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Lenalidomide affect expression level of cereblon protein in multiple myeloma cell line RPMI8226

D.Y. Yang, J.H. Ren, X.N. Guo, X.L. Guo, X.Y. Cai, X.F. Guo and J.N. Zhang

Department of Hematology, Secondary Hospital of HeBei Medical University, HeBei Hematology Institute, Shijiazhuang, HeBei Province, China

Corresponding author: J.H. Ren
E-mail: JinhaiRencn@163.com

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ABSTRACT. We investigated the mechanisms of action of immunomodulatory drug (lenalidomide) on the protein expression of cereblon (CRBN) and their therapeutic targets in the multiple myeloma cell line RPMI8226. The multiple myeloma cell line RPMI8226 was cultured and treated with different concentrations of lenalidomide and bortezomib to determine the proliferation inhibition rate, apoptosis rate, and protein expression of CRBN. The results revealed that both lenalidomide and bortezomib inhibited the proliferation of RPMI8226 and promoted cell apoptosis. However, the protein expression of CRBN decreased significantly after treatment with lenalidomide, while bortezomib had no effect on the expression of CRBN. We confirmed that CRBN may be a target of lenalidomide.

Key words: Apoptosis; Bortezomib; Cereblon; Lenalidomide; Proliferation

INTRODUCTION

Immunomodulatory drugs, such as lenalidomide and thalidomide, play important therapeutic roles in hematological malignancies; however, the mechanisms of action of immunomodulatory drugs remain unclear (Quach et al., 2010). A recent study confirmed that cereblon (CRBN) is a primary target of thalidomide teratogenicity. Thalidomide inhibited the function of the associated E3 ubiquitin ligase complex by binding to wild-type CRBN in a zebrafish model. E3 ubiquitin ligase complex is necessary for growth, development, and fibroblast growth factor 8 expression; however, whether CRBN is a target of immunomoduatory drugs that exert anti-tumor activity remained unknown (Ito et al., 2010, 2011; Ito and Handa, 2012). Subsequent studies confirmed that CRBN was a direct protein target for immunomodulatory and anti-proliferation activities of immunomodulatory drugs (Lopez et al., 2012; Heintel et al., 2013). Moreover, CRBN expression was associated with survival in patients with multiple myeloma (Broyl et al., 2013). In this study, the multiple myeloma cell line RPMI8226 was treated with different concentrations of immunomodulatory drugs, followed by detection of changes in CRBN protein expression to determine the relationship between CRBN expression and lenalidomide concentration.

MATERIAL AND METHODS

Cell culture

The human myeloma cell line RPMI8226 was purchased from The Cell Center of Basic Medical Institute of the Chinese Academy of Medical Sciences and cultured in 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified incubator at 37°C under 5% CO_2. The medium was changed every 3 days and each experiment used cells in the logarithmic phase.

Drug storage

Lenalidomide was purchased from Selleck Chemicals (Houston, TX, USA) and a final concentration of 40 mM was used. Lenalidomide was stored at -80°C. Bortezomib was obtained from Janssen Pharmaceutica (Beerse, Belgium) and stored at -20°C in a dark room.

Groups

Two groups of cells were used, including one in which different concentrations of lenalidomide (0, 200, 800, 3200 nM) were used to treat RPMI8226 cells for 24 h and another in which different concentrations of bortezomib (0, 10, 20, 40 nM) were used to treat RPMI8226 cells for 24 h.

CCK8 assay to detect cell growth inhibiting ratio

The Cell counting Kit-8 was purchased from Dojindo Laboratories Company (Kumamotor, Japan) and used according to the manufacturer protocol.

Flow cytometry was used to detect cell apoptosis. RPMI8226 cells were prepared for 24 h after treatment with drugs. Cell density was 5-6 x 10^5/mL. A 1-mL cell suspension was centrifuged at 800 *g*, 4°C for 10 min, and then supernatant was removed. To the cells, we added 1 mL cold

phosphate-buffered saline and then gently mixed the tube. The tubes were centrifuged at 800 g, 4°C for 10 min and the supernatant was removed. The washing with phosphate buffered saline and removal of the supernatant were repeated twice. The cells were resuspended in 100 mL binding buffer solution and then detected by flow cytometry. The cells were then treated with 5 mL annexin-V-fluorescein isothiocyanate and stained with 5 mL propidium iodide, followed by incubation at 37°C for 15 min in the dark, after which 400 mL binding buffer was added. All samples were analyzed using a flow cytometer according to the manufacturer instructions.

