Hepatocyte Growth Factor and Its Receptor c-Met in Multiple Myeloma

By Magne Børset, Henrik Hjorth-Hansen, Carina Seidel, Anders Sundan, and Anders Waage

We have examined whether the hepatocyte growth factor (HGF)/c-met receptor-ligand pair is expressed in freshly isolated and highly purified myeloma cells and whether HGF can be found in the sera of myeloma patients. Myeloma cells were purified with an immunomagnetic method using the syndecan 1-specific antibody B-B4. HGF and c-met mRNA in these cells were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). HGF and c-met proteins were detected by enzyme-linked immunosorbent assay (ELISA) and Western blot, respectively. Serum from 13 myeloma patients was obtained at diagnosis and the levels of HGF were determined by ELISA. HGF and c-met mRNA were expressed in all examined samples (n = 7). HGF was detected in the supernatants of 17 of 20 primary cultures of myeloma cells, whereas bone marrow mononuclear cells from normal controls did not produce detectable amounts of HGF (n = 3). The mean HGF level in serum of myeloma patients at diagnosis was more than fourfold higher than the mean level in normal controls. Possible implications of HGF/c-met expression for the pathophysiology of multiple myeloma are discussed.

© 1996 by The American Society of Hematology.

MULTIPLE MYELOMA (MM) is an uncontrolled growth of cells with the phenotype of terminally differentiated B cells (plasma cells). The disease usually occurs in the bone marrow (BM) and is associated with skeletal pathology, which in most patients appears as multiple lytic bone lesions and general osteoporosis. The mechanisms leading to this bone pathology are still not fully understood. Cytokines with bone-resorbing properties such as interleukin-1 (IL-1) and IL-6 are known to be produced by BM cells in MM, but recent research suggests that these cytokines are secreted largely by stroma cells rather than by the myeloma cells.

Hepatocyte growth factor (HGF) is a cytokine (for a review, see Zarnegar and Michalopoulos) that recently was shown to promote formation of osteoclasts from hematopoietic precursor cells, to attract osteoclasts to sites of bone resorption, and, in coculture with osteoblasts, to increase the level of resorption. The HGF receptor c-met is expressed by both the bone-forming osteoblasts and the bone-resorbing osteoclasts, and HGF stimulated the growth of both these cell types. It is therefore of interest to study whether HGF is produced by myeloma cells and hence could be responsible for the osteoclast activation in MM.

HGF was originally purified by its ability to cause growth stimulation of hepatocytes but is now known as a pleiotropic cytokine that acts on epithelial cells in several organs, including endothelial cells, β cells in pancreas, epithelium in kidney and lung. Among HGF-producing cells are mesenchymal cells such as fibroblasts, macrophages, and smooth muscle cells. Recently, HGF production by B-lineage cell lines was also reported. In vitro HGF has the ability to cause destruction of tight junctions, thereby inducing the spread of coherent cells. This property of HGF led to the designation scatter factor, a name that is still used synonymously with HGF. Because of HGF’s ability to cause blood vessel formation and to promote cell proliferation, invasion, and motility, it is proposed to be involved in the process of cancer growth and metastasis.

The receptor for HGF is a transmembrane tyrosine kinase that is encoded by the proto-oncogene c-met. In normal tissue, c-met is expressed primarily in epithelial cells, but it has also been detected on a small fraction of cells in the BM, half of which were identified as hematopoietic precursor cells because of their expression of CD34. In normal lymphatic tissue, such as the spleen or tonsils, c-met expression was not found. However, the effect of HGF on the immune response of B cells has been reported. Whether this was a direct effect of HGF on the B cells or required the presence of other c-met-expressing cells is unclear at present.

Several findings support a link between activation of c-met and carcinogenesis. The met gene was originally identified as an oncogene responsible for the chemically induced transformation of a human osteosarcoma cell line. Several groups have reported overexpression of the c-met proto-oncogene in neoplastic tissues such as ovarian cancer and thyroid carcinomas. In hematologic malignancies, c-met has been found in some cell lines from patients with Hodgkin’s disease.

