A natural peptide, dolastatin 15, induces G2/M cell cycle arrest and apoptosis of human multiple myeloma cells

MASANORI SATO, MORIHICO SAGAWA, TOMONORI NAKAZATO, YASUO IKEDA and MASAHIRO KIZAKI

Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Received January 18, 2007; Accepted February 16, 2007

Abstract. Several anti-cancer agents are derivative from natural products and microorganisms. The dolastatins are natural peptides derived from the marine mollusc Dolabella auricularia, which have recently been reported as an anti-cancer agent. Dolastatin 10 and 15 are small peptides; most preclinical studies have used dolastatin 10. It has been reported that dolastatins have cytotoxic activity by inhibiting microtubule assembly, and several clinical studies have already begun for solid tumors. However, the effects of dolastatin 15 against hematological malignancies such as myeloma cells have never been reported. We demonstrate here for the first time that dolastatin 15 induces cell cycle arrest at the G2/M phase followed by apoptosis in various human myeloma cell lines (RPMI8226, U266, and IM9), suggesting that it has effects on mitotic spindles. In addition, we showed that dolastatin 15 induces apoptosis of myeloma cells via activation of both mitochondrial- and Fas (CD95)/Fas-L (CD95-L)-mediated pathways. Our investigations have identified a novel inhibitor of microtubule assembly that induces mitotic arrest and apoptosis of myeloma cells. Therefore, it is possible that dolastatin 15 might be a novel and safe therapeutic agent for patients with multiple myeloma.

Introduction

Multiple myeloma is one of the B-cell malignancies that remain incurable despite the use of high-dose chemotherapy with hematopoietic stem cell transplantation; therefore, novel therapeutic approaches are urgently needed for the treatment of patients with multiple myeloma (1). Recent understanding that has been gained of the biology of multiple myeloma has led to the development of biological treatments including thalidomide and its derivatives, arsenic trioxide, histone deacetylase inhibitors, and bortezomib, which target the myeloma cell and its microenvironment. These agents have shown remarkable activity against refractory multiple myeloma in early clinical trials, but prolonged drug exposure may result in the development of de novo drug resistance (1-3) and unexpected side effects such as serious interstitial pneumonitis induced by bortezomib (4). Therefore, the identification and validation of additional novel targeted therapies to overcome drug resistance and improve patient outcome are urgently needed.

Dolastatins are a unique peptide derived from the marine mollusc Dolabella auricularia; dolastatin 10 and 15 are small peptides. Dolastatins have been reported to have cytotoxic effects with low doses against breast cancer, lung cancer, leukemia, and lymphoma by inhibiting microtubule assembly (5-8). Dolastatin 10 has been selected for clinical studies. It inhibits microtubule assembly, which induces cells to accumulate in the metaphase (9,10). However, dolastatin 10 has been reported to have bone marrow toxicity in early clinical trials, and local irritations at the injection site and peripheral neuropathy have also been reported (11-14). In contrast, dolastatin 15 has not been used for preclinical studies and detailed effects on tumor cells have not been clarified. In addition, the activity of dolastatin 15 has never been evaluated in hematological malignancies such as multiple myeloma. In this study, we investigated for the first time the effect of dolastatin 15 against human myeloma cells.

Materials and methods

Cells and reagents. Human multiple myeloma cell lines RPMI8226, U266, and IM9 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Cells were cultured in RPMI-1640 culture medium (Sigma Chemical Co., 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% CO2. The morphology was evaluated by cytospin slide preparations with Giemsa staining. Dolastatin 15 was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO maintained at -30˚C at a stock concentration of 100 μM.

MTS assay. Growth inhibition was detected in myeloma cells by incubating 1x10^5 cells/well with dolastatin 15 in 96-well
plates for 24 and 48 h. The cells were treated with a range of concentrations of dolastatin 15 from 0.1 to 5 nM. Seeding the cells in 96-well plates, growth inhibition was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's procedures. All experiments were performed at least three times, and data were confirmed to be reproducible.

