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Structure-Activity Relationship of Anthocyanidins as an Inhibitory Effect on Osteoclast Differentiation

Narumi Hirata,[a] Tsukasa Tominari,[b] Ryota Ichimaru,[a] Keita Taniguchi,[b] Chiho Matsumoto,[b] Kenta Watanabe,[c] Michiko Hirata,[b] Sihui Ma,[d] Katsuhiko Suzuki,[e] Florian M.W. Grundler,[c,f] Chisato Miyaura,[a,b,c] and Masaki Inada*[a,b,c]

[a]*Cooperative Major of Advanced Health Science, Tokyo University of Agriculture and Technology, 2–24–16 Nakacho, Koganei-shi, Tokyo 184–8588, Japan;* [b]*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2–24–16 Nakacho, Koganei-shi, Tokyo 184–8588, Japan;* [c]*Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 2–24–16 Nakacho, Koganei-shi, Tokyo 184–8588, Japan;* [d]*Graduate School of Sport Sciences, Waseda University, 2–579–15 Mikajima Tokorozawa-shi, Tokyo 359–1192;* [e]*Faculty of Sport Sciences, Waseda University, 2–579–15 Mikajima Tokorozawa-shi, Tokyo 359–1192;* [f]*Institute of Crop Science and Resource Conservation, University of Bonn, Karlrobert-Kreiten-Strasse 13, 53115 Bonn, Germany*

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Anthocyanins are plant-derived pigments, and their aglycons are called anthocyanidin. Anthocyanidins have shown to exhibit various biological functions, such as anti-oxidant effects. However, their structure-activity relationship in bone tissue is not known. In this study, we examined the effects of three anthocyanidins, delphinidin, cyanidin and pelargonidin, on osteoclast differentiation and bone resorption to elucidate the structure-activity relationship. Anthocyanidins suppressed both IL-1 and LPS induced osteoclast differentiation in cocultures of bone marrow cells and primary osteoblasts, and bone resorbing activity in calvarial organ cultures. In osteoblasts, anthocyanidins inhibited prostaglandin (PG) E_2 production via the downregulation of membrane-bound PGE synthase (mPGES)-1, leading to the suppression of PGE_2-mediated receptor-activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) expression. In osteoclasts, anthocyanidins inhibited RANKL-induced osteoclast differentiation through the downregulation of osteoclast differentiation marker genes, nuclear factor of activated T-cells 1 (NFATc1), cathepsin K and tartrate-resistant acid phosphatase (TRAP). We further found that anthocyanidins suppressed the inhibitor of NF-κB kinase (IKK) activity *in vitro* assay, a signal component of NF-κB pathway, suggesting IKK was a novel target molecule of anthocyanidins. We found that delphinidin exerted the most potent inhibitory activity in these experiments, compared with cyanidin and pelargonidin. Anthocyanidins exhibits inhibitory activity in bone resorption, which may depend on the number of hydroxide residues.

Key words anthocyanidins, osteoclast, bone resorption, lipopolysaccharide

INTRODUCTION

Bone remodeling is precisely regulated by osteoblastic bone formation and osteoclastic bone resorption. Osteoclasts are the bone-resorbing cells differentiated from macrophage lineage cells, and excess activation of osteoclasts induced by inflammatory molecules results in bone-related diseases including periodontitis.[1)] Osteoclast differentiation is regulated by the interaction of receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) expressed on osteoblasts and RANK expressed osteoclast precursor cells. RANKL-RANK signalling activates various molecules including NF-κB and nuclear factor of activated T cells (NFATc) 1, a master transcription factor for osteoclast differentiation and downstream component of NF-κB.[2)] RANKL expression in osteoblasts is induced by various inflammatory molecules, such as interleukin (IL)-1 and lipopolysaccharide (LPS). LPS, an outer membrane component of gram-negative bacteria, is a major pathogenic molecule of the periodontitis and identified as a ligand of toll-like receptor (TLR) 4.[3)] We previously reported that LPS-TLR4 signalling induced membrane-bound prostaglandin E synthase (mPGES)-1-mediated PGE_2 production via NF-κB pathway, and PGE_2 induced RANKL expression in osteoblasts, leading to inflammatory bone resorption of alveolar bone in a model of periodontitis.[4)] PGE_2 is a major inflammatory mediator synthesized thorough arachidonic acid cascade in osteoblasts. In this cascade, free arachidonic acid is released from plasma membrane phospholipid by phospholipase (PL) A_2, and cyclooxygenases (COXs) converts free arachidonic acid into PGH_2. Subsequently, PGE_2 is synthesized from PGH_2 by PGE synthases (PGESs) and is secreted from cells. We also previously reported that PGE_2 recognized by PGE receptor (EP) 4 in osteoblasts induced RANKL expression and osteoclast differentiation.[5,6)]

