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**Epigallocatechin-3-gallate inhibits osteoclastogenesis
by down-regulating c-Fos expression and suppressing the NF- κ B signal**

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ABBREVIATIONS: ALP, alkaline phosphatase; BMM, bone marrow macrophage; CT, computed tomography; EGCG, epigallocatechin-3-gallate; EMSA, electrophoretic mobility-shift assay; IKK, inhibitory κ B kinase; JNK, c-Jun N-terminal protein kinase; M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factor; NFATc, nuclear factor of activated T cells; NF- κ B, nuclear factor κ B; OC, osteoclast; OPG, osteoprotegerin; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor κ B ligand; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase; VitD₃, 1,25-dihydroxyvitamin D₃.

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ABSTRACT

Epigallocatechin-3-gallate (EGCG), the major anti-inflammatory compound in green tea, has been shown to suppress osteoclast differentiation. However, the precise molecular mechanisms underlying the inhibitory action of EGCG in osteoclastogenesis and the effect of EGCG on inflammation-mediated bone destruction remain unclear. In this study, we found that EGCG inhibited osteoclast formation induced by osteoclastogenic factors in bone marrow cell-osteoblast cocultures but did not affect the ratio of receptor activator of nuclear factor κ B (NF- κ B) ligand (RANKL) to osteoprotegerin induced by osteoclastogenic factors in osteoblasts. We also found that EGCG inhibited osteoclast formation from bone marrow macrophages (BMMs) induced by macrophage colony-stimulating factor plus RANKL in a dose-dependent manner without cytotoxicity. Pretreatment with EGCG significantly inhibited RANKL-induced the gene expression of c-Fos and Nuclear factor of activated T cells (NFATc1), essential transcription factors for osteoclast development. EGCG suppressed RANKL-induced activation of JNK pathway, among the three well-known MAPKs and also inhibited RANKL-induced phosphorylation of the NF- κ B p65 subunit at Ser276 and NF- κ B transcriptional activity, without affecting the degradation of I κ B α and NF- κ B DNA-binding in BMMs. The inhibitory effect of EGCG on osteoclast formation was somewhat reversed by retroviral c-Fos overexpression, suggesting that c-Fos is a downstream target for anti-osteoclastogenic action of EGCG. Additionally, EGCG treatment reduced interleukin-1-induced osteoclast formation and bone destruction in mouse calvarial bone in vivo. Taken together, our data suggest that EGCG has an anti-osteoclastogenic effect by inhibiting RANKL-induced the activation of JNK/c-Jun and NF- κ B pathways, thereby suppressing the gene expression of c-Fos and NFATc1 in osteoclast precursors.

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Introduction

Bone mass homeostasis is regulated by the coupled actions of bone-forming osteoblasts and bone-resorbing osteoclasts, a process termed remodeling. Many pathologic and osteopenic diseases, including postmenopausal osteoporosis, lytic bone metastasis, rheumatoid arthritis, periodontitis, and Paget disease, are characterized by progressive and excessive bone resorption by osteoclasts, which are multinucleated cells derived from the monocyte/macrophage lineage precursors (Boyle et al., 2003). Macrophage colony-stimulating factor (M-CSF), which is produced by osteoblasts, plays an important role in proliferation and subsequent osteoclast differentiation in mouse bone marrow cultures (Biskobing et al., 1995). A tumor necrosis factor (TNF) family member, receptor activator of nuclear factor κ B (NF- κ B) ligand (RANKL), is expressed as a membrane-bound protein in osteoblasts and stromal cells or is released as a soluble factor by activated T cells, which promotes osteoclast differentiation and activation (Takayanagi, 2007). Osteoblasts and stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation by interrupting the interaction between RANKL and RANK, resulting in an increase in bone density and bone volume (Simonet et al., 1997). Several osteoclastogenic factors, including 1,25-dihydroxyvitamin D₃ (VitD₃), parathyroid hormone (PTH), and proinflammatory cytokines, can stimulate osteoclast formation by upregulating the ratio of RANKL to OPG in osteoblasts or stromal cells (Takayanagi, 2007). Thus, in many conditions, osteoclast formation is regulated directly or indirectly by environmental cells.

