Hepatoma-derived Growth Factor Is a Neurotrophic Factor Harbored in the Nucleus*

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Hepatoma-derived growth factor (HDGF) is a heparin-binding proliferating factor originally isolated from conditioned medium of the hepatoma-derived cell line HuH-7. HDGF has greatest homology in an amino acid sequence with high mobility group 1 (HMG1), which has been characterized as a DNA-binding, inflammatory, and potent neurite outgrowth molecule. HDGF is reported to be widely expressed and act as a growth factor in many kinds of cells. However, it has not been investigated in the nervous system. Here, we show by Western blot analysis that HDGF is present in the mouse brain from the embryonic period until adulthood. In situ hybridization and immunohistochemical analyses revealed that HDGF was expressed mainly in neurons, and HDGF protein was localized to the nucleus. HDGF and high mobility group 1 were secreted under physiological conditions and released extracellularly in necrotic conditions. Furthermore, we showed that exogenously supplied HDGF had a neurotrophic effect and was able to partially prevent the cell death of neurons in which endogenous HDGF was suppressed. Therefore, we propose that HDGF is a novel type of neurotrophic factor, on account of its localization in the nucleus and its potential to function in an autocrine manner under both physiological and pathological conditions throughout life.

MDG1 was originally isolated from the conditioned medium of a human hepatoma-derived cell line, HuH-7, as a heparin-binding proliferating factor of Swiss 3T3 fibroblasts and HuH-7 cells (1). HDGF represents a new family of growth factors called HDGF-related proteins (HRPs), which includes HRP1, HRP2, HRP3, HRP4, and lens epithelium-derived growth factor (LEDGFp75/p52 (2–6). LEDGFp75/p52 has been characterized as a coactivator of both transcription and pre-mRNA splicing (7, 8) and as an autocrine and paracrine growth and survival factor (9). All of these factors have in common the following features: 1) homology in the N-terminal 98 amino acids, 2) bipartite nuclear localization signals, and 3) absence of signal peptide. Recent studies showed that HDGF is widely expressed. Exogenously supplied HDGF stimulates the growth of fibroblasts (1), endothelial cells (10), vascular smooth muscle cells (11), and some hepatoma cells (12), and nuclear targeting of HDGF was reported to be required for mitogenesis (13, 14). HDGF has also been investigated in terms of development. Expression of HDGF was described during nephrogenesis (10), cardiogenesis (15), and hepatogenesis (16).

HDGF has greatest homology in amino acid sequence (32%) to high mobility group 1 (HMG1), which has been reported to have intranuclear and extracellular functions (17). HMG1 is an abundant component of mammalian nuclei (18, 19). It binds the double helix on binding through the minor groove and binds with high affinity to DNA that is already sharply bent, such as linker DNA at the entry and exit of nucleosomes (20, 21). HMG1, also called amphoterin, has been identified as an extracellular neurite growth factor (22), as a secreted late mediator of endotoxin shock (23), as a mediator of acute inflammation (24), and furthermore as a molecule related to tumor metastasis (25), supporting the notion that it functions in the extracellular space. In HDGF, the absence of the “HMG box,” which is essential for binding DNA, also strongly suggests that HDGF has extracellular roles.

Although intensively studied in many tissues and cells, HDGF has not been investigated in the nervous system. We examined the localization of HDGF in the mouse nervous system and found that it is abundantly expressed in neurons. Since neurons do not replicate, HDGF cannot be considered a proliferation-inducing factor for neurons. In the present report, we describe the neurotrophic effects of HDGF harbored in nuclei.

**MATERIALS AND METHODS**

Western Blot Analysis—Western blot analysis was carried out according to the method of Sambrook et al. (26). Protein was solubilized in radioimmune precipitation assay buffer consisting of 10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.1% Triton X-100. The concentration of the protein was determined using a Bio-Rad protein assay kit (Bio-Rad). The protein was subjected to 15% SDS-PAGE and electroblotted to an Immobilon membrane (Millipore Corp., Bedford, MA). The protein was subjected to 15% SDS-PAGE and electroblotted to an Immobilon membrane (Millipore Corp., Bedford, MA). The protein was then transferred to the membrane using a semidry blotting apparatus (Bio-Rad). The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cappel, Aurora, OH) at a dilution of 1:5000 in blocking buffer for 1 h at room temperature. After being washed with TBS-Tween/Triton buffer (0.05% Tween 20, 0.2% Triton X-100), the membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cappel, Aurora, OH) at a dilution of 1:5000 in blocking buffer.
for 1 h at room temperature. After another wash, the membrane was developed using the ECL system (Amersham Biosciences).

