Caveolin-1 Is Required for Vascular Endothelial Growth Factor-Trigged Multiple Myeloma Cell Migration and Is Targeted by Bortezomib

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ABSTRACT

We recently demonstrated that caveolae, vesicular flask-shaped invaginations of the plasma membrane, represent novel therapeutic targets in multiple myeloma. In the present study, we demonstrate that vascular endothelial growth factor (VEGF) triggers Src-dependent phosphorylation of caveolin-1, which is required for p130Cas phosphorylation and multiple myeloma cell migration. Conversely, depletion of caveolin-1 by antisense methodology abrogates p130Cas phosphorylation and VEGF-triggered multiple myeloma cell migration. The proteasome inhibitor bortezomib both inhibited VEGF-triggered caveolin-1 phosphorylation and markedly decreased caveolin-1 expression. Consequently, bortezomib inhibited VEGF-induced multiple myeloma cell migration. Bortezomib also decreased VEGF secretion in the bone marrow microenvironment and inhibited VEGF-triggered tyrosine phosphorylation of caveolin-1, migration, and survival in human umbilical vascular endothelial cells. Taken together, these studies demonstrate the requirement of caveolae for VEGF-triggered multiple myeloma cell migration and identify caveolin-1 in multiple myeloma cells and human umbilical vascular endothelial cells as a molecular target of bortezomib.

INTRODUCTION

Multiple myeloma (MM) is an incurable disease characterized by the clonal proliferation of malignant plasma cells in the bone marrow (BM). MM affects ~14,700 new individuals in the United States per year and is almost twice as common in the black versus Caucasian population. Despite conventional, high-dose therapy and novel therapy regimens the median survival remains at 3 to 5 years. Novel biologically based therapies are therefore urgently needed to improve patient outcome.

We recently demonstrated that caveolae ("little caves") represent potential novel therapeutic targets in MM (1). Caveolae are vesicular flask-shaped invaginations (50 to 100 nm) of the plasma membrane, which have been implicated in transcytosis, potocytosis, and signaling processes, including apoptosis. They are composed of cholesterol, sphingolipids, and integral membrane proteins, termed caveolins. Specifically, caveolin-1 (Cav-1) is required for caveolae formation, and its phosphorylation facilitates recruitment of Src-homology 2 domain-containing proteins to initiate downstream signaling cascades (2). Diseases, including cancer, diabetes, Alzheimer’s disease, atherosclerosis, and muscular dystrophy, are associated with caveolae (3). A number of studies show the absence of caveolin isoforms in human peripheral blood cells, including myeloid, lymphoid, and erythrocyte cell lines (4–6). Moreover, the requirement of Cav-1 for caveolae formation was shown by transfection with Cav-1 cDNA into lymphocytes, which are Cav-1 deficient (5). In addition to our previous findings that Cav-1 is expressed by MM cells (1), Cav-1 was found only in a subline of human T-cell leukemia Jurkat cell lines (7). Furthermore, in MM cells, Cav-1 colocalizes with interleukin 6 (IL-6) receptor signal transducing chain gp130 and with insulin-like growth factor I (IGF-I) receptor (1). Cav-1 directly binds cholesterol, a cofactor in formation of caveolae, forming homo- and hetero-oligomers; therefore, cholesterol binding substances such as β-cyclo-dextrin or hydroxymethylglutaryl-CoA-reductase inhibitors such as lovastatin prevent caveolae formation required for Cav-1 function. The caveolin-containing caveolar coat initially remains as a precipitate on the flattened membrane and is then lost from membranes (8). Importantly, cholesterol depletion by either β-cyclo-dextrin or the hydroxymethylglutaryl-CoA-reductase inhibitor lovastatin blocked phosphorylation of Cav-1 and signaling downstream of the IGF-I and IL-6 receptor, e.g., insulin-responsive substrate-1, signal transducers and activators of transcription 3, and phosphatidylinositol 3'-kinase /Akt-1. Taken together, these studies therefore confirmed that Cav-1 is required for both IL-6– and IGF-I–mediated growth and survival of MM cells (1).

