

Emodin has a cytotoxic activity against human multiple myeloma as a Janus-activated kinase 2 inhibitor

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Abstract

Emodin is an active component of a traditional Chinese and Japanese medicine isolated from the root and rhizomes of *Rheum palmatum* L. Here, we show that emodin significantly induces cytotoxicity in the human myeloma cells through the elimination of myeloid cell leukemia 1 (Mcl-1). Emodin inhibited interleukin-6–induced activation of Janus-activated kinase 2 (JAK2) and phosphorylation of signal transducer and activator of transcription 3 (STAT3), followed by the decreased expression of Mcl-1. Activation of caspase-3 and caspase-9 was triggered by emodin, but the expression of other antiapoptotic Bcl-2 family members, except Mcl-1, did not change in the presence of emodin. To clarify the importance of Mcl-1 in emodin-induced apoptosis, the Mcl-1 expression vector was introduced into the human myeloma cells by electroporation. Induction of apoptosis by emodin was almost abrogated in Mcl-1–overexpressing myeloma cells as the same level as in parental cells, which were not treated with emodin. In conclusion, emodin inhibits interleukin-6–induced JAK2/STAT3 pathway selectively and induces apoptosis in myeloma cells via down-regulation of Mcl-1, which is a good target for treating myeloma. Taken together, our results show emodin as a new potent anticancer agent for the treatment of multiple myeloma patients. [Mol Cancer Ther 2007;6(3):987–94]

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Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active component in the root and rhizome of *Rheum palmatum* L. (Polygonaceae). This herb has been used as a traditional Chinese and Japanese medicine for the treatment of skin burn, infection, gallstone, hepatitis, inflammation, and osteomyelitis. Emodin is reported to have antimicrobial, antiviral, antiinflammatory, antiulcerogenic, immunosuppressive, and chemopreventive activities. Emodin has also been reported to exert antiproliferative effects in many cancer cell lines; HER-2/neu–overexpressing breast (1) or lung cancer (2), leukemic HL-60 (3), human hepatocellular carcinoma (4), human cervical cancer, and prostate cancer cell lines through the activation of caspase-3 (3, 4) and up-regulation of TP-53 and p21 (4). Moreover, emodin inhibits the kinase activity of p56lck, HER2/neu (1), and casein kinase II (5). However, the actual molecular mechanisms of emodin-mediated tumor regression have not been yet fully defined.

Multiple myeloma (MM) is a plasma cell malignancy characterized by the monoclonal proliferation of malignant plasma cells usually in the bone marrow. MM is still incurable with conventional chemotherapy (6), except for only few patients that received the benefit of the hematopoietic stem cell transplantation (7, 8). Recently, many advances in the knowledge of myeloma cell biology could develop new treatment strategies based upon molecular targeting, such as thalidomide and proteasome inhibitors (9, 10). These agents could be expected to have responses even against refractory relapsed myeloma in early clinical trials; however, the responses are only temporary, and the development of *de novo* drug resistance with prolonged exposure have become a new problem (8, 11, 12). Therefore, novel molecular targeting therapeutic approaches are urgently needed to overcome drug resistance and to improve clinical outcome.

Cytokines from bone marrow microenvironment support MM cell proliferation. Among the cytokines, interleukin-6 (IL-6) plays an important role for the growth (13). IL-6 triggers the proliferation via the Ras/Raf/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/MAP kinase (MAPK) pathway (14), and the phosphoinositide-3-kinase (PI3K)/AKT pathway (15) protects against dexamethasone-induced apoptosis by protein-tyrosine phosphatase SHP2 (16) and promotes survival through the Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway (17). IL-6–induced phosphorylation of STAT3 results in up-regulation of antiapoptotic proteins myeloid cell leukemia 1 (Mcl-1; refs. 18, 19), Bcl-xL (19), and c-myc (20). Mcl-1, a member of the antiapoptotic Bcl-2 family, was identified by a gene expressed during phorbol ester–induced myeloid cell differentiation (21). In myeloma cells,

Mcl-1 is tightly regulated by IL-6 and is one of the key proteins for survival (22–25).

In this study, we examined whether emodin could inhibit an IL-6 signal pathway in MM cells and evaluated the therapeutic potential of emodin against MM *in vitro*. We showed here that emodin induced apoptosis in MM cells at clinically achievable concentrations. Apoptosis is associated with suppression of kinase activity of JAK2, which resulted in the inhibition of phosphorylation of STAT3 and decrease in the expression of Mcl-1. These results could provide a novel molecular targeting strategy in the treatment of MM.

