Human myeloma cells adhere to fibronectin in response to hepatocyte growth factor

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Background and Objectives. Multiple myeloma is characterized by an accumulation of malignant plasma cells in the bone marrow. Inside the bone marrow, adhesion of myeloma cells to extracellular matrix proteins such as fibronectin may promote cell survival and induce drug resistance. In this work we examined the effect of hepatocyte growth factor (HGF) on the adhesion of myeloma cells and the signaling pathways involved.

Design and Methods. Cell adhesion experiments were performed with the human myeloma cell line INA-6 and primary myeloma cells. The HGF signaling pathway was studied in INA-6 cells with the use of antibodies against VLA-4 integrin, and with inhibitors of various intracellular signaling molecules.

Results. We found that HGF stimulated adhesion of myeloma cells to fibronectin. This event was dependent on the α4 and β1 integrin subunits (VLA-4), but HGF did not increase the expression of integrins on the cell surface. Our findings suggest that HGF promotes myeloma cells to adhere via activation of the phosphatidylinositol 3-kinase (PI3K) pathway independently of AKT, but possibly through the involvement of nuclear factor κ B (NF-κB). INA-6 cells adhered to fibronectin after stimulation by insulin-like growth factor or stromal cell-derived factor 1α, but this adhesion was less dependent on PI3K than HGF-mediated adhesion.

Interpretation and Conclusions. This work points to HGF as a pro-adhesive factor in cell adherence to the bone marrow matrix protein fibronectin, an event known to promote cancer cell survival and drug resistance. Inhibiting HGF, its receptor c-Met or the VLA-4 integrin may be beneficial to the myeloma patient.

Key words: multiple myeloma, 16F-1, VLA-4, HGF, SDF-1α.

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Multiple myeloma (MM) cells are usually found in one particular location, the bone marrow (BM). This tropism can be explained if factors present only in the BM are absolutely critical for the survival and growth of MM cells. Such factors can be cell adhesion sites in the bone extracellular matrix or cytokines secreted by cells in the marrow microenvironment. Adhesion between MM cells and the microenvironment might be beneficial to MM cells in several ways. For instance, adhesion is reported to induce the secretion of myeloma growth factors, such as interleukin-6 (IL-6)[1] and also to induce secretion of osteoclast activating factors.[2] Furthermore, adhesion to the bone marrow milieu may allow drug-resistant cells to emerge[3] by activating signal transduction pathways that block drug-induced apoptosis.[1,4] The cell signaling pathways activated by growth factor binding and by adhesion of cells may be interdependent and involve similar mechanisms.[5] The integrins are a family of cell adhesion molecules forming heterodimer (α and β) cell surface receptors for extracellular matrix (ECM) proteins and for other cells.[6] The most abundant integrin found on MM cells is usually very late antigen (VLA-4) (α4β1),[7-10] but other integrins such as α5β1,[8] α4β7[11] and αvβ3[12] may also play a part in adhesion of MM cells. Integrins on lymphocytes are often expressed in an inactive form.[13] Cytokines and chemokines may activate integrins by changing their affinity or their avidity, thereby increasing the ability of the cells to interact with extracellular ligands.[14] Increased affinity is the result of signaling events altering the cytoplasmic tail and subsequently the three-dimensional conformation of the extracellular domain of the integrin, so-called *inside-out-signaling*. *In vitro*, integrins can be converted to a high-affinity state by exposing the cells to divalent cations (e.g. Mn^{2+}),[15,16] or by using certain monoclonal antibodies (e.g. mAb24).[6] Increased integrin avidity is caused by lateral diffusion and/or clustering of integrins leading to increased integrin density in the cell-ligand contact

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zone.[17] Several cytokines, such as insulin-like growth factor-1 (IGF-1)[18] and stromal cell-derived factor-1α (SDF-1α),[19] are known to influence adhesion of MM cells and other lymphoid cells. HGF promotes the adhesion of malignant B-cell lymphoma to fibronectin;[20] and in the present study we investigated HGF promotion of adhesion of MM cells to this ECM protein and the mechanisms involved.

Design and Methods

Antibodies, cytokines and other reagents

Phycoerythrin (PE)-labeled antibodies against integrin α4 (CD49d), β1 (CD29), irrelevant mouse IgG1 and IgG2, as well as neutralizing anti-human CD49 (α4) and anti-human CD29 (β1) were all from Pharmingen (Bedford, MA, USA). Human plasma fibronectin was from BD Biosciences (Bedford, MA, USA). We used the following inhibitors: SU-011274 (SUGEN, San Francisco, CA, USA), pertussis toxin, rapamycin and wortmannin (Sigma, St. Louis, MO, USA), Ro 31-8220, Bisindolylmaleimide (Bis-1), LY294002, PD98059 and U0126 (LC Laboratories, San Diego, CA, USA), the AKT inhibitors SH-6 and SH-5 (Calbiochem La Jolla, CA, USA) and the NF-κB inhibitors bortezomib and PS-1145 (gifts from Millennium Pharmaceuticals Cambridge, MA, USA). Recombinant human IGF-1 was from R&D Systems Inc. (Minneapolis, MN, USA), IL-6 from Biosource (Camarillo, CA, USA), HGF was purified in our own laboratory,[21] SDF-1α was from Peprotech (London, UK) and phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich (Oslo, Norway). All compounds were diluted to final concentrations in RPMI-1640 (Gibco, Paisley, UK) supplemented with l-glutamine (2 mM) and gentamicin (40 μg/mL) (from now on referred to as RPMI).