In MM, migration [e.g., triggered by vascular endothelial growth factor (VEGF; ref. 9) or IGF-I (10)] is necessary for tumor cell homing to the BM, expansion within the BM microenvironment, and egress into the peripheral blood. Increased microvessel density has been observed in the BM of patients with MM, correlated with disease progression and poor prognosis (11). These data provided the rationale for the use of thalidomide and the immunomodulatory drugs to treat MM (12).

Our and other previous studies showed that VEGF is expressed and secreted by MM cells, as well as BM stromal cells. VEGF secreted by MM cells triggers IL-6 production by BM stromal cells, thereby augmenting paracrine MM cell growth; conversely, IL-6 enhances the production and secretion of VEGF by MM cells (13). VEGF also triggers BM angiogenesis (11), inhibits dendritic cell maturation (14), and increases osteoclastic bone-resorbing activity (15). Importantly, VEGF also directly triggers both MM cell migration and proliferation (9, 16), and we have shown that tyrosine kinase inhibitor PTK787 (Novartis, Basel, Switzerland) and the indazolylpyrimidine GW654652 (GlaxoSmithKline, Research Triangle Park, NC) inhibit VEGF-triggered migration and proliferation of patient MM cells and MM cell lines, as well as IL-6 and VEGF secretion induced by MM cell binding to BM stromal cells (1, 17). Cav-1 also has an important role in mediating cell motility; specifically, Cav-1 accumulates at the leading edge of cultured fibroblasts (8), human umbilical vein smooth muscle cells (18), and Cav-1–deficient FRT cells expressing recombinant Cav-1 (19); and at the trailing edge of bovine aortic endothelial cells (20). To date, however, the role of Cav-1 in modulating MM cell migration is undefined.

Bortezomib (formerly PS-34; Velcade, Millennium Pharmaceuticals, Inc., Cambridge, MA) is a boronic acid dipeptide recently approved by the United States Food and Drug Administration for the therapy of MM patients with progressive myeloma after previous treatment (21). Our previous studies have shown that bortezomib inhibits growth, induces apoptosis, and overcomes drug resistance in
human MM cells both in vitro and in vivo. Specifically, bortezomib induces apoptosis in MM cells by activation of caspase-8, caspase-9, and caspase-3, down-regulates expression of adhesion molecules by MM cells and BM stromal cells, inhibits IL-6- or cell-adhesion-mediated drug resistance, and decreases transcription and secretion of cytokines in the BM milieu (22). The antiangiogenic effect of bortezomib (23, 24) represents another mechanism of its anti-MM activity: we recently demonstrated that bortezomib prolongs survival in a human plasma cytoma xenograft mouse model (25), in part, associated with decreasing tumor-associated microvessel density. Importantly, bortezomib induced clinically significant responses in patients with relapsed, refractory myeloma with an acceptable toxicity profile (22).

In this report, we demonstrate that Cav-1 expression and phosphorylation is required for VEGF-triggered MM cell migration and, furthermore, that bortezomib reduces Cav-1 expression and induces marked down-regulation of VEGF-triggered Cav-1 phosphorylation in MM cells, thereby inhibiting VEGF-induced migration. Moreover, we show that bortezomib also inhibits Cav-1 tyrosine phosphorylation, which is required for migration in human umbilical vein endothelial cells (HUVECs). Taken together, this study further delineates the importance of caveolae in MM pathogenesis and identifies Cav-1 as an important target of bortezomib-triggered anti-MM activity.

MATERIALS AND METHODS

Materials. Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). Human plasma fibroconnectin was obtained from Life Technologies, Inc. (Grand Island, NY). Antibodies raised against Flt-1, Cav-1, p130 src, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine 4G10 (Millipore, Billerica, MA) was kindly provided by Dr. Tom Roberts (Dana-Farber Cancer Institute, Boston, MA). PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine; Calbiochem-Novabiochem, San Diego, CA)], a potent inhibitor of the Src family tyrosine kinases, was dissolved DMSO and stored as 5 mmol/L stock solutions at −20°C. DMSO, β-cyclodextrin, and CdCl₂ were purchased from Sigma (St. Louis, MO). Bortezomib (Millennium Pharmaceuticals, Cambridge, MA) was dissolved in DMSO, stored at −20°C, and used in the concentrations indicated.