Western blotting assay to detect protein expression of CRBN

Standard methods were used to extract total protein, which was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein bands were transferred onto polyvinylidene fluoride membranes and then samples were incubated with primary antibody and secondary horseradish peroxidase-conjugated IgG. CRBN antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). All data were analyzed using the image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and all results are reported as means ± standard deviation. Analysis of variance was used for same-group comparisons. P value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Inhibition of cell proliferation was detected using the CCK8 assay in RPMI8226 cells. The cells were treated with different concentrations of lenalidomide (200, 800, and 3200 nM) for 24 h, and the cell growth inhibition ratios were 16.67 ± 0.25, 26.35 ± 1.21, and 36.26 ± 1.03%, respectively (P = 0.00) (Figure 1). RPMI8226 cells were also treated with different concentrations of bortezomib (10, 20, 40 nM) for 24 h, and the cell growth inhibition ratios were 8.13 ± 1.14, 18.46 ± 0.21, and 31.43 ± 1.64%, respectively (P = 0.00) Figure 2).

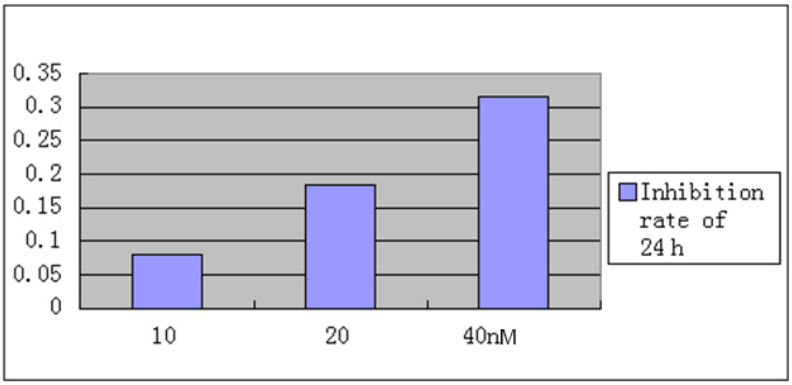

Figure 1. Inhibition rates of RPMI8226 cells treated by different concentrations of lenalidomide for 24 h.

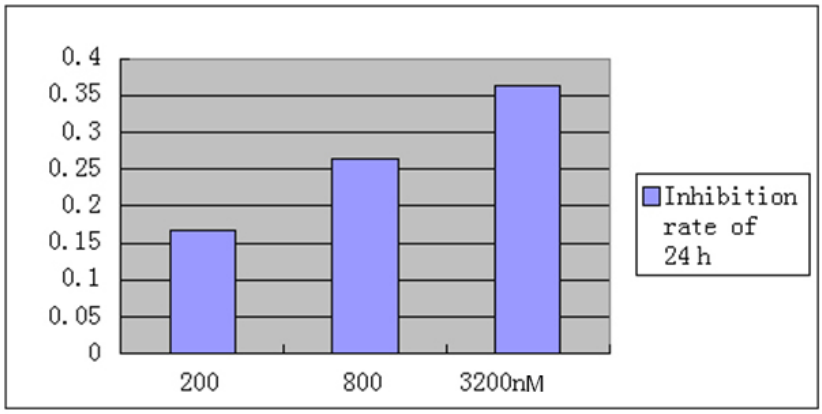

Figure 2. Inhibition rates of RPMI8226 cells treated by different concentrations of bortezomib for 24 h.

The effect of lenalidomide and bortezomib on promoting apoptosis was determined using a flow cytometry assay. RPMI8226 cells were treated with different concentrations of lenalidomide (0,200, 800, and 3200 nM) for 24 h, and cell apoptosis rates were 7.12 ± 0.59, 17.89 ± 0.31, 29.4 ± 0.71, and 40.33 ± 1.25%, respectively (P = 0.00) (Figure 3). RPMI8226 cells were treated with different concentrations of bortezomib (0,10, 20, and 40 nM) for 24 h, and cell apoptosis rates were 7.82 ± 0.33, 14.26 ± 0.60, 25.22 ± 0.26, and 34.54 ± 1.24%, respectively (P = 0.00) (Figure 4).

