Hepatocyte Growth Factor/Scatter Factor Facilitates Migration of GN-11 Immortalized LHRH Neurons

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The molecular cues regulating the migratory process of LHRH neurons from the olfactory placode into the brain are not well known, but gradients of chemotropic and chemorepellent factors secreted by the targets are likely to play a key role in guidance mechanisms.

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic cytokine inducing cell migration. It is involved in a variety of developmental processes through interaction with its receptor c-Met. Here we show that c-Met-antibody labels LHRH migrating neurons in the olfactory mesenchyme of E12 mouse and analyze the potential chemotropic effect of HGF/SF on two immortalized LHRH cell lines, GT1-7 and GN11, isolated from tumors developed in the hypothalamus and in the olfactory bulb, respectively.

By RT-PCR analysis, Western blotting, and immunocytochemistry, we provide evidence for a high level of c-Met expression in GN11, but not in GT1-7, cells. In addition, HGF/SF treatment promotes specific migratory activity of GN11 cells, as demonstrated by collagen gel assay, time-lapse video microscopy, and Boyden's chamber experiments. Such promiscuity is inhibited by the neutralizing antibody.

The data reported here represent the first direct evidence of a chemotactic effect of HGF/SF on immortalized LHRH neurons. (Endocrinology 143: 3306–3315, 2002)
cell line was derived from a hypotalamic tumor, whereas GN cells were obtained from a tumor developed in the OB. This different derivation is indicative of different maturational stages of the two cell lines, as demonstrated by the fact that GT1 cells retain many characteristics of the mature hypothalamic LHRH neurons (34–36). On the contrary, it has been found recently that GN11 cells retain the phenotypic characteristics of immature LHRH neurons and show high migratory activity in vitro, responding to fetal bovine serum (FBS) as a chemotactic stimulus (36, 37).

In the present paper, we show that HGF/SF specifically promotes the migratory activity of GN11, but not GT1-7, cells.

**Materials and Methods**

**Animals and tissue**

The experiments were carried out on CD-1 mice purchased from Charles River Laboratories, Inc. (Calco, Italy). Timed pregnant mice (plug day, E0) were deeply anesthetized and killed at embryonic d 12 (E12). The embryos were harvested and washed in ice-cold Dulbecco’s PBS (Sigma, St. Louis, MO). The heads were fixed in fresh 4% paraformaldehyde (PAF, 4% formaldehyde 3-phosphate dehydrogenase (GAPDH) amplification corresponding to nucleotides 544–732 (accession no. M232599). 5’-GGGACTCCACGCA-CAAAGC-3’ and 5’-GTCTGACGCTACTAGATTTCCC-3’ for c-Met amplification corresponding to nucleotides 296–815 (38). The amplification of GAPDH served as a control, with respect to the quality and quantity of RNA that had been retrotranscribed into cDNA. The number of cycles and the annealing temperature used for each primer pair were 25 cycles and 62°C for c-Met, 25 cycles and 60°C for GAPDH. Amplification products were separated by 1.5% agarose gel electrophoresis and DNA bands visualized by ethidium bromide staining.

For immunoprecipitation and Western-blotting analysis, GT1-7 and GN-11 cells were solubilized in lysis buffer [Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgCl₂, 0.5% NaDOC, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 mM orthovanadate] on ice. Lysates were clarified by centrifugation at 16,000 × g for 15 min, and protein content was determined using a bichinchoninic acid kit for protein determination (Sigma). For c-Met immunoprecipitation, 1 mg protein extract was incubated with polyclonal c-Met antisera (Santa Cruz Biotechnology, Inc.; 1:100) for 1 h at 4°C. Immune complexes were collected with protein-A-Sepharose (Sigma) and washed three times with extraction buffer and once with TNE buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA). Precipitated proteins were released by boiling in Laemmli buffer [2% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4), 20 mM mercaptoethanol, 20% glycerol] and subjected to 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes in TBS-T buffer (20 mM Tris; 150 mM NaCl; 0.1% Tween 20, pH 7.4), filters were probed with 1:500 polyclonal c-Met antisera or 1:500 monoclonal antiphosphotyrosine antibody (Santa Cruz Biotechnology, Inc.) and visualized with the appropriate peroxidase-coupled secondary antibodies using an ECL detection system (Amersham Biosciences).

