Hepatocyte growth factor promotes migration of human myeloma cells

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ABSTRACT

Multiple myeloma is characterized by the accumulation and dissemination of malignant plasma cells in the bone marrow. Cell migration is thought to be important for these events. We studied migration in a Transwell two-chamber assay and tested the motogenic effect of various cytokines. In addition to insulin-like growth factor-1 and stromal cell-derived growth factor-1α, previously known as chemoattractants for myeloma cells, we identified hepatocyte growth factor as a potent attractant for myeloma cells. Hepatocyte growth factor-mediated migration was dependent on phosphatidylinositol-3-kinase, involved the MAPK/Erk signaling cascade and VLA-4 integrins, but did not involve Akt, mTOR or G proteins.

Key words: myeloma, cell migration, hepatocyte growth factor, stromal cell-derived growth factor-1.

Citation: Holt RU, Fagerli U-M, Baykov V, Rø TB, Hov H, Waage A, Sundan A, and Børset M. Hepatocyte growth factor promotes migration of human myeloma cells. Haematologica 2008 Apr; 94(4):619-622. doi: 10.3324/haematol.11867

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Introduction

Multiple myeloma (MM) is an incurable malignant clonal plasma cell disorder.[1] It is believed that MM precursor cells migrate from blood vessels into the BM where they adhere to stromal cells and matrix proteins, receiving signals supporting the long life of MM cells. As the disease progresses, the MM cells disseminate by migration through endothelial barriers.[1]

Several cytokines that are involved in the pathogenesis of MM disease, such as IGF-1, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and SDF-1α, promote MM cell migration.[2-6]

The 68 kDa glycoprotein HGF, produced by stromal cells in the BM, is a pleiotropic cytokine with mitogenic, motogenic and morphogenic properties.[7] The HGF receptor c-Met is a tyrosine kinase type receptor. In hematologic cancer, HGF/c-Met seem to be of particular importance for MM.[8,9] The concentration of HGF is significantly higher in serum[10] and in the bone marrow plasma of myeloma patients than in control samples from healthy subjects. Besides, HGF is a negative prognostic factor for MM patients.[11,12] We showed that HGF promoted adhesion of MM cells to the matrix protein fibronectin, which increased cell proliferation.[13] HGF is also reported to facilitate invasiveness of MM cells,[14] and to inhibit bone formation in multiple myeloma.[15]

We studied chemotaxis of MM cells, and found myeloma cells to be attracted to HGF in concentrations known to be present in the BM of myeloma patients.

Design and Methods

Cytokines, antibodies and other reagents

HGF was purified in our own laboratory.[16] NK1 and tumor necrosis factor (TNF) were from Genentech Inc. (San Francisco, CA, USA). Recombinant human FGF2, IGF-1, interleukin (IL)-15 and VEGF were from R&D Systems Inc. (Minneapolis, MN, USA), IL-6 was from Biosource (Camarillo, CA, USA) and SDF-1α from Peprotech (London, UK). The neutralizing antibody against the VLA-4 integrin subunit α4 and irrelevant IgGs came from Becton and Dickinson (San José, CA, USA). Antibodies against human HGF and c-Met were from R&D Systems Inc. (Minneapolis, MN, USA). The

Acknowledgments: we thank Berit Størdal, Hanne Hella and Toril Holien for their excellent technical help.
Funding: this work is supported by grants from The Norwegian Cancer Society, The Cancer Fund of St.Olavs Hospital, Familien Blix' fond, Rakel og Otto Kr. Bruuns legat and The Research Council of Norway.
Manuscript received June 21, 2007. Revised version arrived on October 30, 2007. Manuscript accepted December 21, 2007.
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The online version of this article contains a supplemental appendix.

c-Met inhibitor PHA-665752 was a gift from Dr. James Christensen, Pfizer (San Diego, CA, USA). Pertussis toxin, rapamycin and wortmannin were from Sigma-Aldrich (St. Louis, MO, USA). LY294002, PD98059 and U0126 were from LC Laboratories (San Diego, CA, USA). The JAK2/STAT3 inhibitor AG 490 and the Akt inhibitor SH-5 were from Calbiochem (CNbiosciences, Inc., La Jolla, CA, USA). Compounds were diluted to final concentrations in RPMI-1640 (Gibco, Paisley, UK) supplemented with l-glutamine (2 mM) and gentamicin (40 μg/mL).

