Chlorogenic Acid Inhibits Osteoclast Differentiation and Bone Resorption by Down-Regulation of Receptor Activator of Nuclear Factor Kappa-B Ligand-Induced Nuclear Factor of Activated T Cells c1 Expression

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Excessive osteoclastic bone resorption plays a critical role in inflammation-induced bone loss such as rheumatoid arthritis and periodontal bone erosion. Therefore, identification of osteoclast targeted-agents may be a therapeutic approach to the treatment of pathological bone loss. In this study, we isolated chlorogenic acid (CGA) from fructus of Gardenia jasminoides to discover anti-bone resorptive agents. CGA is a polyphenol with anti-inflammatory and anti-oxidant activities, however, its effects on osteoclast differentiation is unknown. Thus, we investigated the effect of CGA in receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL)-induced osteoclast differentiation and RANKL signaling. CGA dose-dependently inhibited RANKL-mediated osteoclast differentiation in bone marrow macrophages (BMMs) without any evidence of cytotoxicity. CGA inhibited the phosphorylation of p38, Akt, extracellular signal-regulated kinase (ERK), and inhibitor of nuclear factor-kappa B (IkB), and IkB degradation by RANKL treatment. CGA suppressed the mRNA expression of nuclear factor of activated T cells c1 (NFATc1), TRAP and OSCAR in RANKL-treated bone marrow macrophages (BMMs). Also, overexpression of NFATc1 in BMMs blocked the inhibitory effect of CGA on RANKL-mediated osteoclast differentiation. Furthermore, to evaluate the effects of CGA in vivo, lipopolysaccharide (LPS)-induced bone erosion study was carried out. CGA remarkably attenuated LPS-induced bone loss based on micro-computed tomography and histologic analysis of femurs. Taken together, our findings suggest that CGA may be a potential treatment option for osteoclast-related diseases with inflammatory bone destruction.

Key words chlorogenic acid; osteoclast; differentiation; nuclear factor of activated T cells c1; receptor activator of nuclear factor kappa B ligand; lipopolysaccharide-induced bone destruction

The control of bone loss is a key way ameliorating the quality of life of patients with disorders related to excessive bone resorption.Bone remodeling is maintained by the delicate balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption.1,2 The disruption of balance causes most often by over-activated osteoclast, results in fragile bones.2,3 Through the bone resorbing activity, osteoclasts play a crucial physiological role in bone remodeling and also a pathological role in disease involving abnormal inflammation-induced bone lysis such as rheumatoid arthritis and periodontal bone erosion.2,3)

Osteoclasts are bone-resolving multinucleated giant cells derived from monocyte lineage of hematopoietic cells. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) are essential for osteoclast formation from osteoclast precursors. RANKL, a member of the tumor necrosis factor (TNF) family, promotes osteoclast formation from osteoclast precursors in the presence of M-CSF.4 Binding of RANKL to its receptor RANK activates multiple downstream signaling pathways, including NF-κB, mitogen activated protein (MAP) kinases, and activates two major transcription factors, c-Fos and nuclear factor of activated T cells c1 (NFATc1), that required for successful osteoclast differentiation.5,6)

Several compounds derived from natural products have been recently reported to possess inhibitory effects on osteoclast differentiation and function, leading to decreased bone loss in vivo. Examples from the current literature include curcumin (a compound derived from Curcuma aromatica),7,8 Stewartia koreana extract (Danshen extract known as tanshino JIA),9,10 an undefined chloroform extract of young deer antlers10 and berberine (an element of Coptidis Rhizoma).11,12 To discover new compounds that can act as anti-resorptive agents, we screened natural compounds that regulate osteoclast differentiation and identified chlorogenic acid (CGA). CGA is the ester of caffeic acid and quinic acid in shikimate pathway, which is commonly found in some plants, such as honeysuckle, Cortex Eucommiae, Semen Coffea Arabica, and green tea. We isolated from fructus of Gardenia (G.) jasminoides and it is a type of polyphenol with anti-inflammatory and antioxidant activities. However, the effects of CGA on RANKL-induced osteoclast differentiation are not been studied.

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In the present study, we investigated the effects of CGA on signaling pathways involved in osteoclast differentiation and activation and the in vivo effects of CGA in mice with lipo-poly saccharide (LPS)-induced bone erosion.

