GRP-78 secreted by tumor cells blocks the antiangiogenic activity of bortezomib

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Abstract

Antiangiogenic effects of the proteasome inhibitor bortezomib were analyzed on tumor xenografts in vivo. Bortezomib strongly inhibited angiogenesis and vascularization in the chicken chorioallantoic membrane. Bortezomib's inhibitory effects on chorioallantoic membrane vascularization were abrogated in the presence of distinct tumor xenografts, thanks to a soluble factor secreted by tumor cells. Through size-exclusion and ion-exchange chromatography as well as mass spectroscopy, we identified GRP-78, a chaperone protein of the unfolded protein response, as being responsible for bortezomib resistance. Indeed, a variety of bortezomib-resistant solid tumor cell lines (PC-3, HRT-18), but not myeloma cell lines (U266, OPM-2), were able to secrete high amounts of GRP-78. Recombinant GRP-78 conferred bortezomib resistance to endothelial cells and OPM-2 myeloma cells. Knockdown of GRP78 gene expression in tumor cells and immunodepletion of GRP-78 protein from tumor cell supernatants restored bortezomib sensitivity. GRP-78 did not bind or complex bortezomib but induced pro-survival signals by phosphorylation of extracellular signal-related kinase and inhibited p53-mediated expression of pro-apoptotic Bok and Noxa proteins in endothelial cells. From our data, we conclude that distinct solid tumor cells are able to secrete GRP-78 into the tumor microenvironment, thus demonstrating a hitherto unknown mechanism of resistance to bortezomib. (Blood. 2009;114:3960-3967)

Introduction

Bortezomib (Velcade, formerly PS-341), a boronic acid dipeptide, is a selective but reversible proteasome inhibitor. It has been approved for clinical use in humans, in particular for treatment of multiple myeloma and mantle cell lymphoma. Besides its direct antitumor effects, antiangiogenic actions of bortezomib have recently been described in vitro and in vivo. Angiogenesis is critical for tumor growth, invasion, and metastasis. Thus, inhibiting angiogenesis in tumor tissue is a strategy by which solid tumor progression can be slowed down or even stopped.

In contrast to other antiangiogenic compounds, such as thalidomide or bevacizumab, the clinical use of bortezomib is not associated with vascular complications, such as bleeding and thrombosis. In this study, we aimed to analyze its effects on growth and angiogenesis of tumor xenografts in vivo and to characterize molecular targets in vitro on human endothelial cells. Bortezomib has been shown to interfere in the ubiquitin-proteasome pathway by inhibiting the 26S proteasome at its "chymotrypsin-like" site. The 26S proteasome is responsible for degrading a variety of regulatory proteins involved in proliferation and cell cycle regulation. Moreover, misfolded proteins, such as immunoglobulins in myeloma, are degraded by the proteasome. Accumulation of misfolded proteins after proteasome inhibition results in activation of the unfolded protein response (UPR). The UPR is an evolutionarily conserved mechanism that activates survival pathways to allow eukaryotic cells to adapt to endoplasmic reticulum (ER) stress. One of the main UPR responses is induction of the chaperone protein GRP-78. This protein belonging to the heat shock protein family has a C-terminal ER retention signal and is up-regulated under stress conditions, such as hypoxia, glucose deprivation, or the presence of cytotoxic substances. In this study, we were able to demonstrate that GRP-78 is released from the ER by solid tumor but not by myeloma cells, and protects endothelial cells from the antiangiogenic action of bortezomib. This is a novel, hitherto unknown, mechanism of resistance to bortezomib.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated according to the Jaffe et al protocol. HUVECs were cultivated in endothelial growth medium-2 medium (Cambrex) on type I collagen (from kangaroo; Sigma-Alrich)–coated plastic. Expanded HUVECs were detached using collagenase-I (Sigma-Alrich) and trypsin-ethylenediaminetetraacetic acid (Invitro- gen) and split in a 1:3 ratio.

