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Derksen, P.W.B.

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CHAPTER 2
The hepatocyte growth factor/MET pathway controls proliferation and apoptosis in multiple myeloma

Patrick WB Derksen*, David JJ de Gorter*, Helen P Meijer*, Richard J Bende*, Mirjam van Dijk†, Henk M Lokhorst†, Andries C Bloem†, Marcel Spaargaren* and Steven T Pals*

From the Department of *Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, and the Departments of †Hematology and Immunology University Medical Center, University of Utrecht, Utrecht, The Netherlands.

The evolution of multiple myeloma (MM) depends on complex signals from the bone marrow (BM) microenvironment, supporting the proliferation and survival of malignant plasma cells. An interesting candidate signal is hepatocyte growth factor/scatter factor (HGF), since its receptor MET is expressed on MM cells, while HGF is produced by BM stromal cells and by some MM cell lines, enabling paracrine- or autocrine- interaction. To explore this hypothesis, we studied the biological effects of HGF stimulation on MM cell lines and on primary MMs. We observed that MET is expressed by the majority of MM cell lines and by approximately half of the primary plasma cell neoplasms tested. Stimulation of MM cells with HGF led to activation of the RAS/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) pathways, signaling routes that have been implicated in the regulation of cell proliferation and survival. Indeed, functional studies demonstrated that HGF has strong proliferative and anti-apoptotic effects on both MM cell lines and primary MM cells. Furthermore, by applying specific signal-transduction inhibitors, we demonstrated that MEK is required for HGF-induced proliferation, whereas activation of PI3K is required for both HGF-induced proliferation and for rescue of MM cells from apoptosis. Taken together, our data indicate that HGF is a potent myeloma growth- and survival-factor and suggest that the HGF/MET pathway is a potential therapeutic target in MM.

INTRODUCTION

Multiple myeloma (MM) is a B-cell neoplasm characterized by clonal expansion of malignant plasma cells in the bone marrow (BM). Here, the tumor cells proliferate and acquire resistance to apoptosis, ultimately leading to osteolytic bone destruction, renal dysfunction, and anemia. The disease is still incurable with a median survival of approximately 3 to 4 years (1,2). Malignant transformation in MM evolves through different stages from monoclonal gammopathy of undetermined significance (MGUS) to expansive- and aggressive-, plasmablastic- MM. Most of the tumor evolution in MM takes place in the BM, indicating that signals from the BM microenvironment play a critical role in sustaining the growth and survival of MM cells during tumor progression. To date, these signals and the corresponding intracellular signaling cascades, which present potential targets for therapeutic intervention, have only been incompletely identified. Important candidate signals are direct physical contact of myeloma cells with BM stromal cells via integrin adhesion receptors, which can mediate outside-in growth and survival signals (3-5), as well as a number of cytokines/growth factors, including interleukin 6 (IL6), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF1) (5-8). Of these cytokines, the role of IL6 in the pathogenesis of MM has been most extensively documented. Clinical and experimental studies support an important role for this cytokine in the biology of MM. However, they also indicate that IL6 is not obligatory for MM development and progression, implying that its role in the pathogenesis of MM can be substituted by other signals (1,9). Studies from our own and other laboratories have identified the hepatocyte growth factor (HGF)/MET pathway as a potentially important signaling route in the pathogenesis of MM (10-12).

HGF is a pleiotropic cytokine that induces complex biological responses in target cells, including motility, growth, and morphogenesis. All known biological effects of HGF are transduced via the transmembrane tyrosine kinase MET. Whereas a functional HGF/MET pathway is indispensable for
mammalian development, uncontrolled activation of MET is oncogenic and has been implicated in growth, invasion, and metastasis of a variety of tumors (reviewed by van der Voort et al (13)). This uncontrolled MET activation may involve a variety of mechanisms including translocation, mutation, or amplification of the MET gene, and autocrine- or paracrine MET stimulation. In B-cell malignancies, the HGF/MET pathway might promote tumorgenesis through both autocrine and paracrine mechanisms. In primary effusion lymphoma (PEL), as well as in MM, co-expression of HGF and MET has been observed, suggesting autocrine stimulation (10,11,14). Since bone marrow stromal cells produce HGF (15), paracrine stimulation of MM cells within the bone marrow microenvironment can also take place. Consistent with a role for HGF/MET in MM progression, high serum levels of HGF were reported to be associated with unfavorable prognosis in patients with MM (16). Taken together, these data suggest the involvement of the HGF/MET pathway in the pathogenesis of MM. Indeed, we have recently shown that HGF stimulation of MM cells triggers signaling routes implicated in the regulation of cell proliferation and survival. These signals are amplified by syndecan1 (CD138), which binds HGF and acts as a functional co-receptor for HGF (12). In the present study, we have assessed the functional impact of HGF/MET signaling on MM cells. We show that MET is expressed by the majority of MMs, and that HGF is a potent myeloma growth factor, stimulating proliferation and protecting MM cells from apoptosis via RAS/MAPK and PI3K/PKB signaling.

