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Research Paper

Synergistic Cytotoxicity of Lenalidomide and Dexamethasone in Mantle Cell Lymphoma *via* Cereblon-dependent Targeting of the IL-6/STAT3/PI3K Axis

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ABSTRACT

At our center, relapsed mantle cell lymphoma (MCL) can be treated with maintenance therapy composed of consecutive low-dose lenalidomide and short-term, high-dose dexamethasone (LD regimen), which achieves good responses (longer overall survival and progression-free survival) and low toxicity. Cereblon is probably targeted by both lenalidomide and dexamethasone, which leads to synergistic cytotoxicity in MCL by inhibiting the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3), phosphatidylinositol 3-kinase (PI3K)/AKT and AKT2/Forkhead box O3 (FOXO3A)/BCL2-like 11 (BIM) pathways. The two drugs synergistically inhibit the same pathways, but through different sites. Cereblon was found expressed in most of the MCL tissues (91.3% positivity). Moreover, cereblon expression is positively correlated with LD regimen sensitivity: long-term lenalidomide exposure downregulates cereblon and induces multi-drug resistance against lenalidomide, dexamethasone, cytarabine, cisplatin, and methotrexate *in vitro*. Removal of lenalidomide resensitizes lenalidomide-resistant MCL cells to lenalidomide and dexamethasone. Our work suggests that rotating the LD regimen with other regimens would improve MCL maintenance therapy.

1. Introduction

Mantle cell lymphoma (MCL) is a distinct subtype of B cell lymphoma composed of small to medium lymphoid cells originating from CD5-positive follicular mantle B cells (Pérez-Galán et al., 2011; McKay et al., 2012). Due to an aggressive clinical disease course and incurability with standard chemotherapy, especially in the relapsed/refractory setting in which median overall survival (OS) is approximately 1–2 years with current therapies, MCL has the worst prognosis of B cell lymphomas (McKay et al., 2012; Goy and Kahl, 2011; Vose, 2012). MCL therapy has progressed in the past decade due to clinical experimentation with novel agents and drug combinations. Most novel drugs were tested in relapsed/refractory patients. Lenalidomide showed particularly promising antitumor activities in MCL patients (Habermann et al., 2009; Zinzani et al., 2013; Goy et al., 2013). Several clinical trials on lenalidomide for treating MCL are underway (NCT01035463, NCT01865110, NCT01996865) (Leonard et al., 2012; Martin et al., 2013; Zaja et al., 2012; Morrison et al., 2014). To date, there is no consensus on the best treatment approach for MCL.

At our center, a retrospective study revealed that a small cohort of patients with relapsed MCL who received lenalidomide and dexamethasone (LD regimen: for every 28 days, 10 mg lenalidomide was administered daily from d1 to d21, together with 20 mg dexamethasone at d1, d8, d15, and d22) as maintenance therapy had prolonged OS and progression-free survival (PFS), and showed low toxicity. Lenalidomide and dexamethasone had an obvious synergistic effect on the induction of apoptosis and inhibition of the cell growth of MCL cell lines *in vitro* that was better than that in myeloma lymphoma. However, the mechanism of the lenalidomide and dexamethasone synergy was unclear.

Based on the excellent synergistic effect of lenalidomide and dexamethasone, their mechanisms of action might share some common targets. Cereblon (CRBN) is a direct and therapeutically important molecular target of lenalidomide (Broyl et al., 2013; Lopez-Girona et al., 2012), while the target of dexamethasone in MCL is unknown. In addition, the signaling pathways involved in regulating apoptosis and cell cycle that are responsive to lenalidomide and dexamethasone are unclear. Several signaling pathways have been implicated in MCL cell growth including Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3), phosphatidylinositol 3-kinase (PI3K)/AKT, and AKT2/FOXO3A/BIM. A major driver of STAT3 activation is the

Abbreviations: MCL, mantle cell lymphoma; CRBN, cereblon; Lenalidomide, Lena; Dexamethasone, Dex; LD, lenalidomide and dexamethasone; OS, overall survival; Ara-C, cytarabine; MTX, methotrexate; DDP, cisplatin; CTX, cyclophosphamide; THP, therarubicin.

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cytokine interleukin-6 (IL-6), which signals through a heterodimeric IL-6 receptor (IL-6Rα/IL-6Rβ) to activate JAKs and induce STAT3 tyrosine phosphorylation. STAT3 activation in turn promotes IL-6 production and IL-6R expression, completing the positive feedback loop of the IL-6/STAT3 axis in MCL cells (Sansone and Bromberg, 2012; Snyder et al., 2014; Wang et al., 2009; Carbone et al., 2015; Zhang et al., 2012). AKT activation decreases cells in G0/G1 by phosphorylating the cell cycle inhibitory proteins $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Zhang et al., 2012). Activation of the AKT isoform AKT2 phosphorylates Forkhead box O3 (FOXO3A), inducing FOXO3A inactivation and reducing apoptosis.

In this study, we used CRBN short interfering RNA (siRNA) to show that CRBN was likely involved in the synergy between lenalidomide and dexamethasone. We detected CRBN expression in most of the MCL patients we examined, which together with low toxicity of the drugs probably underlied the effectiveness of the LD regimen as maintenance therapy. We explored how lenalidomide and dexamethasone might affect the IL-6/STAT3, PI3K/AKT and AKT2/FOXO3A pathways. We found that inhibition of IL-6/STAT3, PI3K/AKT and AKT2/FOXO3A/BIM activities, which are crucial for lenalidomide's inhibition of cell growth and promotion of apoptosis were also involved in dexamethasone-induced cell cycle arrest. We also found that CRBN expression correlated positively with LD regimen sensitivity, whereas long-term lenalidomide and dexamethasone exposure downregulated CRBN and induced multi-drug resistance. Removing lenalidomide re-upregulated CRBN and restored the LD regimen sensitivity, which provides a rationale for the intermittent use of the LD regimen to avoid drug resistance in MCL treatment.

