Concomitant Expression of Hepatocyte Growth Factor/Scatter Factor and the Receptor c-MET in Human Myeloma Cell Lines*

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Myeloma cell line supernatants were screened for their ability to inhibit the activity of transforming growth factor-β (TGFβ) in the mink lung cell (Mv-1-Lu) bioassay. Supernatant from the human myeloma cell line JJN-3 contained potent TGFβ antagonistic activity. This activity was isolated and found to be associated with a 72–78-kDa glycoprotein. Specific polyclonal and monoclonal antibodies were generated toward the 72–78-kDa protein, and these antibodies precipitated the TGFβ inhibitory activity from JJN-3 supernatant. Upon amino acid sequencing the protein appeared to be identical to hepatocyte growth factor (HGF), and some of the generated antibodies directly blocked the action of recombinant HGF in various assays. By HGF-specific polymerase chain reaction we demonstrated that HGF mRNA was expressed in five out of five tested myeloma cell lines. The level of HGF protein in supernatants showed great variation from >500 ng/ml in JJN-3 supernatant to a few ng/ml in the supernatants from other myeloma cell lines. The same five cell lines were also screened for expression the HGF receptor c-MET. Four of them expressed the receptor as shown by reverse transcriptase-polymerase chain reaction and Western blot. The receptor was shown to be constitutively phosphorylated in the human myeloma cell line JJN-3. This receptor could be dephosphorylated by anti-HGF antibodies, indicating the existence of an autocrine HGF loop in this cell line. We propose that HGF/c-MET may play a role in multiple myeloma.

Multiple myeloma, a malignant disease that involves proliferation of monoclonal plasma cells, is associated with several clinical manifestations such as skeletal pathology, anemia, hypercalcemia, and renal dysfunction. The cause of these features is only partly understood, but production of soluble factors by the myeloma cells is likely to be involved. In screening for unknown myeloma-produced factors, we noticed that the fetal calf serum was replaced by human A (11–13).

By reverse transcriptase polymerase chain reaction (RT-PCR), we showed that five out of five human myeloma cell lines express HGF mRNA, and HGF protein was found in supernatants from four of these cell lines. To determine whether HGF has the potential to exert autocrine effects on the myeloma cells, we looked for HGF receptor expression on the same cells. By RT-PCR and Western blots we showed that four out of the five cell lines also expressed the HGF receptor, c-MET. Autocrine HGF-mediated tyrosine phosphorylation of c-MET in the cell line JJN-3 could be blocked by anti-HGF. We propose that HGF is one of the long sought cytokines with pathophysiological functions in multiple myeloma.

MATERIALS AND METHODS

Cell Lines and Materials—The human myeloma cell line JJN-3 (14) was a gift from Jennifer Ball, Department of Immunology, University of Birmingham, UK. The OH-2 (15) and JW cell lines were established in our laboratory from pleural effusions of two myeloma patients. The Mv-1-Lu (CCL-64) (16), SW-480, RPMI 8226 (17), and U-266 (18) cell lines were purchased at American Type Culture Collection (Rockville, MA). Cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2×10−3 glutamine, and 40 μg/ml gentamicin (complete medium (CM)), except for the OH-2 cell line, where the fetal calf serum was replaced by human A+ serum (The BloodBank, Regionsykehuset, Trondheim, Norway). For estimation of HGF content in supernatants from various cell lines by enzyme-linked immunosorbent assay as described below, cells were seeded at a concentration of 7.5 × 10³ (OH-2 and U-266 cells), 5 × 10⁴ (JJN-3) or 2.5 × 10⁴ (RPMI 8226 and JW) cells/ml, and the supernatants were harvested after 72 h of culture and stored at −20°C. NSO cells were generously provided by Z. Edahar, Weizmann Institute of Science, Rehovot, Israel (19). Porcine platelet-derived TGFβ1 and βs human platelet-derived

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TGFB1, and recombinant human HGF were from R & D Systems (Minneapolis, MN).

