Abstract. Effect of bortezomib on proliferation and apoptosis of myeloma cells by activating Wnt/β-catenin signaling pathway was investigated. Myeloma cells RPMI-8226 treated with different concentrations of bortezomib were used as experimental groups, and the untreated cells were used as the control group. The proliferation and apoptosis in all groups of cells were detected, as well as the expression levels of Wnt/β-catenin signaling pathway-related proteins, β-catenin and c-Myc. The results revealed that bortezomib could inhibit the proliferation of myeloma cells. The apoptotic rates of RPMI-8226 cells in the groups treated with 20, 50 and 80 nmol/l of bortezomib were 12.08±0.61, 35.97±3.11 and 57.22±5.47%, respectively, which were significantly higher than that in the control group (8.28±0.39%) (P<0.05). The expression levels of β-catenin and c-Myc in the experimental groups were significantly lower than those in the control group (P<0.05). Bortezomib can reduce the expression level of Wnt/β-catenin signaling pathway-related proteins, β-catenin and c-Myc, and may inhibit cell proliferation and accelerate apoptosis by activating the Wnt/β-catenin signaling pathway.

Introduction

Myeloma is also called plasmacytoma. Multiple myeloma (MM), a malignant monoclonal plasma cell disease (1), is most common in clinic and usually occurs in middle-aged and elderly individuals. MM accounts for ~10% of blood tumors and 1% of systemic malignant tumors (2), and its incidence increases with age (3). Chemotherapy and autologous hematopoietic stem cell transplantation (4) are currently the two most commonly used treatment methods for MM patients. As the pathogenesis of MM is not clear yet (5), although the survival rate of MM patients has improved, the recurrence rate is still very high (6). Wnt/β-catenin signaling pathway has the effects of regulating gene expression, cell invasion, migration, proliferation and differentiation, and is involved in the occurrence and development of bone cancer (7). A previous study by Savvidou et al (8) has shown that Wnt/β-catenin signaling pathway is abnormally regulated in the advanced stage of MM disease. However, bortezomib is a proteasome inhibitor (9). Qiang et al (10) have shown that bortezomib can induce activation of Wnt/β-catenin pathway and differentiation of mesenchymal stem cells into osteoblasts. At present, research has proven that bortezomib has good therapeutic effect on MM; however, the exact therapeutic mechanism of bortezomib has not been fully understood. In the present study, the effect of bortezomib on the proliferation and apoptosis of myeloma cells by activating the Wnt/β-catenin signaling pathway was investigated, aiming to uncover the mechanism of bortezomib in the treatment of MM and provide reference and guidance for the clinical treatment of such diseases.

Materials and methods

Reagents and materials. Human MM cell line RPMI-8226 was provided by the BeNa Culture Collection (BNCC338295). The following kits were used: CCK-8 kit (IC-CCK-Hu; Shanghai Yu Bo Biotech Co., Ltd.), TRizol® kit (5301100; Shanghai Mingjin Biology Co., Ltd.), RNasin Inhibitor (R8060; Beijing Solarbio Science & Technology Co.), RT kit (CD-102539GM) and Dual-Luciferase Reporter Assay kit (CDLG-4997) (both from ChunduBio), RIPA (JN0190-HBJ; Beijing Biolab Science and Technology Co., Ltd.), BCA Protein Assay kit (QC12533-A; Shanghai Qincheng Biotechnology Co., Ltd.), ECL kit (H-E-60/H-E-125/H-E-250; Shanghai Chuan Qiu Biotechnology Co., Ltd.), Annexin V/PI Apoptosis Detection kit (AD10-2; Shanghai Jingke Chemical Technology Co., Ltd.), RNA Amplification kit (HZ-051021; Zhen Shanghai and Shanghai Industrial Co., Ltd.), SYBR Green I (KS26757; Shanghai Keshun Biological Technology Co., Ltd.), β-catenin and c-Myc antibodies (YT656 and K12862, respectively; both from Beijing Biolab Science and Technology Co., Ltd.), GAPDH antibody (10900R; Shanghai Caiyou Industrial Co., Ltd.), HRP-labeled secondary antibody (YDJ3235; Shanghai Yuduo Biological Technology Co., Ltd.), microplate reader (BioTek

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Instruments, Inc.), flow cytometer (FACScanto II; Becton, Dickinson and Company), GAPDH (Cell Signaling Technology, Inc.). All primers were designed and synthesized by the Shanghai GenePharma Co., Ltd.

The study was approved by the Ethics Committee of Chuxiong Medical College (Chuxiong, China).

