Bortezomib Inhibits Cell-Cell Adhesion and Cell Migration and Enhances Epidermal Growth Factor Receptor Inhibitor–Induced Cell Death in Squamous Cell Cancer

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Abstract

The lack of cell-cell adhesion and increased migration are key characteristics of cancer cells. The loss of expression of cell adhesion components and overexpression of components critical for cell migration, such as focal adhesion kinase (FAK), correlate with poor prognosis. Because alteration of protein turnover affects the expression levels and, in turn, may influence protein function, we investigated the effects of the proteasome inhibitor bortezomib on cell adhesion and migration in oral squamous cell cancer cell lines SCC68 and SCC15. Following treatment with bortezomib, protein levels of adherens junction components such as E-cadherin were unchanged. The desmosomal linker protein desmoplakin level was increased, whereas the protein level of the desmosomal cadherin, desmoglein 2, was diminished. Reduced desmoglein 2 levels correlated with the diminished strength of mechanical cell-cell adhesion. The protein level of the epidermal growth factor receptor (EGFR) increased after proteasome inhibition and EGFR inhibition with the EGFR-specific tyrosine kinase inhibitor PKI166 was able to restore cell-cell adhesion. Furthermore, we found that the combination of PKI166 with bortezomib enhanced the rate of cell death. Although the FAK protein level was unchanged following bortezomib treatment, recruitment of FAK phosphorylated at tyrosine residue 397 to the periphery of the cell was induced. Migration was reduced following treatment with bortezomib, which could potentially be explained by a prominent but disorganized actin fiber network revealed through immunofluorescence. Collectively, our results suggest that proteasome inhibition using bortezomib affects cell adhesion and cell migration profoundly and provides a rationale for its clinical use in conjunction with an EGFR inhibitor. [Cancer Res 2007;67(2):727–34]

Introduction

The lack of cell-cell adhesion is an important characteristic of cancer cells, and the lack of expression of cell adhesion components, such as cadherins or catenins, is linked to tumor invasion, progression, and poor prognosis (1–3). Adherens junctions and desmosomes are key mediators of intercellular adhesion in epithelial tissue. In adherens junctions, classic cadherin molecules such as E-cadherin are linked to the actin cytoskeleton through linker molecules such as α- or β-catenin. In desmosomes, specialized cadherin molecules, desmoglein(s) and desmocollin(s), are linked to the cytokeratin intermediate filament network through their distinctive set of adapter proteins such as desmoplakin. Although the loss of adherens junctions and their components has frequently been described for various tumors, less attention has been focused on possible alterations in desmosome-dependent adhesion or signaling, which may contribute to tumor progression or invasion (4).

The degradation of cell adhesion molecules is tightly regulated. Most studies have concentrated on the regulation of adherens junction molecules, such as E-cadherin and β-catenin, whereas relatively little is known about the regulation of desmosomal proteins. The inhibition of proteasomal breakdown reduced E-cadherin turnover and prevented the dissolution of adherens junctions (5). In addition to proteasomal degradation, lysosomal breakdown is involved in the regulation of E-cadherin as well (6). The degradation of other adherens junction components such as β-catenin also involves ubiquitination followed by proteasomal breakdown (7) and recent evidence suggests that ubiquitination is a key step in the regulation of the wnt-β-catenin pathway (8).

Much less is known about the turnover of desmosomal components, although evidence suggests that the regulation is equally complex. We have previously shown that one particular desmosomal cadherin, desmoglein 2 (Dsg2), is a substrate for matrix metalloproteinase (MMP)–dependent cleavage, and MMP inhibition may contribute to the up-regulation of Dsg2 and the increase in cell-cell adhesion following inhibition of the epidermal growth factor receptor (EGFR) (9). EGFR inhibition may also diminish Dsg2 internalization, possibly requiring MMP activity (10). No studies thus far have addressed proteasomal degradation of desmosomal proteins.

Cell migration, like cell-cell adhesion, is an important mechanism in tumor invasion and progression. It is a coordinated process that involves changes in the dynamics of actin filaments, together with the formation and disassembly of focal adhesion complexes, which are crucial for coordinated movement and are major factors influencing cell signaling and cell survival (11, 12). Focal adhesion complexes link the basal surface of cells to the intracellular actin cytoskeleton and the extracellular matrix through specialized transmembrane receptors called integrins. Within the adhesion complex, the focal adhesion kinase (FAK) plays a critical role, acting as the anchor for the adhesion complex.

FAK expression is increased in many cancer types (13–17). Several studies have shown that FAK inhibition blocks the response to cell motility cues (18). FAK activity is regulated by phosphorylation of one or more tyrosine residues. The tyrosine

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-2162

phosphorylation at residue 397 (FAK PY397) has been extensively studied and can be induced through a number of conditions such as integrin clustering and growth signals affecting cell migration and cell survival (19). The process by which FAK is degraded is not clearly understood. It seems that FAK is subject to ubiquitination and subsequent proteolytic breakdown (20), and that this process depends on the suppressor of cytokine signaling proteins and their interaction with FAK PY397 (21).

Focal adhesion-dependent cell migration and the dissolution of cadherin-based intracellular adhesion are both necessary processes in cancer progression. FAK signaling seems to play a crucial role in regulating both these processes. FAK PY397 phosphorylation promotes the disruption of E-cadherin–based adherens junctions in colon carcinoma cells through the kinase activity of the FAK-Src complex (22). Overexpression of a kinase-dependent mutant of FAK blocked the accumulation of peripheral E-cadherin in endothelial cells (23). Conversely, the disassembly of adherens junctions and endocytosis of E-cadherin is also required for the formation of integrin-based focal adhesions (24). Taken together, these studies suggest an intricate signaling network between adherens junctions and focal adhesion in which endocytosis and protein degradation may play a crucial role.