Myeloma Cell Lines Produce HGF and c-MET

Mu-1-Lu Bioassay—The amount of TGFB-inhibiting activity was estimated by ability to counteract the TGFB3 inhibition of DNA synthesis in Mu-1-Lu cells (16). Cells were seeded at 10^4 cells/well in microplates and incubated in the presence of 350 pg/ml porcine TGFB3, with or without serial dilutions of samples in a final volume of 0.2 ml. After 20 h the cells were pulsed with 1 Ci/well of methyl-[3H]thymidine (Amer sham Corp.). They were harvested 4 h later on a Microvativ 1396 cell harvester (Packard, Meriden, CT), and β-radiation was measured. In some experiments porcine TGFB3 or human TGFB3 was used.

Purification of TGFB3-inhibiting Activity—After washing JNJ-3 cells three times in Hank's balanced salt solution to eliminate serum proteins, the cells were seeded in protein-free hybridoma medium (Life Technologies, Inc.) at a concentration of 5 × 10^5/ml in 225-mm^2 cell culture flasks (Costar, Cambridge, MA) in a total volume of 45 ml. The protein-free hybridoma medium was supplemented with 2.5 × 10^-5 M mercaptoethanol, 2 mM t-glutamine, and 40 μg/ml gentamicin. We incubated the cultures at 37°C in 5% CO2 humidified atmosphere for 72 h, at which time the cells were pelleted and supernatant was frozen at -20°C. The cell pellet was dispersed in protein-free hybridoma medium, and cells were reused for supernatant production up to five times.

JNJ-3-conditioned protein-free hybridoma medium was concentrated on a Q-Sepharose HP anion exchange column (Pharmacia Biotech Inc.) that was equilibrated with 10 mM Tris/Cl, pH 7.4. Bound material was eluted with 1 M NaCl. The activity was precipitated within 27–50% bodies were labeled with digoxigenin (digoxigenin antibody-labeling kit, normal human or mouse serum nor by the presence of 1 mg/ml of monoclonal antibodies, denoted 3F4 and 2B5, as catching antibodies.

sandwich enzyme-linked immunosorbent assay used to detect amount of the antigen which eventually turned out to be HGF) consisted of two monoclonal antibodies, denoted 3F4 and 2B5, as catching antibodies. Detection of bound antigen was done by a rabbit polyclonal serum toward the protein. The sensitivity of this assay was approximately 500 pg/ml HGF, and the assay was not affected by the presence of 10% normal human or mouse serum nor by the presence of 1 mg/ml of plasminogen, which has about 38% amino acid similarity to HGF (21) (data not shown).

Metabolic Labeling and Immuno-purification of HGF—JNJ-3 cells were seeded in 24-well culture plates (Costar) at a concentration of 2 × 10^5 cells/ml medium. Each well contained 0.5 ml of RPMI medium without methionine or cysteine but supplemented with 2 μM glutamine, 0.05 μCi of Tran35S-label (ICN Biomedicals, Irvine, CA), and 10% fetal calf serum that had been dialyzed against PBS. In some experiments the cells were grown in the presence of a Mitomycin C of 10 μg/ml as indicated. After 4 h the cells were collected by centrifugation, and the supernatant was incubated for 1 h at 4°C, either without antibodies or with 2 μg/ml of the monoclonal antibody 3F4, which specifically recognizes HGF, or the control antibody 6H8 (mouse monoclonal antibody, which recognizes a 180-kDa surface antigen of activated NR cells). Aliquots of 0.5 ml were further incubated for 30 min with 50 μl (50% v/v) of anti-mouse Ig-coated Sepharose (Pharmacia). The Sepharose particles were subsequently washed twice in 10 mM Tris, 1 mM EDTA, 0.5 M NaCl, 0.1% Nonidet P-40, pH 7.5, and once in the same buffer without NaCl. After the addition of 50 μl of SDS sample buffer, the suspension was boiled for 1 min and analyzed by SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by fluorography.

In other experiments designed to remove TGFB-inhibiting activity from JNJ-3 supernatant, 100 μl of anti-mouse Ig-coated Sepharose was preincubated with 50 μg of 3F4 or 6H8 for 2 h, washed in PBS containing 0.1% bovine serum albumin, and rotated overnight with 1 M of JNJ-3 supernatant. The Sepharose was pelleted, and the JNJ-3 supernatant were tested for TGFB-inhibiting activity in the Mu-1-Lu bioassay.