**Cell aggregates (collagen gel assay)**

Collagen gel has been found to allow optimal physiological culture conditions for many tissue fragments and normal isolated cells and to study cell migration (39). Cell aggregates were prepared by the so-called hanging drop technique (40). Subconfluent cells were collected by trypsinization, resuspended in complete culture medium, and seeded in 20-µl drops (200,000 cells for both cell lines) on the lid of a culture dish; the lid was then placed on a 35-mm dish filled with 2 ml culture medium and incubated at 37°C for 48 h.

Collagen gel was obtained as previously described (37), and 200 µl were pipetted onto the bottom of a 24-well culture dish and left to set at room temperature. Cells aggregates were transferred over the cushion and then overlaid with an additional 200 µl collagen. As the overlaid collagen was set, it was covered with 400 µl DMEM (0% FBS), or supplemented with recombinant human HGF/SF (50 ng/ml), Sigma), or with HGF/SF 50 ng/ml preincubated overnight with the blocking antibody to HGF/SF (10–20 µg/ml) (anti-human HGF/SF monoclonal
reactive cells emerged from the developing olfactory epithelium and migrate through the olfactory mesenchyme (increased random cell motility) was distinguished from chemotaxis by of the lower compartment of the chamber. Chemokinesis (stimulation of factors) experiments, the chemoattractants (DMEM FBS 1%, DMEM FBS 0%, HGF/SF at 6.2, 12.5, 25, 50, 100 ng/ml) were placed into the wells precoated with gelatin (0.2 mg/ml in PBS). For chemotaxis (the directed fluence were harvested, and the suspension (10⁵ cells/50 μl) was placed in the open-bottom wells of the upper compartment. Each pair of wells were separated by a polycarbonate porous membrane (8-μm pores) precoated with gelatin (0.2 mg/ml in PBS). For chemotaxis (the directed migration of cells toward regions of higher concentration of chemotactic factors) experiments, the chemoattractants (DMEM FBS 1%, DMEM FBS 0%, HGF/SF at 6.2, 12.5, 25, 50, 100 ng/ml) were placed into the wells of the lower compartment of the chamber. Chemokinesis (stimulation of increased random cell motility) was distinguished from chemotaxis by placing the same concentration of chemoattractant in both the upper and lower wells of the Boyden’s chamber, thereby eliminating the chemical gradient.

The Boyden’s chamber was then kept for 3 h in the cell culture incubator. The cells were allowed to migrate through the membrane for 3 h. After incubation, the cells were fixed and stained (Diff-Quick stain kit, Dade Behring AG, Dudingen, Switzerland) and mounted onto glass slides. For quantitative analysis, the cells were observed using an oil immersion 100× objective on a light microscope. Six random objective fields of stained cells were counted for each well, and the mean number of migrating cells per square millimeter was calculated. The number of migrated cells, obtained from 10 independent wells for each group, was compared by ANOVA and Dunnett’s tests.

Results

c-Met expression in E12 primary olfactory system and in GT1-7 and GN11 cell lines

Immunohistochemistry indicated c-Met protein expression in the olfactory neuroepithelium as well as in cells migrating through the nasal mesenchyme of E12 embryos (Fig. 1). To establish whether c-Met-positive cells were LHRH-migrating neurons, single immunohistochemical stainings were performed on consecutive sagittal sections (Fig. 1).

Even though it was not possible to perform simultaneous double-labeling experiments, c-Met expression seems localized to the LHRH neuronal migratory pathway, and the neuroendocrine cells migrating through the nasal regions seem to express the c-Met-receptor.