To achieve cell cycle–arrested cells, HUVECs were grown to confluence. Cell-cycle arrest was assessed by flow cytometry using propidium iodide staining. Murine B16F10 melanoma cells, the glioblastoma cell line U87, the prostate cancer cell line PC-3, and the breast cancer cell line MCF-7 were purchased from ATCC. The multiple myeloma cell lines U266 and OPM-2 were a generous gift of Dr Albert Amberger (both Tyrolean Cancer Research Institute). All cell lines were grown in the recommended cell media (RPMI 1640; PAA Laboratories GmbH) supplemented with 10% FBS.

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fetal calf serum, 105 IU/L penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine in the presence of 5% CO2.

**Generation of GFP-transgenic U373 and HRT-18 cells**

The cDNA for green fluorescent protein (GFP) was subcloned in a pT2 transposon vector with a strong cytomegalovirus promoter as described elsewhere.17 Thereafter, nucleotide sequence was confirmed by double-strand sequencing. U373 and HRT18 cells were transfected with the pT2 GFP vector and a vector encoding the sleeping beauty transposase SB11. Clones resistant to neomycin were selected and propagated in the ClonCell medium (Stem Cell Technologies) and then analyzed for GFP expression.

**Tumor cell supernatants and bortezomib**

Bortezomib (Velcade, PS341) was purchased from Janssen-Cilag GmbH and diluted with fresh medium immediately before use.

For Western blot analysis and to test their potential to inhibit bortezomib-conditioned supernatants of various tumor cell lines were obtained by culturing the cells with RPMI containing 1% fetal calf serum for 48 hours. For chromatographic analysis of tumor cell supernatants, PC3 were cultured after extensive washing for 72 hours in serum-free RPMI medium containing 105 IU/L penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. Supernatants were harvested, centrifuged at 12 000g for 30 minutes to remove cell debris, and sterile-filtered (Millepore GP, 0.2 μm; Millipore) to remove residual precipitates. Supernatants were concentrated using Vivaspin 20 tubes (Sartorius) according to the manufacturer’s instructions.

**GRP-78 denaturation, knockdown, and immunoabsorption**

Recombinant GRP-78 protein purified from bacterial overexpression was purchased from Acris Antibodies Ltd. Mannitol was solved in EBM-2 medium. Knockdown was controlled after 48 hours by Western blots of the respective supernatants with a rabbit anti–GRP-78 polyclonal antibody (Abnova).

Moreover, CAM xenografts of HRT-18 and U373 cells expressing the transgene GFP were generated. This allows visualization of the tumor by fluorescence stereomicroscope with a connected digital camera (Olympus E410) and flexible cold light (KL200; Olympus).

**BrdU incorporation assay**

A total of 5000 cells/well were seeded in triplicates into a 96-well plate (Nunc) in culture medium and were allowed to adhere overnight before the culture medium was changed. Thereafter, cells were incubated with the respective purified proteins. After 1 day, cells were pulsed for 24 hours with a 10μM dilution of bromodeoxyuridine (BrdU; GE Healthcare) in culture medium. Then cells were washed, fixed, and incorporated BrdU detected by a specific enzyme-linked immunosorbent assay (GE Healthcare) in an enzyme-linked immunosorbent assay reader (ELx808; BioTek).

**Measurement of apoptosis by flow cytometry**

Apoptotic cells were determined by annexin V–fluorescein isothiocyanate (Alexis) and 7-amino-actinomycin D (7-AAD; Beckman Coulter) staining in a binding buffer consisting of 2.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 40 mM NaCl, and 0.8 mM CaCl2. Stained cells were then analyzed in the Cytomics FC-500 cytometer using Cytomics RXP software (Beckman Coulter).

