Quinone methide tripterine, celastrol, induces apoptosis in human myeloma cells via NF-κB pathway

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Abstract. Multiple myeloma is still an incurable hematological malignancy despite the development of high-dose chemotherapy with stem cell transplantation. However, the therapeutic approach for multiple myeloma has progressed significantly in the last decade. Novel agents such as bortezomib, thalidomide and lenalidomide have been introduced in clinics as expanded treatment options and have improved the outcomes of patients with multiple myeloma. More recently, the development of novel agents with better effects and lower side-effects for the treatment of multiple myeloma has became necessary in the clinical setting. Celastrol is a quinone methide triterpene derived from the medicinal plant *Tripterygium wilfordii*, which has been used to treat chronic inflammatory and autoimmune diseases. It also has been reported that celastrol has potential as an anticancer agent; however, the effects of celastrol against myeloma have never been reported. It has been reported that the mechanisms of action occur via the NF-κB pathway. However, the effects of celastrol against multiple myeloma have never been reported. The recent clinical success of proteasome inhibitor bortezomib, which acts by inhibiting the NF-κB activity in patients with multiple myeloma led us to investigate the effects of celastrol on myeloma cells. Here we found for the first time that celastrol induces cell cycle arrest at the G1 phase followed by apoptosis in human myeloma cell line U266 cells. In addition, we showed that celastrol induces apoptosis of myeloma cells via activation of the caspase-3 and NF-κB pathways. These results suggest that celastrol would be an effective therapeutic agent in signal transduction therapy for the treatment of patients with multiple myeloma.

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Introduction

Multiple myeloma is characterized by the proliferation of malignant plasma cells and is associated with an increased level of monoclonal protein in the blood and urine. Multiple myeloma is still an incurable hematological malignancy despite the use of high-dose chemotherapy with hematopoietic stem cell transplantation (1). Although recent studies have shown that novel agents such as bortezomib, lenalidomide and thalidomide can offer important treatment options for patients with multiple myeloma and have dramatically improved outcomes for multiple myeloma patients (1-4), the development of *de novo* drug resistance and unexpected side-effects such as peripheral neuropathy and pulmonary complication due to the use of those agents has been reported (5,6). In addition, there is still no cure, and multiple myeloma remains a fatal disease with a median survival of 4 years (7). It has been reported that the detail of molecular mechanisms that lead to multiple myeloma and its progression (8). These findings have lead to clarification of the molecular targets of this disease and may contribute to the development of new biological targeted therapies for multiple myeloma.

Celastrol (Fig. 1), also known as a quinone methide tripterine, is an active compound extracted from the root bark of the traditional Chinese medicine 'Thunder of God vine' (*Tripterygium wilfordii Hook F*), which has been effectively used in the treatment of autoimmune diseases, chronic inflammation and neurodegenerative diseases such as arthritis, lupus erythematosus, lateral sclerosis and Alzheimer's disease (9-11). In addition, celastrol was reported to inhibit cancer cell proliferation and induce cell death *in vitro* and *in vivo* (12,13). Although celastrol has been shown to be promising in tumor prevention, the direct targets and molecular mechanisms of celastrol-induced apoptosis in cancer cells remain unknown. In this study, we investigated the effect of celastrol against myeloma cells and its molecular mechanism of action for the first time.

Material and methods

Cells and cultures. Human multiple myeloma cell line U266 was obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in RPMI-1640 culture

medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere with 5% CO_2. Morphology was examined on cytospin slides stained with Giemsa.

Reagents. Celastrol was purchased from Sigma and dissolved in DMSO at a stock concentration of 10 mM.

Assays for cellular viability and proliferation. Cellular viability was studied by counting the viable cells using trypan blue exclusions, and cellular proliferation was measured using CellTiter 96® AQueous One Solution cell proliferation assay (Promega, Madison, WI). For the assay, cells were plated on 96-well tissue culture plates at $5x10^4$/ml in a total volume of 500 μl with a range of concentrations of celastrol from 0.1 to 1 μM, and assayed according to the manufacturer's instructions. The absorbance at 490 nm was expressed as a relative value of the control culture.

