

Momordin I, an inhibitor of AP-1, suppressed osteoclastogenesis through inhibition of NF- κ B and AP-1 and also reduced osteoclast activity and survival[☆]

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Abstract

Osteoclasts originating from hematopoietic precursor cells differentiate into multinucleated cells through multiple steps. The essential roles of NF- κ B and AP-1 in osteoclast differentiation have been clearly demonstrated in numerous studies. c-Fos, a component of AP-1 transcription factor, plays a key role in osteoclast differentiation. Recently, we found a strong inhibitor of AP-1 transcriptional activity, named momordin I, based on the structure of oleanolic acid glycosides and originally isolated from *Ampelopsis radix*. So, we hypothesized that momordin I might be able to regulate osteoclast formation, activity, and survival. Here, we report the ability of momordin I to suppress osteoclastogenesis in a co-cultured system and a RANKL-induced osteoclast precursor system. Momordin I remarkably inhibited the activation of NF- κ B as well as AP-1 in RANKL-induced RAW264.7 cells, in which momordin I appeared to target I κ B degradation and c-Fos expression, respectively, but not MAPK signaling pathways. The ability of momordin I to change the ratio of RANKL and OPG in primary osteoblasts was partially responsible for the reduction of osteoclast formation. Furthermore, pit formation on dentin slices was suppressed by momordin I with stimulating actin ring disruption. Our results also showed that momordin I highly shortened osteoclast lifespan and induced osteoclast apoptosis. In conclusion, the present results demonstrate for the first time that momordin I is a potent inhibitor of osteoclast differentiation via the reduction of NF- κ B and AP-1, and also suppresses osteoclast function and survival.

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The maintenance of the integrity and strength of bones is regulated through bone remodeling and homeostasis that are controlled by the activities of specialized cells within the bone: bone synthesis by osteoblasts and bone resorption by osteoclasts. Bone formation and resorption processes are

well coordinated and are regulated by several factors under normal physiological and pathological conditions. Loss of the delicate balance between them leads to bone diseases including osteoporosis and rheumatoid arthritis.

Osteoclasts originate from hematopoietic cells and are capable of resorbing bone. Osteoclastogenesis takes place through multiple steps such as differentiation, fusion, and activation of mature osteoclasts by cell-to-cell contact with osteoblast lineages that express the factors regulating osteoclast differentiation including RANKL and OPG, members of the TNF family [1,2]. RANKL that binds to

[☆] Abbreviations: AP-1, activating protein-1; NF- κ B, nuclear factor- κ B; RANK, receptor activator of nuclear factor- κ B; RANKL, RANK ligand; OPG, osteoprotegerin; MAPK, mitogen-activated protein kinase.

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its receptor, RANK, and stimulates intracellular signals is the most critical molecule for osteoclastogenesis as well as the activation and survival of osteoclasts [3]. OPG, a secreted decoy receptor, binds to RANKL and prevents osteoclast formation. Thus, the RANKL/RANK/OPG axis provides a means of controlling the balance in bone formation and resorption [4].

Intracellular RANK signaling by the interaction with RANKL induces recruitment and activation of cytoplasmic tumor necrosis factor receptor-associated factors (TRAFs) such as TRAF6, leading to the activation of multiple signaling cascades such as ERK, p38, JNK, and IKK [1,5]. The ERK inhibitors PD98059 and U0126 accelerate RANKL-induced osteoclast differentiation, suggesting that the ERK pathway is involved in the negative regulation of osteoclastogenesis [6]. However, the p38 and JNK pathways have been shown to play a critical role during RANKL-induced osteoclast differentiation [7]. TRAF6 also activates transcription factors such as NF- κ B and AP-1, followed by the induction of gene expression necessary for osteoclast formation and function [1,8]. The essential roles of NF- κ B and AP-1 in osteoclast differentiation have been clearly demonstrated in gene knockout mice that showed osteopetrotic phenotypes due to the blocking of osteoclastogenesis [9–12]. The key function of mature osteoclasts, characterized by multinuclearity, tartrate-resistant acid phosphatase (TRAP) staining, an actin ring structure, ruffled border, and acidic cell condition during resorption is bone resorptive activity, following which the differentiated osteoclasts have only a short lifespan and undergo apoptotic cell death [1]. Osteoclast apoptosis is controlled by several signaling molecules involved in apoptotic processes, including TRAF6, Src, PI3K/Akt, ERK, and NF- κ B pathway, and is positively and negatively regulated by cytokines, growth factors, and hormones [5,13–15].

On the basis of these observations, there have been several approaches to the development of drugs for the treatment of bone disease using inhibitors of transcription factor NF- κ B or/and AP-1 as natural molecules [16–18]. For example, curcumin, a powerful inhibitor of NF- κ B and AP-1, has been shown to suppress osteoclastogenesis and pit formation and stimulate osteoclast apoptosis [18,19]. Recently, we found a strong inhibitor of AP-1 transcriptional activity, momordin I, based on the structure of oleanolic acid glycosides originally isolated from *Ampelopsis radix* [20]. Previous studies showed that momordin I suppressed AP-1 activity by inhibiting the interaction between Jun/Fos dimers and the DNA AP-1 site and also inhibited cancer cell proliferation by inducing apoptosis [21,22]. However, the effect of momordin I on NF- κ B activity has not been studied yet. In this study, we examined the effect of momordin I on osteoclast bone resorption processes including the differentiation, activity, and survival of osteoclasts for the first time. Osteoclastogenesis was highly inhibited by momordin I that suppressed RANKL-induced NF- κ B and AP-1 activity in osteoclast precursors and exerted an influence on RANKL

and OPG levels in osteoblasts. Also, we explored the inhibitory effect of momordin I on bone resorbing activity and the stimulatory effect on osteoclast apoptosis.