Anthocyanins (ACNs), a member of flavonoids, are abundantly contained in reddish vegetables and fruits such as red

*To whom correspondence should be addressed. e-mail: m-inada@cc.tuat.ac.jp

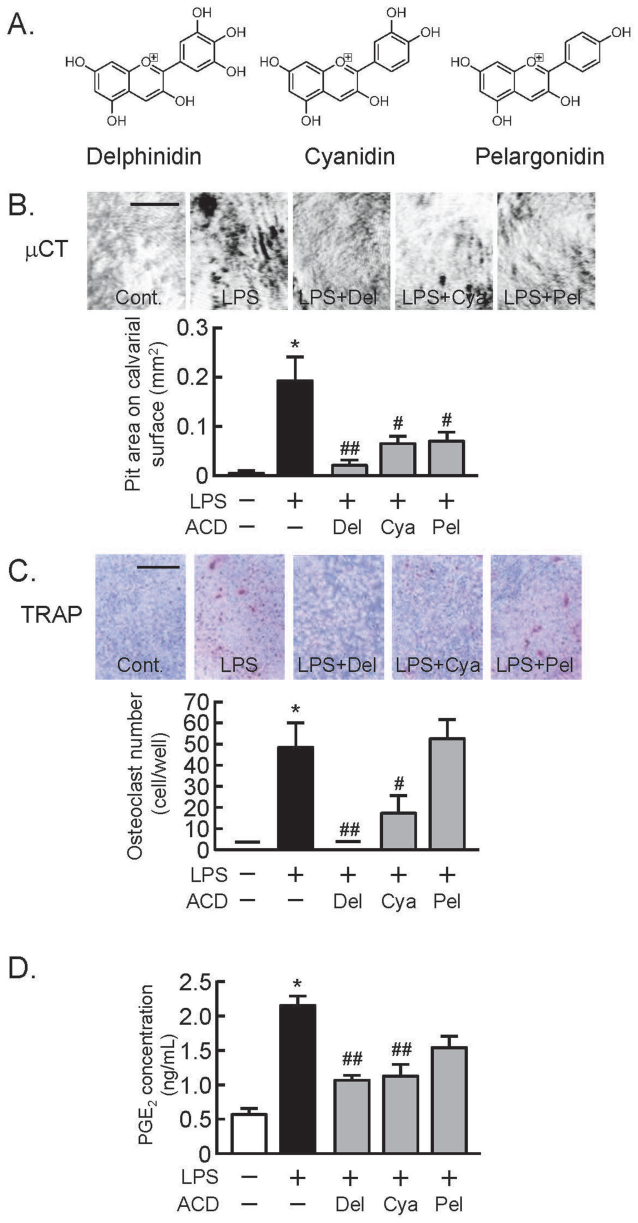

Fig. 1. Effects of ACDs on LPS-induced Bone Resorption and Osteoclast Differentiation

(A) Chemical structures of ACDs. (B) Mouse calvariae were cultured with or without LPS (1 μg/mL) and ACDs (120 μM) for 5 d. The area of bone pit on the calvarial surface was measured using μCT 3D images of calvaria. Bar = 0.5 mm. (C) Mouse BMCs and POBs were cocultured for 7 d with or without 120 μM ACDs and LPS (1 μg/mL). The number of TRAP-positive osteoclasts was counted. Bar = 1mm. (D) The level of PGE_2 in the conditioned medium of cocultures of BMCs and POBs was measured using EIA. Data are expressed as the mean ± S.E.M. of 3-4 independent wells. A significant difference between the two groups is indicated; *$P<0.01$ vs. Control, #$P<0.05$, ##$P<0.01$ vs. LPS.

