Expression of c-Kit isoforms in multiple myeloma: differences in signaling and drug sensitivity

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ABSTRACT

Background

c-Kit is expressed in the plasma cells from 30% of patients with multiple myeloma. Two different isoforms of c-Kit, characterized by the presence or absence of the tetrapeptide sequence GNNK in the extracellular domain, have been described. However, their expression and function in myeloma cells are unknown. We explored the function and expression of these c-Kit isoforms in myeloma cells.

Design and Methods

Expression of c-Kit isoforms was investigated by reverse transcriptase polymerase chain reaction in fresh plasma cells from patients and cell lines. The function of these c-Kit isoforms was analyzed upon expression in myeloma cells. Signaling was investigated by western blotting using antibodies specific for activated forms of several signaling proteins. The impact of c-Kit on the action of drugs commonly used in the treatment of multiple myeloma was investigated by MTT proliferation assays.

Results

Fresh plasma cells from patients as well as myeloma cell lines expressed the two isoforms of c-Kit. Retroviral infection of myeloma cells with vectors that code for c-Kit-GNNK$^+$ or c-Kit-GNNK$^-$ forms demonstrated differences in the kinetics of phosphorylation between these isoforms. Stem cell factor-induced activation of the GNNK$^-$ form was faster and more pronounced than that of the GNNK$^+$ form, whose activation, however, lasted for longer. The c-Kit receptors weakly activated the Erk1/2 and Erk5 pathways. Both receptors, however, efficiently coupled to the PI3K/Akt pathway, and stimulated p70S6K activation. The latter was sensitive to the mTOR inhibitor, rapamycin. Studies of drug sensitivity indicated that cells expressing the GNNK$^-$ form were more resistant to the anti-myeloma action of bortezomib and melphalan.

Conclusions

Our data indicate that c-Kit expression in multiple myeloma cells is functional, and coupled to survival pathways that may modulate cell death in response to therapeutic compounds used in the treatment of this disease.

Key words: c-Kit isoforms, multiple myeloma, signaling, drug-sensitivity.

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by the accumulation of monoclonal plasma cells in the bone marrow.[1] A subset of patients with MM expresses c-Kit (CD117) in tumor plasma cells, while this antigen is absent in normal plasma cells.[2] c-Kit is a transmembrane glycoprotein that belongs to the subclass III receptor tyrosine kinase family, which also includes PDGF-R, CSF-1 and Flt-3.[3,4] c-Kit is expressed in hematopoietic progenitor cells, mast cells, germ cells, melanocytes and interstitial cells of Cajal.[5] Abnormal expression or function of c-Kit is found in mast cell leukemia, mastocytosis, acute myeloid leukemia, gastrointestinal stromal tumors, breast carcinoma, germ cell tumors and MM.[2,5,6] Moreover, drugs that inhibit the action of c-Kit have demonstrated clinical benefit in malignancies, such as gastrointestinal stromal tumors, in which this receptor tyrosine kinase may have a pathogenetic role.[7,8] Physiological activation of c-Kit occurs upon binding to the ligand stem cell factor (SCF). Ligand binding induces dimerization of the receptor and activation of its tyrosine kinase activity.[9] The activated receptor then becomes autophosphorylated in several tyrosine residues, which serve as docking sites for signal transduction molecules containing Src homology 2 (SH2) or phosphotyrosine binding domains.[10] Binding of specific substrates to these phosphorylated residues initiates a signaling cascade responsible for the cellular responses to c-Kit activation.[11]

Two isoforms of c-Kit, produced by alternative mRNA splicing, have been identified in humans.[12,13] These isoforms are characterized by the presence or absence of a tetrapeptide sequence (GNNK) in the juxtamembrane extracellular region (Figure 1A). The two isoforms, termed $GNNK^+$ and $GNNK^-$, are co-expressed in several tissues with a predominant presence of the $GNNK^-$ isoform.[13-17] These isoforms display distinct signaling characteristics, and also different transforming activity in NIH3T3 cells.[18] In these cells, tyrosine phosphorylation of c-Kit and activation of Erk1/2 was more efficient for the $GNNK^-$ than for the $GNNK^+$ isoform. In addition the $GNNK^-$ isoform strongly promoted anchorage-independent growth, loss of contact inhibition, and led to increased tumorigenicity in nude mice.[18]

In this study, we investigated the expression and biological role of the c-Kit isoforms in MM.

Design and Methods

Reagents and immunochemicals

Cell culture media, sera, and penicillin-streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). Protein A-sepharose was from Amersham-Pharmacia (Piscataway, NJ, USA). Immobilon P membranes were from Millipore (Bedford, MA, USA). Interleukin-6, SCF and insulin-like growth factor 1 were from Strathmann (Hamburg, Germany). STI571 was from Novartis Pharma (Basel, Switzerland), PP2, LY294002, and rapamycin were from Calbiochem (La Jolla, CA, USA). Dexamethasone, melphalan, and 3(4,5 dimethiylthiazol-2-yl)-2,5-dipheniltetrazolium bromide (MTT) were from Sigma Chemical (St Louis, MO, USA). PS341 (bortezomib) was from Millenium Pharmaceuticals Ltd. (Cambridge, MA, USA). Other generic chemicals were purchased from Sigma Chemical (St. Louis, MO, USA), Roche Biochemicals (Mannheim, Germany), or Merck (Darmstadt, Germany). The anti-pGSK3β, and anti-pp70S6 kinase antibodies were from Cell Signaling (Beverly, MA, USA). The anti-Erk1/2, anti-pErk1/2, anti-Erk5, and anti-PY99 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-pAkt, anti-Akt, anti-c-Kit, and anti-CD38 antibodies were from BD Biosciences (San Diego, CA, USA). The anti-c-Kit anti-endodomain antibody was generously provided by Dr L Rönnstrand (Lund University, Malmö, Sweden). The horseradish peroxidase–conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA). The anti-Erk5 antibody has been described previously.[19]