Cells and Cell Culture. Human MM cell line MM.1S cells, as well as primary MM cells, were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Harlan, Indianapolis, IN), 100 units/mL penicillin, 10 μg/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro, Herndon, VA). Before stimulation of cells with VEGF, they were incubated overnight in RPMI 1640 with 2% fetal bovine serum, followed by an additional 3 hours in RPMI 1640 without fetal bovine serum. HUVECs from a pool of five healthy donors (Clonetics BioWhittaker, Walkersville, MD) were maintained in EGM-2MV medium (Clonetics BioWhittaker).

Generation and Transfection of Caveolin-1 cDNA and Antisense Constructs. Human Cav-1 cDNA was subcloned into the inducible expression vector pML1 (26) in an inverted order. MM.1S cells were transfected using Nucleofector Kit V, according to the manufacturer’s instructions (Amazex Biosystems, Cologne, Germany); clones were selected in hygromycin (Roche Diagnostics GmbH, Mannheim, Germany). Suppression of Cav-1 expression was induced by 3 μmol/L CdCl₂. Transfected cells were screened for protein expression by immunoblot with an antibody specific for Cav-1.

Isolation of Patient’s Tumor Cells. After appropriate informed consent, MM patient cells (96% CD38−CD56−) were obtained from patient BM samples by antibody-mediated negative selection using RosetteSep (StemCell Technologies, Vancouver, British Columbia, Canada), as described previously (27). Purified MM cells were additionally labeled with magnetically CD138 MicroBeads and positively selected using a magnetic field of a MACS separator (Miltenyi Biotec, Auburn, CA). Purity of myeloma cells was >95%.

Cell Lysis, Immunoprecipitation, and Western Blotting. Cells were washed three times with 1× PBS and lysed with either lysing buffer (10 mmol/L Tris, 50 mmol/L NaCl, Na PP) or radioimmunoprecipitation assay lysis buffer supplemented with 1% Triton, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor mixture (Boehringer Mannheim, Mannheim, Germany), and additionally processed for immunoprecipitation and immunoblotting, as in previous studies (9). As a control for immunoprecipitation, nonspecific protein binding and detection were excluded by incubating protein A-Sepharose beads with lysis buffer and specific antibody only.

Transwell Migration Assay. Cell migration was assayed using a modified Boyden chamber assay, as described previously (9).

Microarray Assay. Total RNA was isolated from bortezomib pretreated or untreated MM.1S cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). The analysis was performed as described previously (28). The DNA Chip Analyzer (Dchip, ref. 29) was used to normalize all CEL files to a baseline array with overall median intensity, and the model-based expression (perfect match only) was used to compute the expression values. Analysis identified signals varying by ≥2-fold (lower bound) with a 90% confidence interval.

Real-Time PCR. Total RNA was extracted from untreated or bortezomib-treated MM.1S cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was treated with RNase free DNase using the MessageClean kit (GenHunter Corp., Nashville, TN), phenol-chloroform extracted, and etomidate precipitated. cDNA was synthesized at a concentration of 0.05 μg/μL with random primers using the Reverse Transcription System (Promega, Madison, WI). Quantitative real-time PCR studies were performed using an iCycler detection system (Bio-Rad, Hercules, CA). cDNA were amplified in 25-μL reactions containing iQ SYBR Green supermix (Bio-Rad), 50 ng of cDNA, and 0.2 mol/L of each primer. Amplification was done with an initial soak at 95°C for 2 minutes followed by 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for a total of 40 cycles. MELT curves were determined to ensure the same PCR product was synthesized across all wells using identical primers through the product’s melting temperature. The comparative threshold cycle method was used to quantify the relative fold mRNA levels. The efficiency of amplification by the primers was confirmed to be equivalent for the housekeeping gene and the gene of interest. The value for efficiency is used in the comparative threshold cycle equation as described. Water samples or RNA samples containing no reverse transcriptase were amplified in parallel to ensure that no contaminating DNA was present during PCR. All experiments were repeated at least twice (n = 2). The following primer sequences were used: (a) Cav-1, forward primer (5′-GGCTGTCTTTCTGCTTCGGA-3′); (b) Cav-1, reverse primer (5′-TGGCGTATGGTCACACTAC-3′); (c) actin, forward primer (5′-GTTTGCTTATGATGTCGGA-3′); and (d) actin, reverse primer (5′-ACTGGAAGCGTTAGGAGC-3′).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT). The inhibitory effect of bortezomib on patient MM and HUVEC growth was assessed using MTT (Chemicon International, Temecula, CA), which is reduced by viable cells to yield a dark blue formazan product, as described previously (1).

