Mast cells promote the growth of Hodgkin's lymphoma cell tumor by modifying the tumor microenvironment that can be perturbed by bortezomib

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Abstract

Hodgkin's lymphoma is frequently associated with mast cell infiltration that correlates directly with disease severity, but the mechanisms underlying this relationship remain unclear. Here, we report that mast cells promote the growth of Hodgkin's tumor by modifying the tumor microenvironment. A transplantation assay shows that primary murine mast cells accelerate tumor growth by established Hodgkin's cell lines, and promote marked neovascularization and fibrosis. Both mast cells and Hodgkin's cells were sensitive to bortezomib, but mast cells were more resistant to bortezomib. However, bortezomib inhibited degranulation, PGE_2-induced rapid release of CCL2, and continuous release of VEGF-A from mast cells even at the concentration that did not induce cell death. Bortezomib-treated MCs lost the ability to induce neovasculization and fibrosis, and did not promote the growth of Hodgkin tumor *in vivo*. These results provide further evidence supporting causal relationships between inflammation and tumor growth, and demonstrate that bortezomib can target the tumor microenvironment.

Keywords: Hodgkin's lymphoma, mast cells, angiogenesis, fibrosis, bortezomib

Introduction

Considerable evidence supports an initiating role of chronic inflammation in a proportion of malignancies (1) (2). In man, inflammatory bowel diseases predispose to colorectal cancer (3), chronic *Helicobacter pylori* infection is the leading cause of gastric cancer (4), and hepatitis virus B or C infection predisposes to liver carcinoma (5). Once established, many malignancies maintain an inflammatory component, perhaps due to persistence of the inflammation-initiating factors or to recruitment of inflammatory cells from the blood stream, resulting in various inflammatory cytokines being present at the tumor site (2). Mast cells are commonly recognized at the margins of diverse tumors in man and rodents (6-8). Mast cells can release numerous factors stored or newly synthesized after activation, resulting in modifying the tumor microenvironment.

Hodgkin's lymphoma (HL) is characterized by a few tumor cells, the Hodgkin and Reed–Sternberg (HRS) cells, surrounded by many benign inflammatory cells including mast cells (9). It has been reported that mast cell infiltration correlates with poor prognosis in Hodgkin's lymphoma (10)(11). Compelling studies have revealed that CD30 ligand secreted from MCs could directly proliferate certain portions of Hodgkin’s lymphoma cells (12). However, the roles of MCs in Hodgkin's lymphoma have not been fully evaluated so far.

Bortezomib, a proteasome inhibitor, has emerged as an effective anticancer therapy toward a broad range of malignant hematological disorders and may have far-reaching potential in autoimmune disease including GVHD because bortezomib can possess immuno-modulatory effects (13)(14). However, the effects of bortezomib on mast cells are completely unknown so far.

In this study, we report that mast cells could promote the growth of Hodgkin tumor

indirectly by modulating the tumor microenvironment and that bortezomib can target the mast cell- functions by inhibiting secretion of mast cells products.

Materials and Methods

Animal studies

The animal experiments were approved by the institutional ethics committee for Laboratory Animal Research, Nagoya University School of Medicine and were performed according to the guidelines of the Institute.

Cells and Reagents

The human Hodgkin's lymphoma cell lines L428 and KMH2 were a kind gift from Hayashibara Biochemical Labs Inc. (Okayama, Japan) and purchased from the Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSMZ) (Braunschweig, Germany). The human promyelocytic leukemia cell line HL-60 was obtained from the American Type Culture Collection (Rockville, MD). Bone marrow-derived mast cells (BMMCs) and spleen-derived mast cells (SPMCs) were established from C57BL/6 mice (National Cancer Institute, Frederick, MD) with murine IL-3 (PeproTech Inc., Rocky Hill, NJ), as described elsewhere (15)(16). Bortezomib (Velcade®) was obtained from LC laboratories (Woburn, MA).

In vitro cocultures

The proliferative effect of MCs on Hodgkin's lymphoma cells was assessed by a colorimetric assay (TetraColor One; Seikagaku Co., Tokyo, Japan) as described elsewhere (17) Briefly, L428 cells, KMH2 cells or HDLM2 cells were incubated alone, together with BMMCs or together with SPMCs in culture medium (RPMI containing 10% FBS and 10 ng/mL IL-3) in 96-well plates (L428, 4.0×10^3 cells/well; KMH2, 4.0×10^3 cells/well; HDLM2, 4.0×10^3 cells/well; BMMCs, 4.0×10^3 cells/well; SPMCs, $4.0 \times$

10^3 cells/well in 0.2 mL culture medium, four independent wells/group). After 72 hours of incubation, 10 μL of TetraColor One reagent was added to each well, and absorbance at 450 nm was measured 4 hours later. Proliferation of Hodgkin's lymphoma cells was calculated as follows: (OD value of the mixture of Hodgkin's lymphoma cells and MCs - OD value of MCs alone)/ OD value of Hodgkin's lymphoma cells alone.