Cell culture and transfections

The myeloma cell lines MM1S (from Dr S T Rosen, Chicago, IL, USA), MM144, OPM2 (from Dr S Rudikoff, Bethesda, MD, USA), U266, and RPMI8226 (from Dr W Dalton, Tampa, FL, USA) U266-LR7, and MGG were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2-95% air. Cells were grown in RPMI 1640 medium with L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% (cell lines) or 20% (patients' cells) fetal bovine serum (FBS). Unless otherwise indicated, for the biochemical experiments, myeloma cells were treated in complete media that had been in contact with the cells for at least 24 hours. Patients' MM cells were isolated from bone marrow aspirates as previously described.[20]

Generation of retroviruses and infection

293T cells were plated in 60-mm–diameter dishes (1.8 x10^6 cells in 3 mL of Dulbecco's modified Eagle's medium with 10% FBS) and allowed to attach overnight. Five minutes prior to transfection, 25 μM chloroquine were added to each plate. The transfection solution contained DNA (2.5 μg pMDGVSV, 5 μg pNGVL-MLV-gag-pol, 3 μg retroviral vector [pLZR-IRES-GFP, pLZR-c-Kit-GNNK(+)-IRES-GFP, or pLZR-c-Kit-GNNK(-)-IRES-GFP]), 61 μL 2 M $CaCl_2$, and doubled-distilled H_2O to make a volume of 500 μL. After mixing, 0.5 mL of 2x Hepes-buffered saline solution (pH 7.0) were added, and the solution was bubbled for 15 seconds. The Hepes-buffered saline solution-DNA complex was dropped onto cells. Eight hours later, this medium was replaced with complete culture medium that was replaced 24 to 32 hours after transfection with 3 mL fresh virus-collecting medium. Twenty-four hours later, the supernatant from transfected cells was centrifuged at 1,000 x g for 5 minutes. MM1S cells were infected with 1 mL viral supernatants containing Polybrene at 6 μg/mL, to which 9 mL RPMI 1640 with 10% FBS medium were added 1 hour later. The following day, the medium was changed to overnight-infected MM cells.

Cell proliferation assays

The analysis of MM cell proliferation using the MTT assay has been previously described.[21] Four wells were analyzed for each condition, and the results are presented as the mean±SD of quadruplicates of a representative experiment that was repeated at least twice.

Immunoprecipitation and western blotting

Cells were collected and centrifuged at 10,000 x g for 2 minutes. The cells were then washed with PBS and lysed in ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris, pH 7.0, 1 μM pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM sodium orthovanadate). Samples were centrifuged at 10,000 x g at 4°C for 10 minutes and supernatants were transferred to new tubes with the corresponding antibody and protein A-sepharose. The rest of the immunoprecipitation and western blotting protocol was carried out as previously described.[22] For quantitation of the bands in the western blots we used the NIH Image 1.61 program, and the intensities are indicted as the fold change with respect to the resting unstimulated values.

Reverse transcriptase polymerase chain reaction (RT-PCR) assay

RNA was isolated from MM1S cells, and an equal amount (2 μg) from the different samples was primed with poly-T. cDNA was synthesized with M-MLV reverse transcriptase (RT; Promega). The following specific primers were used to amplify the cDNA: 5´-ATGTGGGCAAGACTTCTGCCTA-3´ and 5´-ATCATGCCAGCTACGATTA-3´.

Quantitative estimation of phosphorylation intensity of c-Kit isoforms stimulated by SCF

The phosphorylation intensity in western blots was quantified by using NIH image 1.61 software. The maximal intensity band was taken as 100%, and the percentage intensity of each band was refered to that value. Data show the mean ± SD for three different experiments.

Flow cytometry

Immunophenotypic analyses were performed on MM cell lines and a bone marrow sample obtained from a patient with MM. A total of 2×10^6 cells/tube were stained with $CD38^-$ and CD117 using a direct immunofluorescence technique. Plasma cells were identified in the side scatter/$CD38^+$ zone of the cytogram where they are located, and CD117 expression of this population was analyzed using the Paint-a-Gate program (BD Biosciences).

Results

Expression of the c-kit isoforms $GNNK^+$ and $GNNK^-$ in MM cells

To analyze the expression of the c-Kit isoforms $GNNK^+$ and $GNNK^-$ in MM cell lines and in fresh plasma cells from patients, we performed RT-PCR with oligonucleotides that are able to distinguish between both c-Kit isoforms (Figure 1A). All patients and five out of the seven MM cell lines expressed both isoforms, with the $GNNK^-$ isoform being predominant (Figure 1B). In two MM cell lines (OPM2 and MGG), as well as in a promyelocytic leukemia-derived cell line (NB4) that was used as control, only the c-Kit $GNNK^-$ isoform was expressed.

