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Bortezomib influences the expression of malignant plasma cells membrane antigens

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ABSTRACT

Multiple myeloma cells can be characterized immunophenotypically as the expression levels of several membrane antigens differ from those of normal plasma cells. These antigens are important for making a diagnostic of multiple myeloma; they have a significant role in survival and proliferation of multiple myeloma cells. Analyzing the effect of bortezomib on the expression of surface antigens CD138, CD56, CD27, CD28, CD45 and CD221 and xenograft models, we have found that bortezomib increases the level of CD45 and decreases all other antigens. Bortezomib induces the reduction of IGF-1R (CD221) and syndecan 1 (CD138). This effect was associated with the reduced activation of Ras/MAPK, mTOR/p70S6K and JAK/STAT pathways in response to IGF-1 and IL-6. These results suggest that bortezomib may influence the sensitivity of myeloma cells to soluble growth factors by down-regulation of membrane receptors.

1. Introduction

Multiple myeloma is a neoplastic plasma cell disorder, characterized by proliferation of malignant plasma cells in bone marrow microenvironment, and associated with monoclonal antibody secretion. Multiple myeloma remains an incurable disease and represents the most common primary bone malignancy. The median age at diagnosis is 70 years with more than 16,000 new cases diagnosed annually in United States. Immunophenotyping of multiple myeloma cells by flow cytometry is performed at diagnosis and used for the detection of minimal residual disease (Almeida et al., 1999). To distinguish between malignant and normal plasma cells the antigens most frequently used CD19, CD56, CD27, CD28 and CD45. Neural Cell Adhesion Molecule (CD56) and CD19 are the basic markers used to distinction between normal and malignant plasma cells (Rawstron et al., 2008). The presence of CD56 or the negativity of CD19 on plasma cells is considered a diagnostic criterion of multiple myeloma. Levels of the CD45 antigen, a key molecule in the activation of lymphocytes (Trowbridge and Thomas, 1994), can classify myeloma cells into discrete sub-populations (Kumar et al., 2005). Multiple myeloma patient cells expressing CD45 are predominant in early stages of the disease and decrease with disease progression. Furthermore, CD45− malignant plasma cells are associated with higher levels of the anti-apoptotic protein Bcl-2 and the secretion of vascular endothelial growth factor (VEGF) (Pellat-Deceunynck and Bataille, 2004). CD45, which possesses phosphatase activity, negatively regulates the IGF-1α receptor (Descamps et al., 2009). Thus, the transition of multiple myeloma patient cells from a CD45+ to CD45− status is associated with a switch of these cells from a CD221− (IGF-1R−) low to a CD221+ (IGF-1R+) (Descamps et al., 2004). Increased IGF-1R expression leads to up-regulation of the above mentioned signaling pathways, leading to further multiple myeloma cell proliferation and decreased apoptosis. Thus, the activation of IGF-1R induces survival and proliferation signaling pathways such as PI-3K/Akt, Ras/MAPK and nuclear factor-kappaB (NF-κB) (Guo and Chen, 2006; Mitsiades et al., 2002).

Syndecan 1 (adhesion molecule CD138) is considered as another prognostic factor in multiple myeloma. Clinically, high expression of CD138 is correlated with a high percentage of plasma cells in the bone marrow (Dhodapkar et al., 1998). CD138 is a co-receptor of interleukin-6 (IL-6) and epidermal growth factor (EGF), and is considered to be involved in the proliferation and survival of multiple myeloma cells (De Vos et al., 2006).

Bortezomib is the first proteasome inhibitor introduced in treatment of multiple myeloma patients (Chauhan et al., 2005; Richardson et al., 2005). Bortezomib affects cell-cycle regulation, DNA damage response and the up-regulation of pro-apoptotic proteins (Adams et al., 1999; Hideshima et al., 2001). This anti-tumor activity of bortezomib is associated with the accumulation of

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polyubiquitinated proteins in the cytosol, which may be responsible for the induction of ER stress and an unfolded protein response (Obeng et al., 2006).

In this study, we have investigated the effects of bortezomib on the expression of surface antigens present on multiple myeloma cells. We show that bortezomib reduces the expression of several of these antigens both in myeloma cell lines and fresh human myeloma cells. In contrast, antigen CD45 was significantly increased. Reduction of CD221 and CD138 was shown to be associated with a reduced response to IGF-1 and IL-6, respectively.

2. Material and methods

2.1. Reagents

Bortezomib was obtained from Janssen Cilag (Issy-Les-Moulineaux, France). Lenalidomide was obtained from (Celgene) and dexamethasone was purchased from Mylan. Human recombinant IGF-1 was kindly supplied by Tercica Inc (CA, USA). Fluorescent monoclonal antibodies anti-CD38, anti-CD19, anti-CD56, anti-CD37, anti-CD20, anti-CD27, and anti-CD33 and Mouse fluorochrome-conjugated isotype control were purchased from Becton Dickinson. Isotypes antibodies and anti-CD221 were obtained from BD Pharmingen, and anti-CD45, CD138 from Beckman Coulter (Supplementary Table 1). Monoclonal antibodies used for western blots were purchased from Cell Signalling and Imgenex (Supplementary Table 1).