SDS-Polyacrylamide Gel Electrophoresis and Amino Acid Sequencing of Proteins—SDS-polyacrylamide gels were fixed and silver-stained by the method described (22). For sequencing purposes, proteins separated by gel electrophoresis were electrophoretically transferred to Immobiline-PSQ membranes (Millipore, Burlington, MA). Membranes were stained in Coomasie Blue before bands were cut out and subjected to amino acid sequencing using an Applied Biosystems A471 automatic sequencer coupled to an on-line amino acid identification system (Applied Biosystems, Foster City, CA). Alternatively, the purified protein was subjected to proteolytic degradation by V8 (Endo-Glu) protease (Pierce) at 37°C over night before separation of fragments by electrophoresis and sequencing as described above.

Detection of HGF and c-met mRNA by RT-PCR—HGF and c-met-specific primers were synthesized in our laboratory with a Bechman DNA SM synthesizer on the basis of the published HGF (21, 23) and c-met (24) DNA sequences. The HGF primer sequences were: forward primer, 5'-TCCCCATCGCCATCCC-3', and reverse primer, 5'-CACATCCGTCGCTGGA-3'. Sequences were thereafter primer, 5'-TGGAGAATGCTCGCTGGA-3', and reverse primer, 5'-CCA- GAGGAGCCGCCAAA-3'. Total RNA was isolated as described by Gough (25). Briefly, 10^6 cells were lysed in 0.65% Nonidet P-40, 10 mM Tris/HCl, pH 7.5, 0.15 mM NaCl, 1.5 mM MgCl2, and the nuclei were pelleted. 100 μl of 7 M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, and 10 mM Tris/HCl, pH 7.5, was added, followed by extraction with phenol/ chloroform and ethanol precipitation. cDNA was synthesized from 2 μg of RNA by incubating for 1 h with 0.5 μM dNTP, 1.5 μg oligo(dT)15 primer, 1 unit/μl RNasin (Promega Corp., Madison, WI), 0.25% Nonidet P-40, 10 mM dithiothreitol, 10 mM Tris/HCl, pH 8.3, 15 mM KCl, 0.6 mM MgCl2, and 400 units of Moloney murine leukemia virus RT (Life Technologies, Inc.) in a total volume of 30 μl. RT was inactivated at 90°C for 2 min, and the cDNA was frozen at -70°C until further use. PCR was performed as follows: 1 μl (cDNA from JNJ-3 cells in HGF PCR) or 5 μl (all other cell lines and JNJ-3 in c-met PCR) of cDNA was added to a reaction mixture containing 30 pmol of 5' and 3' primers, 50 mM KCl, 10 mM Tris/HCl, pH 8.8, 0.22 mM dNTP, 2.5 mM MgCl2, 1 mM EDTA, and 0.25% Nonidet P-40 in a total volume of 50 μl. The samples were covered with 50 μl of mineral oil and heated to 90°C on a Techne Dri-block DB-3A (Techne Ltd., Cambridge, UK), and 1 unit of Taq polymerase (Boehringer Mannheim) was added through the oil layer. The PCR was through 36 cycles on a Biometra Trio-Thermoblock TB-1 (Biorad, Göttingen, Germany), each cycle comprising of 1 min at 94°C, 2 min at 68°C (HGF PCR) or 58°C (c-met PCR), each cycle comprising of 1 min at 94°C, 2 min at 68°C (HGF PCR) or 58°C (c-met PCR), followed by 30 s of primer extension at 72°C. The expected PCR product of 749 HGF product was detected as 385 (c-met) band. PCR products were detected as 2 agarose gels containing 0.5 μg/ml ethidium bromide. Control primers amplified a 510-base pair fragment of β-actin (Clontech, Palo Alto, CA).

* T. Espevik and A. Sundan, unpublished results.
HGF PCR fragments were cloned into the pCR®II vector (Invitrogen, San Diego, CA). Inserts were sequenced on both strands applying M13 universal forward and reverse primers and analyzed on an Applied Biosystems automatic sequencing machine according to methods provided by the manufacturer (Perkin-Elmer). The HGF-specific fragment was transcribed in vitro by SP6 RNA polymerase (Promega) in the presence of [γ-32P]CTP, and the transcribed RNA was used as a probe on Northern blots of total RNA or mRNA from myeloma cell lines by standard procedures (26). mRNA for this purpose was isolated using a Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway).