We next investigated whether expression of c-Kit in MM cells was functional. For these exploratory experiments, we used the RPMI8226 cell line, because it showed a high level of CD117 staining by flow cytometry when compared to other MM cells lines (Figure 1C, and *data not shown*). Addition of SCF to these cells resulted in receptor activation, as indicated by an increase in tyrosine phosphorylation of c-Kit (Figure 1D). While these results demonstrated that endogenously expressed c-Kit was functional, as RPMI8226 cells expressed both c-Kit receptors, the contribution of each isoform to the action of SCF could not be assessed.

To analyze the biochemical and biological functions of each of the c-Kit isoforms in MM, we decided to use MM1S cells. Even though RT-PCR experiments indicated that these cells expressed c-Kit, flow cytometry or western blotting studies failed to reveal detectable levels of the c-Kit protein (Figure 2A and B, and *data not shown*). We infected MM1S cells with the retroviral vector PLZR-IRES-GFP containing the coding sequence for c-Kit-$GNNK^+$ or c-Kit-$GNNK^-$. We also infected MM1S cells with the empty vector, which was used as a control. FACS analyses of infected MM1S populations indicated that MM1S-$GNNK^-$ and MM1S-$GNNK^+$ were expressed in more than 85% of the cells. However, we did not observe c-Kit staining in cells infected with the empty vector (MM1S-PLZR) (Figure 2A). The mean fluorescence intensity obtained in these transfectants was close to that observed in patients' $CD117^+$ plasma cells (*data not shown*). Expression of the two c-kit isoforms, $GNNK^+$ and $GNKK^-$, in MM1S was also analyzed by western blotting using an anti-C-terminus antibody. As shown in Figure 2B, $GNNK^+$ and $GNNK^-$ were clearly detected in MM1S cells infected with the respective receptors.

Dose-dependence and time-course of activation of the c-Kit isoforms

Previous reports on NIH3T3 cells described that the kinetics of phosphorylation of the c-Kit isoforms $GNNK^+$ and $GNNK^-$ differ.[18] In those cells, SCF stimulated the $GNNK^-$ isoform more rapidly and to a greater extent than the $GNNK^+$ isoform. To check whether this also applied to MM cells, we explored the kinetics of activation of both iso. We first performed dose-response analyses to select an adequate dose of SCF. For this purpose, MM1S-$GNNK^+$ and MM1S-$GNNK^-$ were treated with different doses of SCF, and anti c-Kit immunoprecipitates were then probed with anti-phosphotyrosine antibodies. As shown in Figure 2C, SCF stimulated tyrosine phosphorylation of both isoforms in a dose-dependent manner. Activation was detected at 0.5 nM SCF, and reached a maximum at 10 nM.

