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The hepatocyte growth factor isoform NK2 activates motogenesis and survival but not proliferation due to lack of Akt activation

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ABSTRACT

Hepatocyte growth factor (HGF) is a pleiotrophic factor involved in cellular proliferation, migration and morphogenesis. HGF is required for normal tissue and organ development during embryogenesis, but in the adult HGF has been demonstrated to drive normal tissue repair and inhibit fibrotic remodeling. HGF has two naturally occurring human isoforms as a result of alternative splicing, NK1 and NK2. While NK1 has been defined as an agonist for HGF receptor, Met, NK2 is defined as a partial Met antagonist. Furthermore, under conditions of fibrotic remodeling, NK2 is still expressed while full length HGF is suppressed. Furthermore, the mechanism by which NK2 partially signals through Met is not completely understood. Here, we investigated the mitogenic, motogenic, and anti-apoptotic activities of NK2 compared with full length HGF in primary human bronchial epithelial cells (BEpC) and bovine pulmonary artery endothelial cells (PAEC). In human BEpC, NK2 partial activated Met, inducing Met phosphorylation at Y1234/1235 in the tyrosine-kinase domain but not at Y1349 site in the multifunctional docking domain. Partial phosphorylation of Met by NK2 resulted in activation of MAPK and STAT3, but not AKT. This correlated with motogenesis and survival in a MAPK-dependent manner, but not cell proliferation. Overexpression of a constitutively active AKT complemented NK2 signaling, allowing NK2 to induce cell proliferation. These data indicate that NK2 and HGF drive motogenic and anti-apoptotic signaling but only HGF drives cell proliferation by activating AKT-pathway signaling. These results have implications for the biological consequences of differential regulation of the two isoforms under pro-fibrotic conditions.

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1. Introduction

Hepatocyte growth factor (HGF) is a mitogenic, motogenic, morphogenic, and anti-apoptotic factor that is primarily synthesized and secreted by cells from mesenchymal origin and acts on cells expressing its receptor, Met [1]. HGF is required for normal organ and tissue development during embryogenesis; in the adult, HGF plays a key role in tissue homeostasis and repair in many organs including lung, heart, kidney, liver, skin, and brain [2–9]. Administration of HGF protein or the use of HGF gene therapy promotes normal tissue repair animal models for injury in the lung, liver, kidney, heart, and brain and prevents fibrotic remodeling [10–16].

Full-length HGF is a multidomain protein, synthesized and secreted primarily by cells of mesenchymal origins as a biologically inert 90 kDa precursor and converted extracellularly into the active form through a single cleavage by a tightly regulated serine protease [17,18]. The active form of HGF is a heterodimer consisting of disulfide-linked α- and β-chains (55–60 and 32–34 kDa, respectively). The α-chain possesses the ability to bind to and activate the receptor and is composed of an amino-terminal hairpin loop (N) domain and four kringle domains (K1–K4). The β-chain has strong homology to the protease domain of serine proteases, although lacking any protease activity, and contains a secondary Met binding site [19,20].

Upon binding to the receptor, HGF induces activation of Met by autophosphorylation of a several critical tyrosine residues: juxtamembrane Y1003 that is responsible for Met endocytic processing and degradation [21,22]; Y1234 and Y1235 in the kinase domain that are required for kinase activity of the receptor [23]; and Y1349 and Y1356 in the multisubstrate docking domain that are responsible for recruitment of multiple adaptor proteins upon phosphorylation [24]. Depending on the proteins recruited to the receptor in a specific cell type, Met activates a variety of signaling cascades, including Ras/p42/p44 mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, signal transducer and activator of transcription 3 (STAT3), src homology phosphatase 2 (SHP2), phospholipase C-gamma (PLC-γ), src kinase, protein kinase C (PKC) isoforms, and/or small GTP-binding proteins Rap1, Rac, and Rho [25–36]. The identification of individual signaling pathways required for the specific biological activities of HGF is complicated and appears to have substantial overlap [32,33,37–40].

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There are two other natural isoforms of HGF, known as NK1 and NK2, which are truncated peptides resulting from alternative splicing [41]. NK1 contains the *N*-terminal hairpin and the first kringle domain; the NK2 variant extends through the second kringle (K2) domain. Compared to HGF, relatively little is known about how the two other isoforms elicit biological effects. NK1 was demonstrated to induce motility, survival, and proliferation, although its potency is lower than that of HGF [42]. In contrast, NK2 has motogenic activity but antagonizes the mitogenic activity of HGF [43,44], and probably thereby inhibits normal tissue repair in vivo and leading to increased fibrosis [45].

We previously demonstrated that the pro-fibrotic factor transforming growth factor-beta1 (TGF-β1) inhibits the expression of full length HGF, but not that of the NK2 splice variant. The differential regulation occurs through the regulation of miR-199 binding in the 3′ UTR of full length HGF, which differs from the 3′UTR of NK2 [46]. Thus, expression of the NK2 isoform predominates under pro-fibrotic conditions, when TGF-β1 expression is high. The physiological significance of the differential regulation of HGF and the NK2 isoform are not known.