Materials and methods

Reagents and animals. Momordin I was isolated from *A. radix* at the Central Research Laboratories of Dong-Wha Pharmaceutical Company and dissolved in DMSO. Recombinant RANKL and M-CSF were purchased from PeproTech. (Rocky Hill, NJ, USA). α -MEM, DMEM, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin–EDTA used for cell culture were purchased from Gibco-BRL (Grand Island, NY, USA). 1,25(OH) $_2$ D $_3$, dexamethasone, all reagents for TRAP staining, bisbenzamide for staining nuclei, and any other chemicals were purchased from Sigma–Aldrich. Anti-I κ B- α , anti-c-Fos, anti-p-MAPKs, and anti-MAPKs were obtained from Santa Cruz Biotechnology (San Diego, CA) and anti-caspase-3 was from Cell Signaling Technology (Beverly, MA). Newborn ICR mice were purchased from Biolinks (Umsung, Korea) and 6-week-old ddY mice were from Charles River Japan Lab (Japan).

Osteoclast formation using co-culture and osteoclast progenitor cell culture. An index of osteoclast formation in the culture was determined by counting TRAP-positive multinucleated osteoclast cells (TRAP(+) MNCs). For co-culture, bone marrow cells (2.5×10^5 cells/cm 2) isolated from the femora of 6-week-old ddY mice and primary calvarial cells isolated from calvariae of newborn ICR mice (4×10^4 cells/cm 2) were co-cultured in the presence of 1,25(OH) $_2$ D $_3$ (10^{-8} M) and dexamethasone (10^{-7} M) with α -MEM containing 10% FBS, and the medium was changed every two days. On day 6, the cells were fixed with 10% formaldehyde and stained for TRAP. The number of TRAP(+) MNCs containing more than 6–7 nuclei was counted under a microscope (ZEISS Axiovert 25, Switzerland).

The creation of a culture using bone marrow-derived monocyte/macrophage precursor cells (BMMs) was performed as below. Briefly, bone marrow cells were cultured in α -MEM plus 10% FBS containing M-CSF (5 ng/ml). After 24 h, non-adherent cells containing BMMs were collected, seeded at an initial density of 2.5×10^4 cm $^{-2}$, and cultured in the presence of RANKL (50 ng/ml) and M-CSF (30 ng/ml) for 6 days. For the culture using RAW264.7 (ATCC, Manassas, VA), the cells (1×10^4 cells/cm 2) were plated into DMEM supplemented with 10% FBS. To generate osteoclasts, the medium was changed to α -MEM supplemented with 10% FBS and 50 ng/ml RANKL, and the cultures were fed every 2 days by replacing with fresh medium. After a culturing period of the desired length, the cells were fixed in 10% formalin and stained for TRAP activity.

Bone resorption activity and actin ring staining. Multinucleated osteoclasts with bone resorbing activity were obtained from co-cultured cells as described above. The cells were suspended in α -MEM containing 10% FBS and were placed on a dentine slice (4 mm diameter) in a 96-well culture plate. After 2 h, the dentine slice was transferred to another plate containing momordin I and vehicle and incubated for 24 h more. Some slices were fixed for TRAP staining and actin ring staining with rhodamine-phalloidin (5 U). Other slices were placed in 1 M NH $_4$ OH to remove adherent cells, and resorption pits formed on the slices were visualized by staining with Mayer's hematoxylin. Areas of resorption pits were measured with an image analyzer and compared to the control group.

Osteoclast survival and apoptosis activity. For the study of osteoclast survival, more pure osteoclasts were prepared by removing osteoblasts from the co-cultured cells as below. The harvested co-cultured cells were replated and incubated for 1 day. To remove osteoblasts, the cells were treated with 0.1% collagenase (Wako Pure Chemical, Osaka, Japan) and briefly pipetted. At this time (0 h), the purified osteoclasts were subjected to TRAP staining and other cultures were further incubated in the presence of momordin I or RANKL (100 ng/ml) with or without momordin I for 24 h. The remaining living cells were stained with TRAP. The survival rate was calculated as the percentage of TRAP-positive multinuclear cells remaining after 24 h treatment. To evaluate the effects of momordin I on osteoclast apoptosis, purified osteoclasts were treated with momordin I in α -MEM containing 0.1% FBS for 6 h. The cells were fixed and incubated

with 5 µg/ml bisbenzimidazole for 30 min at RT and then TRAP staining was carried out on the cells. Cells showing fragmented chromatin and condensed chromosome morphology were considered apoptotic. The percentage of apoptotic cells was determined as the number of apoptotic multinucleated cells/total number of multinucleated cells by a fluorescence microscope (ZEISS Axiovert25, mbq 52 ac). At the same time, caspase-3 activity, as a marker of apoptosis, was measured with cell lysates by Western blotting.