Cell culture and treatment

We used the non-adherent, IL-6-dependent human myeloma cell line INA-6, which was a gift from Dr. M. Gramazki (University of Erlangen-Nuremberg, Erlangen, Germany). Cells were grown in RPMI with IL-6 (1 ng/mL) and 10% heat-inactivated fetal calf serum (FCS). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2, and growth media were replenished twice weekly.

We also studied myeloma cells from patients admitted to the Section of Hematology, St. Olav's Hospital, Trondheim, Norway, after obtaining approval from the regional ethics committee and informed consent from patients. Myeloma cells from bone marrow aspirates were purified by immunomagnetic separation using Macs CD138 Micro Beads (Miltenyi Biotec, CA, USA). Purity above 95% MM cells was obtained by this method. The purity was tested by cytospin preparation and microscopy.

Cell adhesion assay

Round-bottomed, 96-well plates (Sarstedt, Newston, NC, USA) were coated overnight at 4°C with fibronectin (20 μg/mL in PBS, 80 μL/well), blocked with bovine serum albumin (BSA) (10 mg/mL, 100 μL/well) for 1 h at room temperature, and finally washed 3 times in Hanks' balanced salt solution (HBSS). BSA and HBSS were from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors and cells were added to the plates immediately before cytokines. Experiments were performed with cytokines added either before or after the cells had been seeded. There were no significant differences in the results obtained by these two protocols. Cells were washed 3 times in HBSS, resuspended in 5 mL RPMI with 0.1% BSA and incubated for 1 h at room temperature with 5 μM of acetoxymethyl ester-2', 7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescin (BCECF-AM) (Sigma-Aldrich) with occasional agitation. Subsequently, the cells were washed twice in HBSS, seeded 5×10^4 per well in a total volume of 100 μL and incubated for 1 h at 37°C in 5 % CO_2. Following incubation, the wells were washed in HBSS to remove non-adherent cells. Remaining cells were lysed by adding 50 μL/well of 1% Triton X-100. The fluorescence intensity at 538 nm, using an excitation wavelength of 485 nm, was determined with a Fluoroskan II fluorescence reader (Labsystems, Helsinki, Finland), before and after removal of non-adherent cells. We used the trypan blue exclusion test to verify that cell detachment was not caused by induction of cell death.

Flow cytometry

Cells were washed four times in HBSS and seeded in RPMI with 0.1% BSA and IL-6 (0.1 ng/mL) at a density of 2.5×10^5 cells/300 μL/well in flat-bottomed 24-well culture plates (Nunc, Brand product, Denmark). Cells were stimulated with HGF (100 ng/mL) and incubated for 25 minutes at 37°C. Non-stimulated cells were used as controls. Thereafter cells were washed twice in PBS with 0.1% BSA and incubated for 30 minutes on ice with 5 μL PE-conjugated anti α4 or β1, or PE-conjugated irrelevant IgG-antibody. The cells were subsequently washed in PBS with 0.1% BSA, and analyzed immediately or fixed in PBS/1% formaldehyde. Samples were analyzed using a Coulter Epics XL-MCL flow cytometer (Beckton) with EXPO32 ADC software.

Proliferation assay

Proliferation was measured by [^{3}H]-thymidine incorporation. Cells were seeded in flat-bottomed, 96-well plastic culture plates (Corning Costar, Corning, NY, USA) at a density of 2×10^4 cells/well in 200 μL RPMI

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with 0.1% BSA. After 48 hours, cells were pulsed with 0.75 μCi methyl-[^{3}H]-thymidine (NEN Life Science Products, Boston, MA, USA) per well, and harvested 18 hours later with a Micromate 96-well harvester (Packard, Meriden, CT, USA). β-radiation was measured using a Matrix 96 β counter (Packard).

Immunoblotting

Cells were washed 4 times in HBSS, seeded in 1 mL RPMI at 2×10^6 cell/well, starved for 3 hours and pre-treated with inhibitors for 60 minutes. After cytokine stimulation for the indicated times cells were washed with ice-cold PBS and resuspended in 80 μL lysis buffer [Tris-HCl (50 mM) pH 7.5, NaCl (150 mM), glycerol (10 %), Triton-X 100 (1 %), EDTA (2 mM), NaF (100 mM), Na_3VO_4 (1 mM), β-glycerophosphate (40 mM), PMSF (1 mM), leupeptin (10 μg/mL) and pepstatin (1 μM)]. After 20 minutes on ice, the nuclei were removed by centrifugation at 12000 ×g, 4°C for 5 minutes. Aliquots of 25 μL were mixed with 8 μL 3.6×LDS sample buffer (Invitrogen, Oslo, Norway) with 100 mM DTT, heated for 5 minutes at 98°C and then separated on 10 % NuPAGE Bis-tris gels (Invitrogen, Oslo, Norway), followed by transfer to 0.45 μm nitrocellulose membranes (BIO-RAD Laboratories, Oslo, Norway). Membranes were blocked with 4% BSA in Tris-buffered saline-Tween 20 (TBS-T) and incubated overnight at 4°C with anti-phospho-AKT (Ser473), anti-total AKT, antibody against phospho mitogen-activated protein kinase (MAPK) (p44/42, Thr202/Tyr204) or anti-total MAPK (p44/p42) (Cell Signaling Technology, Beverly, MA, USA). Detection was performed using horseradish peroxidase-conjugated antibodies (DAKO Cytomation, Glostrup, Denmark) and ECL chemiluminescense (Amersham, Oslo, Norway).

