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Original Articles

Inhibition of autophagy with chloroquine potentiates carfilzomib-induced apoptosis in myeloma cells *in vitro* and *in vivo*

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ABSTRACT

The proteasome inhibitor bortezomib is now the cornerstone of combination therapy of multiple myeloma (MM). Carfilzomib, a second-generation inhibitor, has shown a substantial benefit *vs* bortezomib in combination regimes. Here we have analyzed in detail the mechanism of cell death induced by carfilzomib and its crosstalk with autophagy and applied the results to the *in vivo* treatment of MM in a mouse model. Carfilzomib induced apoptosis essentially by the intrinsic pathway, through the up-regulation of Puma and Noxa proteins followed by the interaction of Puma, Noxa and Bim with Bax and of Noxa with Bak. Carfilzomib also produces an increase in the formation of autophagosomes but, as apoptosis progresses, autophagy is disrupted, probably owing to Beclin 1 and p62 inactivation. Cotreatment with chloroquine, which blocks autophagy, strongly potentiated apoptosis *in vitro* and *in vivo*. Accordingly, combination therapy with carfilzomib plus chloroquine was highly effective in the treatment of MM in a mouse xenograft model. Chloroquine also enhanced carfilzomib-induced calreticulin exposure in MM cells undergoing apoptosis, increasing the immunogenic ability of carfilzomib. These results support design of trials combining carfilzomib with chloroquine to improve MM therapy.

Introduction

Multiple myeloma (MM) is a clonal plasma cell malignancy that accounts for around 13% of all hematological cancers with about 30,330 new cases expected in USA in 2016 [1]. MM still remains an incurable disease, with most patients experiencing relapse after first-line treatment. Median overall survival for patients with standard-risk myeloma is now 7–10 years, while for those with high-risk disease it is less than 2 years [2,3]. In recent years, the introduction of new drugs, such as the proteasome inhibitor bortezomib, has significantly improved myeloma management, but a significant number of patients show resistance to bortezomib [4] or experience neuropathic secondary effects [5]. Carfilzomib is a second-generation inhibitor, which irreversibly binds to the chymotrypsin-like sites of proteasome and immunoproteasome and displays improved selectivity and therapeutic efficacy [6]. Carfilzomib is cytotoxic for different types of cancer cells [7–10] including MM [6,11], but the mechanism of cell death induction, though supposed to be analogous to that of bortezomib, is only known in part. Recently, carfilzomib has been approved by the FDA for the treatment of relapsed MM based on a demonstration of improved progression-free survival in a multicenter, open-label trial [12].

We have studied here in detail the mechanism of carfilzomib-induced death in MM cells. At clinically relevant doses, apoptosis induced by carfilzomib in MM cell lines is mainly triggered by the interaction of Puma and Noxa with Bax and Bak, which cause their pro-apoptotic conformational change, $\Delta\Psi_m$ loss, cytochrome c release from mitochondria and caspase activation. Carfilzomib also induced autophagy and chloroquine, an autophagy inhibitor, strongly potentiated the anti-myeloma effect of carfilzomib *in vitro* as well as *in vivo*, in a mouse MM xenograft model. Our results provide new data on the cellular mechanism of action of carfilzomib and support the combined use of this proteasome inhibitor together with autophagy inhibitors for an improved therapy of patients suffering multiple myeloma.

Materials and methods

Materials

Carfilzomib was purchased from Selleckchem (USA) and bortezomib was a gift from Millennium Pharmaceuticals. Stock solutions were made in DMSO at 10 mM concentration. Final DMSO concentration in experiments was lower than 0.01%. Chloroquine and mitomycin C were from Sigma (Spain) and tetramethylrhodamine ethyl ester (TMRE) from Invitrogen (Spain). Recombinant TRAIL, expressed from a cloned plasmid (pET28A) encoding the extracellular region (amino acids 114–281) with a

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6× His-tag, was kindly provided by Dr. Marion MacFarlane [13] and recombinant Fas-Fc chimera was from R&D Systems (MN, USA). Cytotoxic IgM anti-Fas mAb (clone CH11) was from Merck-Millipore (Germany).

Cell lines

Human MM.1S, U266, H929 and RPMI 8226 multiple myeloma cells, Jurkat T-leukemia and HeLa cells (all from the ATCC, USA) were routinely cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine and penicillin/streptomycin (hereafter, complete medium). RPMI 8226 cells overexpressing Mcl-1 (8226/Mcl-1) or Bcl-x_L (8226/Bcl-x_L) or silenced for Noxa (8226 shNoxa), as well as Jurkat cells not expressing Bax and Bak (Jurkat sh/Bak), were generated in our laboratory, as previously described [14–16]. In some experiments, MM.1S or H929 cells were co-cultured with human HS-5 stromal cells [17]. HS5 stromal cells were seeded to near 50% confluence and incubated overnight at 37 °C in 96-well plates. MM cells were added on the stromal cell layer and allowed to adhere for 24 h in complete medium. Non-adhered cells were washed out and mixed cultures were incubated in 200 µl complete medium with different concentrations of carfilzomib.