**MATERIALS AND METHODS**

**ANTIBODIES**

Mouse monoclonal antibodies used were; anti-CD38, BB4 (IgG1)(Instruchemie, Hilversum, The Netherlands); anti-MET, DO24 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); APC-conjugated anti-CD38, (IgG1); FITC-conjugated anti-CD45RA (IgG2b); anti-RAS (IgG1) (all BD Biosciences, Erkembodegem, Belgium). Polyclonal antibodies used were; micobead-conjugated goat anti-mouse IgG (Miltenyi, Bergisch Gladbach, Germany); rabbit anti-MET, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho PKB/AKT (Ser 473); rabbit anti-phospho p44/42 MAP kinase (Thr 202/Tyr 204) (both New England Biolabs, Hitchin, UK); HRP-conjugated rabbit anti-mouse; (DAKO, Carpinteria, CA); and HRP-conjugated goat anti-rabbit (DAKO).

**PLASMA CELL NEOPLASMS AND CELL LINES**

BM-derived patient MM cells (n=13) and tissue samples of extra-medullary plasmacytoma (n=8) were obtained during routine diagnostic procedures. Tissue samples were frozen at -80°C until further used. The patient myeloma cells (PPM 1 and 2) used in our functional studies were obtained from the pleural effusions of a 67 year old male, and a 62 year old female MM patient. FACS analysis of both patients showed >95% CD138(HIGH), CD38(HIGH) cells. PPM1 was expressing IgD only. Mononuclear cells were harvested by standard Ficoll-Paque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden) and kept on an irradiated mouse embryonic fibroblast feeder-layer in Iscove's medium (Life technologies, Breda, The Netherlands) containing 10% fetal calf serum (Intergro, Zaandam, The Netherlands), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Life Technologies), and 500 pg/ml IL6 (R&D systems, Abington, UK). MM cell line XG1 (17), LME1(12) and UM6 (18) were cultured in Iscove's medium (Life technologies) containing 10% fetal calf serum (HyClone Laboratories, Logan, UT), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Life Technologies). 20 μg/ml human recombinant transferrin (Sigma, Bornem, Belgium), 50μM β-mercapto ethanol, XG1 and UM6 were cultured in the presence of 500 pg/ml IL6 (R&D systems). MM cell lines U1M and UM3 (18), L363 (19), NCI H929 (20) and OPM1 (21) were cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands) containing 10% fetal calf serum (Integro). Burkitt parental Namalwa and the MET-transfected cell line Namalwa (NamMET, (22)) were cultured in RPMI 1640 supplemented with 10% Fetal Clone I serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all Life Technologies).

**MAGNETIC ACTIVATED CELL SORTING (MACS) AND FLUORESCENT ACTIVATED CELL SORTING (FACS)**

Mononuclear cells from BM biopsies were obtained by standard Ficoll-Paque density gradient centrifugation. Plasma cells were sorted by positive selection using anti-CD138 (clone BB4, Immunotech). Cell suspensions were incubated with saturating concentrations of anti-CD138 antibody, washed, and subsequently incubated with microbead-conjugated goat anti-mouse IgG. Microbead-labeled cells were recovered with a magnet, and the purity of the collected cell populations was determined by FACS analysis. A double staining using antibodies against CD38 and CD45RA was done as described (23). Staining was measured by using a FACScalibur flow cytometer (BD Biosciences). Positive sorting yielded populations plasma cells that were >95% pure (CD38+, CD45RA-).