Detection of c-MET Protein by Western Blot—Cells were washed in PBS and extracted with 10 mM Tris/HCl, pH 6.8, 1% Triton X-100. Nuclei and insoluble material were pelleted, and the protein content of the extracts was measured with the Bio-Rad protein assay. 40 µg of protein was solubilized in SDS sample buffer containing 1% β-mercaptoethanol, and run on 10% polyacrylamide gels. The gels were blotted onto nitrocellulose filters (Bio-Rad), and the filters were developed by rabbit polyclonal antibodies toward c-MET and goat anti-rabbit HRP conjugates and Enhanced Chemiluminescence (ECL) detection (Amer sham Corp.). As anti-c-MET we used two different antibodies raised toward synthetic peptides comprising of either the 28 or the 12 COOH-terminal amino acids of the c-MET β-chain (Santa Biotechnology Inc., Santa Cruz, CA). The appropriate synthetic peptide was added in excess to control reactions to check the specificity of the antibodies.

Immunoprecipitation of c-MET and Detection of Tyrosine-phosphorylated c-MET—JJN-3 cells were removed from culture bottles after 3 days of culture in CM and washed once in Hank’s balanced salt solution with 100 µg/ml heparin. Samples of 2.5 × 10^7 cells were used in immunoprecipitation of c-MET, either without further incubation or after pretreatment for 30 min at 37°C in CM with 1:100 dilution of rabbit anti-HGF serum or 100 ng/ml HGF. The cells were washed in PBS and lysed in 70 µl of modified RIPA buffer containing 50 mM Tris, pH 7.4, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1% Nonidet P-40, and 1 µg/ml aprotinin. Nuclei were pelleted, and the supernatants were diluted 1:3 in RIPA buffer with 0.1% Nonidet P-40. The supernatants were further incubated at 4°C for 1 h with a mouse monoclonal antibody directed against the extracellular domain of c-MET (Upstate Biotechnology Incorporated, Lake Placid, NY) and subsequently for 1 h with 50 µl (50% v/v) of anti-mouse Ig-coated Sepharose. The Sepharose particles were washed three times in RIPA with 0.1% Nonidet P-40 and pelleted. Sample buffer with 1% mercaptoethanol was added. SDS-polyacrylamide gels were run and blotted, and tyrosine-phosphorylated proteins were detected by ECL essentially as described above. HRP-conjugated anti-phosphotyrosine antibodies were purchased from Transduction Laboratories (Lexington, KY).

RESULTS

Purification and Identification of the TGFβ-inhibiting Activity—Supernatant from the JJN-3 cells was antagonistic to the growth inhibiting effects of TGFβ on Mv-1-Lu cells (Fig. 1). In order to purify this inhibitory activity, JJN-3 cells were adapted to grow in the absence of serum. After 3 days of culture, the cell supernatants contained approximately 500 units/ml of this activity (Table I). We defined one unit as the amount of activity that in 200 µl restored the thymidine incorporation in Mv-1-Lu cells treated with 350 pg/ml TGFβ to 50% of the thymidine incorporation in untreated cells. Porcine TGFβ1 and platelet-derived human TGFβ1 were inhibited in the same manner by the supernatant as porcine TGF β1 (data not shown).

The substance inhibiting TGFβ was isolated by a series of chromatographic steps as shown in Table I. With respect to protein, the overall purification was 84-fold, and about 10% of the total activity in the supernatant was recovered. The reason for the apparent increase in activity during the ammonium sulfate precipitation step is unclear. From results obtained in the Mv-1-Lu assay, it can be seen that 1 unit corresponded to about 1 ng of purified material (Table I), indicating that the activity was due to a highly potent protein. Thus, the TGFβ antagonist had a specific activity of similar order as the specific activity of TGFβ in the Mv-1-Lu cells. However, binding experiments indicated that the purified inhibitor of TGFβ did not inhibit the binding of iodinated TGFβ to these cells (data not shown), suggesting that the purified protein had its own activity on the cells.

The final purification step consisted of reverse phase chromatography, and a typical chromatogram is shown in Fig. 2. The activity eluted as a peak at about 40% acetonitrile. When the peak from the reverse phase column was analyzed by SDS-polyacrylamide gel electrophoresis, the protein eluted from the RPC column appeared as a broad band at 72–78 kDa under nonreducing conditions (Fig. 3, lane E). Under reducing conditions, three main bands could be detected, migrating in the gels corresponding to molecular masses of approximately 90, 63, and 32–34 kDa (Fig. 3, lane F), suggesting that the broad 72–78-kDa band consisted of at least two subunits linked by disulfide bonds. After the generation of monoclonal antibodies against the 72–78-kDa protein, the inhibitory activity was purified by affinity chromatography before a final reverse phase column as shown in Fig. 2. This purification scheme resulted in the isolation of inhibitory activity with similar electrophoretic mobility (Fig. 3, lane G versus lane E).