Materials and Methods

Cells and Reagents

Human MM cell lines, RPMI8226, U266, and IM-9 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan), and KMS-12-PE (JCRB0430) was from the Health Science Research Resources Bank (Osaka, Japan). After informed consent, fresh peripheral blood mononuclear cells (PBMC) from healthy subjects were collected by Ficoll-Hypaque density sedimentation. Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. The morphology and cytochemistry of the cells were evaluated from cytospin slide preparations with Giemsa staining. Emodin and doxorubicin were purchased from Sigma Chemical (St. Louis, MO), and IL-6 was from R&D Systems (Minneapolis, MN). Emodin was dissolved in DMSO and maintained at -30°C as a 50 mmol/L stock protected from the light. Doxorubicin was dissolved in sterile water as a 5 mmol/L stock and stored at 4°C .

Cell Viability and Apoptosis Assay

Cell viability was assessed by XTT [sodium 3'-(1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro)-benzene sulfonic acid hydrate] cell proliferation assay kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Cell viability was also evaluated by counting trypan blue dye excluded cell number. All experiments were done at least thrice, and the data were confirmed to be reproducible.

Apoptotic cells were determined by morphologic changes, by an annexin V staining and by a DNA fragmentation analysis. Staining with annexin V-FITC (Bio Vision Inc., Mountain View, CA) and propidium iodide (PI) labeling was done according to manufacturer's instructions. These stained cells were analyzed immediately using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA). The progress of apoptosis was detected by a DNA fragmentation assay in total genomic DNA as described elsewhere (26). Apoptotic cells were also determined by colorimetric ELISA method with cell death detection ELISA (Roche Diagnostics).

Western Blotting

Western blotting was done as described previously (27). Antibodies used were anti-caspase-9, caspase-3, STAT3,

and phospho-STAT3 (Cell Signaling, Beverly, MA); caspase-8, BAX (Medical & Biological Laboratories, Nagoya, Japan); Bcl-2 (Roche Diagnostics); Mcl-1, Bim, Bcl-xL/S, gp130, phospho-gp130, JAK2 (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-JAK2 (Upstate, Waltham, MA) as a primary antibody, and anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ). Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) kit for Western blotting detection with hyper-ECL film (GE Healthcare Bio-Sciences). Equal loading was confirmed by probing the blots with the anti- β -actin antibody (Santa Cruz Biotechnology).

Reverse Transcription-PCR

Expression of Mcl-1 mRNA was evaluated with reverse transcription-PCR (RT-PCR) method. Total RNA was extracted from emodin-treated RPMI8226 cells with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by means of Transcriptor Reverse Transcriptase (Roche Diagnostics) with Taq DNA Polymerase (Qiagen Inc., Valencia, CA). Primers used were Mcl-1S, 5'-CAC GAG ACG GTC TTC CAA GGC ATG CT-3', and Mcl-1AS, 5'-CTA GGT TGC TAG GGT GCA ACT CTA GGA-3'. The expected size of the amplification product was 496 bp. Expression of β -actin was used as a control to measure the integrity of the RNA samples.

Plasmid and Transfection

The full-length human Mcl-1 cDNA expression plasmid (pSR α -neo-EAT/Mcl-1; ref. 28), a generous gift from Dr. Akihiro Umezawa and Dr. Hajime Okita (National Research Institute for Child Health and Development, Tokyo, Japan), was used as the template in a PCR to amplify the human Mcl-1 cDNA fragment. The cDNA fragment was cloned into the *Hind*III and *Eco*RI sites of the pEGFP-C1 expression vector (Clontech, Palo Alto, CA). The resultant plasmid was designated pEGFP-Mcl-1. The pEGFP-neo was used as a control plasmid. Exponentially growing cells were suspended in RPMI 1640 at a density of 1×10^7 /mL. Cells were transfected with plasmids by electroporation using a Gene Pulser II (Bio-Rad) set at 280 V, 960 μF . After incubation with 10% FBS-RPMI 1640 for 24 h, enhanced green fluorescent protein (EGFP)-positive cells were collected by a fluorescence-activated cell sorter (EPICS ALTRA HyPerSort, Beckman Coulter, Miami, FL). After the collected cells were incubated with or without emodin, apoptotic cells were evaluated with the methods described above.

