Differential effects of proteasome inhibition by bortezomib on murine acute graft-versus-host disease (GVHD): delayed administration of bortezomib results in increased GVHD-dependent gastrointestinal toxicity

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We have recently demonstrated that the proteasome inhibitor, bortezomib, administered immediately following murine allogeneic bone marrow transplantation (BMT) resulted in marked inhibition of acute graft-versus-host disease (GVHD) with retention of graft-versus-tumor effects. We now assessed the effects of delayed bortezomib administration (5 or more days after BMT) on GVHD. Recipient C57BL/6 (H2b) mice were lethally irradiated and given transplants of bone marrow cells and splenocytes from major histocompatibility complex (MHC)–disparate BALB/c (H2d) donors. In marked contrast to the effects of bortezomib on GVHD prevention when administered immediately after BMT, delayed bortezomib administration resulted in significant acceleration of GVHD-dependent morbidity. No toxicity was observed following delayed bortezomib administration in models where donor T cells were not coadministered, indicating that these deleterious effects were critically dependent on GVHD induction. The increase in GVHD susceptibility even occurred when late administration of bortezomib was preceded by early administration. Pathologic assessment revealed that significant increases in gastrointestinal lesions occurred following delayed bortezomib administration during GVHD. This pathology correlated with significant increases of type 1 tumor necrosis factor α (TNF-α) receptor transcription in gastrointestinal cells and with significant increases of TNF-α, interleukin 1β (IL-1β), and IL-6 levels in the serum. These results indicate that the differential effects of proteasome inhibition with bortezomib on GVHD are critically dependent on the timing of bortezomib administration.

Introduction

The occurrence of acute graft-versus-host disease (GVHD) remains one of the most significant causes of morbidity following allogeneic bone marrow transplantation (BMT). GVHD is caused by administration of donor T cells into a genetically disparate recipient. The pathophysiology of GVHD is a complex process that can be conceptualized in 3 phases.1-3 In the first phase, the cytoreductive conditioning regimen causes immunosuppression of the recipients and damage to host tissues, including a self-limited burst of inflammatory cytokines. In the second phase, donor T cells recognize alloantigens on host antigen-presenting cells (APCs) and these activated T cells then proliferate and differentiate into effector cells. The second phase is critical for the amplification of the systemic inflammatory response, in which donor T cells also contribute to the inflammatory cytokine network. In the third phase, target tissues undergo apoptosis mediated by cellular effectors and inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interferon γ, and further host tissue injury establishes a positive inflammatory feedback loop. Solid organs attacked during acute GVHD include the gut, liver, lungs, and skin.1-3

The proteasome is a multicatalytic protease complex responsible for the degradation of most intracellular proteins, including proteins crucial to cell-cycle regulation and apoptosis. Bortezomib (Velcade, formerly PS-341) is the first of its class of proteasome inhibitors to be tested in humans and has shown promising activity in several tumor types, especially in hematologic malignancies.4 It has recently been approved as a therapy in multiple myeloma.5 Bortezomib exerts numerous biologic effects that include blocking the activation of the transcription factor, nuclear factor-κB (NF-κB).6-8 NF-κB is implicated in the regulation of many genes that code for mediators of the immune and inflammatory responses.9,10

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We have recently demonstrated that proteasome inhibition using bortezomib can markedly inhibit the generation of acute GVHD in mice after allogeneic BMT.\textsuperscript{11} Importantly, significant antitumor effects were maintained, suggesting that bortezomib may be of use to improve the efficacy of BMT. In that study, we found that bortezomib administered immediately after BMT reduced donor-derived T-cell expansion by inhibiting cell proliferation and selectively inducing apoptosis in the activated donor alloreactive T-cell population.\textsuperscript{11} Another mechanism by which bortezomib can limit GVHD may involve the blockade of NF-κB activity, resulting in a decrease in inflammatory cytokines produced by donor T cells and damaged host tissues after cytoreductive conditioning. However, NF-κB has both proapoptotic and anti-apoptotic functions\textsuperscript{12} and can affect both proinflammatory and anti-inflammatory responses.\textsuperscript{13,14} It is possible that GVHD progression could also be enhanced by blocking NF-κB activity and thereby augmenting TNF-mediated cellular injury.\textsuperscript{15,16} Therefore, it was important to investigate the effects of bortezomib administration during ongoing GVHD.