cabbages, berries and red grapes. Their aglycons are called anthocyanidins (ACDs), and there are some structure variants including delphinidin, cyanidin, pelargonidin. Fig. 1A showed the structure of delphinidin, cyanidin and pelargonidin having 3, 2 and 1 hydroxyl groups on B ring, respectively. ACDs have been reported to exhibit anti-inflammatory and anti-oxidative effects, and the strength of their activities could relate to their structure.[7–9] Recently, Cheng *et al*.[10] have reported that cyanidin inhibited RANKL-induced osteoclast bone resorption via inhibiting RANKL-induced NF-κB, ERK (extracellular signal-regulated kinase) and NFAT activation and calcium oscillation, and intraperitoneally injection of cyanidin ameliorated estrogen deficiency-induced bone loss in ovariectomized (OVX) mice, an animal model for postmenopausal osteoporosis. Moriwaki *et al*.[11] have reported that RANKL-induced osteoclast differentiation was suppressed by delphinidin and cyanidin, and that activity of delphinidin were more potent than that of cyanidin. They also indicated that dietary delphinidin attenuated the bone loss in mouse models of osteoporosis induced by both soluble RANKL (sRANKL) injection and ovariectomy.[11] However, the molecular mechanism and structure-activity relationship of anthocyanidins are still unknown. In this study, we compared the effects of delphinidin, cyanidin and pelargonidin on osteoclast differentiation, and examined the molecular mechanisms of the ACDs in bone resorption.

MATERIALS AND METHODS

Animals and Reagents Newborn and 6-week-old *ddy* mice were obtained from Japan SLC Inc. (Shizuoka, Japan). Cyanidin chloride and delphinidin chloride were obtained from Tokiwa phytochemical. Co., Ltd. (Chiba, Japan). LPS and pelargonidin chloride were purchased from Sigma-Aldrich Co. LLC (MO, USA). A PGE_2 enzyme immunoassay (EIA) kit was obtained from GE healthcare UK Co., Ltd. (Buckinghamshire, UK). IKKβ assay kit was purchased from CycLex Co., Ltd. (Nagano, Japan). Soluble RANK ligand (sRANKL) was obtained from PeproTech Inc. (NJ, USA).

Organ Cultures of Mouse Calvariae Calvariae of newborn mouse were collected and precultured for 1 d in BGJb medium supplemented with 1 mg/mL of bovine serum albumin (BSA) at 37 °C under 5% CO_2 in air. Calvariae were treated with or without LPS (1 μg/mL) and ACDs (120 μM, each) and cultured for 5 d. The bone-resorbing activity was determined by measuring the calcium concentration in the conditioned medium by *o*-Cresolphtalein-Complexone (OCPC) methods. The images of absorbed pit areas were scanned by microfocus X-ray CT system (inspXio SMX-90T, Shimadzu Co., Ltd., Kyoto, Japan) and quantified using imaging software (Image J).

Cultures of Mouse Primary Osteoblasts Primary osteoblastic cells (POBs) were isolated from newborn mouse calvariae by 5 routine sequential digestions with enzyme cocktail of 0.1% collagenase (Roche Diagnostics GmbH, Mannheim, Germany) and 0.2% dispase (Roche Applied Science, Mannheim, Germany). POBs were cultured in α-modified MEM (αMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 in air.

Cocultures of POBs and Bone Marrow Cells Mouse bone marrow cells (BMCs) were collected from tibiae of 6-week-old mice. BMCs were cocultured with POBs with or without LPS (1 μg/mL) and ACDs (120 μM, each) for 7 d, and osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated osteoclasts were counted as osteoclasts.

Measurement of PGE_2 Concentration PGE_2 concentration in conditioned medium were measured by an enzyme immunoassay system (EIA) kit (GE Healthcare UK, Ltd., Little Chalfont, UK). The cross-reactivity of the antibody in the EIA was calculated as follows: PGE_2 100%; PGE_1 7%; 6-keto-$PGF_{1\alpha}$ 5.4%; $PGF_{2\alpha}$ 4.3%; and PGD_2 1%.

Quantitative PCR Analysis To determine the mRNA expression, total RNA was extracted from cells and cDNA

was synthesized by reverse transcriptase (Superscript II Preamplification System, Thermo Fisher Scientific Inc., CA, USA) and amplified using quantitative real-time PCR (qPCR). The qPCR analysis (ΔΔCT methods) was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., CA, USA). The sequences of primers described in the following; mouse RANKL, (forward) 5'-AGGCTGGGCCAAGATCTC-TA-3', (reverse) 5'-GTCTGTAGGTACGCTTCCCG-3'; mouse NFATc1, (forward) 5'-AGTCTCTTTCCCCGACATCA-3', (reverse) 5'-CACCTCGATCCGAAGCTC-3'; mouse cathepsin K (CTSK), (forward) 5'-GCCTAGCGAACAGATTCTCAA-3', (reverse) 5'-CACTGGGTGTCCAGCATTT-3'; mouse TRAP, (forward) 5'-CTTCCCCAGCCCTTACTACC-3', (reverse) 5'-GAGTTGCCACACAGCATCAC-3'; mouse β-actin, (forward) 5'-CCCCATTGAACATGGCATTG-3', (reverse) 5'-ACGACCAGAGGCATACAGG-3'. Beta-actin was used as normalized gene.