Hodgkin's lymphoma transplantation assays

Six week-old female NOD/SCID mice (Charles River Japan, Yokohama, Japan), maintained were inoculated subcutaneously into the flank region above the hind leg with L428 (1.0×10^7 cells in RPMI), KMH2 (1.0×10^7 cells in RPMI) either alone or mixed with BMMC (1.0×10^7 cells in RPMI). Total injection volume was 200 μl. To study the effects of bortezomib on mast cell functions to facilitate tumor growth, BMMCs were incubated for 96 hours at 37°C in complete culture medium alone or with the addition of bortezomib (5.0 nM). DMSO (0.1%) was used as a relevant control for bortezomib. BMMCs (5.0×10^6 in RPMI) were washed twice with phosphate-buffered saline (PBS), and then were inoculated subcutaneously into six week-old female NOD/SCID mice (6 mice/group) together with intact L428 cells (5.0×10^6 in RPMI). All animals were examined twice weekly; tumor size was calculated in mm^3 using the formula (length × $width^2$ / 2) as previously described (18). The % tumor size was calculated as follows: (tumor size with bortezomib-treated BMMCs / tumor size with intact BMMCs) ×100. After measurement, Hodgkin's lymphoma cell tumors were removed, fixed in 10% neutral-buffered formalin solution (Sigma-Aldrich), and embedded in paraffin. Tissues were sectioned (5 μm thickness), and slides were stained with Masson's trichrome and H&E (Biopathology Institute Co., Ltd, Oita, Japan).

Human angiogenesis antibody array

The expression profile of angiogenesis-related proteins was detected and analyzed using human angiogenesis array kit (ARY007; R&D Systems). This array contains duplicate spots of 55 angiogenesis-related proteins. A weakly angiogenic HL-60 cell line (19) was adopted as a reference. Briefly, the membrane containing immobilized angiogenesis-related antibodies was incubated with cell lysates from 1.0×10^7 of L428 cells, KMH2 cells or HL60 cells. Bound protein was detected with detection antibody cocktail (Amersham Pharmacia Biotech) and a chemiluminescence detection system (ECL kit; Amersham Pharmacia Biotech). The membrane was scanned and the mean relative protein production level (target protein/GAPDH) from two individual measurements was calculated by densitometric scanning using NIH image software (http://rsb.info.nih.gov/nih-image/). The obtained values were adjusted by protein loading control level in each membrane and shown expressed as the mean spot pixel densities.

Mouse angiogenesis antibody array

The expression profile of angiogenesis-related proteins in BMMCs before and after the bortezomib treatment was analyzed using mouse angiogenesis array kit (ARY015; R&D Systems). Briefly, BMMCs (1.0×10^7) were treated with/without bortezomib (5.0 nM) for 72 hours and then were solubilized in lysis buffer. Hybridization of angiogenesis-related proteins to the membrane and detection of bound protein were similarly performed as described above. The obtained values were adjusted by protein

loading control level in each membrane and shown expressed as the mean spot pixel densities.

In vitro and in vivo measurement of mast cell and Hodgkin's lymphoma cell viability after exposure to bortezomib

MC and Hodgkin's lymphoma cell viability after exposure to bortezomib was assessed by trypan blue exclusion. Briefly, cells were washed twice with PBS, suspended in culture medium (RPMI containing 10% FBS), plated (3.0×10^4 cells/well in 0.2 mL culture medium) in four independent determinations with various concentrations (0-50 nM) of bortezomib onto 96-well plates, and incubated for 96 hours. Viable cells were determined as Trypan blue- negative cells. *In vivo* MC viability after exposed to bortezomib was analyzed by using matrigel (20). MCs (0.5×10^6) were treated with/without bortezomib (5.0 nM) for 72 hours and then mixed with 0.5ml of Matrigel, a crude extract of the Engelbreth-Holm-Swarm tumor (BD Biosciences - Discovery Labware). The mixtures were injected subcutaneously into the mid-abdominal region of female NOD/SCID 6- to 8-week-old mice (5 mice per group). After 7 days, Matrigel plugs were removed, fixed in 10% neutral-buffered formalin solution (Sigma-Aldrich), and embedded in paraffin. Tissues were sectioned (5 μm thickness), and slides were stained with toluidine blue. To measure the number of viable MCs, we counted the number of toluidine blue metachromatic cells in randomly selected 5 fields under a microscope (200× magnification). The results were averaged per each plug (1 plug section per mouse). Group values reflect the average ± SD readings from all sections in the group (5 plugs per group).

In vitro functional studies of bortezomib-treated MCs

Mast cells can release numerous factors stored or newly synthesized after activation through three different pathways: piecemeal degranulation (21), rapid secretion without degranulation (22) (23) and degranulation (24).

To evaluate the effect of bortezomib on piecemeal degranulation, we measured VEGF-A secretion. MCs (1.0×10^6 cells/mL) were incubated in culture medium (RPMI 1640 containing 10% FCS) with/without bortezomib (5.0 nM). MC culture supernatants (4 wells/subgroup) were obtained after cell culture for a period of 48, 72 and 96 hour, and were used for VEGF-A detection by a specific ELISA (R&D Systems, Minneapolis, MN).

To study the effect of bortezomib on rapid secretion without degranulation and degranulation, we measured CCL2 secretion and ß-Hexosaminidase release, respectively. MCs (1.0×10^6 cells/mL) were pretreated with bortezomib at 5.0 nM in complete culture medium for 72 hour before addition of PGE_2 (1000 nM) or calcium ionophore A23187 (5 μM). After 1-hour incubation with calcium ionophore A23187 or 6-hour incubation with PGE_2, cell-free supernatants were tested for CCL2 and β-Hexosaminidase release. All experiments were performed in triplicate. The percent release of MC products was calculated as follows: (MC product release in bortezomib group/ CCL2 release in control group) ×100 [%].

Statistical Analysis

Statistical significance of group differences was evaluated by Student's t-test using Excel software.