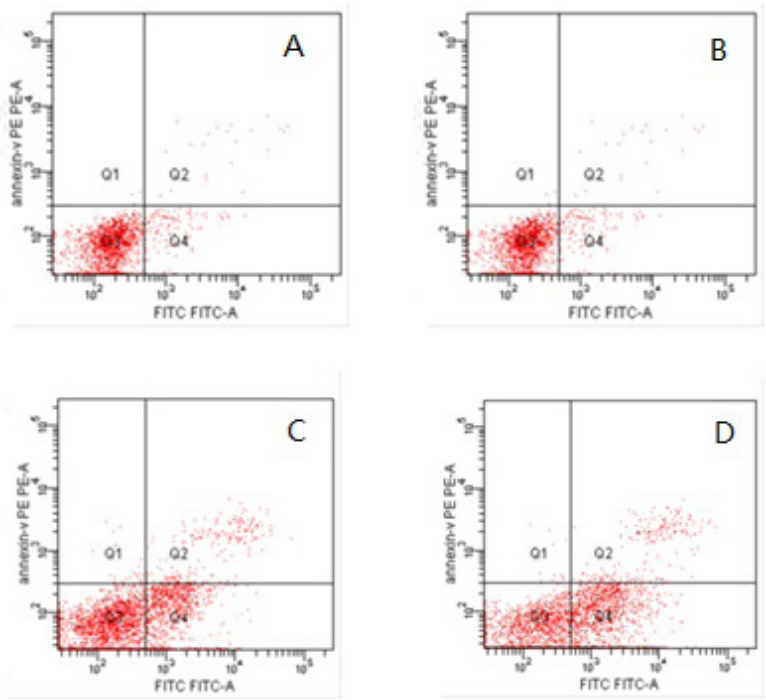

Figure 3. Apoptosis of RPMI8226 cells treated with lenalidomide (0, 200, 800, and 3200 nM) for 24 h.

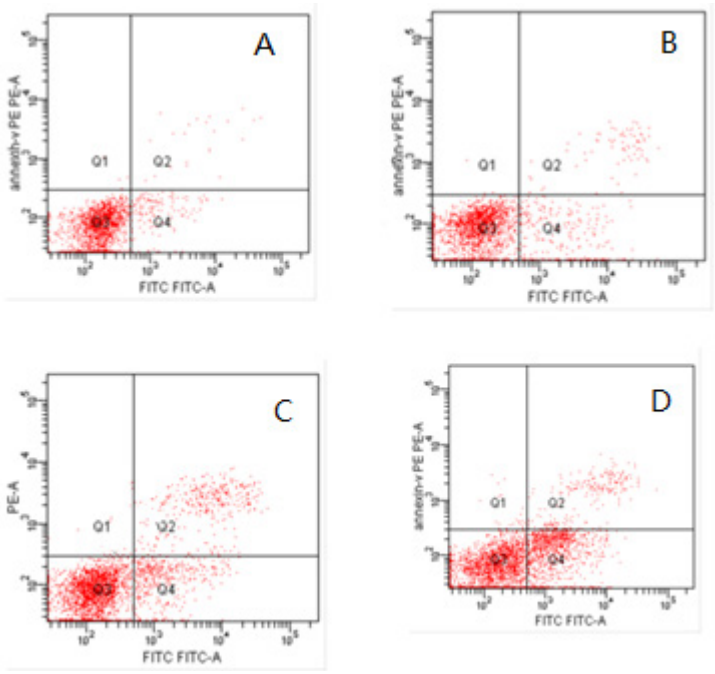

Figure 4. Apoptosis of RPMI8226 cells treated with bortezomib (0, 10, 20, and 40 nM) for 24 h.

Changes in CRBN protein expression in RPMI8226 cells was detected using a western blot assay. The results showed that the expression level of CRBN in RPMI8226 cells after treatment with lenalidomide (0, 200, 800, and 3200 nM) for 24 h was 1.48 ± 0.03, 1.14 ± 0.01, 0.95 ± 0.01, and 0.56 ± 0.02, respectively (P = 0.00) (Figure 5), and the expression level of CRBN in RPMI8226 cells after treatment with bortezomib (0,10, 20, and 40 nM) for 24 h was 1.39 ± 0.1, 1.32 ± 0.1, 1.36 ± 0.2, and 1.34 ± 0.2, respectively (P = 0.92) (Figure 6).

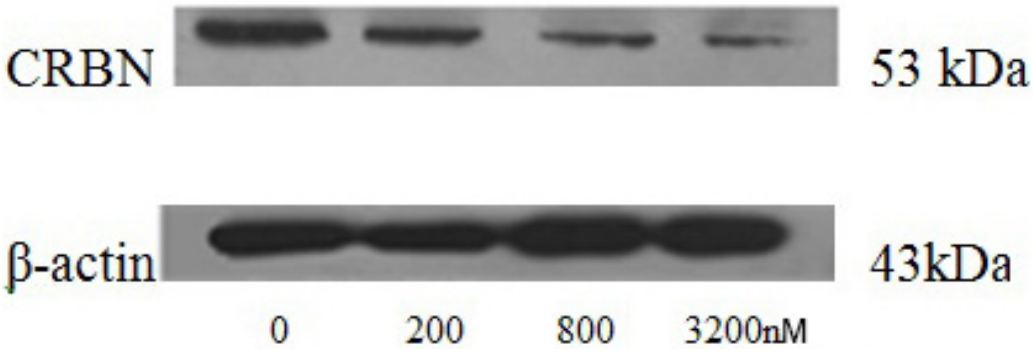

Figure 5. Expression level of CRBN in RPMI8226 cells after treated with lenalidomide for 24 h.