Proteasomal degradation is a key regulator of intracellular protein function and is recognized as a target for cancer therapy. Bortezomib is a selective inhibitor of the 26S proteasome and has been approved for the treatment of multiple myeloma (25). Preclinical and early clinical data suggest that bortezomib also has significant antitumor activity in other types of cancer, including squamous cell cancer (SCC) of the head and neck (HNSCC; ref. 26). Although the mechanism of action likely involves multiple pathways, its effect on HNSCC has been linked to endoplasmic stress, inducing the activation of caspases and, subsequently, cell death (27). Other mechanisms leading to programmed cell death in SCC cells include the effects on the nuclear factor κB and the cyclin-dependent kinase, respectively (26). To date, no studies have addressed the effects of bortezomib on cell-cell adhesion or cell migration despite the central role of intercellular and focal adhesion complex formation in invasion and metastasis.

In this study, we investigated the effects of bortezomib on cell adhesion and migration in SCC15 and SCC68 cell lines, which are derived from human SCC of the tongue. We found that intercellular adhesion diminished as a result of bortezomib exposure and was accompanied by decreased levels of Dsg2, whereas the levels of other desmosomal and adherens junction proteins remained unchanged or increased as a result of proteasome inhibition. EGFR levels were up-regulated, and the addition of an EGFR inhibitor was able to restore cell-cell adhesion while enhancing the induction of cell death compared with either one of these substances alone. Tumor cell migration was reduced, and we were able to show increased FAK PY397 at the cell border following proteasome inhibition with bortezomib.

Materials and Methods

Cell culture. SCC68 and SCC15 cell lines were obtained from James Rheinwald (Harvard University, Cambridge, MA) and grown in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA), supplemented with 1 mL of bovine pituitary extract and epidermal growth factor (0.3 ng/mL) as provided by the supplier. SCC68 cells were grown to 80% confluence in 100-mm culture dishes before passaging. Before treatment, cells were trypsinized and replated into growth medium containing 0.09 mmol/L Ca^{2+} for 24 h before the medium was changed and the cells were treated. In the case of PKI166, the drug was added to a final concentration of 2.5 and 5 μmol/L as indicated; in the case of bortezomib, the concentration ranged between 0.1 ng and 10 μg/mL as indicated. DMSO (0.05%) was used as vehicle control. Cells were harvested at the indicated time points in urea sample buffer as described previously (28). The EGFR-specific small molecule tyrosine kinase inhibitor PKI166 was a gift from Novartis AG (Basel, Switzerland) and stored in DMSO at −20°C. The proteasome inhibitor bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA) and stored at +4°C in aqueous solution.

Antibodies. The following antibodies used in this study were described previously. Mouse monoclonal antibodies 6D8 against Dsg2 was a gift from Dr. Margaret Wheelock (University of Nebraska, Omaha, NE). NW6, a rabbit polyclonal antibody to detect desmoplakin, was a gift from Dr. Kathy Green (Northwestern University, Chicago, IL). The monoclonal mouse anti-E-cadherin antibody HECD-1 was purchased from Research Diagnostics (Flanders, NJ). Rabbit α-catenin antibody 2081 and the β-catenin antibody 2206 were purchased from Sigma (St. Louis, MO). Antiphosphotyrosine mouse monoclonal antibody 4G10 was from Santa Cruz Biotechnology (Santa Cruz, CA). The EGFR monoclonal antibody Ab12 was purchased from Neomarkers (Fremont, CA). The FAK and the FAK PY397 monoclonal antibodies were obtained from BD Biosciences (San Jose, CA). The anti-FAK rabbit polyclonal antibody was purchased from Calbiochem (San Diego, CA). The phalloidin Alexa Fluor conjugate was obtained from Invitrogen. For immunoblotting, the following dilutions were used: NW6 (1:2,500), Ab12 (1:500), 6D8 ascites (1:1,000), 4G10 (1:1,000), and FAK and FAK PY397 (1:1,000). Primary antibodies were diluted in 5% milk powder in PBS. Secondary antibodies, goat anti-mouse peroxidase, goat anti-rabbit peroxidase, and goat anti-chicken peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used at a dilution of 1:5,000 in 5% milk powder in PBS. Antibodies were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). For immunofluorescence, the following antibodies were diluted (1:100): 4G10, FAK, and FAK PY397. Phalloidin staining was done according to the manufacturer's instructions (1:40).

Immunoblotting. For immunoblot analysis of whole cell lysates, cells were harvested in a urea sample buffer, and immunoblotting of protein gels on nitrocellulose membranes was done as described previously (28). To quantify border staining, 50 borders from five fields were randomly selected, and the length occupied by the staining for FAK was scored as follows: one third (black), two thirds (white), and the entire border (checkered) as described previously (9).

Immunofluorescence. Cells were plated on glass coverslips for 24 h in regular growth medium. Following the change of the cell culture medium, bortezomib, PKI166, or DMSO was added. For immunofluorescence, coverslips were rinsed in PBS and fixed in 3.3% paraformaldehyde (EMS, Hartfield, PA), permeabilized with Triton X 1% and incubated with the primary antibody. Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used (1:250). Processed coverslips were examined with a Nikon Eclipse E600 microscope equipped with a Nikon FDX-35 camera. Statistical analyses were done using a two-tailed Student's t test.

