Hepatocyte growth factor promotes migration of human hepatocellular carcinoma via phosphatidylinositol 3-kinase

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Abstract

Hepatocyte growth factor (HGF) is known to be a potent mitogen and motogen for epithelial cells. Hepatocellular carcinoma (HCC) often metastasizes, and the c-Met/HGF receptor is highly expressed by HCC cells. The aim of this study was to investigate the signaling pathways associated with the motogenic effect of HGF on HCC cells via c-Met. HCC cell lines (Hep3B, HepG2, PLC, and Huh-7) and HCC cells harvested from patients were used for the Boyden chamber assay of chemotactic activity as well as for immunoprecipitation and immunoblotting studies. HGF stimulated the motility of Hep3B, HepG2, and Huh-7 cells in a dose-dependent manner in association with tyrosine phosphorylation of c-Met and activation of phosphatidylinositol 3-kinase (PI3-K). A tyrosine kinase inhibitor (genistein) and a PI3-K inhibitor (wortmannin) prevented the migration of HCC cells. However, migration was not prevented by calphostin C, an inhibitor of protein kinase C (PKC), which is a downstream target of phospholipase C \( \gamma \) (PLC \( \gamma \)). HGF also stimulated the migration of HCC cells obtained from three patients, while wortmannin prevented the migration of these cells. These results indicate that HGF stimulates the migration of HCC cells through the tyrosine phosphorylation of c-Met via activation of PI3-K.

Abbreviations: HGF – hepatocyte growth factor; HCC – hepatocellular carcinoma; PI3-K – phosphatidylinositol 3-kinase; PKC – protein kinase C; PLC \( \gamma \) – phospholipase C \( \gamma \); FAK – focal adhesion kinase

Introduction

Hepatocyte growth factor (HGF) is a multifunctional cytokine with mitogenic, motogenic, and/or morphogenic effects on a wide variety of normal and neoplastic cells [1]. It has been shown that HGF increases the growth and repair of the liver in vivo using transgenic mice [2]. HGF is a motogen and motility factor for many epithelial cells [3, 4], and it stimulates tubulogenesis by tubular epithelial cells [5] as well as stimulating the invasive characteristics of various cancer cells [6, 7]. The HGF receptor is a tyrosine kinase receptor encoded by the c-met proto-oncogene [8]. This proto-oncogene product (c-Met) is expressed by both normal and malignant epithelial cells as well as several other cell types [9]. c-Met is a heterodimer of two disulfide-linked chains, 50-kDa \( \alpha \) and 145-kDa \( \beta \) subunits that are generated by cleavage of a single 190-kDa precursor [10]. Binding of HGF induces activation of the kinase and auto/transphosphorylation of specific tyrosine residues in the receptor \( \beta \) subunit [7, 8].

Phosphorylation of the tyrosine residues of a receptor generally provides binding sites for molecules containing SH2 groups that act to transduce extracellular signals [11]. Several intracellular proteins are phosphorylated after association with activated c-Met. These include phospholipase C \( \gamma \) (PLC \( \gamma \)), phosphatidylinositol 3-kinase (PI3-K), Grb2, Ras, and focal adhesion kinase (FAK) [12,13]. Two tyrosine residues (1234 and 1235) within the tyrosine kinase domain of the receptor are essential for its catalytic activity [14]. Also, phosphorylation of tyrosine 1356 within the carboxyl terminus of the \( \beta \) subunit is essential for the recruitment of various substrates, including PI3-K, PLC\( \gamma \), and Grb2 [12–15].

Although the signal transduction pathways of these molecules have been studied, whether HGF has a motogenic effect on human HCC cells or whether its signal transduction pathway is initiated after phosphorylation of c-Met has not been clarified. Intrahepatic metastasis is one of the characteristics of HCC [16]. Several authors have reported on c-Met expression in human HCC, including a correlation of c-Met with tumor differentiation [17] or with the propensity to metastases [18]. These reports suggest that c-met expression is common in HCC and that HGF may play an active role in the development and metastasis of this cancer through...
its receptor, c-Met. In the present study, we investigated the motogenic effect of HGF using four established human HCC cell lines and tumor cells obtained from three patients with HCC who underwent surgical resection. We found that HGF increased the motility of human HCC cells and that PI3-K, but not protein kinase C (PKC), was essential for HCC cell migration.

