaki J, Ogihara T: Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: Up-regulation of essential transcription factor for angiogenesis. Gene Ther 2000;7: 417–427.

2 Hayashi S, Morishita R, Nakamura S, Yamamoto K, Moriguchi A, Nagano T, Taizi M, Noguchi H, Matsumoto K, Nakamura T, Higaki J, Ogihara T: Potential role of hepatocyte growth factor, a novel angiogenic growth factor, in peripheral arterial disease: Down-regulation of HGF in response to hypoxia in vascular cells. Circulation 1999;100:11301–11308.

3 Morishita R, Nakamura S, Hayashi S, Taniyama Y, Moriguchi A, Nagano T, Taiji M, Noguchi H, Takeshita S, Matsumoto K, Nakamura T, Higaki J, Ogihara T: Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 1999;33:1379–1384.

4 Hayashi T, Abe K, Sakurai M, Itoyama Y: Inductions of hepatocyte growth factor and its activator in rat brain with permanent middle cerebral artery occlusion. Brain Res 1998;799: 311–316.

5 Miyazawa T, Matsumoto K, Ohmichi H, Katoh H, Yamashima T, Nakamura T: Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: A novel neurotrophic factor. J Cereb Blood Flow Metab 1998;18:345–348.

6 Yamada T, Yoshiyama Y, Tsuboi Y, Shimomura T: Astroglial expression of hepatocyte growth factor and hepatocyte growth factor activator in human brain tissues. Brain Res 1997; 762:251–255.

7 Jung W, Castrén E, Odenthal M, Vande Woude G, Dienes HP, Lindholm D, Schirmacher P: Expression and functional interaction of hepatocyte growth factor-scatter factor (HGF-SF) and its receptor c-met in mammalian brain. J Cell Biol 1994 126:485–494.

8 Achim CL, Katyal S, Wiley CA, Shiratori M, Wang G, Oshika E, Petersen BE, Li J-M, Michalopoulos GK: Expression of HGF and c-met in the developing and adult brain. Brain Res Dev Brain Res 1997;102:299–303.

9 Honda S, Kagoshima M, Wanaka A, Tohyama M, Matsumoto K, Nakamura T: Localization and functional coupling of HGF and c-met/HGF receptor in rat brain: Implication as neurotrophic factor. Brain Res Mol Brain Res 1995;32:197–210.

10 Jung W, Castren E, Odenthal M, Vande Woude GF, Ishii T, Dienes HP, Lindholm D, Schirmacher P: Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. J Cell Biol 1994;126:485–494.

11 Hamanoue M, Takemoto N, Matsumoto K, Nakamura T, Nakajima K, Kohsaka S: Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J Neurosci Res 1996;43:554–564.

12 Korhonen L, Sjoholm U, Takei N, Kern MA, Schirmacher P, Castren E, Lindholm D: Expression of c-Met in developing rat hippocampus: Evidence for HGF as a neurotrophic factor for calbindin D-expressing neurons. Eur J Neurosci 2000;12:3453–3461.

13 Hayashi K, Morishita R, Nakagami H, Yoshimura S, Hara A, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T, Sakai N: Gene therapy for preventing neuronal death using hepatocyte growth factor: In vivo gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. Gene Ther 2001;8:1167–1173.

14 Powell EM, Campbell DB, Stanwood GD, Davis C, Noebels JL, Levitt P: Genetic disruption of cortical interneuron development causes region- and GABA cell type-specific deficits, epilepsy, and behavioral dysfunction. J Neurosci 2003;23:622–631.

15 Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K: Hepatocyte growth factor reduces the infarct volume after transient focal cerebral ischemia in rats. Neurol Res 2001;23: 417–424.

16 LeDoux JE: The Emotional Brain. New York, Simon and Schuster, 1996.

17 Rosen EM, Nigam SK, Goldberg ID: Scatter factor and the c-met receptor: A paradigm for mesenchymal epithelial interaction. J Cell Biol 1994;127:1783–1787.

18 Zarnegar R: Michalopoulos G: The many faces of hepatocyte growth factor: From hepatopoiesis to hematopoiesis. J Cell Biol 1995;129: 1177–1180.

19 Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude GF: Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci USA 1987;84: 6379–6383.

20 Bowers DC, Fan S, Walter K, Abounader R, Williams JA, Rosen EM, Laterra J: Scatter factor/hepatocyte growth factor activates AKT and protects against cytotoxic death in human glioblastoma via PI3-kinase and AKT-dependent pathways. Cancer Res 2000;60:4277–4283.

21 Fan S, Ma YX, Wang J, Yuan R, Meng Q, Cao Y, Laterra J, Goldberg ID, Rosen EM: The cytokine scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3′ kinase. Oncogene 2000;19:2212–2223.

22 Yamada K, Moriguchi A, Morishita R, Aoki M, Nakamura Y, Mikami H, Oshima T, Ninomiya M, Kaneda Y, Higaki J, Ogihara T: Efficient oligonucleotide delivery using the HVJ-liposome method in the central nervous system. Am J Physiol 1996;271:R1212–R1220.

23 Tsuboi Y, Kakimoto K, Nakajima M, Akatsu H, Yamamoto T, Ogawa K, Ohnishi T, Daikuhara Y, Yamada T: Increased hepatocyte growth factor level in cerebrospinal fluid in Alzheimer's disease. Acta Neurol Scand 2003; 107:81–86.

24 Furge KA, Zhang YW, Vande Woude GF: Met receptor tyrosine kinase: Enhanced signaling through adapter proteins. Oncogene 2000;19: 5582–5589.