Cytotoxicity assays

For toxicity assays, cells ($3–5 \times 10^5$ cells/ml) were treated in flat-bottom, 24-well (1 ml/well) or 96-well plates (100 µl/well) with carfilzomib (2–10 nM) in complete medium for the indicated times. Cell viability was estimated by the Trypan-blue exclusion test and apoptosis by determining PS exposure and $\Delta\Psi_m$ loss by flow cytometry, as described previously [18] and as summarized later. In experiments evaluating the induction of immunogenic cell death, cells were incubated with either mitomycin (20 µg/ml), bortezomib (7 nM), chloroquine (20 µM), carfilzomib (7 nM) or chloroquine + carfilzomib and apoptosis and calreticulin exposure was evaluated by flow cytometry. For apoptosis inhibition assays, cells were preincubated for 1 h with one of the following caspase inhibitors (50 µM each): Z-VAD-fmk, Z-DEVD-fmk, Z-IETD-fmk (all from Bachem, Germany) or Z-LEHD-fmk (BD Biosciences) prior to the addition of carfilzomib. In some experiments, the RIPK1 inhibitor necrostatin 1 (30 µM) was also used. Toxicity of carfilzomib in co-cultures of MM.1S and H929 cells with stromal cells was determined after trypsinization of cells and labeling with anti-CD38-APC (BD Biosciences), to identify myeloma cells and annexin V-FITC (Immunostep, Spain) followed by flow cytometry. A total of 50,000 $CD38^+$ gated cells as well as $CD38^-$ cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and independently analyzed with the CellQuest and WEASEL software. The percentage of annexin V-FITC positivity in each cell population was determined as described below.

MM xenografts

NU/NU immunodeficient nude mice (Charles River) were subcutaneously inoculated into the right flank with 3×10^6 H929 cells in 100 µl of RPMI 1640 medium and 100 µl of Matrigel (BD Biosciences). When tumor size was around 100 mm^3, mice received either chloroquine, carfilzomib, chloroquine plus carfilzomib or vehicle alone, as indicated. Treatment with chloroquine (n = 5) was given by gavage [10 mg/kg], twice a week for 3 weeks. Carfilzomib (n = 6) was given i.p. at 0.425 mg/kg, for three consecutive days followed by a 4-day rest period, for 3 weeks. The control group (n = 5) received vehicle alone (10 mM sodium citrate, 10% (2-hydroxypropyl)-β-cyclodextrin, pH 3.5) using the same schedule. Caliper measurements of tumor diameters were performed each day and the tumor volume was estimated as the volume of an ellipse, using the following formula: $V = 4/3\ \pi \times [a/2] \times [b/2]^2$, where 'a' and 'b' correspond to the longest and shortest diameter, respectively. Animals were euthanized when their tumors reached 1 cm^3. This protocol was approved by the Comité de Experimentación Animal, University of Zaragoza.

BiFC assays

To detect interactions between pairs of proteins of the Bcl-2 family in intact cells, the Bimolecular Fluorescence Complementation (BiFC) technique was used [19]. In brief, HeLa cells were transfected with the appropriate amount of each vector by using Lipofectamine 2000 (Invitrogen). Vectors containing the coding sequences for human Mcl-1, Bcl-x_L, Bim, Bak, Bax, Puma, and Noxa were subcloned by standard PCR strategies into BiFC plasmids containing the N/C-terminal fragment of Venus protein (FLAG/VN173 or HA/VC155). Vector pAL2-mRFP was used to normalize fluorescence intensities and transfection efficiency. The amount of each vector was adjusted so that fusion proteins were expressed at similar levels than that of endogenous proteins, to avoid random complementation of Venus protein fragments. Addition of Z-VAD-fmk (30 µM) was needed to inhibit caspases and maintain cell integrity. Transfected cells were cultured at 37 °C for 1 h, treated with carfilzomib (20 nM) for 24 h and BiFC complexes formed were analyzed by flow cytometry. Apoptosis was evaluated when necessary by measuring phosphatidylserine (PS) exposure by flow cytometry, as described below.