Dispase assay. Confluent SCC68 monolayers were seeded in triplicate onto 60-mm dishes and treated with DMSO, PKI, or bortezomib in the presence of 0.09 mmol/L calcium. After 20 h, cultures were washed twice in Dulbecco's PBS+ and incubated in 1.5 mL of dispase (1.2 units/mL; Roche Diagnostics, Mannheim, Germany) for 30 min. Released monolayer fragments were quantified based on size and total number. Large fragments were defined as fragments >5 mm in size; medium fragments were between 1 and 5 mm, and small fragments were <1 mm in size. Under experimental conditions where fragmentation was excessive, a maximum of 750 fragments was counted. Statistical significance was determined for three experiments using the Student's t test.

Cytotoxicity assay. The cytotoxicity assay was done according to the manufacturer's instructions (Roche Diagnostics). Briefly, cells were grown and treated as described above. At the indicated time points, 150 μL of supernatant was removed and centrifuged at 2,000 rpm, and

100 μL of the supernatant was transferred into a fresh tube. Lactate dehydrogenase (LDH) activity as a measure for cytotoxicity was carried out according to the manufacturer's instructions, and the absorption was measured at 490 and 620 nm wavelength. Statistical significance was determined for three independent experiments using the Student's *t* test.

Results

The proteasome is a major pathway of intracellular protein degradation and is involved in the regulation of a large number of proteins. Bortezomib is a selective inhibitor of the 26S proteasome and has shown promising activity in a variety of human cancers including HNSCC (26). We used SCC68 and SCC15 cell lines derived from SCC of the tongue based on their previously documented invasive characteristics to study the effects of bortezomib treatment on cell adhesion and cell migration.

We first examined the protein levels of desmosomal and adherens junction proteins at different doses of bortezomib. After proteasome inhibition, we expected protein levels to remain stable or to increase in proteins subject to proteasomal degradation. The protein level of adherens junction components, such as E-cadherin and α-catenin, remained stable. The armadillo family protein β-catenin did not change visibly and could be visualized as two bands, a ubiquitinated band of 100 kDa and the full-length β-catenin at 80 kDa. β-Catenin protein increased in intensity with higher doses of bortezomib (Fig. 1, *arrow*) before weakening at the highest dose level of 5 μg/mL. Ubiquitinated β-catenin has previously been identified following pharmacologic proteasome inhibition (7). No similar additional bands were observed for any other tested proteins. Protein levels of both splice forms of the desmosomal linker protein desmoplakin increased markedly. Interestingly, the protein level of Dsg2 actually diminished in a dose-dependent manner (Fig. 1, *top*).

Because proteasome inhibition does affect protein levels of cell adhesion molecules, we examined whether the mechanical stability of cell-cell adhesion was also influenced by proteasome inhibition. To address this question, we used the dispase assay as described previously (9). Briefly, in this assay, a confluent monolayer of cells is

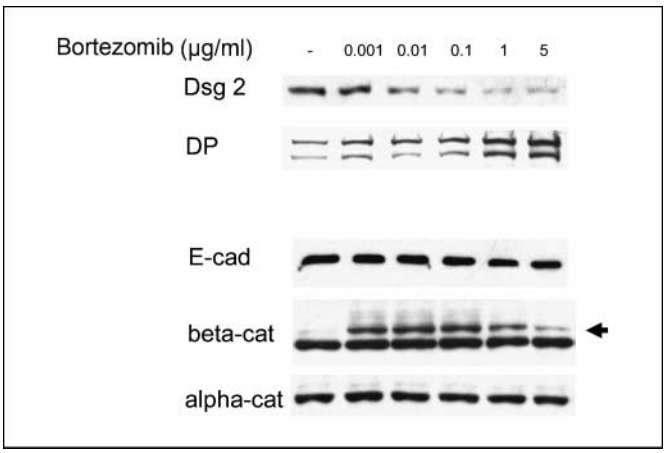

Figure 1. Protein levels of cell adhesion molecules are influenced by proteasome inhibition. SCC68 cells were treated with bortezomib with doses ranging between 1 ng/mL and 5 μg/mL overnight. Equal amounts of protein were loaded. The amount of Dsg2 diminishes (*top*), whereas desmoplakin increases as a result of proteasome inhibition. The protein level of adherens junction proteins, such as E-cadherin, α-catenin, and β-catenin, remains unchanged. Ubiquitinated β-catenin can be visualized as a distinct band at 100 kDa (*bottom, arrow*).

treated with dispase, a nonspecific proteinase, releasing cells from the bottom of the Petri dish while leaving intercellular adhesion intact. The number and the size of these fragments are a good indicator of the mechanical strength of intercellular adhesive forces (29). We found that following treatment with bortezomib, there was a significant increase in small-sized fragments, whereas the number of large fragments diminished significantly, indicating diminished intercellular adhesion (*, $P > 0.05$). This effect was dose dependent, and the mechanical stability of the monolayer diminished with increasing doses of bortezomib in SCC68 cells (Fig. 2*A*). The number of medium-sized fragments did not follow a clear pattern but were included for completeness. Results with SCC15 cells were comparable (data not shown). The correlation of decreased cell-cell adhesion with diminished levels of Dsg2 could indicate a direct functional link between Dsg2 level and cell-cell adhesion, thus providing further evidence to support our previous results that in this model system, Dsg2 might be a particularly important provider and determinant of cell adhesion (9).