In vitro Kinase Assay

RPMI8226 cells were cultured with 0.4 ng/mL of IL-6 for 10 min and lysed in a lysis buffer. Whole cell extracts were immunoprecipitated with anti-JAK2 antibody (Santa Cruz Biotechnology) and protein G sepharose 4B beads (Invitrogen) at 4°C overnight. The immune complex in the presence or absence of emodin was assayed for the kinase activity using kinase assay buffer containing 20 mmol/L Tris-HCl (pH 7.6), 10 mmol/L MgCl_2 , 3 mmol/L MnCl_2 , 2 $\mu\text{mol/L}$ unlabeled ATP, 5 mCi (185 kBq) [γ - ^{32}P]ATP, and 5 μg Histone H2 (Sigma) per sample. After incubation at 30°C for 30 min, the reaction was stopped by boiling

the solution in Laemmli sample buffer, and the reaction mixtures were resolved on 12% SDS-PAGE. The radioactive bands of the dried gel were visualized by Typhoon 9410 PhosphorImager (GE Healthcare Bio-Sciences).

Measurement of DNA Binding Activity of STAT3

The effect of emodin on DNA binding capacity of STAT3 was analyzed using electrophoretic mobility gel shift assays (EMSA). After 18 h of treatment, cells were washed twice with ice-cold PBS and were incubated on ice with 0.6 mL of NP40 lysis buffer [10 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, and 0.1% NP40]. After 10 min, nuclei were separated by centrifugation at 5,000 rpm for 10 min and resuspended in the nuclear extraction buffer [50 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 20% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L DTT]. Nuclear lysates were incubated with ³²P-labeled double-stranded probe (forward, 5'-TTT CCC CTT TTA CGG GAA GTC CT-3'; reverse, 5'-AGG ACT TCC CGT AAA AGG GGA AA-3') at room temperature for 30 min. Samples were run on 5% Tris-borate EDTA gels and visualized by Typhoon 9410 PhosphorImager.

Results

Emodin Inhibits the Cellular Growth and Promotes Apoptosis in Myeloma Cell Lines

We first examined the effect of emodin on the growth of RPMI8226 MM cells at the concentration of 1, 10, 20, 50, and 100 μ mol/L by 24 h using the XTT assay (Fig. 1A). The cellular growth was inhibited dose-dependently and IC₅₀ was observed at 24 h of 37.7 μ mol/L. Incubation with 50 μ mol/L of emodin for up to 120 h resulted in the growth suppression in a time-dependent manner (Fig. 1B). We also determined the proliferation of RPMI8226 cells by trypan blue exclusion method, whereas RPMI8226 cells grew faster in the presence of IL-6, emodin suppressed the proliferation in a dose-dependent manner, and IL-6 could not overcome the suppression (Fig. 1C). The effect of emodin on the growth suppression of other MM cell lines, U266 and IM-9 cells, as well as RPMI8226 cells, was confirmed by counting trypan blue unstained viable cells (Fig. 1D). However, emodin did not change the growth of normal human PBMCs (Fig. 1E), normal mice bone marrow mononuclear cells or normal mice splenic plasma cells (data not shown). To elucidate the mechanism of emodin-induced growth suppression of myeloma cells, we next examined