Results

Effects of mast cells on the proliferation of Hodgkin's lymphoma cells in vitro

In vitro coculture assays showed that BMMCs weakly proliferated only KMH2 cell, and that SPMCs did not proliferate any Hodgkin lymphoma cells (Figure 1a).

Mast cells promote the growth of Hodgkin's lymphoma cell tumors in vivo

We tested for the potential contribution of MCs to tumor formation of Hodgkin's lymphoma cells *in vivo*. L428, KMH2 cells and HDLM2 cells gave rise to tumors significantly more rapidly in NOD/SCID mice when they were inoculated subcutaneously together with 5×10^6 of BMMCs (5.0×10^6) (Figure 1b). The mean size of tumors derived from inoculation of either L428 plus BMMC, KMH2 plus BMMC or HDLM2 plus BMMC were significantly greater ($P<0.01$ all comparisons) than the size of tumors derived from inoculation of L428 alone, KMH2 alone or HDLM2 alone, respectively. We had confirmed that BMMC alone did not form tumors (20). Microscopically, tumors derived from inoculation of L428, KMH2 and HDLM2 Hodgkin's lymphoma cells together with mast cells possessed increased vasculature identified by their content of red blood cells and fibrosis whereas tumors derived from inoculation of Hodgkin's lymphoma cells alone were generally hypovascularized with less fibrosis and were necrotic in most of areas (Figure 1c). Interestingly, MCs, identified by cytochemical staining with toluidine blue (purple), localized in the proximity of vessels (Figure 1C, insets).

L428 and KMH2 Hodgkin lymphoma cells minimally produce proangiogenic factors

To determine proangiogenic factors released by Hodgkin's lymphoma cell lines, we

performed an antibody array using cell lysates from L428 cells, KMH2 cells or HL60 cells. A weakly angiogenic HL-60 cell line was adopted as a reference. As shown in Table 1, L428 cells and KMH2 cells rarely produced proangiogenic factors except for VEGF-A and FGF2. L428 cells and KMH2 cells weakly produce VEGF-A compared to HL-60. Both L428 cells and KMH2 produce FGF-2 in similar levels of VEGF.

Mast cells are sensitive to bortezomib, but are more resistant than Hodgkin's lymphoma cells

We exposed MCs and Hodgkin's lymphoma cells continuously to increasing concentrations of bortezomib and quantified the viabilities by trypan blue exclusion 96 hours later. Bortezomib induced a minimal cell death in MCs at concentrations as low as 5 nM with about 50% of cell death observed at a concentration of 10 nM (Figure 2a). However, more than 90% of Hodgkin's lymphoma cells were dead at the 10 nM bortezomib (Figure 2b). These results clearly suggest that MCs are more resistant to bortezomib than Hodgkin's lymphoma cells.

Bortezomib perturbs mast cell functions at the dose that minimally affects on mast cell viabilities

We tested effects of bortezomib on mast cell functions to release numerous factors stored or newly synthesized after activation. After 72-hour incubation with bortezomib (5 nM), calcium ionophore A23187- and PGE_2-activated BMMC and SPMCs released significantly less ß-Hexosaminidase and CCL2, respectively, compared with BMMC and SPMCs not treated with bortezomib (Figure 3a and 3b). Using a specific ELISA to measure VEGF-A in culture supernatants, we found that bortezomib can decrease by

approximately 50% the levels of VEGF-A released by BMMCs and SPMCs (Figure 3c). We also tested effects of bortezomib on expression of other proangiogenic factors in BMMC by using an antibody array using cell lysates from BMMCs before and after bortezomib treatment (5 nM for 96 hours). As summarized in Table 2, bortezomib treatment profoundly decreased expression of Angiopoietin-1, Endoglin, HB-EGF and VEGF-B, but not PAI-1. These decreased levels of proangiogenic factors in MCs culture supernatants and/or cytoplasm could not be attributed to decreased MC numbers, as 96 hour-treatment with bortezomib at a 5 nM minimally affected on mast cell viabilities (Figure 2a).

Bortezomib-treated MCs lost the abilities to promote the growth of Hodgkin's lymphoma cell tumors in vivo

We tested for the contribution of mast cell functions to the tumor growth of Hodgkin's lymphoma cells *in vivo* by using bortezomib. First, we analyzed *in vivo* MC viability after exposed to bortezomib by using matrigel. MCs (0.5×10^6) were treated with/without Bortezomib (5.0 nM) for 72 hour and then mixed with 0.5ml of Matrigel. The mixtures were injected subcutaneously into the mid-abdominal region of female NOD/SCID 6- to 8-week-old mice (5 mice per group). After 7 days, Matrigel plugs were removed. Toluidine blue staining showed that there was no significant difference in the number of viable MCs treated with/without bortezomib (Figure 4a). Next, NOD/SCID mice (6 mice per group) were inoculated subcutaneously with L428 cells or KMH2 cells (5.0×10^6) in conjunction with BMMCs (5.0×10^6) treated with/without bortezomib (5.0 nM). The mean size of tumors derived from inoculation of L428 or KMH2 plus intact BMMCs were significantly greater than the size and weight of tumors derived

from inoculation of L428 or KMH2 plus bortezomib-treated BMMCs (Figure 4b). Microscopically, tumors derived from inoculation of L428 or KMH2 Hodgkin's lymphoma cells together with intact MCs were highly vascularized and fibrotic, whereas tumors derived from inoculation of L428 or KMH2 cells plus bortezomib-treated MCs were generally not (Figure 4c). These results confirm that bortezomib is effective at inhibiting the MC-induced growth of Hodgkin's cell tumors *in vivo* by blocking the release of secretary granules from MCs.