MATERIALS AND METHODS

Isolation and Purification of Chlorogenic Acid (CGA) from *Gardenia fructus* The dried fructus of *G. jasminoides* (18 kg) were purchased from a herbal store in Seoul, Korea. The fructus of *G. jasminoides* were extracted with MeOH for 7 d at room temperature. The MeOH extract was concentrated in a rotary evaporator under reduced pressure and suspended in distilled water and then successively fractionated into hexane, ethyl acetate and aqueous fractions. The ethyl acetate fraction was chromatographed on a Sephadex LH-20 (GE Healthcare, U.S.A.) using a MeOH solvent to give 9 fractions. For further purification, the subfraction F 4e was subjected to reverse phase column chromatography (Merek, LiChroPrep RP18) with step gradient elution using MeOH/H2O (40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0) to give chlorogenic acid. The 1H-NMR (500MHz) and 13C-NMR (125 MHz) spectra were obtained on a JEOL ECS400 spectrometer, with CD3OD and pyridine-d6 as a solvent. The electrospray ionization-mass spectrum (ESI-MS) was determined using an Agilent 6430 LC/MS/MS and 1100 LC/MS spectrometer.

Mice and Reagents Male, 5-week-old ICR mice were purchased from Damul Science (Daejeon, Korea) and housed in controlled temperature (22–24°C) and humidity (55–60%) with 12 h light/dark cycles. All experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University. Recombinant soluble human M-CSF and human RANKL were obtained from PeproTech EC Ltd. (London, U.K.). Anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-ERK 1/2, anti-p38, and anti-phospho-p38, anti-IκB, and anti-phospho-IκB antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal α-actin antibody was obtained from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS), α-minimum essential medium (α-MEM), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, U.S.A.). All other chemicals were of analytical grade or complied with the standards required for cell culture experiments.

Mouse Bone Marrow Macrophage Preparation and Osteoclast Differentiation Bone marrow cells were obtained by flushing the femurs and tibiae of 5-week-old ICR mice with α-minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, U.S.A.) and suspended in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, U.S.A.). Non-adherent cells were collected and cultured for 3 d in the presence of M-CSF (30 ng/mL). Floating cells were discarded and adherent cells on dish bottoms were classified as bone marrow derived macrophages (BMMs). BMMs were seeded at 3.5 × 10⁴ cells/well in α-MEM/10% FBS and cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 d in the presence or absence of CGA. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) activity, as described below. TRAP-positive multinucleated cells with greater than three nuclei were counted as osteoclasts.

Cytotoxicity Assay BMMs were plated in 96-well plates at a density of 1 × 10⁵ cells/well in triplicate. Cells were treated with M-CSF (30 ng/mL) and increasing concentrations of CGA were added to the mix. After 3 d, XTT reagent (50 μL) was added to each well. Wells were incubated for 4 h. The optical density at 450 nm was analyzed with an enzyme-linked immunosorbent assay (ELISA) reader.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was isolated with QIAzo1 reagent (Qiagen, Valencia, CA, U.S.A.) according to manufacturer’s instructions. RNA (1 μg) was reverse transcribed using oligo dT primers (10 μg) and deoxyribonucleotide triphosphates (dNTPs) (10 μM). The mixture was incubated at 65°C for 5 min, and cDNA was produced by incubating at 42°C for 50 min with first strand buffer (50 μM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 100 μM dithiothreitol (DTT), RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). The cDNA was amplified using the following primer sets: NFA Tc1, 5’-TCG AAA GAC GAC ACT GGA GAC-3’ (forward) and 5’-GCT GCC TCT CGT CTC ATG-3’ (reverse); TRAP, 5’-GGA GTG CAC GAT GGC ACC GAC A-3’ (forward) and 5’-CCG TGC TCG GCG ATG GAC CAG A-3’ (reverse); OSCAR, 5’-CTG CTC GTG GTA ACG GAT CAG CCT CCC AGA-3’ (forward) and 5’-CCA AGG AGC CAC AAC CTT CGA AACT-3’ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-ACC ACA CAG TCT GAC ATC AC-3’ (forward) and 5’-TCC ACC ACC TGG TCT GGT GA-3’ (reverse). Real-time RT-PCR was conducted using a Exicyclerm1™ 96 Real-Time Quantitative Thermal Block (Bioneer Co., Daejeon, Korea) in a 20 × 1 reaction mixture containing 10 × SYBR Green Premix (Bioneer Co.), 10 pmol forward primer, 10 pmol reverse primer, and 1 μg cDNA. The amplification parameters consisted of initial denaturation at 95°C for 5 min and 40 cycles of 3-step PCR (denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min). The fluorescence resulting from the incorporation of SYBR Green 1 dye into the double-stranded DNA produced during the PCR and the emission data were quantified using the threshold cycle (Ct) value. Relative levels of NFA Tc1, TRAP, and OSCAR were normalized to GAPDH.