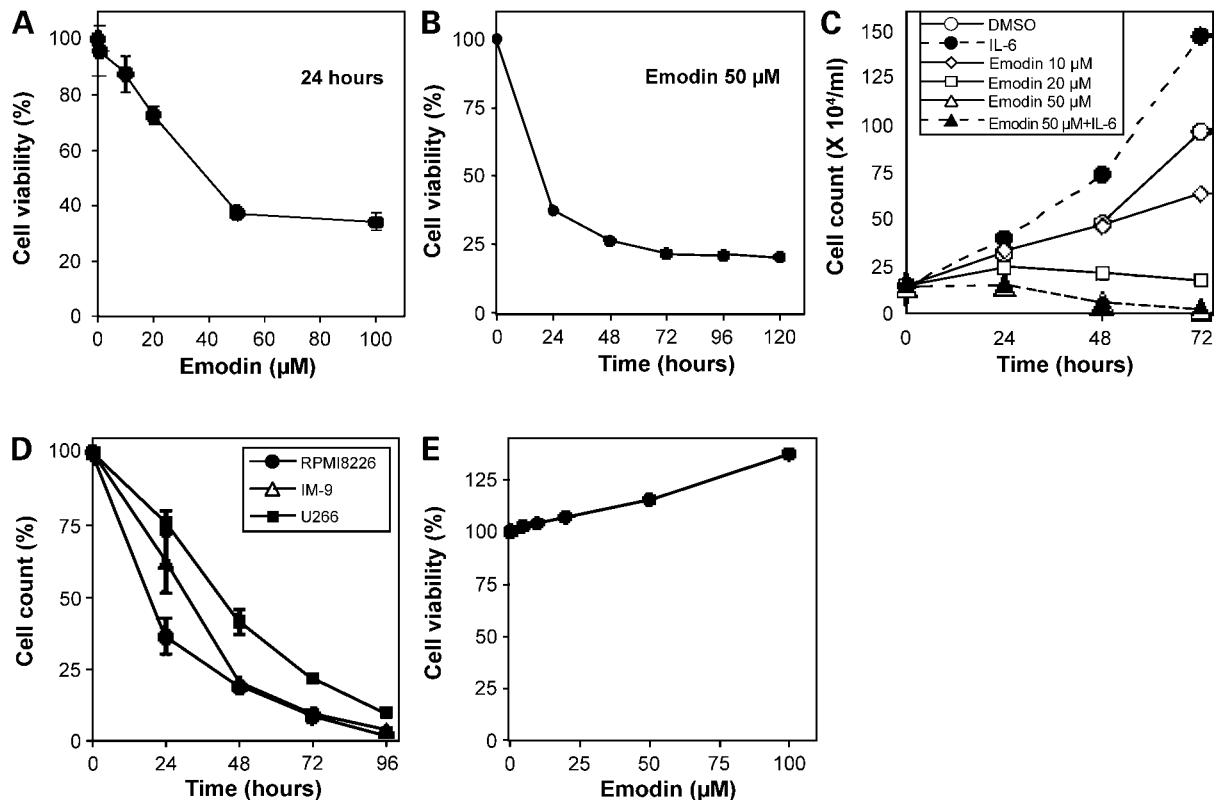


Figure 1. Emodin has cytotoxicity in MM cell lines, but not in normal PBMCs. **A** and **B**, RPMI8226 cells were cultured with various concentrations of emodin for 24 h (**A**) or with 50 μ mol/L emodin for various times (**B**). Cell viability was assessed by the XTT assay. **C**, cells were incubated with DMSO (\circ) or 10 μ mol/L (\diamond), 20 μ mol/L (\square), and 50 μ mol/L (\triangle) emodin. The cells were treated in the presence of 4 ng/mL IL-6 with DMSO (\bullet) or 50 μ mol/L emodin (\blacktriangle). Cell proliferation was assessed by trypan blue exclusion method. **D**, growth inhibition in various MM cell lines [RPMI8226 (\bullet), IM-9 (\blacktriangle), and U266 (\blacksquare)] by 50 μ mol/L of emodin for the indicated time was determined by the counting number of trypan blue excluded cells. **E**, viability of PBMCs was assessed by the XTT assay after 48 h culture. Points, mean; bars, SD.

their morphologic changes. Exposure of RPMI8226 cells to 50 $\mu\text{mol/L}$ emodin resulted in the typical morphologic appearance of apoptosis (Fig. 2A). To confirm these results further, genomic DNA from myeloma cells exposed to 50 $\mu\text{mol/L}$ emodin for 48 h exhibited a DNA ladder formation in RPMI8226 cells (Fig. 2B). Furthermore, RPMI8226 cells cultured with 50 $\mu\text{mol/L}$ emodin for 18 h were stained with annexin V to detect the externalization of phosphatidylserine on the cell membrane. As shown in Fig. 2C, the proportion of annexin V–positive and PI–negative cells increased strikingly.

We next investigated whether the apoptotic effects of emodin were caspase dependent. After RPMI8226 cells were treated with emodin for 24 h, whole-cell extracts were prepared and analyzed for the activation of caspase-9, caspase-8, and caspase-3 by Western blotting. In response to 50 $\mu\text{mol/L}$ emodin for 24 h, expression levels of caspase-9 and caspase-3 precursors were decreased, and cleaved products of lower molecular weight appeared, whereas