Discussion

Evidence that pathological changes associated with inflammation in the tumor microenvironment promote tumorigenesis has been obtained in experimental model systems and clinical settings (2) (25) (26). It has been reported that mast cell infiltration correlates with poor prognosis in Hodgkin's lymphoma (10)(11). However, mouse MCs do not directly proliferate all of Hodgkin's lymphoma cells (Figure 1a). A previous study has shown that human MCs, when the same number of MCs and Hodgkin's lymphoma cells were cocultured, proliferated weakly but significantly Hodgkin's lymphoma cells (27). This discrepancy of effectiveness may result from different origins of MCs. Interestingly, when the same number of murine MCs and Hodgkin's lymphoma cells were inoculated subcutaneously in NOD/SCID, L428, KMH2 cells and HDLM2 cells gave rise to significantly larger tumors compared to Hodgkin's lymphoma cells alone (Figure 1b). Microscopic analysis showed that tumors derived from inoculation of Hodgkin's lymphoma cells together with mast cells possessed increased vasculature whereas tumors derived from inoculation of Hodgkin's lymphoma cell alone generally did not (Figure 1c). Thus, we provide direct evidence that mast cells have an ability to promote the growth of Hodgkin's lymphoma cell tumors indirectly through modulating the microenvironment. The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel (28). Protein array analysis showed that L428 and KMH2 Hodgkin's lymphoma cells minimally produce proangiogenic factors (Table 1), while mast cells are rich in proangiogenic factors (20). Thus, MCs must be indispensable for Hodgkin's lymphoma cells to propagate themselves. Mast cells also induced fibrosis in Hodgkin's lymphoma cell tumors (Figure 1c) as reported in various tissues in human diseases (29)

(30). Quantitative studies of mast cells in human specimens of Hodgkin's lymphoma showed that nodular sclersosis contained many more mast cells than the other subtypes (31) (32). Physical forces such as compression and tension forces induced by fibrosis can help cancer progression and tumor cell invasion through promoting angiogenesis, accumulating the fluid from leaky blood and lymphatic vessels and increasing the local concentration of growth factors and cytokines to facilitate autocrine and paracrine signalling (33). Taken together, fibrosis induced by mast cells could not only determine the subtype of Hodgkin's lymphoma but also contribute to Hodgkin's lymphoma progression.

Bortezomib, a proteasome inhibitor, exerts anticancer activity mainly by inhibiting NF-kB which a pivotal role in synthesis of antiapoptotic factors, such as c-Flip, inhibitor of apoptosis (IAP)1/2, and Bcl-2, and of angiogenic factors (34). In addition to their anticancer properties, bortezomib modulates inflammatory and immune responses by affecting function and survival of immune cells such as lymphocytes and dendritic cells (35). However, unique contributions of bortezomib to mast cell functions have not been previously demonstrated. Here we show that bortezomib not only inhibits the three distinct ways of release of secretary granules from MCs but also decrease expression of proangiogenic factors probably due to blocking the NF-kB pathway since compelling studies revealed that NF-kB activation can be involved in production and/or secretion of cytokines, chemokines and chemical mediators from MCs (36)(37)(38).

Bortezomib was approved for the treatment of multiple myeloma at first and then applied to malignant lymphoma (39). Bortezomib also has been evaluated in patients with relapsed/ refractory Hodgkin's lymphoma, but none achieved a clinical response with bortezomib monotherapy (40) or with bortezomib plus dexamethasone (41) on the

contrary to our results. In these clinical studies, the dose of bortezomib was as the same as that used in multiple myeloma (1.3mg/m^2 on days 1, 4, 8, 11 every 21 days). Inflammatory cells such as mast cell and macrophages have been proved to be durable to even high dose chemotherapy and irradiation (42) (30). We also confirmed that MCs are more resistant to bortezomib than Hodgkin's lymphoma cells (Figure 2a). Pharmacokinetic Analysis showed that bortezomib, when administrated intravenously even at the dose of 1.45mg/m^2, was rapidly eliminated from the circulation and the plasma level 5 hour after administration was below 5 ng/mL (43). In this study, bortezomib at 5 ng/mL perturbed mast cell functions, but the viabilities of MCs were virtually 100 % even after 72-hour treatment (Figure 2a). The effects of bortezomib on inflammatory cells can be transient because bortezomib is a reversible enzyme inhibitor. These evidence and results suggest that bortezomib monotherapy at the dose of 1.3mg/m^2 is insufficient to target the tumor microenvironment to support Hodgkin's lymphoma cells and that higher dose of bortezomib or combination with other anticancer drugs must be required to treat relapsed or refractory Hodgkin's lymphoma.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Effects of mast cells on development of Hodgkin's lymphoma cell tumors. (**a**) Direct effects of mast cells on proliferation of Hodgkin's lymphoma cells. Human Hodgkin's lymphoma cell lines (L428 cells and KMH2, 2.5×10^4 cells each) were mixed with or without primary mast cells from bone marrow (BMMC, 2.5×10^4 cells) and incubated in 0.5 mL culture medium (four independent wells/group) for 7 days. The total number of viable cells was determined by Trypan blue exclusion. The number of Hodgkin's lymphoma cells in the mixture was determined as c-kit negative cells analyzed by FACS. The results represent the mean ± SD of four replicates (representative experiment of three performed). (**b**) Effects of mast cells on development of Hodgkin's lymphoma cell tumors. Six week-old female NOD/SCID mice were inoculated subcutaneously into the flank region above the hind leg with L428 (10×10^6 cells in RPMI), KMH2 (10×10^6 cells in RPMI) either alone or mixed with BMMC (10×10^6 cells in RPMI) (5 mice/group). Total injection volume was 200 μl. All animals were examined twice weekly; tumor size was calculated in mm^3 using the formula (length × $width^2$ / 2). The results represent the mean ± SD of tumor size derived from 5 animals (left panel). Statistical significance: $^{**}P < 0.01$. All animals were sacrificed on day 12 and tumors were removed (right panel). Representative results from 2 independent experiments are shown. (**c**) Representative microscopic histology of Hodgkin's lymphoma cell tumors. Tumors derived from inoculation of L428 and KMH2 with/without BMMCs were stained with H&E (upper panel) and Masson Trichrome (lower panel). Masson Trichrome staining turns red blood cells and collagen bundles into orange and blue, respectively. MCs were identified by cytochemical staining with toluidine blue (purple, arrowhead, insets).