ELISA. Cytokine levels were measured in supernatants from coculture systems as described previously (9). VEGF and IL-6 concentrations were measured using commercially available ELISA kits (R&D Systems). The absorbance of each well was detected by a microtiter plate reader at 450 nm. Each well was analyzed in triplicate.

Statistical Analysis. Statistical significance of differences observed in VEGF-treated versus control cultures was determined using an unpaired Student’s t test. The minimal level of significance was P < 0.05.

RESULTS

VEGF Triggers Src-Kinase Recruitment to a Flt-1/Caveolin-1–Containing Complex and Tyrosine Phosphorylation of Caveolin-1. Cav-1 is tyrosine phosphorylated in response to insulin, angiotensin II, osmotic shock, oxidative stress, IGF-I, IL-6, and VEGF (30–37) and interacts with several proteins, including Src (3, 34). Importantly, VEGF markedly increases Src-dependent tyrosine phosphorylation of Cav-1 in endothelial cells, thereby mediating endothelial cell migration (36, 37). In MM, VEGF is expressed and secreted by tumor cells, as well as BM stromal cells. Besides stimulating angiogenesis, inhibiting dendritic cell maturation (14, 15), and increasing bone-resorbing activity of osteoclasts (38), VEGF promotes MM cell proliferation and migration (9, 16). On the basis of these
observations, we determined whether functional Cav-1 is required for VEGF-triggered MM cell migration. We first tested whether Cav-1 colocalizes with Fli-1 and Src and whether VEGF induces Cav-1 tyrosine phosphorylation in MM cells. Cav-1 immunoprecipitation and immunoblotting studies showed that Fli-1 constitutively associates with Cav-1 and that VEGF induces recruitment of Src to this complex, as well as Cav-1 tyrosine phosphorylation (Fig. 1A).

VEGF-induced Multiple Myeloma Cell Migration Is Dependent on Intact Caveola Structure and Src-Kinase Activity. Cav-1 directly binds cholesterol, the most important cofactor in caveolar morphogenesis (8, 39, 40); conversely, β-cyclodextrin disrupts caveola-like structures by binding to and sequestering cholesterol from the plasma membrane of intact cells (41–43). Pretreatment of MM cells with β-cyclodextrin depletes caveolae at an ultrastructural level and changes the distribution of Cav-1 within a discontinuous sucrose gradient (1). We next therefore tested whether intact caveolae are required for VEGF-triggered MM cell migration. Multiple myeloma cell migration was evaluated using a modified Boyden chamber. As shown in Fig. 1B, inhibition of VEGF-dependent MM cell migration was observed in the presence of β-cyclodextrin. Importantly, β-cyclodextrin at the concentrations used in migration assays did not affect survival of MM cells, as assessed by MTT assay (data not shown). Furthermore, treatment of MM cells with the potent Src-kinase inhibitor PP2 also markedly decreased VEGF-triggered MM cell migration. Taken together, these data reflect the importance of intact caveolae and Src-kinase activity for VEGF-triggered MM cell migration.

VEGF-Triggered Phosphorylation of p130Cas Is Dependent on Intact Caveola Structure and Src-Kinase Activity. The cytoskeletal protein p130Cas is an assembling molecule of actin filaments promoting cell movement, migration, and spreading (44). To additionally verify that intact caveolae and Src activity are required for VEGF-triggered MM cell migration, we next investigated (a) whether p130Cas tyrosine phosphorylation is triggered by VEGF and (b) whether this tyrosine phosphorylation is dependent on both intact caveolae and Src activation, respectively. As shown in Fig. 1C, VEGF-induced p130Cas tyrosine phosphorylation is abrogated after either cholesterol depletion or PP2 treatment.