RANKL binding to RANK, a receptor of RANKL that is expressed in osteoclast precursors, mediates the biological effects of RANKL and leads to recruitment of TNF receptor-associated factor 6, which results in the activation of downstream signaling pathways, including NF- κ B, c-Jun N-terminal protein kinase (JNK), p38, ERK, and PI3K/AKT (Darnay et al., 1998; Darnay et al.,

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1999; Lee et al., 2002; Li et al., 2000). RANKL activation of JNK phosphorylates the transcription factor c-Jun (Kobayashi et al., 2001), which forms activator protein-1 complexes with c-Fos, an essential transcription factor for osteoclast formation (Grigoriadis et al., 1994). Genetic disruption experiments have revealed that mice lacking both of the p50 and p52 NF- κ B subunits develop osteopetrosis caused by the arrested generation of osteoclasts (Franzoso et al., 1997; Iotsova et al., 1997). Thus, NF- κ B genes play an indispensable role in regulating the differentiation and function of osteoclasts. Recently, it was revealed that NF- κ B functions upstream of c-Fos expression during RANKL-induced osteoclastogenesis. RANKL-induced c-Fos expression is abolished in NF- κ B p50/p52 double-knockout osteoclast precursors, and RANKL can induce osteoclast formation from NF- κ B p50/p52 double-knockout osteoclast precursors when c-Fos is overexpressed (Yamashita et al., 2007). Nuclear factor of activated T cells (NFATc1), which also plays an important role in osteoclastogenesis, is upregulated by RANKL in osteoclast precursors through mechanisms that are dependent on NF- κ B and c-Fos (Matsuo et al., 2004; Yamashita et al., 2007). Furthermore, overexpression of NFATc1 in osteoclast precursors causes proficient induction of osteoclast formation even without RANKL stimulation (Takayanagi et al., 2002). These discoveries imply that NFATc1 may be a master transcription factor for osteoclast differentiation.

Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea. It has recently attracted interest for its emerging biological activities, such as anti-arthritic, anti-inflammatory, and cancer chemoprevention effects, in a variety of experimental models (Ahmed et al., 2004; Ahmed et al., 2006; Doss et al., 2005). Furthermore, it has been reported that EGCG can induce the apoptotic cell death of osteoclasts (Nakagawa et al., 2002). Although EGCG has been shown to inhibit osteoclast formation (Yun et al., 2004), the precise molecular mechanisms of EGCG action remain unclear. In addition, whether EGCG has a therapeutic effect on pathological bone destruction has not been

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studied. In the present study, we investigated the effects of EGCG on osteoclast development and the molecular mechanisms for these effects in vitro and in vivo.

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Materials and Methods

Reagents and Antibodies. Penicillin, streptomycin, α -MEM, and fetal bovine serum were purchased from Invitrogen Life Technologies (Carlsbad, CA). Recombinant soluble human M-CSF, human RANKL, mouse IL-1, and TNF- α were obtained from PeproTech (Rocky Hill, NJ). VitD₃, prostaglandin E₂ (PGE₂), and EGCG were purchased from Sigma (St. Louis, MO). SP600125 was from Calbiochem (La Jolla, CA). Specific antibodies for phospho-ERK1/2 (Thr202/Tyr204), ERK, phospho-JNK1/2 (Thr183/Tyr185), JNK, phospho-p38 (Thr180/Tyr182), p38, phospho-p65 (Ser536 and Ser276), p65, phospho-c-Jun (Ser73), c-Jun, phospho-AKT (Thr308 and Ser473), AKT, phospho-I κ B α (Ser32), and I κ B α were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for c-Fos, NFATc1, and β -actin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Preparation of Osteoclast Precursors. Mouse bone marrow cells were obtained from femurs and tibias of 5-week-old ICR mice and were incubated in α -MEM complete media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin on 10-cm culture dishes in the presence of M-CSF (10 ng/mL) overnight. Nonadherent bone marrow cells were transferred to 10-cm bacterial culture dishes and were cultured in the presence of M-CSF (30 ng/mL) for 3 days. Adherent cells were used as bone marrow macrophages (BMMs) as osteoclast precursors after nonadherent cells were washed out.