Figure 2. Measurement of mast cell and Hodgkin's lymphoma cell viability after exposure to bortezomib.

(**a**) (**b**) MCs (bone marrow-derived MCs and spleen-derived MCs, 3.0×10^4 cells each, left panel) and human Hodgkin's lymphoma cell lines (L428 cells and KMH2, 3.0×10^4 cells each, right panel) were incubated in 0.2 mL culture medium with various concentrations (0-50 nM) of bortezomib for 96 hours (four independent determinations/each bortezomib concentration/subgroup). The number of viable and dead cells was determined by Trypan blue exclusion. Histograms show total number of viable cell at each concentration of bortezomib. Line graphs show % viability of cells at each concentration of bortezomib. The percentage of viable cells was calculated as (the number of viable cells/the number of viable plus dead cells) × 100 (%). The results shown reflect the mean ± SD of four independent determinations, and the results are representative of three independent experiments.

Figure 3. *In vitro* functional studies of bortezomib-treated mast cells.

Mast cells (MCs) can release numerous factors stored or newly synthesized after activation through three different pathways: degranulation, rapid secretion without degranulation and piecemeal degranulation. (**a**) (**b**) To study the effect of Bortezomib on degranulation and rapid secretion without degranulation, ß-Hexosaminidase release and CCL2 secretion were evaluated, respectively. MCs (bone marrow-derived MCs and spleen-derived MCs, 1.0×10^6 cells/mL each, left panel) were pretreated with bortezomib at 5.0 nM in complete culture medium for 72 hours before addition of calcium ionophore A23187 (5μM) or PGE_2 (1000 nM). After 1-hour incubation with calcium ionophore

A23187 or 6-hour incubation with PGE_2, cell-free supernatants were tested for β-Hexosaminidase and CCL2 release. All experiments were performed in triplicate. The percent release of MC products was calculated as follows: (MC product release in bortezomib group/ MC product release in control group) ×100 [%]. The results shown reflect the mean ± SD of three independent determinations, and the results are representative of three independent experiments. Statistical significance: $*P < 0.05$.

(**c**) To evaluate the effect of Bortezomib on piecemeal degranulation, we measured VEGF-A secretion. MCs (10^6 cells/mL) were incubated in culture medium with/without bortezomib (5.0 nM). MC culture supernatants (4 wells/subgroup) were obtained after cell culture for a period of 48, 72 and 96 hour, and were used for VEGF-A detection by a specific ELISA. The results shown reflect the mean ± SD of four independent determinations, and the results are representative of three independent experiments. Statistical significance: $**P < 0.01$.

Figure 4. *In vivo* functional studies of bortezomib-treated mast cells.

(**a**) Bone marrow-derived MCs (BMMCs, 0.5×10^6 cells) were treated with/without Bortezomib (5.0 nM) for 72 hours and then mixed with 0.5 ml of Matrigel. The mixtures were injected subcutaneously into the mid-abdominal region of female NOD/SCID 6- to 8-week-old mice (5 mice per group). After 7 days, Matrigel plugs were removed and stained with toluidine blue. To measure the number of viable MCs, the number of toluidine blue metachromatic cells was counted in randomly selected 5 fields under a microscope (200× magnification). The results were averaged per each plug (1 plug section per mouse). Group values reflect the average ± SD readings from all sections in the group (5 plugs per group). (**b**) NOD/SCID mice (6 mice per group) were inoculated

subcutaneously with L428 cells (5.0×10^6) in conjunction with BMMCs (5.0×10^6) treated with/without bortezomib (5.0 nM). All animals were sacrificed on day 10 and tumors were removed. The % tumor size was calculated as follows: (tumors with bortezomib-treated BMMCs / tumors with intact BMMCs) ×100 [%]. Statistical significance: $*P < 0.05$. Representative results of two independent experiments are shown. (**c**) Representative microscopic histology of Hodgkin's lymphoma cell tumors. Tumors derived from inoculation of L428 with intact or bortezomib-treated BMMCs were stained with H&E (upper panel) and Masson Trichrome (lower panel). Masson Trichrome staining turns red blood cells and collagen bundles into orange and blue, respectively.