Caveolin-1 Is Required for VEGF-Induced Multiple Myeloma Cell Migration and p130Cas Phosphorylation. To directly confirm the importance of Cav-1 on MM cell migration, MM.1S cells were transfected with antisense full-length mRNA for Cav-1 under the influence of an inducible metallothionein promoter. A total of 3 μmol/L cadmium (16 to 18 hours) was used for inducible suppression of Cav-1 expression, detected by Western blot analysis. Clone 3 with normal expression levels of Cav-1 was compared with clones 7 and 25, which showed a 60 to 70% decrease of Cav-1 expression (Fig. 2, A and B). Moreover, induced suppression of Cav-1 expression in clones 7 and 25 was associated with a marked decrease in VEGF-triggered MM cell migration (Fig. 2C). We next tested whether Cav-1 is required for p130Cas tyrosine phosphorylation. As shown in Fig. 2D, induced suppression of Cav-1 abrogated VEGF-triggered p130Cas tyrosine phosphorylation.

Bortezomib Decreases Caveolin-1 Expression Profile in Multiple Myeloma Cells. The proteasome inhibitor bortezomib is a promising novel agent, which induces apoptosis in drug-resistant MM cells, inhibits binding of MM cells in the BM microenvironment, and decreases both production and secretion of cytokines mediating MM cell growth and survival. Antiangiogenesis (23, 24) represents another potential mechanism of its anti-MM activity (25). To define molecular targets of proteasome inhibition, we performed cDNA microarray profiling on MM cells. In contrast to a previous study that characterized gene expression profiles in MM cells treated with 100 nmol/L bortezomib using the human U95Av2 Affymetrix GeneChip (12,600 genes; ref. 45), the present study evaluated gene expression profiles on MM cells treated with only 20 nmol/L bortezomib for 6 hours versus control cells, using the U133A Affymetrix GeneChip containing ~45,000 genes. Bortezomib induced significant (~3.14-fold) suppression of Cav-1 transcription (Fig. 3, A and C). To confirm this data, we performed real-time PCR analysis using gene-specific primers for Cav-1 and actin (Fig. 3B). After normalizing to expression of actin, real-time PCR analysis showed a 2-fold suppression of Cav-1 mRNA (Fig. 3C), additionally confirming bortezomib-induced down-regulation of Cav-1 expression. Consequently, protein levels of Cav-1 in bortezomib-treated MM cells were decreased after 6 hours (~33% inhibition) and 12 hours (~86% inhibition) compared with untreated MM cells, without significant changes in protein levels of actin (Fig. 3D). Taken together, these data show that bortezomib inhibits Cav-1 expression.

Bortezomib Decreases VEGF-Induced Phosphorylation of Caveolin-1 and Migration. We have previously demonstrated that pretreatment with bortezomib inhibits IL-6–triggered phosphorylation of extracellular signal-regulated kinase, thereby inhibiting MM cell growth (22). Having shown that VEGF-triggered tyrosine phosphorylation of Cav-1 is required for MM cell motility, we next sought to determine whether bortezomib pretreatment of MM cells can also abrogate VEGF-induced Cav-1 phosphorylation and related MM cell

![Fig. 1](image-url)
Fig. 2. Cav-1 is required for VEGF-induced multiple myeloma cell migration and p130Cas phosphorylation. A and B, induced suppression of Cav-1 expression in MM cells. Lysates of MM.1S cells (clones 3, 7, and 25) expressing antisense Cav-1 before (−) and after (+) cadmium (CdCl₂) induction were immunoblotted for Cav-1 and reprobed for actin as a control (A). Relative Cav-1 and actin levels among clones were quantified by densitometry and confirmed in three separate experiments (B). C. Multiple myeloma cell migration is inhibited after suppression of Cav-1 expression. Growth factor-deprived MM.1S cells (clones 3, 7, and 25) were either pretreated with cadmium or left untreated. Cells were then analyzed as described in Fig. 1B. Data represent means ± SEM for duplicate samples. Results shown are representative of three independent experiments. D. VEGF- triggered phosphorylation of p130Cas is dependent on Cav-1 expression. Growth factor-deprived MM.1S cells (clone 25) were either pretreated with cadmium or left untreated and then stimulated with VEGF for the indicated intervals. Cell lysates were immunoprecipitated with anti-p130Cas antibody and analyzed by immunoblotting with the indicated antibodies. C, immunoprecipitation (IP) control.