Western Blot Analysis Cells were lysed in a buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. The lysates were centrifuged at 14000 × g for 20 min and supernatants were collected. Protein concentrations of supernatants were determined and resolved by 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). Non-specific interactions were blocked with 5% skim milk for 1 h and were then probed with the appropriate primary antibodies. Membranes were incubated with the appropriate secondary antibodies attached to horseradish peroxidase, and immunoreactivity was detected with enhanced chemiluminescence reagents (Millipore). Densitometric values were quantified for each band with the Image Pro-plus program version 4.0.
Retrovirus Preparation and Infection. The retroviral vectors pMX-internal ribosome entry site-enhanced green fluorescent protein (pMX-IRES-EGFP), and pMX-constitutively active (CA)-NFATc1-IRES-EGFP packaging were performed by transient transfection of these pMX vectors into Plat-E retroviral packaging cells using X-tremeGENE 9 (Roche, Nutley, NJ, U.S.A.) according to the manufacturer’s protocol. After incubation in fresh medium for 2 d, the culture supernatants of the retrovirus-producing cells were collected. For retroviral infection, non-adherent BMCs were cultured in M-CSF (30 ng/mL) for 2 d. The BMMS were incubated with viral superna-

Fig. 1. The Structure of Chlorogenic Acid

Fig. 2. CGA Inhibited RANKL-Induced Osteoclast Differentiation

(A) BMMs were cultured for 4 d with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence of varying concentrations of CGA. Cells were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP solution. (B) TRAP-positive cells were counted as osteoclasts. (C) CGA was treated at indicated time point for 1 d with M-CSF (30 ng/mL) and RANKL (50 ng/mL) and TRAP staining was carried out. (D) TRAP-positive cells were counted as osteoclasts. (E) BMMs were cultured for 3 d at the indicated doses of CGA in the presence of M-CSF (30 ng/mL). Cell viability was determined by XTT assay. *p<0.05, **p<0.01, ***p<0.001 versus the control.
tant pMX-IRES-EGFP, and pMX-CA-NFATc1-IRES-EGFP virus-producing Plat-E cells together with polybrene (10 μg/mL) and M-CSF (30 ng/mL) for 6 h. The infection efficiency of the retrovirus was determined by green fluorescent protein expression, and was always >80%. After infection, the BMMs were induced to differentiate in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 d. The forced expression of each construct and subsequent osteoclast formation were detected using a fluorescence microscope and TRAP staining, respectively.

**Mouse Model of Lipopolysaccharide-Mediated Bone Erosion and Micro-Computed Tomography and Histology**

The mice were divided into phosphate-buffered saline (PBS)-treated (Control), LPS-treated and LPS and CGA-treated. CGA (10 mg/kg) or PBS was administered orally one day before LPS injection (5 mg/kg) and continued for further every other day for 8 d. LPS was injected intraperitoneally on days 1 and 4. The mice were euthanized after 8 d of LPS injection and the left femurs were subjected to high-resolution microcomputed tomography (micro-CT) analysis. The images were analyzed using VGStudio MAX 1.2 software (Volume Graphics Inc., Germany) to calculate bone volume and bone parameters. The bone volume ratio, represented by bone volume/tissue volume (BV/TV) was used to assess the trabecular bone microstructure of the femurs. Tissues were removed and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 1 d at 4°C and were then decalcified in 12% EDTA. Decalcified bones were paraffin-embedded and sectioned. For histologic examination, sections were stained with hematoxylin and eosin (H&E) and another section was stained with TRAP to identify osteoclasts on the bone surface. Parameters for bone resorption included eroded surface per bone surface (ES/BS, %), number of osteoclasts per field of tissue were quantified by using the Image Pro-Plus program (version 4.0; Media Cybernetics).

**Statistical Analysis**

Each experiment was performed at least three times and all quantitative data are presented as mean±standard deviation (S.D.). All statistical analyses were performed using SPSS (Korean version 14.0). Student’s t-test was used to compare the parameters between 2 groups, while the analysis of variance (ANOVA) test, followed by the Tukey post-hoc test was used to compare the parameters among 3 groups. A p<0.05 was considered statistically significant.

