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Hepatocyte Growth Factor Is a Mitogen for Olfactory Ensheathing Cells

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Abstract

Hepatocyte growth factor (HGF) is a potent mitogen for mature hepatocytes, and it has multi-functional effects in a variety of cells in various organs. HGF stimulates DNA synthesis and promotes cell migration and morphogenesis in several cell types including the olfactory system. To characterize the potential mitogenic activity of HGF that might contribute to olfactory ensheathing cell (OEC) proliferation, we tested the ability of HGF to stimulate OEC division in vitro. OECs were obtained from adult rat olfactory bulbs and cultured in serum-free medium, and were identified by double immunostaining for p75 and S-100 antibodies. DNA synthesis assayed by pulsing BrdU for 24 hr showed that HGF at the concentration of 5–100 ng/ml elicited a 5–10-fold increase of OEC proliferation. By immunocytochemical analysis, we demonstrated that c-Met-immunoreactivity was present in cultured OECs, and c-Met anti-serum significantly sequestered the activity of HGF on OECs proliferation, suggesting that HGF-induced proliferation of OECs is mediated by the c-Met receptor. The mitogenic activity of HGF was potentiated by addition of heregulin (HRG), but inhibited by addition of forskolin. These results demonstrate that HGF is a novel mitogen for rat OECs in vitro, and HGF/c-Met system is involved in regulating OECs growth and development.

Keywords

cell culture; growth factor; proliferation; c-Met

There is increasing interest in recent years in olfactory ensheathing cells (OECs) that are thought to derive from precursor cells in the olfactory epithelium (Mendoza et al., 1982). During development, primitive olfactory neurons extend their axons from the olfactory placode, as they exit the developing epithelium; OECs migrate out from olfactory epithelium and accompany the olfactory nerve fibers, and some of them eventually reside in the olfactory nerve layer of the olfactory bulb (Chuah and Au, 1991; Ramón-Cueto and Valverde, 1995; Ramón-Cueto and Avila, 1998a). OECs share many properties with Schwann cells (SCs) including expression of low-affinity nerve growth factor receptor (p75), L1, S-100 and, N-CAM (Miragall et al., 1989; Pixley, 1992; Ramón-Cueto and Nieto-Sampedro, 1992; Franklin and Barnett, 1997) and respond to growth factors such as basic fibroblast growth factor (bFGF) and heregulin (HRG) (Chuah and Teague, 1999; Pollock et al., 1999; Yan et al., 1999; Yan et al., 2001).

Several reports demonstrated that transplanted OECs promote peripheral nervous system (PNS) and central nervous system (CNS) axonal regeneration (Ramón-Cueto et al., 1994, 1998b, 2000; Li et al., 1997, 1998; Verdú et al., 1999; Imaizumi et al., 2000a,b) and remyelinate

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demyelinated CNS axons (Franklin et al., 1996; Imaizumi et al., 1998; Barnett et al., 2000; Kato et al., 2000). To study the potential of OEC transplantation as a strategy in the repair of CNS injury or demyelination requires the availability of a large number of highly enriched populations of OECs. Rat OECs proliferate very slowly in vitro, even in serum-containing medium. Several identified polypeptide growth factors act as OEC mitogens in vitro including bFGF and HRG (Pollock et al., 1999; Chuah and Teague, 1999; Chuah et al., 2000). Our previous work demonstrated that PDGF and IGF-I also stimulate OECs proliferation and the action of these growth factors can be potentiated by forskolin, which elevates intracellular cAMP (Yan et al., 1999, 2001).