Assays for apoptosis. Apoptosis was determined by morphological changes as well as by staining with Annexin V-FITC and propidium iodide (PI)-double labeling. The cells were treated with celastrol (0.25 and 0.5 μM) for the indicated times (0-48 h). Apoptotic cells were quantified by Annexin V-FITC and PI-double staining using a staining kit purchased from Bio Vision (Mountain View, CA). The cells were analyzed on a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA).

Cell cycle analysis. The celastrol-treated cells were washed twice with PBS and suspended in hypotonic solution [0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, 0.1 mM EDTA, and PI 50 μg/ml] and stained with 50 μg/ml of PI. The DNA content was analyzed by FACSCalibur. The population of cells in each cycle phase was determined using Cell ModiFIT software (Becton-Dickinson).

Assay for caspase-3 activity and mitochondrial transmembrane potential (MMP). Caspase-3 activity was determined by using a commercially available kit (caspase-3 assay kit; PharMingen, San Diego, CA) according to the manufacturer's instructions. Briefly, the cells ($1x10^5$) were washed twice with chilled PBS and fixed using Cytofix/Ctytoperm™ for 20 min on ice; the cells were then pelletted and washed in Perm/Wash™ buffer (PharMingen). The cells were then stained with FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (PharMingen) for 30 min at room temperature, and were analyzed by flow cytometry. The mitochondrial transmembrane potential (MMP) was determined by FACSCalibur (Becton-Dickinson). The cells were washed once with PBS and incubated with 40 μM DioC6 (Sigma) at 37˚C for 20 min. DioC6 intensity was determined by flow cytometry.

NF-κB transcription factor assay. The cells were pre-treated with 0.5 μM of celastrol for 3, 6 and 12 h, and collected by centrifugation at 500 x g for 5 min. The pellets were resuspended in a lysis buffer [1% NP40, 1 mM phenylmethylsulfonyl fluoride (MPSF), 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM NaOV] at 4˚C for 15 min. The level of NF-κB was assessed by ELISA using monoclonal antibodies and the procedure recommended by the manufacturer (TransAM NF-κB p65 transcription factor assay kits; Active Motif, Carlsbad, CA).

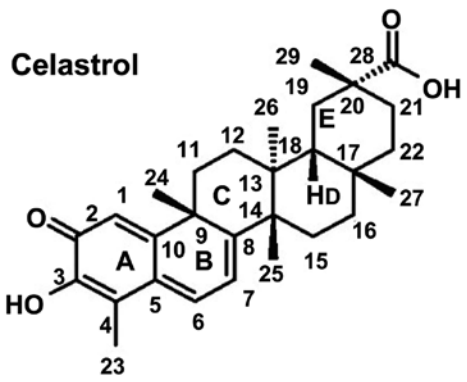

Figure 1. Chemical structure of celastrol.

Cell lysate preparation and Western blotting. Cells were collected by centrifugation at 700 x g for 10 min and then the pellets were resuspended in lysis buffer [1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Tris-HCl (pH 8.0), 150 mM NaCl] at 4˚C for 15 min. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA). Cell lysates (15 μg protein per lane) were fractionated in 12.5% SDS-polyacrylamide gels prior to transfer to the membranes (Immobilon-P membranes; Millipore, Bedford, MA) using a standard protocol. Antibody binding was detected using an enhanced chemiluminescence kit for Western blotting detection with hyper-ECL film (Amersham, Buckinghamshire, UK). Blots were stained with Coomassie brilliant blue to confirm that there were equal amounts of protein extract on each lane. The following antibodies were used in this study: NF-κB p65 and IκB-α purchased from Cell Signaling Technology (Beverly, MA), and Oct-1 (C-21) and β-actin (I-19) from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis. Results are expressed as mean ± standard deviation (SD). Student's t-test was used to compare quantitative data population with normal distribution and equal variance. $P<0.05$ was considered statistically significant otherwise specified.