We have shown previously that inhibition of the EGFR is able to induce the formation of desmosomes and promote cell adhesion in SCC cells. Therefore, we tested whether the addition of the EGFR-specific small molecule tyrosine kinase inhibitor PKI166 was able to restore cell-cell adhesion following bortezomib treatment. The EGFR itself is subject to ubiquitination and subsequent proteolytic breakdown and may thus be affected by proteasome inhibition (30). We found that treatment with bortezomib increased EGFR levels depending on the dose up to 4-fold (Fig. 2*B*). This effect occurred as early as 8 h following treatment and remained stable for at least 24 h (data not shown). In addition to the increase of EGFR protein, there was also an increase in EGFR phosphorylation, indicating increased EGFR activation (Fig. 2*B, middle*). To test whether there was a measurable increase in mechanical cell-cell adhesion with the addition of an EGFR inhibitor, we did the dispase assay as described above using bortezomib at 0, 0.001, 0.1, and 1 μg/mL dose levels. Similar to the data in Fig. 2*A*, cell adhesion diminished as a result of proteasome inhibition, leading to a significant reduction in the number of large-sized fragments and production of a significantly higher number of smaller fragments (Fig. 2*C, left*). The number of large fragments increased significantly with the addition of the EGFR inhibitor PKI166 compared with treatment with bortezomib alone, and there was a significant decrease in the number of small fragments (*, $P < 0.05$), indicating that cell-cell adhesion was enhanced. This also suggested that SCC15 and SCC68 cells were able to assemble functional cell adhesion complexes in response to EGFR inhibition in the presence of bortezomib. As indicated by the overall higher number of fragments in this assay, however, cell adhesion could not be restored to the same level as in untreated controls, possibly due to cell damage that occurred as a result of treatment with the EGFR and proteasome inhibitors. We also observed a higher number of dead cancer cells floating in the supernatant when the two substances were combined. To address this question in a semiquantitative way, we did the LDH assay, which has previously been shown to be a sensitive indicator of cell death (31). As shown in Fig. 2*D*, we found an additive effect on cell death when both substances were combined. There was a higher baseline rate of cell death in the sample in which the EGFR inhibitor had been added resulting in higher absorption measurements than in the samples, which were treated with bortezomib alone. Bortezomib did not influence the rate of cell death at the 0.001 and 0.01 μg/mL dose level as indicated by the flat part on