Concomitant expression of both HGF and its receptor seems to be uncommon in normal cells, but has been observed in tumors such as non-small cell lung cancers, various sarcomas, and pancreatic adenocarcinomas. Murine NIH 3T3 fibroblasts that were cotransfected to express human HGF and the proto-oncogene c-met became highly tumorigenic in nude mice. Transfection of c-met-expressing NBT-II rat bladder carcinoma cells with human HGF created an autocrine loop, and the cells became more tumorigenic and invasive than did their normal counterparts. Simultaneous expression of HGF and its receptor thus seems to correlate with increased malignancy.

In a recent study we found that HGF and c-met were simultaneously expressed in the human myeloma cell lines JIN-3, U-266, OH-2, and JW. In the present report, we show that this expression pattern is also found in freshly isolated myeloma cells.
MATERIALS AND METHODS

Patient samples. Serum and BM samples were obtained from myeloma patients as part of the diagnostic procedure. Leftover material was used for research after informed consent had been obtained. In 3 cases, myeloma cells were separated from pleural fluid that had been evacuated as part of palliative treatment. Control BM samples came from patients who underwent diagnostic BM sampling for indications such as elevated sedimentation rate. These samples were assessed as morphologically normal in smears stained with May-Grünwald-Giemsa. Blood samples for separation of chronic lymphocytic leukemia (CLL) B cells were taken from CLL patients after informed consent. Normal peripheral B cells were separated from buffy coats of healthy donors (Bodemann, Regional Hospital, Trondheim, Norway). Serum from age- and sex-matched donors was obtained from healthy volunteers.

Cell separation and culture. Myeloma cells from BM or pleural fluid samples were purified by positive immunomagnetic selection using the plasma-cell-specific monoclonal antibody (MoAb) B-446 (Serotec, Oxford, UK). B-4 recognizes the adhesion molecule syndecan 1, a molecule that in normal BM is specifically expressed by progenitors of B cells and by plasma cells.39 The B-4 antibody stains less than 1% of the leukocytes in normal BM and the majority of the stained cells have a plasma-cell-like morphology (unpublished results). The separation method is described elsewhere.5 In short, mononuclear cells were isolated by density gradient centrifugation, incubated with B-4, washed, and incubated with Dynabeads coated with sheep antimouse Ig (Dynal, Oslo, Norway). B-4+ cells attached to the beads and were recovered with a magnet. After an overnight incubation, a majority of the cells spontaneously detached from the beads and could be used for culture or RNA isolation. This purification method gives samples of myeloma cells that are more than 98% pure, as estimated by morphologic evaluation of May-Grünwald-Giemsa–stained cytospin smears.3

At the end of the myeloma cell separation, a portion of the B-4+ myeloma cells and of the cells that did not attach to the magnetic beads during the separation procedure (B-4- BM mononuclear cells [BMMC]) were washed and seeded in 24-well culture plates (Costar, Cambridge, MA) at a concentration of 106 cells/mL in complete medium (CM) consisting of RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10% fetal calf serum (GIBCO), 2 mmol/L L-glutamine, and 40 pg/mL gentamicin and incubated at 37°C for 24 to 72 hours in a humidified atmosphere containing 5% CO2. The conditioned medium was harvested and kept frozen at -70°C until cytokine determination.

Malignant B cells from CLL patients and peripheral B cells from normal donors were separated from blood samples by a similar immunomagnetic method using Dynabeads coated with anti-CD19 antibodies, as described.39 B cells were either harvested for RNA isolation immediately after cell separation or grown for 3 days in CM supplemented with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1% phytohemagglutinin (PHA).