We report here that the differential effects of proteasome inhibition with bortezomib on a murine GVHD model is determined by the timing of bortezomib administration. Delayed bortezomib administration resulted in marked acceleration and manifestation of GVHD-dependent pathology, particularly in the gut following BMT. This was correlated with increased transcription of type 1 TNF-α receptor (TNFR1) in the gut and the presence of proinflammatory cytokines in the serum. These results suggest that extreme caution must be used with delayed administration of bortezomib in combination with T cell–replete allogeneic BMT.

Materials and methods

Animals

Female BALB/c (H2\textsuperscript{a}), C57BL/6 (B6, H2\textsuperscript{b}), and immunodeficient C.B-17\textsuperscript{scid/scid} (SCID, H2\textsuperscript{c}) mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were kept in specific pathogen-free conditions. All animal protocols were approved and in vivo studies were performed at each of the 2 animal facilities (National Cancer Institute, Frederick, MD, and University of Nevada, Reno). Mice were between 8 and 12 weeks of age at the start of the experiments.

Reagent

The proteasome inhibitor, bortezomib, was kindly provided by Millennium Pharmaceuticals (Cambridge, MA). Stock bortezomib solution (1 mg/mL) was prepared in Dulbecco phosphate-buffered saline solution (PBS) and stored at −70°C for up to 2 months prior to use. Bortezomib solutions were protected from light at all times. The stock solutions were thawed and diluted to 0.075 mg/mL in PBS immediately prior to use.

Cell preparation

Bone marrow cell (BMC) suspensions were prepared by gently releasing cells from the backbones, femurs, and tibiae into PBS with a mortar and pestle, filtering through a mesh filter to remove particulates, and washing the cell suspensions twice. Spleen-cell preparations were prepared by gently crushing the tissues to release the cells. Preparations were filtered to remove debris and washed twice in PBS for injection. Cell counts were determined by multiplex analysis on a Luminex (Austin, TX), using mouse cytokine-specific bead sets and standards according to the manufacturer’s instructions. Serum samples from 3 independent experiments with 3 to 5 serum samples per group were analyzed in each independent experiment.

In vivo studies

Induction of GVHD studies were performed at each of the 2 animal facilities (National Cancer Institute and University of Nevada) according to the guidelines of the Animal Care and Use Committees of each institution.
scores were evaluated with the Mann-Whitney test. *P* less than .05 was considered significant.

### Results

**Delayed administration of bortezomib results in accelerated GVHD morbidity following allogeneic BMT**

We have previously observed that bortezomib, administered immediately after allogeneic BMT, resulted in increased survival and protection from GVHD. Therefore, we next assessed the effects of bortezomib given at later time points when GVHD was ongoing. Using a fully major histocompatibility complex (MHC)–mismatched BMT model (BALB/c (H2d) BMCs and SCs lethally irradiated C57BL/6 (H2b) recipients), we found that early administration of bortezomib (day 0-2 after BMT) consistently resulted in significant protection from GVHD (Figure 1A-B). In marked contrast, when bortezomib was administered at later time points (day 5-7 after BMT), the mice rapidly died due to acute GVHD.

Because the delayed administration of bortezomib results in a rapid induction of GVHD mortality, we next performed dose responses of donor SCs to ascertain the extent of GVHD augmentation with delayed bortezomib administration. The results demonstrate that even at low doses of SCs, in which no outward signs of GVHD were apparent, the delayed administration of bortezomib resulted in significantly (*P* < .005) increased GVHD morbidity (Figure 2C-E). Thus, delayed bortezomib administration can result in the occurrence of acute GVHD in instances where no evidence of overt GVHD would normally be detected.