Initial attempts to identify the TGFβ-inhibiting activity by NH2-terminal amino acid sequencing of the protein comprising the 72–78-kDa band were unsuccessful. However, treatment of the purified protein by V8 protease (Endo-Glu) generated several fragments that could be sequenced after separation by SDS-polyacrylamide gel electrophoresis and transferal to PVDF membranes. Two of the fragments generated NH2-terminal sequences that were identical to sequences in the human hepatocyte growth factor/scatter factor (21, 23). Thus, in one of the fragments, 17 consecutive amino acids were identified that were identical to the reported sequence of human HGF for amino acids 45–61 in Ref. 21. It also became clear that the electrophoretic mobility of the purified TGFβ antagonist corresponded closely to the earlier published data for HGF, both under reducing and nonreducing conditions (27).

To demonstrate that the TGFβ-inhibiting activity produced by the JJN-3 myeloma cells was due to the purified 72–78-kDa protein and that this protein was identical to HGF, monoclonal antibodies were raised toward the protein. As shown in Fig. 3, these antibodies recognized a protein of 72–78 kDa from the supernatants of JJN-3 cells. Furthermore, as shown in Fig. 4,
these antibodies removed the TGFβ-inhibiting activity from the supernatants of JJN-3 cells, establishing that the TGFβ-antagonistic activity was associated with the 72–78-kDa band. And finally, these antibodies precipitated recombinant human HGF, which in itself had identical TGFβ-inhibiting activity in the MV-1-Lu cells (data not shown). Taken together, these data demonstrate that the potent TGFβ-antagonistic protein purified from the cell supernatant of the myeloma cell line JJN-3 was HGF.

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Biological Activity of the Purified Protein—As shown above,
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HGF-specific mRNA in normal human peripheral B-cells (data not shown). Molecular cloning and sequencing of the 749-base pair HGF PCR fragment revealed that the sequence of this fragment, which encodes most of the α-chain, was identical to the previously published HGF cDNA sequence obtained from human placenta (23). When the mRNA from the two myeloma cell lines JJN3 and U-266 was analyzed by Northern blot, applying in vitro transcribed HGF antisense RNA as a probe, four species of mRNA with the approximate sizes of 6.2, 3.0, 2.3, and 1.5 kilobases could be detected in both cell lines as shown in Fig. 7. The presence of these mRNA species, as well as the relative abundance of the bands as apparent in Fig. 7, corresponds closely with what has earlier been shown for human placenta mRNA (31), indicating that the transcription mechanism and RNA processing for the HGF gene in myeloma cells resembles the process taking place in placenta.

The ability of myeloma cells to produce HGF was further analyzed by assaying cell supernatants for the presence of HGF by sandwich enzyme-linked immunosorbent assay. Table II shows the content of HGF in the supernatants from five myeloma cell lines. JJN-3 produces up to 0.5 ng/ml of HGF and is unique among these cell line in its ability to secrete very high amounts of HGF. However, significant amounts of HGF could also be detected in the supernatants of three out of four other myeloma cell lines (Table II).

Expression of c-met in Myeloma Cell Lines—RT-PCR was performed with c-met-specific primers amplifying an mRNA sequence coding for part of the intracellular portion of the β-chain of c-met. c-met-specific mRNA could be detected in four of the five tested cell lines (Fig. 6, lane B). The only exception was the RPMI 8226 cell line, in which no PCR amplification was achieved.

Protein extracts from the human myeloma cell lines U-266, JJN-3, JW, and RPMI 8226, as well as from the adenocarcinoma cell line SW-480 (positive control), were run in SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Rabbit polyclonal antibodies against the carboxy-terminal end of the β-chain of the c-met product were used to detect the protein. U-266, JJN-3, SW-480 (Fig. 8), and JW (data not shown) cells all contained c-met protein. As shown by other groups, both the precursor protein, p170met, and the β-chain, p145met, were detected by this antibody (32). In RPMI 8226 cells the protein could not be detected (Fig. 8). Three other bands, present in all three myeloma cell lines but not in SW-480, were also recognized by the antibody (Fig. 8). When excess soluble antigen was added, not only the antibody binding to the two proteins of expected size was reduced, but also the binding of the three other bands. However, any relationship between these three bands and the p170met or p145met has not yet been found.