**Cell line culture, grouping and administration.** RPMI-8226 human myeloma cells were transferred to a medium containing 10% fetal bovine serum and were cultured in a constant temperature incubator at 37˚C with 5% CO₂ for 24 h. Next, 20, 50 and 80 nmol/l of bortezomib were added, respectively. No drugs were added to the control group. The cells of each group were collected after 24 h of treatment.

**CCK-8 detection of cell proliferation.** After treatment, the cells of the experimental and control groups were collected, inoculated on 96-well plates, and then cultured for 24, 48, 72 and 96 h. The cells were cultured in a 5% CO₂ incubator at 37˚C, 10 µl of CCK-8 solution was added to each well, and the culture was continued for 1-4 h. The OD value of each group of cells was measured under 450 nm absorbance by using an enzyme-labeled instrument.

**Detection of apoptosis by flow cytometry.** The treated cells were digested with pancreatin, washed with PBS, added with 100 µl of binding buffer, and then prepared into 1x10⁶/ml suspension. Annexin V-FITC and PI were added, and the cells were incubated at room temperature in the dark for 20 min. The apoptotic rate of the cells was analyzed using a flow cytometer and FACScanto II software.

**Detection of β-catenin and c-Myc gene expression by reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from the RPMI-8226 cells of each group using TRIzol® reagent. Total RNA was reverse transcribed into cDNA. Reaction system: 1 µl M-MLV, 1 µl Oligo(dT), 0.5 µl RNasin Inhibitor, 1 µl dNTPs, and RNAse-free water was added to a final volume of 15 µl. Following incubation at 38˚C for 60 min, 1 µl of cDNA was collected at 85˚C for 5 sec. The synthesized cDNA was used as template for the RT-qPCR amplification. GAPDH was used as internal reference for β-catenin and c-Myc, and the reaction conditions were: Pre-denaturation at 95˚C for 30 sec, denaturation at 95˚C for 5 sec, 60˚C for 20 sec, for 40 cycles. Each experiment was repeated 3 times and the relative expression of the genes was detected by 2⁻ΔΔCq method (11). Primer sequences are shown in Table I.

**Detection of β-catenin and c-Myc protein expression by western blot analysis.** RIPA was used to lyse the cells and extract total protein. Protein concentration was quantified by BCA. Samples of 0, 1, 2, 3 and 4 µl were added to the wells, respectively, and the concentration was adjusted to 20 µg/µl. A total of 40 µg protein/lane were separated via 12% SDS-PAGE and transferred onto a PVDF membrane. The corresponding bands were selected according to the target protein. The separated proteins were blocked with 5% of skim milk powder at room temperature for 1.5 h. TBST was used to wash the membranes for 3 min. After washing, 2 ml of western primary antibody solution consisting of β-catenin (1:1,000), c-Myc (1:500) and GAPDH (1:1,000) was added and the membranes...
were stored at 4˚C overnight. On the next day, the membranes were incubated with primary antibodies for 30 min. Following the primary incubation, the membranes were incubated with HRP-labeled secondary antibody (1:1,000) for 1 h at 37˚C and were rinsed 3 times with PBS for 5 min each time. Protein bands were visualized in a dark room. ECL was carried out and the protein expression levels were determined. GAPDH was used as an internal reference for the analysis of the relative expression of each indicator. The results were analyzed by Image Lab™ software (Bio-Rad Laboratories, Inc.).

Observational indexes. The proliferation and apoptosis of cells in the experimental and control groups were observed. The expression levels of β-catenin and c-Myc in each group of cells were also observed.

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used for data analysis. Measurement data were expressed as the mean ± SD. The comparison of measurement data between two groups was made by independent samples t-test, and among multiple groups by one-way ANOVA (expressed as F-value). LSD post hoc test was used for pairwise comparisons. Repeated measures ANOVA was used for multi time-point expression (expressed as F-value) and the Bonferroni test was used as the post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of proliferation ability among groups. The results of CCK-8 detection revealed that, compared with the control group, the proliferation ability of the cells treated with 20, 50 and 80 nmol/l of bortezomib was reduced. There was no significant difference between the proliferation ability of the cells treated with 20 nmol/l of bortezomib and that of the cells in the control group, and both were higher than the proliferation ability of the cells treated with 50 and 80 nmol/l of bortezomib (P<0.05) (Fig. 1).