migration. As shown in A and B of Fig. 4, Cav-1 immunoprecipitation and immunoblotting studies demonstrate that VEGF-triggered tyrosine phosphorylation of Cav-1 is abrogated after short pretreatment of MM.1S cells with bortezomib (40 nmol/L; 1 hour), without changes in Cav-1 expression. Moreover, bortezomib decreased VEGF-triggered MM.1S cell migration in a dose-dependent fashion (Fig. 4C). Bortezomib similarly inhibited VEGF-triggered migration in the MM cell lines OPM-2, RPMI Dox-40, and MM.1R (Fig. 4D), as well as in patient MM cells (Fig. 5A). Importantly, bortezomib at the concentrations used in migration assays did not affect survival of patient MM cells, as assessed by MTT assay (Fig. 5B). Taken together, these data identify Cav-1 as one of many cellular proteins targeted by bortezomib. Specifically, our data show blockage of VEGF-triggered MM cell migration by bortezomib is associated with both inhibition of functional (phosphorylated) Cav-1 and later down-regulation of Cav-1 expression.

Bortezomib Decreases VEGF Secretion in the Bone Marrow Microenvironment. Our own and others’ studies have shown that MM cells express and secrete VEGF, thereby stimulating production and secretion of IL-6 in BM stromal cells. Conversely, the production and secretion of VEGF by MM cells is enhanced by IL-6 (13). VEGF, in turn, directly triggers endothelial cell proliferation, growth and
migration of MM cells (9, 16), inhibition of dendritic cell maturation (14), and bone-resorbing activity of osteoclasts (15). We next therefore tested the effect of bortezomib on VEGF secretion in MM cell lines and MM patient cells. Bortezomib triggered a significant concentration-dependent decrease in VEGF production and secretion in both MM cell lines (Fig. 5C) and MM patient cells (Fig. 5D).

**Bortezomib Inhibits VEGF-Triggered Tyrosine Phosphorylation of Cav-1, As Well as Migration and Survival of HUVECs.** We have previously shown that the antiangiogenic (23, 24) effect of bortezomib is a component of its anti-MM activity (25). As in MM cells, VEGF markedly increases Src-dependent tyrosine phosphorylation of Cav-1 in endothelial cells, thereby mediating endothelial cell migration (36, 37). Because bortezomib inhibited VEGF-triggered Cav-1 mediated MM cell migration, we next tested whether bortezomib can similarly inhibit (a) VEGF-triggered phosphorylation of Cav-1 and (b) migration in HUVECs. Bortezomib abrogated VEGF-triggered Cav-1 tyrosine phosphorylation and subsequent phosphorylation of extracellular signal-regulated kinase and Akt1 (Fig. 6A). Furthermore, bortezomib inhibited HUVEC migration in a dose-dependent fashion (Fig. 6B) without affecting survival (4 hours; Fig. 6C). Importantly, exposure to bortezomib for 24 and 48 hours significantly inhibited HUVEC survival, analyzed by MTT assay (Fig. 6D). Taken together, these data show that bortezomib blocks VEGF-triggered Cav-1 phosphorylation and migration in HUVECs early and alters HUVEC survival only after longer drug exposure.

**DISCUSSION**

Previous studies have shown that Cav-1 mediates cell motility. Specifically, Cav-1 accumulates at the leading edge of cultured fibroblasts (8), human umbilical vein smooth muscle cells (18), and Cav-1–deficient FRT cells expressing recombinant Cav-1 (19) and at the...
trailing edge of bovine aortic endothelial cells (20). However, the role of Caveolin-1 in VEGF-triggered MM cell migration (9, 16) has not been determined. In the present study, we show that (a) caveolae are required for VEGF-triggered MM cell migration and (b) this effect can be targeted by the proteasome-inhibitor bortezomib.