Electrophoretic mobility shift assay (EMSA). RAW264.7 cells were washed with cold PBS and suspended in 0.2 ml hypotonic lysis buffer (10 mM Hepes (pH 7.9), 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT) containing protease inhibitors. After incubation on ice for 10 min, the cell lysates were centrifuged for 10 min at 5000 rpm and the supernatant containing the cytoplasmic extract was stored at –80 °C. The nuclear pellets were resuspended in ice-cold nuclear extraction buffer (20 mM Hepes, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors) and incubated on ice for 30 min with occasional agitation. The extract was subjected to centrifugation for 10 min at 12,000 rpm and supernatant containing nuclear extracts was separated, whereupon the protein content was measured by a BCA kit. EMSA for NF-κB and AP-1 activation was undertaken as described [3]. Extracts of 10 µg were incubated with binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 25% glycerol, and poly(dI-dC)), and [α -³²P]ATP-end labeled NF-κB oligonucleotide (5'-AGTTGAGGG GACTTCCAGGC-3') or AP-1 (5'-CGCTTGATGAGTCAGCCG GAA-3') (Promega, Madison, WI, USA) for 30 min at RT. The DNA-protein complex was separated on 6% polyacrylamide/0.25× TBE gels. The gels were dried and exposed to an X-ray film.

Western blot analysis. Cytoplasmic or nuclear extracts (20 µg) and cell lysates (40 µg) were subjected to 10% or 12% SDS-PAGE and the proteins separated in the gel were transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was incubated with anti-IκB-α, anti-c-Fos, anti-p-MAPKs, and anti-caspase-3 antibodies and, subsequently, with peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody. Immunoreactive proteins were visualized with Western blot chemiluminescence reagents (Amersham Biosciences) following the manufacturer's instructions.

RT-PCR analysis. The total RNA (1 µg) extracted from the cultured cells was converted to complementary DNA (cDNA) by reverse transcriptase and amplified by PCR using a PCR kit (Bioneer, Daejeon, Korea). PCR amplification was done by gene-specific PCR primer as follows: murine RANKL (antisense: 5'-GGGAATTACAAAGTGCACCAG-3', sense: 5'-GGTCGGGCAATTCTGAATT-3') and murine GAPDH (antisense: 5'-ACCACAGTCCATGCCATCAC-3', sense: 5'-TCCACCA CCCTGTTGCTGTA-3'). Amplification was conducted for 32 cycles, each at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The amplified samples were run in a 1.5% agarose gel with ethidium bromide and the bands were visualized under UV illumination.

Determination of secreted OPG levels. For the quantitative determination of OPG amounts secreted in cell culture medium, primary calvarial cells (1 × 10⁵ cells/well) were plated into a six-well tissue culture plate and allowed to grow with 1,25(OH)₂D₃ with and without momordin I for 3 days. The secreted OPG content was analyzed using a sandwich enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. The results are expressed as means ± SD. All the experiments have been performed at least two times independently. Statistical significance was determined by Student's *t* test.

Results

Momordin I inhibits osteoclast differentiation from co-culture and osteoclast precursor culture

In co-cultures, the addition of momordin I at 0.1 ~ 5 µM significantly reduced the number of TRAP(+) osteoclasts in a dose-dependent manner (Figs. 1A and B).

Momordin I inhibited osteoclast formation by more than 82% while maintaining >95% cell viability, determined by the MTT assay (data not shown). We also examined the direct effect on osteoclast progenitors using mouse RAW264.7 cells and BMMs that do not require supporting cells. In RANKL-stimulated RAW264.7 cells, we confirmed the inhibitory effect of momordin I on osteoclast formation (Fig. 1C). Consistent with results in RAW264.7 cells, momordin I dramatically reduced the number of TRAP(+) osteoclasts and showed inhibitory effects of 76%, 53%, and 35% at 5, 2, and 1 µM, respectively (Fig. 1D). From these results, we concluded that momordin I significantly inhibited osteoclastogenesis in both co-cultured cells and osteoclast progenitor cells and that it might be a potent inhibitor of osteoclast differentiation.

In order to determine which stage of osteoclast formation was affected by momordin I, cells were treated with momordin I every 2 days during a 6-day culture period in BMM culture. The osteoclastogenesis was suppressed when momordin I was added at the early stage (0–2 days), a stage for the commitment step of osteoclast differentiation, and such inhibition was partly observed at the late stage (4–6 day) (Fig. 1E). These results indicated that momordin I preferentially inhibited osteoclast differentiation during the early stage after RANKL and M-CSF treatment.

Momordin I stimulates secretion of OPG and inhibits expression of RANKL mRNA in primary osteoblasts

To determine whether primary osteoblasts were involved in the inhibitory effect of momordin I during osteoclastogenesis, we analyzed RANKL expression and OPG secretion in 1,25(OH)₂D₃-treated cells. RT-PCR results showed that the addition of momordin I down-regulated RANKL mRNA expression—increased by 1,25(OH)₂D₃—in a dose-dependent manner (Fig. 2). Furthermore, ELISA using conditioned medium showed that momordin I rescued 1,25(OH)₂D₃-attenuated OPG secretion, not dramatically but significantly (Table 1). These findings suggested that momordin I could inhibit, at least partially, osteoclast formation through the stimulation of OPG secretion and the reduction of RANKL levels in osteoblast/stromal cells.