**Chick CAM assay**

The assay was performed as described elsewhere.18 In brief, fertilized white leghorn chicken eggs (SFP eggs) were purchased from Charles River (Kieslegg) and incubated at 37°C with 60% humidity (Compact S84; Grumbach) for 4 days. Then a Thermanox ring (Nunc) was placed on the chorioallantoic membrane (CAM), and after sealing (Durapor tape) eggs were incubated for 24 hours. Thereafter, the ring was opened and various concentrations of bortezomib, dissolved in a 10 mM Tris-glucose buffer solution (pH 7.4), or buffer solution alone were applied to the ring area. After 3 days of incubation, blood vessels were analyzed using the Olympus SZX10 stereomicroscope (Olympus) equipped with an Olympus DPFL 2X-4 objective lens (numerical aperture 0.2) connected with a digital camera (Olympus E410) and flexible cold light (KL200; Olympus).

**Tumor xenografts in the CAM**

B16F10 xenografts were generated by grafting 10⁶ melanoma cells within the ring area on the CAM of a 4-day-old embryo. One day after grafting, various concentrations of bortezomib were applied daily to the area within the ring. Dark-pigmented melanoma cells and the subjacent CAM were analyzed on developmental day 8 (ie, 72 hours after incubation).

Moreover, CAM xenografts of HRT-18 and U373 cells expressing the transgene GFP were generated. This allows visualization of the tumor by fluorescence stereomicroscope with a connected digital camera (same Olympus system as described in “Chick CAM assay”). For this purpose, 10⁶ tumor cells were seeded in the ring area of 7-day-old embryos. After 2 days, the rings were treated daily with 0.5 ng of bortezomib. On day 12, tumors and subjacent CAM were analyzed.

**Western blot analysis**

A total of 30 μg of total protein was denaturated, separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Lonza Walkersville), and transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking, membranes were probed with primary antibodies directed against alpha tubulin (Sigma-Aldrich), phospho-p53 (S46; R&D Systems), Noxa (GeneTex), 20S proteasome core subunits
elsewhere. In brief, supernatants were buffered with a 0.020 M Tris-HCl solution (pH 9.0) before loading on a BioSuite Q10 AXC column (Waters). The proteins were eluted using 1 M NaCl/0.020 M Tris-HCl, pH 9.0. The elution gradient proceeded from 0% to 70% of 1 M NaCl within 70 minutes. Isoelectric points (pI) were estimated with the broad pI kit (3.6-9.3; GE Healthcare).

Ion-exchange chromatography (IEC) was performed as described elsewhere. In brief, supernatants were buffered with a 0.020 M Tris-HCl solution to pH 9.0 before loading on a BioSuite Q10 AXC column (Waters). The proteins were eluted using 1 M NaCl/0.020 M Tris-HCl, pH 9.0. The elution gradient proceeded from 0% to 70% of 1 M NaCl within 70 minutes. Isoelectric points (pI) were estimated with the broad pI kit (3.6-9.3; GE Healthcare).

Two-dimensional gel electrophoresis and mass spectrometry

Proteins in the active fractions from SEC and IEC were precipitated in a 10% trichloroacetic acid solution (Roth) as described previously. The precipitated proteins were resuspended in an isoelectric focusing sample solution (8 M urea, 1% 3-(cholamidopropyl)dimethylammonio-1-propanesulfonate, 0.010 M dithiothreitol, 0.1% [wt/vol] amphotelys [pH 3-10]; Bio-Rad). First-dimension isoelectric focusing was performed with the Ettan IPGphor system (GE Healthcare) with Immobiline DryStrip gels (GE Healthcare) having a pH gradient of 3 to 10. SDS-PAGE in the second dimension was performed with 4% to 16% gradient gels (Lonza Walkersville) in the Mine PROTEAN 3 system (Bio-Rad) as described previously. Protein spots were visualized using Page Blue (Fermentas) or SyproRuby (Lonza Walkersville) according to the manufacturer’s protocol. Spots of interest were excised from gels and subjected to mass spectrometry (MALDI TOF/TOF 4800 plus Analyzer; Applied Biosystems), and fragments were analyzed with ProteinPilot Version 2.0 software (Applied Biosystems).

Statistical analysis

Statistical analysis of all in vitro assays was performed with Prism 5 software for Windows (GraphPad Software Inc) using the 2-sided Mann-Whitney test.