Statistical analysis

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

Results

HGF induced adhesion of human myeloma cells to fibronectin

We stimulated INA-6 myeloma cells with HGF (150 ng/mL), IGF-1 (100 ng/mL) or SDF-1α (75 ng/mL) and measured adhesion. The basal adhesion of INA-6 cells to fibronectin was low. However, HGF, IGF-1 and SDF-1α increased cell adhesion 7-8 fold (Figure 1A). HGF, IGF-1 and SDF-1α also made two out of five primary MM cell samples adhere to fibronectin (Figure 1B and C). Compared with non-stimulated cells, adhesion of MM cells was 2 to 3-fold higher after HGF stimulation. A third sample adhered better to fibronectin than to BSA, but did not respond to cytokines, while two other primary myeloma cell samples did not adhere to fibronectin regardless of cytokine stimulation (*data not shown*). INA-6 cells adhered to fibronectin in response to HGF in a dose-dependent manner. The 50% effective dose (ED_{50}) was about 20 ng/mL and maximum adhesion was reached around 100 ng/mL (Figure 1D). In further experiments concentrations of HGF from 100-150 ng/mL were used.

In experiments to assess the kinetics of cytokine-induced adhesion, adherent cells were quantified at different time points (1–120 minutes). At 8 minutes, HGF stimulation gave no adhesion of INA-6 cells to fibronectin. Thereafter a linear increase in cell adhesion was registered until 45 minutes, when it levelled off. Cells remained firmly attached at 120 minutes (Figure 1E), and even at 22 hours no decline in cell adhesion was observed (*data not shown*). The kinetics of HGF-stimulated cell adhesion followed the same pattern as that after IGF-1 or SDF-1α stimulation (Figure 1E).

Adhesion to fibronectin sensitized INA-6 cells to proliferate in response to HGF

INA-6 cells were seeded in 96-well plates pre-coated with either fibronectin or BSA and stimulated for three days with various concentrations of HGF. Proliferation was then examined by measuring thymidine incorporation. A moderate, but significant increase in HGF-stimulated proliferation was seen when cells adhered to fibronectin compared to cells grown on BSA (Figure 2).

HGF-induced adhesion of myeloma cells was dependent on α4β1 integrin

To examine whether the HGF-induced adhesion of cells to fibronectin was mediated through α4β1 integrin, INA-6 cells were pre-incubated with neutralizing antibodies against either α4 or β1 integrin subunits and incubated with or without HGF. The adhesion of cells pre-treated with anti-α4 or β1 was close to the control level (Figure 3A). When anti-α4 was added to HGF-stimulated cells 21 hours after seeding, the adhesion to fibronectin decreased below the level of cells not stimulated by HGF (*data not shown*). This indicates that VLA-4 is responsible for HGF-stimulated adhesion throughout a 22-hour period. We analyzed integrin expression by flow cytometry using fluorescence-labeled antibodies against integrin subunits. Interestingly, we found that HGF did not increase the α4β1 integrin expression level, but a minor decrease in α4 expression level was seen (Figure 3B).

Only HGF-induced cell adhesion was dependent on the HGF receptor, c-Met. SU-011274 is a novel

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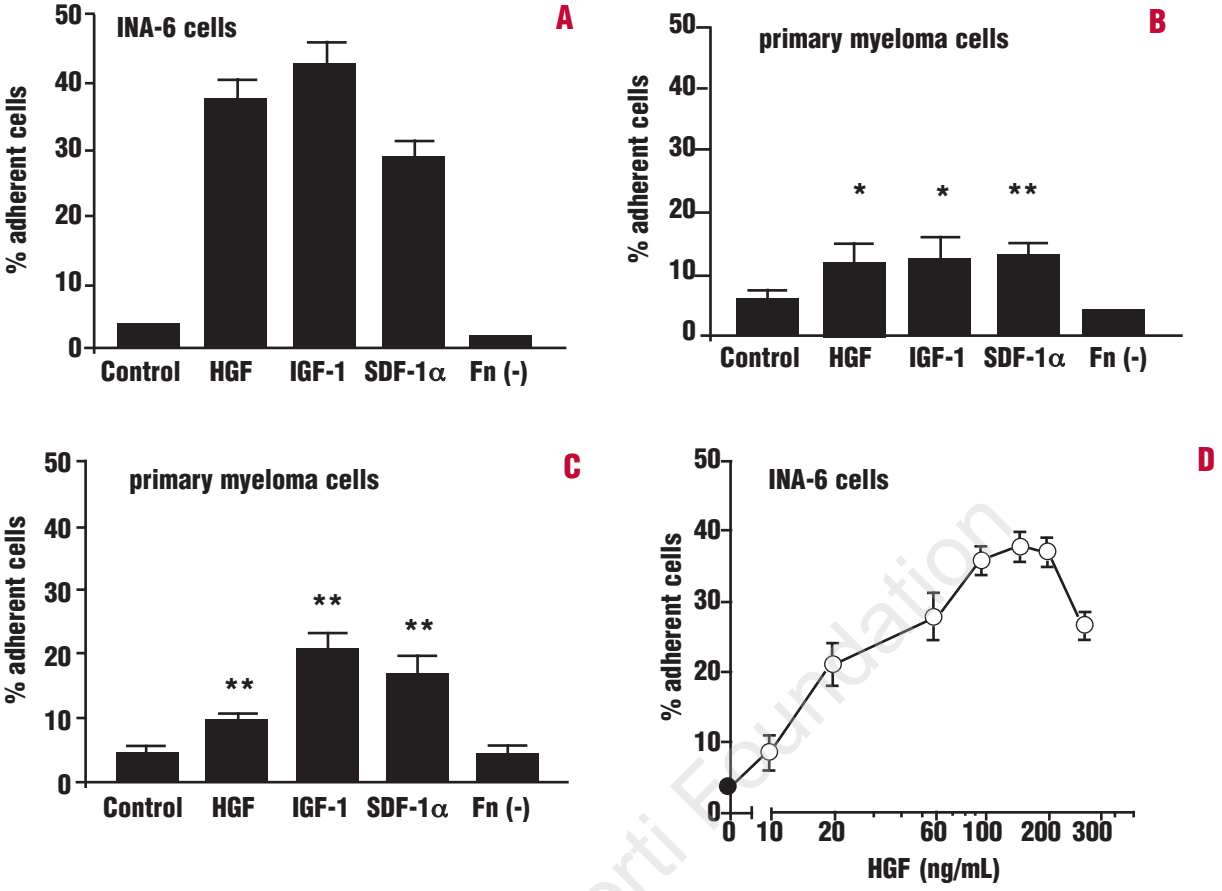