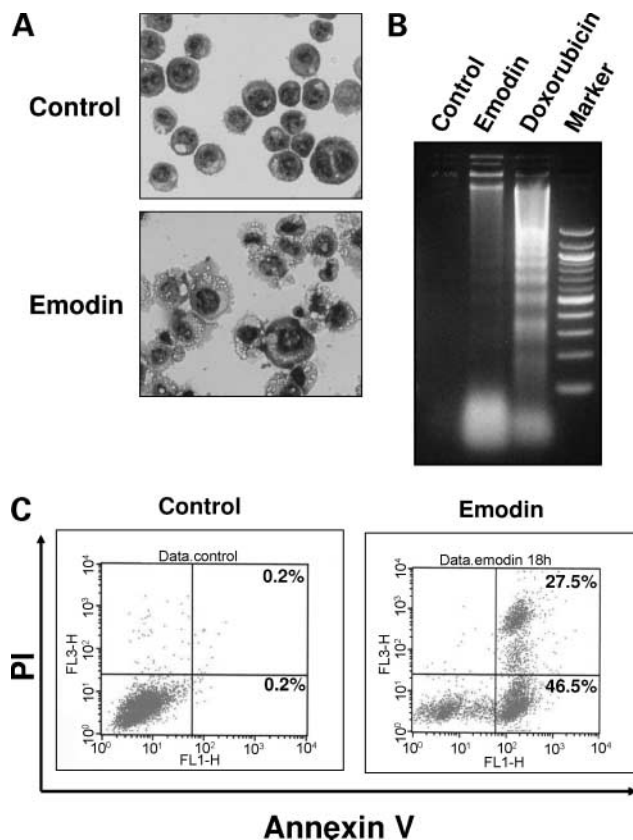


Figure 2. Effect of emodin on apoptosis induction in RPMI8226 cells. **A**, morphologic changes characteristic of apoptosis in RPMI8226 cells. Cells were incubated with or without 50 $\mu\text{mol/L}$ of emodin for 48 h and then stained with Giemsa. Original magnification, $\times 1000$. **B**, after RPMI8226 cells were treated with 50 $\mu\text{mol/L}$ emodin or 100 ng/mL doxorubicin for 48 h, DNA fragmentation assay was done. **C**, induction of annexin V–positive cells by emodin. RPMI8226 cells incubated with 50 $\mu\text{mol/L}$ emodin for 18 h were stained with annexin V and PI. The percentage of annexin V–positive cells was determined by flow cytometry.

caspase-8 was not cleaved into the active form (Fig. 3A). Caspase-9 activation usually occurs after mitochondria injury by a number of cytotoxic agents and Bcl-2 superfamily proteins, which play a central role in this apoptotic pathway. We next examined the expression change of Bcl-2 family proteins in emodin-treated cells. Western blot analysis revealed that treatment with emodin eliminated the expression of Mcl-1 (Fig. 3B), but did not significantly affect to the level of Bim, Bcl-2, Bcl-xL/S, and Bax, whereas doxorubicin induced the expression of Bax and decreased the level of Bcl-xL (Fig. 3B). The reduction in Mcl-1 expression level by emodin was observed in U266, IM-9, and KMS-12-PE cells as well as RPMI8226 cells (Supplementary Fig. S1).³ Furthermore, the expression levels of Mcl-1 transcripts were evaluated in emodin-treated myeloma cells by RT-PCR assay. Treatment with emodin caused a decrease in the expression level of Mcl-1 mRNA in a time-dependent manner (Fig. 3C and Supplementary Fig. S1).³ These results suggested that emodin could inhibit myeloma cell proliferation through the down-regulation of Mcl-1 expression.

Emodin Inhibits JAK2-STAT3 Signal Pathway

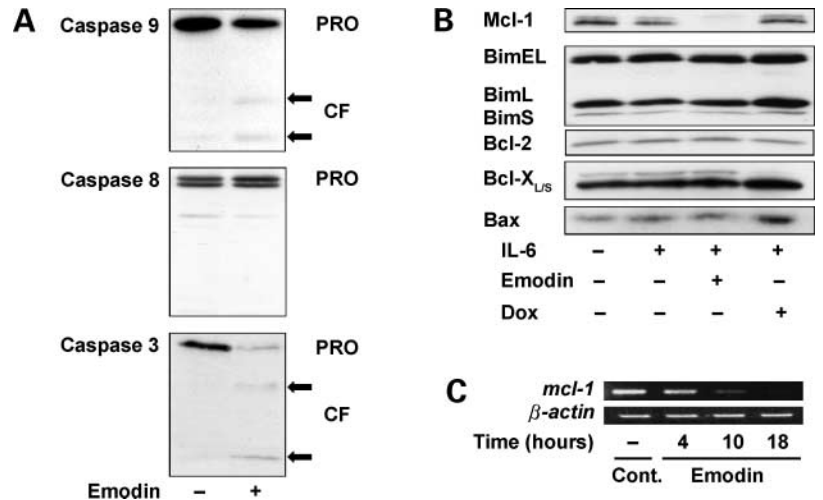
In myeloma cells, several cytokines, IL-6, insulin-like growth factor I, tumor necrosis factor- α , and vascular endothelial growth factor, mediate proliferation, drug resistance, and migration among these cytokines; IL-6 especially plays a pivotal role in myeloma cell proliferation. IL-6 stimulates three major survival pathways; the JAK2/STAT3 pathway, the Ras/Raf/MEK/MAPK pathway, and the PI3K/AKT pathway. The Mcl-1 expression is reported to be regulated by JAK2/STAT3 pathway; therefore, we next examined the activation of IL-6–induced signal molecules by Western blot analysis. IL-6–induced phosphorylation of gp130, JAK2, and STAT3 was diminished by emodin (Fig. 4A), but the phosphorylation of AKT, ERK, or p38 was not affected (data not shown). *In vitro* kinase assay showed that kinase activity of JAK2 was abrogated by emodin in a dose-dependent manner (Fig. 4B). As shown in Fig. 4C, IL-6–induced STAT3 binding capacity to STAT recognizing consensus sequence was diminished in the presence of emodin. Therefore, it was suggested that emodin could inhibit the IL-6–induced JAK2 kinase activity important for the phosphorylation of STAT3 protein, and which resulted in diminished STAT3 activity and decrease in Mcl-1 expression.