In Vitro Osteoclast Cultures. To generate osteoclasts, BMMs (4×10^4 cells/well) were cultured for 4 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in 48-well (1 mL/well) tissue culture plates. To generate osteoclasts from the coculture of primary osteoblasts and bone marrow cells, primary osteoblasts from newborn ICR mouse calvariae were prepared as previously described (Ha

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et al., 2006). Bone marrow cells (3×10^5 cells/well) and primary osteoblasts (2.5×10^4 cells/well) were cocultured for 6–7 days in 1 mL of α -MEM complete medium in 48-well tissue culture plates. The complete medium was changed every third day. At the end of the culture period, the cells were fixed in 10% formalin for 10 min, permeabilized with 0.1% Triton X-100, and then stained for tartrate-resistant acid phosphatase (TRAP) by using the Leukocyte Acid Phosphatase Assay Kit (Sigma, St. Louis, MO).

Cell Viability Assay. The XTT assay was performed to examine the effect of EGCG on the cytotoxicity of BMMs by using a Cell Proliferation Kit (Roche, Nutley, NJ) according to the manufacturer's instructions. BMMs (1×10^2 cells/well) were cultured with EGCG at various concentrations (1–50 μ M) for 48 h in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) on 96-well plates (200 μ L/well). Then, a medium containing 100 μ L XTT solution (XTT labeling reagent + electron-coupling reagent) was added. After 6 h of incubation, the plate was read at 450 nm (650 nm reference) by using a 96-well plate recorder.

Western Blot Analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were lysed in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.2% deoxycholate, and protease and phosphatase inhibitors for 30 min on ice. Protein concentrations of cell lysates were determined by using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). An equal amount of proteins (30 μ g/lane) was resolved by SDS-PAGE and was then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway NJ). The membrane was probed with the indicated primary antibody. Blots were finally developed by using horseradish peroxidase-conjugated secondary antibodies and were visualized by using enhanced chemiluminescence (ECL kit, Amersham Biosciences, UK).

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Nuclear Fraction and Electrophoretic Mobility-Shift Assay. Cells were washed twice with ice-cold PBS, lysed in C buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.5% NP-40, 20% glycerol, and 0.5 mM PMSF) for 5 min on ice, and were then microfuged at 1,500g for 5 min. The pellet was lysed in N buffer (20 mM HEPES [pH 7.6], 420 mM NaCl, 2 mM EDTA, 1% Triton X-100, 20% glycerol, 25 mM β -glycerophosphate, and 0.5 mM PMSF) for 30 min on ice and was then microfuged at 13,400g for 15 min. Supernatants were used as nuclear extracts. Electrophoretic mobility-shift assay (EMSA) was performed as previously described (Lee et al., 2001). Briefly, nuclear extracts (10 μ g) were incubated with the reaction buffer (10 mM Tris-HCL, 50 mM KCl, 1 mM EDTA, 5% glycerol, 2 mM DTT, and 1 μ g of poly[dI-dC]) containing approximately 20,000 cpm of P-labeled NF- κ B (5'-AGTTGAGGGGACTTTCCCAGGC-3', Santa Cruz Biotechnology) binding site oligomer for 30 min at 20°C. The DNA-bound proteins were separated on 4%-polyacrylamide gels. The gels were dried and subjected to autoradiography.

Quantitative PCR Analysis. Total RNA was prepared by using an RNeasy Mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions, and cDNA was synthesized from 2 μ g of total RNA by use of reverse transcriptase (Superscript II Preamplification System; Invitrogen). Real-time PCR was performed on an ABI Prism 7500 sequence detection system with SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, Cheshire, UK) by following the manufacturer's protocols. The ABI 7500 sequence detector was programmed with the following PCR conditions: 40 cycles of 15 s of denaturation at 95°C and 1 min of amplification at 60°C. All reactions were run in triplicate and were normalized to the housekeeping gene HPRT. Relative differences in PCR results were evaluated by using the comparative cycle threshold method. The

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following primer sets were used: mouse TNF- α forward, 5'-GACGTGGAAGTGGCAGAAGAG-3'; reverse, 5'-TGCCACAAGCAGGAATGAGA-3'; mouse ICAM-1 forward, 5'-GCCTAAGGAAGACATGATA-3'; reverse, 5'-CAAGAAGAGTTGGGGACAAT-3'; mouse Nfkb2 forward, 5'-TACAAGCTGGCTGGTGGGGA-3'; reverse, 5'-GTCGCGGGTCTCAGGACCTT-3'; mouse c-Fos forward, 5'-ACTTCTTGTTTCCGGC-3'; reverse, 5'-AGCTTCAGGGTAGGTG-3'; mouse NFATc1 forward, 5'-CCGTTGCTTCCAGAAAATAACA-3'; reverse, 5'-TGTGGGATGTGAACTCGGAA-3'; mouse β -HPRT forward, 5'-CCTAAGATGAGCGCAAGTTGAA-3'; reverse, 5'-CCACAGGGACTAGAACACCTGCTA-3'.