Figure 1. Adhesion of INA-6 cells (A, D and E) and primary MM cells (B and C) to fibronectin. After pre-incubation with a fluorescent dye, cells were incubated for 1 hour (A–D), or for various periods (E) with or without HGF (150 ng/mL), IGF-1 (100 ng/mL) or SDF-1α (75 ng/mL), or with various concentrations of HGF (D). The fluorescence intensity of adherent cells was measured and calculated as the percentage of the fluorescence intensity from all seeded cells. Error bars for INA-6 cells represent ± 1 standard error of the mean (SEM) of three individual experiments, and ± 1 SEM in D. Six independent measurements were made for each experiment. For primary myeloma cells (B and C) error bars represent ± 1 standard deviation (SD) of six independent measurements in one experiment. The level of adherent cells was significantly increased after cytokine stimulation, according to an unpaired Student's two-tailed t test. *(p<0.05) and **(p<0.01) denote significantly different from non-stimulated cells.

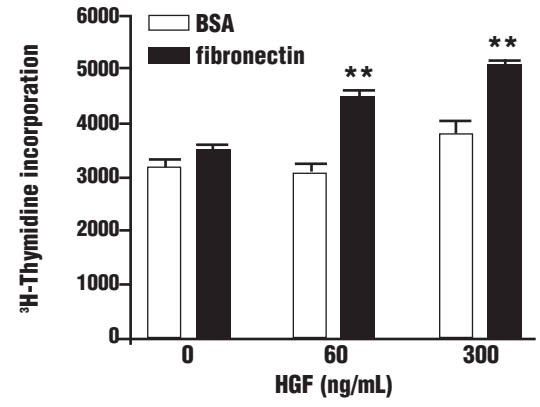

Figure 2 [left]. Proliferation of INA-6 cells stimulated with or without HGF and grown on fibronectin or BSA. [^{3}H]-thymidine incorporation was measured for the final 18 hours of a 72-hour incubation period. Results from one out of three similar experiments are shown. Error bars represent ±1 SD of triplicate data. **denotes significant difference from cells on fibronectin without HGF (p<0.01), according to an unpaired Student's two-tailed t-test.

receptor tyrosine kinase inhibitor of c-Met,[22] the receptor for HGF. Adding SU-011274 to INA-6 cells stimulated with IGF-1 or SDF-1α did not affect the adhesion of cells to fibronectin, whereas HGF-mediated cell adhesion was blocked almost completely at

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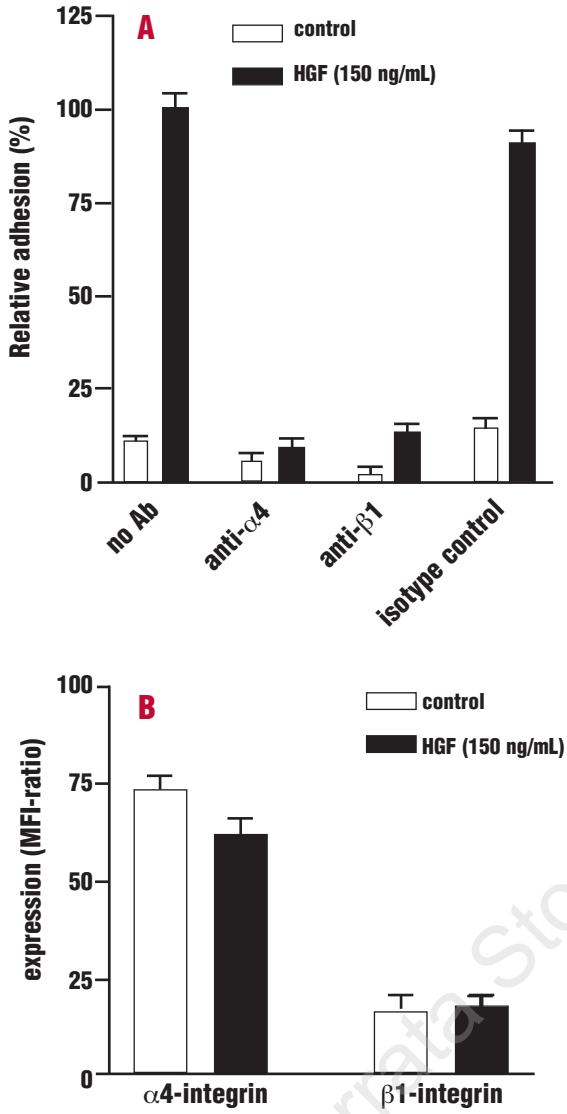

Figure 3. A. Neutralizing antibodies against VLA-4 integrin inhibited HGF-mediated adhesion of INA-6 cells to fibronectin. Antibodies against the integrin α4 (0.1 μg/mL) and β1 (10 μg/mL) sub-units were tested in a fluorescence adhesion assay performed as described in the methods section. Isotype control antibody at 1 μg/mL was used. HGF-stimulated adhesion was normalized to 100%. Compiled data from three experiments are shown and error bars represent +1 SEM. Six independent measurements were made for each experiment. B. Expression level of α4 and β1 integrin subunits on INA-6 cells before and after HGF (150 ng/mL) stimulation was analyzed by fluorescence-labeled antibodies and flow cytometry. MFI ratios are between fluorescence signal from cells labeled with integrin antibodies and the signal from cells labeled with isotype-specific control antibodies. All experiments were repeated three times and the values for one representative experiment are reported. Error bars represent ± 1 SD of four individual measurements.