Results

Induction of apoptosis by celastrol in myeloma cells. We first examined the effects of celastrol on the cellular proliferation of human multiple myeloma cell line U266. Celastrol inhibited the cellular proliferation of myeloma cells in a dose-dependent manner with IC_{50} values of 0.47 μM at 24 h (Fig. 2A). To investigate the mechanisms of celastrol-induced apoptosis of myeloma cells, we next examined the morphological changes of myeloma cells. There was a typical morphological appearance of apoptosis in U266 cells exposed to celastrol, including condensed chromatin and fragmented nuclei with apoptotic bodies (Fig. 2B). At the same time, we measured the apoptosis using Annexin-V and PI-double staining. U266 cells cultured

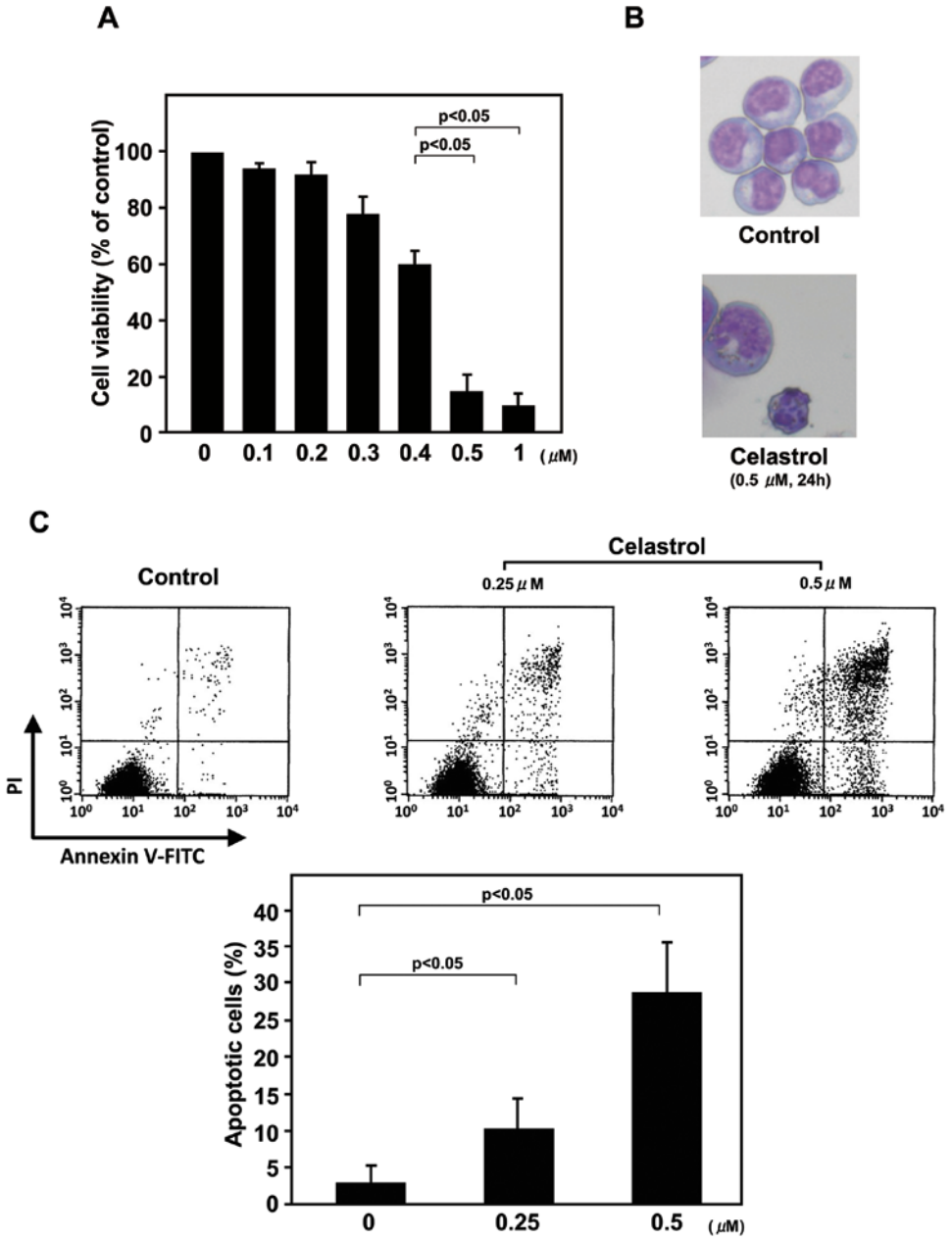

Figure 2. Growth inhibition of U266 myeloma cells by celastrol. (A) U266 cells were treated with various concentrations (0-1 μM) of celastrol for 24 h. Growth inhibition was assessed by MTS growth inhibition assay. Results are expressed as the mean ± SD of three different experiments. The IC_{50} value at 24 h is 0.47 μM. (B) Morphologic changes characteristic of apoptosis in U266 cells. Cells were treated with 0.5 μM celastrol for 24 h, and cytospin slides were then prepared and stained with Giemsa. Original magnification, x1000. (C) Induction of apoptosis was examined by annexin V/PI-double staining using flow cytometry analysis (upper panel). U266 cells were treated with celastrol (0-0.5 μM) for 24 h, and representative of three duplicate experiments is shown. The percentage of apoptotic cells is expressed in the histogram as mean ± SD. P-value showed statistical significance.