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Expression of Hepatocyte Growth Factor in Rat Skeletal Muscle

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Abstract. The present study examined the localization of hepatocyte growth factor in rat skeletal muscle, and investigated whether levels of hepatocyte growth factor differ between skeletal muscles. Levels of hepatocyte growth factor in soleus and tibialis anterior muscles were measured using enzyme-linked immunosorbent assay. Localization of hepatocyte growth factor and proliferating cell nuclear antigen in the soleus muscle was visualized using immunofluorescence analysis. Level of hepatocyte growth factor was 3.2 ± 1.4 ng/g tissue in the soleus muscle and 3.4 ± 0.4 ng/g tissue in the tibialis anterior muscle. No significant differences were identified between muscles with differential contractile characteristics. Existence of hepatocyte growth factor was observed in cytoplasm of small cells conterminous to muscle fibers. Cells in a similar position displayed reactivity to proliferating cell nuclear antigen, suggesting that they represented activated skeletal muscle satellite cells. Hepatocyte growth factor is produced in normal rat skeletal muscle by activated skeletal muscle satellite cells.
Key words: Skeletal muscle, HGF, PCNA

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INTRODUCTION

Skeletal muscle cells play important roles in muscle regeneration and hyperplasia[1]. Skeletal muscle satellite cells are usually present in a quiescent state between the plasma membrane and basal lamina[2], but become activated following muscular injury or mechanical stretch[3–5]. Activated satellite cells enter into a cycle of proliferation and division, and differentiate into myoblasts[6, 7]. These myoblasts undergo coalescence and maturation, finishing with repair and hyperplasia. Growth factors such as hepatocyte growth factor (HGF)[5], fibroblast growth factor[8, 9] and insulin-like growth factor I[10] are associated with the proliferation and differentiation of satellite cells. However, each factor plays a different role. While HGF can cause precocious entry into the cell cycle for satellite cells, the actions of HGF in skeletal muscle *in vivo* remain unclear. Although the contractile function of skeletal muscles differs between specific muscles, relationships between the contractile properties of skeletal muscle and concentrations of HGF are unknown. Clarification of these mechanisms could prove very useful in determining physical therapy to achieve hypertrophy or hyperplasia. Furthermore,

repair reactions of specific skeletal muscles may differ with function. The present study investigated associations between production of HGF and contractile properties in rat skeletal muscle.

METHODS

Animals and materials

The present study used 6 female, 11-week-old Wistar rats (body weight, 196–220 g). Deep anesthesia was induced in all animals by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight). For quantitative analysis of HGF levels in tissue, 5 of the rats were exsanguinated. The right soleus and tibialis anterior (TA) muscles were then excised and quick-frozen in liquid nitrogen. For immunofluorescence analysis, right soleus muscle was excised from the other normal rat and oriented for cross-section in embedding medium (Tissue Tek OCT compound; Miles, Elkhart, IN, USA), then quick-frozen in isopentane chilled with liquid nitrogen. Samples were stored at –70°C until use. At the end of the study, all animals were sacrificed. All procedures for animal care and treatment were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University.

Enzyme-linked immunosorbent assay for hepatocyte growth factor

For detection of HGF levels, tissues were completely homogenized in lysis buffer (pH 7.5). Samples containing HGF were separated by centrifugation for 60 min at 16,100 × g and 4°C. Measurement of HGF levels was performed using an enzyme-linked immunosorbent assay (ELISA) kit (Institute of Immunology, Tokyo, Japan).

Histological analysis

Sections (10 μm thick) were cut on a cryostat, then dried for 1 h at room temperature. For morphological observation, cross sections were stained using hematoxylin and eosin.

Immunofluorescence staining for hepatocyte growth factor and proliferating cell nuclear antigen

Sections (6 μm thick) were cut using a cryostat, and dried for 1 h at room temperature. For detection of HGF, sections were fixed in methanol for 5 min at 4°C. For detection of HGF and proliferating cell nuclear antigen (PCNA; Dako Cytomation Japan, Kyoto, Japan), sections were treated with phosphate-buffered saline (PBS) containing 0.1% TritonX-100 (pH 7.4) for 5 min at room temperature. Non-specific binding sites were blocked using normal swine serum and bovine serum albumin (BSA) in PBS for 10 min. Sections were incubated with each primary antibody, polyclonal anti-rat HGF antibody (Institute of Immunology) diluted 1:10 in PBS and monoclonal anti-mouse PCNA antibody (Dako Cytomation Japan), each for 90 min at 37°C then 30 min at room temperature. Sections were covered with secondary antibody for 20 min at 37°C, using goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) diluted 1:300 in PBS and goat anti-mouse Alexa Fluor 546 (Molecular Probes) diluted 1:500 in PBS. All nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). Negative controls were incubated with each rabbit serum and mouse IgG. Fluorescein signals in sections were observed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan).

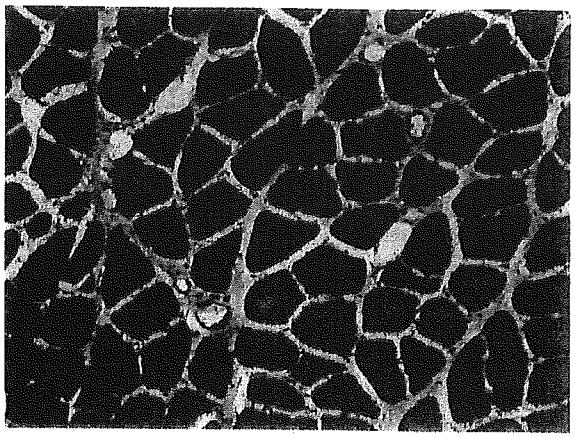

Fig. 1. Soleus muscle. Hematoxylin and eosin, × 200. Scale bar: 50 μm.

