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Bortezomib suppresses focal adhesion kinase expression via interrupting nuclear factor-kappa B

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ABSTRACT

Aims: Bortezomib is a potent proteasome inhibitor currently used to treat various malignancies with promising results. To explore the role of bortezomib in reducing cancer cell migration and inducing apoptosis, we evaluated the effects of bortezomib on the expression of focal adhesion kinase (FAK).
Main methods: Various types of cancer cells including lung cancer A549, H1299; a breast cancer MCF7; a hepatocellular carcinoma Huh7, and a tongue squamous cell carcinoma SCC-25 were treated with different concentrations of bortezomib or MG-132 as indicated for 24 h. Protein and mRNA levels were determined by Western blotting and real-time PCR. Apoptosis was analyzed by caspase 3 cleavage and activity. FAK promoter and NFκB binding activities were measured by luciferase-reporter method. NFκB subunit p65 binding capacity was determined by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis.
Key findings: Both bortezomib and another proteasome inhibitor, MG-132, significantly reduced FAK expression, suppressed cancer cell migration and increased cell apoptosis. Results of real-time PCR and promoter activity assay revealed that bortezomib decreased FAK expression through transcriptional inactivation. Results of FAK promoter activity and ChIP assays in A549 and H1299 cells indicated that bortezomib suppressed FAK activity through a p53-independent pathway. Furthermore, reduction of NFκB binding capacity demonstrated by EMSA and ChIP assay suggested that NFκB plays an important role in bortezomib suppressing FAK expression.
Significance: These results suggested that FAK is downregulated by bortezomib through a proteasome-dependent NFκB inhibitory mechanism. Thus, FAK could be a potential molecular target of bortezomib for therapeutic strategy.

Introduction

Focal adhesion kinase (FAK) was originally identified as a protein tyrosine kinase that localizes to cell focal adhesion contacts and adhesion sites (McLean et al. 2005; van Nimwegen and van de Water 2007; Wozniak et al. 2004). FAK interacts with a cluster of transmembrane integrins and plays a prominent role in regulating integrin-mediated signaling (Toutant et al. 2002). Activated FAK recruits proteins to form a complex that regulates multiple signaling downstream pathways implicated in various cellular processes, including cell adhesion, motility, division, proliferation, and survival. Downregulation of FAK inhibits cell spreading and the response to exogenous stimuli of migration (Ilic et al. 1995; Owen et al. 1999; Sieg et al. 2000), whereas overexpression of FAK enhances cell motility, and reconstituting FAK-deficient cells with wild-type FAK restores cell migration (Richardson et al. 1997; Taylor et al. 2001). Attenuation or inhibition of FAK expression is associated with loss of cell adhesion and anoikis, which results in induction of tumor-cell and fibroblast apoptosis (Frisch et al. 1996; Hungerford et al. 1996; Xu et al. 1996). Moreover, a large

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number of reports describe high expression or activities of FAK in a wide variety of human primary and metastatic tumor tissues (Aronsohn et al. 2003; Cance et al. 2000; Gabriel et al. 2004; Kahana et al. 2002; Kornberg 1998a,b; Lark et al. 2003; McCormack et al. 1997; Owens et al. 1995, 1996; Tremblay et al. 1996). These results provide evidence for the role of FAK in carcinogenesis and metastasis.

Several potential binding sites for transcription factors, including p53 and nuclear factor NFκB, were identified within the human FAK promoter region (Golubovskaya et al. 2004). Analysis of the FAK promoter activities by serial deletion constructs revealed that NFκB induces but p53 inhibits promoter activities (Golubovskaya et al. 2004). Moreover, a recent report showed similar NFκB and p53 binding domains in human and mouse FAK promoter regions (Corsi et al. 2006). These studies suggest that FAK expression is tightly controlled by these two transcriptional regulators (Corsi et al. 2006; Golubovskaya et al. 2004).

Bortezomib (Velcade® or PS-341) was the first proteasome inhibitor used in clinical settings and has been approved for multiple myeloma treatment (Gilmore 2007; Hideshima et al. 2004). In vitro studies showed that bortezomib induces apoptosis of cancer cells through inhibition of IκB degradation, which is followed by inactivation of NFκB and stabilization of proapoptotic proteins (Hideshima et al. 2001; Sunwoo et al. 2001; Tan and Waldmann 2002). Furthermore, bortezomib was demonstrated to have excellent tumoricidal effects in animal studies, and clinical trials of bortezomib for various solid and hematological malignancies are ongoing (Davies et al. 2007; Mackay et al. 2005). However, the effects of bortezomib on cancer cell migration and metastasis have not been well explored. In this study, we examined the effect of bortezomib on suppressing FAK expression via a proteasome-dependent NFκB inhibitory mechanism. Our findings suggest that bortezomib might be a potential agent in adjuvant or neo-adjuvant therapies for cancer patients with high rates of distant metastasis.