RANKL and OPG Protein Expression Assays in Osteoblasts. Primary osteoblasts (4×10^5 cells/well) were pretreated with or without EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h and were then stimulated with IL-1 (10 ng/mL), TNF- α (20 ng/mL), and VitD₃ (10 nM) plus PGE₂ (100 nM) for 24 h. The amounts of RANKL proteins in cell lysate and OPG secretion in cell culture media were determined by using RANKL and OPG ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Retroviral Gene Transduction. The retroviral vectors pMX-IRES-EGFP and pMX-c-Fos-IRES-EGFP were kindly provided by Dr. Nacksung Kim (University of Chonnam, Gwangju, Korea). Retrovirus packaging was performed by transient transfection of these pMX vectors into Plat-E retroviral packaging cells. After incubation in fresh medium for 2 days, culture supernatants of the retrovirus-producing cells were collected. For retroviral infection, nonadherent bone marrow cells were cultured in M-CSF (30 ng/mL) for 48 h. Media were removed and replaced with culture supernatants of pMX-IRES-EGFP and pMX-c-Fos-IRES-EGFP virus-producing Plat-E cells

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together with polybrene (6 $\mu\text{g/mL}$) and M-CSF (30 ng/mL) for 12 h. Infected cells were then cultured in the presence of M-CSF for 1 day and then further cultured with or without EGCG (20 μM) in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days.

Luciferase Reporter Assays. HEK293T cells were plated at a density of 5×10^4 cells/well in 24-well plates. The next day, cells were transfected with 0.8 μg of RANK and NF- κB luciferase reporter vector by using 2 μL of Lipofectamine 2000 (Invitrogen) for 6 h in DMEM., and then the medium was replaced by DMEM complete medium. After incubation for 12 h at 37°C in 5% CO_2 , the cells were pretreated with EGCG (20 μM) or vehicle (dimethylsulfoxide) for 12 h and were then stimulated with RANKL (100 ng/mL) for the indicated times. The cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI), and luciferase activity was measured by using a luminometer (EG&G Berthold, Bad Wildbad, Germany).

In Vivo Experiments. An IL-1-induced mouse calvarial bone loss model was used as previously described (Ha et al., 2006). In brief, a collagen sponge treated with vehicle (PBS) or IL-1 (2 μg) was implanted over calvarial bone in groups of 10 mice (6-week-old male ICR mice). Mice were intraperitoneally administered EGCG (15 $\mu\text{g/g}$ of body weight) or vehicle (dimethylsulfoxide) daily beginning on day 0. The mice were sacrificed 7 days after the implantation, and whole calvariae were fixed in 4% paraformaldehyde and stained for TRAP. Three-dimensional images of calvarial bone were obtained by micro-computed tomography (micro-CT) scanning (SMX-90CT, Shimadzu, Japan). BMC was determined by using TRI 3D-BON (RACTOC system Engineering Co., Japan) program. For histological analysis, calvarial tissues were fixed in 4% paraformaldehyde, decalcified in 12% EDTA, and then embedded in paraffin. After that, histological sections (5 μm) were prepared, stained for TRAP, and counterstained by using hematoxylin. Image analysis (Image J,

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NIH) was further employed to quantify the osteoclasts number and the percentage of osteoclast surface. All animal experiments were reviewed and approved by the Seoul National University School of Dentistry Animal Care Committee.

Statistical Analysis. Values are presented as the mean \pm S.D from three or more experiments. Data were analyzed with the Student's *t* test for comparisons between two mean values. A value of *P* < 0.05 was considered significant.