Statistical analysis

Student's t-test was used for comparisons between HGF levels in soleus and TA muscles. Values of $P<0.05$ were considered statistically significant.

RESULTS

Tissue HGF level was 3.2 ± 1.4 ng/g of tissue in the soleus muscle and 3.4 ± 0.4 ng/g of tissue in the

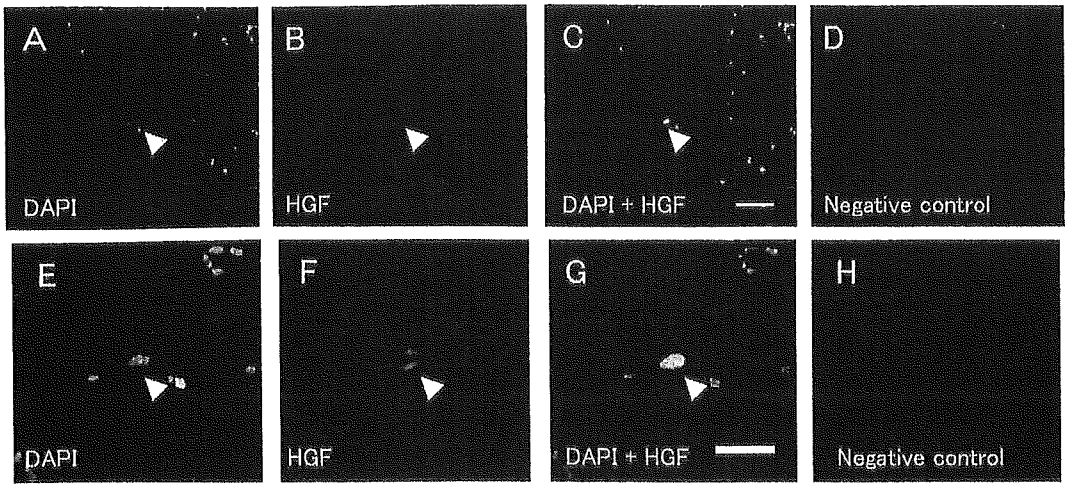

Fig. 2. Immunofluorescence staining for HGF and nuclei in soleus muscle. Many nuclei were observed in general sections (A, C, E, G). HGF was observed in cytoplasm of small cells conterminous to muscle fibers (F). Background in the negative control section was much lower (D, H). Scale bar: A–D, 50 μm; E–H, 10 μm. Magnification × 200.

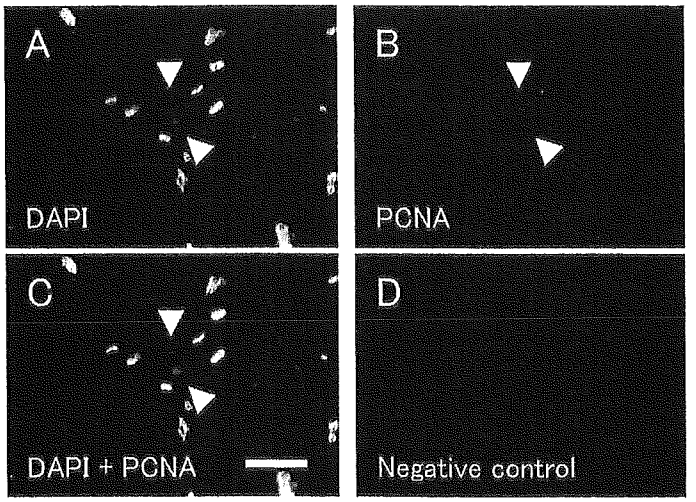

Fig. 3. Immunofluorescence staining for PCNA and nuclei in soleus muscle. Many nuclei are apparent in general sections, but few PCNA-labeled cells are present (A–C). PCNA-labeled cells are present conterminous to muscle fibers (B, C). Background in the negative control section was much lower (D). Scale bar: 20 μm. Magnification × 200.

TA muscle, with no significant differences noted between muscles.

HGF-positive cells were identified as small cells conterminous to muscle fibers (Fig. 2C, G), and HGF signals were localized to the cytoplasm of these small cells (Fig. 2B, C, F, G). Nuclei displayed no positive staining for HGF. Negative control sections displayed lower background levels of normal rabbit serum (Fig. 2D, H). Some cells in a similar position to HGF-positive cells displayed positive reactivity for PCNA (Fig. 3B, C).

DISCUSSION

C-Met is an HGF receptor, which is expressed in normal adult rat TA muscle satellite cells, and HGF is released from satellite cells by mechanical stretch *in vivo*[5, 11]. Expression of slow myosin heavy chains represents 78% of total myosin heavy chain isoforms expressed in the rat soleus, compared to 5% in TA[12]. The number of satellite cells proliferating in the soleus muscle is elevated after functional loading[13]. Thus, in the soleus muscles, which act as "antigravity" postural muscles, HGF concentrations might be assumed to be higher than in TA muscles. However, no significant differences were identified for HGF levels in soleus and TA muscles in the present study. This result suggests that HGF levels in soleus and TA muscles under stationary conditions are around 3.2–3.4 ng/g tissue, with no real difference between muscles displaying differing contractile characteristics.