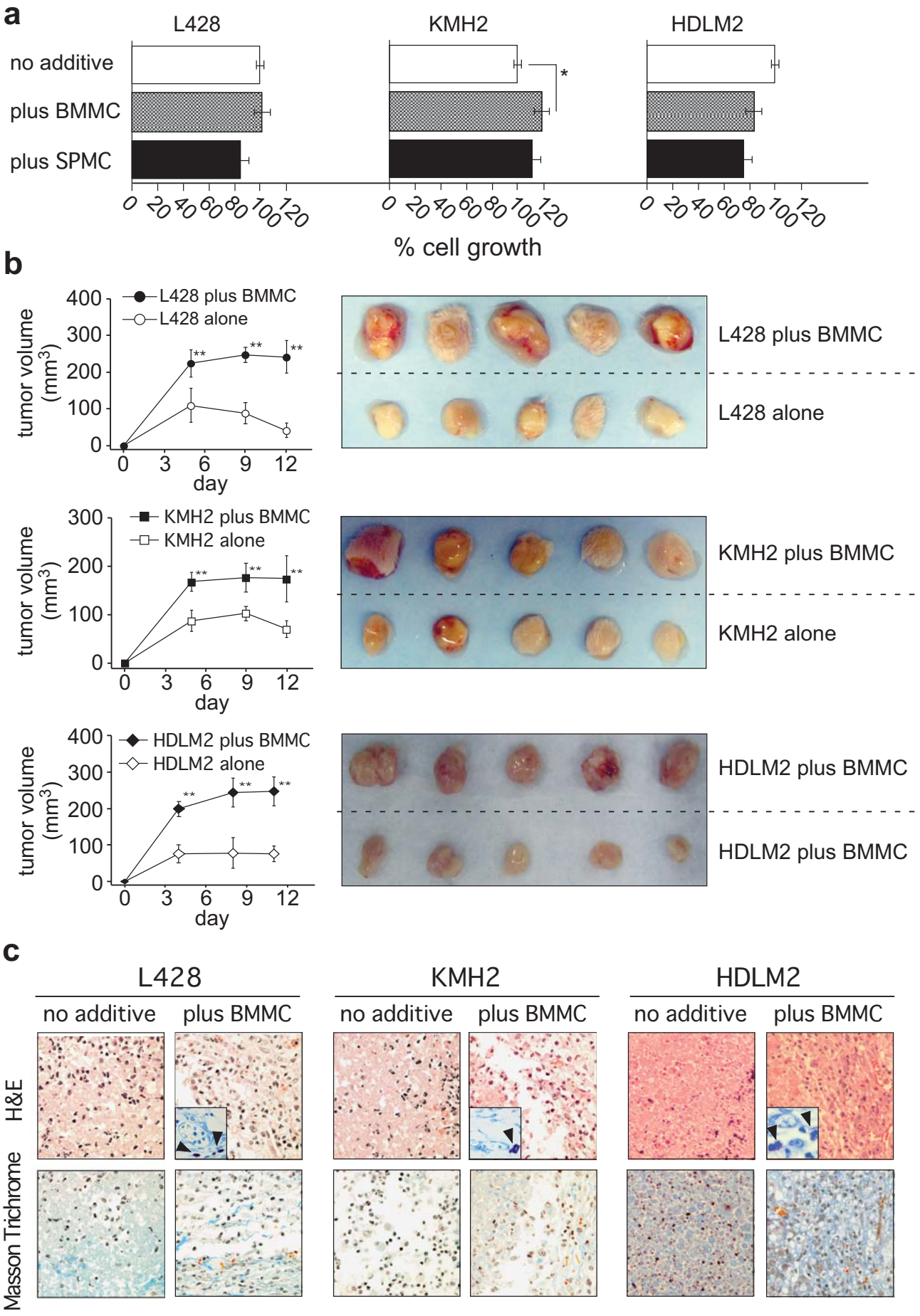
a
L428
KMH2
HDLM2
no additive
plus BMMC
plus SPMC
*
0 20 40 60 80 100 120
% cell growth
b
L428 plus BMMC
L428 alone
tumor volume (mm3)
day
KMH2 plus BMMC
KMH2 alone
HDLM2 plus BMMC
HDLM2 alone
c
H&E
Masson Trichrome

Figure 1

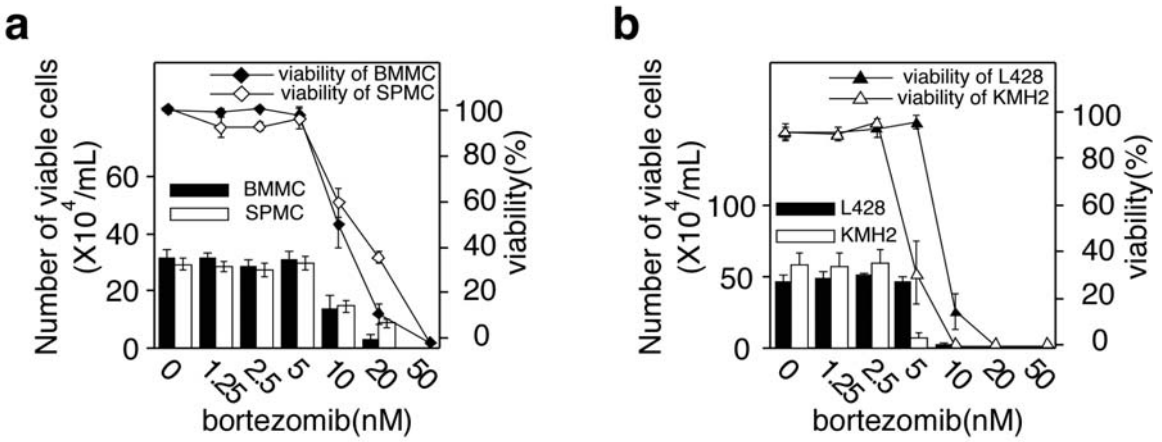
a
viability of BMMC
viability of SPMC
BMMC
SPMC
Number of viable cells
(X10 4 /mL)
viability(%)
bortezomib(nM)
b
viability of L428
viability of KMH2
L428
KMH2
Number of viable cells
(X10 4 /mL)
viability(%)
bortezomib(nM)