Momordin I inhibits RANKL-induced NF-κB and AP-1 activation, c-Fos and IκB degradation, but not MAPKs in osteoclast precursors

To investigate the molecular mechanisms of momordin I in osteoclast precursors, we primarily examined NF-κB and AP-1 activity by EMSA in RAW264.7 cells. After pretreatment with momordin I for 2 h, the cells were treated with RANKL (100 ng/ml) for 30 min. As shown in Figs. 3A and B, RANKL up-regulated both NF-κB and AP-1 activities within 30 min, whereas unstimulated cells had a low basal level of NF-κB and AP-1 activity. Interestingly, momordin I greatly suppressed RANKL-in-

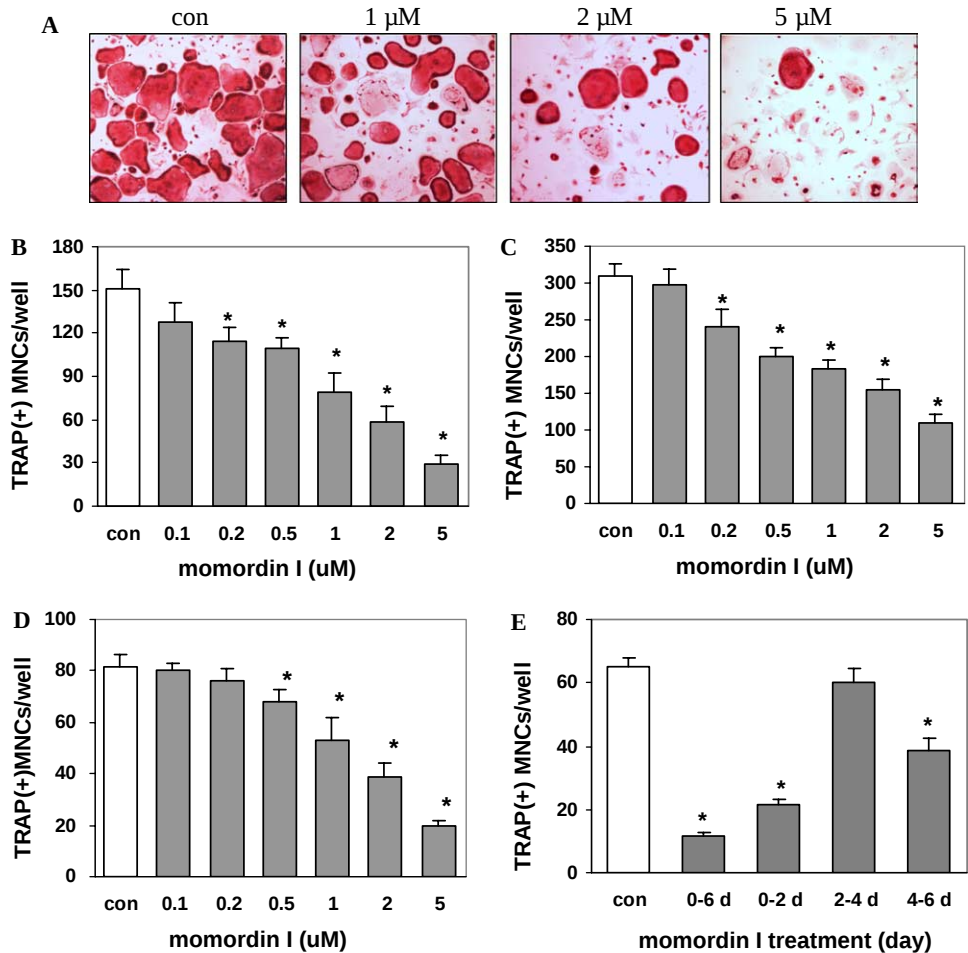


Fig. 1. Inhibitory effects of momordin I on osteoclastogenesis. (A,B) Momordin I inhibited osteoclast formation in co-culture. Mouse bone marrow cells and calvarial osteoblasts were cocultured in the presence of 1,25(OH)₂D₃ and dexamethasone with indicated concentrations of momordin I for 6 days. Cells were stained for TRAP and photographed. TRAP(+) MNCs with six or more nuclei were counted. (C,D) Momordin I inhibited RANKL-induced osteoclastogenesis. RAW264.7 cells induced by RANKL (50 ng/ml) (C) and BMM cells induced by RANKL (50 ng/ml) and M-CSF (30 ng/ml) (D) were cultured in the presence of momordin I for 4 days and 6 days, respectively. (E) Momordin I inhibited RANKL-induced osteoclast formation predominantly during the early stage. BMM cells were cultured with momordin (5 μ M) for a 2-day period, for 6 days in the presence of RANKL and M-CSF. Results are represented as means $\pm$ SD (n = 5) of three dependent experiments. * p < 0.01 compared to control.