Fluorescence microscopy and flow cytometry

Nuclear morphology in cells labeled with Hoechst 33342 (1 µg/ml) was analyzed by fluorescence microscopy (Nikon Eclipse 50i). Apoptosis was evaluated by the simultaneous determination of phosphatidylserine PS exposure and mitochondrial membrane potential ($\Delta\Psi_m$) in the same cells, as indicated [20]. Briefly, cells (2.5×10^5) were incubated with 2 nM $DiOC_6(3)$ at 37 °C for 10 min in 200 µl annexin-binding buffer (ABB, 140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.4). Then, 0.5 µl annexin V-PE (Immunostep) was added and incubated at room temperature for 15 min. In GFP-expressing cells, $\Delta\Psi_m$ was determined by incubation with 60 nM TMRE at 37 °C for 20 min in complete medium. In some cases, the integrity of cell membrane during apoptosis was monitored by adding 5 µl (0.25 µg) of 7-amino-actinomycin D (7-AAD, Immunostep) per 10^6 cells. In all cases, cell suspensions were diluted to 0.5 ml with ABB or culture media and analyzed by flow cytometry. Immunogenic ability of apoptotic cells was determined by measuring surface exposure of calreticulin [21]. Cells (1×10^5 in 200 µl) were incubated with 0.5 µl of anti-calreticulin-DY488 mAb (Enzo) for 30 min at 4 °C. Intracellular amount of active caspase-3 was determined by immunolabeling with an anti-active caspase-3-FITC mAb (C92-605, BD Biosciences). Cells (1×10^6) were fixed in 4% paraformaldehyde in PBS (15 min, 4 °C), permeabilized with 0.1% saponin in PBS containing 5% goat serum (15 min) and incubated with the mAb at 20 °C for 30 min. Cells were resuspended in 500 µl PBS and analyzed by flow cytometry. Intracellular levels of Puma and Noxa were determined with rabbit and mouse mAbs (clone EP512Y and 114C307, respectively) from Abcam. Conformational changes of Bax and Bak proteins were assessed by using specific antibodies (6A7, BD Biosciences, and TC100, Calbiochem, respectively), recognizing only the pro-apoptotic conformation of these proteins [20]. Cells (1×10^6) were cultured in the presence or absence or 7 nM carfilzomib for 24 h. Then, cells were fixed with 1% paraformaldehyde in PBS (15 min, 4 °C) and incubated for 30 min with the corresponding mAb in 100 µl of PBS containing 0.1% saponin and 5% goat serum. Cells were washed with 0.03% saponin in PBS, incubated with a FITC-labeled anti-rabbit or anti-mouse IgG antibody (Invitrogen) and analyzed in the flow cytometer. Autophagosome generation was evaluated by incubation of cells (3×10^5 in 100 µl) with the Cyto-ID probe (Enzo Life Sciences) at 37 °C for 30 min. For cell cycle analysis, cells (1×10^6) were washed with PBS, pH 7.4, containing 1 mg/ml glucose and fixed with 70% ethanol at –20 °C for 24 h. Cells were next incubated in PBS containing 0.5 µg/ml RNase A and 20 µg/ml propidium iodide for 1 h in the dark at room temperature and analyzed by flow cytometry using the ModFit software.

Western blot analysis

Changes in the amount of cytosolic proteins involved in apoptosis or in autophagy were determined by Western blotting of cell extracts. After the corresponding treatments, cells were lysed in 50 mM Tris/HCl pH 7.4 buffer containing 0.15 M NaCl, 10% glycerol, 1 mM EDTA, 10 mg/ml leupeptin, 1 mM PMSF and 1% Triton X-100. Solubilized proteins from equal numbers of Trypan-blue negative cells (3×10^5/lane) were resolved by SDS-12% PAGE, transferred to PVDF membranes and incubated with primary antibodies diluted in TBS-T (10 mM Tris/HCl pH 8.0, 0.12 M NaCl, 0.1% Tween-20) containing 5% skimmed milk. In general, membranes were sequentially analyzed for several proteins by a multiple blotting assay method, as described [18]. Primary antibodies anti human proteins used were: anti-Bcl-x_L (sc-1041) and anti-Mcl-1 (sc-819) from Santa Cruz Biotechnology; anti-Bim (clone 22–40, Calbiochem); anti-Beclin 1 (clone 20, BD Biosciences); anti-Atg5 (clone D5F5U, Cell Signaling) and anti-LC3B (#L7543) and anti-p62/SQSTM1 (#P0067) from Sigma. Membranes were washed with TBS-T and incubated with 0.2 µg/ml of the corresponding peroxidase-labeled secondary antibody (Sigma) and revealed by ECL (Millipore). Control of protein loading was achieved by reprobing with anti-actin or anti-tubulin mAb (Sigma).

Statistical methods

Statistical analysis was performed by analysis of variance (ANOVA) or *t* test using the GraphPad Prism 4.0 software.

Results

Characterization of apoptosis induced by carfilzomib

Carfilzomib induced apoptosis in MM cell lines and was able to circumvent the pro-survival signals provided by stromal cells to MM cells (Fig. 1A). Co-culture of MM cell lines with stromal HS-5 cells have no significant effect (H929) or mitigated toxicity caused by carfilzomib at doses lower than 7 nM but not at higher concentrations (MM.1S, Fig. 1A). Cell cycle analysis revealed that carfilzomib increased the proportion of cells in the G_2/M phase prior to entry in apoptosis (Fig. 1B). MM cells exhibited typical characteristics of apoptosis such as cell shrinking and chromatin condensation (Fig. 1C) as well as PS exposure and $\Delta\Psi_m$ loss (Fig. 1D). Carfilzomib treatment of MM cell lines induced proapoptotic conformational changes