Detection of HGF and c-met mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). HGF- and c-met–specific primers were synthesized in our laboratory with a Beckman DNA SM synthesizer (Beckman Instruments Inc, Fullerton, CA) on the basis of the published HGF39 and c-met41 cDNA sequences. The HGF primer sequences were as follows: forward primer, 5'-CTC CCC ATC GGC ATC CCC-3'; reverse primer, 5'-CAC CAT GGC CTC GGC TGG-3'. The c-met primer sequences were as follows: forward primer, 5'-TGG GAA TTC TGC GTC GAA-3'; reverse primer, 5'-CCA GAG GAC GAC GAC AAA-3'. Total RNA was isolated as described by Gough.39 Briefly, 107 to 108 cells were lysed in 0.65% NP-40, 10 mmol/L Tris-HCl, pH 7.5, 0.15 mmol/L NaCl, and 1.5 mmol/L MgCl2 and the nuclei were pelleted. One hundred microliters of 7 mol/L urea, 1% sodium dodecyl sulfate (SDS), 0.35 mol/L NaCl, 10 mmol/L EDTA, and 10 mmol/L Tris-HCl, pH 7.5 was added, followed by extraction with phenol/chloroform and ethanol precipitation. cDNA was synthesized from 1 to 2 pg RNA by incubating for 1 hour with 0.5 mmol/L dNTP, 1.5 mmol/L oligo(dT)12 primer, 1 U/µL RNasin (Promega Corp, Madison, WI), 0.25% NP-40, 10 mmol/L dithiothreitol (DTT), 10 mmol/L Tris-HCl, pH 8.3, 15 mmol/L KCl, 0.6 mmol/L MgCl2, and 400 U Moloney murine leukemia virus (M-MLV) RT in a total volume of 30 µL. RT was inactivated at 90°C for 2 minutes, and the cDNA was frozen at -20°C until further use. PCR was performed as follows: 5 µL of cDNA was added to a reaction mixture containing 30 mmol of 5' and 3' primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.8, 0.22 mmol/L dNTP, 2.5 mmol/L MgCl2, 1 mmol/L EDTA, and 0.25% NP-40 in a total volume of 50 µL. The samples were covered with 50 µL mineral oil and heated to 90°C on a Techne Dri-block DB-3A (Techne Ltd, Cambridge, UK), and 1 U of Taq-polymerase (Boehringer Mannheim, Mannheim, Germany) was added through the oil layer. The PCR was run for 36 (HGF) or 40 cycles (c-met) on a Biometra Trio-Thermoblock TB-1 (Biometra, Göttingen, Germany), with each cycle composed of 1 minute at 94°C and 2 minutes at 68°C (HGF PCR) or 58°C (c-met), followed by 30 seconds of primer extension at 72°C. Samples giving no PCR product after the indicated number of cycles were rerun for 48 cycles. The expected PCR products of 749 (HGF product) or 395 (c-met) bp were detected by electrophoresis in 2% agarose gels containing 0.5 µg/mL ethidium bromide. Control primers amplified a 510-bp fragment of β-actin (Clontech, Palo Alto, CA).

HGF enzyme-linked immunosorbent assay (ELISA) and IL-1 bioassay. A sandwich HGF ELISA was developed in our laboratory. Two mouse MoAbs, denoted 3F4 and 2B5, were established as described39 and used as capturing antibodies. Detection of bound HGF was performed by polyclonal rabbit anti-HGF serum and horseradish peroxidase-labeled goat antirabbit IgG (H + L; Zymed, San Francisco, CA), with ortho-phenylene diamine (OPD) as substrate. The sensitivity of this assay was approximately 50 pg/mL HGF, and the assay was not affected by the presence of 10% normal human or mouse serum or by the presence of 1 mg/mL of plasminogen, which has about 38% amino acid similarity to HGF40 (data not shown).

