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Brief communication

Alkylating agents induce activation of NFκB in multiple myeloma cells

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Abstract

Multiple myeloma is still not curable and drug combination strategies are currently being evaluated in order to achieve high remission rates with tolerable toxicity. Bortezomib has been shown to exert inhibitory effects on NFκB activity. NFκB in turn is known to be activated by cytokines, growth factors and by cellular adhesion to bone marrow stromal cells and represents an important mediator of primary and secondary drug resistance in multiple myeloma that confers to proliferation and survival.

In this study we confirm that bortezomib sensitized MM cells to the DNA-damaging drugs melphalan and doxorubicin. Further, we demonstrate that the sole incubation of MM cells with melphalan or doxorubicin leads to a vast activation of NFκB activity. Additionally, we show that the co-incubation of bortezomib with melphalan or doxorubicin reduces activation of NFκB. These data suggest that the drug-sensitizing effect of bortezomib on MM cells is due to inhibition of melphalan- and doxorubicin-induced activation of NFκB activity.

This study, therefore, supports the idea of combining a NFκB inhibitor with alkylating drugs in the therapy of multiple myeloma.

Keywords: Multiple myeloma; Melphalan; Doxorubicin; Bortezomib; NFκB

1. Introduction

The alkylating drug melphalan has been the standard agent in myeloma therapy for more than 30 years. Dose intensification of melphalan – enabled by autologous stem cell transplantation – has proven to prolong overall survival. However, despite high dose regimes and combination therapies, multiple myeloma remains incurable.

The proteasome inhibitor bortezomib has been shown to induce apoptosis in myeloma cells even at nanomolar concentrations [1] and importantly, it shows a tolerable toxicity profile. The mechanisms by which bortezomib induces apoptosis has been revealed by several groups. Besides showing effect on DNA-repair pathways, cell cycle proteins, p53 mediated apoptosis and caspase-induced apoptosis, it inhibits NFκB. The transcriptional factor NFκB is activated not only by a wide variety of cytokines which are found in the bone marrow milieu, but also by cellular adhesion to bone marrow stromal cells. Once activated, it leads to cell proliferation, and most importantly, to drug resistance in MM cells [2]. When bortezomib was combined with alkylating agents like melphalan, strong synergistic effects in human MM cell lines and primary MM cells have been observed [3]. These studies hold the rationale for combining melphalan and bortezomib.

In order to achieve higher response rates, to avoid drug related side effects and to overcome primary and secondary drug resistance in myeloma cells, combination chemotherapies have been established and their efficacy has been evaluated in clinical studies. Regimens containing alkylating agents and bortezomib show a high rate of complete (CR) and very good partial remissions (VGPR), indicating

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that bortezomib-containing combinations are highly active against myeloma cells *in vivo* [5,6]. The molecular mechanism how bortezomib sensitizes myeloma cells to common drugs is not yet fully understood.

2. Methods

2.1. Cells

The human MM cell lines U266 and OPM-2 were obtained from the American Type Culture Collection (Rockville, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated fetal calf serum (Boehringer, Ingelheim, Germany) in a humidified atmosphere (37 °C; 5% CO_2), and seeded at a concentration of 1×10^5 cells/ml. All cell lines have been regularly tested for mycoplasma and are free of this contamination.

2.2. Reagents

Melphalan and doxorubicin were purchased from Calbiochem (Schwalbach, Germany), bortezomib from Millennium Pharmaceuticals (Boston, MA), and the cell reagent WST-1 from Roche (Penzberg, Germany).

2.3. Cell growth assay

For quantification of the cells in suspension a WST-1 viability assay protocol was used as recommended by the manufacturer (Roche, Penzberg, Germany). Briefly, 4×10^3 cells/100 μl were seeded to 96-well-plates and were incubated with the test compounds for 48 h. For the last 2 h of culture, cells were incubated with 10 μl of WST-1. Absorbance at 440 nm was measured using a microplate ELISA reader to detect metabolically intact cells (reference wavelength: 680 nm). Additionally, cells were counted in cell chambers after trypan blue staining.