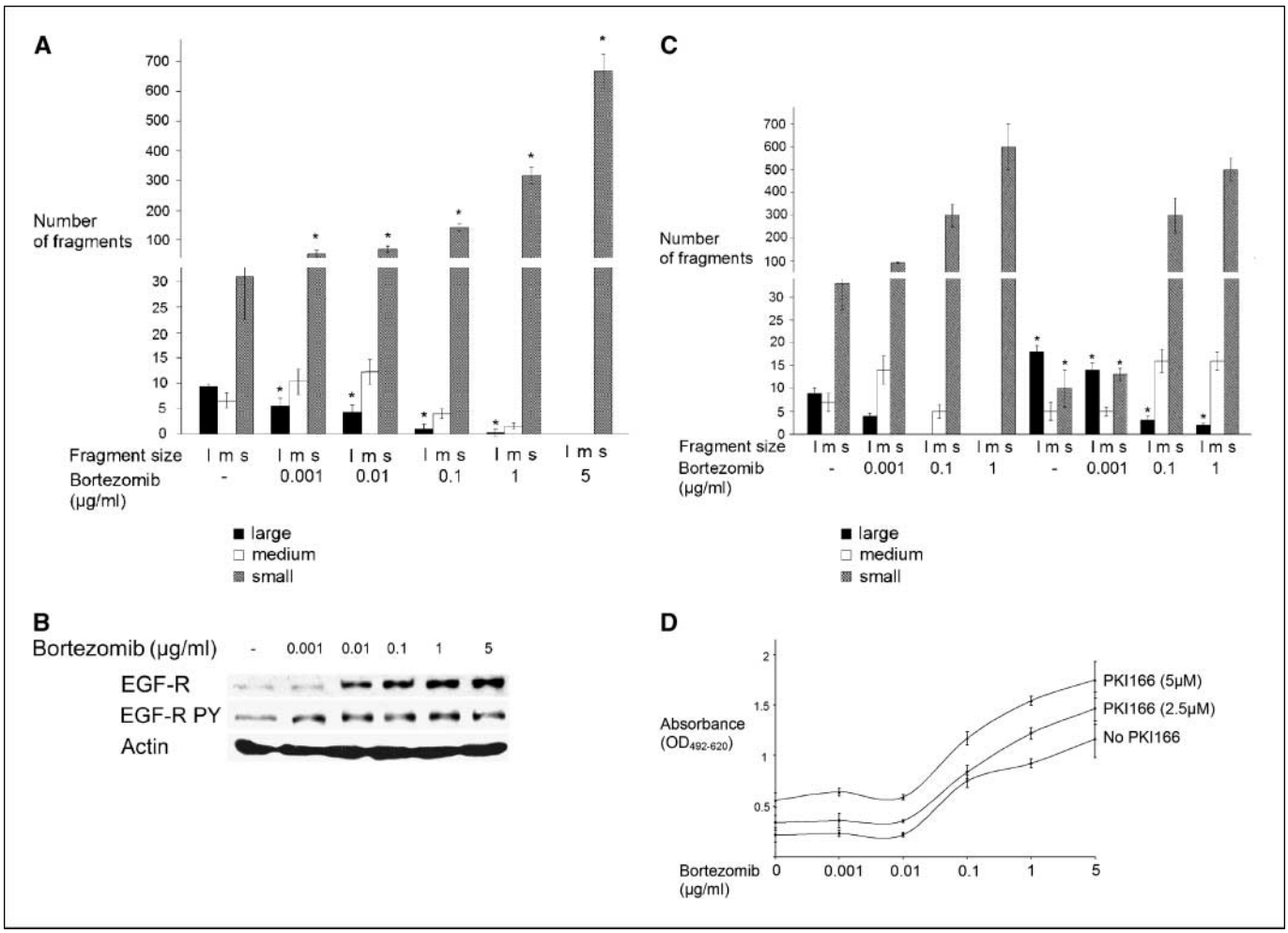

Figure 2. *A,* bortezomib diminishes mechanical cell-cell adhesion. In the dispase assay, a confluent monolayer of SCC68 cells was treated with bortezomib overnight at the indicated dose levels (μg/mL). The monolayer was removed from the surface of a 60-mm Petri dish with the proteinase dispase and was released as fragments of varying sizes. The number of fragments was counted using a semiquantitative scale of large (*l*, >5 mm in greatest diameter), medium (*m*, 1–5 mm in largest diameter), and small (*s*, up to 1 mm in greatest diameter). Following proteasome inhibition, there were a significantly smaller number of large fragments and a significantly higher number of small fragments ($P < 0.05$ on a two-tailed *t* test, $n = 3$), indicating diminished mechanical strength of cell-cell adhesion. *Columns*, mean; *bars*, SD. *B,* bortezomib increases EGF-R protein level: SCC68 cells were treated with bortezomib at the indicated dose levels (μg/mL) overnight. Equal amounts of whole cell lysate were blotted using an anti-EGFR antibody (AB12) and show an up to 4-fold increase in EGFR protein level as measured by pixel count. The amount of tyrosine phosphorylated EGFR (EGFR PY) also increased, indicating a larger amount of activated EGFR in cells treated with bortezomib. Actin staining is provided for loading control. *C,* the EGFR inhibitor PKI166 restores cell adhesion in SCC68 cells treated with the proteasome inhibitor bortezomib: The dispase assay reveals diminished cell adhesion following treatment with bortezomib alone (*left*) in a dose-dependent manner at three different dose levels of bortezomib similar to results shown in (*A*). The addition of the EGFR inhibitor PKI166 (5 μmol/L, *right*) results in the generation of a significantly higher proportion of large fragments and a significantly higher number of small fragments at 0 and 0.001 μg/mL bortezomib dose levels compared with controls without the addition of PKI166 (*, $P > 0.05$, $n = 3$). At the 0.1 and 1 μg/mL dose level, no large fragments were found without the addition of the EGFR inhibitor. These data indicate that EGFR adhesion is able to restore cell-cell adhesion and counteract the effects of bortezomib on cell adhesion. *Columns*, mean; *bars*, SD. *D,* EGFR and proteasome inhibition enhances cell death in SCC68 cells. SCC68 cells were grown to 80% confluence in 60-mm Petri dishes and treated with bortezomib at the indicated dose levels with and without the presence of the EGFR inhibitor, PKI166, at two dose levels (2.5 and 5 μmol/L). Lactate dehydrogenase activity in the supernatant was analyzed using spectrophotometric analysis. EGFR inhibition added significantly to cell death in the presence of bortezomib at all dose levels.

the left of the graph regardless of whether PKI166 had been added. At doses higher than 0.1 μg/mL, there was a linear increase in cell death reflected in the increased LDH activity. The additional cell death was also dependent on the dose of PKI166, with more cell death measured at the 5 μmol/L dose level than at 2.5 μmol/L. Comparison with a standard curve obtained during the same experiment showed that the measured extinction was within the linear part of the absorbance curve. This standard curve had been obtained by spectrophotometric analysis of defined dilutions of a sample in which all cells had been destroyed by adding Triton X (Supplementary Fig. S2*E*). Comparison with this standard curve showed that PKI increased the cell death rate by 15% at the 2.5 μmol/L dose level and by 30% at 5 μmol/L. These data suggest that bortezomib may act as a sensitizer for PKI166 and could provide a rationale for the combined clinical use of bortezomib in conjunction with an EGFR inhibitor.

Migration is a key process in tumor cell invasion and is frequently increased in cancer biogenesis. It occurs in part via the assembly of focal adhesion complexes along the leading edge of the cell membrane linking the tumor cell to the actin cytoskeleton allowing for coordinated movement. Within the adhesion complex, FAK plays a critical role, acting as the anchor for the adhesion

complex, and FAK expression is increased in many cancer types (15–17). There is evidence that FAK is subject to ubiquitination and subsequent proteolytic breakdown (20), suggesting that its regulation is key to the process of tumor progression. We assessed the impact of proteasome inhibition on protein levels of FAK using various dose levels of bortezomib. Although the total amount of full-length FAK protein increased only minimally following proteasome inhibition (Fig. 3), a distinct band of 40 kDa increased in intensity at higher doses of bortezomib and likely represents the accumulation of a FAK breakdown product (Fig. 3, *arrowhead*), whereas a smaller, separate band diminished in intensity, possibly indicating the accumulation of ubiquitinated FAK breakdown products similar to the additional bands seen with β-catenin. Staining with a polyclonal FAK antibody directed against the COOH-terminal domain of the FAK molecule showed the same additional bands (data not shown), suggesting that these breakdown products were specific to FAK following bortezomib treatment. The protein levels of the cytoskeletal protein actin remained unchanged on Western blotting (Fig. 3, *bottom*). On immunofluorescence, however, we observed a marked increase in FAK staining along the basal surface of the cell in SCC68 cells (Fig. 4, *right*). In addition, the staining pattern was changed compared with untreated controls. There was a marked increase in staining encompassing the entire circumference of the cell as opposed to staining preferentially seen on one side of the cell (Fig. 4, *left*). Because FAK autophosphorylation is critical for focal adhesion assembly and disassembly and plays a crucial role in tumor cell migration, we did immunofluorescence to assess the phosphorylation status of FAK. Using an antibody directed against phosphorylated tyrosine residues (4G10), we detected an increase of phosphotyrosine along the basal cell surface, which colocalized with FAK staining (data not shown). Immunofluorescence staining with an antibody specific to phosphorylated FAK at tyrosine residue 397 revealed identical staining that was increased along the basal surface of the cell following treatment with bortezomib (Fig. 4, *bottom*). To quantify these changes, we selected 50 cell borders randomly from five fields and scored the cell border staining according to the amount of FAK staining on immunofluorescence. The staining was quantified based on whether FAK staining was seen at one third, two thirds, or the entire cell border (Fig. 4, *bottom*). We found that the amount of cell border staining