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Results

EGCG suppresses osteoclast formation in bone marrow cell-osteoblast cocultures and primary BMMs

We first examined the effect of EGCG on osteoclast formation induced by osteoclastogenic factors in cocultures of bone marrow cells and primary osteoblasts in vitro. Stimulation of IL-1 (10 ng/mL), TNF α (20 ng/mL), and VitD₃ (10 nM) plus PGE₂ (100 nM) for 6 days formed TRAP-positive multinucleated osteoclasts in the cocultures and, under these conditions, treatment with EGCG (at 20 μ M) significantly inhibited osteoclast formation (Fig. 1A). In these culture systems, osteoblasts support osteoclastogenesis from osteoclast precursors by regulating the expression of RANKL and OPG (Takayanagi, 2007). We thus evaluated by ELISA whether EGCG affects the expression of RANKL and OPG in osteoblasts. The addition of osteoclastogenic factors, including IL-1, TNF α , and VitD₃ plus PGE₂, increased RANKL expression and decreased OPG expression at 24 h in osteoblasts. Pretreatment with EGCG did not alter the expression of RANKL or OPG (Fig. 1B). We next examined the effects of EGCG on RANKL-induced osteoclast formation from osteoclast precursor BMMs. RANKL (100 ng/mL) generated numerous TRAP-positive multinucleated osteoclasts in the presence of M-CSF (30 ng/mL) for 4 days. Treatment of the same cultures with EGCG suppressed osteoclast formation in a dose-dependent manner (Fig. 1C). Complete inhibition of osteoclast formation was achieved with 20 μ M of EGCG, and this concentration was subsequently used unless otherwise noted. The anti-osteoclastogenic effect of EGCG was not mediated through cellular toxicity or cell proliferation, even at high doses (50 μ M) (Fig. 1D).

EGCG down-regulates c-Fos and NFATc1 expression induced by RANKL

To determine the molecular mechanisms of the action of EGCG in osteoclastogenesis, we first examined the effect of EGCG on the expression of the key transcription factors, c-Fos and NFATc1.

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As previously reported, the expression of c-Fos and NFATc1 was upregulated in BMMs by RANKL stimulation. Pretreatment with EGCG for 12 h strongly inhibited the RANKL-induced mRNA expression of c-Fos and NFATc1 (Fig. 2A) and also suppressed RANKL-induced the two protein expression in a dose-dependent manner (Fig. 2B and 2C). To investigate whether c-Fos is a downstream target for the anti-osteoclastogenic action of EGCG, we ectopically expressed the c-Fos gene in BMMs by use of a retroviral system. By forced expression of c-Fos (Fig. 2D), the inhibitory effect of EGCG on RANKL-induced osteoclast formation was somewhat reversed (Fig. 2E).

EGCG inhibits the RANKL-induced phosphorylation of JNK and c-Jun in BMMs

To further investigate the molecular mechanisms underlying the inhibitory effects of EGCG on RANKL-induced c-Fos expression and osteoclast formation, we examined the effects of EGCG on RANKL-induced early signaling pathways including ERK, JNK, p38, and AKT. Phosphorylation of these signaling molecules was observed at 5 min after RANKL treatment in BMMs. Among these pathways, phosphorylation of JNK and its downstream target c-Jun was only suppressed by pretreatment with EGCG (Fig. 3A). Thus, we examined whether JNK/c-Jun activation is required for RANKL-induced c-Fos induction in BMMs. Pretreatment of BMMs with SP600125, a specific JNK inhibitor, at 5 μ M suppressed RANKL-induced JNK phosphorylation and subsequent c-Jun phosphorylation (Fig. 3B) without changing in RANKL-induced p-ERK, p-p38, p-Akt, and p-I κ B α levels (data not shown). Moreover, the RANKL-induced expression of the key transcription factors c-Fos and NFATc1 was inhibited by SP600125 (Fig. 3C). Consistent with these effects, osteoclast formation was strongly suppressed (Fig. 3D).

EGCG inhibits RANKL-induced NF- κ B transcriptional activity, without affecting its DNA binding activity

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RANKL stimulation leads to activation of NF- κ B as well as MAPKs and AKT (Darnay et al., 1998; Darnay et al., 1999; Lee et al., 2002; Li et al., 2000). Thus, next assessed the effect of EGCG on RANKL-induced NF- κ B activation. In HEK 293T cells cotransfected with RANK and a NF- κ B reporter gene, stimulation of RANKL augmented NF- κ B transcriptional activation, which was significantly inhibited by EGCG treatment (Fig 4A). To examine whether EGCG inhibits RANKL-induced NF- κ B-dependent transcription in BMMs, we evaluated mRNA levels of several NF- κ B-regulated genes by using real-time quantitative PCR. There was an increase in ICAM-1, Nfkb2, and TNF α mRNA expression after RANKL stimulation. Consistent with the results of NF- κ B reporter assay, pretreatment with EGCG lessened RANKL-induced upregulation of these genes (Fig 4B). The primary regulatory point of NF- κ B activity is at the level of I κ B protein degradation. RANKL stimulation led to the phosphorylation and almost complete degradation of I κ B α within 15 min as assessed by Western blotting and pretreatment with EGCG did not alter either phosphorylation or degradation of I κ B α (Fig 4C). Furthermore, EGCG did not impair the RANKL-stimulated DNA-binding activity of NF- κ B in the EMSA assay (Fig 4D). Interestingly, we found that EGCG inhibits RANKL-stimulated phosphorylation of the NF- κ B p65 subunit at Ser276 but not Ser536 (Fig. 4C) which has been shown to be involved in NF- κ B transcriptional activity (Vermeulen et al., 2004; Dong et al., 2008).

