Constitutively Active FGFR3 with Lys650Glu Mutation Enhances Bortezomib Sensitivity in Plasma Cell Malignancy

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Abstract. The ectopically expressed fibroblast growth factor receptor 3 (FGFR3) and its constitutively active mutations have been detected in patients with multiple myeloma (MM). This study investigated whether the cytotoxic effects of bortezomib on malignant plasma cells are associated with FGFR3 expression and the existence of mutations of FGFR3. Materials and Methods: Cell apoptosis assays were performed in a plasmacytoma cell line, FR4 cells and a myeloma cell line, RPMI8226 cells overexpressing wild-type FGFR3 (FGFR3WT) or two different mutants, FGFR3K650E or FGFR3Y373C, and the induction of endoplasmic reticulum (ER) stress protein was compared between each type of cell. Results: FR4 cells with FGFR3K650E showed enhanced sensitivity to bortezomib together with increased induction of ER stress proteins, compared to FR4 cells with mock, FGFR3WT or FGFR3Y373C. RPMI8226 cells with FGFR3K650E also showed enhanced bortezomib sensitivity. Conclusion: This study indicated that FGFR3K650E is associated with bortezomib sensitivity in malignant plasma cells via ER stress pathways.

The t(4;14)(p16.3;q32.3) translocation occurs in 15-25% of multiple myeloma (MM) patients and leads to the deregulation of FGFR3 gene (1). This results in ectopic expression of FGFR3, which promotes the proliferation and survival in myeloma cells (2, 3). Clinical data indicate that patients with this translocation demonstrate resistance to conventional chemotherapy and early progression after a response occurs, leading to poor prognosis. It has been reported that activating mutations of FGFR3 are found in bladder and cervical cancer (4, 5) and overexpression of FGFR3 has been observed in Hodgkin’s and T-cell lymphomas (6-8), suggesting that FGFR3 is associated with the oncogenic transformation. FGFR3 is a receptor tyrosine kinase which is composed of extracellular three immunoglobulin-like domains, a single transmembrane helix and a cytoplasmic tyrosine kinase domain (9). Activating mutations of FGFR3, such as Lys650Glu (K650E) and Tyr373Cys (Y373C) have been identified in MM patients with t(4;14) (10). The former exists in the activation loop of the tyrosine kinase domain and the latter in the transmembrane domain. These mutations have been reported in tumor cells from patients in the terminal therapy-refractory phase of the disease or in cell lines established from advanced diseases, suggesting that the presence of these mutations are associated with the worst prognosis (10).

Bortezomib, which was the first proteasome inhibitor to be used clinically (11, 12), was initially reported to induce apoptosis by inhibiting the activation of NF-kappaB. However, the mechanism is now revealed to be more complicated. Recently, it has been reported that the balance between proteasome workload and degradative capacity is a critical determinant of sensitivity of MM cells to proteasome inhibitors (13). In order to examine whether FGFR3 and its mutations are associated with the cytotoxic effect of bortezomib, this study established MM cells which overexpressed wild-type FGFR3, K650E or Y373C-mutated FGFR3 and analysed their bortezomib sensitivity in relation to the ER stress.

Materials and Methods

DNA constructs. pMSCV-neo plasmid and two pMSCV-neo plasmids containing the full-length human complementary DNA (cDNA) of wild-type or K650E mutant form of FGFR3 were constructed, as previously described (14). The Y373C mutant form was created from wild-type FGFR3 with QuikChange-XL Site-
Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Primer sequences for Y373C were: 5’ primer GAGCCCGGACTGGTG TGGCAGGATCCATCAGC and 3’ primer GCTGAGGATCCCTG CACACACACTGCCCGGCTC. Mutations were confirmed by sequencing.