Here, we compared the biological responses of HGF and NK2 in primary lung cell types, to determine whether this preferential expression might alter the biological outcome of NK2 activation of Met. We now demonstrate that although NK2 can prevent apoptosis of endothelial and epithelial cells and promote the migration of these cells, NK2 is not able to stimulate cell proliferation which is essential for normal tissue repair. We further demonstrate that NK2 activates downstream signaling from Met in a manner which fails to activate AKT, a signaling pathway that is pivotal for the proliferative response.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS; 100–106) was purchased from Gemini Bio-Products (Woodland, CA). RPMI-1640 medium, fungizone, penicillin/streptomycin, and Dulbecco's PBS were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO), U0126 MEK1/2 inhibitor from Cell Signaling Technology (Danvers, MA), Angiotensin II from Bachem, Inc. (Torrance, CA), and recombinant human HGF from ProSpec-Tany Technogene Ltd. (East Brunswick, NJ). NK2 protein was produced as previously described [47]. The CA-AKT construct was previously described [48].

2.2. Cell culture

Human BEpC were purchased from Cell Applications, Inc. and cultured in the prepared B/TEpC growth medium (#511–500; Cell Applications, Inc., San Diego, CA). A starving medium was prepared from this medium by mixing it (1:100 v/v) with the basal medium (#510–500; Cell Applications, Inc.). Bovine PAEC were purchased from ATCC (Manassas, VA). Passage 2–8 cells were used for all experiments. PAEC were cultured in RPMI 1640 with 10% FBS (0.1% for low serum media), 1% penicillin/streptomycin and 0.5% fungizone. Cells were grown in 5% CO_2 at 37 °C in a humidified atmosphere cell culture incubator. At least two isolates of each primary cell type were used to confirm the data.

2.3. Cell proliferation assay

Cells were seeded on 30 mm dishes at 50% confluence for BEpC and 30% confluence for PAEC. The following day, medium was replaced with low serum medium. After 24 h, cells were treated with HGF (25 ng/ml) or NK2 (100 ng/ml) for 2–3 days, then trypsinized and counted using an TC20 ™ Automated Cell Counter (BioRad Laboratories Inc., Hercules, CA). Control cells were treated with the same amount of HGF dilution buffer (1 × PBS containing 0.1% BSA).

2.4. Neutral comet assay

The neutral comet assay was used to measure double stranded DNA breaks as an indication of apoptosis. Confluent cells were treated with Ang II (10 μM) for 24 h. Cells were embedded in 1% low-melting point agarose and placed on a comet slide according to the manufacturer's protocol (Trevigen, Gaithersburg, MD). Slides were then placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Na-lauryl sarcosinate, 1% Triton X-100, and pH 9.9) for 20 min, washed by immersion in 1 × TBE buffer (0.089 M Tris, 0.089 M Boric acid, and 0.002 M EDTA pH 8.0). The nuclei were subsequently subjected to electrophoresis for 10 min at 6 mAmp in a horizontal mini-electrophoresis apparatus (BioRad Laboratories Inc.) with 1 × TBE buffer. Then cells were fixed with 75% ethanol for 10 min and air-dried overnight. Cells were stained with 1 × Sybr® Green (Molecular Probes, Eugene, OR) and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA) using 10× magnification at 478 nm excitation and 507 nm emission wavelengths. Approximately 100 cells were randomly selected and microscopically scored according to tail length. Comets were defined as apoptotic cells as described by Krown et al., 1996 [49].

2.5. Cell migration assay

Cells were trypsinized, washed, and plated at a density of 3×10^5 cells/well in 12 well/plate Transwell (Corning, Corning, NY) dishes (12-μm pore size). Cells were allowed to attach for 3 h, then treated in both upper and lower chambers with HGF (25 ng/ml) or NK2 (100 ng/ml) in triplicate wells. After 16 h, cells on the upper side of the membrane was cleaned using a cotton swab and cells that were migrated through the Transwell insert were fixed using 100% methanol for 15 min, stained with crystal violet (Fisher, Fairlawn, NJ), 0.94% in 20% methanol. After staining for 1 h, cells were counted using microscopy.

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using an RNeasy Kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RNase-Free DNAse Set (Qiagen, Valencia, CA). RNA was quantified spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE) and integrity was assessed by capillary electrophoresis (Experion, Bio-Rad, Hercules, CA). RNA (1.0 μg) was subjected to reverse transcription with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). RT-PCR was performed in triplicates with 1 μl cDNA using 4 μM each forward and reverse primer, 200 μM each dNTP and 1 U i*Taq* DNA polymerase according to the manufacture's protocol (BioRad Laboratories Inc.). The PCR primers for Bcl-x_L mRNA were: 5′-GGTATTGGTGAGTCGGATCG and 5′-GCTGCATTGTTCCCGTAGAG. Reactions were carried out for 25 cycles using the following conditions: 95 °C 1 min; 60 °C 45 s; and 72 °C 1.5 min. The last cycle extension was for 10 min at 65 °C. PCR primers for GAPDH were: 5′- GAAGCTCGTCATCAATGGAAA-3′ and 5′-CCACTTGATGTTGGCAGGAT-3′. PCR reactions were analyzed by gel electrophoresis using 1.5% agarose, PCR products were visualized using ethidium bromide (0.1 μg/ml).

2.7. Determination of mRNA half-life

PAEC were treated with HGF (25 ng/ml) or NK2 (100 ng/ml) for 30 min prior to Ang II (10 μM) exposure. After 8 h, actinomycin D (5 μg/ml) was added to the medium to block the transcription. Cells were harvested at the indicated times and total RNA was isolated and the level of Bcl-x_L mRNA was determined by semi-quantitative RT-PCR. Determination of internal GAPDH mRNA level was used as control.