HGF acted as an activator of quiescent satellite cells *in vivo*[14]. Cells labeled with PCNA, which can be used to detect entry into the cell cycle, were usable as markers for satellite cell activation[15]. We therefore assumed that HGF and PCNA were present in activated satellite cells. In the present study, HGF- and PCNA-positive cells were observed in the same region of the soleus muscle. This indicates that HGF is expressed and activated satellite cells are present in normal rat soleus muscles.

Soleus muscle activity can increase to about 3-fold higher than TA muscle activity during exercise[16]. Muscles of the rat hindlimb were injured by downhill exercise on a treadmill, and the percentage of morphologically altered fibers was 4–8% in soleus muscles, and 1–2% in TA muscles[12, 13]. The number of proliferating satellite cells was then seen to increase within 2 days of injury[12]. Running exercise might thus account for up-regulation of HGF in the soleus muscle.

The present results reveal that HGF levels in normal rat soleus and TA muscles are 3.2–3.4 ng/g of tissue, with no significant differences between muscles. Furthermore, HGF appears to be present in the normal rat soleus muscle, produced by muscle satellite cells. Further research is required to clarify the mechanisms of hypertrophy, hyperplasia and muscle remodeling.

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REFERENCES

1) Allen RE, Rankin LL: Regulation of satellite cells during skeletal muscle growth and development. Proc Soc Exp Biol Med, 1990, 194: 81–86.
2) Schultz E, Gibson MC, Champion T: Satellite cell are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. J Exp Zool, 1978, 206: 451–456.
3) Anderson JE: A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol Biol Cell, 2000, 11: 1859–1874.
4) Sheehan SM, Tatsumi R, Temm-Grove CJ, et al.: HGF is an autocrine growth factor for skeletal muscle satellite cells *in vivo*. Muscle Nerve, 2000, 23: 239–245.
5) Tatsumi R, Anderson JE, Nevoret CJ, et al.: HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol, 1998, 194: 114–128.
6) Bischoff R: Proliferation of muscle satellite cells on intact myofibers in culture. Dev Biol, 1986, 115: 129–139.
7) Bladt F, Riethmacher D, Isenmann S, et al.: Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature, 1995, 376: 768–771.
8) Yablonka-Reuveni Z, Seger R, Rivera AJ: Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. J Histochem Cytochem, 1999, 47: 23–42.
9) Kastner S, Elias MC, Rivera AJ, et al.: Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells J Histochem Cytochem, 2000, 48: 1079–1096.
10) Chakravarthy MV, Davis BS, Booth FW: IGF-restores satellite cell proliferative potential in immobilized old skeletal muscle. J Appl Physiol, 2000 89:1365–1379.
11) Tatsumi R, Sheehan SM, Iwasaki H, et al.: Mechanical stretch induces activation of skeletal muscle satellit cells *in vitro*. Exp Cell Res, 2001, 267: 107–114.
12) Smith HK, Maxwell L, Rodgers CD, et al.: Exercise enhanced satellite cell proliferation and new myonuclear accretion in rat skeletal muscle. J Appl Physiol, 2001, 90: 1407–1414.
13) Smith HK, Plyley MJ, Rodgers CD, et al.: Skeletal muscle damage in the hindlimb following single or repeated daily bouts of downhill exercise. Int J Sport Med, 1997, 18: 94–100.

14) Allen RE, Sheehan SM, Taylor RG, et al.: Hepatocyte growth factor activates quiescent skeletal muscle satellite cells *in vitro*. J Cell Physiol, 1995, 165: 307–312.
15) Johnson SE, Allen RE: Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. J Cell Physiol, 1993, 154: 39–43.
16) Canu MH, Falempin M: Effect of hindlimb unloading on two hindlimb muscles during treadmill locomotion in rats. Eur J Appl Physiol Occup Physiol, 1997, 75: 283–288.

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広範囲 血液・尿化学検査 免疫学的検査

—その数値をどう読むか—

［第6版］

(4)

Ⅸ. プロスタノイド，サイトカイン，増殖因子，ケモカイン

肝細胞増殖因子（HGF）

大谷若菜　　船越　洋　　中村敏一

肝細胞増殖因子(HGF)

Hepatocyte growth factor(HGF)

大谷若菜　船越　洋
中村敏一

Key words: 肝細胞増殖因子，c-Met，劇症肝炎，血清

1. 概　　説

肝細胞増殖因子(hepatocyte growth factor: HGF)は1984年当研究室の中村らにより肝細胞増殖活性を指標にラットの血小板より精製され[1]，1989年にラットならびにヒトHGFがクローニングされた[2]．その構造は，クリングルドメインを4つ含むα鎖(69kDa)と，セリンプロテアーゼ様構造をもつβ鎖(34kDa)からなる．細胞からは一本鎖のプロ体として分泌され，HGF converting enzymeもしくはHGF activator，u-PA(urokinase-type plasminogen activator)，t-PA(tissue-type PA)，matriptaseなどによりα鎖とβ鎖間のArg-Val部位で切断され二本鎖HGFとなり初めてc-Met/HGF受容体との結合活性をもつ(図1-a)．

HGFの生物活性は当初の肝細胞増殖活性のみでなく，肝細胞以外にも多数の上皮細胞，内皮細胞，一部の間葉細胞に増殖活性作用を，更には細胞分化，細胞遊走，器官形成，抗アポトーシス，血管新生作用をももつことが明らかとなってきている[4]．このように多彩な作用を兼ね備えたHGFは組織傷害に対して生体の再生機構の主役を果たすと考えられている．