Cell culture and transfections. The human plasma myeloma cell line FR4 (15) and the MM cell line RPMI8226 were used in this study. These cell lines are negative for either t(4;14) or expression of endogenous FGFR3 (2). FR4 and RPMI8226 cells were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) plus fetal calf serum (FCS) (ICN Biomedicals Inc., Aurora, Ohio, USA) and penicillin-streptomycin (100 μg/ml), and cultured at 37°C in a humidified atmosphere containing 5% CO2. FR4 cells and RPMI8226 cells were transfected with the plasmid encoding a full length FGFR3 cDNA of wild-type, K650E or Y373C mutations, or vector alone (mock). FR4 cells were transfected using FuGENE6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s procedure. One day before the transfection, 2×105 cells in 2 ml of medium were plated to each well of a six-well plate. The mixture of 97 μl of serum-free medium, 3 μl of FuGENE6 Reagent and 2 μg of plasmid DNA was added to each well. RPMI8226 cells were transfected by electroporation. Briefly, 10 μg plasmid DNA was added to 6×106 cells, and then electroporation was performed at 300 V, 975 μF. Two days after the transfection with FuGENE or electroporation, the cells were placed in RPMI medium containing 0.8 mg/ml neomycin for selection. The cells stably expressing FGFR3 were then collected after the culture in selection medium by using BD FACSAria cell sorter with BD FACS Diva software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blots. Human anti-phosphoFGFR3 (Tyr-724) polyclonal antibody and anti-FGFR3 polyclonal antibody which recognizes the C-terminal FGFR3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were rinsed with cold phosphate-buffered saline (PBS), and lysed on ice in a lysis buffer (0.5 mM HEPES pH 7.5, 1% (v/v) Triton X, 10% (v/v) glycerol, 150 mM NaCl, 1.5 mM MgCl2, 5 mM EGTA, 100 mM NaF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM Na-orthovanadate). The samples were electrophoresed through a sodium dodecyl sulfate-polyacrylamide gel by loading equivalent amounts of protein and transferred to a nitrocellulose membrane (Immobilon-FL Transfer Membrane; Millipore, Billerica, MA, USA). The membrane was incubated with antibodies according to the manufacturer’s protocol. Immunoblots were detected by Western Lightning (Becton Dickinson). The relative amounts of protein were evaluated by the densitometry software Image Quant version 5.2 (Molecular Dynamics, Sunnyvale, CA, USA).

Immunocytochemistry. FR4 cells were rinsed once with PBS and incubated for 15 min in 4% paraformaldehyde. Then the cells were permeabilized by 0.1% Triton X in PBS. After the procedure of blocking by 1% bovine serum albumin, the first antibody, which was either anti-FGFR3 antibody (Santa Cruz), anti-phosphoFGFR3 antibody (Santa Cruz) or anti-KDEL antibody (Stressgen, Brussels, Belgium), was added and incubated overnight. Then, the cells were washed with PBS and reacted with the second antibody which was either Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) and together with Hoechst (1 ng/ml). After washing, analyses were performed using confocal microscopy, Zeiss LSM 5 PASCAL Laser Module 405 (Carl Zeiss, Oberkochen, Germany). Two treatments were considered before analysis: (i) Brefeldin A (Sigma, St. Louis, MO, USA) treatment with a dose of 5 μg/ml for 2 hours at 37°C and (ii) FGF ligand treatment with FGF9 (100 ng/ml) and heparin (100 μg/ml) for 10 minutes at 37°C before analysed.

Apoptosis analysis. Annexin V assay was performed to evaluate the cytotoxicity of several compounds, including melphalan (Sigma), dexamethasone (Sigma) or bortezomib (Millenium Pharmaceuticals, Cambridge, MA, USA). The cells were seeded into 12-well microculture plates at 1×105 cells/well and incubated with each compound. After 48 hours, the cells were washed and resuspended in the binding buffer. Then 5 μl mixture of annexin V and propidium iodide was added to the cell suspension and incubated at room temperature for 5 min in the dark (Annexin V-FITC Apoptosis Detection Kit; BioVision, Mountain View, CA, USA). The cells were analyzed using BD FACScanto II Flow Cytometer with BD FACS Diva software (Becton Dickinson).

Real-time polymerase chain reaction (PCR). Total RNA was extracted using RNEasy Mini kits (Qiagen, Germany) according to the manufacturer’s instructions. cDNA was synthesized using Transcripter First Strand cDNA Synthesis Kit (Roche). SYBR Green real-time PCR (Applied Biosystems, Carlsbad, CA, USA) was performed on cDNA extracted from cells after bortezomib treatment. The housekeeping gene GAPDH served as a control for the cDNA quality. The expression levels of BIP, CHOP and EDEM were divided by the GAPDH expression levels, which were then used as the relative mRNA expression. Primer sequences were as follows: BIP, forward primer 5’-CAATCAAGGTCTTGAGGTTGAAGA-3’, reverse primer 5’-CACATCCTACTCTAAAGTGCATCTAATC-3’; CHOP, forward primer 5’-TGGAAAATGAGAGAGGAAATCAA-3’, reverse primer 5’-CAGCCAAGGCCAGGAAGA-3’; EDEM1, forward primer 5’-ACTCAGGTCCTCAAATCCT-3’, reverse primer 5’-GTATTGAAGAAGAGGAAATCAA-3’. Thermal cycling conditions were one cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data analysis was completed using the 7500 Sequence Detection software (Applied Biosystems).