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 dolastatin 15 (5 nM) for the indicated times (0-24 h). Apoptotic cells were quantified by Annexin V-FITC and PI double staining by using a staining kit purchased from BioVision (Mountain View, CA). The cells were analyzed on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA). The mitochondrial transmembrane potential (ΔΨm) was determined by FACS Calibur (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.

Cell cycle analysis. The dolastatin 15-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 FACS Calibur. The population of cells in each cycle phase was determined using Cell ModFit software (Becton Dickinson).

Western blotting. The cells were collected by centrifugation at 500 x g for 5 min, and the pellets were resuspended in a lysis buffer [1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM NaOV] at 4°C for 15 min. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA). Cell lysates (20 μg protein per lane) were fractionated on 12.5% SDS-polyacrylamide gels before being transferred to the membrane (Immobilon-P membranes, Millipore, Bedford, MA) according to standard protocol. Antibody binding was detected by using the enhanced chemiluminescence kit with hyper-ECL film (Amersham, Buckinghamshire, UK). The blots were stripped and reprobed with β-actin antibody as an indicator of the equality of lane loading. Primary antibodies for the following proteins were used (1:1000 dilution): phospho-p53 (Ser15), phospho-p53 (Ser20), cdc2, phospho-cdc2 (Tyr15), phospho-cdc25C (Thr48), chk2, phospho-chk2 (Thr68), caspase-3, -8, -9 (Cell Signaling Technology Inc. Beverly, MA); bcl-2, bcl-Xl, p53, cyclin B1, and β-actin (Santa Cruz Biotech., Santa Cruz, CA); Bax, and Bid (MBL, Nagoya, Japan). Secondary antibodies (1:3000 dilution) conjugated with horseradish peroxidase were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Results

Effect of dolastatin 15 on cell growth in myeloma cells. To investigate the ability of dolastatin 15 to control the growth of myeloma cells, we first examined the effect of dolastatin 15 on the cell growth of human multiple myeloma cell lines (RPMI8226, U266, and IM9) by MTS assay. Myeloma cells were cultured with various concentrations of dolastatin 15 (0-5 nM) for the indicated times (24 or 48 h). Dolastatin 15 inhibited the cellular growth of all myeloma cells in dose- and time-dependent manners in all myeloma cell lines (Fig. 1). Therefore, dolastatin 15 was a potent inhibitor of cell proliferation, with IC50 values in the nanomolar range. IC50 values of dolastatin 15 against myeloma cells were 0.5-1 nM (Fig. 1).

Induction of apoptosis by dolastatin in myeloma cells. To investigate the mechanism of the dolastatin-induced growth suppression of myeloma cells, we next examined the morphological changes of myeloma cells. Exposure of RPMI8226 cells to dolastatin 15 (5 nM) for 24 h resulted in a typical
morphological appearance of apoptosis, including condensed chromatin and fragmented nuclei with apoptotic bodies (Fig. 2). In parallel, apoptosis was measured by means of Annexin V and PI double staining. RPMI8226 cells cultured with 5 nM dolastatin for the indicated times (0, 6, 12, and 24 h) were stained with Annexin V to detect externalization of phosphatidylserine on the cell membrane. As shown in Fig. 3, dolastatin 15 induced apoptosis upon RPMI8226 cells in a time-dependent manner.

Dolastatin 15 induced G2/M cell cycle arrest in RPMI8226 cells. The effects of dolastatin 15 on cell cycle progression were investigated using RPMI8226 cells. RPMI8226 cells were treated with dolastatin 15 (0-5 nM) for 24 h and then the cells were analyzed in terms of cell cycle distribution by means of flow cytometry. The cell cycle analysis demonstrated that dolastatin 15 induced a depletion of cells in the G1 phase and a concomitant accumulation of cells in the G2/M phase (Fig. 4). After treatment, an increase was observed in the fraction of hypodiploid cells, which indicated that these cells had undergone apoptosis (Fig. 4). These results suggested that dolastatin 15 induced G2/M cell cycle arrest followed by apoptosis in RPMI8226 cells.