Hepatocyte growth factor (HGF) is a pleiotropic cytokine that triggers proliferation, migration, and morphogenesis in a variety of epithelial cell types during normal development and in pathophysiological conditions (Brinkman et al., 1995; Matsumoto and Nakamura, 1997; Birchmeier and Gherardi, 1998; Stella and Comoglio, 1999). Although HGF was initially identified to be a potent mitogen for mature hepatocytes in primary culture (Nalamura et al., 1984), an increasing amount of evidence indicates the roles of HGF in the nervous system. HGF promotes survival of many different types of neuronal cells such as hippocampal neurons, midbrain dopaminergic neurons, spinal motor neurons, sensory neurons and sympathetic neuroblasts (Honda et al., 1995; Ebens et al., 1996; Hamanoue et al., 1996; Thewke and Seeds, 1999; Bottara et al., 2001; Davey et al., 2000). HGF and its receptor, c-Met, are highly expressed during development of the brain including the olfactory system (Thewke and Seeds, 1996, 1999).

Although HGF has been fairly extensively evaluated in mesenchymal-derived cells, the understanding of its role in the nervous system is largely incomplete. Little is known regarding HGF's activity on OEC growth. To determine if HGF interacts with the c-Met receptor to influence proliferation in OECs, we studied the expression c-Met on OECs and the proliferative effect of HGF on purified OEC cultures. Our results demonstrate that HGF is a novel mitogen for OECs and OECs expressed HGF receptor protein, c-Met, suggesting that the HGF/c-Met system can regulate OECs growth and development.

MATERIALS AND METHODS

Materials

The following sources and materials were used: Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixtures, Leibovitz L-15 medium, trypsin/EDTA, N2 medium (GIBCO, Grand Island, NY); fetal bovine serum (Hyclone Laboratories Inc., Logan, UT); forskolin, poly-L-lysine, recombinant human HGF, anti-GFAP polyclonal antibody (Sigma Chemical Company, St. Louis, MO); trypsin (Boehringer-Mannheim Biochemicals, Indianapolis, IN); Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Eugene, OR); goat anti-mouse immunoglobulins (Capell/Organnon Teknika Corp., West Chester, PA); 192 hybridoma cell line (gift of Dr. Eugene M. Johnson); recombinant heregulin $\beta 1$ (R&D, Minneapolis, MN); bromodeoxyuridine (BrdU, Amersham Pharmacia Biotech, UK); S-100 polyclonal antibody (Dakopatts, Glostrup, Denmark); Falcon 8-well chambers (Becton Dickinson Labware, NJ); anti-c-Met antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

OECs Culture and Purification

Primary olfactory bulb cultures were prepared as previously described by Ramón-Cueto et al. (1998b). Briefly, 3–4-month-old adult female Wistar rats (Harlan Sprague-Dawley) were deeply anesthetized and killed by decapitation and the olfactory bulbs were dissected and placed in Leibovitz L-15 medium. After carefully removing the pia, the olfactory nerve fiber

and glomerular layers were dissected away from the rest of the bulb. The tissues were cut into 1 mm³ pieces and incubated with 0.1% trypsin at 37°C for 15 min with shaking. Trypsinization was stopped by adding DMEM and Ham's F-12 (D/F-12, 1:1 mixture) supplemented with 10% heat-inactivated fetal bovine serum (D/F-10S). Cells were seeded on 100-mm culture dishes coated with 50 µg/ml poly-L-lysine and maintained in D/F-10S. The dishes were incubated at 37°C, 5% CO₂ and the medium were changed every 2 days.

The method used to purify OECs from primary cultures was as described by Ramón-Cueto et al. (1998b). Briefly, 6–7 days after plating, OECs were separated from other cell types in primary cultures by immunoaffinity, using an antibody against p75. Cells from primary cultures were detached with 0.05% trypsin and 0.02% EDTA, centrifuged, and washed two times with D/F-10S. Cells were suspended in L-15 medium and plated on 100 mm Petri dishes that have been prepared as follows. Dishes were incubated with 1:100 goat anti-mouse IgG antibody overnight at 4°C and washed with L-15 medium. They were then incubated with supernatant of cultured 192 hybridoma cells containing p75 antibody at a 1:10 dilution in L-15 medium with 5% FBS for 2 hr at 4°C. After the dishes were washed with L-15, 10 ml of a suspension containing cells from olfactory bulb primary cultures were plated on the antibody-treated dishes, at a density of 4×10^5 cells per dish for 45 min at 37°C, 5% CO₂. For removing unbound cells, dishes were washed five times with L-15 medium. Bound cells were detached from the dishes with a cell scraper, centrifuged, and resuspended in D/F-10S. Cells were seeded onto poly-L-lysine-coated 8-well cell culture chambers at a density of 1.5×10^4 cells and were incubated at 37°C in 5% CO₂.