Cell culture conditions

We used the IL-6-dependent human myeloma cell lines INA-6 and ANBL-6, and myeloma cells from 11 randomly selected patients admitted to the hematology department of St. Olavs Hospital, Trondheim. Cells were harvested and cell lines were grown as previously described.[13] The study was approved by the regional ethics commitee and patients gave their informed consent.

Migration assay

INA-6 and ANBL-6 cells were washed in Hanks' balanced salt solution (HBSS), and resuspended in RPMI-1640 with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) and IL-6 (0.1 ng/mL) (referred to as RPMI-medium). Cells were seeded ($2x10^5$ cells in 100 μL RPMI-medium) in the upper compartments of polycarbonate Transwell two-chamber migration plates (pore size: 5 μm) (Costar, Corning, NY, USA). The volume of RPMI-medium in the lower compartments was 600 μl. All experiments were performed in duplicates and repeated at least three times. After 22 h, at 37°C and 5% CO_2, the number of cells that had migrated through the membrane to the lower chamber was determined by a Coulter Counter Z1 (Beckman Coulter, Fullerton, CA, USA)

Statistics

Statistical significance was determined using a two-tailed, unpaired Student's t-test. The minimal level of significance was p=0.05.

Results and Discussion

HGF promoted myeloma cell migration

The dissemination of malignant plasma cells throughout the bone marrow is one of the hallmarks of multiple myeloma. In our study selected cytokines known to induce biological responses in MM cells were assessed for their influence on MM cell migration. Compared with non-stimulated cell locomotion, migration was increased 5-fold by HGF or SDF-1α, and 2 to 3-fold by IGF-1. FGF2, IL-15, TNF or VEGF gave no significant increase in INA-6 cell migration above control level (Figure 1A).

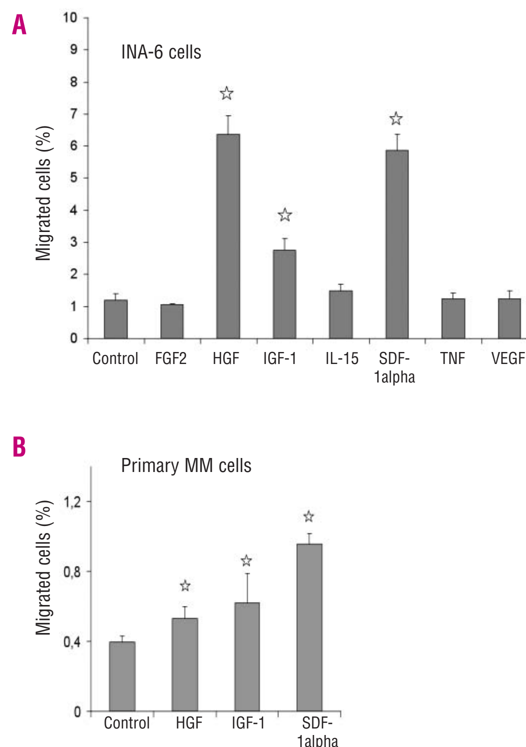

Figure 1. INA-6 cells (A) or primary MM cells (B) were seeded in the upper compartment of a two-chamber Transwell migration assay. To the bottom compartment was added RPMI-medium with or without FGF2 (10 ng/mL), HGF (150 ng/mL), IGF-1 (100 ng/mL), IL-15 (20 ng/mL), SDF-1α (75 ng/mL), TNF (20 ng/mL) or VEGF (100 ng/mL). After 22 hrs. at 37°C, cells in the bottom compartments were counted and percentage of migrated cells calculated. Error bars represent +1 standard deviation (SD) of three repeated counts in two independent measurements. A is representative for three similar experiments. B represents 1 out of 5 primary cell samples. Migration increased significantly when cells were subjected to specific cytokines, according to an unpaired Student's two-tailed test, *(p<0.01).