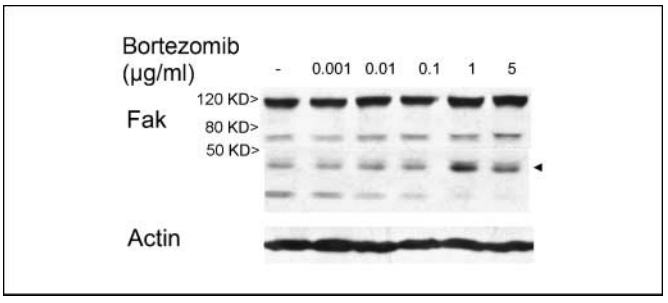

Figure 3. Bortezomib influences full-length FAK levels minimally and leads to the accumulation of a 40-kDa FAK fragment. Western blot analysis of whole cell lysates from SCC68 cells was treated with bortezomib and immunoblotted for FAK and actin. Protein levels of FAK remained unchanged at lower doses of bortezomib (1–100 ng/mL) and increased only slightly at higher dose levels (1 and 5 μg/mL). There was a considerable accumulation of a 40-kDa fragment (*arrowhead*) and diminishing amounts of a smaller FAK fragment. Actin levels were unaltered and show similar loading.

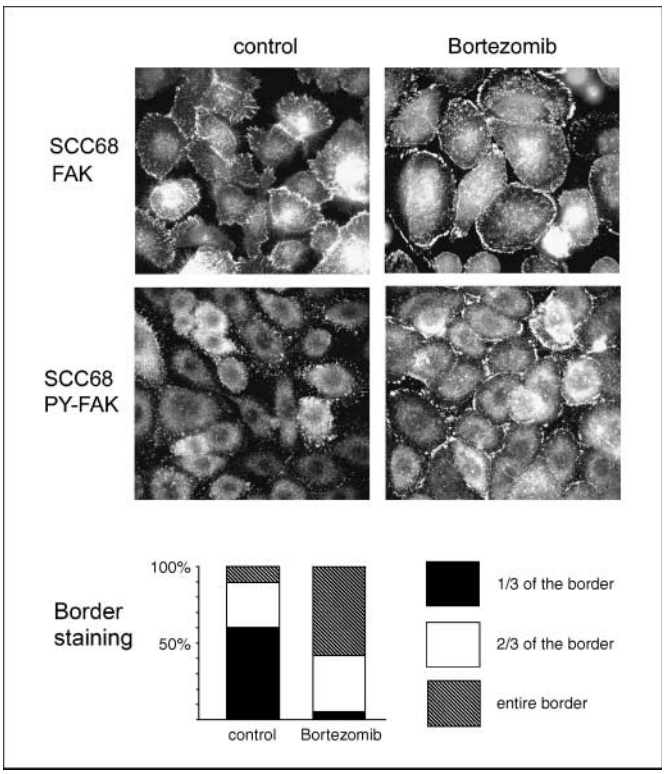

Figure 4. Bortezomib induces recruitment of FAK to the cell border and of phosphorylated FAK Y397 in SCC68 cells. SCC68 cells were treated with 0.5 μg/mL bortezomib overnight, and immunofluorescence was done using antibodies against FAK and FAK PY397. Compared with controls, the amount of FAK and phosphorylated FAK was increased along the basolateral cell border. FAK recruitment to the basolateral border was scored by determining the amount of FAK staining on immunofluorescence. The amount of FAK staining was significantly increased in SCC68 exposed to bortezomib compared with controls ($P < 0.05$). The same experiment with SCC15 cells yielded comparable results (Supplementary Fig. S4*B*).

was significantly increased following bortezomib treatment. These changes were comparable in SCC15 cells (Supplementary Fig. S4*B*).

FAK Y397 phosphorylation has been linked to integrin clustering and Src activation and is viewed as a critical step in the initiation of cell migration (18). In most model systems, the presence of FAK and FAK autophosphorylation seems to be necessary in initiating cell migration. Other studies suggest, however, that depending on the cell type studied, autophosphorylation might also have inhibitory effects on migration. To address this question, a wound-healing assay was done. In this assay, cells were plated on coverslips and grown to 80% confluence. A wound was scratched into the layer of cells using a sterile 10-μL pipette tip. Cells were treated with different dose levels of bortezomib, and the migration of cells across this artificial wound was assessed. In the untreated controls, cell spreading from the edges of the wound could be shown at 8 h after the initiation of the wound, and the almost complete closure of the wound edges occurred at the 24-h time point. Increasing doses of bortezomib reduced migration, and there was virtually no migration at the 1 μg/mL dose level at the 8- and 24-h time points (Fig. 5, *bottom*).