Cell Growth Assay

The growth of OECs was examined by bromodeoxyuridine (BrdU) incorporation. Purified OECs were cultured for 2 days in D/F-10S medium. After washing three times with DMEM/F-12 medium, the medium was replaced by N2 chemical defined medium. After culturing for 2 days in serum-free medium, cells were exposed to varying concentrations of mitogens for 48 hr. To label nuclei that were synthesizing DNA, 10⁻⁵ M BrdU was administered to the cultures for the last 24 hr before staining. In a second series experiments, cell chambers containing 1.5×10^4 OECs were fed with N2 medium alone or N2 medium with c-Met polyclonal antibody at the concentration of 0.5–10 µg/ml for 30 min at room temperature. Without rinsing the cultures, they were then fed with 1 µg/ml HGF (1/100 vol) to give a final concentration of 10 ng/ml. The cells were maintained at 37°C, 5% CO₂ for an additional 24 hr. Cells were pulsed labeled for the subsequent 24 hr using medium containing BrdU after reapplying antibody followed by HGF (10 ng/ml) as described above.

Immunocytochemistry

The cultures were washed with L-15 medium two times and then fixed with 4% paraformaldehyde for 15 min followed by with acid-ethanol for 30 min at room temperature. The cultures were permeabilized with 0.3% Triton X-100 in 0.1 M PBS for 30 min and incubated with monoclonal anti-BrdU (1:100), monoclonal anti-p75 supernatant (1:10), polyclonal S-100 (1:200), GFAP (1:200), or c-Met (1:20–40) antibody plus 10% normal goat serum overnight at 4°C. After washing three times with 0.1 M PBS (pH7.4), cells were incubated with Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488-goat anti-rabbit IgG for 90 min at room temperature. The coverslips were mounted on glass slides with mounting medium with DAPI, which binds DNA and was used to image all the nuclei by fluorescence microscopy. OECs were identified as p75 and S-100 positive. All S-100 positive cells and BrdU labeled cells of per coverslip were scored using a 40× fluorescent filter appropriate for the fluorescent dyes conjugated to the secondary antibodies. Each coverslip was scanned in the same manner by counting adjacent fields until at least 20 fields had been counted:

$$\text{Cell labeling index (\%)} = \frac{\text{number of BrdU}^+/\text{S } 100^+}{\text{number of S } 100} \times 100\%$$

RESULTS

Characterization of EG Cultures in Serum-Free Medium

OECs were characterized morphologically and immunocytochemically to detect cell-type specific antigens. After immunopanning, OECs were grown in chemically defined medium (N2) for 4 days. Double immunocytochemical staining revealed that about 90% cells were p75⁺/S100⁺ or p75⁺/GFAP⁺, which is characteristic of OECs (Barber and Lidsay, 1982; Chuah and Au, 1991; Franklin and Barnett 1997; Ramón-Cueto and Avila, 1998). Approximately 5–7% of cells appeared p75[−]/GFAP⁺; these cells were defined as astrocytes. Another 5% cells were undefined cells. Purified OECs exhibited large flattened cells, bipolar, tripolar or multipolar morphology. When OECs were exposed to growth factors for two days, their antigenic phenotype and morphology were not changed (Fig. 1A–D).