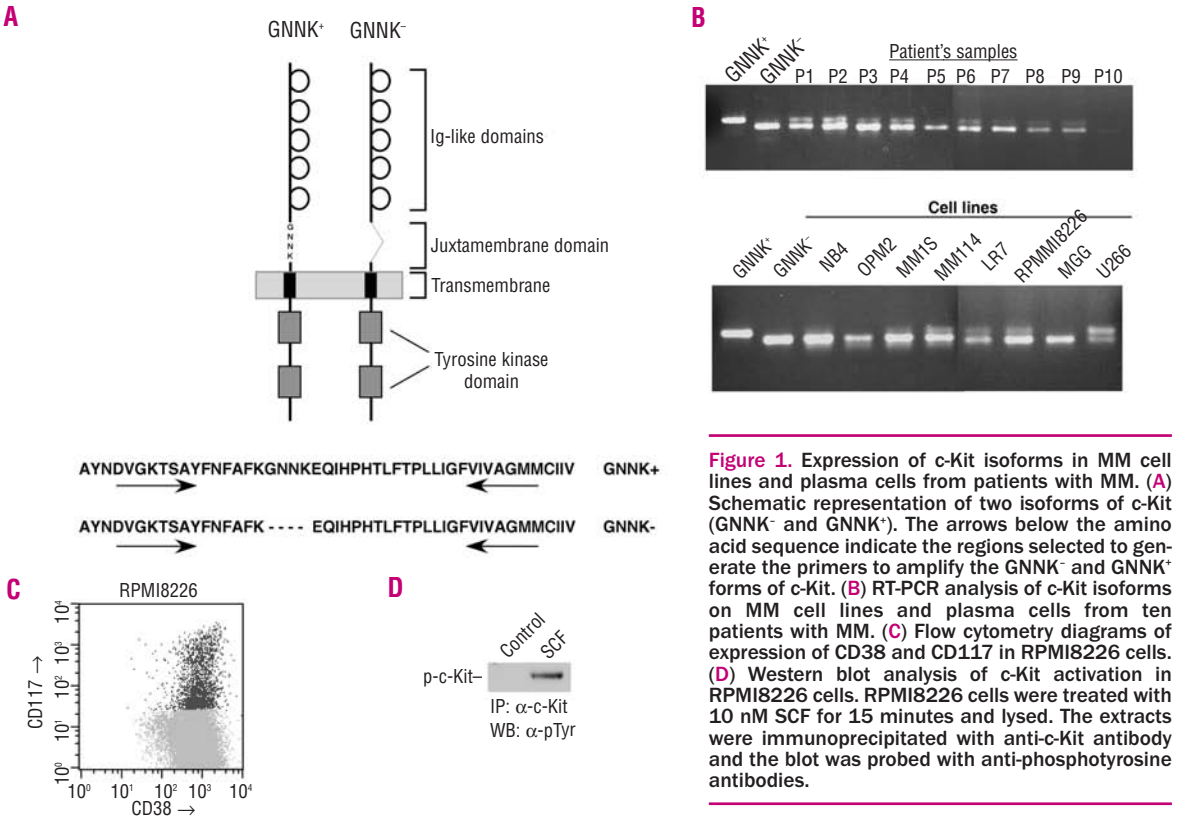

Figure 1. Expression of c-Kit isoforms in MM cell lines and plasma cells from patients with MM. (A) Schematic representation of two isoforms of c-Kit (GNNK⁻ and GNNK⁺). The arrows below the amino acid sequence indicate the regions selected to generate the primers to amplify the GNNK⁻ and GNNK⁺ forms of c-Kit. (B) RT-PCR analysis of c-Kit isoforms on MM cell lines and plasma cells from ten patients with MM. (C) Flow cytometry diagrams of expression of CD38 and CD117 in RPMI8226 cells. (D) Western blot analysis of c-Kit activation in RPMI8226 cells. RPMI8226 cells were treated with 10 nM SCF for 15 minutes and lysed. The extracts were immunoprecipitated with anti-c-Kit antibody and the blot was probed with anti-phosphotyrosine antibodies.

Interestingly, the level of tyrosine phosphorylation of the GNNK⁻ form obtained with 1 nM of SCF was substantial, and higher than that observed in GNNK⁺ cells treated with the same dose of SCF.

To analyze the kinetics of receptor activation, MM1S-GNNK⁺ and MM1S-GNNK⁻ were treated with 10 nM SCF, and tyrosine phosphorylation of c-Kit was followed at different times (Figure 2D). SCF-induced c-Kit phosphorylation was faster and more pronounced in the GNNK⁻ cells than in the GNNK⁺ ones, peaking at 15 and 30 minutes for the GNNK⁻ and GNKK⁺ forms, respectively. However, the GNNK⁺ form remained phosphorylated for longer (Figure 2D and E).

STI-571 and Src inhibitors block SCF-induced tyrosine phosphorylation of both c-Kit isoforms

STI-571 (imatinib mesylate, Gleevec®) is an important protein kinase inhibitor that has reached the clinic for the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors, and has been reported to inhibit c-Kit.[7,8] To investigate the action of this drug on the activation of both c-Kit receptor isoforms in MM1S cells, the cells were pretreated with STI-571 for 1 hour, and then SCF was added for 15 minutes. As shown in Figure 2F, STI-571 blocked SCF-induced tyrosine phosphorylation of both receptors. Because Src has been involved in the differential signaling by both c-Kit isoforms, and its inhibition has been shown to cause a decrease in phosphorylation of both receptors,[23] we decided to explore whether the Src inhibitor PP2 also affected c-Kit phosphorylation in MM1S cells. As shown in Figure 2G, pretreatment with this inhibitor also blocked SCF-induced tyrosine phosphorylation of c-Kit GNNK⁻ and GNNK⁺.

Signaling downstream of c-Kit in myeloma cells

We also investigated whether the c-Kit isoforms in MM cells differed in the activation of downstream signaling pathways. In MM1S-GNNK⁺ and MM1S-GNNK⁻ cells SCF stimulation of c-Kit tyrosine phosphorylation was accompanied by Akt phosphorylation at Ser 473 (Figure 3A). SCF was also able to induce a small increase in Erk1/2 dual phosphorylation. We also analyzed whether SCF was able to activate the Erk5 pathway. Erk5 is expressed in plasma cells from patients with MM and myeloma cell lines, is activated by the myeloma growth factor interleukin-6, and participates in the control of MM proliferation and apoptosis.[24] MM1S-GNNK⁻ and MM1S-GNNK⁺ cells were treated with interleukin-6 (used as a control for Erk5 activation in MM cells) or SCF, lysed, and the extracts were immunoprecipitated with the anti-Erk5 antibody, followed by western blotting with the same antibody (Figure 3B). As previously reported,[24] interleukin-6 activated Erk5, indicated as gel retardation. SCF activated Erk5 in both MM1S-GNNK⁻ and MM1S-GNNK⁺ cells, this activation being more pronounced in the MM1S-GNNK⁻ cells.

Akt and Erk1/2 were phosphorylated in a time-dependent manner and the peak activation occurred at 15 minutes in MM1S-GNNK⁻ cells and at 30 minutes in MM1S-GNNK⁺ cells (Figure 3C). These kinetics of acti-