To ascertain whether GT1-7 and GN11 cell lines express the c-Met receptor, RT-PCR analysis was undertaken. Because previous studies revealed that c-Met is expressed in the murine adult OB (30), OB extracts were used as positive control (for the negative control, sterilized water was used to replace the DNA sample).

RT-PCR reactions, carried out using specific c-Met oli-

![Fig. 1. LHRH and c-Met immunohistochemistry performed on consecutive sagittal sections of a 12-d mouse embryo (E12). LHRH-immunoreactive cells emerged from the developing olfactory epithelium and migrate through the olfactory mesenchyme (arrows) toward the forebrain. Met-immunoreactivity in the adjacent section resembles the staining pattern of LHRH. Note that the Met-antibody (a-Met) likely labels the same groups of migrating elements (arrows) stained with the LHRH antiserum (a-LHRH). OE, Olfactory epithelium; L, lumen. Scale bar, 250 μm.](https://academic.oup.com/endo/article-abstract/143/9/3306/2880324?image)
Immunoprecipitation and Western blot experiments were performed to identify the expression of the c-Met protein in both cell lines. c-Met was immunoprecipitated, using a rabbit polyclonal antibody, from cells either treated or not with HGF/SF (50 ng/ml for 15 min). The same antibody was also used to detect the protein (Fig. 2B). GN11 cells express HGF/SF receptor. As shown by other groups, both the precursor protein p170\textsuperscript{met} and the p145\textsuperscript{met} were detected (41).

A band of 145-kDa molecular mass was barely detectable in GT1-7 cells (Fig. 2B).

Following the immunoprecipitation protocol, an antiphosphotyrosine antibody was also used to detect the phosphorylation state of the receptor in both cell lines. c-Met was found to be constitutively tyrosine-phosphorylated in GN11 cells, even though at barely detectable levels (Fig. 2C). Subsequent incubation with HGF enhanced the phosphorylation in GN11 cells, whereas antiphosphotyrosine did not detect any specific band in GT1-7 cells (Fig. 2C).

As a further test of the plasma-membrane localization of c-Met in GN11 and GT1-7 cells, immunocytochemical experiments were performed by incubating nonpermeabilized cells with the c-Met antibody. A plasma-membrane-associated staining pattern was clearly evident in GN11 cells (Fig. 3A). According to RT-PCR and Western blot analyses, GT1-7 cells did show only a weak c-Met immunoreactivity, near to background levels (Fig. 3B).

### Collagen gel assay

Previous studies have shown that GN11, but not GT1-7, cells show significant migratory activity and actively respond to the chemotactic stimulus represented by FBS (37).

Considering that the expression of c-Met in GN11 cells might be linked to their ability to respond to HGF/SF, a series of migratory assays were performed. The collagen gel assay was chosen as the first good experimental tool, because it is a generally accepted procedure to study cell migration in a three-dimensional matrix. In these experiments, both the morphology of migrating cells and the distance which these cells cover under 24 h of HGF/SF stimulation were evaluated. Cellular aggregates of GT1-7 and GN11 cells were prepared by the hanging-drop technique (40) and incubated for 24 h in collagen gel prepared in SFM (DMEM 0% FBS) with or without 50 ng/ml HGF/SF. In addition, GN11 cell aggregates were also exposed to HGF/SF preincubated with a specific anti-HGF/SF (20 μg/ml).