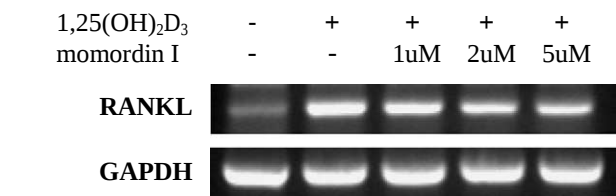


Fig. 2. Momordin I inhibited RANKL expression in osteoblasts. 1,25(OH)₂D₃-treated calvarial cells were incubated with momordin I for 4 days at the indicated concentrations. Total RNA was isolated from the cells and RANKL mRNA expression was determined by RT-PCR.

duced NF- κ B activation in a dose-dependent manner. As expected, momordin I strongly abrogated the RANKL-induced AP-1 activation and complete inhibition was observed at 2 μ M concentration of momordin I. These results indicated that the suppressive effect of momordin

Table 1		
Momordin I stimulated OPG secretion in osteoblasts		
Momordin I (μ M)	1,25(OH) ₂ D ₃	OPG secretion (ng/1 $\times$ 10 ⁵ cells)
—	—	55.75 $\pm$ 0.77
—	+	5.76 $\pm$ 0.13
1	+	6.16 $\pm$ 0.09
2	+	6.67 $\pm$ 0.08*
5	+	6.78 $\pm$ 0.27*

Primary calvarial cells were treated in the presence of 1,25(OH)₂D₃ with momordin I for 4 days and the conditioned medium was collected. The amount of OPG secreted into medium was analyzed using quantitative ELISA. Results are expressed as means $\pm$ SD (n = 3) of two dependent experiments. * p < 0.05 compared to only 1,25(OH)₂D₃-treated cells.

I on RANKL-induced osteoclastogenesis resulted from the inhibition of NF- κ B and AP-1 activation. We further looked at the mechanisms of its effect on NF- κ B and AP-