HGF and SDF-1α also induced migration of ANBL-6 cells significantly above the control level (*Online Supplementary Figure S1A*). Furthermore, HGF significantly promoted cell migration in 5 out of 11 patient MM cell samples tested (represented by Figure 1B), showing the clinical relevance of HGF-mediated migration. Cells migrated in response to HGF in a dose-dependent manner (*Online Supplementary Figure S1B*). The 50% effective dose (ED_{50}) was calculated to 38 ng/mL, which is equal to or lower than the concentration of HGF found in the BM plasma of 30% of MM patients.[12] Migration reached its maximum at 100-150 ng/mL and decreased at concentrations above 200 ng/mL. In further experiments, 150 ng/mL of HGF was used. Migration increased only as long as a positive HGF gradient was maintained (data not shown). We confirmed SDF-1α to be a strong promoter of myeloma cell migration. However, in cell lines, we found HGF to be an equally strong chemoattractant for MM cells. Cell migration was an active process enabling cells to migrate through pores (5 μm) half the size of a cell diameter (11 μm).

HGF stimulated cell migration through c-Met

HGF stimulated migration through its high-affinity receptor c-Met, since migration decreased almost to control level when cells were given antibodies against HGF or against c-Met, or when given the c-Met inhibitor PHA665752 at concentrations known to fully inhibit c-Met phosphorylation. NK1, a natural splice variant of HGF containing the HGF receptor-binding sites,[7] was previously found to have varying effects on cell behaviour.[17] In our study, NK1 did not promote cell migration. On the contrary, it partly (42%) inhibited HGF-induced migration (Figure 2A).

HGF-mediated cell migration was dependent on the intracellular signaling molecules PI-3K and involved the MAPK/Erk signaling pathway and the VLA-4 integrin

In order to delineate signaling pathways involved in HGF-stimulated migration, we subjected INA-6 cells to selected inhibitors of intracellular signaling molecules and to antibodies against the VLA-4 integrin. Lipid products of PI3K are known to activate the serin/threonine kinase Akt,[18] and mTOR is a signaling mediator downstream of Akt. We have previously shown that HGF activates Akt, and have thereby indirectly shown PI3K activation in INA-6 cells.[13] The PI3K inhibitors wortmannin and LY2940002 blocked HGF-mediated migration completely (Figure 2B) showing that HGF-mediated migration was fully dependent on PI3K. HGF has previously been shown to activate the MAPK/Erk pathway in INA-6 cells.[13] PD98059 and U0126, in concentrations that fully inhibit phosphorylation of p44/p42 MAPK in INA-6 cells,[13] decreased HGF-mediated migration by 42% and 47%, respectively (Figure 2C). This suggests that cytokine-induced migration, in contrast to HGF-induced cell adhesion,[13] does involve the MAPK/Erk signaling pathway in MM cells. Here MM cells could be similar to carcinoma cells, in which MAP kinases are known to increase the activation of actin/myosin motors by enhancing the activity of myosin light chain kinases and thereby promoting motility.[19]

We have previously found VLA-4 to be an abundant integrin on MM cells and shown that HGF increases VLA-4-dependent adhesion of MM cells to fibronectin.[13] Use of neutralizing antibodies against the integrin subunit α4 decreased migration by 47% (Figure 2B). These data indicate that adhesion was an important part of migration and that cell migration, not surprisingly, is a complex event in which additional adhesion molecules, besides VLA-4, may participate.

We found that the Akt inhibitor SH-5, the mTOR inhibitor rapamycin, as well as the Jak2/STAT3 inhibitor AG490 did not influence HGF-mediated migration (Figure 2B and C). The efficacy of SH-5, AG490 and rapamycin was proven in a methyl-3H-thymidine proliferation assay (*Online Supplementary text*) where all inhibitors, at concentrations used in our experiments, decreased INA-6 cell proliferation (*Online Supplementary Figure S2*). The efficacy of PD98059 and U0126 has previously been tested elsewhere.[13]

All inhibitors used in our experiments were tested for their influence on cell viability after 22 h using an Apoptest-FITC kit (*Online Supplementary text*). No significant difference in viability was seen in treated compared with non-treated cells at concentrations of