これまでにHGFは，肝臓をはじめ，腎臓，肺，心臓，脳といった様々な臓器疾患，特に治療法がなかった難治性疾患に対してもダイナミックな治療効果をあらわし，一方でHGFアンタゴニストとして機能するNK4(HGFα鎖)は，癌の浸潤，転移，血管新生の阻害による抗癌作用をあらわすことが動物レベルで多数報告されている．上記疾患に反応して生体はHGFを放出し傷害を治癒しようとすることで血中HGFは変動するが，その際内在性HGF量が不十分な場合は補充療法による治療が有効となるだろう．本稿では，これら様々な疾患によるHGFの血中変動を中心に解説し，診断・治療を行う際の一助となることを目的としている．

2. 試料の採取方法，保存条件

採血は溶血を避け，速やかに血清分離を行う．検体の保存には，ポリプレン製かポリエチレン製チューブまたはシリコンコートしたガラス製チューブを使用し，4℃で1週間，1週間以上の際は，−20℃以下で保存する．検体の凍結融解の繰り返しは避ける．

3. 測定法—ELISA法によるHGF蛋白質量の定量法

著者らの研究室で行っているhuman HGF ELISA法は，固相(ELISA plate)に固定化した抗HGF 1次抗体に抗原であるHGFを捕捉させ，更にビオチン化抗HGF 2次抗体と結合後，酵素反応を経て試料中の抗原分子濃度を定量する方法である．この方法により簡便な操作で高感度(HGF検出濃度；0.1ng/ml)かつ再現性の高い結果が得られる[5](ヒト，ラットHGF ELISA kit: 株式会社特殊免疫研究所；Tel: 03-3814-4081)．また，活性型HGFのみを検出するELISA法が大西らにより報告されている[6]．

4. HGFの血中動態

^{125}I-HGF静脈注射3分後の組織分布で，HGFは肝臓，副腎，脾臓，腎臓，肺，胃，小腸に分

Wakana Ohya, Hiroshi Funakoshi, Toshikazu Nakamura: Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine　大阪大学大学院医学系研究科 未来医療開発専攻組織再生医学講座 分子組織再生分野

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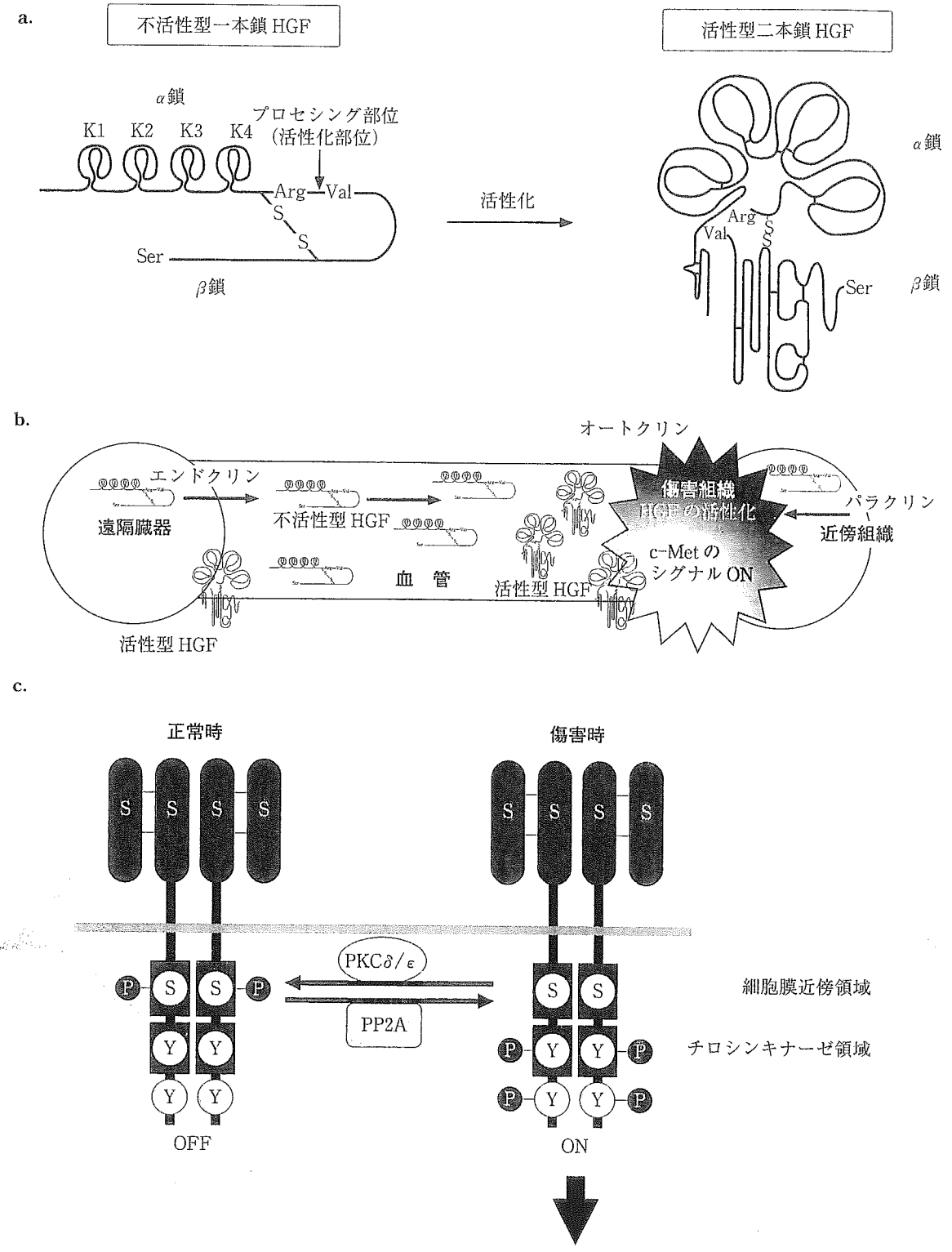