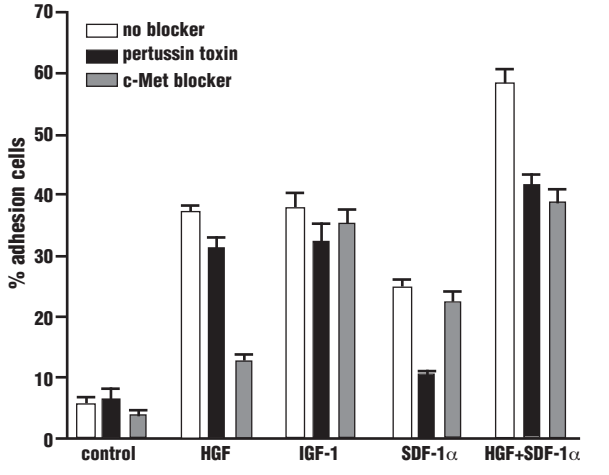

Figure 4. The effect of inhibiting c-Met and G protein-coupled receptors on INA-6 cell adhesion. INA-6 cells were stimulated with or without HGF, IGF-1, or SDF-1α or with HGF and SDF-1α in combination and analyzed by the adhesion assay described in the methods section. Cells were given inhibitors for 15 minutes prior to the cytokines. The results shown represent one of three similar experiments, and error bars represent +1 SD of four parallel measurements.

2 μM of SU-011274 (Figure 4). Next, we used pertussis toxin, an inhibitor of G protein-coupled receptors such as CXCR4 (SDF-1α receptor) and other chemokine receptors. As expected, this inhibitor blocked SDF-1α-induced adhesion (by 80%), whereas HGF-or IGF-1-induced adhesion was reduced by only 15% (p=0.04 for HGF and p=0.26 for IGF-1). When INA-6 cells were treated simultaneously with HGF and SDF-1α, there was an additive effect on cell adhesion. Neither pertussis toxin nor the c-Met inhibitor SU-11274 was able to decrease the additive cell adhesion to a level below that induced by HGF or SDF-1α alone (Figure 4).

PI3K inhibition, but not AKT inhibition, decreased HGF-stimulated adhesion of cells. The PI3K inhibitors LY294002 (20 μM) and wortmannin (1 μM) reduced cytokine-induced cell adherence to fibronectin (Figure 5A). The blockade was complete for HGF-stimulated adhesion. Wortmannin blocked IGF-1-induced adhesion of cells while LY294002 reduced it by about 55%. SDF-1α-induced adhesion was reduced by about 50% and 35% by wortmannin and LY294002, respectively. Thus it seemed that HGF signaling was more dependent on PI3K activation than IGF-1 and SDF-1α. The difference in use of the PI3K pathway was confirmed by Western blot analysis in which HGF and IGF-1 activated AKT, one of the messenger proteins downstream of PI3K more rapidly (within 5 minutes) and more strongly than SDF-1α did (after 45 minutes) (Figure 5B).

The mammalian target of rapamycin (mTOR) is one of the messenger proteins downstream of PI3K, and this regulator of cell growth is inhibited by rapamycin. Rapamycin at 100 ng/mL, a concentration that inhibited MM cell proliferation (*data not*