Autocrine Tyrosine Phosphorylation of c-MET in the Myeloma Cell Line JJN-3—The HGF receptor c-MET was found to be constitutively tyrosine-phosphorylated in JJN-3 cells after 3 days of culture in CM (Fig. 9, lane A). Some cell samples were taken from the culture bottles and washed in heparin-containing solution to deplete surface-bound HGF. Subsequent incubation with exogenously added HGF maintained the phosphorylation (Fig. 9, lane B), whereas incubation with antibodies to HGF caused dephosphorylation of c-MET (Fig. 9, lane C). The lack of tyrosine phosphorylation in the anti-HGF-treated samples was not due to a general down-regulation of the receptor, because in parallel blots probed with anti-c-MET, the receptor was detected to approximately the same level in cells treated with anti-HGF as in cells stimulated with HGF (Fig. 9, lane E versus lane F).

| Cell line | HGF in supernatant after 72 h |
|-----------|-------------------------------|
| JJN-3     | 500                           |
| U-266     | 24                            |
| JW        | ND                            |
| OH-2      | 5                             |
| RPMI 8226 | 5                             |

*Estimated by sandwich immunoassay. The detection limit of the assay was about 0.5 ng/ml. ND, not detected.
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Fig. 8. c-MET protein from myeloma cell lines detected by Western blot. Extracts of total protein from the human myeloma cell lines JJN-3, U-266, and RPMI 8226 were run in gel electrophoresis and blotted onto nitrocellulose membrane. The adenocarcinoma cell line SW-480, which was known to express c-MET, was used as a positive control. A rabbit antisera against the carboxyl-terminal portion of the c-MET β-chain was used for detection. Bound antibody was labeled with HRP-conjugated goat anti-rabbit antibodies and developing was done with ECL. The upper arrow points to the c-MET precursor protein, p170\textsuperscript{met}. The lower arrow indicates the β-chain, p145\textsuperscript{met}. The four right-hand lanes show controls to which excess of the C-28 synthetic antigen had been added to check the specificity of the antibody.

Fig. 9. Autocrine phosphorylation of c-MET in JJN-3 cells. JJN-3 cells were cultured in CM for 3 days, and c-MET was immuno-precipitated from the cells either after no further incubation (lanes A and D) or after additional incubation for 30 min in 100 ng/ml HGF (lanes B and E) or in 1:100 dilution of anti-HGF rabbit serum (lanes C and F). Western blots of the precipitated protein were made as described under "Materials and Methods." Lanes A–C are probed with anti-phosphotyrosine, and lanes D–F are probed with anti-c-MET. The arrow points to the β-chain of c-MET, p145\textsuperscript{met}.

DISCUSSION

The most important finding presented here is that human myeloma cell lines produce HGF and its receptor c-met and that the receptor is stimulated in an autocrine manner. All but one cell line expressed both HGF and c-MET, suggesting that concomitant expression of these proteins is the rule in myeloma cell lines. In healthy tissue c-MET and HGF expression seems to be uncommon in normal cells, it has been observed in tumor specimens such as nonsmall cell lung cancers, various sarcomas, and pancreatic adenocarcinomas (33–36). Furthermore, murine NIH 3T3 fibroblasts that were co-transfected to express human HGF and the protooncogene c-MET became highly tumorigenic in nude mice (12, 13). Transfection of human NBT-II rat bladder carcinoma cells with HGF created an autocrine loop, and the cells became more tumorigenic and invasive than their normal counterparts (11). Simultaneous expression of HGF and its receptor seems to correlate with increased malignancy. Interestingly, high levels of HGF (>1 ng/ml) were found in blood plasma of patients with hematological malignancies, notably 4 out of 21 patients with acute myeloblastic leukemia and 1 out of 2 multiple myeloma patients (37). To our knowledge, the present study shows the first cancer cell lines of hematological origin where simultaneous expression of this ligand receptor pair has been detected, and these findings raise the possibility of an autocrine effect of HGF in multiple myeloma. Further experiments showed that both HGF and c-MET were also simultaneously expressed in highly purified fresh patient samples of myeloma cells, suggesting that the results presented here are not due to a trait that developed after prolonged in vivo culture of the cell lines.\textsuperscript{3}