Results

Bortezomib inhibits angiogenesis in vivo in the CAM assay

The CAM assay is an established model for studying proangiogenic and antiangiogenic substances in vivo. Effects can be directly monitored by studying the formation of the vascular plexus in the CAM after treatment with bortezomib. Even lowest concentrations of bortezomib (0.1 ng/ring) were found to inhibit the formation of vascular structures in the treated area (Figure 1A). Doses of up to 10 ng were also tested but were toxic to the embryo, that is, caused growth retardation and/or death (data not shown).

Bortezomib does not inhibit growth or angiogenesis of melanoma xenografts

To study the effects of bortezomib on tumor growth and vascularization in vivo, we used a xenograft tumor model in the chicken embryo. B16F10 melanoma cells were used because dark melanin pigments allow easy detection of tumor cells on the CAM. Moreover, the generated melanomas have a strong proangiogenic phenotype. Interestingly, even highest dosages of bortezomib (10 ng/ring) did not affect tumor growth or vascularization of the subjacent CAM (Figure 1B). We thus hypothesized that tumors secrete substances able to block the inhibitory effects of bortezomib on the subjacent vascular plexus. Indeed, application of purified B16F10 melanoma cell supernatants blocked the antiangiogenic activity of bortezomib on the CAM (Figure 1C).
endothelial cells (Figure 2A). Neither myeloma cell lines U266 or OPM-2 nor the breast cancer cell line MCF-7 or the glioblastoma cell line U373 were able to block the antiangiogenic activity of bortezomib. However, the colorectal carcinoma cell line HRT-18 and the prostate carcinoma cell line PC-3 strongly inhibited the antiangiogenic action of bortezomib.

To monitor tumor growth in vivo, bortezomib-sensitive U373 and bortezomib-resistant HRT-18 cells were genetically modified to stably overexpress GFP. This transgene allows tumors to be monitored after xenotransplantation in the CAM. Growth of HRT-18 tumors was not affected by bortezomib treatment. By contrast, the growth of U373 tumors was markedly inhibited by bortezomib (Figure 2B).

**Figure 2.** Screening for human tumor cell supernatants that block the antiangiogenic activity of bortezomib. (A) HUVECs were incubated with either control solution (-) or 10 ng/mL bortezomib (+) in the presence of the respective tumor cell culture supernatant. Cell viability was determined after 24 hours in a WST-1 assay monitoring the mitochondrial succinate reductase activity at 450 nm. Cells in standard culture medium served as control. Among the analyzed cell lines were: myeloma (U266 and OPM-2), breast carcinoma (MCF-7), glioblastoma (U373), colorectal cancer (HRT-18), and prostate carcinoma (PC-3). Bars represent mean ± SEM. *P < .05. (B) Growth and vascularization of human tumor xenografts in the CAM assay. Bortezomib-sensitive glioblastoma (U373) and bortezomib-resistant colorectal cancer (HRT-18) cells with stable overexpression of GFP were grown on the CAM for 2 days. Then xenografts were treated daily with control solution (-) or with 5 ng/mL bortezomib (+). After 3 days, tumors were analyzed under the fluorescence stereomicroscope to visualize GFP-positive tumor cells on the CAM. Bortezomib displayed marked antitumor effects against U373 but not against HRT-18 xenografts on the CAM. Original magnification ×10. Images were acquired using an Olympus SZX10 stereomicroscope (2×/0.2 NA objective) with an Olympus E410 digital camera.