Materials and methods

Cell culture and reagents

H1299, A549, MCF-7, Huh7 and SCC-25 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The proteasome inhibitor MG-132 and permeable caspase 3 inhibitor (DEVD-CHO) were purchased from Calbiochem. Bortezomib (PS-341) was from Millennium Pharmaceuticals (Cambridge, MA, USA) and dissolved in phosphate buffered saline (PBS).

Western blot analysis

Extracted protein (30 μg) was subjected to Western blot analysis as described previously (Liou et al. 2007). Rabbit polyclonal antibodies against human FAK, NFκB (RelA or p65) and goat polyclonal antibody against actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against human caspase 3, p53 and poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signaling (Cell Signaling Technology Inc., Danvers, MA). Rabbit polyclonal antibody against phospho-FAK (Y397) was purchased from Chemicon (Chemicon International Inc., Temecula, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and mouse IgG secondary antibody were purchased from Santa Cruz Biotechnology. Protein bands were visualized by use of an enhanced chemiluminescence system (Pierce, Rockford, IL).

Cell migration assay

H1299 cells were cultured on 12-well plates in complete medium and replaced with serum-free media when cells reached confluence. Cross streaks from the center of wells were drawn with a yellow tip. Cells were further treated with control vehicle, 5 μM MG-132 or 2 μM bortezomib for additional 24 h or 48 h. The migration of cells toward the well center was examined with a light microscope.

TUNEL assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) technique was performed to detect apoptotic cells using the In situ Cell Death Detection Kit (Roche) according to the manufacturer's instruction. Briefly, cells on chamber slides were fixed with 4% paraformaldehyde for 1 h and permeabilized in solution of 0.1% Triton-100 with 0.1% sodium citrate for 2 min. The slides were incubated with TUNEL reaction mixture for 1 h followed with alkaline phosphatase-conjugated anti-fluorescein antibody for 30 min. Results of TUNEL positive cells were examined by fluorescent microscopy.

Caspase-3 activity

Cells of each treatment were homogenized and caspase 3 activity was determined with a caspase-3 colorimetric activity assay kit (Chemicon). Results were measured at wave length of 405 nm in a microtiter plate reader and standardized with total protein concentration determined by a Bio-Rad Protein Assay kit.

Real-time PCR

Total RNA was isolated by use of Trizol reagent (Invitrogen, Carlsbad, CA). An amount of 1.5 μg RNA was used for cDNA synthesis with oligo-dT and SuperScript II reverse transcriptase (Invitrogen). Synthesized cDNAs were used as templates for PCR amplification with the FAK (186 bp) primers forward, 5′-CAATCCCACACATCTTGCTGA-3′, and reverse, 5′-AGCCGGCAGTACCCATCTATT-3′; primers for actin (238 bp) used as a loading control were forward, 5′-GGGRCAGAAGGATTCCTATG-3′, and reverse, 5′-GGTCTCAAACATGATCTGGG-3′. Quantitative real-time PCR was performed in 25-μL reaction volume with the standard protocols of the ABI PRISM 7900 system. FAK gene expression was obtained by ΔCT and ΔΔCT assay [$\Delta CT = CT_{(\text{Target gene})} - CT_{(\beta\text{-actin})}$ and

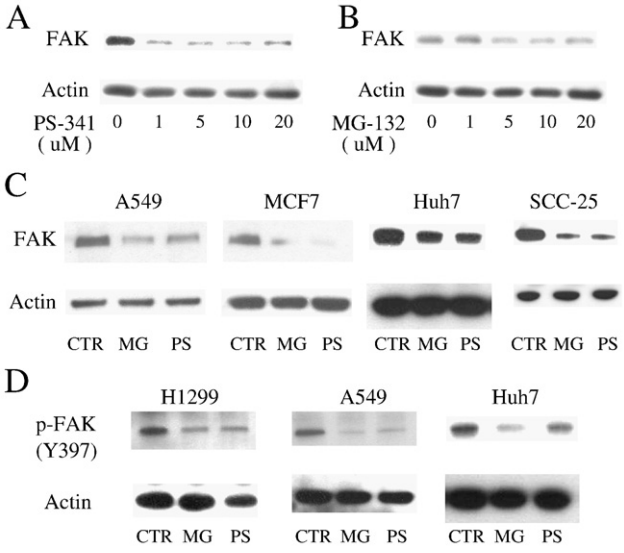

Fig. 1. Bortezomib and MG-132 suppress FAK expression. Western blot analysis of FAK protein levels in H1299 cells treated with 0 to 20 μM (A) PS-341 (bortezomib) and (B) MG-132 for 24 h. Actin was used as a protein loading control. (C) Protein levels of FAK in human cancer cell lines treated with MG (10 μM MG-132) and PS (2 μM PS-341). (D) Protein levels of p-FAK in human cancer cell lines treated with MG-132 and PS-341. These blots are representative of three experiments with similar results.