with 0.25 and 0.5 μM of celastrol for 24 and 48 h were stained with Annexin-V to detect externalization of phosphatidylserine on the cell membrane (Fig. 2C). Celastrol was found to induce apoptosis of U266 cells in a dose- and time-dependent manner.

Celastrol induced G1 cell-cycle arrest in myeloma cells. To investigate the effect of celastrol on the cell cycle progression of myeloma cells, we analyzed the cell cycle distribution of U266 treated with 0.5 μM of celastrol for 6, 12 and 24 h using flow cytometry. There was an increase in the cells in the G1 phase (Fig. 3), which suggested that celastrol induced G1-phase arrest followed by apoptosis in U266 cells.

Celastrol-induced apoptosis is mediated through the caspase-3 pathway. Caspase-3 is known to be a crucial mediator of apoptosis, catalyzing the specific cleavage of many key cellular proteins. Therefore, we investigated the activity of caspase-3 in U266 cells treated with 0.5 μM of celastrol for 9, 24 and 48 h. The activity of caspase-3 increased in a time-dependent manner (Fig. 4A), suggesting that the caspase-3 pathway plays an essential role in celastrol-induced apoptosis in myeloma cells.

Celastrol-induced apoptosis is mediated through the mitochondrial pathway. We examined the MMP by flow cytometry using DioC6 to address the apoptotic pathway through the mitochondria. After treatment with 0.5 μM celastrol for the

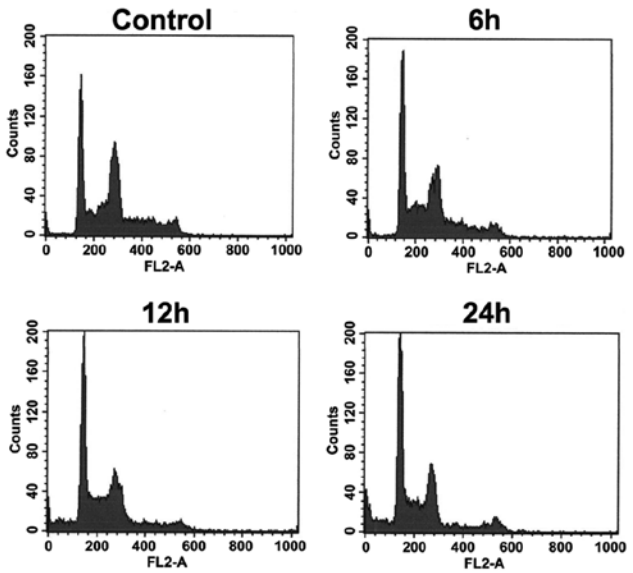

Figure 3. Celastrol-induced G1 cell cycle arrest. U266 cells were treated with 0.5 μM celastrol for the indicated times and then stained with PI as described in Materials and methods. The DNA content was analyzed by means of flow cytometry.

indicated time, DioC6 staining of myeloma cells indicated an increase in the loss of MMP (Fig. 4B). These results suggest that celastrol-induced apoptosis is mediated through a mitochondria-dependent pathway.