2.4. Noshift NFκ B binding assay

Treated cells were washed with PBS and nuclear extracts were prepared by using the NucBuster Protein Extraction Kit (Novagen, Merck Biosciences, Bad Soden, Germany) according to the manufacturer's instructions. The binding affinity of myeloma cell nuclear extracts to NFκB (p65) DNA was determined with the NoShift Transcriptional Factor Assay Kit (Novagen, Merck Biosciences, Bad Soden, Germany). Each reaction mixture contained 5 μl 4× NoShift Bind buffer, 1 μl poly(dI-dC)·poly(dI-dC), 1 μl salmon sperm DNA, 1 μl biotinylated (10-pmol/μl) NFκB WT DNA and 5 μl nuclear extract DNA in a total reaction volume of 20 μl. After 30 min of incubation on ice, 80 μl of 1× NoShift Bind buffer were added to each reaction mixture. The mixtures were transferred into a prewashed 96-well streptavidin-coated plate and incubated for 1 h at 37 °C. After washing the wells, 100 μl of diluted (1:1000 in NoShift antibody dilution buffer) anti-NFκB (p65) primary antibody were added to each well and incubated for 1 h at 37 °C. The wells were washed again and 100 μl diluted (1:1000) horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody were added. After 30 min of incubation at 37 °C, 100 μl of TMB (tetramethyl benzidine) substrate were added to the washed plate and the wells were incubated for 30 min at room temperature in the dark. The reaction was stopped by the addition of 100 μl of 1N HCl to each well, and absorbance was measured at 450 nm.

2.5. Statistics

Mean values with standard deviations from representative experiments are shown in the figures. Data were confirmed by at least two independent experiments. Wilcoxon analysis was used to compare different groups. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Bortezomib sensitizes myeloma cells to melphalan

We re-evaluated the sensitizing effect of bortezomib on myeloma cells. U266 myeloma cells were incubated with 4 nM bortezomib and none, 5 μM or 10 μM melphalan and cell growth inhibition was determined by the WST-1 assay.

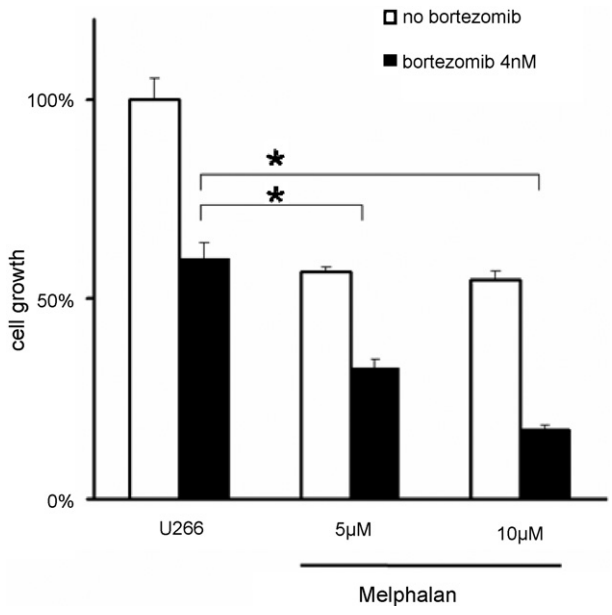

Fig. 1. Bortezomib sensitizes myeloma cells to melphalan. U266 myeloma cells were incubated for 48 with none or 4 nM of bortezomib. One hour after preincubation with bortezomib, none, 5 μM or 10 μM of melphalan was added and cells were incubated for 48 h. Then, cell growth inhibition was determined by the WST-1 assay. Mean values and standard deviations are shown. *$P < 0.05$ versus control. Data obtained from these experiments were analysed using the Calcusyn software in order to show additive/synergistic effects.