**IMMUNOHISTOCHEMISTRY**

Immunohistochemical stainings were performed
on acetone-fixed cryostat sections (MET) or formalin fixed paraffin embedded sections (CD138). For single staining, fixed sections were washed in PBS and pre-incubated with 10% normal goat serum (Sera Lab, Sussex, UK) in PBS for 15 min. After incubating with the primary antibody for 1 h, endogenous peroxidases were blocked with 0.1% 
\[ \text{NaNO}_2 \] \text{H}_2\text{O}_2 \] PBS for 10 min. Subsequently, the sections were stained with biotin-conjugated rabbit anti-mouse for 30 min, followed by an incubation with HRP-conjugated avidin-biotin complex for 30 min. Substrate was developed with either 3,3'-diaminobenzidine (Sigma) (anti-MET staining), or 3,3'-diaminobenzidine (Sigma) (anti-CD138 staining) for 10 minutes.

**PULL-DOWN AND WESTERN BLOT ANALYSIS**

For precipitation of GTP-bound RAS, a fusion protein of glutathione S-transferase (GST) with the minimal RAS-binding domain of RAS (RAF-RBD) was used as described (24). Briefly, 1 - 10³ cells were stimulated with HGF (100 ng/mL) for 2 minutes at 37 °C. GST-RAF-RBD fusion proteins were coupled to glutathione-Sepharose beads for 30 minutes at 4°C, after which cell lysates were added and incubated for 30 minutes at 4°C. Bound proteins were eluted with sample buffer, separated by 10% SDS-polyacrylamid gel electrophoresis, and blotted. For analysis of phosphorylation of PKB and the MAP kinases ERK1 and -2, after the indicated treatments with 100 ng/mL human recombinant HGF (R&D) and the PI3K inhibitor LY294002 or the MEK inhibitor PD98059 (both Biomal, Plymouth Meeting, PA), 3 x 10⁵ cells were directly lysed in sample buffer, separated by 10% SDS-polyacrylamid gel electrophoresis, and blotted. Equal loading was confirmed by staining the part of the blot above 130 kDa with anti-MET (C12). The middle part (50-130 kDa) was stained with anti-phospho PKB and the bottom part (below 50 kDa) was stained with anti-phospho MAP kinase antiserum (both New England Biolabs). Primary antibodies were detected by HRP-conjugated goat anti-rabbit antibodies.

**GROWTH, SURVIVAL AND APOPTOSIS ASSAYS**

Cells were plated in 96 flat bottom tissue culture plates (Costar, Cambridge, MA) at a density of approximately 100,000 cells/mL (200 x L total) in the absence of serum, in supplemented Iscove's as described above. HGF was added, and cells were cultured for four days. Viable cell numbers were determined by adding propidium iodide (PI) and analysis on a FACScanLibur (BD Biosciences). For proliferation, the cell culture was pulsed with 0.5 µCi (methyl-³H) thymidine (87 Ci/mmol, Amersham Life Science, Little Chalfont, UK) during the last 4 hours of culture. Results are expressed as counts per minute (cpm). Apoptosis was measured using FITC-conjugated AnnexinV (IQ Products, Groningen, The Netherlands) binding to phosphatidylserine, PI incorporation and FACS analysis as described (25).

**Results**

**Expression of MET on primary plasma cell neoplasm and MM cell lines**

Previous studies have indicated that MET, the receptor tyrosine kinase for HGF, can be expressed on MM cells (10, 11). Here, we confirm and extend these observations by studying a panel of primary MM, extramedullary plasmacytomas, and MM cell lines. In 6 of 8 MM cell lines tested, a 145 kDa band, corresponding to MET, was detected by immunoblotting (Fig 1A). This band was also present in the cell lysates of frozen stored BM aspirates from 7 of 13 MM patients, but not in normal control BM (Table 1). Importantly, in BM cell populations enriched for neoplastic (CD138+) plasma cells by means of MACS, the intensity of the MET bands was proportionally increased, indicating that MET was indeed expressed by the tumor cells (Fig 1B). In addition to MM, we also assessed MET expression on a panel of extramedullary plasmacytomas. Immunohistochemistry on frozen tissue sections revealed MET expression in 3 of 8 cases. Taken together, our findings demonstrate that MET is expressed by most myeloma cell lines and that MET expression is common on primary MMs as well as on extramedullary plasmacytomas (Table 1).