Statistical analysis. All experiments were repeated at least three times. The values reported here is the mean±standard deviation of triplicate experiments. Student’s t-test was used to examine the statistical significance in cell apoptosis assay and real-time PCR assay. Microsoft Excel software (Microsoft, Redmond, WA, USA) was used for statistical analyses.

Results

Overexpression of wild-type, K650E and Y373C FGFR3 in FR4 and RPMI8226 cells. FR4 and RPMI8226 cells were transfected with the vector alone, or the plasmid encoding the human wild-type FGFR3, or FGFR3 with K650E or Y373C mutation. These cells were named as mock-FR4, wt-FR4, KE-FR4, YC-FR4, mock-RPMI, wt-RPMI, KE-RPMI and YC-RPMI, respectively. The expression of FGFR3 was confirmed by both Western blots (Figure 1A, B) and flow
cytometry (data not shown). Cells were starved overnight and treated with or without FGF9 (100 ng/ml) and heparin (10 μg/ml) for 10 minutes before being lysed, and protein extracts were immunoprecipitated with anti-FGFR3 antibody and analyzed. As shown in Figure 1A and B, FGFR3 was not detected by Western blot in mock cells, whereas it was highly expressed in wild-type, and K650E and Y373C transfectants. This result indicated that overexpression of FGFR3 may be achieved in FGFR3-deficient cells through plasmid transfection. Western blots stained with anti-phospho-FGFR3 antibody demonstrated that K650E FGFR3 (FGFR3K650E) and Y373C FGFR3 (FGFR3Y373C) were autophosphorylated constitutively, while wild-type FGFR3 (FGFR3WT) was phosphorylated only in the presence of ligand.

**Localization of the FGFR3 within the cells.** Immunocytochemical analysis was performed in order to visualize the localization of the FGFR3 receptors in the cells. The ER was visualized with anti-KDEL antibody which detected ER-resident proteins specifically. The images of Figure 2A show that receptors of the FGFR3K650E mutant were preferentially located in the ER, whereas FGFR3WT and FGFR3Y373C receptors existed on the cell surface. When the cells were treated with brefeldin A, an inhibitor of trafficking of proteins from ER-Golgi to cell surface, all FGFR3 receptors remained at the ER (Figure 2B). Since the receptor tyrosine kinase is glycosylated in the ER and the Golgi for maturation after the synthesis in the ER, the FGFR3 receptors localized in the ER are supposed to be immature forms. Positive staining with phospho-FGFR3 antibody was detected in KE- and YC-FR4 cells in the absence of the ligand, which is consistent with their constitutive activation, while it was observed in wild-type FR4 cells only after the stimulation by FGF ligand (Figure 2C, D). In addition, phospho-FGFR3 was mostly localized at the ER in KE-FR4 cells, while it was predominantly detected at the cell membrane in YC-FR4 cells and ligand-stimulated wild-type FR4 cells. These results indicated that the trafficking of FGFR3K650E receptor is specifically impaired and the receptor is aberrantly activated in the ER.

**Bortezomib sensitivity in mock cells and FGFR3 transfectants.** To evaluate the sensitivity against melphalan, dexamethasone, bortezomib and irradiation, each transfectant, as well as the mock cells, were treated with the respective agent or irradiation, and then the annexin V assay was performed. As shown in Figure 3, the cell survival decreased in a dose-dependent manner after treatment with each agent or irradiation in FGFR3-transfected and mock cells. There was no significant difference in the ratio of apoptosis between each cell type after the treatment with melphalan or dexamethasone (Figure 3A, B). In addition, there was no significant difference after irradiation (Figure 3C). On the contrary, the survival of KE-FR4 cells declined more rapidly after exposure to bortezomib as compared to the wt- and YC-FR4 cells. As shown in Figure 3D, 52% of wt-FR4 cells and 59% of YC-FR4 cells survived 48 hours after exposure to 15 nM bortezomib, whereas only 35% of mock-FR4 cells and 19% of KE-FR4 cells survived under the same conditions. Similar results were obtained when RPMI8226 cells were used in place of FR4 cells (Figure 3E, F). These data suggest that the FGFR3K650E expressing cells are notably sensitive to bortezomib as compared to the cells with FGFR3WT and FGFR3Y373, indicating that the effect of bortezomib is associated with the expression of FGFR3 mutation.