Pathologic evaluation indicated that recipients of SCs and delayed bortezomib treatment had marked increases in gut lesions in the small intestine and colon (Figures 3-4) compared with control recipients. The small intestine had greater villous blunting and fusion, ulceration, and inflammation. Crypt-cell apoptosis and hyperplasia were also more extensive than in the recipients receiving SCs alone. Sections of colon tissue showed evidence of goblet-cell depletion with sloughed cells in crypt lumens, multifocal ulceration, and inflammation. As in the small intestine, crypt-cell hyperplasia was more extensive than in the recipients receiving SCs alone. The presence of microscopic lesions was contingent on the administration of both SCs and delayed bortezomib administration because no pathologic damage or GVHD mortality was observed when bortezomib was administered at this or any other time point without SCs (Figures 2A-B and 3). In the liver, there were no significant differences in the lesions present between the 2 treatment groups that received SCs alone. This suggests that the gut is the primary target for this increased mortality after delayed bortezomib administration following allogeneic BMT. Thus, the data indicate that the timing of bortezomib administration
Delayed administration of bortezomib is associated with increased TNFR1 mRNA levels in the gut of GVHD mice

Because histopathologic examination had revealed that the gut was particularly affected with delayed bortezomib administration, we analyzed the tissue for expression of genes associated with killing pathways. At 6 hours after delayed (day 12-13 after BMT) bortezomib administration, the small intestine and liver were assessed by RPA for mRNA levels of Fas, FasL, and TNFR1 (p55). The results demonstrate that significant increases in TNFR1, the principal proinflammatory and proapoptotic receptor for TNF-α, were observed in the small intestine, but not the liver, of mice receiving SCs and delayed bortezomib treatment compared to recipients receiving either delayed bortezomib treatment (data not shown) or SCs alone (Figure 5A-C). No significant differences in Fas or FasL were observed (data not shown) suggesting that the TNF pathway may be particularly targeted in the gut of recipients receiving SCs after delayed bortezomib administration. Thus, up-regulating TNFR1 expression in small intestine may be, in part, the mechanism of increased susceptibility of GVHD mice to immune-mediated target damage and lethality after delayed bortezomib administration.

Increases in serum cytokine levels in GVHD mice after delayed bortezomib administration

We next assessed the levels of proinflammatory cytokines at 6 hours after delayed (day 12-13 after BMT) bortezomib administration. Serum levels of the proinflammatory cytokines TNF-α, interleukin 1β (IL-1β), and IL-6 were all significantly (P < .05) increased in the recipients receiving SCs and delayed bortezomib treatment compared to recipients receiving SCs only, suggesting that the TNF pathway increases only in the recipients with SCs but not in the recipients without SCs (Figure 6). These results indicate that despite the ability of proteasome inhibition by bortezomib to block NF-κB, increased proinflammatory cytokines were detected after delayed administration of bortezomib during ongoing GVHD. Consistent with the histopathology data, increases in TNF-α, IL-1β, and IL-6 levels as well as gut tissue TNFR1 levels after delayed bortezomib administration were critically dependent on GVHD induction.
Discussion

The results presented here definitively demonstrate that proteasome inhibition with bortezomib can prevent acute GVHD when administered immediately after BMT but can also promote GVHD-dependent toxicity when administration is delayed. This increase in GVHD mortality was striking in that it could be observed even when lower amounts of donor T cells were administered such that no outward manifestations of GVHD (ie, diarrhea, posture, or grooming changes) were observed in the recipients not receiving delayed bortezomib and when GVHD was prevented by early administration of bortezomib. These results are consistent with our previous study that GVHD prevention by early administration of bortezomib is due to a lowering or dampening of the donor T-cell responses. Reduced T-cell responses could still be augmented by...
the later administration of bortezomib. This may also explain how graft-versus-tumor responses were still maintained following early bortezomib treatment.