**RESULTS**

**CGA Inhibits RANKL-Mediated Osteoclast Differentiation in BMMs**

As shown in Fig. 1, CGA is a phenolic compound formed by the esterification of cinnamic acid, such as caffeic, ferulic, and p-coumaric acid, with (−)-quinic acid. To investigate the effects of CGA on osteoclastogenesis, we treated primary BMMs with CGA in the presence of RANKL and M-CSF. While the BMMs of the control group differentiated into mature TRAP-positive multinucleated osteoclasts, CGA dose-dependently reduced the formation and numbers of TRAP-positive multinucleated cells (Figs. 2A, B). To study the time-course effects of CGA on RANKL-mediated osteoclast differentiation, BMMs were challenged with CGA at indicated time points after RANKL treatment. It was found that the inhibitory effect of CGA on osteoclast differentiation could be obtained when BMM cells treated with CGA at all-time points after RANKL stimulation. In particular, CGA most effectively inhibited RANKL-mediated osteoclast differentiation when treated 2 d after RANKL stimuli (Figs. 2C, D). Next, we checked the effects of CGA on bone marrow cells with the XTT assay to exclude the possibility that the inhibition was due to the reduced viability and/or proliferation of the osteoclast precursor cells. The results revealed that CGA did not exhibit cytotoxic effects at the doses that effectively inhibited osteoclast differentiation (Fig. 2E).

**CGA Inhibits RANKL-Mediated Signals in BMMs**

RANKL activates a variety of signal transducers that are involved in osteoclastogenesis including p38, JNK, Erk, and NF-κB which are recognized as the key transcriptional factor of osteoclast differentiation. To investigate the mechanism underlying the inhibition of RANKL-induced osteoclast differentiation by CGA, we investigated the effect of CGA on RANKL-induced early signaling pathways, including p38, JNK, ERK, and transcriptional factor, NF-κB. Different signaling pathways were observed after osteoclast precursors were pretreated with indicated concentrations of CGA for 1 h and stimulated with RANKL for 7 min. We found that phosphorylation of p38, JNK, Akt, and IκB, and degradation of IκB by RANKL were all significantly inhibited by CGA (Fig. 3).

**CGA Inhibits Osteoclast Differentiation through NFATc1 Suppression**

NFATc1 has an essential role in the osteoclast differentiation and regulates the expression of osteoclast-specific genes, such as OSCAR and TRAP. To assess whether CGA have any effects on NFATc1 expression, the effects of CGA on the RANKL-induced regulation of NFATc1 expression was examined. Osteoclast precursors were treated with CGA and further stimulated with RANKL at indicated time points. We found that mRNA and protein
levels of NFATc1 were increased in response to RANKL and the increased NFATc1 expression was significantly inhibited by CGA (Figs. 4A, B). TRAP and OSCAR mRNA expression was significantly inhibited by CGA (Fig. 4A). Furthermore, to examine whether ectopic NFATc1 expression can reverse the effect of CGA on osteoclast differentiation, we overexpressed the constant active form of NFATc1 (CA-NFATc1) in BMMs. Overexpression of CA-NFATc1 reversed the CGA-mediated inhibition of osteoclast differentiation (Fig. 4C). These results suggest that the CGA suppresses transcription of NFATc1 during RANKL-mediated osteoclastogenesis in BMMs.

**CGA Suppresses LPS-Mediated Bone Loss in Mice** We investigated the in vivo effects of CGA with an experimental animal model of bone erosion. LPS was injected into peritoneum and CGA was orally administered to mice. The mice were euthanized 8d after the first LPS injection with/without CGA treatment and femurs underwent micro-CT analyses. A 3-dimensional visualization of the femoral area revealed the massive loss of trabecular bone following LPS treatment while LPS-induced bone loss was remarkably suppressed in the femurs of CGA-treated, LPS-injected mice (Fig. 5A). On morphometric analyses of the femurs from LPS-treated mice, the trabecular bone volume per tissue volume (BV/TV) and the trabecular bone thickness (Tb.Th), except trabecular bone number (Tb.N), were significantly recovered by CGA treatment (Fig. 5B). On histological analysis, it was found that the LPS-induced bone loss was greatly ameliorated in the femurs of CGA-treated mice (Fig. 5C). These results suggest that CGA inhibits inflammation-mediated bone loss in vivo.
The prevention or therapeutic treatment of loss of bone mass is an important means of improving the quality of life for patients with disorders related to osteoclast-mediated bone loss. The major modalities currently used in treatment of bone loss primarily include estrogen replacement therapy along with bisphosphonates (e.g., alendronate, risedronate), selective estrogen receptor modulators (SERM) and calcitonin. However, owing to such therapies are associated with adverse effects, including breast cancer, endometritis, thromboembolism, hypercalcemia, GI problems and hypertension, efforts have been to find natural products with no adverse side effect on prevention and/or treatment of bone loss. Due to the abundance of polyphenol in dietary products and their beneficial pharmacological properties, many polyphenols are of considerable interest as therapeutic agents as well as health food supplements. Also, several reports have shown that bone destruction was associated with inflammatory diseases such as periodontitis and rheumatoid arthritis. Inflammatory cytokines and prostaglandins up-regulates RANKL in osteoblasts, synovial fibroblasts and activated T cells. The enhanced RANKL expression then stimulates osteoclast formation, resorption activity, and survival. Therefore, we screened for anti-osteoclastogenic components from a focused pool of anti-inflammatory compounds.