図 1　HGF の構造と傷害組織特異的作用分子機構の模式図

a: HGF の構造と活性化機構．b: 傷害臓器への活性化 HGF の供給．弱い傷害では HGF はパラクリンやオートクリンによって局所的に供給されるが，強い傷害や慢性傷害では，遠隔の臓器によるエンドクリンによる供給も行われる．主に傷害組織で不活性型 HGF は活性型 HGF へ変換される．c: 傷害認知機構としての c-Met のシグナルスイッチ．正常組織では c-Met 細胞膜近傍領域のセリンがリン酸化されていることで HGF による c-Met のチロシンのリン酸化が阻害されている（シグナル OFF）．一方で，傷害組織ではこのセリンが PP2A によって脱リン酸化されているため HGF により c-Met のチロシンのリン酸化が起こりシグナルが伝達される（シグナル ON＝c-Met 活性化）．つまり傷害特異的な c-Met のセリンリン酸化により HGF は傷害組織特異的に c-Met を活性化する．

布する．一方，心臓や脳への分布は大部分が細胞外スペースへの見かけの分布である．部分肝切除，肝硬変ラットにおいては，ヒトHGF静脈注射後少なくとも2時間は正常ラットの10倍の血清ヒトHGF量が維持される[7]．

血液中のHGFのクリアランスの約70％は肝臓（他，腎臓で10％以下）で行われる．ここでは，c-Metを介した内在化機構（エンドサイトーシス）とともに，ヘパラン硫酸との結合による低親和性クリアランスの両者が想定されている．肝傷害時やリコンビナントHGF大量投与時のように血液中HGFが高濃度に存在すると，肝臓におけるc-Metの発現が低下し内在化機能低下によるクリアランスが低下する．劇症肝炎発症時の血清HGF値の著明な増加は，HGF産生の増加に加え，c-Metの発現低下，肝細胞数の減少によるクリアランスの低下も大きく関与すると考えられる[4]．

5. HGFの生理的変動

正常血清レベルは，年齢による変化は少ないが20歳代でピークになり以後徐々に減少し，男女間で有意差はない．ただし女性の場合，子宮内膜の増殖，再生，修復を反映し，月経時に高く排卵時に低いという性周期に伴った変化をする．また妊娠時には，母体血液中HGF濃度は妊娠経過とともに上昇し，妊娠後期でピークを示すのに対して，羊水中では妊娠中期にピークを示し（胎盤でのHGF発現も同様）このとき母体の100倍となり胎児肺，腸管形態形成に強く関与することが推察される[4]（**表1**）．また肥満のように，HGFを産生する細胞（脂肪細胞）の過増殖による場合も増加する．

6. HGFのその他の因子による変動

IL-1β，グルココルチコイドなど様々な因子，薬物がHGF産生を誘導，あるいは抑制することが報告されており[9]，これらの投与によるHGF値変動も忘れてはならない．

表1 HGF検査値の生理的変動

	HGF(ng/ml)		
年 齢	女性血清中		男性血清中
10代	0.36±0.16		0.35±0.25
20代	0.39±0.25		0.37±0.22
30代	0.37±0.19		0.33±0.19
40代	0.33±0.17		0.29±0.17
50代	0.26±0.17		0.29±0.15
妊娠時期	血清中	羊水中	臍帯血中
妊娠初期	0.30以下	15±8	0.30以下
妊娠中期	0.41±0.21	48±23	
妊娠後期	0.48±0.25	6±3	

7. HGFの正常および各種疾患における検査値と臨床的意義（表2[4]）

a. 傷害時血清HGF値の変動

器官再生促進作用をもつHGFの血中濃度は様々な臓器の傷害時に敏感に反応変動する．器官に傷害が発生したとき，傷害臓器のみならず遠隔の正常臓器でも傷害が認知され即座にHGFが産生，放出され，このHGFはエンドクリン，パラクリンを駆使して傷害組織に供給される（**図1-b**）．血中に豊富に供給されたHGFのシグナルが傷害臓器でのみ強く受け取られるという巧妙な仕組みは，HGFの活性化とc-Metの傷害認知機構としてのシグナルスイッチ[8]，c-Metの発現誘導によって支えられている（**図1-b，c**）．また，傷害に依存したc-Metの特異的シグナルスイッチは活性型リコンビナントHGF蛋白が傷害組織で効率よく機能することに有利に働いている．

b. 各種疾患による変動

1）肝 疾 患

急性肝炎，慢性肝炎，肝硬変，肝細胞癌などでは肝傷害の重症度に相関してHGF値のレベルが増加する．このことは急性肝炎ではビリルビン，AST，γ-GTP，慢性肝炎では組織活性インデックスといった肝機能検査との相関によって明らかである．一方で，劇症肝炎では，肝細胞の著減によるc-Met依存的なHGFのクリアランスの極度低下という因子も加わり血清HGF値