Because migration requires the coordinated interplay between assembly and disassembly of focal adhesion and the attachment to actin fiber, we did double label staining with FAK and actin. In the untreated controls, a delicate network of actin stretched between the focal adhesions and was found predominantly on one side of

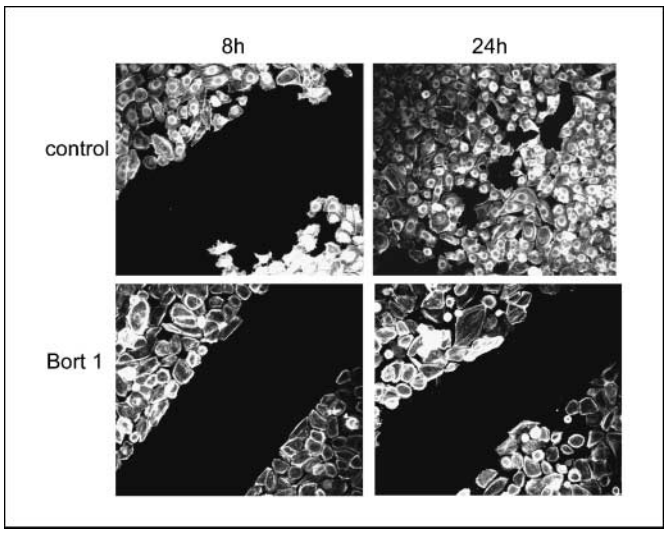

Figure 5. Bortezomib inhibits cancer cell migration. SCC69 cells were grown to 80% confluence on coverslips, and a wound was inflicted using a sterile pipette tip. After treatment with bortezomib at the indicated dose levels (μg/mL) for 8 and 24 h, staining for actin was done using phalloidin. In the untreated controls, the wound edges started to close at the 8-h time point, and at 24 h, the wound was barely visible (*top*). Bortezomib treatment reduced migration noticeably at the 1 ng/mL dose level, and migration was virtually stopped without any significant wound closure at 8 and 24 h time points (*bottom*).

the cell corresponding to the leading edge, whereas in cells treated with the proteasome inhibitor, the actin network was prominent but showed a ringlike, circular distribution within the cells (Fig. 6). These effects were also dose dependent and suggest a poorly organized interaction between focal adhesions and the cytoskeleton and may account for the reduced cell motility.

Discussion

In preclinical and early clinical studies, bortezomib has shown activity in many cancer types, including HNSCC (26). Currently, it is approved for the treatment of multiple myeloma (25, 32, 33). Bortezomib's mechanism of antitumor activity, however, is still poorly understood. Because a multitude of proteins are subject to ubiquitination and proteosomal breakdown, it is not surprising that bortezomib affects numerous cellular pathways. Bortezomib treatment results in the stabilization of p21, p25, p53, and transforming growth factor α, as well as the abolition of prosurvival nuclear factor κB (34). In head and neck cancer, bortezomib might directly induce apoptosis by activating proapoptotic free radical formation (27). Thus far, no studies have addressed the effect of bortezomib on tumor cell adhesion and migration. There is growing evidence that adhesion complex proteins are involved in signaling and regulate important cellular functions beyond providing mechanical stability (4). The effect of bortezomib on these processes may facilitate an understanding of tumor response to targeted therapy.

In this study, we found that treatment with bortezomib in SCC68 cells resulted in a dose-dependent down-regulation of Dsg2 and an increase in desmoplakin, whereas adherens junction proteins were unaffected. The down-regulation of Dsg2 correlates with diminished mechanical cell adhesion. Bortezomib up-regulates the EGFR in SCC cells, and the addition of an EGFR inhibitor induces a measurable increase in cell-cell adhesion. Furthermore, the addition of PKI166 adds to the cytotoxic properties of bortezomib. We show that bortezomib leaves the protein levels of FAK and actin unchanged; interestingly, bortezomib treatment leads to the recruitment of activated FAK to the basolateral cell membrane consistent with focal adhesion formation. Tumor cell migration is reduced significantly, providing a new rationale for the clinical use of bortezomib in head and neck cancer.

Ubiquitination and subsequent proteasomal degradation is a major pathway in the regulation of E-cadherin and β-catenin among other cell adhesion molecules (35, 36). In addition to the proteasomal pathway, endosomal degradation is also involved in the regulation of E-cadherin and plays a role in the breakdown of β-catenin (6). Much less is known about the regulation of desmosomal cell adhesion molecules despite the crucial role of desmosomal complexes in cell adhesion and invasion.

Dsg2 is a desmosomal cadherin, which has been shown to be a major contributor to mechanical stability of intercellular adhesions and may also have signaling properties (37–39). We previously showed that the inhibition of the EGFR results in the formation of desmosomes, and that increased stability of cell-cell adhesion correlates with elevated protein levels of Dsg2 (9). Here, we show that the loss of cell adhesion following proteasome inhibition correlates with diminished Dsg2 protein levels, confirming the important role of this protein in maintaining mechanical stability. This finding was unexpected because the inhibition of proteasomal degradation should have resulted in an increased or at least stabilized protein level as was the case with its adherens junction equivalent, E-cadherin. Our results suggest that a number of breakdown mechanisms besides proteasomal degradation participate in the regulation of Dsg2 levels. These could include lysosomes and endosomal pathways as has been shown for the closely related adherens junction protein E-cadherin (6, 40).