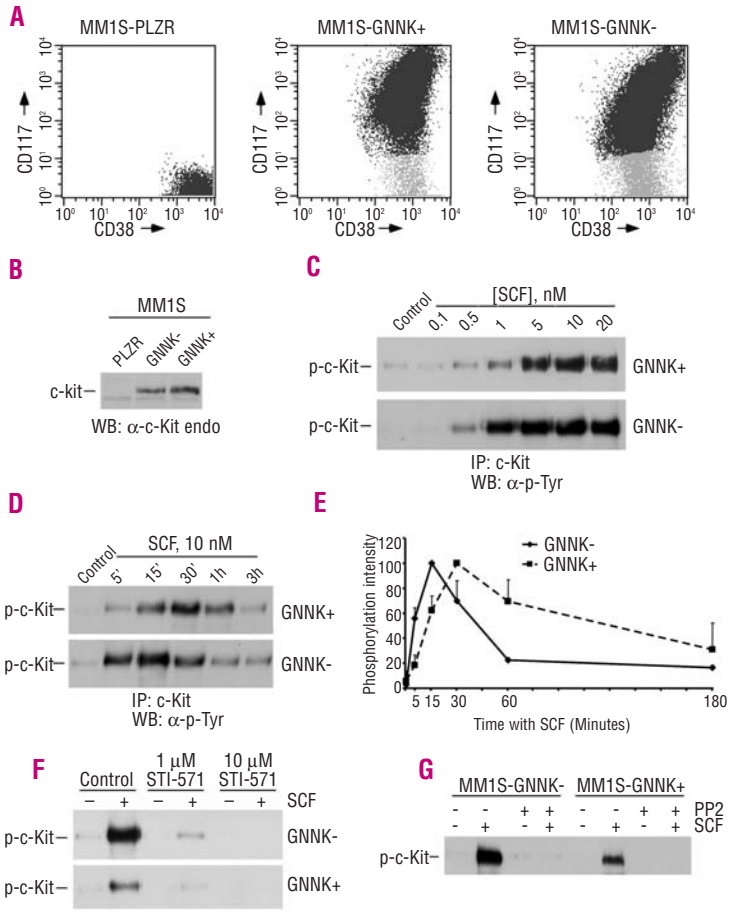

Figure 2. Expression of c-Kit isoforms in MM1S cells, and their activation by SCF. (A) Flow cytometry diagrams of c-Kit isoforms in MM1S cells. (B) Western blot analysis of expression of the c-Kit isoforms in MM1S-PLZR, MM1S-GNNK$^-$, and MM1S-GNNK$^+$ cells. Cell extracts from the indicated cell lines were prepared, and c-Kit analyzed by western blotting using the anti-c-Kit endodomain antibody. (C) Activation of c-Kit isoforms in MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells by SCF. MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells were incubated with the indicated doses of SCF for 15 minutes. The cells lysates were immunoprecipitated with the anti-c-Kit antibody, and the blot was probed with the anti-phosphotyrosine antibody. (D) Time course of the action of SCF on MM1S-GNNK$^-$, and MM1S-GNNK$^+$ cells. Cells were treated with 10 nM SCF, and then the phosphorylated receptor analyzed as above. (E) The graph shows the phosphorylation intensity of both c-Kit isoforms at different times of treatment with SCF. The maximal intensity band was taken as 100%, and the percentage intensity of each band was referred to this value. Data show the mean ± SD for three different experiments. (F) MM1S cells expressing both c-Kit isoforms were preincubated with STI-571 1 μM and 10 μM, or PP2 20 μM (G) for 1 hour and, where indicated, SCF was added for the last 15 minutes. Cells lysates were immunoprecipitated with the anti-c-Kit antibody, and the blot was probed with anti-phosphotyrosine antibody.

vation were similar to those obtained when analyzing the phosphorylation of both receptors (see Figure 2D). Activation of Akt was also dose-dependent and the peak activation was obtained with 10 nM SCF (Figure 3D). Activation of Erk1/2 dual phosphorylation was less evident and difficult to detect in MM1S-GNNK$^+$ cells.

We next investigated whether the SCF-induced activation of Akt in MM cells occurred through the canonical PI3-kinase pathway. MM1S-GNNK$^+$ cells were pretreated with the PI3-kinase inhibitor LY294002, and then treated with interleukin-6, insulin-like growth factor-I (two factors that are important in the biology of the MM)[25-28] or SCF (Figure 4A). All these treatments activated Akt, and this activation was prevented by pre-treatment with the PI3-kinase inhibitor LY294002. This indicated that the activation of Akt by SCF in myeloma cells is dependent on PI3-kinase. The inhibitor of PI3-kinase slightly affected interleukin-6 and SCF-induced Erk1/2 activation.

The p70S6K is activated in MM1S-GNNK$^+$ and MM1S-GNNK$^-$ cells by SCF

As Akt was activated by SCF in MM1S-GNNK$^+$ cells, we wanted to test whether different well-known substrates and downstream targets of Akt may be activated by SCF. MM1S-PLZR and MM1S-GNNK$^+$ cells were stimulated with SCF or insulin-like growth factor-I (used as a control),[29] lysed, and the extracts were analyzed by western blotting using antibodies phosphospecific for different Akt substrates. Insulin-like growth factor-I stimulated the phosphorylation of the Akt subtrate p70S6K in both cell types (Figure 4B). SCF increased phosphorylation in the MM1S-GNNK$^+$ cells, but not in MM1S-PLZR. Insulin-like growth factor-I and SCF also provoked small increases in the phosphorylation of Bad and GSK3β, two additional Akt substrates (*data not shown*). As activation of p70S6K is caused by mTOR,[30] we analyzed whether the mTOR inhibitor, rapamycin, was able to inhibit the activation of p70S6K by SCF. Cells were pretreated with rapamycin, and then the effect of SCF on p70S6K was analyzed by western blotting. As shown in Figure 4C, p70S6K phosphorylation was prevented when cells were preincubated with rapamycin.