NF-κB pathway is crucial in celastrol-induced apoptosis. The NF-κB pathway is a key regulator of cytokine stimulation, the cell cycle, apoptosis and angiogenesis (14,15). It is also critical in the progression and apoptosis of cancer cells, including multiple myeloma. Recently, inhibition of the NF-κB pathway using the proteasome inhibitor bortezomib was found to be pivotal in the treatment of untreated and relapse/refractory myeloma (1,4). We then examined the NF-κB pathway both by the ELISA method and Western blotting. The expression of NF-κB in the nucleus increased after TNF-α stimulation (Fig. 5A); however, as shown by ELISA, celastrol inhibited the migration of NF-κB into the nucleus in U266 cells (Fig. 5A). It was also shown by Western blotting that the expression of NF-κB in the nucleus increased after TNF-α stimulation; however, celastrol inhibited the migration of NF-κB into the nucleus as well as IκB-α cleavage in a time-dependent manner (Fig. 5B). These results indicated that celastrol inhibited the NF-κB pathway via the inhibition of the migration of NF-κB into the nucleus.

Discussion

Multiple myeloma is characterized by the accumulation of secretary plasma cells with a low proliferative index and an extended life span in the bone marrow. Conventional therapy for multiple myeloma involves combinations of vincristine, melphalan, cyclophosphamide, doxorubicin and prednisone or dexamethasone (16). Patients younger than 65 years are usually given high-dose melphalan with autologous stem cell support, and older patients or those who cannot tolerate such intensive treatment are given standard-dose oral melphalan and dexamethasone. However, these treatments are associated with low remission rates, short survival times and the development of drug resistance (17). Recently, novel agents such as bortezomib, thalidomide and lenalidomide, which target myeloma cells and their microenvironments, have shown remarkable activity against refractory