Dual-luciferase Reporter Gene Assay For reporter gene assay, plasmid pNF-κB-TA-Luc (0.4 μg) containing four tandem copies of the NF-κB consensus sequence with the firefly luciferase reporter gene (Clontech Laboratories, Inc., CA, USA) and the pGL4.74 [hLuc/TK] plasmid (40 ng) containing the renilla luciferase reporter gene (Promega Corp., WI, USA) as an internal control reporter vector were transfected into POBs using Lipofectamine 2000 (Thermo Fisher Scientific Inc.). The luciferase activity was measured with the Dual-luciferase reporter assay system (Promega Corp.) using ARVO MX multilabel/luminescence counter (Perkin Elmer Co., Ltd., MA, USA).

IκB Kinase Activity *In Vitro* Assay The activities of inhibitor of NF-κB kinase (IKK) was measured using IKKα and β Kinase Assay/Inhibitor Screening Kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

Protein Structure Preparation The three-dimensional X-ray crystal structure of IKKβ was obtained from protein data bank (PDB ID:4KIK, 2.83 Å resolution).[12] For docking simulations, default parameters (H-atoms) were added into the protein structures using AutoDock Tools (Molecular Graphics Laboratory, Torrey Pines Rd. LaJolla, CA, USA).

Ligand Structure Preparation The chemical structure of delphinidin was built and optimized using an online compound editor InDraw (http://in.indraw.integle.com/; Integle Chemistry, Inc., Shanghai, China). All the 2D structures were converted into a three-dimensional structure in the form of pdb format and saved as mol format using Open Babel (http://www.openbabel.org/).[13]

Molecular Docking Study The protein–ligand molecular docking study was performed by using AutoDock Vina.[14] Subsequently, the AutoDock Vina was employed to implement fast docking of inhibitor ligand into the active pocket of both IKKβ and kinase domains, which considered flexibility and mobility of the ligand molecules and protein active-site residues, and used Lamarckian genetic algorithm to fully explore conformational space for IKKβ inhibitor interactions. The rotational bonds of the protein were kept rigid,while those of the ligands were treated as flexible. Grid box was set at 60 × 60 × 60 on the x-axis,y-axis, and z-axis. The amino acids Leu21- Tyr 23, Val29, Ala42, Lys44, Glu61, Val74, Met96, Glu97, Tyr98, Cys99, Gly102, Asp103, Glu149, Asn150, Val152, Ile165, Asp166 and the surrounding residues within the distance range of 6.5 Å were defined as active sites.

For the analysisand visualization of protein–ligand interaction, Pymol (http://apbs.sourceforge.net) was used. Amino acids of active sites and the surrounding residues was selected to show, other parts are hided.

Cultures of Raw264.7 Cells To examine the differentiation of osteoclast precursor cells into mature osteoclasts, Raw264.7 cells, mouse macrophage cell line, were cultured with or without sRANKL (100 ng/mL) and ACDs (120 μM, each) for 5 d in 96 well plate. To examine the effects of ACDs on the survival of mature osteoclasts, Raw264.7 cells were cultured with sRANKL for 5 d to form mature osteoclasts in 24 well plate, and further cultured with sRANKL and ACDs for 1 d. Osteoclasts were detected by TRAP staining.

Statistical Analysis All data were analysed using one-way ANOVA, followed by Tukey's test for *post hoc* analysis using IBM SPSS Statistics Ver.23 software (NY, USA).