表2 正常および各種疾患における血清，組織などのHGF値

	疾患名	検査値(ng/ml; ng/mg)	
	正常	0.27±0.08*	
各種疾患の患者血清	アルコール中毒	0.78	
	肥満	2.46±0.18(0.77正常)	Rehman Jら(2003)
	急性肝炎	0.45±0.23*	
	慢性肝炎	0.40±0.16*	
	肝硬変	1.05±0.64*	
	肝細胞癌	1.06±1.45*	
	原発性胆汁性肝硬変	0.44±0.22*	
	劇症肝炎	16.40±14.67*	
	肝臓移植後順調な回復時	0.33±0.04	
	肝臓移植後プロトロンビン時間の異常上昇時	2.01±0.99	
	胆管閉塞	0.32±0.13(0.17±0.03)	Kimura Fら(2000)
	間質性肺炎	1.16±0.22(p<0.01)	
	細菌性肺炎	0.96±0.27(p<0.01)	
	肺線維症	0.34±0.002(p<0.01)	
	急性腎不全(急相期)	0.55±0.24	
	慢性尿細管間質性腎炎	0.44±0.37	
	慢性腎不全(非透析時)	0.33±0.1	
	慢性腎不全(透析1年未満)	0.33±0.13	
	慢性腎不全(透析5年から10年)	0.45±0.13	
	移植後急性腎拒絶	2.17±1.14	
	腎異系移植片機能良好時ピーク	2.48-5.63	
	高血圧(WHO stage I)	0.48±0.03(p<0.01)	
	高血圧(WHO stage II, III)	0.88±0.1(p<0.01)	
	動脈硬化症	0.35±0.11	
	狭心症	0.3±0.1	
	急性心筋梗塞(6時間以内)	10.4±8.8	
	急性心筋梗塞(6-12時間)	6.7±4.5	
	軽，中等度急性膵炎	0.63±0.06	
	重度急性膵炎	2.30±0.61	
	インスリン治療前値	0.74±0.14	
	I型糖尿病罹患短期(発症半年から3年)	0.78±0.40	
	I型糖尿病罹患長期(腎障害を伴わない)	0.86±0.42	
	I型糖尿病罹患長期(腎障害を伴う)	0.79±0.27	
	多発性筋炎	0.63±0.11	
	皮膚筋炎	0.58±0.07	
	不活性型全身性エリテマトーデス	0.79	
	活性型全身性エリテマトーデス	1.02	
	潰瘍性大腸炎	1.38±0.11	
	Crohn病	1.44±0.08	
	HELLP症候群	1.79±0.35	
	アミロイドーシス	2.26±2.73(0.18±0.07)	Shikano Mら(2000)
	生存1年以上，1年未満	0.46±0.26，2.83±2.85	
気管支肺胞洗浄液	正常	0.23±0.09	
	特発性肺線維症	0.77±0.88(p<0.001)	
	リウマチ性関節炎	0.50±0.64(p<0.001)	
	サルコイドーシス	0.41±0.61(p<0.05)	
大脳皮質	正常(平均72.0歳)	9.60±4.62	
	Alzheimer病(平均78.7歳)	33.7±18.47	
	進行性Parkinson病(平均78.5歳)	20.23±13.55	
	Huntington病(平均73.8歳)	36.15±11.98	

( ): 正常対照群，*: RIA(radioimmunoassay)による値，
*以外：ELISAによる値，**: 活性化型HGF量．(文献[3,10])

(次ページにつづく)

（表2つづき）

	疾患名	検査値（ng/ml; ng/mg）	
脳脊髄液	正常	0.35±0.126（0.034±0.012**）	
	非感染性髄膜炎	0.42±0.07	
	細菌性髄膜炎	6.10±5.20	
	筋萎縮性側索硬化症	0.58	
	Alzheimer病	0.06±0.017**	Tsuboi Yら（2003）
	もやもや病	0.87±0.32	Namba Rら（2004）
尿	正常**	19.3±7.1（pg/mg creatinine**）	
	急性尿細管壊死（正常尿量時）	6.9±0.7（ng/g creatinine）	
	急性尿細管壊死（乏尿時）	19.1±4.2（ng/g creatinine）	
胆汁	正常	0.8±0.1	
	肝切除後1日目（非糖尿病）	4.0±0.4（p＜0.05）	
溝歯肉液	正常	7.37±1.46（1.70±0.73**）	Oshima Mら（2004）
	歯周病	117.3±16.9（3.23±1.01**）	Kakimoto Kら（2004）
涙	正常	0.19-0.29	Li Qら（1996）
	手術1日後（白内障，角膜手術）	0.45-0.62	
硝子体	正常	2.16±1.39	
	裂孔原性網膜剥離	2.02±0.84	
	増殖性硝子体網膜症	3.94±2.29	
	非糖尿病例	1.6	
	糖尿病性増殖性網膜症（ルベオーシス－）	4.2（p＜0.05）	
	糖尿病性増殖性網膜症（ルベオーシス＋）	7.2（p＜0.01）	
関節液	変形性関節症	0.19	
	細菌性関節炎	0.18	
	リウマチ性関節炎	1.21	
胎盤	正常	6.16±3.32（ng/mg）	
	妊娠中毒症	4.05±1.44（ng/mg）（p＜0.05）	
癌患者の血清	食道癌（stage I/II）	0.47±0.13	
	食道癌（stage III/IV）	0.88±1.05	
	食道癌（再発性）	1.51±1.62	
	胃癌（stage I/II）	0.32±0.15	
	胃癌（stage III/IV）	0.49±0.46	
	胃癌（再発性）	0.44±0.29	
	肝細胞癌	1.06±1.45	
	肝芽腫（治療前）	0.89	
	肝芽腫（化学療法後）	0.46	
	結腸直腸癌（stage I/II）	0.35±0.15	
	結腸直腸癌（stage III/IV）	0.38±0.19	
	結腸直腸癌（stage V）	0.50±0.25	
	結腸直腸癌（再発性）	0.44±0.14	
	乳癌（原発性）	0.38±0.31	
	乳癌（再発性）	0.59±0.42	
	前立腺癌（転移なし）	0.97	
	前立腺癌（転移あり）	2.12	
	小細胞肺癌（平均）	0.40±0.17	
	小細胞肺癌（限局性）	0.34±0.12	
	小細胞肺癌（広範囲）	0.47±0.20	
	急性骨髄芽球性白血病	2.03（1.055）	
	多発性骨髄腫（stage I）	1.43	Alexandrakis MGら（2003）
	多発性骨髄腫（stage II）	1.74	
	多発性骨髄腫（stage III）	1.99	
	Hodgkin病	1.40±0.09（0.67±0.03）	Teofili Lら（2001）
	Hodgkin病回復時	0.62±0.03	
	Hodgkin病再発時	1.50±0.24	
	リンパ腫（Hodgkin病以外）	1.02（0.69）	Hsiao LTら（2003）
腫瘍	正常乳房	0.11	
	乳癌	0.35	

