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Original Article

Synergistic effects of curcumin and bortezomib on multiple myeloma cells

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Abstract: This study is to investigate the therapeutic effects of curcumin and bortezomib combination on multiple myeloma cells, and the related mechanisms. Human multiple myeloma Dex-resistant (MM.1R) cells were treated with curcumin and/or bortezomib. Cell proliferation was assessed with MTT assay, and apoptosis was detected with flow cytometry. Expression levels of apoptosis-regulated proteins were assessed with Western blot analysis. MTT assay showed that, curcumin treatment alone significantly inhibited the proliferation and enhanced the apoptosis of MM1.R cells, while the bortezomib monotherapy did not significantly alter the cell proliferation and apoptotic process. When the cells were treated with curcumin and bortezomib combination, the cell proliferation was further significantly declined, and the apoptosis was further enhanced, compared with the curcumin treatment alone at corresponding concentrations. In addition, the bortezomib monotherapy did not significantly change the expression levels of apoptosis-related proteins, including caspase-3 and -9, NF-κB, and HSP-90, in MM1.R cells. Curcumin treatment alone significantly stimulated the activation of caspase-3 and -9, and declined the expression levels of NF-κB and HSP-90, in MM1.R cells. When these cells were treated with the curcumin and bortezomib combination, the activation of caspase-3 and -9 was further enhanced, while the expression levels of NF-κB and HSP-90 were further decreased. Curcumin and bortezomib could exert synergistic effects on MM1.R cells, inhibiting cell proliferation and enhancing cellular apoptosis, which involves the regulation of the expression levels of NF-κB and HSP-90 in these cells.

Keywords: Multiple myeloma, curcumin, bortezomib, NF-κB, HSP-90

Introduction

Multiple myeloma (MM) is the second most commonly seen hematologic malignancy, which has been characterized by the malignant proliferation of monoclonal plasma cells [1]. Heat shock proteins (HSPs) are a group of proteins whose expression is significantly increased when the cells are exposed to stimuli such as high temperature, radiation, hypoxia, and toxins [1, 2]. It has been shown that, the expression level of HSP-90 in the myeloma cells is 2-10 times higher than the normal cells, indicating that HSP might be involved in the disease pathogenesis, especially concerning the elevated proliferation of these tumor cells [3, 4].

In recent years, studies concerning MM have been mainly focusing on the target treatment with proteasome inhibitors. Bortezomib, a proteasome inhibitor, is currently one of the most widely used and effective drugs for the treatment of MM [5]. However, the utility of bortezomib has been largely limited in clinic due to its toxicity to nervous and hematopoietic systems. Therefore, it is of great importance to investigate the combination therapy to improve the survival and prognosis of MM patients treated with bortezomib. Recent studies have shown that curcumin could effectively inhibit the proliferation and induce the apoptosis of MM cells [6], which might be a potential candidate for the combination therapy with bortezomib for MM.

In this study, the therapeutic effects of curcumin and bortezomib on MM cells were investigated, and the related molecular mechanisms were also studied. Human multiple myeloma Dex-resistant (MM.1R) cells were treated with curcumin and/or bortezomib, and the cell proliferation and apoptosis were assessed. The effects of drug treatments on the expression levels of apoptosis-related proteins were as well investigated.

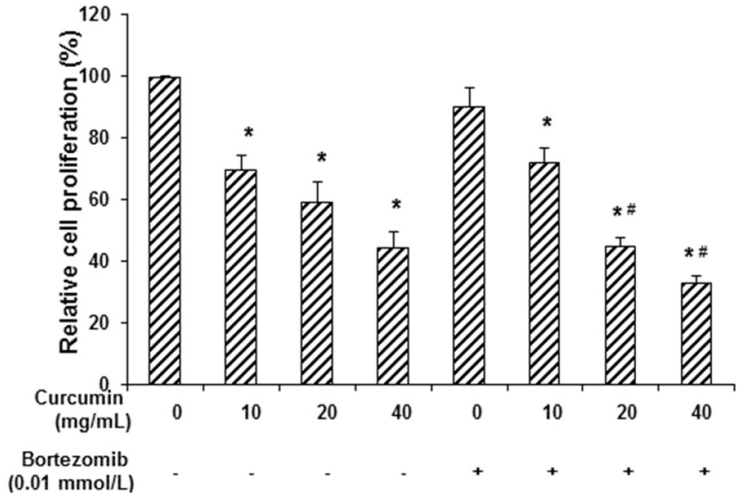

Figure 1. Effects of curcumin and bortezomib on MM1.R cell proliferation. Half of the MM1.R cells were treated with curcumin alone at 0, 10, 20, and 40 mg/mL, respectively, for 24 h, while the other half were treated with bortezomib (0.01 mmol/L) combined with curcumin at indicated concentrations. Cell proliferation was assessed with MTT assay. Compared with the control group (without treatment), $^{*}P < 0.05$; compared with the corresponding curcumin monotherapy group, $^{\#}P < 0.05$.