Materials and methods

Reagents and cell culture

Recombinant human hepatocyte growth factor (HGF) was purchased from Toyobo (Tokyo, Japan). The antibodies used included anti-c-Met (c-met, c-12), and an antiphosphotyrosine monoclonal antibody (PY-20) from Santa Cruz Biotechnology Inc. (Santa Cruz, California). Anti-PI3-K, anti-PLCγ, and anti-FAK antibodies were purchased from Transduction Lab. (Lexington, Kentucky). The following protein inhibitors were used: genistein (TopoGEN Inc. Columbus, Ohio), wortmannin (Biomol. Plymouth, Pennsylvania), and calphostin C (Kyowa Medex Tokyo, Japan).

Four human HCC cell lines, Hep3B, HepG2, PLC (ATCC, Rockville, Maryland), and Huh-7, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and were maintained at 37 °C in a humidified atmosphere of 5% CO2. Cells were washed three times with phosphate-buffered saline (PBS) and incubated in DMEM without serum for 24 h. Then the cells were pretreated with indicated concentrations of genistein, calphostin C, or wortmannin for 3 h in serum-free medium.

Migration assay and inhibition experiments

Cell migration was assayed in a modified Boyden chamber system [6]. We used a two-chamber apparatus containing a polycarbonate filter with 8 μm pores (Coster Co., Cambridge, Massachusetts). The filter was precoated with 0.5 mg/cm2 of Matrigel (Becton Dickinson Labware, Bedford, Massachusetts). Human HCC cells were incubated without serum for 24 h and detached from culture dishes with 2 mM EDTA. The cells were suspended in DMEM supplemented with 0.1% (w/v) bovine serum albumin at a final concentration of 1 × 106 cells ml−1. The cells were placed in the upper compartment of each well and the lower compartment was immediately filled with serum-free DMEM in the presence or absence of 20 ng ml−1 recombinant HGF for 36 h, after which cells were fixed and stained with 5% Giemsa.

Wound assay

Hep3B and PLC cells were grown to confluence in six-well tissue culture plates, after which the cells on half of each plate were scraped off with a sterile razor blade and washed three times with PBS [22]. The cells were incubated with serum-free DMEM in the presence or absence of 20 ng ml−1 recombinant HGF for 36 h, after which cells were fixed and stained with 5% Giemsa.

Immunoprecipitation and immunoblotting

Subconfluent monolayers of HCC cells were washed with PBS and lysed in 1 ml of solubilizing buffer (10 mM Tris-HCL, pH 7.8/1% NP 40/0.15 M NaCl/1 mM EDTA/10 μg ml−1 aprotinin/0.5 mM sodium orthovanadate/5 μg leupeptin/100 μg ml−1 PMSF). The lysate was centrifuged at 10,000 × g for 30 min at 4 °C. Equal amounts of protein (15 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Laemmli’s buffer. Immunoblot analysis was carried out using an Amersham ECL immunoblot detection system (Little Chalfont, Buckinghamshire, UK) [18].

For immunoprecipitation, subconfluent monolayers of HCC cells were incubated without serum for 24 h. The cells were pretreated with 50 μM genistein, 150 nM calphostin C, or 30 nM wortmannin for 3 h, and then were stimulated with 20 ng ml−1 of HGF for 7 min. Next, the cells were washed twice with ice-cold PBS containing 0.1 mM sodium orthovanadate, lysed in 1.4 ml of solubilizing buffer (described above), and let stand for 1 h at 4 °C. The lysates were precleared by incubation with protein A agarose (Sigma, St. Louis, Missouri) for 1 h at 4 °C, and centrifuged for 5 min at 2000 × g. The supernatants were incubated with antibodies (anti-c-Met, anti-PI3-K, anti-PLCγ, or anti-FAK) overnight at 4 °C, and then incubated with protein A agarose beads to precipitate antigen-antibody complexes. The beads were washed three times with 50 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X and 2 mM sodium orthovanadate, resuspended in sample buffer, and boiled at 100 °C for 3 min. After SDS-PAGE, protein was transferred to polyvinylidene difluoride membranes (PVDF-plus, Micron Separations Inc., Westbourough, Massachusetts). The membranes were subsequently probed with anti-phosphotyrosine antibody (PY-20) and immunoreactive bands were visualized by an Amersham ECL immunoblot detection system.