Emodin-Induced Apoptosis Is Abrogated in Mcl-1–Overexpressed Myeloma Cells

To confirm that emodin could inhibit JAK2/STAT3/Mcl-1 survival pathway, we introduced EGFP–Mcl-1 expression vector, pEGFP–Mcl-1, into KMS-12-PE MM cells by electroporation. EGFP–neo– or EGFP–Mcl-1–expressed cells, KMS-12-PE/neo or KMS-12-PE/Mcl-1 cells, respectively, were separated by cell sorter, and the effects of emodin on the separated cells were evaluated. Induction of

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Figure 3. Induction of caspase-dependent apoptosis in emodin-treated myeloma cells. **A**, cleavages of caspases were observed after treatment of 50 $\mu\text{mol/L}$ emodin for 24 h. *Arrows*, caspase proform (*PRO*) and cleaved forms (*CF*), respectively. **B**, changes in the expression of Bcl-2 family proteins in MM cells. **C**, decrease of Mcl-1 mRNA expression in emodin-treated MM cells. Cells were incubated with 50 $\mu\text{mol/L}$ emodin, and the expression of Mcl-1 mRNA was determined by RT-PCR.



apoptosis was examined by ELISA detecting mono- or oligonucleosome. Figure 5A shows that apoptosis was induced by emodin to the same extent in both parental and KMS-12-PE/neo cells; however, induction of apoptosis by emodin was almost completely abrogated in KMS-12-PE/Mcl-1 cells. Western blot revealed that intrinsic Mcl-1 was almost disappeared in both KMS-12-PE/neo and KMS-12-PE/Mcl-1 cells, whereas the force-expressed EGFP-Mcl-1 protein in KMS-12-PE/Mcl-1 cells was not affected either with or without emodin (Fig. 5B). Escape from emodin-induced apoptosis was also observed by fluorescent immunostaining for annexin V (Fig. 5C). EGFP-neo-expressed cells became positive for phycoerythrin-conjugated annexin V in the presence of emodin, whereas EGFP-Mcl-1-expressed cells were still negative in the same condition. The observation that forced expression of Mcl-1 in MM cells could abrogate the emodin-induced apoptosis suggests emodin could induce apoptosis on myeloma cells through the inhibition of the JAK2/STAT3/Mcl-1 pathway.

Discussion

In this study, we showed that emodin inhibited the growth of MM cells and induced apoptosis. The molecular mechanisms of emodin-induced apoptosis were via the inhibition of JAK2 kinase activity and the rapid down-regulation of Mcl-1 expression, which is required for myeloma cell survival (22–25).

It has been reported that emodin also induces apoptosis in several cancer cell lines (1–4) through the inhibition of p56lck (29), HER2/neu (30), and casein kinase II (5). Therefore, we hypothesized that emodin could be expected to suppress some kinases, which have important roles for the growth of MM cells. IL-6 from the bone marrow microenvironment and myeloma itself plays a pivotal role in the pathogenesis of myeloma cells (13) through the signal transduction pathways. The proliferation of myeloma cells even in the presence of IL-6 was completely abrogated by emodin; therefore, we thought that emodin might suppress IL-6 signal pathways. Although emodin did not block phosphorylation of AKT or ERK, the phosphorylation