2. Materials and Methods

2.1. Cell Lines and Antibodies

The JeKo-1 cell line was obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The Z138 and REC-1 cell lines were obtained from the Biology Corporation of Meiyan. JeKo-1 cells were cultured in RPMI 1640 medium (Gibco) containing 20% fetal bovine serum (FBS; HyClone), 1% antibiotics/antimycotics in a humidified 5% CO_2 incubator at 37 °C. Z138 and REC-1 cells were similarly cultured except 10% FBS was added in medium. The CRBN antibody was purchased from Sigma-Aldrich. Other antibodies used for western blot analysis were purchased from Cell Signaling Technology. The apoptosis and cell cycle detection kits were purchased from Sigma-Aldrich. The antibodies for flow cytometry, including those against CD126 and CD130, were purchased from eBioscience. The IL-6 enzyme-linked immunoassay (ELISA) kit was purchased from R&D Systems.

2.2. Lenalidomide and Dexamethasone Treatment

Dexamethasone (Sigma-Aldrich) was dissolved as previously described (Zhang et al., 2012). Lenalidomide (Selleckchem) was dissolved in dimethyl sulfoxide. JeKo-1, Z138, and REC-1 cells were treated with either control reagents or with lenalidomide for 72 h and/or dexamethasone for 24 h. Following incubation, the cells were harvested as previously described to assess apoptosis, cell cycle status, and for western blot analysis (Wang et al., 2009). For the treatment of CRBN knockdown cells, lenalidomide was added to the cell suspension 16 h after siRNA transfection, and dexamethasone was added 20 h before cell cycle and apoptosis detection. The cell cycle and apoptosis were detected 72 h after siRNA transfection.

2.3. Drug Combination Analysis

The effect of drug combination was analyzed using the CI method, as defined by the following equation: CI = (OD490)AB / [(OD490)A + (OD490)B], where (OD490)AB was the absorbance of the LD regimen treatment group, and (OD490)A and (OD490)B were the absorbance of the groups treated with lenalidomide and dexamethasone alone, respectively. CI > 1 indicated antagonism, CI = 1 indicated additivity, CI < 1 indicated synergy, and CI < 0.7 indicated significant synergy. Each CI ratio was the mean value calculated from at least three independent experiments.

2.4. IL-6 and IL-6R Detection

JeKo-1 cells were treated with 200 ng/mL lenalidomide for 1 week before the IL-6 concentration was measured using ELISA. IL-6R (CD130/CD126) was detected by flow cytometry after one-week treatment of the cells with 200 ng/mL lenalidomide and 2-day treatment with 20 μM dexamethasone.

2.5. SiRNA Constructs and Transfection

Three CRBN siRNAs were synthesized by GenePharma.; the siRNA information is available in the Supplemental materials. The CRBN siRNAs were transfected into JeKo-1 cells using the transfection reagent INTERFERin (Dakewe Biotech) according to the manufacturer's protocol. Transfection efficiency was determined by flow cytometry (BD Biosciences) 8 h post-transfection using 5-carboxyfluorescein (FAM)-labeled siRNA (GenePharma). 12 h and 24 h post-transfection, proteins were analyzed by western blotting.

2.6. Development of Lenalidomide-resistant Cell Lines

Lenalidomide-resistant JeKo-1 cells were selected by 2-week treatment with 0.5 μM lenalidomide. Then, lenalidomide treatment was discontinued for 1 week in one group of cells. For viability assays, cultures were initiated with 2×10^5 cells/mL and treated with 200 ng/mL lenalidomide for 3 days. Apoptosis and cell cycle status were then detected using apoptosis and cell cycle kits, respectively (Sigma-Aldrich). The cells were harvested at 3, 7, 10, and 14 days to detect CRBN expression by western blotting. Following the 1-week discontinuation of lenalidomide treatment, the cells were again incubated with lenalidomide for 3 days and subjected to apoptosis, cell cycle status, and CRBN expression analyses.

2.7. Treatment of Cells With Chemotherapeutic Agents

JeKo-1 cells were separated into lenalidomide-susceptible, lenalidomide-resistant, and lenalidomide-resensitized groups. The cells were treated for 24 h with ara-C (20 μM), CTX (50 μM), DDP (20 μM), MTX (10 μM), or THP (1 μM), and apoptosis was measured following the treatment.

2.8. Retrospective Study of Patient Outcomes and Immunohistochemistry

Our study involved the examination of the data of patients with MCL collected at Huadong Hospital affiliated with Fudan University, Shanghai, China, from 2009 to 2016, which received the approval of the Huadong Hospital ethical committee. We obtained forms of written informed consent from 23 participants prior to their inclusion in the study. CRBN expression in the lymph nodes was detected by immunohistochemistry and bone marrow pathology. In the LD maintenance therapy group, patients received 10 mg lenalidomide orally on days 1–21 of a 28-day cycle. Dexamethasone (20 mg) was given orally on day 1, 8, 15, and 22 every 28 days after complete or partial remission following chemotherapy. The first-line therapy was induction chemotherapy consisting of ara-C and autologous hematopoietic stem cell transplantation. The LD regimen was administered in relapsed or refractory patients until they achieved complete response or partial response. The patient follow-up methods are available in the Supplemental materials.

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