HGF Stimulated OECs Division

To determine whether HGF is mitogenic for OECs derived from adult rat olfactory bulbs, cells were cultured for 2 days in the presence or absence of HGF in serum-free medium. The proliferation rate was determined by counting the number of BrdU labeled S100-positive cells (Fig. 2). OECs cultured in serum-free medium without growth factors for 3 days after immunopanning have a very low cell labeling index (Fig. 3). The addition of HGF at various concentrations (1–100 ng/ml) increased BrdU uptake. HGF elicited about 5–10-fold increases in the cell-labeling index at concentrations of 5–10 ng/ml (Fig. 3). At higher concentrations (20–100 ng/ml), however, HGF produced no further increase in proliferation of OECs.

Forskolin Inhibited the Mitogenic Effect of HGF

Previous studies have shown that forskolin, a cyclic AMP (cAMP) elevating agent, potentiates mitogenic responses of bFGF, IGF-1 and PDGF on SCs (Davis and Stroobant, 1990; Chen et al., 1991; Schumacher et al., 1993) and OECs (Yan et al., 1999, 2001). To determine whether activity of HGF could be enhanced by elevation of intracellular cAMP, HGF was added to OECs in combination of forskolin. Two μM forskolin alone did not stimulate DNA synthesis in serum-free medium, however, it potentiated the mitogenic effect of HRG in serum-free medium (Fig. 4). In contrast, when added to OECs in combination of HGF (10 ng/ml), forskolin significantly attenuated the ability of HGF to stimulate OECs proliferation. This decrease was not a result of FSK toxicity, because 2 μM FSK could slightly stimulate DNA synthesis in serum-containing medium (data not shown). HRG is a potent mitogen for Schwann cells (SCs) (Levi et al., 1995) and in this study we tested whether HRG synergizes with HGF in stimulation of OECs proliferation. OECs were exposed to HGF (10 ng/ml) in combination with HRG (10 ng/ml). This combination of HRG and HGF robustly promoted OECs proliferation in an additive manner (Fig. 4).

OECs Express c-Met Protein and c-Met AntiSerum Blocked Activity of HGF

Previous studies showed that HGF is mitogenic for astrocytes and SCs. As described above, HGF stimulated OECs proliferation and we hypothesized that these responsive cells should possess HGF receptor. To test this hypothesis, double immunostaining for p75 and c-Met was performed (Fig. 5). OECs were immunopositive for c-Met antibody. It should be noted that some p75 negative cells also were c-Met positive in our culture system. In our culture system, there are approximately 5–10% p75 negative cells; a portion of these cells may be astrocytes based on morphology. We could not clearly define astrocytes because both of OECs and astrocytes are immunopositive for GFAP.

To determine if the effect of HGF on OEC proliferation is mediated by HGF receptor, we looked for differences in the cell labeling index in the presence and absence of c-Met anti-serum (Fig. 6). c-Met anti-serum significantly ($P < 0.01$) attenuated the HGF-induced BrdU labeling of OECs. With the increase of c-Met anti-serum concentration, the cell labeling index decreased; 10 $\mu\text{g/ml}$ c-Met anti-serum maximally inhibited for HGF-induced proliferation. This result strongly suggests that the HGF effect on OECs proliferation is mediated by c-Met receptor.

DISCUSSION

HGF, also known as scatter factor, is a mesenchyme-derived pleiotropic protein composed of a 69 kDa α -chain and 34 kDa β -chain (Rubin et al., 1993). HGF receptor is the c-Met proto-oncogene product, a transmembrane tyrosine kinase that consists of a transmembrane-spanning 145 kDa beta chain and a 50 kDa alpha chain (Bottara et al., 1991) and autophosphorylated in response to HGF binding (Matsumoto and Nakamura, 1996). HGF has a wide range of cellular effects including stimulation of DNA synthesis, cell migration, and cellular morphogenesis (Matsumoto and Nakamura, 1996; Cai et al., 2000). Recent studies have shown that both HGF and c-Met are expressed in the nervous system (Jung et al., 1994; Thewke and Seeds, 1996; Yamada et al., 1997; Fenton et al., 1998). In accordance with this, various types of neurons and glia respond to HGF in vivo and in vitro (Krasnoselsky et al., 1994; Okura et al., 1999; Caton et al., 2000; Zhang et al., 2000).