が正常時の60倍となる．またこのときのHGFはほとんどが不活性型である[4]．予後因子としてのHGFは，予後が極めて良くない肝炎の劇症化(脳症の発現)を血清HGFが1ng/m*l*を超えた時点で診断し，早期に治療を開始する指標として重要であるとされている．

2) 腎疾患

腎不全では，血清HGF値は正常の2-3倍程度に上昇する．このうち急性腎不全ではほとんどが活性型になっているのに対して，慢性腎不全ではほとんどが不活性型であり，腎臓よりむしろ肝臓，脾臓でHGF濃度が上昇しておりエンドクリン的供給が行われている．透析の際，静脈内注射，体外循環液中にヘパリンを用いた場合，HGFとの親和性の差により組織中のヘパラン硫酸と低親和性に結合しているHGFが流出するため，血中HGF値が上昇する．また移植腎の急性拒絶反応でも，免疫反応による腎障害によってHGFが産生され血中HGF値は上昇するが，腎毒性をもつ免疫抑制剤やHGF産生を抑制するデキサメタゾンを使用した際はHGF値が修飾されるため，その点に留意した診断が必要である[4]．

3) 肺疾患

血清HGF値が間質性肺炎と細菌性肺炎で高値を示す．肺炎治療に応答した患者では血清HGF値は低下，改善するが，死亡患者では不変であり，血清HGF値と肺炎の予後に相関を認める[4]．

4) 膵疾患

膵炎の重症度評価に血清HGF値は血清CRP値と同程度，IL-6値より有用と報告されている[4]．

5) 血管性疾患

血管障害でのHGFの供給は特にエンドクリンの要素が強いため，血中HGF値にあらわれやすい．HGFは血管内皮細胞増殖作用をもつことから，高血圧においても，血清HGF値は上昇しており，収縮期，拡張期血圧のいずれとも相関を示し重症度を反映している．また糖尿病では，グルコース毒性により内皮細胞が傷害され合併症として高血圧，動脈硬化につながることが多いが，この際HGF値は糖尿病でわずかに減少するも高血圧を合併した場合は上昇し，糖尿病における合併症進展への診断につながる．更に糖尿病性閉塞性動脈硬化症(ASO)，増殖性網膜症でも血中HGF値は高値を示すが，閉塞血管では減少していることから，HGFの補充療法は治療効果を示す[4]．ヒトASOに対するHGF遺伝子治療も大阪大学病院で開始され，高い治療効果が証明されている．

6) 神経疾患

脳は，血液の供給において脳血液関門が存在するため他臓器と切り離された環境にある．したがって脳疾患は血清HGF値に反映されにくい．脳内の傷害を反映すると考えられる脳脊髄液では，HGF値は髄膜炎のうち非感染性では変化しないのに対して細菌性で著増する．筋萎縮性側索硬化症(ALS)，もやもや病で2倍程度に増加する．更にAlzheimer病では活性型HGFが2倍に増加する．このとき．HGFアクチベーター阻害因子(HGF activator inhibitor)の脳内発現が低下することが報告されている[4]．HGFは多くの難治性神経疾患の標的神経細胞に対する強力な神経栄養因子でありALSなどの治療に大きな期待が寄せられている．またHGFが抗不安作用をもつことが明らかとなり，今後は精神神経疾患におけるHGF値の測定も重要となると考えられる．

7) 心疾患

血清HGF値は狭心症では増加しないのに対し，急性心筋梗塞においては血清CKおよびCK-MB値と経時的に相関して高値を示す．特にHGF値の増加は他の2者より早く，狭心痛発作後3時間以内の増加率が高い．このため血清HGF値は心筋梗塞の早期マーカーとしても有効と考えられる．著者らの研究室では*in vivo*で心筋細胞死阻止および血管新生促進作用の両作用を確認しており，これら二重効果を利用したHGFの心筋梗塞への臨床応用が期待できる[4]．

8) 癌

正常組織において主に線維芽細胞，内皮細胞，マクロファージなどの間質細胞により産生されるHGFは，癌組織においては，癌細胞自身が産生し自身で受け取るというオートクリンループを形成する場合もみられる．血中HGF値は様々な細胞種の癌で増加が認められる．**表2**で示したいずれの癌においても，悪性化(ステージの進