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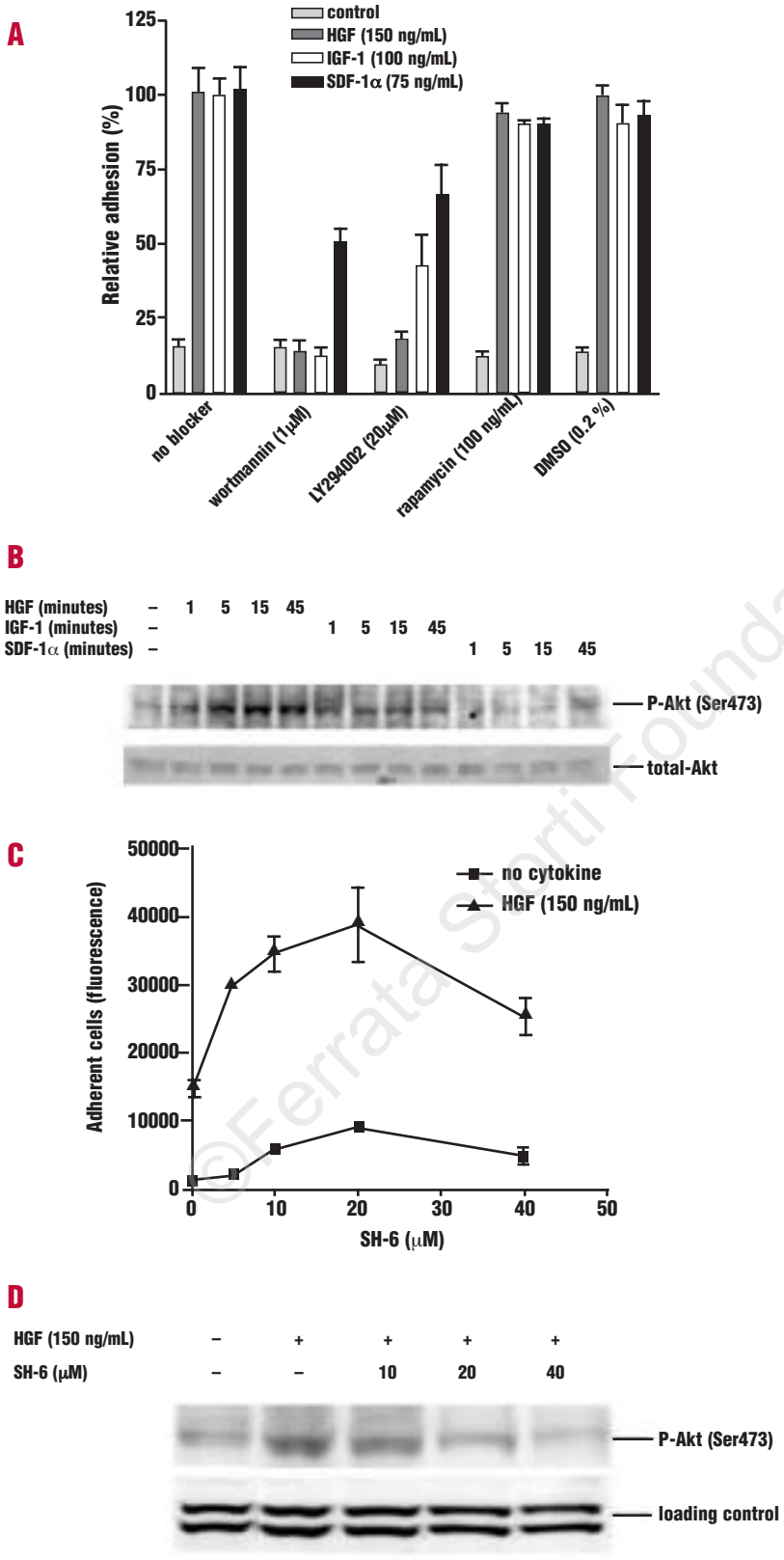

Figure 5. HGF, IGF-1 and SDF-1--induced cell adhesion are not equally dependent on PI3K activation. A. The PI3K inhibitors wortmanin and LY294002 blocked or decreased cytokine-stimulated adhesion of INA-6 cells to fibronectin, and HGF-stimulated cell adhesion was more dependent on PI3K than was IGF-1- or SDF-1α-stimulated adhesion. The mTOR inhibitor rapamycin (100 ng/mL) had no effect on cytokine-stimulated adhesion. The result shown is one representative out of three. Error bars represent +1 SD of six independent measurements. B. Immunoblots against phosphorylated AKT (upper panel) and loading control (lower panel) show that in serum-starved INA-6 cells HGF (150 ng/mL) and IGF-1 (100 ng/mL) activated AKT within 5 minutes whereas SDF-1α (75 ng/mL) activated AKT after 45 minutes. C. The specific AKT inhibitor SH-6 did not block HGF-mediated adhesion of INA-6 cells to fibronectin. INA-6 cells were given SH-6 at a concentration range from 5–40 μM 15 minutes before the cytokines were given. The fluorescence intensity from adherent cells was measured. The figure shows one representative result from four experiments. The error bars represent ± 1 SD of six independent measurements. D. The efficiency of the AKT inhibitor SH-6 was tested in an immunoblot assay using antibody against phosphorylated Ser473. INA-6 cells were starved in serum-free medium for 3 hours and indicated cells were given SH-6 one hour before being stimulated with HGF for 5 minutes.

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shown), did not block cytokine-induced cell adhesion (Figure 5A). To determine whether HGF-induced adhesion of cells was mediated by AKT, INA-6 cells were treated with the AKT inhibitors, SH-5 or SH-6, in a concentration range from 5-40 μM before HGF stimulation. Interestingly, the inhibitors at concentra-