Comparison of apoptotic rate among groups. Flow cytometry analysis of cell apoptosis revealed that the apoptotic rates of RPMI-8226 cells treated with 20, 50 and 80 nmol/l of bortezomib were 12.08±0.61, 35.97±3.11 and 57.22±5.47%, respectively, which were significantly higher than the apoptotic rate of the cells in the control group (8.28±0.39%), (P<0.05), in a dose-dependent manner (Fig. 2).

Effect of bortezomib on Wnt/β-catenin signaling pathway. The expression of the Wnt/β-catenin signaling pathway-related proteins, β-catenin and c-Myc, was detected by western blot analysis. The protein expression levels of β-catenin and c-Myc in the RPMI-8226 cells of the control group were significantly higher than those in the bortezomib-treated groups (Fig. 3).

The expression of β-catenin and c-Myc in each group of cells was detected by RT-qPCR. The results showed that the expression levels of β-catenin and c-Myc in the RPMI-8226 cells of the control group were significantly higher than those in the bortezomib-treated groups (Fig. 4).

Discussion

MM can lead to osteolytic lesions and fractures in the body (12), and almost all MM cases present painless, pre-malignant lesions, known as uncertain monoclonal hematopathy (13). The annual incidence rate of MM ranks second among all hematological malignancies (14), and MM is especially prevalent
in middle-aged and elderly individuals. Although in recent years a number of studies have been reported on MM and the understanding of myeloma diseases is getting deeper, MM is still an incurable disease due to its unclear pathogenesis, drug resistance and disease recurrence.

At present, the main treatments for MM are chemotherapy, immunotherapy, and autologous stem cell transplantation (15-17). Autologous stem cell transplantation has a huge toxic effect, thus requiring long-term hospitalization. Immunomodulatory drugs and proteasome inhibitors can significantly improve the prognosis of patients (18). Proteome is an enzyme that exists in all cells and plays an important role in cell growth and function. Bortezomib, as a proteasome inhibitor, can cause cancer cells to die or not grow any further (19). However, due to its drug resistance, bortezomib is not the best treatment method for MM. Bortezomib drug resistance is an urgent clinical problem to be solved. It is very important to find a treatment method to overcome bortezomib drug resistance (20). A number of studies have confirmed that the activation of Wnt/β-catenin signaling pathway is positively correlated with the prognosis of various malignant tumors (21,22). Transcription mediated by the β-catenin/Tcf promotes cell proliferation, survival and invasion (23). β-catenin is a downstream effector of Wnt signaling pathway that regulates cell proliferation and differentiation (24). Typical Wnt/β-catenin signaling pathway has been extensively studied as a target for tumor therapy (25). The present study aimed to investigate the effect of bortezomib on proliferation and apoptosis of myeloma cells by activating Wnt/β-catenin signaling pathway, in order to provide a reference for the study of drug resistance.

In the present study, the proliferation abilities of bortezomib-treated and untreated myeloma RPMI-8226 cells were compared, and the results showed that the proliferation ability of myeloma cells treated with 20, 50 and 80 nmol/l of bortezomib was lower than that of the control group after 96 h. In addition, the proliferation ability of myeloma cells treated with 50 and 80 nmol/l of bortezomib was significantly lower than that of the control group (p<0.05). Furthermore, the apoptotic rate of each group of cells was analyzed by Annexin V-FITC/PI flow cytometry. The apoptotic rate of the cells treated with bortezomib was significantly higher than that of the untreated control group (P<0.05), which also showed that bortezomib has the effect of promoting myeloma cell apoptosis. These results are consistent with previous studies (26,27). Finally, the expression levels of Wnt/β-catenin signaling pathway-related proteins β-catenin and c-Myc were detected in each group by two methods. The expression levels of β-catenin and c-Myc in myeloma cells treated with bortezomib were lower than those in the control group, and were decreased in a dose-dependent manner. Jin et al (28) have shown that the sensitivity of myeloma cells to bortezomib can be enhanced by regulating Wnt/β-catenin signaling pathway. At present, numerous studies have confirmed that Wnt/β-catenin signaling pathway is related to hematological tumors (29-31), such as lymphoma, in accordance to the results of this study and supporting our conclusions.

There are some deficiencies in the presented study. First of all, normal cells were not included in this research and the effect of bortezomib on normal cells was not investigated. Secondly, due to the small number of research samples, individual differences are not excluded. Finally, the effect of bortezomib on the regulation of related proteins was not studied and further research is required to improve our results.

In conclusion, bortezomib may reduce myeloma cell proliferation and accelerate cell apoptosis by activating the Wnt/β-catenin signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YD analyzed and interpreted the data, and wrote the manuscript. XG designed the study and performed the experiments.
The authors declare that they have no competing interests.

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