Expression of c-Kit counteracts the anti-myeloma action of melphalan and bortezomib

As expression of growth factor receptors, such as the interleukin-6 or the insulin-like growth factor-I receptors is linked to drug resistance in MM,[29,31] we decided to explore whether expression of the c-Kit isoforms also altered drug-induced cell death in MM cells. The rationale for testing this hypothesis was also supported by the ability of SCF to activate Akt, a pathway linked to cell survival.[32] To explore this aspect, MM1S-GNNK$^+$ and MM1S-GNNK$^-$ cells were treated with increasing

doses of melphalan or bortezomib, two anti-myeloma agents used in the clinic, and their MTT uptakes measured. MM1S and MM1S-GNNK$^+$ cells had similar sensitivity to bortezomib or melphalan (Figure 5A and B). In contrast, the action of bortezomib or melphalan on MM cells was significantly inhibited by expression of the GNNK$^-$ isoform.

Discussion

Expression of c-Kit, as detected using cytometric techniques, has been reported in one-third of patients with MM.[2,33,34] However, the specific c-Kit isoforms expressed in the plasma cells from these patients have not been described. Using RT-PCR we detected expression of the two c-Kit forms GNNK$^+$ and GNNK$^-$ in fresh plasma cells from patients and MM cell lines, the GNNK$^-$ isoform being predominant. This result is consistent with previous findings of predominant expression of the GNNK$^-$ form in leukemic and other cell types.[13-15] The high frequency of expression of c-Kit in patients' plasma cells, determined by RT-PCR using primers that differentiate between the two c-Kit isoforms, raises the interesting question of what functions this receptor have in MM pathophysiology. In an attempt to address this, we expressed each of the two isoforms in MM1S cells. This cell line expresses very low (undetectable by cytometric or western blotting techniques) levels of the c-Kit isoforms, and is a widely used MM cell line model. To achieve significant expression of the c-Kit receptor isoforms in this cell line, MM1S cells were infected with the retroviral polycistronic vector PLZR-IRES-GFP containing the cDNA coding for c-Kit GNNK$^+$ or c-Kit GNNK$^-$. We found interesting differences in the activation of these two c-Kit receptor isoforms by SCF. Thus, even though western blotting data indicated that the expression of the GNNK$^+$ isoform was slightly higher than that of the GNNK$^-$ isoform, the phosphorylation of the GNNK$^-$ isoform by SCF was higher than that induced by this growth factor in the MM1S-GNNK$^+$ cells. The reason for such a different behavior in MM cells is unknown, but may be related to preferential c-Src