Dolastatin 15-induced G2/M arrest is associated with the phosphorylation of cdc2. To gain a better understanding of the mechanism of G2/M arrest in dolastatin 15-treated myeloma cells, we examined the levels of proteins that regulate the G2/M transition (Fig. 5). During the G2 phase, cyclin B1/cdc2 is inactivated by phosphorylation at the Thr14 and Tyr15 residues, which are dephosphorylated by cdc25C phosphatase before entering mitosis (15). Treatment of RPMI8226 cells with dolastatin 15 resulted in a time-dependent increase in the phosphorylation of cdc25C (Thr48) and cdc2 (Thr14
and Thr15) (Fig. 5). Dolastatin was found to induce the phosphorylation of the Thr68 residue of chk2 kinase, which was observed as early as 3-6 h after treatment (Fig. 5). In addition, dolastatin 15 induced phosphorylation of p53 at the Ser15 and Ser20 residues corresponding to the accumulation of p53 in RPMI8226 cells (Fig. 5).

Dolastatin 15-induced apoptosis is mediated through mitochondrial pathway. Recent studies have suggested that mitochondria play an important role in death signal transduction (16). Mitochondrial changes, including permeability transition pore opening and the collapse of the ΔΨm, resulted in the release of cytochrome c into the cytosol, which subsequently caused the activation of caspases (17). We next examined ΔΨm collapse by flow cytometry using DiOC6. After treatment with 5 nM of dolastatin 15, the weak DiOC6 staining of RPMI8226 cells indicated an increase in the loss of ΔΨm in a time-dependent manner (Fig. 6). The loss of ΔΨm appeared to occur in parallel with the activation of caspase-3, -9, -8, and cells undergoing apoptosis (Fig. 7), suggesting that a mitochondria-dependent pathway plays an important role in dolastatin 15-induced apoptosis in myeloma cells.
Expression of apoptosis-related proteins in dolastatin 15-treated myeloma cells. We further examined the expression of apoptosis-associated proteins during treatment with dolastatin 15. The 32-kDa procaspase-3 was cleaved into its active form (17 kDa) after 24 h of exposure to dolastatin 15 (Fig. 7). In addition, we found that dolastatin 15 induces the activation of caspase-9 (Fig. 7). The activation of caspase-8 was also induced by dolastatin 15, which took place at 6-12 h of treatment (Fig. 7). Dolastatin 15 cleaved Bid protein after 12 h of treatment; this occurred in parallel with activation of the Bax protein (Fig. 7). These results suggest that dolastatin 15-induced apoptosis in myeloma cells is also mediated through Fas (CD95)/Fas-L (CD95-L) pathway. In contrast, anti-apoptotic Bcl-2 and Bcl-XL proteins were not modulated in response to dolastatin 15 (Fig. 7 and data not shown).