After a 24-h incubation, no spontaneous motility of GN11 cells was observed in the absence of HGF/SF (Fig. 4A). However, GN11 cells, stimulated with HGF/SF, consistently migrated out of the aggregate and became arranged in chains in the collagen matrix (Fig. 4B). No migration of GN11 cells was observed in the presence of HGF/SF immunoneutralized with the specific antibody (Fig. 4C). Figure 3D shows, at higher magnification, that GN11 migrating cells displayed a generally accepted procedure to study cell migration in a three-dimensional matrix. In these experiments, both the morphology of migrating cells and the distance which these cells cover under 24 h of HGF/SF stimulation were evaluated. Cellular aggregates of GT1-7 and GN11 cells were prepared by the hanging-drop technique (40) and incubated for 24 h in collagen gel prepared in SFM (DMEM 0% FBS) with or without 50 ng/ml HGF/SF. In addition, GN11 cell aggregates were also exposed to HGF/SF preincubated with a specific anti-HGF/SF (20 μg/ml).

A quantitative analysis of the migratory activity of GN11 cells, under the three previously described experimental conditions, was also performed by measuring the mean dis-
FIG. 3. Immunolocalization of c-Met protein in nonpermeabilized GN11 (A) and GT1-7 (B) cells. GN11 cells exhibit a strong immunoreactivity for c-Met, whereas GT1-7 cells show only a weak labeling, near to background levels (see particular). Scale bar, 100 μm.

FIG. 4. GN11 cell aggregates, cultured for 24 h in collagen gel matrix, respectively, in SFM (DMEM 0% FBS, A), in the presence of 50 ng/ml HGF/SF containing medium (B and D) and in the presence of specific anti-HGF/SF antibody (+Ab) (C). Cell migration is prevented in A and C experimental conditions. D, Brightfield picture of GN11 cell aggregate (higher magnification of B), showing the chain of migrating cells that emerge from the aggregate under HGF/SF stimulation (cresyl violet staining). E, GT1-7 cell aggregate, cultured for 24 h in the presence of 50 ng/ml HGF/SF, showing the absence of migration (F, higher magnification of E). Scale bars: A, B, C, and E, 125 μm; D, 12.5 μm; F, 25 μm.
stances between the front of migration of the cells and the border of the aggregate (Fig. 5). When the cells were cultured in the presence of HGF/SF, GN11 cells migrated off the edge of the aggregates 5-fold more, with respect to the control conditions.

All of these results strongly suggest that the observed chemotropic response was specifically attributable to the presence of HGF/SF.

**Time-lapse video microscopy**

Time-lapse video microscopy was used to evaluate the motility response induced by HGF/SF after 24 h of serum deprivation, a procedure that slows down the spontaneous motility of GN11 cells (37). Cells were monitored for 8 h in an incubation chamber at constant temperature and CO₂ regulation. Time-lapse microscopy images of these cells were taken at 1-h intervals. Figure 6 illustrates the behavior of six GN11 cells in control conditions, in the absence of HGF/SF, during an 8-h recording. Time-lapse microscopy images show the lack of spontaneous motility of these cells that kept the same positions during all the time of the monitoring.

Figure 7 shows the behavior of three representative cells exposed to 50 ng/ml HGF/SF. Cells A and B displayed an elongated morphology and moved in opposite directions, sliding over each other. The cell indicated as C in Fig. 7 began to extend a leading process after 1 h of HGF-exposition. Over the next hours, this process continued to extend and broaden, directing toward the other cells. Contraction of the trailing process can be appreciated in the final two frames. These results indicate that HGF was sufficient to induce a motility response in the absence of FBS.

**Microchemotaxis assay**

Boyden’s chamber assay gives quantitative information regarding the cell population that migrates toward a concentration gradient of a chemotropic factor. Therefore, the cellular response of GN11 cells to HGF was tested through a series of microchemotaxis assays using Boyden’s chambers (see Materials and Methods). GN11 cells did not show a significant migratory activity in the absence of any chemotactic factor (Fig. 8), whereas the exposure to 1% FBS induced a significant chemomigration of these cells. The results also show that exposure to a gradient of HGF induced a significant strong chemotactic response of GN11 cells (Fig. 8). HGF exerted its effect in a concentration-dependent manner, with a maximal increase in responsiveness at 50 ng/ml (Fig. 8A). At this concentration, HGF was able to induce a chemotactic response comparable with that obtained by 1% FBS stimulus (Fig. 8A). At higher concentration (100 ng/ml), however, HGF produced no further increase in migration of GN11 cells (Fig. 8A).