Figure 2

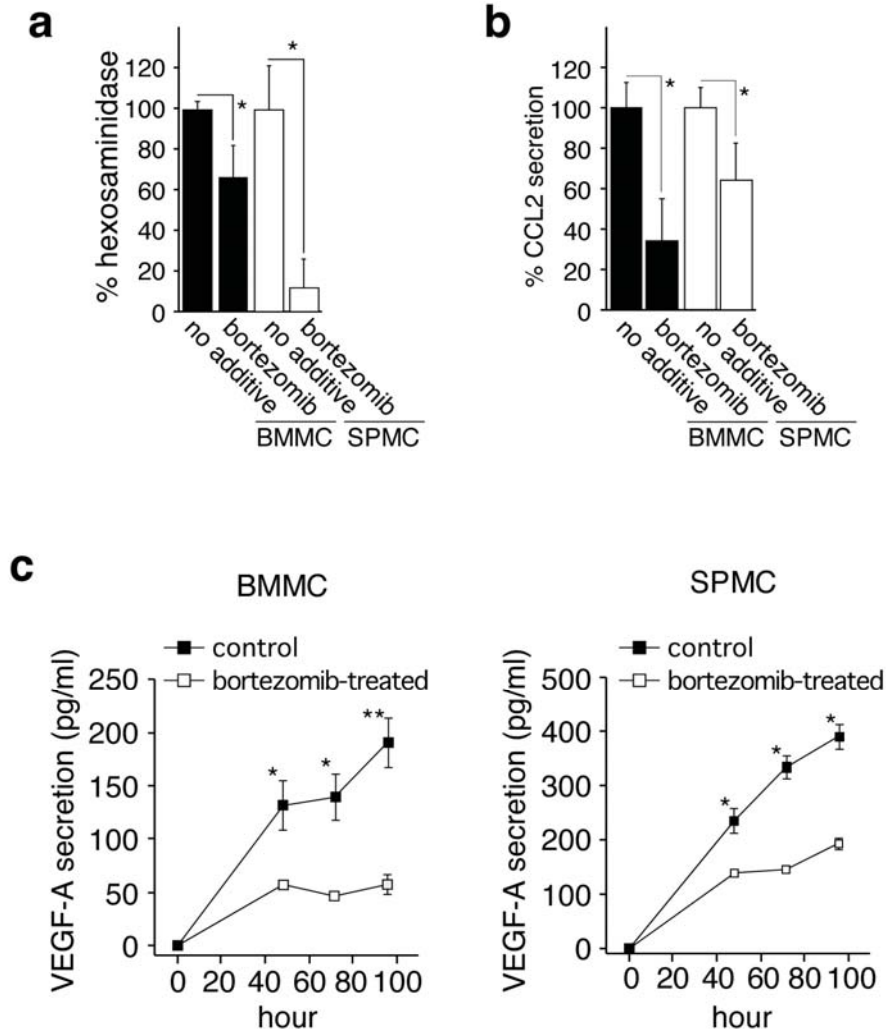
a
% hexosaminidase
120
100
80
60
40
20
0
*
*
no additive
bortezomib
no additive
bortezomib
BMMC
SPMC
b
% CCL2 secretion
120
100
80
60
40
20
0
*
*
no additive
bortezomib
no additive
bortezomib
BMMC
SPMC
c
BMMC
control
bortezomib-treated
VEGF-A secretion (pg/ml)
250
200
150
100
50
0
*
*
**
0 20 40 60 80 100
hour
SPMC
control
bortezomib-treated
VEGF-A secretion (pg/ml)
500
400
300
200
100
0
*
*
*
0 20 40 60 80 100
hour