2.2. Multiple myeloma cell lines

Human multiple myeloma cell lines LP1, U266 and MM.1S were purchased from ATCC-LGC (Molsheim, France), RPMI8226 from ECACC. Multiple myeloma cell lines were maintained in RPMI1640 + L-Glutamine (Invitrogen, Cergy Pontoise, French) supplemented with 10% fetal bovine serum (Invitrogen, Cergy Pontoise, French) and antibiotics (penicillin 100 μM and streptomycin 50 μg/mL) (Invitrogen, Cergy Pontoise, French) at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Immunophenotypic study

2.3.1. Human multiple myeloma cell lines

Cell surface antigen expression of multiple myeloma cell lines was determined by flow cytometry analysis. Briefly, 1×10^6 human myeloma cells were incubated at 37 °C with or without 1.5 nM bortezomib for 24 h. For the Kinetics study, myeloma cells were exposed to 1.5 nM bortezomib at different time (1, 6, 24, 48 or 72 h). Cells were washed with phosphate-buffered saline (PBS) and incubated for 15 min at room temperature in the dark with fluorescent monoclonal antibodies anti-CD38, anti-CD138, anti-CD221, anti-CD19, anti-CD56, anti-CD37, anti-CD20, anti-CD27, and anti-CD33. To analyze the dose response of bortezomib, MM.1S, U266, RPMI8226 and LP1 cell lines were incubated in the presence of different concentrations of bortezomib (0.15 nM, 1.5 nM and 15 nM) for 24 h. After incubation, cells were washed, suspended in PBS and analyzed on a FACS Canto II (Becton Dickinson). Analysis of the data was done with the Cell Quest software program (Becton Dickinson). Mean fluorescence intensity (MFI) was determined by subtracting the signal of isotype-matched antibody staining from the staining observed with the specific primary antibody.

2.3.2. MM.1S and LP1 xenografts

The effect of bortezomib was also evaluated *in vivo* on MM.1S and LP1 xenografts. Female SCID mice (4 weeks old) were obtained from Charles River (France). The animals were housed (6 mice per cage) and treated according to local institutional guidelines. Before initiating the experiment, all mice were acclimated to a pulverized diet for one week. None of the mice exhibited any lesions and all tested pathogen-free. Our experimental protocol was reviewed and approved by the Lyon University Animal Ethics Committee. For xenotransplant experiments, 3×10^6 cells were injected subcutaneously into the right flank of the mice. On day 0, mice were randomly divided into one control group and one group treated with bortezomib (0.5 mg/kg intraperitonally, weekly, for four weeks). Four weeks following multiple myeloma cells injetions tumors were carefully excised. For immunophenotypic study resuspended cells were incubated with fluorescent monoclonal antibodies for 15 min. Cells were washed by PBS1X and analyzed for antigen expression by flow cytometry as previously indicated.

2.3.3. Fresh human myeloma cells

Primary myeloma cells were obtained from bone marrow biopsies of myeloma patients. Samples were incubated at room temperature with a lysis buffer for 10 min. Cells were washed with PBS 1X, centrifuged for 5 min at 600 g at room temperature and suspended in RPMI1640 and 10% FBS. Cells were cultured in 24-well plates at the concentration of 1×10^6 cell per well (Costar, NY, USA) and incubated for 24 h at 37 °C with 1.5 nM bortezomib. After incubation, cells were washed with PBS 1X, centrifuged for 5 min at 600 g and incubated with monoclonal antibody directed against CD38, CD221, CD138, and CD45 for 15 min in the dark at room temperature. Cells were washed with PBS1X, then suspended in 500 μL PBS1X and analyzed on a FACS Canto II (Becton Dickinson). Malignant plasma cells were identified by the high expression of CD138 and CD38. Patient provided informed consent and this protocol was approved by the Hospices Civils de Lyon Ethics Committee.

2.4. Western blot analysis

To analyze the protein content of IGF-1R and study signaling components downstream of IGF-1R and IL-6, 1×10^7 human myeloma cells were incubated with bortezomib (1.5 nM) for 24 h at 37 °C. Cells were washed and incubated with human recombinant IGF-1 (200 ng/mL) or recombinant IL-6 (10 ng/mL) for 15 min at 37 °C. After incubation, cells were washed with phosphate-buffered saline (PBS) and then solubilised in lysis buffer (20 mM Tris–HCl (pH 6.8), 1 M $MgCl_2$, 2 mol/L EGTA, 0.5% NP40) with protease inhibitors (leupeptine, aprotinine, benzamidine, PMSF, TRCK) for 30 min on ice. Cell debris and nuclei were removed by centrifugation at 15,000 rpm for 15 min at 4 °C. Protein concentration was determined by analysis of samples stained with Coomassie blue by Ascent Software for Multiskan. Equal concentrations of total protein (50 μg per lane) combined with Laemmli buffer were heated at 95 °C for 5 min, and then separated on 8% (SDS)-polyacrylamide gels followed by electrophoretic transfer to PVDF or nitrocellulose membranes in iBlot Gel transfer stacks (Invitrogen, Cergy Pontoise, Frensh). Membranes were blocked for 1 h at room temperature with PBS containing Tween and 5% dry milk and incubated overnight at 4 °C with specific antibody. After 16 h, membranes were washed with PBS–Tween with 5% dry milk and then incubated with the secondary antibody (anti mouse peroxydase-conjugated antibody [Sigma] at a dilution of 1:6,000) for 1 h at room temperature. After extensive washing with phosphate-buffered saline, proteins were detected after addition of the staining substrates ECL (Amersham). The proteins were detected by chemiluminescence using Kodak film (Eastman Kodak Company) or using the Odyssey