Materials and methods

Cell line, cell culture, and drug administration

Human multiple myeloma Dex-resistant (MM.1R) cell line was a kind gift from Dr. Steven Rosen from the Northwestern University. These cells were cultured in suspension with RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) in a 37°C, 5% CO_2 incubator. For drug administration, these cells were planted onto 96-well plates, at a density of 1×10^5 cells/mL. Half of the MM1.R cells were treated with curcumin (Sigma, St Louis, MO, USA) alone at 0, 10, 20, and 40 μg/mL, respectively, for 24 h, while the other half were treated with bortezomib (0.01 mmol/L; Xian Janssen Pharmaceuticals, Ltd., Co., Xian, Shaanxi, China) combined with curcumin at indicated concentrations.

MTT assay

Cell proliferation was assessed by the MTT assay. Cells were planted onto the 96-well plate, at the density of 1×10^5 cells/mL. After drug administration, 20 μL MTT was added into each well to incubate the cells in a 37°C, 5% CO_2 atmosphere for 4 h. Then 200 μL dimethylsulfoxide sulfone was added into each well. The absorbance (A) at 570 nm was read with a microplate reader. Survival rate was calculated according to the following formulation: Survival rate = $A_{treatment}/A_{control} \times 100\%$.

Flow cytometry

Cells were planted onto the culture bottle at the density of 5×10^5 cells/mL. After drug administration, these cells were harvested and re-suspended in 1 × binding buffer. 100 μL cell suspension was added into a tube, followed by the addition of 5 μL Annexin V (FITC; BioVision, Milpitas, CA, USA) and 5 μL PI (PE; Sigma), which was then kept in dark for 15 min. After adding another 300 μL 1 × binding buffer, the fluorescence was detected with a flow cytometer and analyzed with the Cellquest I.2 software.

Western blot analysis

Total protein was extracted with the extraction kit (Active Motif, Carlsbad, CA, USA). 40 μg protein was subjected to SDS-PAGE, and then electronically transferred onto a PVDF membrane. After blocked with non-fat milk for 1 h, the membrane was incubated with goat anti-human anti-caspase-3 polyclonal primary antibody (Santa Cruz, Santa Cruz, CA, USA), goat anti-human anti-caspase-9 polyclonal primary antibody (Santa Cruz), mouse anti-human anti-NF-κB monoclonal primary antibody (R&D System; Minneapolis, MN, USA), or mouse anti-human anti-HSP-90 monoclonal primary antibody (Santa Cruz), at 4°C overnight. After washing, the membrane was incubated with HRP-conjugated secondary antibody (1:1000 dilution; Cell Signaling Technology, Beverly, CA, USA) at room temperature for 1 h. Color development was performed with the ECL method (Pierce, Rockford, IL, USA). β-actin was used as control.

Statistical analysis

Data were expressed as mean ± SD. SPSS 15.0 software was used for statistical analysis. ANOVA was performed for the group comparison. $P < 0.05$ was considered statistically significant.