**HGF induces proliferation and suppresses apoptosis of MM cells**

We next explored the functionality of the MET signaling pathway in MM cells. Stimulation with HGF resulted in a rapid increase in the amount of active, GTP-bound RAS in both the MM cell lines as well as in the primary tumor cells from a MM patient (PPM 1) (Fig 2A). Also, the downstream effector MAP kinases ERK1 and -2 were strongly activated in response to HGF stimulation (Fig 2A). In the MM cell lines LME1 and XG1 and in the pri-
primary MM cells, we also observed a strong HGF-induced serine phosphorylation of PKB (also known as AKT). Hence, signaling through MET in MM leads to activation of the PI3K/PKB as well as the RAS/MAP kinase signaling pathways, which have been implicated in the regulation of cell survival and proliferation, respectively (26-28).

To assess whether HGF indeed affects the growth of MM, MM cell lines LME1, XG1, and primary myeloma cells were cultured in the absence or presence of HGF. In LME1 cultures, serum-deprivation resulted in a strong reduction in the number of viable cells, whereas HGF stimulation resulted in a dose dependent exponential increase in cell numbers (Fig 2B, upper left panel). In XG1 cultures deprived of serum, we also observed a rapid decrease in the number of viable cells. Culturing these cells in presence of recombinant HGF resulted in a rescue with stable cell number, rather than in an increased cell number (Fig 2B, upper right panel). Like in LME1, stimulation of primary myeloma cells (PPM1) with HGF led to an exponential, dose-dependent increase in cell numbers (Fig 2B, lower left panel). Also, a second primary MM sample (PPM2) responded to HGF treatment. Like XG1, triggering of these primary MM cells recombinant HGF resulted in a rescue of cell numbers (Fig 2B, lower right panel).

To explore whether the above effects of HGF on MM growth were due to increased cell proliferation or decreased apoptosis (or both), we examined the effects of HGF stimulation on DNA synthesis by MM cells and on their capacity to bind AnnexinV. As is shown in Figure 3, HGF induced a strong increase in $^3$H thymidine uptake in both MM cell lines, and primary MM cells (PPM1 and 2). Moreover, HGF stimulation also induced a strong reduction in the percentage of apoptotic cells, which bind AnnexinV via phosphatidylserine exposed on their outer plasma membrane (Fig 4A and B).
Figure 2. HGF is a growth factor for MMs. A) HGF activates both the RAS/MAP kinase and PI3K/PKB signaling cascades in MM. Cells were incubated for 2 minutes with HGF (100 ng/mL). Activation of RAS was assayed using a pull-down assay with GST-RAF-RBD fusion proteins, blotted and stained with a monoclonal anti-RAS antibody (top panel). Activation of ERK1 and -2 and PKB was assessed by immunoblotting using phospho-specific anti-ERK1 and -2 (α p-MAPK) and anti-PKB (α p-PKB), respectively. Stainings with anti-MET represent loading controls (bottom panel). B) HGF stimulation induces growth of MMs. LME1, XG1, and two primary myeloma cell samples (PPM1 and 2) were grown in the absence of serum, and HGF was added at the concentrations indicated. The number of viable cells was quantified using propidium iodide incorporation and FACS analysis over a 4-day period. Error bars represent the standard deviation of triplicates.
HGF-induced proliferation in MM cells is PI3K- and MEK1-dependent whereas rescue from apoptosis requires PI3K

As shown in Figure 2A, triggering of MET leads to activation of PKB as well as RAS and the MAP kinases ERK1 and -2. PKB, a target of PI3K-derived signals, has been implicated in the maintenance of both growth and survival (26, 28, 29), whereas RAS and its downstream effector components MEK1, and ERK1 and -2 have been directly linked to the regulation of cell proliferation (27). To investigate the functional importance the PKB/PI3K and RAS/MAP kinase cascades in MM growth and survival, we measured ³H thymidine uptake and AnnexinV binding in the presence or absence of the PI3K inhibitor LY294002 (LY) or the MEK inhibitor PD98059 (PD). The specificity of these inhibitors in our system is shown in figure 5A: the PI3K inhibitor LY completely abrogated the HGF stimulated activation of PKB but had no effect on the phosphorylation status of ERK1 and -2. Vise versa, the HGF stimulated activation of ERK1 and -2 was specifically blocked by PD but not affected by LY (Fig 5A).