**GRP-78: an acidic 72-kDa protein of the unfolded protein response is secreted by bortezomib-resistant tumor cells**

The supernatant of the bortezomib-resistant prostate cancer cell line PC-3 was further analyzed to identify factors responsible for blocking the antiangiogenic activity of bortezomib. This was done by separating the serum-free secretome according to the molecular weight using SEC. All fractions were subsequently tested on HUVECs to identify those able to inhibit the antiangiogenic action of bortezomib. A fraction with the molecular size of approximately 70 kDa showed the strongest inhibitory effect on endothelial cell growth (Figure 3A). In parallel, proteins of the same PC-3 supernatant were separated according to pl using anion-exchange chromatography (IEC). Obtained fractions were again tested on HUVECs to identify those that block the antiangiogenic effect of bortezomib on endothelial cells (Figure 3B). Only a very acidic fraction with a pl of less than 4.5 was able to block the antiangiogenic effect on HUVECs. Bars represent mean ± SEM. *P < .05. (C) GRP-78 was the main compound in both biologically active fractions (Fr.8 from SEC and Fr.15 from IEC). Fractions were analyzed with recombinant GRP-78 (100-nM solution) either by SDS-PAGE stained with Coomassie blue (left panels) or by Western blot (right panel) to estimate GRP-78 concentrations and purity.

**Figure 3.** Identification of GRP-78 in the prostate carcinoma cell supernatant. (A) SEC of PC-3 supernatants was performed, and single fractions were tested on HUVECs in the WST-1 cell viability assay. All fractions were tested in the presence of 10 ng/mL bortezomib in direct comparison with unfractionated supernatant (SN). Untreated HUVECs served as positive control (co. +) and HUVECs stimulated with bortezomib in the absence of tumor supernatant as negative control (co. −). The scale indicates the molecular weight (kDa) of the individual fractions. A fraction with the molecular size of approximately 70 kDa showed the strongest inhibitory effect on endothelial cell growth. (B) IEC of PC-3 supernatants was performed, and the fractions were analyzed in HUVECs in the WST-1 assay. The scale indicates the approximate pl of the various fractions. Only a very acidic fraction with a pl of less than 4.5 was able to block the antiangiogenic effect on HUVECs. Bars represent mean ± SEM. *P < .05. (C) GRP-78 was the main compound in both biologically active fractions (Fr.8 from SEC and Fr.15 from IEC). Fractions were analyzed with recombinant GRP-78 (100-nM solution) either by SDS-PAGE stained with Coomassie blue (left panels) or by Western blot (right panel) to estimate GRP-78 concentrations and purity.
transport and recognition. Immunoactive GRP-78 protein was present in both biologically active fractions of SEC and IEC (Figure 3C right panel) at concentrations lower than 100nM. GRP-78 was also the major protein compound in both fractions analyzed as shown by SDS-PAGE and Coomassie staining (Figure 3C left panel).

GRP-78 is up-regulated after bortezomib treatment in solid tumor cells

Although almost all tumor cell lines analyzed and also HUVECs contain GRP-78 intracellularly (Figure 4A), only the bortezomib-resistant lines were found to secrete it into the supernatant. Notably, GRP-78 protein was detectable in high amounts in the supernatants of both bortezomib-resistant cell lines PC-3 and HRT-18 but not or only faintly in the supernatants of MCF7, U373, U266, and OPM-2, respectively (Figure 4B). Whereas U266 myeloma cells down-regulated intracellular GRP-78 expression 24 hours after bortezomib stimulation, PC-3 prostate cancer cells showed a significant up-regulation of cytosolic GRP-78 (CT; Figure 4C). Moreover, PC3 cells also increased GRP-78 secretion into the supernatant on bortezomib treatment (SN, Figure 4C).

GRP-78 confers resistance to OPM-2 myeloma cells

Finally, recombinant full-length GRP-78 was tested for inhibition of the proapoptotic action of bortezomib (10 ng/mL) on solid tumor (MCF7) and myeloma cells (OPM-2, U266) that are not able to secrete this chaperone. When stimulated simultaneously, GRP-78 concentrations of 100 nM were sufficient to completely inhibit bortezomib-induced death in OPM-2 cells but were not effective in U266 or MCF7 cells (Figure 4D). However, when added 12 hours after bortezomib, GRP-78 was not able to rescue cells from bortezomib-induced apoptosis (supplemental Figure 1A, available on the Blood website; see the Supplemental Materials link at the top of the online article).