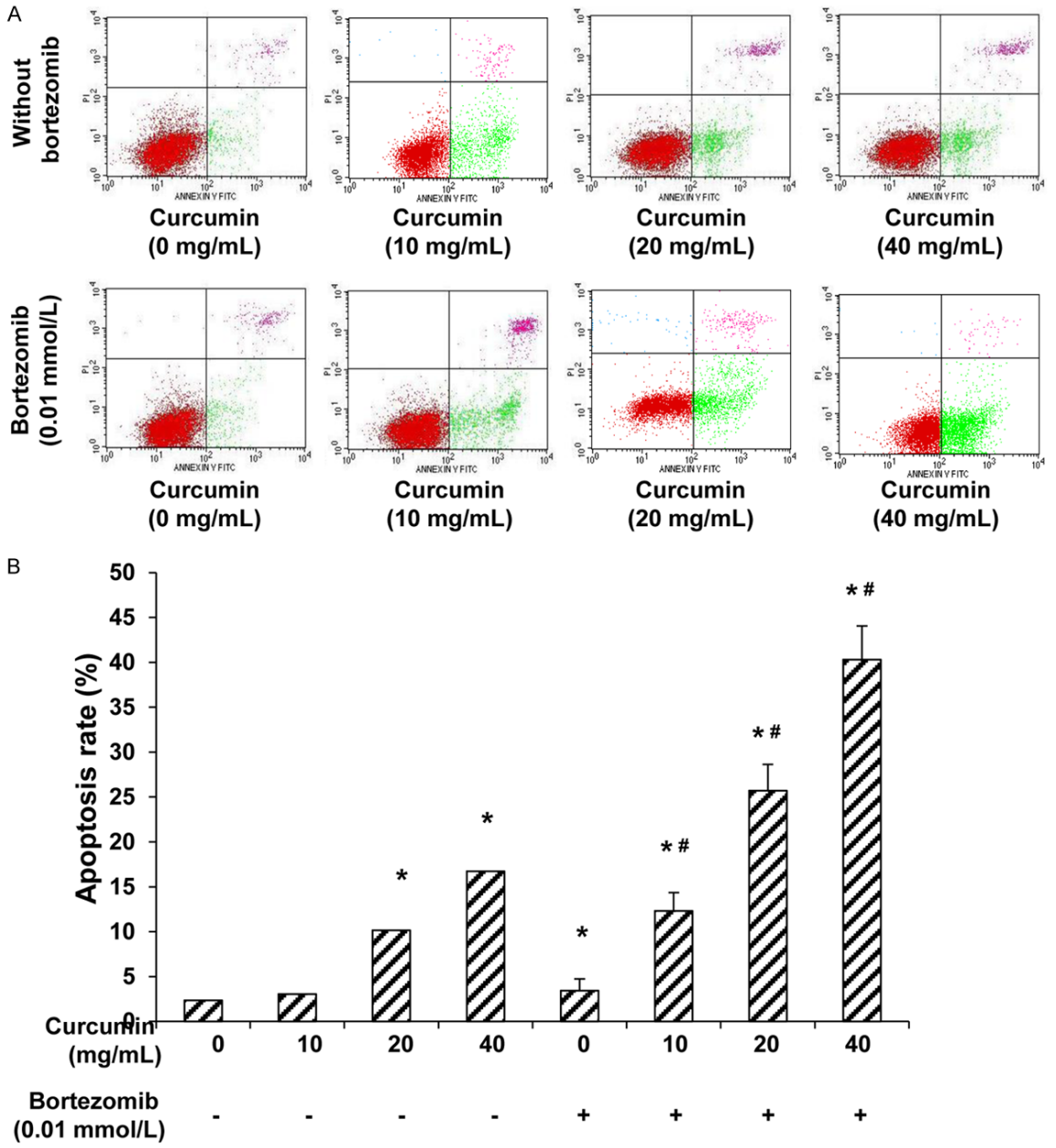

Figure 2. Effects of curcumin and bortezomib on apoptosis of MM1.R cells. Half of the MM1.R cells were treated with curcumin alone at 0, 10, 20, and 40 mg/mL, respectively, for 24 h, while the other half were treated with bortezomib (0.01 mmol/L) combined with curcumin at indicated concentrations. A. Cellular apoptosis was detected with flow cytometry. B. Statistical analysis of the apoptosis rates of MM1.R cells. Compared with the control group (without treatment), $^{*}P < 0.05$; compared with the corresponding curcumin monotherapy group, $^{\#}P < 0.05$.

Results

Effects of curcumin and bortezomib on proliferation of MM1.R cells

To investigate the effects of curcumin and/or bortezomib on the proliferation of MM1.R cells, MTT assay was performed. Our results showed that, curcumin treatment alone significantly inhibited the proliferation of MM1.R cells ($P < 0.05$), in a dose-dependent manner. On the other hand, the monotherapy of 0.01 mmol/L bortezomib did not significantly alter the proliferation of MM1.R cells ($P > 0.05$). However, when the cells were treated with curcumin and bortezomib combination, the cell proliferation