In this study we examined the biological effects of HGF in stimulating OECs proliferation in vitro. The ability to stimulate OEC proliferation was determined in serum-free medium. HGF at a concentration of 10 ng/ml exerted an approximately 10-fold increase of OEC proliferation. At concentrations above 10 ng/ml, HGF showed reduced ability to stimulate OEC proliferation. This range of concentrations has also been found to be effective in stimulating SC proliferation (Krasnoselsky et al., 1994). It is not known from our study why the proliferation of OECs was reduced when the concentration of HGF was increased up to 20–100 ng/ml. We could exclude the possibility of toxicity of HGF at high concentrations, because cell number in the culture kept constant over the duration of the bioassay event at 100 ng/ml.

Previous studies with SCs showed that growth factor mitogenic action including FGF-2, IGF-1, PDGF, is potentiated by the cAMP-elevating agent, forskolin (Davis and Stroobant, 1990; Schumacher et al., 1993; Chen et al., 1994). Also our previous work indicated that forskolin potentiated the mitogenic action of HRG, FGF-2, PDGF, and IGF-1 for OECs (Yan et al., 1999, 2001). In contrast, in this study the presence of forskolin significantly attenuated the mitogenic effect of HGF on OEC growth in serum-free medium (Fig. 4). cAMP can modulate the response of SCs to mitogen by upregulating growth factor receptor such as PDGF receptor (Weinmaster and Lemke, 1990). In this study, however, the intensity of immunostaining for c-Met was not changed when OECs were exposed to forskolin at different concentrations (Fig. 5), suggesting that forskolin did not likely downregulate HGF receptor to abolish HGF activity. FSK attenuated HGF activity of proliferation on OECs is not likely due to toxicity because FSK alone at the concentration that was used in serum-free medium could stimulate OECs proliferation in serum-containing medium and it could potentiate activity of HRG on OECs proliferation in serum-free medium. The combination of HGF, HRG and forskolin elicited the same mitogenic response as HRG plus forskolin alone, suggesting that HGF-induced OEC proliferation was abolished and did not interfere with HRG/forskolin induced OEC proliferation. HGF per se does not affect adenyl cyclase activity or increase intracellular cAMP level (Marker et al., 1992). Elevation of cellular cAMP level resulted in inhibition of HGF-stimulated DNA synthesis (Marker et al., 1992). Our results on OECs are consistent with HGF activity on SCs and hepatocytes (Marker et al., 1992; Krasnoselsky et al., 1994).

It has been shown that HGF and c-Met are expressed in both of neurons and glial cells including SCs, microglia, astrocytes, and oligodendrocytes (Krasnoselsky et al., 1994; Yamada et al., 1994; Achim et al., 1997; Kilpatrick et al., 2000; Korhonen et al., 2000), suggesting that HGF and c-Met may play a role not only in neuronal development but also in neuronalglia interactions. The expression of HGF and its receptor, c-Met, was observed in the olfactory system in adult mice (Thewke and Seeds, 1996), but was not specifically localized to OECs. In the present study, we found that immunoreactivity for p75 and c-Met on isolated purified OECs from adult rat olfactory bulb was strong. When 10 µg/ml c-Met anti-serum was added to the culture medium, the cell labeling index dramatically decreased, suggesting blocking HGF receptor prevented HGF stimulation of OECs proliferation. High-level expression of c-Met immunoreaction in adult rat OECs implicates the role of HGF/c-Met system signaling in the adult, as well as a role during olfactory system development. Based on HGF and the c-Met expression patterns in other regions of the brain, HGF has been proposed to act as a target-derived neurotrophic factor (Jung et al., 1994). HGF has been shown to promote the survival of motor and hippocampal neurons in culture (Honda et al., 1995; Wong et al., 1997). Within the olfactory bulb, the expression pattern of HGF and the c-Met suggest a neurotrophic role in the formation or maintenance of olfactory glomeruli (Thewke and Seeds, 1996). During development, OECs accompany olfactory nerve bundles and migrate from olfactory epithelium to olfactory bulb. Therefore, like SCs and oligodendrocytes, OECs are also motile cells. HGF acts as a chemoattractant for migrating cells (Birchmeier and Gherardi, 1998). The recent observation that the c-Met expression is essential for myoblasts (Bladt et al., 1995) and retinal endothelial cell (Cai et al., 2000) migration and that these appear to migrate up a concentration gradient of HGF, suggests that HGF and the c-Met may play a similar role in the development of olfactory system.