Discussion

Various anti-cancer agents were originally developed from natural sources (18). Epidemiological investigation and laboratory studies have indicated that bioactive natural compounds play an important role in the treatment of many cancers. We have reported that various bioactive agents from natural compounds induce apoptosis of human leukemia and myeloma cells on the basic molecular mechanism that takes place in these cells (19-26). The first marine-derived anticancer agent to be developed for clinical use was cytarabine (18). Cytarabine is currently used for patients with leukemia and lymphoma. In addition, gemcitabine is also derived from a marine organism and has shown significant activity for patients with various solid tumors (27,28). Therefore, the search for new anti-cancer agents derived from marine sources has been performed by many researchers. More than 3000 new compounds from marine sources have been described and entered into clinical trials (29). The dolastatins are cytotoxic peptides derived from the ocean mollusk Dolabella auricularia, found in the Indian Ocean (18). The dolastatins have been reported to induce inhibition of cellular growth in various tumor types in vitro and in vivo (18). Dolastatins 10 and 15 are linear peptides and potent cytotoxins with IC_{50} values towards various tumor cells on the subnanomolar range (30). The mechanism of growth inhibition by dolastatins is induced apoptosis of tumor cells through inhibition of microtubular assembly, binding to the vinca domain on tubulin (8). Until now, dolastatin 10 has been used for preclinical models leading to its evaluation in Phase I/II clinical trials (11-14,31). However, the results of the Phase II studies in several cancers have shown that dolastatin 10 is well tolerated but did not show clinical anti-tumor activity as a single agent (8). In contrast, there have been no preclinical and clinical studies of dolastatin 15 against human cancers, including hematological...
cells was associated with the increased expression of Bax assembly. Following by apoptosis, indicating the inhibition of microtubule formation of abnormal mitotic spindles, leading to mitotic arrest and initiation of apoptosis (8). Consistent with previous reports, dolastatin 15 induces chk2 kinase. It has been demonstrated to be required for the G2/M phase cell cycle arrest following DNA-methylating agents and chemopreventive agents (34, 35). Activated chk2 inhibits cdc25C phosphatase by phosphorylating Thr48 to promote its association with 14-3-3 proteins. Cdc25C plays a critical role in the dephosphorylation of cdc2 on Thr14/Tyr15; the blockage of its activity required for G2/M transition. Therefore, chk2 may be an important molecule for dolastatin 15-induced G2/M cell cycle arrest.

It is well known that anti-microtubule agents cause cell cycle arrest at the G2/M phase (36-38). Dolastatins have been reported to cause microtubule depolymerization and formation of abnormal mitotic spindles, leading to mitotic arrest and initiation of apoptosis (8). Consistent with previous reports, dolastatin 15 induces G2/M cell cycle arrest followed by apoptosis, indicating the inhibition of microtubule assembly.

The induction of apoptosis by dolastatin 15 in myeloma cells was associated with the increased expression of Bax protein, the loss of ΔΨm, and the activation of caspase-3 and -9. These results suggest that dolastatin 15-induced apoptosis is associated with a mitochondrial pathway, a process that is more likely mediated by the cytochrome c/Apaf1/caspase-9 pathway. The alternative pathway of apoptosis is mediated through death receptors such as Fas (CD95) and the TNF-receptor by their ligands, resulting in the activation of caspase-8 (39). The activation of p53 transcriptional activity is well known to be required for the increased expression of Fas (CD95)/Fas-L (CD95-L). We demonstrated that dolastatin 15 induces the phosphorylation of p53 at the Ser15 residue in RPMI8226 cells. In addition, caspase-8 was also cleaved into its active form by treatment with dolastatin 15. Moreover, dolastatin 15 cleaved Bid protein. Bid links the extrinsic death pathway initiated by Fas (CD95) to the intrinsic mitochondrial-mediated pathway in the course of death signaling (40). Taken together with our results and previous reports, we concluded that dolastatin 15 induces apoptosis of myeloma cells via a mitochondrial- and Fas (CD95)-mediated pathway.

In conclusion, dolastatin 15 has a superior cytotoxic activity against myeloma cells via the induction of G2/M cell cycle arrest followed by apoptosis. Of note is the fact that dolastatin 15-induced apoptosis in myeloma cells is mediated by dual pathways through the mitochondrial and Fas (CD95)/Fas-L (CD95-L) system. This is the first report of dolastatin 15-induced apoptosis in multiple myeloma cells; the results of the present study may contribute to the development of new strategies for the treatment of cancer, including the treatment of multiple myeloma.

Acknowledgements

We would like to thank Chika Saito for excellent technical assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Ministry of Health, Labour, and Welfare of Japan, and Chugai Co. Ltd. Fund.

References