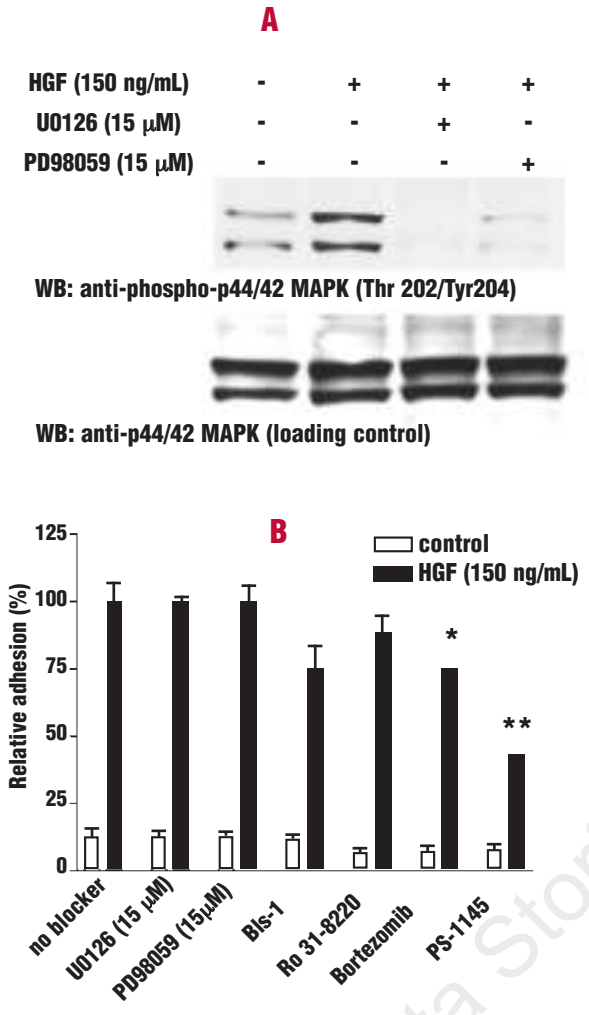

Figure 6. The role of MAPK, PKC and NF-κB in HGF-stimulated adhesion of INA-6 cells to fibronectin. A. INA-6 cells were starved in serum-free medium for 3 hours and subsequently left non-stimulated, or stimulated for 5 minutes with HGF. p44/42 MAPK in INA-6 cells was phosphorylated when cells were subjected to HGF. The p44/42 MAP-kinase phosphorylation decreased when INA-6 cells were given the MAPK-inhibitors U0126 or PD98059 for one hour before adding HGF. B. Adhesion of INA-6 cells to fibronectin was not affected by the MAPK blockers U0126 (15 μM) or PD98059 (15 μM), nor by the PKC blockers Bis-1(1 μM) or Ro 31-8220 (1 μM). Cell adhesion decreased when using the IKK inhibitor PS-1145 (5 μM) or the proteasome inhibitor bortezomib (15 nM). Cell adhesion was significantly inhibited, *$p< 0.05$ and **$p<0.01$, according to the Student's two-tailed t test. INA-6 cells were seeded in fibronectin-coated wells and given inhibitors 15 minutes before the cells were stimulated with or without HGF. HGF-stimulated cell adhesion was normalized to 100 % and the amount of adherent cells at other conditions was calculated as relative to this. The results from one representative experiment out of three are shown. Error bars represent +1 SD of four independent measurements.

tions lower than 15 μM (SH-5) (*data not shown*) and 20 μM (SH-6) (Figure 5C) increased cell adhesion to fibronectin in HGF-stimulated cells. The efficacy of the AKT inhibitors was confirmed by Western blot analysis: cytokine-induced phosphorylation of serine-473 AKT decreased by using SH-5 (*data not shown*) or SH-6 (Figure 5D).

HGF-mediated adhesion of cells was not dependent on signaling through p44/42 MAPK or protein kinase-C (PKC)

HGF activated the p44/42 MAPK pathway in INA-6 cells (Figure 6A). The p44/42 MAPK inhibitors, U0126 and PD98059, as well as the PKCα inhibitors, Ro 31-8220 (1 μM) and Bis-1 (1 μM), did not influence HGF-stimulated adhesion of INA-6 cell to fibronectin (Figure 6B). Phosphorylation of MAPK was almost completely abolished by the use of PD98059 or U0126 (Figure 6A), showing the efficacy of these inhibitors. The efficacy of the PKC inhibitor was confirmed by an assay in which Ro 31-8220, at a concentration of 1 μM, inhibited PKC-dependent IL-11 production in Saos-2 cells (*data not shown*). HGF-stimulated adhesion of cells to fibronectin was not significantly influenced by PMA, an activator of PKC. At the same time, basal adhesion of INA-6 cells to fibronectin increased in a dose-dependent manner when cells were given PMA (*data not shown*).

An inhibitor of Iκ-B kinase reduced HGF-mediated cell adhesion

We treated INA-6 cells with the Iκ-B kinase (IKK) inhibitor, PS-1145, at concentrations from 0 to 25 μM before addition of HGF in the adhesion assay. A dose-dependent decrease in HGF-stimulated adhesion of cells to fibronectin was found (*data not shown*). At a concentration of 5 μM PS-1145, HGF-stimulated cell adhesion was reduced by 60% (Figure 6B). We also tested the proteasome inhibitor bortezomib (15 nM) in adhesion experiments. Bortezomib blocks NF-κB activation by inhibiting degradation of phosphorylated Iκ-B. A 15–20% decrease in HGF-induced cell adhesion was then observed (Figure 6B).

Discussion

The main finding in the present study is that HGF stimulates adhesion of myeloma cells to the bone marrow matrix protein fibronectin in a dose-dependent manner with an ED_{50} of 20 ng/mL, a potency of HGF that is comparable to that found for other cell types.[23,24] Importantly, primary myeloma cell samples adhered to fibronectin after HGF stimulation, indicating that HGF-induced adhesion is not restricted to cell lines, but also pertains to patients with bone marrow HGF concentrations in the range that influences cell adhesion. The concentration of HGF is elevated in the BM of a large proportion of patients with MM, and levels above 20 ng/mL were found in more that 30% of patients in a study from our laboratory.[25] Our study also confirms the recently reported pro-adhesive abilities of IGF-1 and SDF-1α.[18,19] HGF has previously been shown to have a role in MM pathogen-

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esis.[26-28] A raised HGF level in serum is a negative prognostic factor for MM patients.[29-31] HGF can sustain myeloma cell survival and growth[28] and promote invasiveness.[32] Our study establishes HGF as a pro-adhesive cytokine for MM cells, a property that was previously documented for normal and neoplastic cell types other than MM cells.[33] Furthermore, we found a moderate increase in proliferation for INA-6 cells growing on fibronectin in the presence of HGF. For a slowly proliferating disease, over a long period even a modest increase in proliferation can result in a severe tumor burden. Thus, HGF is potentially an important cytokine increasing tumor burden by increasing both the cells' ability to adhere to fibronectin and to proliferate. Neutralizing antibody against the α4 or β1 integrin completely abolished INA-6 cell adhesion to fibronectin, thus confirming previous studies that have pointed to α4β1 as an important integrin for myeloma cells.[8,34] Interestingly, the integrin expression level was not increased by HGF stimulation. Our data indicate that anti VLA-4 therapy could be tried against MM. However, other investigators have shown functional significance of other adhesion molecules on myeloma cells, so VLA-4 is not the only adhesion molecule to be considered as a target for treatment of MM.