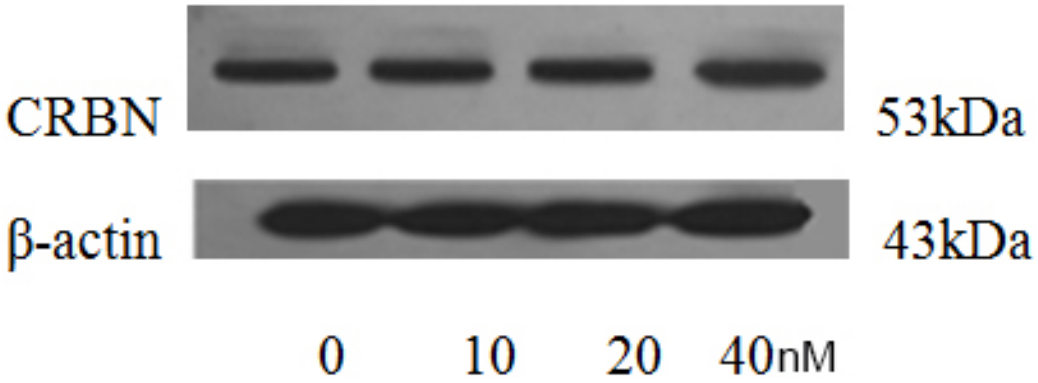

Figure 6. Expression level of CRBN in RPMI8226 cells after treated with bortezomib for 24 h.

DISCUSSION

Lenalidomide is an important derivative of thalidomide and shows more stable chemical properties as well as stronger inhibition of angiogenesis and anti-tumor activities. Furthermore, lenalidomide has fewer side-effects than thalidomide and has activity against various hematological and solid malignancies (Chanan-Khan and Cheson, 2008; Kotla et al., 2009; Jakubikova et al., 2011; Lopez et al., 2011); however, the exact mechanisms and actions of targeting remain unknown (Chang and Stewart, 2011). Gandhi et al. (2014) found that in cell lines resistant to lenalidomide and pomalidomide, CRBN protein was greatly reduced. Additionally, Zhu et al. (2011) found that CRBN knockout cells were highly resistant to lenalidomide and pomalidomide, but not resistant to bortezomib, dexamethasone, and melphalan. This indicates that CRBN is necessary for lenalidomide to exert its anti-tumor activities.

Bortezomib (Velcade) is an alaninyl-boric acid derivative and the boron atom in bortezomib binds the catalytic site of the 26S proteasome with high affinity and specificity. Moreover, bortezomib binds to the active site of the 20S core to intervene chymasr activity and then reduce I-κB expression to inhibit nuclear factor-κb activity. Bortezomib reduces myeloma cell apoptosis and decreases the interleukin-6 and adhesion factor levels (Hideshima et al., 2001), as well as promotes endoplasmic reticulum stress and induces a terminal unfolded protein response to cause myeloma cell apoptosis (Obeng et al., 2006). One study reported that bortezomib inhibited myeloma cell growth triggered by mitogen-activated protein kinase signaling cascades and then affected P27, P53, and P21 expression to suppress the anti-apoptosis of B cell lymphoma-2 (Hideshima et al., 2003).

In this study, the RPMI8826 cell line was treated with different concentrations of lenalidomide and bortezomib. The results showed that both drugs inhibited the proliferation of RPMI8226 cells and promoted cell apoptosis in a dose-dependent manner. However, lenalidomide reduced CRBN protein expression dose-dependently after treatment with lenalidomide, but there was no change in CRBN expression after treatment with bortezomib. This indicates that CRBN is a target of lenalidomide.

Our results showed that CRBN may be a target of lenalidomide, but its exact mechanisms and signal pathways remain unknown. Determination of the changes in CRBN and signal factors levels before and after treatment with lenalidomide in clinical patients is necessary. This will enable therapy in multiple myeloma patients to improve the effectiveness of treatment.

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