RESULTS

Effects of ACDs on LPS-induced Osteoclast Differentiation and Bone Resorption We first examined the effects of delphinidin (Del) on RANKL-induced osteoclast differentiation in Raw264.7 cells, and found that 30-120 μM Del dose-dependently suppressed osteoclast formation (supplemental data). Then, we used 120 μM ACDs in the present study. We examined the effects of three ACDs, Del, cyanidin (Cya) and pelargonidin (Pel) (Fig. 1A), on bone resorption in organ cultures of mouse calvariae and osteoclast differentiation in cocultures of POBs and BMCs. To elucidate the bone resorbing activity, the area of bone pit was measured using μCT imaging of calvarial surface. LPS markedly increased the area of pits on calvarial surface, but the pit area was significantly reduced by ACDs (Fig. 1B). In cocultures of BMCs and POBs, Del and Cya significantly suppressed LPS-induced osteoclast differentiation, however, Pel showed no effects (Fig. 1C). We further measured the levels of PGE_2, an inflammatory mediator, in the conditioned medium of cocultures. ACDs markedly inhibited PGE_2 production induced by LPS (Fig. 1D). These data suggested that ACDs inhibited bone resorption and osteoclast differentiation via the inhibition of PGE_2 production.

Effects of ACDs on PGE_2 Production and RANKL Expression in Osteoblasts To examine the molecular mechanisms of ACDs in osteoblasts, POBs were cultured with or without LPS and ACDs. PGE_2 production was induced by LPS but ACDs significantly inhibited LPS-induced PGE_2 production (Fig. 2A). In qPCR analysis, ACDs significantly downregulated LPS-induced mRNA expression of RANKL (Fig. 2B). In reporter gene assay, LPS enhanced NF-κB activiy, however, ACDs blocked its NF-κB activation (Fig. 2C).

Docking Simulation of Delphinidin and ATP Binding Pocket in IKKβ Some natural compounds including flavonoids might bind to ATP-binding pocket of IKKβ to downregulate its kinase activity.[15,16] IKKβ phosphorelated IκBα, an endogenous NFκB inhibitor, and phospho-IκBα are degradated by ubiquitin-proteasome system, leading to nuclear transport of NFκB . In *in vitro* assay using recombinant IKK without cells, ACDs directly acted on IKK and suppressed its kinase activity (Fig. 2E). In order to get hints about possible binding model of Del, Del was submitted to molecular docking study using a homology model of IKKβ. Fig. 3B showed the overview of the docking simulation workflow. The docking study suggested a blockage caused by Del. As shown in the Fig. 3C, the amino acids Leu21- Tyr 23, Val29, Ala42, Lys44, Glu61,

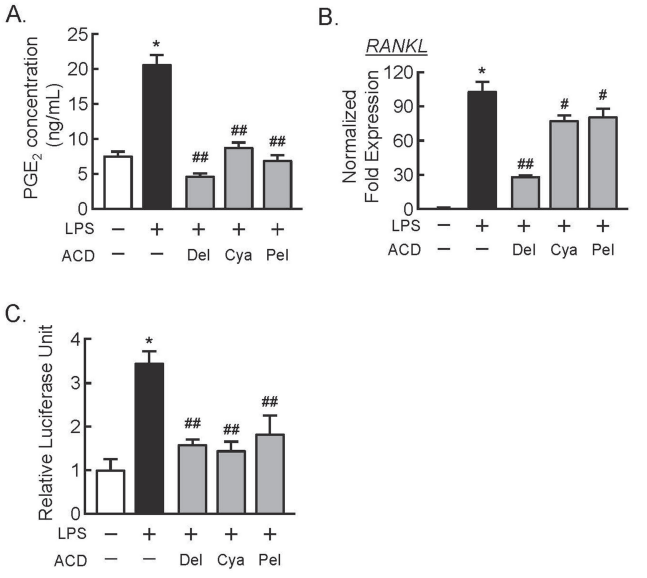

Fig. 2. Effects of ACDs on PGE_2 Production and RANKL Expression in Osteoblasts

(A) Mouse osteoblasts were treated with or without LPS (1 μg/mL) and ACDs (120 μM). The level of PGE_2 in the conditioned medium of cocultures of BMCs and POBs was measured using EIA. The data are expressed as the mean ± S.E.M. of 3 independent wells. (B) Total RNA of POBs was extracted, and the mRNA expression of RANKL was analysed using qPCR analysis. Beta-actin was used as the normalized gene. Data are expressed as the mean ± S.E.M. of 4 replicated wells in quadruplicate. (C) Mouse osteoblasts were transfected with pNFκB-TA-Luc or pGL4.74 [hLuc/TK] vectors. Osteoblasts were cultured with or without LPS (1 μg/mL) and ACDs (120 μM), and NFκB activation was measured by the luciferase assay. The data are expressed as the mean ± S.E.M. of 3-4 independent wells. A significant difference between the two groups is indicated; **P<0.01* vs. Control, *#P<0.05, ##P<0.01* vs. LPS.