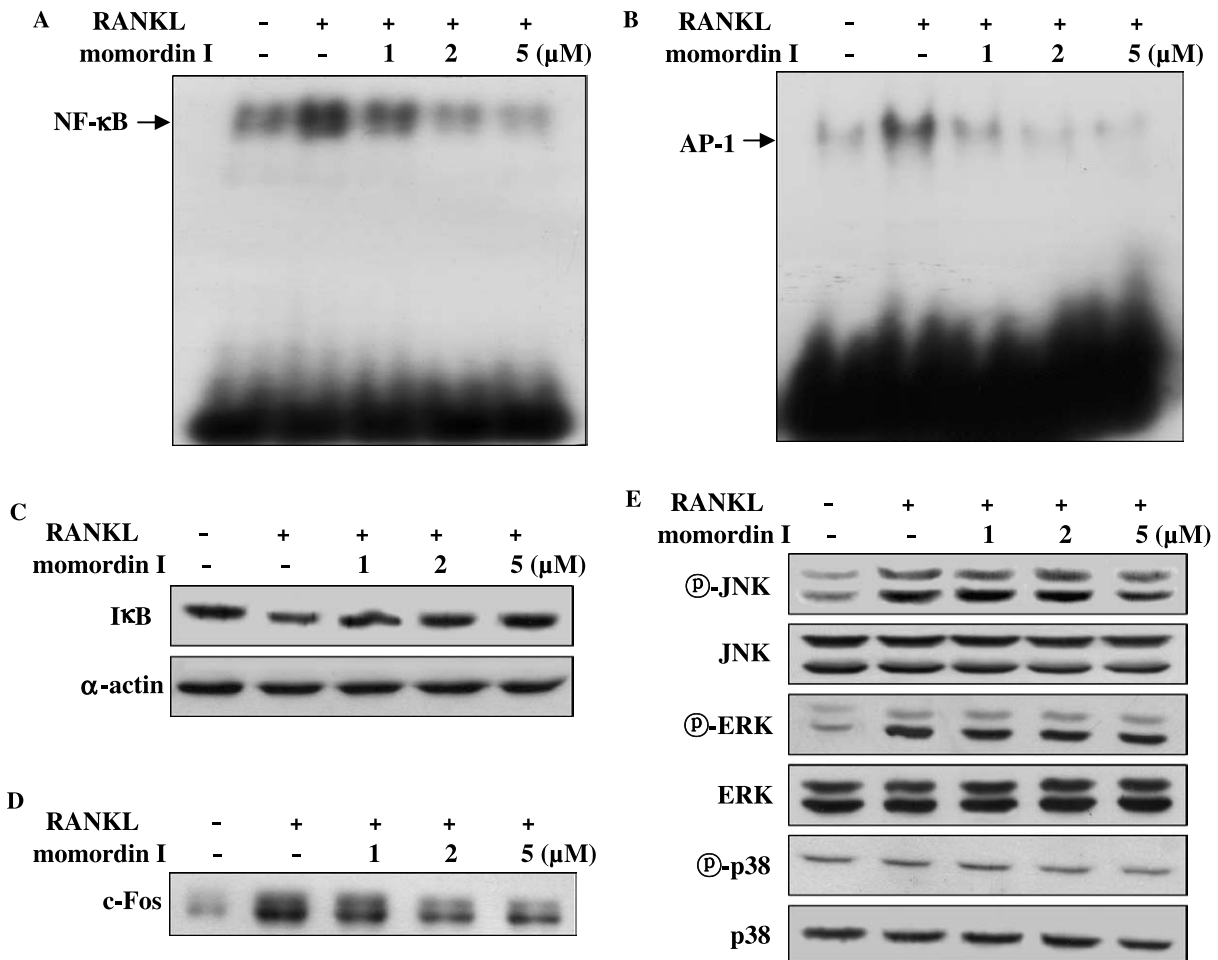


Fig. 3. Inhibitory effects of momordin I on RANKL-induced NF-κB and AP-1 activation in RAW264.7 cells. RAW264.7 cells were incubated either alone or in the presence of momordin for 2 h and treated with RANKL (100 ng/ml) for 30 min. Nuclear and cytosolic extracts were then separated from harvested cells. (A,B) Momordin I inhibited RANKL-activated NF-κB (A) and AP-1 (B) activation. Nuclear extracts were analyzed by EMSA. (C) Momordin I reduced IκB degradation. Cytosol extracts were fractionated on a gel and immunoblotted with anti-IκB and anti-actin. (D) Momordin I suppressed the expression of c-Fos. Nuclear extracts were analyzed by Western blot with anti-c-Fos. (E) Effects on MAPKs activation. Cytosolic extracts were subjected to Western blot with anti-Ⓟ-JNK, anti-Ⓟ-p38, and anti-Ⓟ-ERK. These results were repeated over two or three experiments.

1 activation. NF-κB is known to be activated through the activation of IKK and subsequent phosphorylation and degradation of IκB. Therefore, we studied the effect of momordin I on RANKL-induced IκB degradation. RANKL was found to induce degradation of IκB within 30 min, but momordin I largely inhibited this process (Fig. 3C), suggesting that momordin I inhibits RANKL-induced NF-κB activity by interfering with the degradation of IκB. We also determined the expression level of c-Fos. As shown in Fig. 3D, nuclear c-Fos expression was abrogated by momordin I in a dose-dependent manner (Fig. 3D), indicating that momordin I reduced AP-1 activity by inhibiting the expression of c-Fos. Taken together, we confirmed whether momordin I affected MAPK pathway in the inhibition of osteoclastogenesis. The results of Western blot analysis using antibodies on the phosphorylated forms of ERK, JNK, and p38 showed that momordin I had no or a little effect on these MAPK

(Fig. 3E). It seems that the MAPK pathway is not a primary target of momordin I during RANKL-induced osteoclastogenesis.

Momordin I reduces bone resorbing activity of osteoclasts

A main function of mature osteoclasts is to resorb mineralized bone surface and the formation of actin rings is an essential morphological character of osteoclasts in carrying out their resorption function. We investigated whether momordin I directly inhibits the function of osteoclasts. The results of Figs. 4A and B show that momordin I actively suppressed the number of TRAP(+) osteoclasts and pit areas on a dentine slice in a dose-dependent manner. We also confirmed that actin rings of osteoclasts were disrupted in momordin I-treated cells (Fig. 4C). These results indicate that momordin I suppressed the bone resorption of mature osteoclasts and

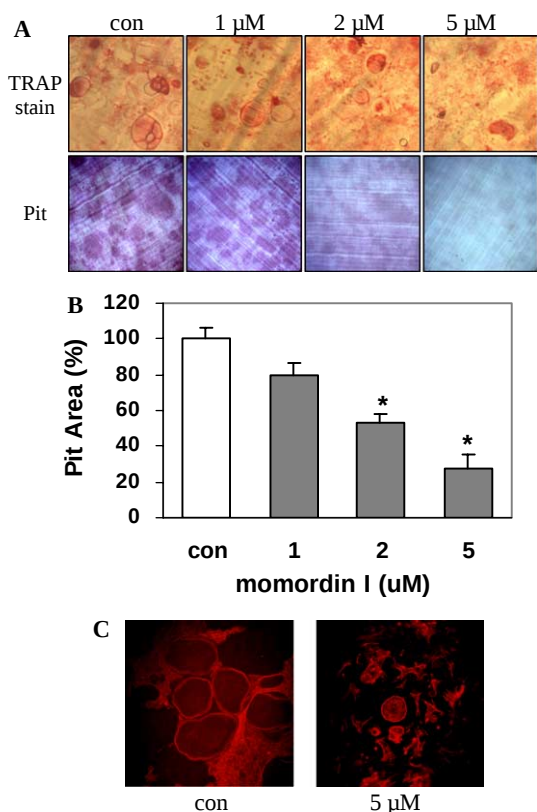


Fig. 4. Effects of momordin I on bone resorption and actin ring disruption. Differentiated osteoclasts were obtained from co-cultured cells as described in Materials and methods. Cells were plated on dentine slices and cultured in the presence or absence of momordin I for 24 h. (A,B) Momordin I suppressed pit formation on dentin slice. After removing the cells, resorption pits were stained with hematoxylin after removing the cells and some osteoclasts on dentin slice were stained with TRAP (A). Area of resorption pit was measured by the image analyzer and represented as a percentage of control (B). (C) Momordin I disrupted the actin rings. Actin rings were stained with rhodamine-phalloidin and photographed at 40 $\times$ magnification.

that the disruptive effects of momordin I on actin rings have mainly contributed to its suppressive effect on bone resorption.