In Situ Hybridization—*In situ* hybridization was carried out according to the method of Yamamoto et al. (27) with some modifications. cDNA encoding mouse HDGF (nucleotides 356–775) (2) was inserted into pBlueScript II (Stratagene). Probes were prepared using a DIGoxigenin RNA Labeling Kit (Roche Applied Science) according to the manufacturer’s instructions. Corresponding cDNA probes were used as controls. Eight-week-old male mice (C57BL/6) were anesthetized with sodium pentobarbital (5 mg/kg) intraperitoneally. Brains were carefully removed, frozen in dry ice powder, and stored at –80 °C until sectioning. Serial sections (15 μm) were cut with a cryostat (Bright; Huntingdon, UK). After being mounted onto slides, sections were dried with a driller and fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature. After being washed with PBS, sections were treated with 10 μg/ml proteinase K containing 50 mM Tris, pH 7.5, and 5 mM EDTA, and the reaction was stopped by treating with 4% paraformaldehyde in PBS for 20 min. Sections were covered overnight at 55 °C with hybridization buffer (50% deionized formamide, 0.3 mM NaCl, 20 mM Tris, pH 8.0, 10% dextran sulfate, 0.2% FeCl2, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 500 μg/ml yeast tRNA, 0.2 ng/ml salmon testes DNA, 0.2% N-lauryl sarcosine sodium salt) containing 0.5 μg/ml antisense or sense probes. The following day, the sections were subjected to a series of stringent washes and RNase treatment before immunological detection with anti-digoxigenin-AP conjugate (1:1000; Roche Applied Science) for 1 h at room temperature. The sections were then washed and exposed to a coloring reaction solution of 0.15 M sodium citrate, 0.05% Triton X-100, 0.1% bovine serum albumin, 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche Applied Science).

**Immunohistochemistry—**Eight-week-old male mice (C57BL/6) were anesthetized with sodium pentobarbital (15 mg/kg) intraperitoneally and then perfused transcardially with 4% paraformaldehyde in PBS, pH 7.4. Brains were carefully removed and postfixed overnight at 4 °C in the same fixative. Tissue blocks were rinsed for 1 h with PBS and dehydrated through a series of increasing concentrations of ethanol. After dehydration, they were cleared with chloroform and xylene and then embedded in paraffin. The sections were then cut as 6-μm-thick sections onto poly-L-lysine-coated slides (Matsunami, Japan). The sections were deparaffinized, and endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were incubated with anti-HDGFL polyclonal antibody (C terminus) (14) or affinity-purified rabbit anti-HDGFL polyclonal antibody (Pharmingen). The sections were either induced to undergo necrosis by treatment with 10 μM ionomycin (Sigma) and 20 μM carbonyl cyanide 3-chlorophenylhydrazone (Sigma) for 24 h or induced to undergo apoptosis by treatment with 300 mM etoposide (Sigma) for 24 h. Neuro2a cells without treatment served as a control. For Western blot analysis, the media from treated and untreated cells in 3.5-cm tissue culture dishes were collected and concentrated 10-fold using a Centricon YM-10 (Millipore). For staining, 5 × 10^5 Neuro2a cells were seeded onto 8-well chamber slides (Nunc) and induced to undergo necrosis or apoptosis and then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 15 min at room temperature. After being washed with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 h with 10% normal goat serum in PBS. Cells were incubated with rabbit-anti-HDGFL polyclonal antibody (C terminus) (14) in PBS containing 5% normal goat serum for 1 h at room temperature. After washing with PBS, the bound primary antibodies were labeled by incubation with goat anti-rabbit Ig conjugated to fluorescein (1:50; Cappel) in PBS containing 5% normal goat serum. After further washing, the cells were treated with propidium iodide (4 μg/ml), mounted, and observed with an Olympus microscope equipped with epifluorescent filters. Laser scanning was performed with a Zeiss LSM510, Axiovert 25 confocal imaging system.