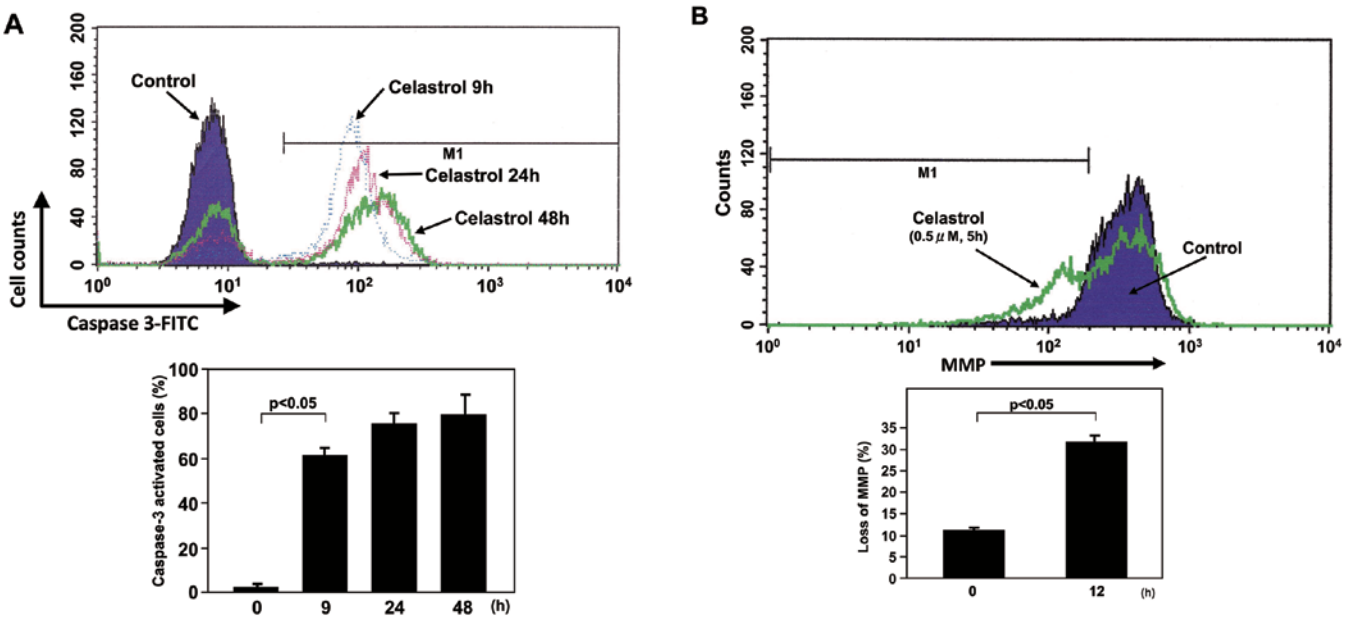

Figure 4. Celastrol-induced caspase-3 activity and loss of mitochondrial membrane potential (MMP). (A) Effect of celastrol on caspase-3 activity. Cell were incubated with 0.5 μM celastrol for various lengths of times (0-48 h) and then analyzed by flow cytometry and colorimetric assay (upper panel). Caspase-activated cells were expressed as a histogram (lower panel). (B) Flow cytometric analysis of MMP as estimated by Rh123 intensity (upper panel) and expressed as a histogram (lower panel). U266 cells were cultured with 0.5 μM celastrol for 12 h, and Rh123 fluorescence was analyzed by flow cytometry. P-values show statistical significance.