EGCG prevents IL-1-induced osteoclast formation and bone destruction in vivo

Finally, having established that EGCG inhibits osteoclastogenesis in vitro, we addressed whether the same effect could be observed in vivo. TRAP staining of whole calvariae and histological sections showed that IL-1 dramatically increased osteoclast number and surface (Fig 5A, 5D, and 5E). In parallel with the effects in vitro, EGCG notably reduced IL-1-induced osteoclast formation when administered systemically. Furthermore, the results of the bone histomorphometric analysis

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after micro-CT also showed that IL-1-induced bone destruction were notably prevented by EGCG treatment (Fig. 5B and 5C).

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Discussion

Excessive RANKL signaling causes enhanced osteoclast formation and bone resorption. As such, the down-regulation of RANKL expression or its downstream signals may be a valuable approach to the treatment of pathological bone loss. In the present study, we found that EGCG prevents osteoclast differentiation from bone marrow cells and primary osteoblast cocultures induced by IL-1, TNF- α , and VitD₃ plus PGE₂ without affecting the expression of RANKL and OPG. We next investigated whether EGCG inhibits RANKL-induced osteoclast formation from its precursors in the absence of osteoblasts or stromal cells. As reported previously (Yun et al., 2004), TRAP-positive multinuclear cells from this culture were decreased in a dose-dependent manner after treatment with EGCG, which was similar to the observations in cocultures. These results clearly suggest that EGCG suppresses osteoclast formation via directly acting on osteoclast precursors.

Several transcription factors, including PU.1, microphthalmia-associated transcription factor (MITF), NF- κ B, c-Fos, and NFATc1 have been shown to play a role in osteoclast development from its precursors. PU.1 and MITF mediate the early nonspecific differentiation along the osteoclast pathway (Teitelbaum and Ross, 2003), whereas NF- κ B, c-Fos, and NFATc1 function downstream of RANKL signaling for osteoclast differentiation. The c-Fos/c-Jun/NFATc1 pathway plays a critical and essential role in osteoclast development, and the lack of any of these three components arrests osteoclastogenesis (Teitelbaum, 2004). In this study, RANKL-induced expression of c-Fos and NFATc1 was dramatically down-regulated with EGCG pretreatment. Furthermore, the forced expression of c-Fos somewhat reversed the EGCG inhibition of osteoclastogenesis, suggesting that c-Fos is a target of EGCG inhibitory effect on osteoclast development.

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Stimulation of RANKL has been reported to activate three well-known MAPKs: ERK, JNK, and p38. Furthermore, each specific inhibitor of MEK and p38 or DN-JNK prevented RANKL-induced osteoclastogenesis, which suggest that these signaling pathways also have a role in osteoclast formation (Ikeda et al., 2004; Kobayashi et al., 2001; Lee et al., 2002). We found that pretreatment with EGCG specifically down-regulates the JNK activation without affecting the activation of ERK and p38. In JNK pathway, MKK7, one of the major JNK upstream kinase, is involved in osteoclastogenesis (Ikeda et al., 2004; Yamamoto et al., 2002), and c-Jun, the best-characterized substrates of JNK, is also required for maximum induction of NFATc1 and osteoclastogenesis in response to RANKL (Ikeda et al., 2004). In our experiments, EGCG inhibited RANKL-induced c-Jun phosphorylation (Fig. 3A) but not MKK7 phosphorylation (data not shown). We also found that blockade of the JNK/c-Jun pathway by the JNK inhibitor SP600125 not only impaired RANKL-induced osteoclast formation but also inhibited RANKL-induced c-Fos and NFATc1 expression. Taken together, these results strongly suggest that EGCG suppresses RANKL-induced osteoclast differentiation and the induction of c-Fos and NFATc1 at least in part by inhibiting JNK/c-Jun pathway in BMMs.