Both PD and LY had dramatic effects on HGF-induced cell proliferation in LME1 as well as in XG1 cells (Fig 5B). With the MEK-inhibitor PD, ³H thymidine uptake was reduced to control levels, whereas an even stronger inhibition, below that
Figure 4. HGF protects MM cells from apoptosis. A) Activation of MET leads to rescue from apoptosis in XG1. Cells were cultured under serum-free conditions and HGF was added at the concentrations indicated. After 36 hours of culture, propidium iodide was added, and apoptotic cells were identified by their binding of FITC-conjugated Annexin V. Apoptotic cells were defined as propidium iodide negative, Annexin V positive cells. B) HGF protects the MM cell lines LME1 (upper left panel), and XG1 (upper right panel), as well as primary myeloma cells (PPM1 and 2)(bottom panels) from apoptosis. Culture conditions and apoptosis assessment as in (A). Error bars represent the standard deviation of triplicate measurements.
Figure 5. HGF-induced proliferation in MM cells is PI3K and MEK1-dependent whereas rescue from apoptosis requires PI3K. A) HGF-mediated activation of PKB and ERK1 and -2 is blocked by inhibition of PI3K and MEK1, respectively. Cells were deprived of serum for 3 hours, and incubated with medium containing LY294002 (20 μM), PD98059 (50μM), or DMSO only, prior to incubation with HGF (100 ng/mL). Activation of PKB and ERK1 and -2 was determined in total cell lysates of LME1 and XG1 by immunoblotting with anti-phospho PKB (top) and anti-phospho ERK1 and -2 (middle) antibodies, respectively. Staining with anti-MET represent loading controls (bottom). B) HGF-induced proliferation is mediated by PI3K and MEK-1. LME-1 and XG-1 cells were incubated with medium containing either LY or PD, and stimulated with HGF. Proliferation was assessed by measuring 3H thymidine incorporation at day 3. C) HGF-induced rescue from apoptosis requires PI3K. LME-1 and XG-1 cells were incubated with medium containing either LY or PD, and stimulated with HGF. Apoptosis was assessed by FACS by measuring binding of FITC-conjugated Annexin V and propidium iodide incorporation. Apoptotic cells were defined as propidium iodide negative, Annexin V positive cells. Error bars represent standard deviations of triplicate measurements.

of the unstimulated controls, was obtained using the PI3K-inhibitor LY (Fig 5B). In contrast to their almost identical effects on cell proliferation, PD and LY differentially inhibited the anti-apoptotic action of HGF. Whereas HGF-induced survival was completely abolished (LME1), or strongly reduced (XG1) by LY, it was hardly affected by PD (Fig 5C). These data indicate that whereas the PI3K/PKB and RAS/MAP kinase pathways are both needed for proliferation, the HGF mediated effects on the survival of MM cells depend on the PI3K/PKB pathway, but not on the RAS/MAP kinase pathway.

DISCUSSION

MM is a still incurable B cell neoplasm characterized by the accumulation of malignant plasma cells in the BM. Since MMs are genetically unstable, and consequently heterogenous, multiple coordinate and overlapping signals from the microenvironment presumably determine the faith of individual subclones during MM progression. For successful therapeutic intervention, it is critical to identify the various signals controlling MM growth and survival. Although there is a vast amount of circumstantial evidence implicating the HGF/MET pathway in the pathogenesis of multiple myeloma (30-32), direct functional support for this role thus far is scarce and comes from a single recent study from our laboratory showing that HGF induces proliferation in the myeloma cell line XG1 (12). The present paper greatly extends this observation by showing that HGF also has strong proliferative effects on another myeloma line and on two primary myeloma cell samples. Moreover, it shows for the first time that HGF is a potent survival factor for both myeloma cell lines and primary myeloma cells and rescues these cells from apoptosis. Finally, it demonstrates that whereas PI3K/PKB pathway is required for both HGF-induced proliferation and rescue from apoptosis, the RAS/MAPK pathway is required for proliferation.