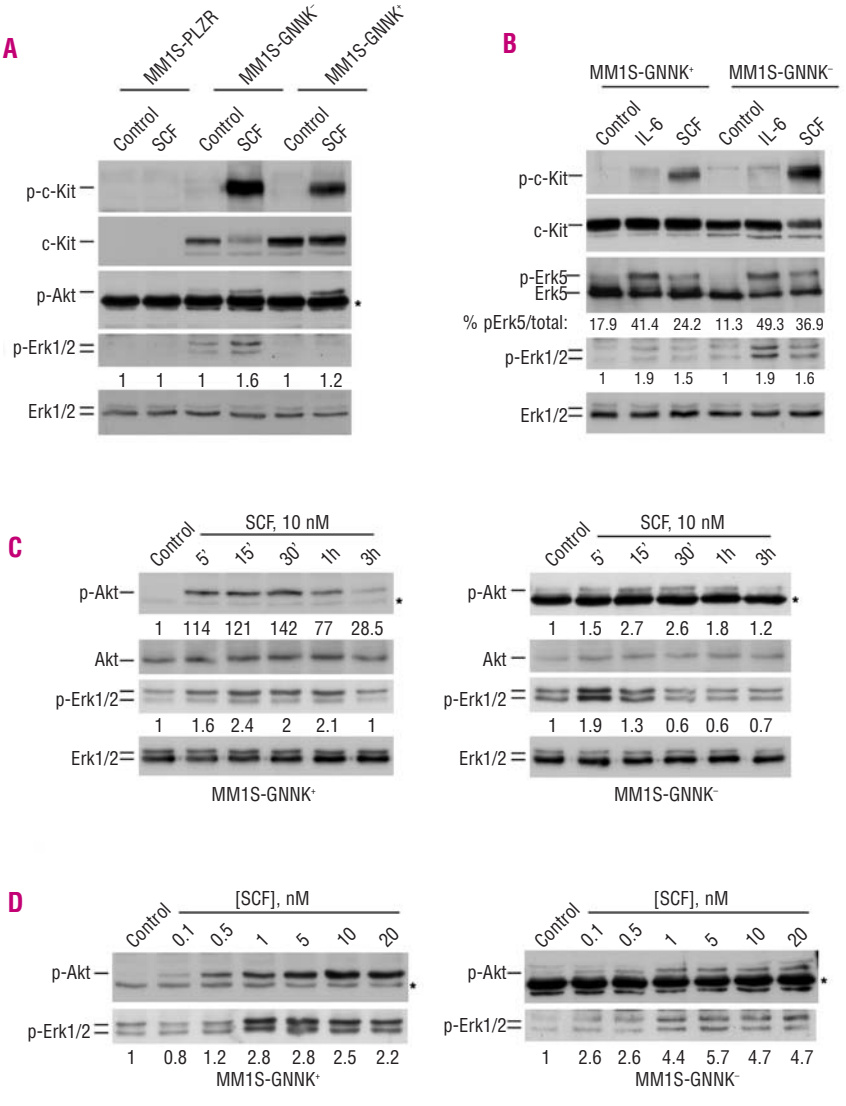

Figure 3. SCF-induced signaling pathways in MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells. (A) Activation of the distinct c-Kit receptors and signaling routes in MM cells. The respective cells were treated with SCF (10 nM), and lysates probed with the indicated antibodies. The asterisk indicates a non-specific band. (B) Activation of Erk5 in MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells by SCF. MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells were treated where indicated with 10 nM SCF and 10 nM interleukin-6 (IL-6) for 15 minutes and lysed. Erk5 was immunoprecipitated and then detected by western blotting using the same antibody. (C) Time course of activation of Akt and Erk1/2 by SCF in MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells. MM1S cells expressing the c-Kit isoforms were incubated with SCF (10 nM) for the indicated time and lysed. Equal parts of the extracts were used to detect phosphorylation of Akt and Erk1/2 by western blotting using anti-pAkt, and anti-pErk1/2 antibodies. To verify loading equality, these membranes were reprobed with antibodies to Akt or Erk1/2. (D) Dose-response analyses of the action of SCF on Akt and Erk1/2 phosphorylation in MM1S-GNNK$^-$ and MM1S-GNNK$^+$ cells. Cells were treated with the indicated doses of SCF for 15 minutes and lysed. The cell extracts were used to detect the phosphorylation of Akt and Erk1/2 by western blotting. The numbers shown below the gels indicate a quantitative measurement of the fold change with respect to untreated samples; except in the case of pErk5, which is represented as the % value of the amount of pErk5 with respect to total Erk5.

association to the GNNK⁻ isoform upon stimulation by SCF, as reported in other cellular systems.[23] Another interesting difference between the c-Kit isoforms was observed in time-course experiments. The GNNK⁻ form was phosphorylated more rapidly and to a greater extent than the GNNK⁺ form; however, the latter remained phosphorylated for longer. Analogous results have been obtained in NIH3T3 cells.[18]

SCF activated several downstream signaling pathways in MM, including the Erk1/2, Erk5, and Akt pathways. It is important to note that although the level of activation of Erk1/2 was low, Erk1/2 dual phosphorylation was higher under resting conditions or after stimulation with SCF in MM1S-GNNK⁻ than in MM1S-GNNK⁺ cells. This result is in agreement with a previous report that suggested that these differences could be due to stronger activation of c-Src by the GNNK⁻ isoform.[23] As this MAPK pathway is mainly linked to cell proliferation in MM cells,[35] and in other cell types, the weak activation of this pathway is indicative of SCF being poorly effective in inducing a proliferative response in MM cells. This was further substantiated by MTT assays performed under different conditions, which failed to demonstrate a clear mitogenic effect of SCF on MM cells (*JCM and AP, unpublished observations*). Similar results were shown for RPMI8226 cells in which SCF also did not have a significant effect on proliferation.[21] Analogously, activation of the Erk5 route was also weak, as compared to that induced by interleukin-6. As this route controls MM proliferation,[24] its low activation by SCF supports, in concert with the Erk1/2 data, the concept that SCF cannot be considered a strong mitogenic factor for MM cells.

Besides Erk1/2 and Erk5, SCF activated the PI3-kinase/Akt route in MM cells, a pathway linked to mitogenesis and survival.[36,37] Furthermore, it has been observed that constitutive activation of the PI3-kinase/Akt and mTOR/p70S6K pathways, and inhibition of the PI3-kinase-Akt route affect the growth and survival of myeloma cells.[38] Stimulation with SCF induced the phosphorylation of Akt in both MM1S-GNNK⁻ and MM1S-GNNK⁺ cells. Akt activation was inhibited by LY294002, indicating that the activation