Effect of momordin I on osteoclast survival and apoptosis

We explored the effect of momordin I on the survival of osteoclasts in the absence of supporting cells. As shown in Figs. 5A and B, mature osteoclasts isolated from osteoblasts showed spontaneous cell death and kept around 40% survival rate within 24 h. Interestingly, the addition of momordin I dose-dependently reduced the survival of osteoclasts. Furthermore, momordin I attenuated the survival activity promoted by RANKL, a positive regulator of osteoclast survival length. To address how momordin I shortens osteoclast survival, we measured the apoptosis of osteoclasts by staining them with bisbenzimidazole that could allow apoptotic cells with condensed chromatin and fragmented DNA to be seen. Consistent with the survival results, momordin I increased

the number of apoptotic cells (Fig. 5C). At the same time, we analyzed cleaved caspase-3 expression as a marker of apoptosis, resulting in the finding that momordin I enhanced the expression of caspase-3 in a dose-dependent manner (Fig. 5D). These data suggested that momordin I shortened the lifespan of osteoclasts by accelerating osteoclast apoptosis.

Discussion

Bone disorder induced by pathological conditions commonly enhances the number of osteoclasts, activates them, and then triggers bone loss [23]. Many approaches have been taken to look into the development of drugs preventing and treating such bone disorders. Among them, two prominent transcription factors in osteoclast differentiation, NF- κ B and AP-1, have been considered good candidates for the treatment of bone resorbing diseases, because they play a critical role during osteoclast differentiation [16,18]. In this study, we applied momordin I, an inhibitor of AP-1, to several osteoclast culture systems and elucidated its action on osteoclast differentiation, activity, and survival.

Osteoclasts were able to differentiate from both a co-culture system stimulated by osteotropic factors, such as PTH, PGE₂, IL-11, and 1,25(OH)₂D₃ and an osteoclast precursor culture system induced by RANKL and M-CSF [1–3,24]. Thus, osteoclast formation in a co-culture system could be influenced either by direct effects on osteoclast progenitors or by indirect effects on osteoblasts that support the differentiation of osteoclast progenitors by expressing and secreting RANKL and M-CSF. Momordin I showed significant inhibition on osteoclastogenesis in both culture models. Interestingly, the extent of such inhibitions in both systems was not remarkably different, suggesting that momordin I predominantly targets the osteoclast precursors during osteoclastogenesis. Although we confirmed that momordin I could change the ratio of RANKL/OPG in osteoblasts, it may have, not completely but at least partially, contributed to the inhibition of osteoclast formation.

To resorb the bone, the mature multinucleated osteoclasts have to maintain the proper actin cytoskeletal organization that facilitates resorptive activity [1]. Also, the number and activity of osteoclasts and programmed cell deaths are believed to largely contribute to the regulation of bone resorption [13,25]. Momordin I suppressed pit formation on dentin slices and induced the disruption of the actin rings. We also had the evidence that momordin I inhibited the survival of osteoclasts. Since osteoclast apoptosis is an influential determinant of bone resorptive activity and bone homeostasis, shortening survival length of osteoclasts must be a good target for the treatment of bone disorders, and we showed the possibility that momordin I is a negative controller on osteoclast survival in this study. Collectively, it is likely that momordin I reduced the pit forming activity of osteoclasts both by triggering the direct