**Induction of ER stress associated protein by bortezomib treatment.** In order to clarify the mechanism of the increased sensitivity of KE-FR4 and KE-RPMI to bortezomib, the ER stress pathway induced by bortezomib was investigated. To confirm the induction of ER stress and activation of unfolded protein response (UPR) by bortezomib treatment, the transcript levels of stress proteins, such as BIP, EDEM1 and CHOP were determined 24 hours after treatment with bortezomib. ER chaperone protein, BIP, was induced in all FR4 cell lines, the induction level of which was most pronounced in KE-FR4 cells. EDEM1, which promotes the ER-associated degradation pathway response, was marginally induced in all cells, while the level of its induction was
Figure 2. continued
Figure 2. Intracellular localization of wild-type (wt) and FGFR3 mutants. Cells were fixed and incubated with fluorescence-conjugated antibody. A: Merge analysis showed the presence of the wild-type and Y373C FGFR3 receptors on the cell surface. On the contrary, FGFR3 of K650E mutant were mostly located in the ER. B: FGFR3 receptors were observed within the cells when treated with brefeldin A. C: Phospho-FGFR3 receptors were detected only in the KE- and YC-FR4 cells without ligand. D: Cells after stimulation of the ligand phospho-FGFR3 receptors were detected also in wt-FR4 cells after FGF stimulation.
highest in KE-FR4 cells. CHOP, a mediator of apoptosis induced by ER stress, was strongly induced by bortezomib in all cell lines, the magnitude of which was significantly increased in FR4-KE cells (Figure 4A-D). Immunoblotting of cell lysates in similar conditions demonstrated an increase of CHOP expression, particularly in KE-transfected cells. These results indicated that high level of bortezomib-induced ER stress in KE-transfected cells mediate bortezomib efficacy.

Alteration of bortezomib sensitivity by co-treatment with tunicamycin or cycloheximide. This study hypothesized that the difference in bortezomib sensitivity between cell types may be caused by the level of bortezomib-induced ER stress. To confirm this hypothesis, the apoptosis assay was performed after treatment with bortezomib alone or together with the ER stressor, tunicamycin, or the reliever of ER stress, cycloheximide, for 24 hours (Figure 5A, B). Tunicamycin inhibits N-linked glycosylation, which results in ER stress (16). Meanwhile, cycloheximide is reported to decrease ER stress by reducing the overall levels of client proteins in the ER (17). The results of this study revealed that the combination of bortezomib and tunicamycin enhanced the cytotoxicity of bortezomib, resulting in the comparable apoptosis in each cell type. In contrast, ameliorating ER stress with cycloheximide reversed the cytotoxic activity of bortezomib, leading to similar survival of each cell type. These results supported the notion that the high sensitivity to bortezomib of malignant plasma cells with FGFR3K650E is mediated by the increased ER stress induced by bortezomib.

Discussion

It has been reported that t(4;14) has poor prognostic value for both the event-free and overall survival in MM patients (18). Recently, patients who expressed FGFR3 responded equally well and had similar outcomes with bortezomib compared...
with FGFR3-negative patients (19). Since it is generally
acknowledged that overexpression of receptor tyrosine kinase
confers resistance to chemotherapy, these reports support the
notion that bortezomib induces apoptosis by a different
pathway compared to classical chemotherapeutic drugs. With
regard to the constitutive active mutations of FGFR3, there
are no reports about the efficacy of bortezomib. A few reports
indicate that bortezomib is effective on myeloma or
lymphoma cells with FGFR3 expression, especially those
with constitutively activated K650E mutation and it is related

Figure 4. A-D: Cellular effects of ER stress induced by bortezomib. Transcriptional up-regulation of BIP, EDEM1 and CHOP were observed
by real-time PCR. E: Left panel: Up-regulation of CHOP in FR4 and RPMI8226 cells occurred in response to bortezomib. Actin was used as loading
control. Right panel: Quantification of CHOP protein in FR4 and RPMI8226 cells treated with bortezomib was performed by image Quant 5.2. The
values are given as fold-change relative to the mock cells.
to signal transduction of FGFR3 (20, 21). However, the details of the mechanism are still unclear.