Bortezomib exerts numerous biologic effects that include blocking the activation of the transcription factor NF-κB. NF-κB is implicated in the regulation of many genes that code for mediators of the immune and inflammatory responses. GVHD progression is accompanied by inflammatory cytokine production and inflammatory cytokine-mediated target tissue damage. Therefore, not only immune cells (donor T cells, recipient dendritic cells) but also nonimmune cells (recipient target tissues) should be affected by delayed bortezomib administration. NF-κB plays a protective role in cell death induced by TNF-α. Therefore, blocking NF-κB activity in GVHD target tissues may enhance GVHD damage by enhancing TNF-mediated cellular injury. Our data indicate that the gut is the primary organ made more susceptible to GVHD attack after delayed bortezomib administration. Other studies demonstrate a strong association with NF-κB and protection from intestinal epithelial apoptosis and suppression of antiapoptotic molecules of the inhibitor of apoptosis protein (IAP) family up-regulation by the proteasome inhibitor MG132 with the sensitization of normal enterocytes to Fas-induced apoptosis. Additionally, it has been demonstrated that NF-κB can protect enterocytes from TNF-mediated death. Consistent with this observation, it has been demonstrated that NF-κB inhibition could prevent inflammation and yet result in increased cell injury in an intestinal ischemia model. NF-κB blockade may play a similar role in GVHD by suppressing the initial phase of inflammation and the later phase by increasing the susceptibility of GVHD target organs to immune-mediated apoptosis. TNFR1 is the principal proinflammatory and proapoptotic receptor for TNF-α, and the data presented here demonstrating increased mRNA levels of TNFR1 would support this pathway as being a potential component of the mechanisms by which bortezomib accelerated GVHD-induced gut pathology. It has been demonstrated that TNFR1-induced apoptosis involves 2 sequential signaling complexes. Failure to activate NF-κB (the initial step in the signaling process) results in cell death. Therefore, delayed bortezomib administration increased susceptibility to GVHD target organ (gut) damage and lethality may involve multiple steps. Donor T cells, as well as cytokines released by donor T cells, are necessary to trigger immune-mediated damage. Delayed bortezomib administration may act, in part, to enhance this damage by up-regulating expression of TNFR1, although bortezomib may induce apoptosis by multiple pathways, some of which may be independent of the NF-κB pathway. No protection from bortezomib-mediated accelerated morbidity was observed when mice deficient in TNFR1 were used (data not shown). This would suggest that TNFR2 or other mediators may also contribute to the toxicity associated with delayed bortezomib administration.

A previous study has shown that NF-κB activation in leukocytes recruited during the onset of inflammation is associated with proinflammatory gene expression, whereas activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis. Inhibition of NF-κB during the resolution of inflammation protracts the inflammatory response and prevents apoptosis. This suggests that NF-κB has an anti-inflammatory role in vivo involving the regulation of inflammatory resolution. A similar phenomenon may be occurring with donor T cells during GVHD progression in the GVHD models presented here.

Even though previous studies demonstrated that NF-κB activation provides radioprotection to the intestinal epithelium, we have shown that delayed bortezomib administration-dependent gut damage in our model is not dependent on radiation damage. The results showing that recipients of grafts with minimal (bone marrow source only) T cells and receiving bortezomib at any time point lack pathologic damage or morbidity indicate that bortezomib-related lethality in our model is dependent on GVHD-related immune-mediated attack. The demonstration that adoptive allogeneic lymphocyte transfer into immunodeficient SCID mice (without conditioning such as TBI) results in increased GVHD susceptibility after delayed bortezomib administration confirms that bortezomib-related lethality is due solely to GVHD processes.

Because GVHD is a complex and multistage disease process, it is perhaps not surprising that bortezomib exhibits differential effects contingent on the timing of administration. The induction phase of GVHD appears to be the most susceptible phase for successful intervention. It has been demonstrated that administration of cytokines such as IL-2, IL-12, administration of cellular therapy such as donor-type natural killer (NK) cells, and the presence of cytokines such as interferon-γ, are all protective only if administered or present early after BMT. If administration or presence of any of the cytokines or immune cells is delayed, accelerated GVHD has been reported. Given that proteasome inhibitors have been demonstrated to sensitize neoplastic cells to chemotherapeutics and immune lytic molecules such as TNF-related apoptosis-inducing ligand (TRAIL) or TNF-α, it is also perhaps not surprising that normal cells and tissues may also be sensitized to subsequent immune attack. It remains to be determined if augmented antitumor responses can also be observed during this stage and if there is a means to prevent the GVHD-dependent toxicity without loss of graft-versus-tumor effect.

The results presented here demonstrate the 2 facets of proteasome inhibition by bortezomib in GVHD, as determined by the timing of bortezomib administration. Our data indicate that bortezomib could be potentially used at any time following T cell–depleted allogeneic BMT. However, extreme caution must be used with delayed administration of bortezomib in combination with T cell–replete allogeneic BMT because exacerbation of GVHD-dependent pathology, particularly in the gut, may occur.

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