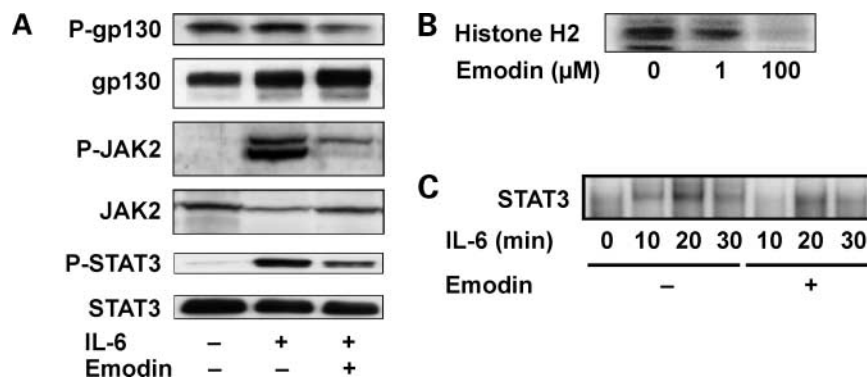


Figure 4. Emodin modulates IL-6-triggered signal pathway. **A**, RPMI8226 cells were pretreated with or without 50 $\mu\text{mol/L}$ of emodin in 2% FBS containing medium overnight, then cells were stimulated with 0.4 ng/mL IL-6 for 10 min. Whole cell lysates were subjected to Western blotting to assess the phosphorylated states or the expression of gp130, JAK2, and STAT3. **B**, cells were incubated with 0.4 ng/mL IL-6 for 10 min. Whole cell lysates were immunoprecipitated with anti-JAK2 antibody. The immune complex samples were pretreated with emodin for 30 min, and then *in vitro* kinase assay was done. **C**, cells were pretreated with or without 50 $\mu\text{mol/L}$ emodin in 2% FBS containing medium overnight and then incubated with 0.4 ng/mL IL-6. Nuclear extracts were used for EMSA.

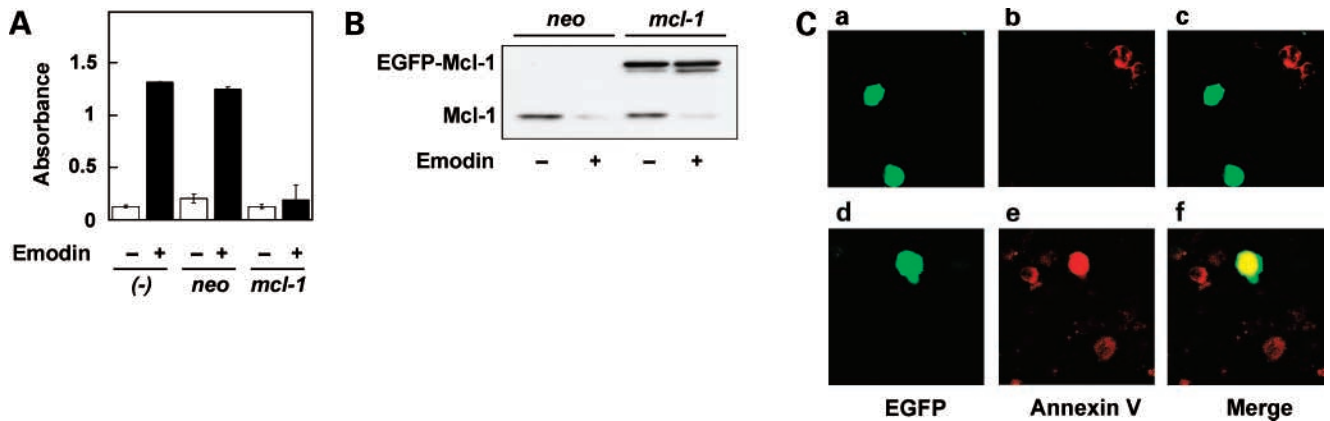


Figure 5. Mcl-1 abrogated emodin-induced apoptosis. **A**, pEGFP-neo or pEGFP-Mcl-1 was introduced into KMS-12 PE cells by electroporation. After 24 h, EGFP-positive cells were sorted. After the sorted cells were treated with or without 50 $\mu\text{mol/L}$ of emodin for 24 h, whole cell lysates were subjected to Western blotting to detect Mcl-1. **B**, pEGFP-neo- or pEGFP-Mcl-1-expressed cells were incubated with 50 $\mu\text{mol/L}$ emodin for 24 h. Induction of apoptosis was determined by ELISA. Columns, mean for three independent experiments; bars, SD. **C**, pEGFP-Mcl-1-transfected cells (a-c) or pEGFP-neo-transfected cells (d-f) were treated with emodin for 24 h. After staining with phycoerythrin-labeled annexin V, EGFP-positive cells (a and d), or phycoerythrin-annexin V-positive cells (b and e) were observed by laser electron microscope. EGFP- and phycoerythrin-annexin V-positive cells were determined as the pictures merged (c and f).

of STAT3 was inhibited by emodin. *In vitro* kinase assay also showed that emodin inhibited JAK2 kinase activity, which is important for the phosphorylation of STAT3. Mcl-1, one of the STAT3-regulated molecule, acts as an antiapoptotic protein and is present in most MM cell lines and patient cells (19, 31). Mcl-1 was isolated from the ML-1 myeloid leukemia cell line during phorbol ester-induced differentiation (21). This protein is essential in regulating apoptosis, lymphoid development, and the maintenance of mature lymphocytes (32).