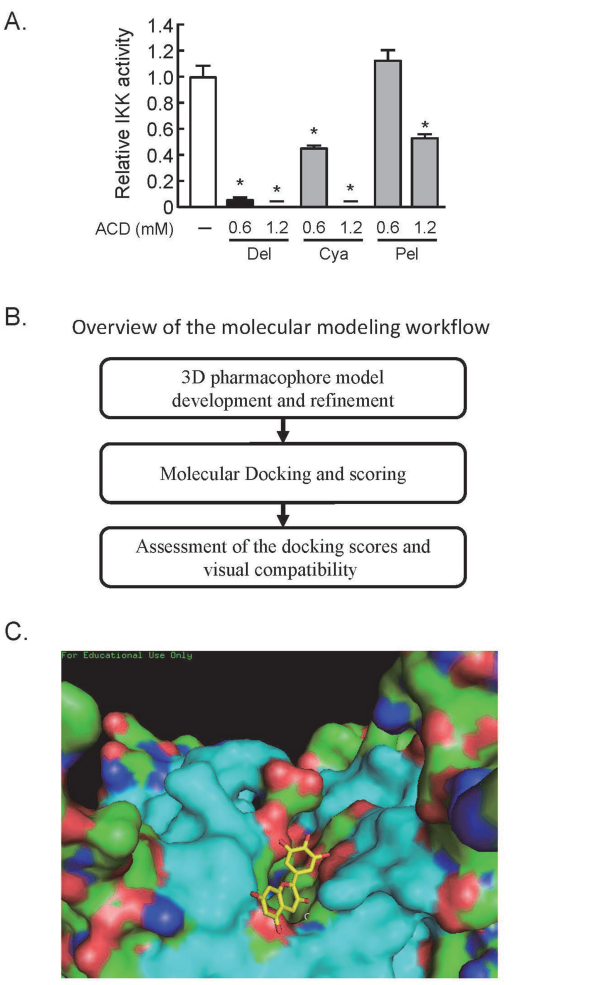

Fig. 3. Docking Simulation of Delphinidin and ATP Binding Pocket in IKKβ

(A) IKK activity was measured in the presence or absence of ACDs (0.6, 1.2 mM) by *in vitro* IKKβ assay kit using IKKβ, IκBα, and anti-phospho-IκBα antibody. IKK activity was expressed as relative activity of the control without ACDs. The data are expressed as the mean ± S.E.M. of 3 independent wells. A significant difference between the two groups is indicated; **P<0.01* vs. Control. (B) Overview of the molecular modelling workflow for docking simulation between Del and IKKβ. (C) Predicted binding pose of Del docked into a homology model of IKKβ. The 3D representation of the ligand binding pose was shown with the binding surface (molecular surface around active pocket was marked as light blue).

Val74, Met96, Glu97, Tyr98, Cys99, Gly102, Asp103, Glu149, Asn150, Val152, Ile165, Asp166 and the surrounding residues within the distance range of 6.5 Å were defined as active sites and the surrouding area was marked as light blue using pymol software. Del was positioned in catalytic center, a pocket-like structure of the assumed binding site (Fig. 3C).

Effects of ACDs on RANKL-induced Osteoclast Differentiation We examined the effects of ACDs on osteoclast precursor cells. In Raw264.7 cell cultures, ACDs significantly inhibited sRANKL-induced osteoclast differentiation (Fig. 4A). To test the effects of ACDs on mature osteoclasts, ACDs added to cultures after sRANKL induced osteoclast differentiation in Raw 264.7 cells. ACDs also blocked sRANKL-induced osteoclast survival (Fig. 4B). In qPCR analysis, sRANKL upregulated mRNA expression of NFATc1, CTSK and TRAP, but ACDs attenuated the expression of these genes in Raw 264.7 cells (Fig. 4C).