IL-1 was measured in a bioassay using the EL-4-NOB-143 and HT-243 cell lines. EL-4-NOB-1 cells respond to IL-1 stimulation with IL-2 production and the HT-2 cells are dependent on IL-2 for growth. Both cell lines were grown in CM, supplemented with 2.5 × 107 cells and 200 µL medium per well) with serial dilutions of test sample. After 24 hours of incubation, the plates were centrifuged and 100 µL of supernatant from each well was transferred to empty microwells, into which HT-2 cells were seeded (1.5 × 106 cells per well; final volume, 200 µL). Cell growth was assayed after 4 hours of incubation with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma, St Louis, MO]. IL-1 activity was calculated using IL-1β (Genzyme, Cambridge, MA) as standard. The detection limit was 5 pg/mL recombinant IL-1 (Rl-1). To control that the proliferation of HT-2 cells was not caused by IL-2 present in the test samples, positive supernatants were added to cultures of HT-2 without prior conditioning by EL-4-NOB-1 cells.

Detection of c-met protein by Western blot and flow cytometry. Mononuclear cells were isolated from pleural fluids of 3 different myeloma patients by density gradient centrifugation. More than 97% of the cells in these samples were estimated to be myeloma cells by morphologic criteria in May-Grünwald-Giemsa–stained smears and by expression of CD38+ as detected by flow cytometry. In Preparation for immunoprecipitation, 20 to 40 × 107 cells were washed in phosphate-buffered saline and extracted with 50 µL of...
modified RIPA buffer containing 50 mmol/L Tris, pH 7.4, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L EDTA, 1% NP 40, 1 μg/mL pepstatin A, and 1 μg/mL aprotinin. Nuclei were pelleted and the supernatants were diluted 1:3 in RIPA buffer with 0.1% NP 40. The supernatants were incubated for 1 hour at 4°C with a mouse MoAb directed against the extracellular domain of c-met (Upstate Biotechnology Inc, Lake Placid, NY) and subsequently for 1 hour with 50 μL (50% vol/vol) of antimiouse- IgG-conjugated sepharose (Zymed). The sepharose particles were washed three times in RIPA with 0.1% NP 40 and pelleted. Sample buffer with 1% mercaptoethanol was added and the samples were boiled for 2 minutes. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run and blotted onto nitrocellulose filters (Bio-Rad, Hercules, CA). The filters were developed by incubation with another MoAb against c-met (Upstate Biotechnology Inc), followed by goat anti-mouse HRP conjugates and enhanced chemiluminescence (ECL) detection (Amersham, Aylesbury, UK). The myeloma cell line JIN-3 (a gift from Jennifer Ball, Department of Immunology, University of Birmingham, Birmingham, UK) and a protein preparation from the carcinoma cell line A431 (provided by Upstate Biotechnology Inc) were used as positive controls for c-met.

Cell surface c-met and CD38 were detected by flow cytometric analysis. Cells (106) were incubated with unconjugated anti-c-met or phycoerythrin-conjugated anti-CD38 (Becton Dickinson, Mountain View, CA) for 1 hour at 4°C. When the primary antibody was unconjugated, subsequent staining was with fluorescein isothiocyanate-conjugated goat antimouse IgG (Becton Dickinson). Before final analysis with a FACScan flow cytometer (Becton Dickinson), the cells were washed three times in phosphate-buffered saline containing 0.1% bovine serum albumin. Five thousand cells from each sample were analyzed by the flow cytometer on a single cell basis and displayed as frequency distribution histograms. Dead cells and debris were gated out on the basis of the forward light scatter signal.

Statistical methods. Calculations were performed with SPSS for Windows (SPSS Inc, Chicago, IL). Analysis was performed with the Student's t-test or with Mann-Whitney U-test where appropriate. Both tests were two-sided. A P value of .05 was defined as the limit for statistical significance.