CGA is a naturally occurring phenolic acid with anti-inflammatory, antioxidant activities in human diets including many types of fruit and coffee. Because there are some reports that inflammatory factors can cause bone loss, we examined the effects of CGA in osteoclast differentiation in the present study. Our data revealed that CGA decreased osteoclast formation compared to the RANKL- and M-CSF-treated control group during all stages of osteoclastogenesis, especially more in late stage expressing the major osteoclastogenic molecules and genes such as OSCAR, TRAP, and NFATc1 without cytotoxic effects (Fig. 2). Binding of RANKL–RANK activates a series of major intracellular signal transducing pathways including NF-κB, JNK, ERK, and p38 MAPK. NF-κB knockout mice showed the defects of osteoclastogenesis and severe osteopetrosis, indicating that NF-κB is a crucial factor in osteoclast differentiation. Inhibitor of nuclear factor-kappa B (IκB) is attached to NF-κB preventing it from migrating into the nucleus. NF-κB phosphorylation and degradation of IκB by IκB kinase (IKK). We found that CGA inhibited activation of NF-κB through suppressing of RANKL-mediated IκBα phosphorylation and degradation (Fig. 3). The involve-

**DISCUSSION**

(A) Mice were sacrificed 8d after the first LPS injection, and radiographs of the longitudinal section of the proximal femurs were obtained with a micro-CT apparatus (top). Dissected femurs were fixed, decalcified, embedded, and sectioned. The sections were stained with H&E (bottom). (B) The BV/TV, Tb.Sp, Tb.Th, and Tb.N of the femurs were determined using the micro-CT data as analyzed with VGStudio MAX 1.2 software. *p<0.05 versus the control group, †p<0.05 versus the LPS group.
ment of MAP kinases signaling pathway in RANKL-induced osteoclast differentiation has been reported in several studies. Especially, the importance of p38 in inflammatory bone destruction has been suggested in several reports, and it is considered to be a potential therapeutic target for inflammatory bone destruction. CGA inhibited the RANKL-induced phosphorylation of p38, ERK, JNK (Fig. 3). RANKL-induced activation of MAP kinases further leads to the activation of transcription factors such as NFATc1. Apparently, NFATc1 play a critical role in the regulation of genes for osteoclast differentiation. After NFATc1 is expressed in the middle or late stages of osteoclast differentiation, it subsequently regulates a number of osteoclast-specific genes, such as OSCAR and TRAP (Fig. 4A). CGA inhibited the expression of NFATc1 at the transcriptional levels in this study (Figs. 4A, B). The involvement of NFATc1 in CGA-inhibited osteoclast differentiation was confirmed by overexpression of the NFATc1 leading to the CGA-induced inhibition of osteoclast differentiation. Inhibited osteoclast differentiation by CGA was almost entirely rescued by NFATc1 induction (Fig. 4C). These results suggest that NFATc1 is involved in the inhibitory effect of CGA on osteoclast differentiation.

LPS elicits inflammatory reactions through the stimulation of monocytes and macrophages. LPS stimulation leads to the intracellular induction of p38, JNK phosphorylation and NF-κB activation in macrophages and monocytes. Production of inflammatory factors induces pre-osteoclast fusion, supports the survival of mature osteoclasts and stimulates osteoclastic bone resorption. CGA inhibits LPS-induced inflammatory response in RAW264.7 cells through attenuating the activation of NF-κB and JNK/AP-1 signaling pathways, this study support our results that CGA ameliorated LPS-mediated bone loss in vivo (Fig. 5).

In summary, our findings clearly show that CGA efficiently prevented RANKL-induced osteoclastogenesis of BMMs as well as osteoclastic bone destruction in vivo. The therapeutic effect of CGA is associated with down-regulation of activation of NF-κB, JNK, ERK, p38 MAPK and suppression of c-Fos and NFATc1 expression, leading to the lowered expression of TRAP and OSCAR. Hence, CGA could be a potential therapeutic candidate for treating diseases with inflammatory bone destruction.

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