In conclusion, we have identified that HGF is a novel mitogen for cultured OECs derived from adult rats, and furthermore OECs express HGF receptor (c-Met protein). These results suggest that the HGF/c-Met system may be involved in regulating the growth and development of OECs.

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Fig. 1. Immunocytochemical staining of OECs cultures shows phenotypic properties and morphology. OECs were cultured for 3 days in serum-free medium after immunopanning. OECs appeared flat multipolar morphology with positive immunoreaction for both of p75 (**A**) and S-100 (**B**) in the same field. (**C,D**) shows that OECs appeared positive immunoreaction for p75 and GFAP respectively. Scale bar = 25 μ m.

Fig. 2. Immunocytochemical staining of OECs cultures shows phenotypic properties and morphology after BrdU uptake. Red stain, BrdU; green stain, S-100; blue nuclear stain, Hoechst dye. OECs cultured in serum-free medium containing: (A) no growth factors; (B), 10 ng/ml HGF. OECs were exposed to growth factors for 48 hr; BrdU was added for the last 24 hr. Double immunostaining for S-100 and BrdU labeled nuclei were considered to identify dividing EG (arrows). Scale bar = 25 μ m.

Fig. 3. Dose-response stimulation of OECs by HGF in serum-free medium. OECs were exposed to HGF for 48 hr and BrdU was added for the last 24 hr. HGF significantly increases OECs labeling index up to 10 ng/ml. Data are the mean $\pm$ SEM.

Fig. 4.

The mitogenic activity of HGF on OECs was inhibited by forskolin. The cultures were incubated in serum-free medium with 2 μ M forskolin, HGF, HRG and HGF, HRG, HGF-HRG plus forskolin. DNA synthesis was measured by the incorporation of BrdU for the last 24 hr. To analyze proliferation, S-100 immunostaining and BrdU labeling were monitored. In each experiment, at least 400 cells were counted and the results presented as the mean $\pm$ SEM ($n = 3$). All value shown was statistically significant as determined by the Student's *t*-test. * $P < 0.01$ vs. HGF alone, ** $P < 0.01$ vs. HGF or HRG alone.

Fig. 5.

OECs express c-Met. OECs derived from adult rat were isolated and cultured for 4 days in serum-free medium alone (**A,B**) or treated with HGF at 10 ng/ml (**C,D**) or forskolin at 4 μ M (**E,F**) before performing immunostaining. Double immunostaining shows multipolar and bipolar OECs staining positively for p75 (**A,C,E**) and c-Met (**B**) in the same field. There is a p75- non-OECs in the culture (**A**), which is c-Met positive (**B**, arrow). Field treated with HGF (**C,D**) or forskolin (**E,F**) labeled with c-Met antibody. Scale bar = 25 μ m.

Fig. 6. c-Met anti-serum blocked HGF-stimulated OECs proliferation. OECs were treated with c-Met anti-serum (0.5–10 $\mu\text{g/ml}$) or HGF (10 ng/ml) alone or c-Met anti-serum plus HGF (10 ng/ml). After 48 hr of treatment, cell proliferation assays were performed by BrdU labeling. Data were presented as a percentage of BrdU labeling. The results are the mean $\pm$ SEM ($n = 3$). All value shown was statistically significant as determined by the Student's t -test. $**P < 0.01$ vs. c-Met anti-serum or HGF alone.