RESULTS

HGF and c-met mRNA in purified myeloma cells as detected by RT-PCR. Highly purified myeloma cells from all the patients (n = 7) contained both HGF and c-met mRNA as detected by RT-PCR (Fig 1, lanes C through I). For comparison, we also tested B cells from CLL patients and normal donors. About one third of freshly separated CD19+ CLL cells contained HGF mRNA, whereas only 1 of 14 such samples contained c-met mRNA (Table 1). Unstimulated peripheral CD19+ B cells from healthy donors contained neither HGF nor c-met mRNA (Fig 1, lanes N through R, and Table 1). Stimulation of the B cells with PMA/PHA for 3 days did not induce HGF expression (data not shown). BMNC from nonmyeloma controls (n = 1) contained neither HGF nor c-met mRNA (Fig 1, lanes J through L).

HGF and IL-1α in supernatants from highly purified myeloma cells. To study HGF production in primary cells, we established cultures of purified myeloma cells (B-B4+ MM) isolated from 19 different patients. BMNC remaining after extraction of MM cells were also cultured from some of these patients (B-B4+ BMNC). Supernatants from B-B4+ MM cells and from B-B4+ BMNC were tested for HGF content in an HGF-specific ELISA. Detectable levels of HGF were found in 85% of the myeloma supernatants and in 77% of the B-B4+ BMNC supernatants, with the values being 243 pg/mL (range, 0 to 29,000 pg/mL) and 212 pg/mL (range, 0 to 5,800 pg/mL), respectively (Fig 2A). Although the average level of HGF was higher in the B-B4+ MM cultures, the difference between the two culture types in HGF expres-

| Table 1. Expression of HGF and c-met mRNA in Purified B-B4+ Myeloma Cells, CD19+ Cells From Patients With CLL, Normal CD19+ Peripheral B Cells, and BMNC, as Detected by RT-PCR |
|-----------------|-----------------|-----------------|
| HGF             | c-met           |
| MM              | 7 (7)           | 7 (7)           |
| CLL             | 5 (17)          | 1 (14)          |
| B cells         | 0 (7)           | 0 (5)           |
| BMNC            | 0 (3)           | 0 (3)           |

All positive samples gave clearly visible bands after 36 (HGF) or 40 (c-met) PCR cycles. In negative samples, no bands could be seen even after 48 cycles. The number of examined samples are given in parentheses.

Fig 1. HGF and c-met mRNA expression in purified myeloma cells, BMNC, and peripheral B cells, as detected by RT-PCR. Agarose gels of RT-PCR products amplified from cDNA of (lanes C through I) primary myeloma cells taken from seven different patients, (lanes J through L) normal BMNC, and (lanes N through R) normal peripheral B cells. Panel 1, HGF; panel 2, c-met; panel 3, β-actin. Lane B, control without RT in the cDNA synthesis; lanes A and M, molecular weight standard (Pst I-digested λ-phage). Five microliters of cDNA was used in all reactions.
HGF and c-Met in Myeloma

Fig 2. HGF and IL-1 in supernatants from cultures of purified B-B4+ myeloma cells (MM B-B4+) and B-B4- BMMC (BMMC B-B4-) from myeloma patients. (A) HGF measured by an HGF-specific ELISA. The detection limit is indicated by the thin line. (B) Bioactive IL-1 measured by the NOB-1/HT-2 bioassay.

Discussion

The data in this report show that expression of HGF and its receptor c-met is a common if not ubiquitous trait for myeloma patients. Serum was taken from 13 myeloma patients at time of diagnosis (before treatment) and from age- and sex-matched controls. The level of HGF in these samples was tested in an HGF-specific ELISA. The HGF concentration in the myeloma and control sera was 1,055 ± 836 pg/mL and 254 ± 178 pg/mL (mean ± SD), respectively (Fig 3), a difference that was statistically significant (P = .00025, t-test). All 13 myeloma patients had HGF levels above the mean of the controls; in 9 of them, the HGF levels were greater than the mean + 2SD of HGF in controls and can thus be considered to be significantly elevated.