The crucial role of NF- κ B signaling in osteoclast development has been demonstrated by genetic experiments including p50/p52 NF- κ B double-knockout mice that displayed severe osteopetrosis (Franzoso et al., 1997; Iotsova et al., 1997) and IKK β knockout mice that exhibited failed osteoclastogenesis (Ruocco et al., 2005). Recently, it has suggested that NF- κ B function upstream of c-Fos expression during RANKL-induced osteoclastogenesis (Yamashita et al., 2007). Similar with JNK/c-Jun pathway, NF- κ B also seems to be involved in RANKL-induced NFATc1 induction as well as c-Fos incution. Takatsuna et al. (2005) showed that an NF-

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κ B inhibitor suppresses RANKL-stimulated induction of NFATc1. In addition, NF- κ B co-operates with NFATc2 to activate the initial induction of NFATc1, followed by an auto-amplification of NFATc1 (Asagiri et al., 2005). In this study, EGCG inhibited RANKL-induced NF- κ B transcriptional activation, suggesting that the down-regulation of NF- κ B-dependent transcription might be involved in the inhibitory effect of EGCG on RANKL induction of c-Fos and NFATc1 during osteoclastogenesis.

NF- κ B proteins can be post-translationally modified, including acetylation (Chen et al., 2001), S-nitrosylation (Marshall et al., 2004), and phosphorylation (Vermeulen et al., 2002; Vermeulen et al., 2003; Zhong et al., 1998), which can affect NF- κ B activity. It has been shown that phosphorylation of the NF- κ B p65 subunit at Ser276 plays a critical role for TNF-induced transactivation of p65 and act as a switch regulating the association with either CBP/p300 or histone deacetylase (HDAC) (Vermeulen et al., 2003; Zhong et al., 1998; Zhong et al., 2002). Recently, Dong et al. (2008) showed that cells from a knock-in mouse containing the nonphosphorylatable p65 S276A variant display a significant reduction of some but not all NF- κ B-responsive gene expression without affecting DNA binding activity, and that unphosphorylated nuclear NF- κ B can affect expression of genes not normally regulated by NF- κ B through epigenetic mechanisms. In this study, RANKL stimulated both phosphorylation of the p65 subunit at Ser276 and Ser536. Interestingly, EGCG specifically inhibited RANKL-induced phosphorylation of the p65 subunit at Ser276 but not Ser536. Thus, these results may imply that RANKL-induced phosphorylation of the p65 subunit at Ser276 plays a role in the osteoclastic gene induction by NF- κ B transcription-dependent and/or -independent mechanisms. However, further studies are required to clarify this possibility.

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IL-1 is a proinflammatory cytokine that is thought to be a potent stimulator of the pathologic bone destruction induced by both estrogen deficiency and inflammation. Mice lacking the type I IL-1 receptor are resistant to bone destruction after ovariectomy (Lorenzo et al., 1998). Blocking IL-1 signaling can also reduce bone loss and cartilage degradation in animal models of rheumatoid arthritis (Abramson and Amin, 2002). In our experiments, EGCG noticeably suppressed IL-1-induced calvarial bone destruction as well as osteoclast formation *in vivo*. The *in vivo* effects are most likely the result of the suppression of RANKL induction of c-Fos and NFATc1 in osteoclast precursors.

Bone homeostasis depends on maintaining a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts (Boyle et al., 2003). Thus, we next examined the effects of EGCG on bone formation by primary mouse calvarial osteoblasts. Osteogenic media containing ascorbic acid, β -glycerophosphate, and BMP2 substantially promoted alkaline phosphatase (ALP) activity (Supplemental Fig. S1A) and bone nodule formation as shown by alizarin red-S (Supplemental Fig. S1B). Treatment of osteoblasts with EGCG up to 20 μ M did not affect ALP activity, while showing marginal effect on bone nodule formation at 20 μ M (Supplemental Fig. S1A and S1B). Consistent with ALP activity, the mRNA levels of osteoblast marker genes, Runx2, ALP, and Osteocalcin (Ducy et al., 1997; Murshed et al., 2005; Owen et al., 1990) were not affected by EGCG treatment (Supplemental Fig. S1C).