The present study found that myeloma cells with \( \text{FGFR3}^{K650E} \) are sensitive to bortezomib compared with \( \text{FGFR3}^{WT} \) and \( \text{FGFR3}^{Y373C} \)-transfected cells. In order to understand the relationship between \( \text{FGFR3} \) mutation and bortezomib sensitivity, this study focused on the mechanism by which bortezomib loads ER stress on MM cells. Plasma cells synthesize and secrete large quantities of monoclonal paraprotein. In this process, immunoglobulins are folded into their tertiary structures within the ER, where UPR maintains proper protein folding. If the UPR is unable to maintain protein production, an ER stress signal is generated and apoptosis ensues. From this point of view, the plasma cell tumor is notably vulnerable to ER stress, in the condition that the UPR response is compromised. Proteasome inhibitor is thought to disrupt the unfolded protein response in MM cells and inhibit cells to handle the proper folding of proteins (22). Though the precise mechanism of ER stress-induced apoptosis is still unclear, the proapoptotic protein CHOP is considered to play a significant role in ER-dependent cell death (23). CHOP protein is induced by unfolded protein response and leads cells to apoptosis (24-26). Certainly, in this study, CHOP was induced by bortezomib, especially in cells with K650E mutant. BIP and EDEM1 genes, both of which are known to code for ER stress proteins and are related to ER reticulum-associated degradation (27, 28), were also induced most prominently in cells with K650E by bortezomib treatment. In the combined treatment of bortezomib and tunicamycin, an ER stressor, tunicamycin increased bortezomib-induced cell death, which resulted in comparable levels of apoptosis between cells with K650E and others. In contrast, relieving ER stress by cycloheximide, a translation inhibitor, reversed the enhanced cytotoxic activity of bortezomib in cells with K560E mutant. Taken together, these results indicate that the increased ER stress loaded by bortezomib is closely related to the enhanced apoptosis of KE-FR4 cells.

There are reports which indicate that constitutively active mutant proteins of receptor tyrosine kinase, such as c-Kit\(^{Asp816Val} \) or FLT3 internal tandem duplication (ITD), are mainly localized in Golgi or ER as an unglycosylated immature forms (29, 30). A report regarding FLT3-ITD also suggested that the aberrant activation of tyrosine phosphorylation itself impedes the maturation of the receptor tyrosine kinase (31). In addition, FLT3-ITD localized in ER may preferentially activate STAT5, but failed to activate PI3K and MAPK signaling (29). Concerning \( \text{FGFR3} \) mutants, it was reported that constitutively active kinase activity also affects the maturation and trafficking of mutant FGFR (32, 33). Consistent with these reports, immunocytochemical analysis revealed that \( \text{FGFR3}^{K650E} \) is localized in the ER as a constitutively active form, different from \( \text{FGFR3}^{Y373C} \) and \( \text{FGFR3}^{WT} \) also in malignant plasma cells. It is hypothesized that deregulated accumulation of mutant FGFR3 in the ER may be a possible cause for the enhanced ER stress, since the ER localization of K650E mutant is the specific characteristic different from Y373C mutant or wild-type. There are some data about the association between ER stress and the activation of ER-localized tyrosine kinase. ABL tyrosine kinase was reported to be localized in the ER and mediate ER stress-induced apoptosis (34), while another report showed that imatinib, an inhibitor of ABL kinase, ameliorates ER stress and induces remission of diabetes in \( \text{db/db} \) mice (35). It is suggested that the aberrant localization of constitutive active tyrosine kinase in the ER may phosphorylate the ER-resident proteins which imitates the function of ABL kinase, thereby augmenting ER

Figure 5. Accumulation of ER stress with tunicamycin enhanced the cytotoxic effect of bortezomib, whereas amelioration of ER stress with cycloheximide reversed the resistance to bortezomib. A: Viable cells after treatment with bortezomib (10 nM) and tunicamycin (1 μg/ml) for 24 hours. B: Surviving fraction of FR4 cells after treatment with bortezomib (10 nM) and cycloheximide (1 μg/ml) for 24 hours.
stress. Another possibility is that the existence of active tyrosine kinase in the ER itself may induce ER stress since, in general, the receptor tyrosine kinase is subject to the degradation after the activation, which requires the disposal at the proteasome. Further studies are necessary for the clarification of the association between enhanced ER stress and ER-localized activation of tyrosine kinase.

This study herein suggested that bortezomib is effective on cells with specific FGFR3 mutation and the increased cytotoxicity is ER stress-dependent and may be related to the localization of mutant receptor tyrosine kinase. Since mutations of receptor tyrosine kinase are prevalent in neoplastic cells other than hematological malignancy, this study suggests a novel possibility that proteasome inhibitor may work as a specific targeting drug for cancer cells with mutant tyrosine kinases.

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