Mcl-1 has Bcl-2 homology domains (BH1, BH2, and BH3), PEST sequences, and immediate response boxes (IRB; refs. 21, 28). The PEST sequences and Arg:Arg motifs that are commonly found in the short half-life proteins (21, 28, 33–35) and Mcl-1 are degraded by proteasome (36) or caspases (37). Moreover, Mcl-1 mRNA has a character as an immediate early gene (28, 33) and is induced by various extracellular stimuli; retinoic acid (28), 12-*O*-tetradecanoylphorbol-13-acetate (21), granulocyte macrophage colony-stimulating factor (33), heat shock (38), or vitamin D₃ (39). Recent studies show that the down-regulation of Mcl-1 by antisense oligonucleotide or small interfering RNA induces apoptosis in myeloma cells (24, 40), and novel biological agents like statins (41), nuclear factor κB inhibitors (42), and cyclin-dependent kinase inhibitors (43) induce apoptosis, at least in part, through the decreased expression level of Mcl-1. Therefore, Mcl-1 is a good molecular target for treating MM (22–25, 31). In our experiments, in EGFP-Mcl-1-overexpressed myeloma cells, which avoided emodin-induced apoptosis, intrinsic Mcl-1 expression was almost completely eliminated as much as in EGFP-neo-transfected myeloma cells, whereas expression level of cytomegalovirus-promoter-driven EGFP-Mcl-1 was not changed. From these findings, emodin might induce apoptosis via the suppression of JAK2 kinase activity, which resulted in the decrease in phosphorylated STAT3 and Mcl-1 expression

level. Although it is reported that Mcl-1 overexpression was not sufficient to protect the IL-6-dependent MM cells from apoptotic death on IL-6 withdrawal (44), most recent study indicates that the balance of antiapoptotic Mcl-1 and proapoptotic Bim expression level is important for the apoptosis control of myeloma cells (23). In the same way, elimination of Mcl-1 protein and unchanged expression of Bim protein in myeloma cells by emodin might result in the induction of apoptosis. Furthermore, activation of STAT3 is also reported to increase the transcription and expression levels of Bcl-2, Bcl-xL, c-myc, and cyclin D in various organ cells (45–47). However, we could not find any expression changes of other Bcl-2 family proteins except Mcl-1 in the presence of emodin. Certainly, although these STAT3-regulated proteins are important for survival and proliferation of myeloma cells, Brocke-Heidrich et al. (44) showed that only Mcl-1 is induced by IL-6 alone among the antiapoptotic members of Bcl-2. The viability of MM cells was not affected by either Bcl-2 or Bcl-xL antisense oligonucleotide, whereas Mcl-1 antisense oligonucleotide induced apoptosis in MM cells. In addition, a synergistic effect of Pim-1 and c-myc is needed for STAT3-mediated Bcl-2 induction and survival of pre-B cells (20). Because emodin-induced apoptosis occurred, unless other antiapoptotic Bcl-2 family proteins remained and were recovered by Mcl-1 overexpression, we concluded that other Bcl-2 families might not participate in the induction of apoptosis by emodin. Even under condition of a long-term diet of emodin, no evidence of carcinogenic activity in rats and mice was observed (48). In the case of rats or mice given emodin during pregnancy period, neither prenatal mortality nor morphologic development was affected, and genotoxicity of emodin would be unlikely (49, 50). Therefore, emodin should be a safe therapeutic agent for MM.

In conclusion, we have shown that emodin induces apoptosis via inhibition of JAK2 and down-regulation of

antiapoptotic Mcl-1 expression. Several agents have been searched for more effective and safe treatment of multiple myeloma. Emodin is a safe agent even in long exposure in mice. Therefore, it might be possible to develop emodin as a new potent anticancer agent for the management of multiple myeloma and as a novel therapeutic agent that can replace the more cytotoxic agents currently used to treat patients with multiple myeloma.

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