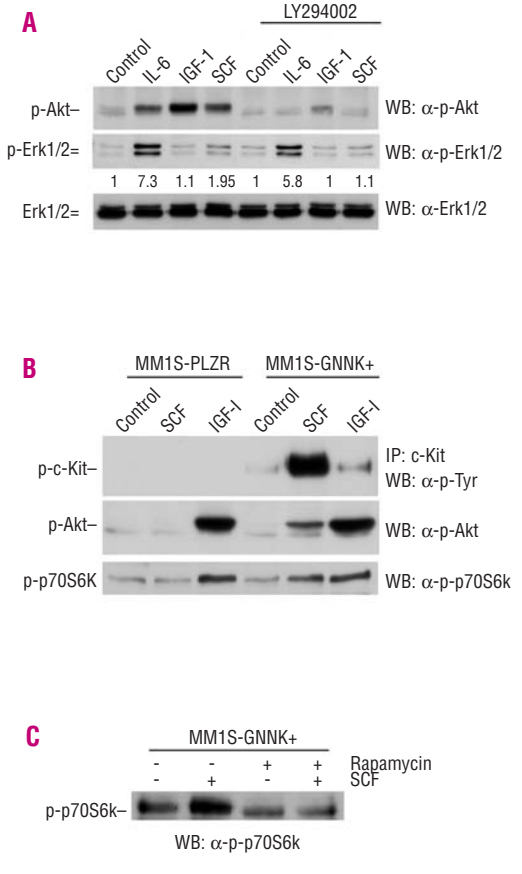

Figure 4. SCF-induced PI3-kinase/Akt signaling in MM cells. (A) Effect of LY294002 on SCF-induced phosphorylation of Akt. MM1S-GNNK⁺ cells were incubated with LY294002 (20 μM) for 1 hour and, where indicated, 10 nM SCF, interleukin-6 (IL-6), or insulin-like growth factor-I (IGF-I) were added for the last 15 minutes. Cell lysates were analyzed by western blotting with anti-pAkt or anti-pErk1/2 antibodies. As a loading control, a blot with anti-Erk1/2 was performed (bottom of panel). The numbers shown below the gel indicate a quantitative measurement of the fold change with respect to the untreated sample. (B) MM1S-PLZR and MM1S-GNNK⁺ cells were incubated with 10 nM of SCF or IGF-I and lysed. The extracts were analyzed by western blotting, with anti-pAkt, or anti-p-p70S6K. (C) Effect of rapamycin on SCF-induced phosphorylation of p70S6K. MM1S-GNNK⁺ cells were pretreated with rapamycin (20 nM) for 1 hour and then stimulated with SCF for 15 minutes. The activation of p70S6K was analyzed by western blotting with an anti-p-p70S6K antibody.

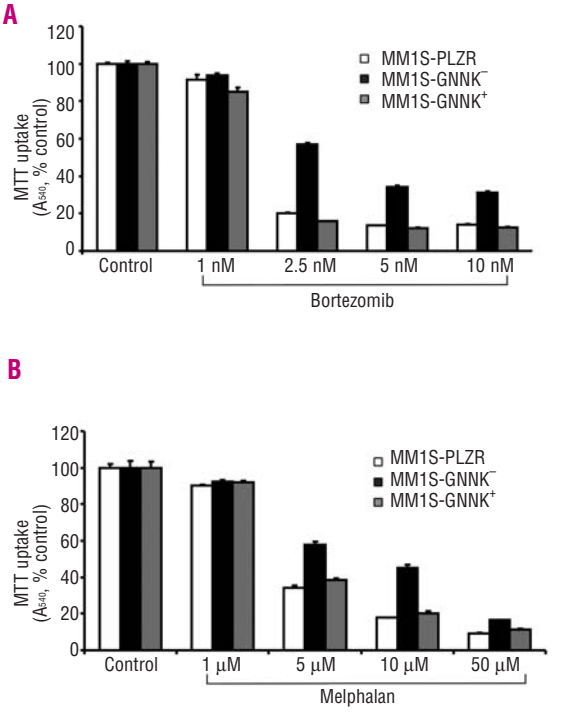

Figure 5. Effect of bortezomib and melphalan on MM1S-GNNK⁻ and MM1S-GNNK⁺ cells. Action of bortezomib (A) and melphalan (B) on MM1S cells expressing c-Kit isoforms. Cells were treated with bortezomib or melphalan at the doses indicated, and MTT uptake was measured 2 days later. The data represent the mean ± SD of quadruplicates of an experiment that was repeated twice.

of Akt by SCF occurred through the canonical PI3-kinase-dependent mechanism. One of the important signaling molecules that is a substrate of Akt is p70S6K. The latter molecule was activated by SCF, and this activation was dependent on mTOR. Activation of p70S6K by SCF has also been observed in primary cultures of spermatogonia.[39]

It should be noted that stimulation of the phosphorylation of Akt, taken as a surrogate of Akt activation, was more clearly observed than the activation of the MAPK pathways. As the Akt pathway is mainly involved in sustaining survival, our data suggest that the action of c-Kit on MM cells could favor the survival of these cells. The drug sensitivity of MM cells expressing the GNNK$^-$ form is in line with these findings. The MM1S-GNNK$^-$ cells were more resistant than MM1S-GNNK$^+$ and MM1S-PLZR cells to the action of melphalan and bortezomib. Moreover, the addition of SCF partially protected against dexamethasone-induced apoptosis of cells expressing either of the two c-Kit isoforms (*JCM and AP, unpublished observations*). Preventing the action of c-Kit may, therefore, act in a synergistic manner with other anti-myeloma agents.

These findings deserve some comments, as they may have important clinical implications from a therapeutic point of view. A phase II trial analyzing the use of STI-571 in MM indicated that this drug was not effective in patients with this disease.[40] Furthermore, our results using STI-571 showed activity of this drug on MM cells in culture, but only at high doses.[21] These data on the limited antimyeloma action of STI-571 preclude its use as a single agent for the treatment of MM. Our current results indicating that c-Kit may restrict the response to clinically relevant antimyeloma drugs do, however, call for a reconsideration of the potential value of drugs that target c-Kit in myeloma. The availability of such agents, for example STI-571,[41] and the multikinase Abl/Src inhibitor dasatinib,[42] makes *in vivo* testing of their efficacy in combination with classical anti-myeloma agents an attractive line of investigation.

In summary, our data indicate that c-Kit expression in MM cells is functional, and is coupled to survival pathways that may modulate cell death in response to therapeutic compounds used in the treatment of this disease.

Authorship and Disclosures

JCM: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; RLP: conception and design, final approval of manuscript; JFSM: conception and design, provision of study material and patients, collection and assembly of data, data analysis and interpretation, final approval of manuscript; AP: conception and design, provision of study material and patients, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

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