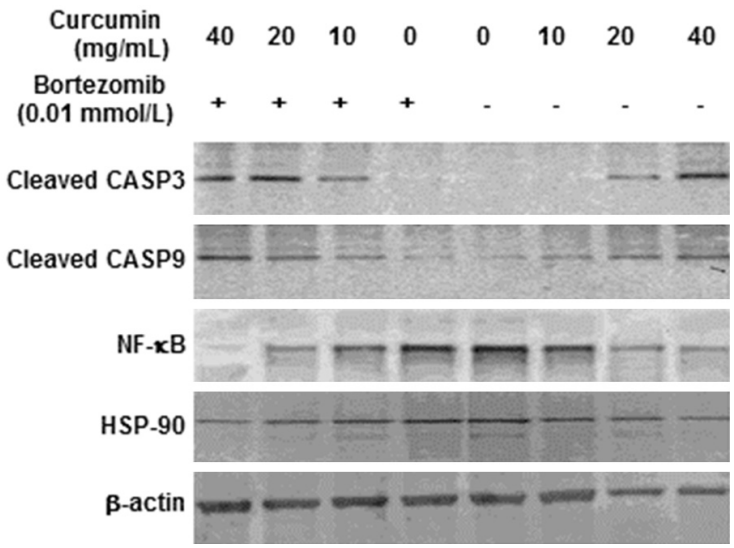

Figure 3. Effects of curcumin and bortezomib on expression of apoptosis-related proteins in MM1.R cells. Half of the MM1.R cells were treated with curcumin alone at 0, 10, 20, and 40 mg/mL, respectively, for 24 h, while the other half were treated with bortezomib (0.01 mmol/L) combined with curcumin at indicated concentrations. The activation of caspase-3 (Cleaved CASP3) and -9 (Cleaved CASP9), and the expression levels of NF-κB, and HSP-90 were detected with Western blot analysis.

was further significantly declined compared with the curcumin treatment alone at corresponding concentrations ($P < 0.05$ for 20 and 40 μg/mL) (**Figure 1**). These results suggest that, combination of curcumin and bortezomib could effectively inhibit the proliferation of MM1.R cells.

Effects of curcumin and bortezomib on apoptosis of MM1.R cells

To investigate the effects of curcumin and/or bortezomib on the apoptosis of MM1.R cells, flow cytometry was performed. Our results showed that, the monotherapy of curcumin significantly enhanced the apoptosis of MM1.R cells ($P < 0.05$), in a dose-dependent manner, while the treatment of bortezomib alone (0.01 mmol/L) did not significantly stimulate the apoptosis of these cells ($P > 0.05$). However, when the cells were treated with the combination therapy, the cellular apoptosis was significantly enhanced compared with the curcumin treatment alone at corresponding concentrations (all $P < 0.05$) (**Figure 2**). These results suggest that, the treatment of curcumin and bortezomib could effectively enhance the apoptosis of MM1.R cells, with synergistic effects.

Effects of curcumin and bortezomib on expression of apoptosis-related proteins in MM1.R cells

To investigate the effects of curcumin and/or bortezomib on apoptosis-related proteins in MM1.R cells, the expression levels of caspase-3 and -9, NF-κB, and HSP-90 were detected with Western blot analysis. Our results showed that, the bortezomib monotherapy (0.01 mmol/L) did not significantly change the expression levels of caspase-3 and -9, NF-κB, or HSP-90 in MM1.R cells ($P > 0.05$). On the other hand, the treatment of curcumin alone significantly elevated the activation of caspase-3 and -9, and significantly declined the expression levels of NF-κB and HSP-90 in these MM1.R cells, indicating enhanced apoptotic process. When these cells were treated with combination treatment of curcumin and bortezomib, the activation of caspase-3 and -9 was further enhanced, while the expression levels of NF-κB and HSP-90 were further decreased, in MM1.R cells (**Figure 3**). Taken together, these results suggest that, the combination treatment of curcumin and bortezomib could significantly affect the expression levels of apoptosis-related proteins in MM1.R cells, which was in line with the enhanced apoptotic process in these cells.

Discussion

Although it has been more than 150 years since multiple myeloma (MM) was first recognized, it is still an incurable disorder so far. At present, the clinical treatment strategies of MM mainly include the conventional chemotherapy, stem cell transplantation, bortezomib combination treatment, and thalidomide combination treatment [7]. Bortezomib is the first proteasome inhibitor which has been widely used in clinic. However, the clinical application of bortezomib has been limited due to the side effects such as nausea, diarrhea, thrombocytopenia, and peripheral neuropathy [8]. Therefore, it is of great importance to discover the ideal

combination agent for bortezomib in the treatment of MM.