DISCUSSION

The structure-activity relationship of natural compounds is important to understand their biological activities. In the present study, we newly indicated that Del, Cya and Pel inhibited bone resorption and osteoclast differentiation with a structure-activity relationship. Recent studies have demonstrated the effects of Del and Cya on osteoclast differentiation and bone resorption. Cheng *et al*.[10] have found that Cya inhibited RANKL-induced osteoclast differentiation via the attenuation of ERK and NFATc1 activation, and further indicated that the intraperitonially injection of cyanidin prevented tibial bone loss in OVX mice. Moriwaki *et al*.[11] have reported that Del and Cya have been reported to inhibit RANKL-induced osteoclast differentiation in Raw264.7 cells. Hou *et al*.[8] have reported to compare the effects of five ACDs on cyclooxygenase (COX)-2 expression in macrophages with a structure-activity relationship. They tested five ACDs, Del (3 hydroxyl groups on B ring), Cya (2 hydroxyl groups on B ring), Pel (1 hydroxyl group on B ring), peonidin (1 hydroxyl and 1 methoxy groups on B ring) and malvidin having two methoxy group on B ring. Although Pel, peonidin and malvidin had no effect, Del and Cya significantly reduced LPS-induced COX2 expression in macrophages, and the effect of Del is higher than that of Cya. Since reactive oxygen species (ROS) is crucial factor for osteoclast differentiation, antioxidative activity of ACDs may contribute to the inhibitory effects of osteoclast differentiation.[17] Ali *et al*. have examined the structure-activity relationships of six ACDs, Del, petunidin, Cya, malvidin, peonidin and Pel, for antioxidative effects.[18] The antiox-