**Assessment of Neuronal Survival and Death—**Brain-derived neurotrophic factor (BDNF) was purchased from R&D Systems (Minneapolis, MA). Recombinant HDGF was purified as previously described (16). Mouse primary hippocampal neurons were prepared as described above. After a 6-h incubation with the serum-free defined medium lacking B27 (Invitrogen), 0.3 μl/glitter glutamine, and penicillin/streptomycin, the medium was replaced with serum-free defined medium lacking B27 (Invitrogen) in order to study neuronal survival (28) but containing BDNF (10 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml, or 100 ng/ml) to determine the optimal BDNF concentration. Half of the medium was exchanged every other day, and after 5 days of incubation, cell viability and death were assessed using the MTT colorimetric assay (Chemicon, Temecula, CA) and the live/dead (THI) Assay Kit (Molecular Probes, respectively). Neuronal survival was almost identical in the presence of BDNF at concentrations of 10 and 100 ng/ml. Finally, to examine the neurotrophic effect of HDGF, we added HDGF (1, 10, 100, or 500 ng/ml) to primary hippocampal neurons. Survival values were normalized, taking the survival value of untreated cells as 0% and that of 10 ng/ml etoposide as 100%. All experiments were carried out in triplicate and repeated four times.

**Small Interfering RNA (siRNA) Technology—**pSUPER, an siRNA vector, was generously provided by Dr. Reuven Agami of the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital (29). The annealed oligonucleotides (5′- gcccttTCACAAGGAAAGTGTGCGGATccttcACCGAACACTTCTCGGA-3′ and 5′- TCCAAGGAAAGTGTGCGGATccttcACCGAACACTTCTCGGA-3′) were ligated into the vector. The 19-nucleotide HDGF target sequence is indicated in capital letters in the oligonucleotide sequence. The plasmid constructed was designated pSUPER-HDGFL.
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RESULTS

HKGF Is Synthesized and Localized Mainly in Neurons in the Nervous System—It has been observed that HKGF mRNA is expressed in the brain using Northern blot analysis (1, 15). To examine the expression of HKGF protein in the nervous system, a developmental study was carried out by Western blot analysis of the mouse brain (Fig. 1). We found that HKGF protein was constantly expressed in the brain from embryonic day 13 until 2 years of age.

To identify cells producing HKGF in the nervous system, in situ hybridization was performed using 8-week-old male mouse brains. HKGF mRNA was highly expressed in neurons, especially in hippocampal neurons and Purkinje cells (Fig. 2, A, C, and E). No significant staining was observed with sense probes (data not shown).

To examine the localization of HKGF protein in the nervous system, immunohistochemical experiments were carried out using 8-week-old male mouse brains. No staining was visible without the primary antibody (data not shown) or with the preabsorbed antibody (Fig. 2H). HKGF was highly stained in the nuclei of most of the hippocampal neurons and Purkinje cells (Fig. 2, B, D, and F). In cerebral cortex, most of the neurons were stained with anti-HKG antibody. Although some glial cells were stained with anti-HKG antibody, most of them were HKGF-negative (Fig. 2B).

These results indicate that HKGF is continuously produced in the nervous system and that in adult mice, HKGF is produced mainly in neurons and retained in their nuclei.

HKGF and HMG1 Are Secreted in Physiological Conditions—We tested whether HKGF and HMG1 were secreted in neuronal cells, using mouse primary hippocampal neurons and the mouse neuroblastoma cell line Neuro2a. The purity of the neurons was confirmed to be more than 90% (Fig. 3A). As shown in Fig. 3, B and C, both HKGF and HMG1 were detected in the conditioned media by Western blot analysis. This result was consistent with previous reports showing that HMG1 was secreted by certain cells (25, 30, 31). We also estimated the amount of HKGF by Western blot analysis using a series of recombinant HKGFs as standards (data not shown). The amount of HKGF in the medium of the hippocampal neuronal culture was estimated to be ∼13 pg/24 h/10^5 cells. The amount in the hippocampal neurons in culture was ∼1.3 ng/10^5 cells. Thus, approximately one-hundredth of the intracellular HKGF in the hippocampal neurons was secreted over 24 h in vitro.