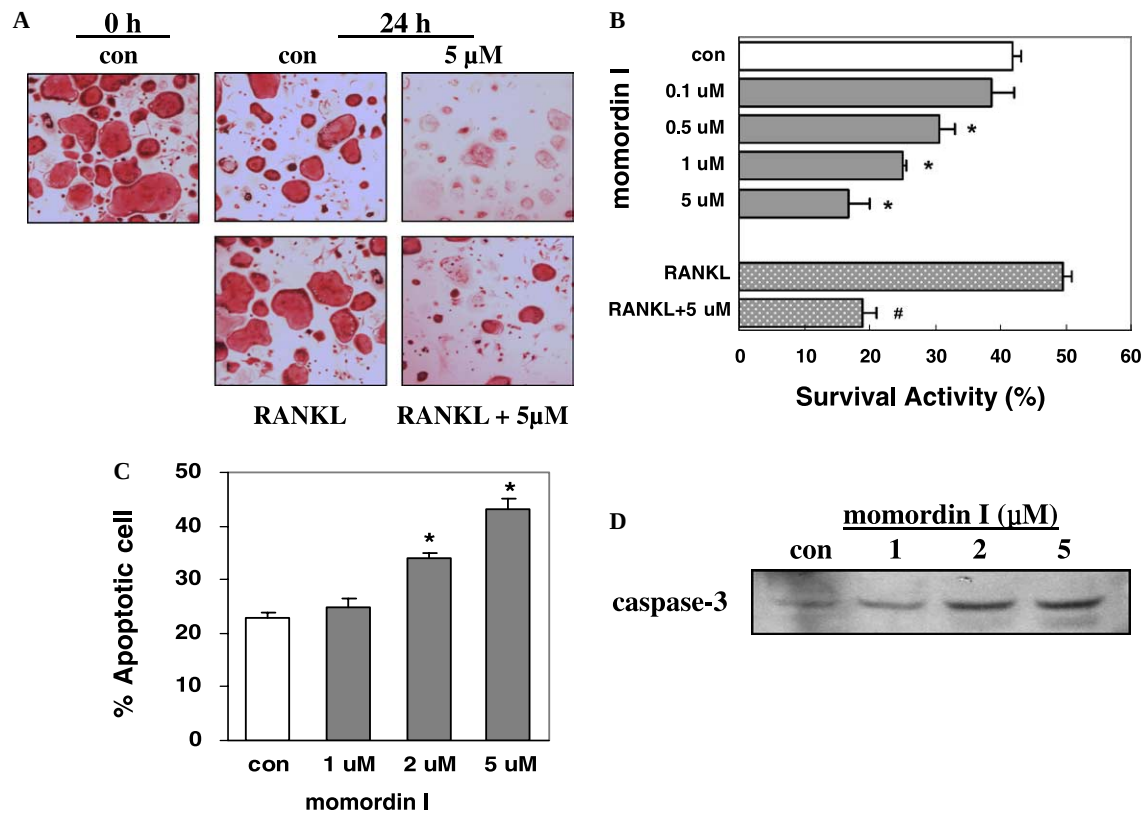


Fig. 5. Effects of momordin I on osteoclast survival and apoptosis. (A,B) Momordin I shortened osteoclast lifespan. Osteoclasts were purified from co-cultured cells as described in Materials and methods, and cultured with momordin I with or without RANKL (100 ng/ml) for 24 h. Cells were stained with TRAP before (0 h) and after (24 h) the addition of momordin I (A). Survival activity was calculated as the percentage of TRAP-positive multinuclear cells remaining after 24 h treatments (B). (C,D) Momordin I induced osteoclast apoptosis. Purified osteoclasts were treated with momordin I in α -MEM containing 0.1% FBS for 6 h. After fixation, cells were incubated with bisbenzimidazole and then TRAP staining was conducted. The quantitative analysis was performed by counting the number of osteoclasts containing apoptotic nuclei (C). Cell lysates were separated by 12% SDS-PAGE and analyzed by Western blot with anti-caspase-3 antibody (D). Data are expressed as means $\pm$ SD of three independent experiments ($n = 5$). * $p < 0.01$ vs. control, # $p < 0.01$ vs. RANKL.

disruption of actin rings and by inhibiting osteoclast formation and the survival of mature osteoclasts.

RANKL–RANK interaction triggers a signal cascade involving recruitment of TRAFs and eventually leads to the activation of two prominent transcription factors, NF- κ B, and AP-1, acting early in monocyte/macrophage lineage [5,9–12]. Interestingly, both transcription factors are regulated at multiple levels by dimerization and phosphorylation and very specifically affect the osteoclast lineage. We elucidated the action mechanisms of momordin I on RANKL-induced osteoclast differentiation in osteoclast precursor cells. Our findings demonstrated that momordin I has a potent inhibitory effect on osteoclastogenesis and strongly reduces RANKL-induced NF- κ B and AP-1 activation with molecular mechanisms inhibiting I κ B degradation and c-Fos expression, respectively. In MAPK signaling pathways, we observed that momordin I showed little or no effects on RANKL-induced phosphorylation of JNK, ERK, and p38. Therefore, we hypothesized that momordin I regulated RANKL-induced osteoclastogenesis by inhibiting NF- κ B and AP-1 activation rather than by controlling MAPK signaling pathways.

Unlike NF- κ B, c-Fos controls the generation of osteoclasts positively as well as negatively. The essential roles of c-Fos in osteoclast differentiation have been explained by the observations in Fos $^{-/-}$ mice showing the severe osteopetrotic phenotype [10,26]. In Fos $^{-/-}$ osteoclast precursors, the expression of all Fos family proteins was down-regulated and other Fos proteins such as Fra1 could rescue the osteoclast formation in vitro as well as in vivo [27,28]. In addition, Fos $^{-/-}$ precursors did not induce the expression of the nuclear factor of activated T cells c1 (NFATc1) that is an essential transcription factor for RANKL-induced osteoclastogenesis, acting downstream of TRAF 6 and c-fos, and that cooperates with c-Fos for induction of osteoclast-specific genes at the terminal differentiation stage [29–31]. It is indicated that roles of c-Fos induce osteoclastogenesis by targeting the transcription of Fos family and NFATc1 genes in RANKL signaling. However, surprisingly, c-Fos could initiate a negative feedback regulation of RANK signaling and eventually inhibit osteoclastogenesis. Recent reports have demonstrated that c-Fos activates interferon- β (IFN- β), which suppressed RANKL-induced c-Fos expression through the induction

of IFN-inducible genes [32]. In respect that RANKL-induced c-Fos expression induces its own inhibitor, c-Fos may be a unique autoregulatory mechanism for controlling bone homeostasis. In the increasing interest about c-Fos in the fields of bone biology [31], we think that our results using an AP-1 inhibitor are helpful in contributing to greater understanding of the role of AP-1 in skeletal systems.

In this study, we have shown for the first time that a naturally isolated AP-1 inhibitor, momordin I, has direct inhibitory effects on osteoclast differentiation, bone resorptive function, and survival. Our study also demonstrated that momordin I could down-regulate NF- κ B as well as AP-1 activation, which plays a critical role in osteoclast differentiation, by inhibiting I κ B degradation and c-Fos expression, respectively. Although further investigations are required to understand precisely which signaling pathway and what signaling components are engaged in the inhibitory effect at each stage of osteoclast differentiation, resorption, and survival responding to momordin I, it is likely that momordin I will prove a good candidate for controlling bone disorders.

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