Receptor tyrosine kinases are reported to sometimes activate G protein-coupled receptors.[35] Our results indicate that IGF-1 as well as SDF-1α work independently of HGF on the receptor level since they are not blocked by an inhibitor of the HGF receptor, c-Met. As expected, SDF-1α signaling is blocked substantially by pertussis toxin, an inhibitor of G protein-coupled receptors. A small (and not significant for IGF-1) decrease in HGF- and IGF-1-stimulated cell adhesion after treatment with pertussis toxin shows that involvement of G proteins in the pathway from these cytokines cannot be ruled out, but is not likely to play a major role.

Two main signaling pathways activated by HGF in myeloma cells are the MAPK pathway and the PI3K pathway.[36] IGF-1- and SDF-1α-stimulated adhesion is suggested to be PI3K-dependent.[18,37] By using the PI3K-blockers wortmannin and LY29002 we found that also HGF-induced adhesion was PI3K-dependent. Blocking PI3K activation abolished, either partially or completely, HGF- and IGF-1-induced adhesion of INA-6 cells to fibronectin. We found a clear, although not complete, inhibition of SDF-1α–induced cell adhesion after PI3K blockade, indicating that HGF/IGF-1-stimulated adhesion is PI3K-dependent, while SDF-1α-stimulated adhesion is less dependent on PI3K. These results were supported by Western blot analysis showing that INA-6 cells were stimulated by HGF, IGF-1 or SDF-1α. We found that PI3K was activated more strongly when cells were stimulated with HGF or IGF-1 than with SDF-1α.

One of the signaling mediators downstream of PI3K is mTOR. Our results using rapamycin in combination with HGF indicate that HGF-stimulated cell adhesion was not mTOR-mediated. AKT, a messenger protein upstream of mTOR and downstream of PI3K, is activated by its translocation to the plasma membrane and phosphorylation at Thr308 and Ser47.[3,38] Our results from Western blot analysis show that HGF activates PI3K and AKT. The adhesion experiments suggest that the signaling pathway from HGF receptor binding to cell adhesion is through PI3K, but not through AKT. SH-5 and SH-6 are two phospdatidylinositol analogs that inhibit phosphorylation of AKT at Ser473 and thereby inhibit full activation of AKT and downstream substrates. Blocking the phosphorylation of AKT with SH-6 (5–40 μM) did not reduce cytokine-induced INA-6 cell adhesion to fibronectin. On the contrary, more cells adhered to fibronectin when AKT inhibitors were added to HGF than with HGF alone. When SH-6 or SH-5 was given in concentrations from 5–40 μM together with HGF, the adhesion increased up to 80% for INA-6, compared with cells given HGF alone. One explanation for these results could be that a negative feedback loop from AKT was inhibited when AKT phosphorylation decreased. We, therefore, suggest that HGF causes cell adhesion through PI3K, but not through AKT. However, our results should be interpreted with some caution, as pharmacological inhibitors may inhibit other signaling pathways, especially at high doses.

HGF is known to stimulate the MAPK pathway, and some cytokines, such as VEGF, stimulate cell adhesion dependent on PKC.[39] We found that HGF-stimulated cell adhesion remained unaffected by the MAPK and PKC inhibitors tested. The data indicated that neither the MAPK nor the PKC pathway participated in the signaling relay from HGF binding to adhesion of cells. At the same time basal adhesion of INA-6 cells increased with PMA in a dose-dependent manner, showing that PKC has the capacity to cause adhesion of INA-6 cells. Landowski *et al*. discovered that cell adhesion to fibronectin alters gene expression and leads to activation of the NF-κB heterodimer RelB/p50, thereby contributing to the drug-resistant phenotypes of MM cells.[40] We found that NF-κB may influence cell adhesion. The ability of HGF-stimulated cells to adhere to fibronectin decreased by 60% when we used PS-1145, a blocker of I-κB kinase, and therefore a blocker of NF-κB activation.[41] This indicates that the adhesion of cytokine-stimulated cells may be dependent on activation of both PI3K and NF-κB. We used PS-1145 at 5 μM, a concentration which does not influence the proliferation rate of INA-6 cells. When the proteasome inhibitor bortezomib was used at high concentrations to block NF-κB activation, HGF-mediated cell adhesion was reduced by only

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20%. These apparently diverse results obtained from using an IKK inhibitor and a blocker of I-κB degradation in adhesion assays may reflect the fact that an IKK blocker works upstream of I-κB and thus may influence other pathways. Alternatively, they may indicate that HGF-mediated cell adhesion involves a type of NF-κB activation that is dependent on IKK but not dependent on the degradation of I-κB. For instance, adhesion-mediated activation of RelB/p50 is not dependent on I-κB degradation (*personal communication from Terry Landowski*).

In conclusion, our study adds HGF to the list of pro-adhesive cytokines for MM cells. The inhibition of HGF or its receptor, c-Met, may be beneficial to myeloma patients. HGF-mediated adhesion was totally dependent on VLA-4 in INA-6 cells and we suggest that anti-VLA-4 therapy could also be considered in myeloma patients.

RUH: primary responsibility for the paper, performed adhesion and Western Blot experiments, analyzed data, created Figures 1A-E, 3A, 4, 5A-D, 6A, B, wrote the final version of the paper; VB, TBR: performed adhesion experiments, contributed to the discussion and writing of the manuscript; SB; performed flow cytometry experiments and made Figure 3B; AW: collected patient's samples and gave advice during the discussion of the work; AS: advised throughout the whole work and discussions; MB: designed the research, performed the proliferation experiments, made figure 2 and wrote the final version of the manuscript.

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