GT1-7 cells did not respond to increasing concentrations of HGF (Fig. 8B); in addition, these cells did not present any chemotactic response, even in the presence of a high concentration of FBS (10% FBS; data not shown).

To better investigate whether the effect of HGF on the motility of GN11 cells was specifically directional (chemotaxis) or caused by the induction of random locomotor activity (chemokinesis), the cells were exposed to HGF present in the lower (chemotaxis) or in both (chemokinesis) compartments of the Boyden’s chamber. In these conditions, the induction of the cell motility was significantly less efficient in chemokinesis experiments than that induced by the same concentration of HGF/SF placed exclusively in the lower compartment of the chamber (chemotaxis; Fig. 8C).

**Discussion**

Although HGF/SF has been shown to be present in the brain in several areas (29, 30, 31, 42), the function of HGF/SF in the nervous system is largely unknown. It has been found to exhibit neurotrophic actions (21, 43, 44) as well as to function as an axonal chemoattractant (21, 45). Recently, HGF/SF has been also proposed as a mitogen in the trans-telencephalic migration of interneurons from the ganglionic eminence to the cerebral cortex (27) and as a novel mitogen for the olfactory ensheathing cells derived from adult rats (28). The data reported here are therefore the first evidence of a direct chemotactic effect of HGF/SF on LHRH-immortalized neurons.

GN11 and GT1-7 neurons displayed different migratory activity under HGF/SF stimulation. This effect is well correlated with the strong c-Met expression found in the GN11 cell line, demonstrated by RT-PCR, Western blot, and immunocytochemical analyses. Immunocytochemical results of c-Met localization in nonpermeabilized GN11 cells confirmed its membrane localization. On the contrary, the same set of experiments indicated that the HGF/SF receptor was absent or only at a barely detectable level in GT1-7 cells.

C-Met expression conferred to GN11 cells chemotactic responsiveness to HGF/SF. A collagen gel assay was used to assess the motility and the phenotype of these cells exposed
to exogenous HGF/SF. After 24 h of HGF/SF exposure, the migration of GN11 cells typically extended from the aggregates 5-fold more than in control conditions. The motility response was blocked by neutralizing antibody as well as in serum-free conditions (DMEM 0% FBS). On the other hand, GT1-7 cells did show a refractory response to the induced motility stimulus of HGF/SF; indeed, they did not leave the aggregates under different concentrations of the growth factor. Time-lapse video microscopy shows that GN11 cells, cultured for 24 h in serum-deprived medium and subsequently recorded for 8 h in the presence of HGF/SF, assumed a typical morphology of migrating neurons with an elongated cell body and the extension of leading processes. In addition, these cells often moved, sliding over each other. This also confirms the importance of cellular contacts during their movements and demonstrates, once more, that preincubation in serum-deprived medium does not influence the chemotactic response of GN11 cell line (37). Time-lapse video microscopy shows that GN11 cells failed to show any spontaneous motility and reinforces the hypothesis of a specific role of HGF/SF in the induction of a migratory response of this LHRH immortalized cell line.

In other model systems, cells expressing c-Met, indeed, seem to move toward a concentration gradient of HGF/SF (27, 46). Therefore, we have investigated the quantitative response of both GT1-7 and GN11 cell lines by microchemotaxis assays, performed using the Boyden’s chamber, under HGF/SF stimulation. We have observed that GN11, but not GT1-7 cells, significantly respond to this chemotactic stimulus in a dose-related fashion. Most important is the finding that HGF/SF was significantly efficient in stimulating chemotaxis, but not chemokinesis, as demonstrated by a strong reduction of cell migration after exposure of the cells to a uniform concentration of the chemoattractant. The magnitude of maximal stimulation was comparable with that obtained using 1% FBS that, to date, represented the strongest stimulus for these cells (37).