By studying a panel of MM cell lines, primary MMs, and extramedullary plasmacytomas, we observed that the receptor tyrosine kinase MET is expressed by the majority of MM cell lines and by approximately half of the primary plasma cell neoplasms tested (Fig 1, Table 1). This observation confirms and extends previous studies, which have also reported MET protein expression on MM cells, albeit on a much smaller number of samples (10, 11). HGF and its receptor tyrosine kinase MET induce complex biological responses in target cells including growth, survival, and motility. In mice, Met or Hgf deficiency results in embryonic death with severe defects in the development of the placenta, liver, and limb muscles, whereas uncontrolled activation of MET, in both mice and humans, has been implicated in tumor growth, invasion, and metastasis (reviewed by van der Voort et al (13)). Of note, the finding of MET mutations in hereditary papillary renal carcinoma has established a causative role of MET in human cancer (33). These mutations result in enhanced kinase activity upon stimulation with HGF and were shown to mediate transformation, invasive growth, and protection from apoptosis (34-37). The HGF/MET pathway has also been implicated in B cell development and neoplasia. During normal B cell differentiation, MET is expressed at the germinatal center and plasma cell stage, whereas HGF is produced by follicular dendritic cells and by bone marrow stromal cells. HGF stimulation of B cells leads to integrin activation, promoting cell adhesion to VCAM1, a major integrin ligand on follicular dendritic cells and bone marrow stromal cells (22). In B cell malignancies, specifically in MM, HGF produced in the tumor microenvironment (15) could promote tumorigenesis in a paracrine fashion, whereas co-expression of HGF and MET has also been observed, suggesting autocrine stimulation (10,11,14). Consistent with a role for HGF/MET in the pathogenesis of MM, elevated serum levels of HGF were reported in MM patients and identified a group of patients with poor response to treatment (16). Whether this HGF represented autocrine production by the tumor cells, or was paracrine-derived, remains to be determined. Since only 1 of the 6 MET positive MM cell lines in our present study expressed detectable
**Figure 6. HGF induces growth and survival in Multiple Myeloma.** HGF is produced either by the bone marrow stromal cells in a paracrine fashion, or autocrine by MM cells, leading to MM growth and survival. Furthermore, direct physical contact of myeloma cells with BM stromal cells via integrin adhesion receptors, can mediate growth and survival signals and trigger cytokine production. INSET: Para- or autocrine produced HGF will be sequestered on the plasma membrane by the heparan sulfate (HS) side chain of syndecan1, thereby promoting ternary-complex formation between HGF, Met, and syndecan1 facilitating activation, leading to enhanced signal transduction. MET autophosphorylation leads to the recruitment of downstream effector molecules GRB2 and GAB1 involved in activation of the RAS/MAPK as well as the PI3K/PKB pathways. Activation of RAS/MAPK will trigger proliferation in multiple myeloma. Direct as well as indirect activation of PI3K can activate PKB, which controls proliferation and prevents apoptosis. Also, additional pathways (depicted as question marks) may be activated by HGF through PI3K, leading to both growth and survival in MM. See text for additional comments.

levels of HGF protein, autocrine growth may not be the most common scenario (data not shown). It is conceivable, however, that during progression of MM a gain of HGF expression may take place, establishing an autocrine HGF/MET activation loop, leading to autonomous growth and to dissemination to extra-medullary sites.