Necrotic Cells Release HKGF—It has been reported that HMG1 is passively released from necrotic cells, whereas apoptotic cells retain HMG1 in their nuclei (32). We tested whether HKGF was released under pathological conditions, using the Neuro2a cell line. More than 50% of the cells underwent necrosis or apoptosis after 24 h of treatment at 37 °C. Western blot analysis with anti-HKG and anti-HMG1 polyclonal antibodies showed that HKGF protein was highly expressed in the conditioned media. Therefore, HKGF is released into the conditioned media from necrotic cells, possibly contributing to the survival of endogenous HKGF-suppressed cells.
HDGF is a Neurotrophic Factor in the Nucleus

HDGF Is a Neurotrophic Factor for Neurons

Many proliferating and/or differentiating factors have been reported to affect the survival of neurons (33–35). We examined the survival-promoting effect of HDGF using mouse primary hippocampal neurons. Mouse primary hippocampal neurons were prepared, and the medium was replaced with the serum-free defined medium without B27 (Invitrogen) in order to study neuronal survival. After confirmation that the optimal concentration of BDNF for neuronal survival was 10 ng/ml (data not shown), neurons were cultured for 5 days in the serum-free defined medium with HDGF (1–500 ng/ml) or BDNF (10 ng/ml) or medium alone. HDGF protein expression was observed in all neurons prepared from embryonic day 17 hippocampus (Fig. 4A). The HDGF concentration in the medium of the hippocampal neuronal culture without trophic factors was estimated to be less than 1.0 ng/ml by Western blot analysis using recombinant HDGF as a standard (data not shown). Cell viability and cell death were assessed using the MTT colorimetric assay and by measuring lactate dehydrogenase activity, respectively. Since the results of these two assays were reciprocal, only those of the MTT assay are shown in Fig. 5B. The absorbance values at 540 nm were 0.443 ± 0.012 (control), 0.444 ± 0.008 (HDGF, 1 ng/ml), 0.611 ± 0.021 (HDGF, 10 ng/ml), 0.747 ± 0.006 (HDGF, 100 ng/ml), 0.717 ± 0.016 (HDGF, 500 ng/ml), and 0.780 ± 0.015 (BDNF, 10 ng/ml). Then, the data were standardized to BDNF (100%) and no growth factors (0%). HDGF had a dose-dependent neurotrophic effect on neurons, although compared with BDNF, a well defined neurotrophic factor, a 10 times higher concentration of HDGF was required for similar activity.

These results combined with the data on the expression of HDGF in neurons and on secretion of HDGF from neurons suggest that HDGF is an autocrine neurotrophic factor.

Exogenously Supplied HDGF Partially Protected Endogenous HDGF-suppressed Neurons against Death—We tested the effect of endogenous HDGF suppression using siRNA technol-
ogy (29). A plasmid, pSUPER-HDGF, was constructed to suppress endogenous HDGF expression. Primary hippocampal neurons were transiently transfected with either pEGFP-C1 alone, pEGFP-C1 and pSUPER, or pEGFP-C1 and pSUPER-HDGF. The plasmid pEGFP-C1 was used to mark the transfected cells as well as assess the transfection efficiency. The transfection efficiency was found to be ~10%. A reproducible reduction in HDGF expression to the background level was confirmed by immunohistochemistry for the neurons co-transfected with pEGFP-C1 and pSUPER-HDGF at 5 days after transfection (Fig. 6A). The time courses of the GFP-positive neurons for each condition are shown in Fig. 6B. In the control where pEGFP-C1 and pSUPER were co-transfected, GFP-positive neurons decreased to 57% after 5 days in culture, whereas in the endogenous HDGF-suppressed neurons, GFP-positive neurons decreased to 32% (Fig. 6C). These results indicate that nuclear HDGF might be important for survival.

Finally, using this paradigm, we examined whether exogenously supplied HDGF rescued endogenous HDGF-suppressed neurons from death. Recombinant HDGF was supplied exogenously to neurons co-transfected with pEGFP-C1 and pSUPER-HDGF. As shown in Fig. 6C, exogenously supplied HDGF partially prevented the cell death that occurred in endogenous HDGF-suppressed neurons.

**DISCUSSION**

HDGF has been intensively studied as a mitogenic factor (1, 10–12). In the present study, we found another role for HDGF as a neurotrophic factor. Peculiar to HDGF among neurotrophic factors is that it is localized in the nucleus and that it has the potential to function in physiological and pathological conditions from the embryonic period through to adulthood.

These features are considered to be derived from the inherent domains of the amino acid sequence of HDGF. First, HDGF does not have a signal peptide-like hydrophobic region (1). Likewise, no signal peptide has been reported in the primary amino acid sequence of acidic or basic fibroblast growth factors (36). These molecules are thought to be secreted via a pathway other than the classical secretory pathway with a signal peptide. Other features are that HDGF possesses nuclear localization signals and heparin binding capacity. We speculate that HDGF protein synthesized in the cytoplasm preferentially moves to the nucleus due to its nuclear localization signals or is secreted via the nonclassical pathway and functions in an autocrine manner via a mechanism involving its heparin-binding capacity. The amount of HDGF in the medium and in the neurons in the hippocampal neuronal culture was estimated to be ~13 pg/24 h/10^5 cells and ~1.3 ng/10^5 cells, respectively.