The JJN-3 cell line was unique among the tested cell lines in the amount of HGF produced. JJN-3 cells grow easily under serum-free conditions, a feature that simplifies the isolation of secreted proteins. Thus, HGF can be purified with high yield and purity by a simple two-step chromatographic procedure. HGF is synthesized as a single polypeptide chain that has to be proteolytically processed into the respective α- and β-chains to become biologically active (27, 38). Recently, the isolation and characterization of serum proteases, which specifically cleaves the HGF precursor, was reported (29, 30). However, it is still unclear whether these particular proteases are expressed in myeloma cells. When JJN-3 cells were grown under serum-free conditions, a substantial part of the HGF in the supernatant could be reduced into α- and β-chains, suggesting that a protease with the ability to cleave HGF precursor is associated with these cells.

In this paper we also show that HGF is able to counteract the biological effect of TGFβ on Mv-1-Lu mink lung cells. This release from TGFβ-induced growth arrest by HGF has recently also been observed by others (39). The inhibition of TGFβ by HGF can be used as a sensitive and reliable bioassay for HGF (40). There are several possibilities for intracellular crosstalk between the TGFβ and HGF signaling pathways. One of the intracellular effects of c-MET is activation of a Ras protein, resulting in a shift in the equilibrium between Ras-GTP and Ras-GDP toward the active, proliferation-inducing GTP-bound state (41). From studies of Mv-1-Lu cells, it is known that TGFβ maintains p21\textsuperscript{ras} in the GDP-bound state (42). This prevention of Ras activation was crucial for TGFβ-induced growth arrest of Mv-1-Lu cells and activation of p21\textsuperscript{ras} was required for progression into S-phase after cell cycle arrest by TGFβ (42). Ras proteins are therefore a possible point of intersection for the intracellular pathways of HGF and TGFβ signaling.

Although HGF counteracted the activity of TGFβ on the Mv-1-Lu cells, further experiments showed that these cytokines have unrelated or cooperative effects in other cell systems. Thus, when the human FS-4 fibroblastic cell line was cultured for 4 days, both TGFβ and HGF stimulated the DNA synthesis in these cells in an additive manner (data not shown). Furthermore, TGFβ causes inhibition of the IL-2-induced proliferation of the murine T-cell line HT-2 (43). We tried to reverse this TGFβ-mediated activity, but HGF did not seem to interfere with this effect of TGFβ (data not shown).

c-MET activation is known to favor cell invasion and migration and to cause angiogenesis and proliferation, all of which are essential in tumor progression. Several of these aspects could be important in multiple myeloma. In vivo, myeloma cells are often found in the bone marrow of most of the skeleton, usually associated with destruction of the bone substance surrounding the cells. This bone destruction is thought to be due to a combination of enhanced bone resorption and diminished

\textsuperscript{3} Barset, M., Hjorth-Hansen, H., Seidel, C., Sundan, A., and Waage, A. (1996) Blood, in press.
bone formation (44, 45). TGFβ has been proposed as a factor which preserves the balance in bone remodeling by controlling that increased resorption is counteracted by both a rise in bone formation and a decrease in bone resorption (46). HGF was recently shown to promote formation of osteoclasts from hematopoietic precursor cells (47), to attract osteoclasts to sites of bone resorption (48), and, in co-culture with osteoblasts, to increase the level of resorption (47, 48). However, whether HGF affects bone remodeling in multiple myeloma directly or as an opponent to TGFβ remains to be tested. We have tested whether the HGF/c-MET receptor-ligand interaction is involved in proliferation in an autocrine or paracrine manner in our myeloma cell lines. However, in a series of experiments with various concentrations of HGF or anti-HGF we have not been able to show such effect on cell proliferation. Nevertheless, the fact that concomitant expression of the ligand and receptor seems to be the rule in multiple myeloma cell lines, whereas it probably is an exception in other hematological cancers, certainly merits further investigation of the role of this cytokine-receptor pair in multiple myeloma.

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