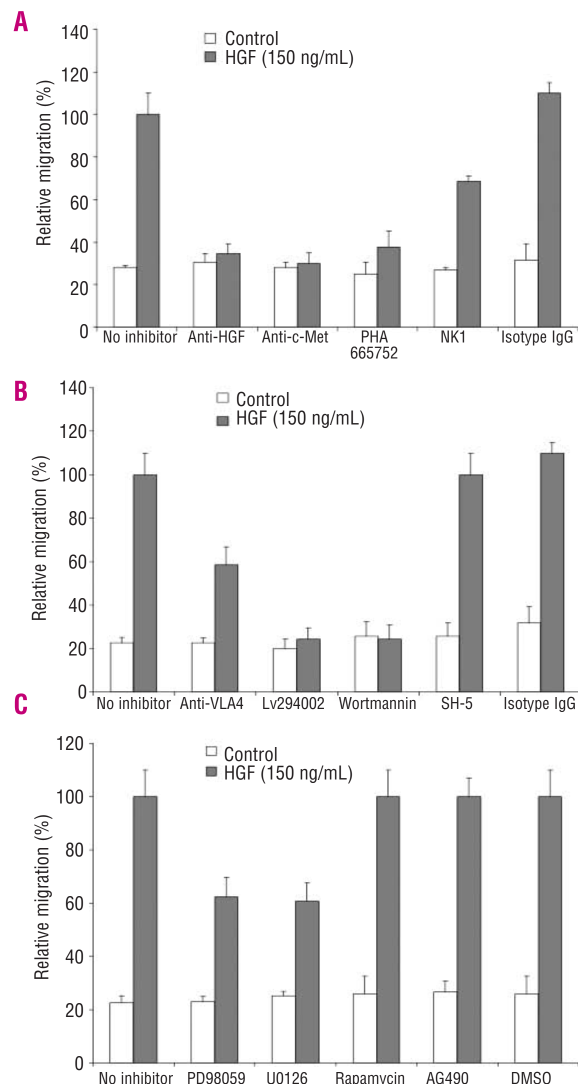

Figure 2. (A) Neutralizing antibodies against HGF (10 μg/mL) or c-Met (2 μg/mL) inhibited INA-6 cell migration to control levels. Isotype control antibody (10 μg/mL) was used. The c-Met inhibitor PHA 665752 (50 nM) and the isotype variant of HGF, NK1 (100 ng/mL), reduced HGF-mediated cell migration. (B) Neutralizing antibody against the VLA4-integrin α4 sub-unit (0.1 μg/mL) reduced HGF-mediated cell migration by 40%. The PI3K inhibitors Ly2940002 (3 μM) and wortmannin (50 nM) reduced migration to control levels. The Akt inhibitor SH-5 (10 μM) did not influence migration. (C) The inhibitors of MAPK, PD98059 (5 μM) and U0126 (5 μM) reduced HGF-mediated migration more than 40%, while HGF-mediated migration was not influenced by rapamycin (5 ng/mL) an inhibitor of mTOR, AG490 (5 μM) an inhibitor of JAK/STAT, or an isotype control IgG (0.1 μg/mL). The error bars represent ± 1 SD of three repeated counts of two independent measurements. The figures show one representative out of three similar experiments.

inhibitors that were effective in the migration assay (*data not shown*).

In some carcinoma cell lines, c-Met can be transactivated by ligand binding to G-protein-coupled receptors (GPCR).[20] Pertussis toxin, an inhibitor of GPCR, did not influence HGF-stimulated INA-6 cell migration, while it decreased SDF-1α-stimulated migration (*Online Supplementary Figure S3*). This indicates that HGF-mediated migration does not signal through GPCRs in our cells.

Taken together, our study shows that HGF-mediated migration was dependent on PI3K, it involved the MAPK/Erk-pathway and the integrin VLA-4, but HGF-mediated migration did not involve JAK/STAT, Akt or its downstream effector mTOR.

In conclusion, HGF, which is a cytokine known to play a role in MM biology, is here shown to be a promoter of MM cell migration with potency similar to that of SDF-1α. HGF may contribute to the dissemination of myeloma cells within the patient's bone marrow and as such could be an attractive target for therapy.

Authorship and Disclosures

RUH: designed research, performed experiments and wrote the paper. UMF: performed experiments and contributed to the writing of the paper. VB, TBR and HH: performed experiments. AW: collected patient samples. AS: advised throughout the work and discussions. MB: contributed in design of the research and wrote the final version of the manuscript

The authors reported no potential conflicts of interest.

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