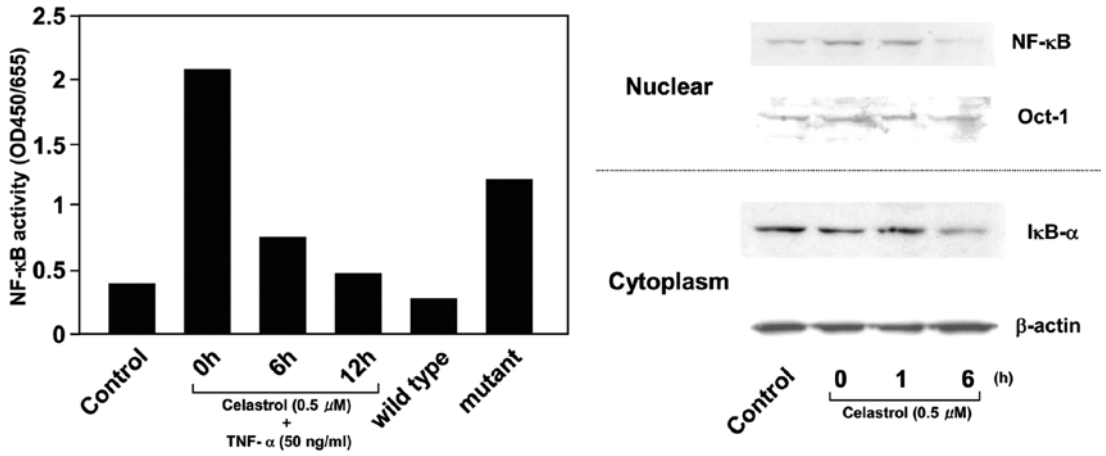

Figure 5. Effects of celastrol on NF-κB activity in myeloma cells. (A) U266 cells were treated with 50 ng/ml TNF-α for 12 h, and then the cells were incubated with 0.5 μM of celastrol for 6 and 12 h. The DNA binding activity of NF-κB in U266 cells was quantified by ELISA with the use of a Trans-AM NF-κB p65 transcription factor assay kit. Celastrol significantly suppressed the stimulatory effect of TNF-α on NF-κB DNA binding activity ($P<0.01$). The control is referred to as no stimulated control, the wild-type as wild-type oligonucleotide, and the mutant as mutant oligonucleotide. (B) Effects of celastrol on the constitutive expression of NF-κB and IκB-α in myeloma cells. U266 cells were treated with celastrol (0.5 μM) for the indicated times. Cell lysates (15 mg protein per lane) were fractionated in 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with an antibody against NF-κB p65 and IκB-α. Nuclear and cytoplasmic extracts were prepared in order to check these proteins by Western blotting.

and chemoresistant cases in early clinical trials, and they have prolonged the progression-free and overall survival of multiple myeloma patients (18,19). Progression and chemoresistance are thought to involve interleukin (IL)-6, the expression of which is induced by NF-κB, via its regulation of the growth and survival of myeloma cells (1). IL-6 leads to the constitutive activation of STAT3, which in turn results in the expression of high levels of anti-apoptotic Bcl-xL and Mcl-1 proteins (20,21). Thus, the constitutive activation of both NF-κB and STAT3 plays an important role in chemoresistance, and it is expected that the inhibition of NF-κB and STAT3 may overcome such chemoresistance. However, many patients have relapsed and have been refractory to each agent. Therefore, novel therapeutic agents that can overcome drug resistance are required.

Many natural products have been developed as anticancer agents in clinical settings (22). For the past 30 years, many natural products have provided lead structures, and these have been used as templates for developing new agents with enhanced biological properties (23). Such new agents are mostly derived from plants and marine products with less toxicity than chemotherapeutic agents. We have previously reported that many natural products induced apoptosis of human leukemia and myeloma cells through the basic molecular mechanisms that take place in these cells (24-30).

Celastrol, also known as a tripterine from Chinese traditional medicine, is an active compound extracted from the root bark of *Tripterygium wilfordii Hook F.* It has been used in the treatment of autoimmune disease, chronic inflammation and neurodegenerative diseases (9-11). Recent studies have shown that celastrol inhibits the proliferation of prostate cancer and leukemia (12,13). However, there have been no reports of celastrol inducing myeloma cell death. In this study, we showed for the first time that celastrol induced apoptosis in myeloma cells through NF-κB inhibition.

We first demonstrated in this study that celastrol inhibited the growth of human myeloma cell line U266 cells via the induction of apoptosis with an IC_{50} of <5 μM. In addition, celastrol induced G1 cell cycle arrest. It is already known that celastrol induces cell cycle arrest at the G1 phase followed by apoptosis in various cancer cells (31,32). The NF-κB pathway is a potential molecular target for cancer therapy (33). Many cancer cells contain aberrantly sustained nuclear NF-κB activity because of aberrant IKK activity, a shorter half-life of IκB-α, mutation of IκB-α, overexpression of IL-1β, and excess production of TNF-α. Several drugs that inhibit NF-κB activation, such as inhibitors of the upstream kinase IKKβ, have already shown anti-myeloma activity in preclinical models (33,34). Moreover, proteasome inhibitor bortezomib, which inhibits NF-κB activation, has been widely been used to treat multiple myeloma patients worldwide (1,4). Therefore, natural products that inhibit NF-κB activation could be novel potential agents for the treatment of multiple myeloma. In this study, we demonstrated that celastrol inhibited the shuttling of NF-κB into the nucleus, which was followed by apoptosis.

Recently, celastrol has been found to be a novel Hsp90 inhibitor (35,36). However, no study has found that the celastrol-induced apoptosis of myeloma cells was induced by Hsp90 inhibition. Further studies are needed to address the effect of celastrol on the inhibition of Hsp90 activity in myeloma cells.

In conclusion, we showed that celastrol induced apoptosis in myeloma cells, and that its main molecular mechanisms is the inhibition of NF-κB activation. This drug could be a novel molecular-targeted therapy for the treatment of multiple myeloma.

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