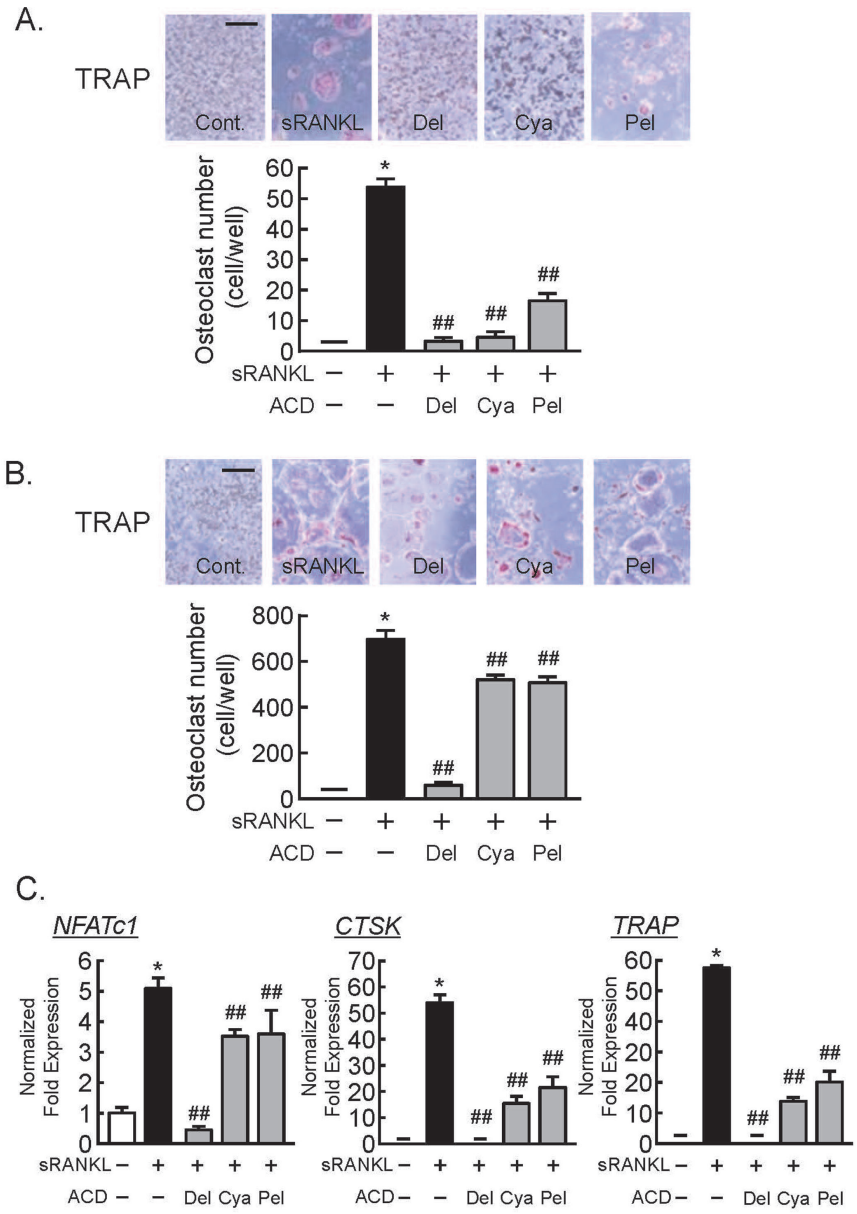

Fig. 4. Effects of ACDs on RANKL-induced Osteoclast Differentiation and Survival

(A) Raw264.7 cells were cultured for 5 d with or without sRANKL (100 ng/mL) and ACDs (120 μM). The number of TRAP-positive osteoclasts was counted. Data are expressed as the mean ± S.E.M. of 4 independent wells. Bar = 200 μm. (B) Raw264.7 cells were cultured for 4 d with sRANKL (100 ng/mL), then added ACDs (120 μM) and cultured for further 1 d. The number of TRAP-positive osteoclasts was counted. Data are expressed as the mean ± S.E.M. of 4 independent wells. Bar = 200 μm. (C) Total RNA was extracted in the cultures of Raw264.7 cells treated with sRANKL (100 ng/mL) and ACDs (120 μM), and the mRNA expression of NFATc1, CTSK and TRAP was analysed using qPCR analysis. Data are expressed as the mean ± S.E.M. of 3 replicated wells in triplicate. A significant difference between the two groups is indicated; A significant difference between the two groups is indicated; **P<0.01* vs. Control, *##P<0.01* vs. sRANKL.

idative activity of these ACDs were depend on the number of hydroxyl and/or methoxy groups, and their strengths of antioxidative activity were described as follows: Del (3 hydroxyl groups on B ring) > petuidin (2 hydroxyl and 1 methoxy groups on B ring) > Cya (2 hydroxyl groups on B ring) > malvidin (1 hydroxyl and 2 methoxy groups on B ring) > peonidin (1 hydroxyl and 1 methoxy groups on B ring) > Pel (1 hydroxyl group on B ring). Our data showed the similar trend: Del showed the most potent activities on inhibition of osteoclast differentiation and bone resorption while that of Pel had lower activities among three ACDs.

In the molecular mechanisms of ACDs, Dou *et al.*[19] have shown that Cya had the stimulatory effect on RANKL-induced osteoclast differentiation and fusion at the low concentration (< 1 μg/mL) by upregulating the expression of osteoclast marker genes (NFATc1, Mitf and c-Fos) and liver X receptor (LXR)-β, whereas high concentration of Cya (> 10 μg/mL) showed the inhibitory effect by downregulating the expression of osteoclast marker genes. LXR-β was reported to be negative regulator of RANKL-induced osteoclast differentiation,[20] and Cya was shown to be direct agonistic ligand of LXR-β.[21] These reports suggest that LXR-β could be an intracellular target molecule of Cya to inhibit osteoclast differentiation. Our present study indicated IKKβ was the novel target molecule of ACDs, and ACDs directly attenuated IKKβ activities with a structure-activity relationship. Kim *et al.*[15] have suggested that luteolin, one of flavonoid, might bind to ATP-binding pocket of IKKβ to downregulate its kinase activity. Our dock-

ing simulation of Del and IKKβ indicated that ACDs could bind to ATP-binding pocket of IKKβ to suppress its kinase activity. Considering the similarity of the structure of Del and K252a, a known ATP-competitive IKKβ inhibitor,[12] Del might contact with the hinge region of IKKβ by forming a hydrogen bond between the Del and IKKβ. Since IKK is required for both signalling of LPS-TLR4-NF-κB and RANKL-RANK-NF-κB, ACDs may block IKK-dependent NF-κB activation in both osteoblasts and osteoclasts, leading to the suppression of osteoclastic bone resorption. Since bone turnover is regulated by osteoblastic bone formation and osteoclastic bone resorption, it is possible that ACDs may act on osteoblasts to regulate bone formation. We will plan to examine the effects of ACDs on osteoblast differentiation in the near future.

In conclusion, we have shown that the activities of ACDs on osteoclastic bone resorption is dependent on the total number of hydroxyl group on B ring, and IKK may be the novel intracellular target molecule of ACDs. Del may be the most beneficial compounds for preventing of bone destructive diseases in three major ACDs, Del, Cya and Pel.

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Conflict of interest The authors declare no conflict of interest.

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