Figure 3

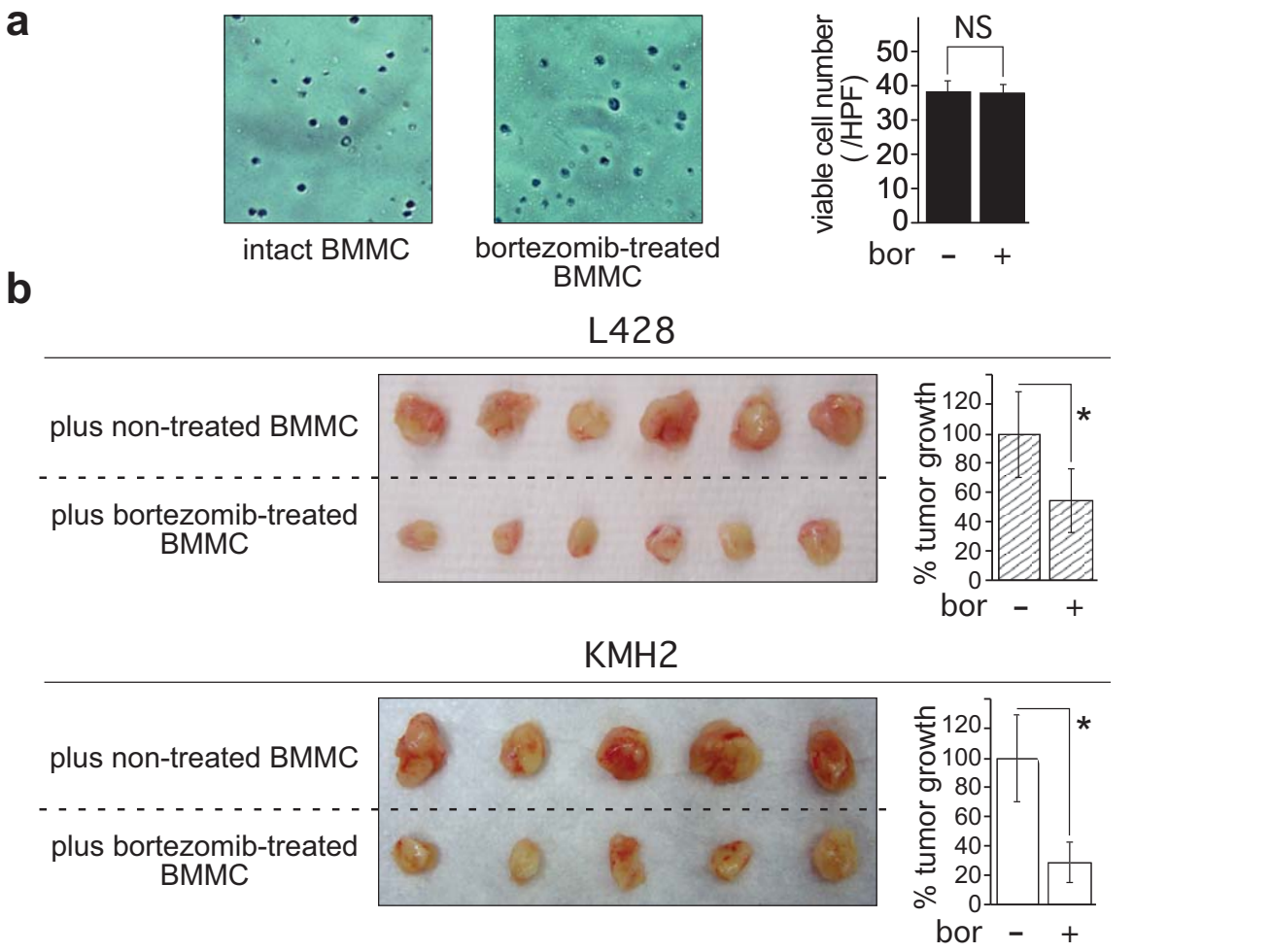
a
intact BMMC
bortezomib-treated BMMC
viable cell number (/HPF)
NS
50
40
30
20
10
0
bor – +
b
L428
plus non-treated BMMC
plus bortezomib-treated BMMC
% tumor growth
120
100
80
60
40
20
0
*
bor – +
KMH2
plus non-treated BMMC
plus bortezomib-treated BMMC
% tumor growth
120
100
80
60
40
20
0
*
bor – +
c
L428 plus BMMC
KMH2 plus BMMC
non-treated
bortezomib-treated
non-treated
bortezomib-treated
H&E
Masson Trichrome

Figure 4

Table 1. Expression profiling of angiogenesis-related proteins in L428, KMH2 and HL-60 cells.

Gene name	UniGene code	L428 (pixel density)	KMH2 (pixel density)	HL-60 (pixel density)
Angiopoietin-1	Hs.369675	ND	ND	ND
Angiopoietin-2	Hs.583870	ND	ND	ND
Tissue Factor	Hs.62192	ND	23040	28560
EGF	Hs.419815	ND	ND	ND
EG-VEGF	Hs.514793	ND	ND	ND
FGF-1	Hs.483635	ND	ND	ND
FGF-2	Hs.284244	33726	46729	ND
FGF-4	Hs.1755	ND	ND	ND
FGF-7	Hs.567268	ND	ND	ND
IL-8	Hs.624	ND	5624	55080
CCL2	Hs.303649	ND	ND	41565
CCL3	Hs.514107	ND	ND	ND
PD-ECGF	Hs.592212	ND	ND	ND
PDGF-AA	Hs.535898	ND	131	ND
PDGF-BB	Hs.1976	ND	ND	ND
CXCL4	Hs.81564	ND	ND	ND
PlGF	Hs.252820	ND	ND	ND
VEGF	Hs.73793	29158	44241	82110
VEGF-C	Hs.435215	ND	ND	ND

ND, not detected

Table 2. Expression profiling of angiogenesis-related proteins in BMMCs before and after bortezomib.

Gene name	UniGene code	before bortezomib (pixel density)	after bortezomib (pixel density)	ratio (after/before)
Angiopoietin-1	Mm.309336	61950	13132	0.21
Endoglin	Mm.225297	32598	4721	0.14
Endostatin	Mm.4352	16249	12335	0.76
HB-EGF	Mm.289681	15900	3718	0.23
CCL2	Mm.290320	117560	82110	0.70
CCL3	Mm.1282	36137	23667	0.65
NOV	Mm.5167	96293	85680	0.89
PDGF-AA	Mm.2675	33154	24735	0.75
PDGF-AB	Mm.144089	24125	13574	0.56
PlGF-2	Mm.4809	56006	37740	0.67
PAI-1	Mm.250422	139619	149302	1.07
VEGF-A	Mm.282184	9919	ND	NA
VEGF-B	Mm.15607	14704	2360	0.16

BMMCs, bone marrow-derived mast cells; ND, not detected; NA, not applicable