GN11 cell line was derived from a tumor developed in the OB (33); therefore, these cells are representative of LHRH neurons arrested during their transit to the brain, and they keep many features of migrating neurons (36, 37). On the
contrary, GT1-7 cells, derived from a hypothalamic tumor, exhibit both morphological and behavioral features of the hypothalamic postmigratory neurons that lost their ability to migrate (36, 37, 47). The unresponsiveness of GT1-7 cells to HGF/SF may be then correlated with a more differentiated phenotype, with respect to GN11, including the development of higher adhesive properties that would block their motility.

Based on the previous assumption that GN-derived cells are representative of immature migrating LHRH neurons, we propose HGF/SF as one of the possible chemical factors inducing the migration of LHRH neurons in vivo as well.

Although the several studies focused on LHRH neuronal migration have identified the preferential association between these neurons and the olfactory fibers in the nasal mesenchyme, the migratory guidance molecules involved in such a process remain mostly unknown. Schwarting et al. (10) have recently presented evidences that a versatile guidance molecule, such as netrin-1, and its receptor DCC, play a role in this migratory process. Loss of DCC function results, indeed, in the migration of many LHRH neurons to inappropriate destinations.

Unfortunately, mice lacking either HGF/SF or its receptor die during early embryogenesis, with defects in placenta,
liver, and muscle (46, 48–50); and this embryonic lethality makes it difficult to study the role of HGF/SF in the LHRH migratory process. Nevertheless, several lines of evidence support the hypothesis that HGF/SF could be involved in the control of the migration of LHRH neurons.

HGF/SF and c-Met mRNA transcripts were identified in the mouse developing OB, by Northern analysis (29, 30, 31). In addition, HGF/SF transcript has been evidenced in the forebrain as early as E11.5 (27), which is a stage corresponding to the beginning of the LHRH migratory process (1, 51). HGF/SF expression in the target tissue of this neuronal migration suggests that molecular signaling between forebrain and the olfactory placodes might influence olfactory pathway development. In the developing olfactory system, HGF/SF and c-Met distributions are distinct. As cited above, both molecules are localized in the developing and adult OB (29–31); and, more precisely, HGF/SF is seemingly expressed in the glomerular layer, whereas c-Met mRNA is distributed in the mitral cell layer (30). In addition, in the embryonic olfactory mucosa, c-Met mRNA is localized in the olfactory epithelium as well as in the olfactory nerve layer (30). Coincident with the appearance of c-Met in the embryonic olfactory epithelium, HGF/SF and its activator, tissue plasminogen activator (tPA), can be detected in the lamina propria underlying the epithelium and in the epithelium, respectively (30). Furthermore, tPA have been found to be expressed in migrating cells of the olfactory neuroepithelium during mice embryogenesis (52). Because LHRH neurons represent a subpopulation of these migrating cells (together with olfactory ensheathing and olfactory marker protein-positive cells), as shown by Tarozzo et al. (53), and tPA expression overlapped with the migratory pathway of these migrating cells, we cannot exclude that tPA is expressed by LHRH cells themselves. We demonstrate here, for the first time, the expression of both the c-Met mRNA and protein in the GN11 LHRH-secreting cell line but not in GT1-7 cells. Moreover, we do show that HGF/SF represents a chemotactic factor for GN11 neurons.

Lastly, an intriguing result was our observation, by immunohistochemical experiments, that, during embryonic development, c-Met expression correlates its temporal and spatial distribution with the LHRH migratory pathway.

Based on the preexisting literature and on the present results, it is possible to postulate that HGF/SF may play a main role as a chemotactic factor for elongating olfactory axons and for migrating neuroendocrine (LHRH) cells. The present study provides a new basis for additional studies aimed at clarifying the roles of HGF/SF in the LHRH migratory process in vivo.

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