Curcumin is a kind of phenolic pigment that is extracted from the curcuma longa (*Zingiberaceae*). Curcumin could exert anti-inflammatory, blood lipid-reducing, and anti-tumor effects under various pathophysiological conditions, with low toxicity. In this study, bortezomib was combined with curcumin to treat MM model cells, and the effects of the combination therapy on the cell proliferation and apoptosis were investigated. Studies have found that the monotherapy of curcumin could inhibit the proliferation and enhance the apoptosis of myeloma H929 and RPMI8226 cells [6]. Our results herein also showed that the curcumin treatment alone could significantly inhibit the proliferation and enhance the apoptosis of MM1.R cells, in a dose-dependent manner. Moreover, our results showed that, the monotherapy of bortezomib at low dosage (0.01 mmol/L) could not dramatically alter the proliferation and apoptosis of the MM1.R cells. However, when bortezomib was combined with curcumin, the effects on cellular proliferation and apoptosis were significantly enhanced, indicating the synergistic effects of curcumin and bortezomib. Mitsiades *et al.* [9] have shown that, bortezomib regulates the apoptotic process via modulating the expression of Fas/FasL in MM cells, which also involves the activation of caspases. Our results showed that the monotherapy of curcumin could elevate the activation of caspase-3 and -9, while the bortezomib treatment alone could not influence the expression of these apoptosis-related proteins.

NF-κB signaling pathway has been shown to be highly expressed in MM cells, and the suppressing effects of bortezomib on myeloma cell growth are achieved via blocking the NF-κB signaling pathway [10]. In this study, our results showed that, the treatments of curcumin could significantly influence the expression of NF-κB in MM1.R cells, which could be further enhanced by the combination with bortezomib. These results suggest that, bortezomib could elevate the sensitivity of the NF-κB signaling pathway to curcumin, resulting in decreased cell proliferation and increased cellular apoptosis.

HSP-90 is one of the most active intracellular chaperone proteins, playing important roles in the cell signaling, hormone responses, and transcriptional regulating processes. It has been shown that, HSP-90 is highly expressed in MM cells, which is closely associated with the disease pathogenesis [11]. Our results showed that, the monotherapy of bortezomib at low concentration could not significantly influence the expression of HSP-90 in MM1.R cells, which however could be significantly down-regulated by the treatment of curcumin alone. Moreover, the combination treatment of curcumin and bortezomib significantly further decreased the expression levels of HSP-90 in MM1.R cells, which was in line with the suppressed proliferation and enhanced apoptosis in these cells. Taken together, all these results demonstrate the synergistic effects between curcumin and bortezomib on MM1.R cells. Angelo *et al.* [12, 13] have found that curcumin could block the activation of heat shock factor 1 (HSF1). Before the mRNA transcription, curcumin could partially block the heat shock responses and down-regulate the HSP-90 expression levels, further inducing apoptosis in MM1.R cells, increasing its sensitivity to bortezomib [14-17]. We suppose that curcumin might decline the activity of NF-κB and reduce the expression of HSP-90, to synergistically enhance the effects of bortezomib on MM1.R cells. Multiple signaling pathways have been shown to be activated in the proliferation of MM cells. Therefore, the efficiencies of single-targeted therapies might be limited due to the drug resistance [18-21]. According to our results, curcumin might fight against the drug resistance with its multiple effects at various levels [22-24].

In conclusion, our results showed that, curcumin treatment alone significantly inhibited the proliferation and enhanced the apoptosis of MM1.R cells, in a dose-dependent manner. The monotherapy of bortezomib did not significantly alter the cell proliferation and apoptotic process. When the cells were treated with the combination of curcumin and bortezomib, further suppressed proliferation and enhanced apoptosis would be observed in these MM1.R cells. In addition, the combination treatment could significantly increase the activation of caspase-3 and -9, and decrease the expression levels of NF-κB and HSP-90, in MM1.R cells. Our findings suggest that, curcumin could enhance the sensitivity of MM1.R cells to bortezomib, and they could exert synergistic effects on MM1.R cells, inhibiting cell proliferation and enhancing cellular apoptosis, via the regulation

of the expression of NF-κB and HSP-90. These findings provide evidence for application of the combination of curcumin and bortezomib in the treatment of MM.

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Disclosure of conflict of interest

None.

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