The key finding of our study is that HGF is a potent growth and survival factor for MMs. In both MM cell lines and primary MM cells, HGF stimulation induced a strong dose-dependent increase of DNA synthesis (Fig 3). Moreover, it has potent anti-apoptotic effects (Fig 4). The HGF-induced proliferation requires activation of both MEK and PI3K, whereas activation of either MEK and MAPK or PI3K and PKB is not sufficient. In contrast, HGF controlled survival requires activation of PI3K only (Fig 5). Activation of the RAS/MAPK pathway by HGF involves recruitment of a complex of the exchange factor SOS and GRB2 to the docking site of Met, resulting in RAS activation (38-40). This will lead to translocation and activation of RAF and the consecutive activation of MEK and the MAP kinases ERK1 and 2 (41, 42). Activation of these MAP kinases results in phosphorylation of transcription factors (e.g. ELK1 and ETS2), which mediates the expression of immediate early genes such as FOS, leading to cell proliferation (27). In MM, several cytokines/growth factors such as IL6, VEGF and IGF1, activate the RAS/MAP kinase cascade, leading to proliferation (5,7,8). Our study is the first to show that HGF is able to activate RAS, MEK and the MAP kinases ERK1 and –2, inducing a proliferative response in MM. Furthermore, we show that activation of MEK is required, but not sufficient for HGF-induced proliferation of MM.

Our data shows that PI3K is required for both HGF-induced proliferation as well as survival of MM (Fig 5B and C). Activation of the PI3K/PKB pathway by HGF involves recruitment of PI3K to the docking site of Met, either by a direct interaction, or indirectly via the docking protein GAB1 (43-45). Studies in a variety of other cell types have also revealed a prominent regulatory role for PI3K in either HGF-induced proliferation (46-48) and survival (49-51). Furthermore, in MM, PI3K has been implicated in IL6-induced proliferation (29, 52) and in the rescue from apoptosis by either IL6 (29,53) or IGF1 (52). The most likely candidate for executing both the PI3K-mediated proliferation and survival signals is the PH domain-containing effector molecule PKB (54), which, upon PI3K-depend-
MET MEDIATES GROWTH AND SURVIVAL IN MULTIPLE MYELOMA

...membrane localization, is phosphorylated and activated by PDK1 (55,56). Indeed, we observed a strong PI3K-dependent phosphorylation of PKB upon HGF stimulation of the MM cells (Fig 2A). Recent studies have revealed that PKB mediates HGF-induced survival responses in other cell types (49,50), whereas in MM, PKB is involved in IL6-induced proliferation (57). PKB can control both survival as well as proliferation by a wide variety of mechanisms, including the phosphorylation of Forkhead transcription factors (58-70), IKKα (61), GSK3 (62), and mTOR (63). Noteworthy, two recently identified substrates of mTOR, p70S6kinase and the translational repressor 4E-BP1, are involved in the IL6-controlled MM growth (64). Likely candidates to directly mediate the HGF-induced anti-apoptotic signal via the PI3K/PKB pathway are caspase 9 and the pro-apoptotic protein BAD, which are both activated upon phosphorylation by PKB. (26, 65, 66). Importantly, recent studies revealed that in MM, IL6, in a PI3K-dependent fashion, controls the activity of Forkhead transcription factors and caspase 9 (29), whereas IGFI stimulation results in BAD phosphorylation (8,29). Further investigation regarding the activation of downstream components of the PI3K/PKB pathway will help to clarify the proliferative and anti-apoptotic responses initiated by HGF in MM.

Interestingly, we have recently shown that HGF/MET signaling in MM is strongly promoted by syndecan-1, the major heparan sulfate proteoglycan (HSPG) on MM cells (12). Cell surface-expressed syndecan1 binds HGF and presumably promotes signaling by increasing the effective concentration of HGF at the plasma membrane, whereas binding of several HGF molecules to syndecan1 may promote dimerization and oligomerization of Met, leading to enhanced receptor activation. Alternatively, by inducing a conformational change, syndecan1 might influence the affinity of HGF for MET. Also, the polarized distribution of syndecan-1, as observed in MM cells (31), may impose a constraint on the spatial distribution of HGF, resulting in clustering of activated MET and MET-associated signaling molecules. Thus, the potentiation of MET signaling may be partially explained by HGF-mediated colocalization of syndecan1 and MET, which may bring relevant intracellular signaling molecules in the proximity of each other. Taken together, our results indicate that the HGF, Met, and syndecan1, form a "ménage a trois" with a key role in controlling the growth and survival of MET positive malignant plasma cells (Fig 6) and suggest these molecules as targets for therapy in MM.

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CHAPTER 2


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