In contrast to Dsg2, protein levels of the desmosomal linker protein desmoplakin increased following proteasome inhibition. This new finding suggests that the 26S proteasome plays an important role in desmoplakin regulation. However, this increase

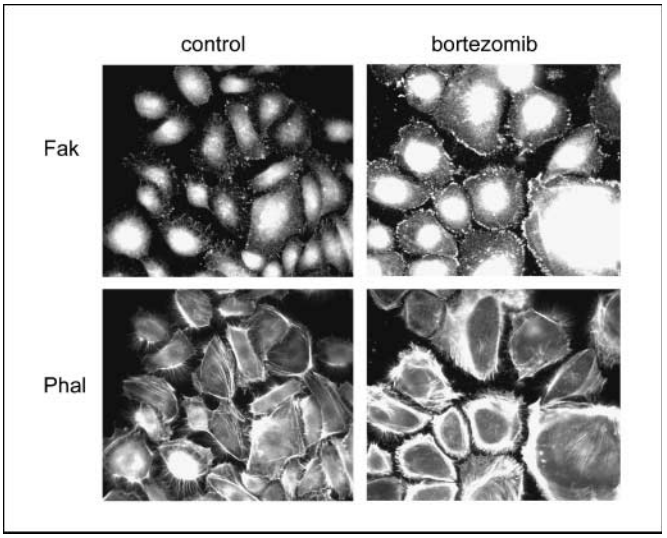

Figure 6. Bortezomib treatment results in the circular, unpolarized distribution of the actin network. SCC68 cells were treated with bortezomib 0.5 μg/mL overnight, and double label immunofluorescence was done using the antibody against FAK and fluorescein-labeled phalloidin. Although in the control, actin appears as a delicate network located predominantly on one side of the cell, actin staining seemed more prominent in cells treated with bortezomib and showed a ringlike distribution, indicating a lack of polarization.

in desmoplakin did not result in increased cell adhesion as one might have expected. The up-regulation of desmoplakin may not solely represent changes in cell mechanical stability because desmoplakin has been shown to play a critical role in embryonic development (41). Our finding that desmoplakin levels are regulated at least in part by proteasomal degradation could help shed some light on the way desmoplakin signaling might be modulated. Indirectly, this also suggests that the decrease in Dsg-2 levels was not a result of general degeneration of cell proteins possibly due to bortezomib-induced cell death, but rather, the result of a specific regulatory step.

As shown in other studies, the ubiquitinated form of β-catenin could be visualized as a distinct band of ~100 kDa following treatment with bortezomib as shown in Fig. 1 (7). β-Catenin was the only molecule among those studied here, in which the accumulation of the ubiquitinated form could be visualized as a distinct band on Western blotting.

The EGFR is in the crossroads of numerous pathways controlling cell function, including cell growth, survival, adhesion, and migration. It is therefore critical that EGFR expression is well coordinated. Cancer cells of many tumor types, including SCC of the head and neck, frequently overexpress the EGFR, and this correlates with aggressive tumor growth and poor survival. Although the regulation of EGFR expression is comparatively well characterized, the steps involved in the breakdown of the EGFR are less well studied. It is clear, however, that the EGFR undergoes ubiquitination and subsequent proteasomal degradation. Other pathways affecting EGFR turnover such as lysosomes and endosomes are involved as well, and it seems that proteasomal processing of the EGFR precedes lysosomal degradation (30).

Treatment with the proteasome inhibitor resulted in an early and impressive increase in EGFR levels. It is unclear whether this is the result of inhibition of EGFR degradation alone or whether the up-regulation is part of a survival strategy of the tumor cells. With the addition of the EGFR inhibitor to the cytotoxic effects of bortezomib, our findings suggest that this drug combination might be a viable therapeutic strategy. Interventions to up-regulate the EGFR have been successfully used in the treatment of head and neck cancer. For example, the combination of radiation and EGFR inhibition is thought to be effective in part due to the radiation-induced up-regulation of the EGFR. It is intriguing to think that bortezomib could possibly imitate the effects of radiation in tumor tissue and sensitize the tumor cells to an EGFR inhibitor. The addition of an EGFR inhibitor may also be an attractive therapeutic intervention because the loss of cell adhesion following bortezomib treatment was restored, which could possibly contribute to reduced invasion and metastasis.

Cell migration is a complex, highly regulated process requiring the continuous formation and disassembly of adhesions and has been implicated in the invasion of tumor cells. FAK controls the dynamic regulation of integrin-linked adhesion, cadherin-dependent cell-cell adhesion, and peripheral actin structures and, thus, is a critical contributor to cell migration and invasion (18, 42). FAK-deficient cells spread more slowly, exhibit an increased number of prominent focal adhesions, and migrate poorly in response to chemotactic stimuli (43). FAK PY397 has been frequently found in different tumor types including laryngeal cancer (44). In one study, there was a high correlation between the phosphorylation of FAK and E-cadherin (45), which may reflect the loss of intercellular adhesion and increased cell migration in cancer progression. Although a direct link between FAK phosphorylation and the dissolution of E-cadherin–based intercellular adhesion has not been shown, FAK PY397 could contribute to weakening cell-cell adhesions through the activation of Src through the dissolution of E-cadherin–based junctions (22, 46). Our findings that FAK PY397 was recruited to the basolateral membrane could therefore be directly linked to the observed decrease in intercellular adhesion. Based on published data on FAK PY397, one should have expected increased migration. However, we found that tumor cell mobility was greatly reduced. Although it has been argued that FAK PY397 may not, in all cases, indicate the activation of FAK, additional bortezomib-induced changes may account for reduced migration. Our finding that the actin cytoskeleton seemed disorganized could provide a clue to support this hypothesis.

Taken together, our results indicate a number of previously undescribed effects of bortezomib on SCC, which can contribute to our understanding of the consequences of proteasome inhibition and providing a rationale for its use in combination with an EGFR inhibitor in the treatment of patients with cancer of the head and neck.

Acknowledgments

Received 6/13/2006; revised 10/16/2006; accepted 11/10/2006.

Grant support: NBL3 Program of the German government.

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We thank all those who have generously contributed reagents and antibodies, including Kathleen Green, Novartis AG, and Millennium Pharmaceuticals; Mechthild Hatzfeld for scientific support and advice; Katrin Reichel for technical assistance; and Gita Maher and David Seldin for helpful comments.

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Cancer Res 2007;67:727-734.