The protective action of GRP-78 on endothelial cells can be significantly blocked by a neutralizing antibody

Application of recombinant GRP-78 was tested on HUVECs. Whereas the control protein (alkaline phosphatase) and mannitol (added as a stabilizer to commercially available bortezomib) had no effect on cell viability, recombinant GRP-78 efficiently prevented loss of cell viability after bortezomib treatment (Figure 5A). Concentrations of 10nM were sufficient to antagonize 26nM bortezomib (10 ng/mL, P < .05). GRP-78 still retained its protective activity even after denaturation and refolding (10 minutes, 100°C). Addition of a GRP-78-neutralizing polyclonal antibody together with GRP-78 rendered cells significantly more sensitive to bortezomib treatment than an isotype control antibody (Figure 5A, P < .05). Exogenous application of the GRP-78–neutralizing polyclonal antibody to PC3 and HRT18 cells did not render cells more sensitive to bortezomib-induced apoptosis (supplemental Figure 1B).

Depletion of GRP-78 protein from tumor supernatants restores sensitivity of endothelial cells to bortezomib

Depletion of GRP-78 from tumor cell supernatant was performed by 2 approaches. First, endogenous gene expression was down-regulated by 100 nM siRNA, resulting in loss of GRP-78 secretion within 72 hours of incubation. Second, GRP-78 was depleted from the tumor cell supernatant by immunoprecipitation (IP) with a GRP-78–specific polyclonal antibody. Compared with the control siRNA and control IP using an isotype antibody, both techniques were successful to eliminate GRP-78 protein from the tumor supernatant (Figure 5B). All supernatants were tested together with 10 ng/mL bortezomib on HUVECs in a cell viability assay. As expected, depletion of GRP-78 resulted in a significant loss of protection from bortezomib-induced cell death (Figure 5D, P < .05).

GRP-78 does not bind bortezomib and does not affect S20 proteasome activity

To study the possibility of a direct interaction of GRP-78 with bortezomib, immunoprecipitation assays using a GRP-78–binding antibody were carried out after incubation of the respective supernatant (HRT-18 and PC-3 cells, respectively) with 10 ng/mL bortezomib (Figure 5C). After effective GRP-78 depletion using Sepharose beads (Figure 5B), the supernatant was tested on endothelial cells. GRP-78 was not found to inhibit bortezomib by direct interaction with the compound itself (eg, binding or complex formation) because GRP-78–depleted supernatants still contained the drug and induced cell death on exposed HUVECs (Figure 5D). Moreover, GRP-78 had no significant impact on the overall
proteasome activity of HUVECs with or without the presence of bortezomib (Figure 5E).

**GRP-78 increased phosphorylation of ERK and AKT and inhibited p53-mediated induction of BOK and NOXA**

Bortezomib induces apoptosis in proliferating but not in confluent endothelial cells as determined by annexin V–fluorescein isothiocyanate staining, not even at concentrations as high as 1000 ng/mL. In contrast to the control protein, GRP-78 (10nM) significantly protected endothelial cells from bortezomib-induced apoptosis (Figure 6A).

Next, the molecular targets of bortezomib were investigated up to 12 hours after bortezomib treatment on confluent and proliferating HUVECs. Moreover, effects of GRP-78 were studied in direct comparison with a control protein on proliferating HUVECs in the presence of bortezomib. Compared with arrested cells, bortezomib induced phosphorylation of p53 (serine 46; 6- to 7-fold) and induced the proapoptotic p53 target proteins BOK (4- to 6-fold) and NOXA (7- to 8-fold) in proliferating HUVECs (Figure 6B). The addition of GRP-78 significantly inhibited p53 phosphorylation (7.4- vs 2.3-fold) and induction of BOK (5.4- vs 0.9-fold) and NOXA (8.4- vs 3.2-fold). However, GRP-78 had no impact on the amount of 26S proteasome subunits in endothelial cells. Notably, the overall amount of 26S proteasome subunits was 6- to 7-fold higher in proliferating than in confluent cells.