**FIG. 5.** Effects of HDGF on the survival of mouse hippocampal neurons. A, phase micrograph of the hippocampal neurons after 1 day of culture. All neurons were stained with anti-HDGF antibody. B, neurons were cultured for 5 days in the serum-free defined medium with HDGF (1–500 ng/ml) or with an optimal concentration of BDNF (10 ng/ml) or medium alone. The cell viability was assessed by an MTT colorimetric assay. Values (mean ± S.E.; n = 3) were normalized, with survival in the serum-free defined medium alone taken as 0% and that in 10 ng/ml BDNF taken as 100%.

**FIG. 6.** Exogenously supplied HDGF partially rescues endogenous HDGF-suppressed neurons. Primary hippocampal neurons were transfected with either pEGFP-C1 alone, pEGFP-C1 and pSUPER, or pEGFP-C1 and pSUPER-HDGF. HDGF (100 ng/ml) was added at the time of transfection. A, a reduction in HDGF expression to the background level was confirmed by immunohistochemistry for the neurons co-transfected with pEGFP-C1 and pSUPER-HDGF. A representative neuron is shown. B, the time courses of GFP-positive neurons for each condition are shown. The percentage of GFP-positive neurons after transfection was calculated in relation to the number of GFP-positive neurons at 1 day after transfection defined as 100% for each condition. C, in the control where pEGFP-C1 and pSUPER were co-transfected, GFP-positive neurons decreased to 57% after 5 days in culture, whereas in the endogenous HDGF-suppressed neurons, GFP-positive neurons decreased to 32%. In the endogenous HDGF-suppressed neurons with exogenously supplied HDGF, GFP-positive cells decreased to 51%. Thus, exogenously supplied HDGF partially rescued endogenous HDGF-suppressed neurons.
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Thus, approximately one-hundredth of the intracellular HDGF in the hippocampal neurons was secreted over 24 h in vitro. Although we have not examined the turnover of HDGF in vivo, these protein movements might be finely controlled under physiological conditions.

Some growth factors including nerve growth factor, epidermal growth factor, and platelet-derived growth factor, have been reported to exert their effects by binding to their receptor, being internalized, and being conveyed to the nucleus associated with their receptor or even without a receptor by targeting to the nucleus (37–39). Exogenously supplied HDGF is reported to induce proliferation of fibroblasts (1), endothelial cells (10), vascular smooth muscle cells (11), and some hepatoma cells (12). Transfection of deletion mutants of HDGF clearly showed that nuclear targeting was required for mitogenesis (13, 14).

Exogenously supplied GFP-HDGF was reported to translocate to the nucleus in 293 cells (14). Therefore, extracellular HDGF is considered to enter into the nucleus and show mitogenic effects. In our experiments on neurons, we showed that exogenously supplied HDGF has a neurotrophic effect on hippocampal neurons (Fig. 5). The death of endogenous HDGF-suppressed neurons in Fig. 6 suggests that nuclear HDGF contributes to the survival. Besides, we showed that the reduction in the survival of endogenous HDGF-suppressed neurons was attenuated by exogenously supplied HDGF (100 ng/ml).

As for neurons, the mechanism behind the effectiveness of exogenously supplied HDGF remains to be elucidated; however, our results support the notion that extracellular HDGF enters the nucleus and shows neurotrophic effects in neurons.

HDGF was shown here to share some characteristics with HMG1. Both molecules are localized to the nucleus, secreted under physiological conditions, and released from necrotic cells, while being retained within apoptotic nuclei (32). We demonstrated that HDGF has neurotrophic activity, whereas HMG1 has been reported to be an extracellular neurite growth factor (22). HMG1 enhances the activity of several transcription factors, including the glucocorticoid receptor, as well as the activity of RAG recombinase (19, 40). One member of the HDGF family, LEDGF/p75/p52, was reported to function as a general transcriptional coactivator that enhanced activated transcription through its binding to the receptor for advanced glycation end products controlled by HDGF in the nucleus and shows neurotrophic effects in neurons.

For neurons, the mechanism behind the effectiveness of exogenously supplied HDGF remains to be elucidated; however, our results support the notion that extracellular HDGF enters the nucleus and shows neurotrophic effects in neurons.

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