Detection of c-met protein by Western blot and flow cytometry. c-met was immunoprecipitated in cell extracts prepared from myeloma cells taken from the pleural fluids of 3 myeloma patients, from the JJN-3 myeloma cell line, and from A431 carcinoma cells. The precipitates were run in SDS-PAGE and electroblotted onto nitro-cellulose membranes. Another MoAb against c-met was used to detect the protein. A 145-kD band was identified as the c-met β-chain p145c-met. This band was present in all three patient samples (Fig 4, lanes A, C, and E). The uncleaved c-met precursor protein, p170c-met, was present in both controls (Fig 4, lanes D and F) and in at least one of the patient samples (Fig 4, lane E). Three other bands of lower molecular weight were also present in all the samples. The nature of these bands is unknown at present.

Cells from these three pleural fluids were also examined by flow cytometry. A major cell fraction expressed c-met, as seen in the histogram presented in Fig 5.
human myeloma cells. Previous work with cell lines suggested the importance of this ligand-receptor pair in MM, and we now show that also freshly isolated myeloma cells express both HGF and c-met at the mRNA and at the protein level. HGF protein was found in 17 of 20 different supernatants from highly purified myeloma cell cultures. The level of HGF production in these cultures varied from barely above the detection limit of about 50 pg/mL to nearly 30 ng/mL. We also show that most myeloma patients have elevated levels of HGF in serum at the time of diagnosis.

The supernatants of cultured BMMC from myeloma patients contain an osteoclast-stimulating factor. Recent work has shown that HGF is one of the factors involved in the process of osteoclast recruitment, growth, motility, and activation. This cytokine could therefore be a candidate in the search for myeloma-derived osteoclast-stimulating factors. It is known that IL-1 and IL-6 have the property to induce bone resorption and these cytokines have been proposed as responsible for the osteoclast stimulation in MM. The origin of IL-1 and IL-6 in myeloma BM has been a matter of debate for the past few years. Growing evidence suggests that IL-1 and IL-6 are produced mainly by nonmyeloma cells. All together, there is reason to search for additional myeloma cell-produced factors that, perhaps in cooperation with IL-1 and IL-6, are involved in the induction of the bone lesion in MM. The observation in the present report that HGF, but not IL-1, is produced in vitro by human myeloma cells indicates that HGF could be involved in the bone pathology in MM.

Good prognostic factors are needed in MM. It is therefore of interest that myeloma patients have increased levels of HGF in serum at diagnosis. Other diseases with elevated HGF levels in serum include acute hepatitis and notably fulminant hepatic failure, whereas the slight elevation observed in liver cirrhosis and chronic hepatitis was not significant. Elevated levels of HGF have also been found in the plasma of patients with acute myeloblastic leukemia, whereas patients with CLL had normal values. Our observation that myeloma cells can produce HGF indicates that part of the HGF in serum of myeloma patients is derived from the malignant cells. Whether there is any correlation between serum HGF and other disease parameters, such as skeletal involvement, stage, M-component, renal disease, hypercalcemia, disease progression, etc, is still unknown. The usefulness of serum HGF as a prognostic factor thus remains to be clarified.

It is noteworthy that myeloma cells express not only HGF, but also the HGF receptor c-met. c-met is found primarily in epithelial cells in normal tissue and its expression in hematologic malignancies has not been extensively studied. The three studies that are known to us from the literature indicate that c-met is not widely expressed in such cancers. In these
HGF AND c-MET IN MYELOMA

ACKNOWLEDGMENT

We are grateful to Siv Helen Moen and Hege Skjellerudsveen for excellent technical assistance and to Bente Moen Rad for oligonucleotide synthesis.

REFERENCES

6. Bafset M, Helseth E, Naune B, Waage A: Lack of IL-1 secre-

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.


47. Weimar IS, Muller EJ, de Gast GC, Nakamura T, de Jung D, Gerritsen WR: Hepatocyte growth factor stimulates adhesion/invasion of c-Met positive lymphoma cells. Blood 86:184a, 1995 (abstr, suppl 1)
Hepatocyte growth factor and its receptor c-met in multiple myeloma

M Borset, H Hjorth-Hansen, C Seidel, A Sundan and A Waage