Treatment with bortezomib significantly reduced DNA synthesis of proliferating endothelial cells (Figure 6C). In contrast to the control protein, the addition of GRP-78 to bortezomib resulted in an increased DNA synthesis, whereas GRP-78 alone had no impact on DNA metabolism.

To test the hypothesis that, in the presence of bortezomib, exogenous GRP-78 may activate prosurvival pathways, we analyzed the expression of ERK and AKT at various time points after exposure to bortezomib in combination with GRP-78 (Figure 6D). Indeed, the addition of GRP-78 to bortezomib resulted in an increase of phosphorylated isoforms of ERK in endothelial cells compared with control protein-treated cells, although, to a lesser extent, phosphorylation of AKT in HUVECs was also increased after coincubation of bortezomib with GRP-78.

**Discussion**

Analysis of various tumor and myeloma cell lines revealed that supernatants of distinct solid tumor cell lines, such as HRT-18 and PC-3, conferred bortezomib resistance to endothelial cells. However, other cell lines, such as MCF-7 and the multiple myeloma cell lines U266 and OPM-2, did not protect endothelial cells from bortezomib-induced apoptosis. The finding that tumor cells secrete factors that render adjacent cells of the tumor microenvironment resistant to bortezomib treatment has not been described to date. However, several other mechanisms that render tumor cells resistant to bortezomib have been found recently. As an example, mutations in the β5 subunit of the proteasome prevented bortezomib from binding to the proteasome.22 Furthermore, tumor cells have been shown to acquire resistance by up-regulating subunits of the proteasome after prolonged sublethal proteasome inhibition.23,24 Moreover, overexpression of heat shock protein (HSP) 27 has been reported in some tumor cell types that inhibited the
Figure 6. GRP-78 inhibits p53-mediated induction of BOK and NOXA and induces phosphorylation of ERK. (A) HUVECs were incubated with increasing concentrations of bortezomib (0.01-1000 ng/mL) for 24 hours. Then the percentage of apoptotic cells was determined by flow cytometry after annexin V and 7-AAD staining. Confluent cells were directly compared with proliferating cells (log phase) and proliferating cells treated with 10nM control protein or GRP-78. *P < .05. (B) Molecular proapoptotic targets of bortezomib and GRP-78 on endothelial cells: p53 phosphorylation and NOXA and BOK expression were analyzed in confluent and log phase growing cells 5 to 12 hours after bortezomib stimulation (10 ng/mL). In contrast to control protein, GRP-78 (10nM) prevented the phosphorylation of p53 (serine 46) and induction of BOK and NOXA. Tubulin-α served as internal loading control. (C) DNA synthesis was studied by the BrdU incorporation assay. In contrast to control cells and cells treated with control protein, 10nM GRP-78 significantly blocked the anti-proliferative effect of bortezomib on endothelial cells. Bars represent mean ± SEM. *P < .05. (D) Phosphorylation of the ERK and protein kinase B (AKT) signaling pathway.30 Moreover, despite the presence of bortezomib, GRP-78 induced a strong phosphorylation of ERK in endothelial cells and, although weakly, also of AKT. Again, GRP-78 might bind to cell surface receptors, such as cripto-1, which is highly expressed on the cell surface of endothelial cells and induces a strong prosurvival signal by activating the ERK and possibly also the AKT signaling pathway.30

In conclusion, distinct tumor but not myeloma cells are able to inhibit bortezomib by secretion of GRP-78 on proteasome inhibitors, thus demonstrating a hitherto unknown mechanism of resistance to bortezomib. Future studies will have to focus on the potential clinical relevance of these findings with regard to the administration of bortezomib, possibly in combination with HSP inhibitors in solid tumors and lymphoproliferative disorders.

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Authorship

Contribution: J.K., G.U., and M.S. designed the study, carried out experiments, data analysis, and statistical evaluation, and wrote the manuscript; C.Z. and B.S. carried out size-exclusion and ion-exchange chromatography and mass spectrometry; G.G., E.G., G.U., and M.S. interpreted study data; and E.G. and M.S. provided funding.

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