We have recently shown that caveolae, which are usually absent in blood cells (4–6), are present in MM cells (1). Specifically, we carried out cDNA profiling of 12,600 genes in CD138+ cells isolated from BM mononuclear cells from eight untreated MM patients and four individuals with monoclonal gamopathy of undetermined significance. Our results showed that Caveolin-1 is up-regulated in MM versus monoclonal gamopathy of undetermined significance samples, suggesting a possible role for caveolae in the transition of monoclonal gamopathy of undetermined significance to MM. Furthermore, we showed that these plasma membrane microdomains play an essential role in IL-6– and IGF-1–triggered Akt-1-mediated survival of MM cells (1). In a subsequent study, we identified a chemotactic effect of IGF-1 on human MM cells mediated via activation of phosphatidylinositol 3′-kinase/Akt-1 and β1 integrin and demonstrated that membrane raft association with IGF-1 and β1 integrin regulates both adhesion and migration of MM cells within the BM milieu (10).

In this article, we show that VEGF triggers Src-kinase recruitment to FIt-1/Caveolin-1 containing complexes and induces tyrosine phosphorylation of Caveolin-1 in MM cells. Moreover, we show that this effect is dependent upon intact caveolae structure and Src-kinase activity. p130Cas was originally identified as a major tyrosine-phosphorylated protein in cells transformed by v-Src or v-Crk (46, 47); it is also tyrosine phosphorylated by various physiologic stimuli, including cell adhesion, cytokine receptor engagement, and growth factor stimulation. Several studies indicate that Src is directly responsible for p130Cas phosphorylation (48, 49). After p130Cas is hyperphosphorylated, it forms a signaling complex with the adapter protein Crk, which mediates cell migration (50). Here, we show that VEGF-triggered phosphorylation of p130Cas is dependent on intact caveolae structure and Src-kinase activity. To more specifically determine whether functional Caveolin-1 is required for these signaling sequences, we induced depletion of Caveolin-1 by antisense methodology in human MM cells. Using this approach, we abrogated VEGF-triggered MM cell migration and downstream p130Cas phosphorylation, confirming that intact Caveolin-1 is required for VEGF-triggered MM cell migration. In ongoing studies, we are additionally evaluating the role of the p130Cas–Crk complex formation in VEGF-mediated MM cell migration.

Bortezomib is a promising novel anti-MM agent recently approved by the United States Food and Drug Administration for therapy of patients with progressive MM after previous treatment (21). It induces apoptosis in drug-resistant MM cells and inhibits both binding of MM cells in the BM microenvironment, as well as production and secretion of cytokines that mediate MM cell growth and survival. IL-6–triggered phosphorylation of extracellular signal-regulated kinase, but not signal transducers and activators of transcription 3, is blocked by bortezomib. Moreover, bortezomib mediates antimyeloma activity by phosphorylating both p53 protein and c-Jun NH2-terminal kinase, cleavage of DNA-PKcs or ATM, and caspase-dependent down-regulation of p53 (22). An antiangiogenic effect of bortezomib (23, 24) represents another potential mechanism of its anti-MM activity (25).

Our data show that bortezomib induces marked inhibition of Caveolin-1 tyrosine phosphorylation. Moreover, using cDNA microarray profiling on MM cells, we demonstrate that bortezomib also induces marked suppression of Caveolin-1 transcription after prolonged treatment. Furthermore, significant concentration-dependent reduction of VEGF production and secretion was observed in both MM cell line and MM patient cells. As in MM cells, VEGF markedly increases Src-dependent tyrosine phosphorylation of Caveolin-1 in endothelial cells, thereby mediating endothelial migration (36, 37). Importantly, we show that bortezomib blocks both VEGF-triggered Caveolin-1 tyrosine phosphorylation and migration early but decreases HUVEC survival only later. The present study demonstrates that both functional (phosphorylated) Caveolin-1 and Caveolin-1 expression are required for VEGF-triggered MM cell migration. Bortezomib has many molecular targets, including proteins related to apoptosis, growth signaling/cell cycle, heat shock proteins, and the proteasome pathway. In this article, we identify Caveolin-1 as an additional molecular target of bortezomib. Specifically, bortezomib inhibits early VEGF-triggered Caveolin-1 phosphorylation and later induces down-regulation of Caveolin-1 expression in MM cells. Furthermore, bortezomib also decreases VEGF secretion in the BM microenvironment and inhibits VEGF-induced Caveolin-1 phosphorylation in endothelial cells, which is required for their migration. We therefore identify Caveolin-1 as an additional molecular target of bortezomib, adding another facet to its antimyeloma activity. Importantly, these data therefore additionally support novel treatment strategies targeting caveolin in MM.

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