In summary, our findings clearly show that EGCG has an anti-osteoclastogenic potential by reducing RANKL induction of c-Fos and NFATc1 in osteoclast precursors. The inhibitory action of EGCG on RANKL-induced activation of NF- κ B and JNK/c-Jun pathways is most likely to be involved in this anti-osteoclastogenic effect. EGCG also prevented IL-1-induced osteoclastic

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bone destruction in vivo. Thus, our findings strongly suggest that EGCG deserves new evaluation as a potential treatment option in various bone diseases associated with excessive osteoclast formation and bone destruction.

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Footnotes

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Figure legends

Fig. 1. EGCG inhibits osteoclast formation from cocultures and BMMs. A, mouse bone marrow cells and primary osteoblasts were cocultured with IL-1 (10 ng/mL), TNF α (20 ng/mL), or VitD₃ (10 nM) plus PGE₂ (100 nM) in the presence of EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 6 days. After culturing, the generated osteoclasts were detected by TRAP staining, and TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts (OC) (**, $P < 0.01$). B, mouse primary osteoblasts were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h and were then stimulated with IL-1 (10 ng/mL), TNF α (20 ng/mL), or VitD₃ (10 nM) plus PGE₂ (100 nM) for 24 h. The amounts of RANKL and OPG were determined by using ELISA kits in cell lysates and in cell culture media, respectively. C, Bone marrow macrophages (BMMs) were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) at the indicated doses of EGCG for 4 days. After culturing, cells were fixed and TRAP staining was performed (upper). The number of TRAP-positive multinucleated osteoclasts (OC) was counted (lower) (*, $P < 0.05$, **, $P < 0.01$ versus untreated control). D, BMMs were cultured for 48 h with M-CSF (30 ng/mL) and RANKL (100 ng/mL) at the indicated doses of EGCG. Then, cell viability was determined by XTT assay as described in Materials and Methods.

Fig. 2. EGCG suppresses RANKL-induced expression of c-Fos and NFATc1 in BMMs. A and C, BMMs were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) in the presence of M-CSF for 12 h and were then stimulated with RANKL (100 ng/mL) for the indicated times. B, BMMs were pretreated with EGCG (5-20 μ M) or vehicle (dimethylsulfoxide) in the presence of M-CSF for 12 h and were then stimulated with RANKL for 24 h. Expression of mRNA for c-Fos and NFATc1 was analyzed by real-time PCR with HPRT mRNA as an endogenous control (A). Western blotting was performed with the indicated antibodies (B and C). Actin served as an internal control.

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D and E, BMMs were infected with retroviruses expressing pMX-IRES-EGFP (Vector) or pMX-c-Fos-EGFP (c-Fos). Infected BMMs were cultured for 24 h, and Western blotting was performed for c-Fos expression levels (D). Infected BMMs were cultured with or without EGCG (20 μ M) in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days. After culturing, the cells were fixed, and the cells were stained for TRAP (E, left). TRAP-positive multinucleated osteoclasts (OC) were counted (E, right; **, $P < 0.01$, versus untreated control; ##, $P < 0.01$, versus Vector group treated with EGCG).

Fig. 3. EGCG down-regulates the JNK/c-Jun pathway in BMMs. A, BMMs were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h in the presence of M-CSF (30 ng/mL) and were then stimulated with RANKL (100 ng/mL) for the indicated times. Whole-cell lysates were subjected to Western blotting with the indicated antibodies. Actin served as an internal control. B, BMMs were pretreated with SP600125 (5 μ M) or vehicle (dimethylsulfoxide) for 3 h in the presence of M-CSF (30 ng/mL) and were then stimulated with RANKL (100 ng/mL) for the indicated times. Whole-cell lysates were subjected to Western blotting with the indicated antibodies. Actin served as an internal control. C, BMMs were pretreated with SP600125 (0.5-5 μ M) or vehicle (dimethylsulfoxide) for 3 h and were then stimulated with RANKL for 24 h. Western blotting was performed with the indicated antibodies. Actin served as an internal control. D, BMMs were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) at the indicated doses of SP600125 for 4 days. After culturing, cells were fixed and TRAP staining was performed. The number of TRAP-positive multinucleated osteoclasts (OC) was counted (*, $P < 0.05$, **, $P < 0.01$ versus untreated control).

Fig. 4. EGCG impairs RANKL-induced transcriptional activity of NF- κ B in BMMs. A, HEK

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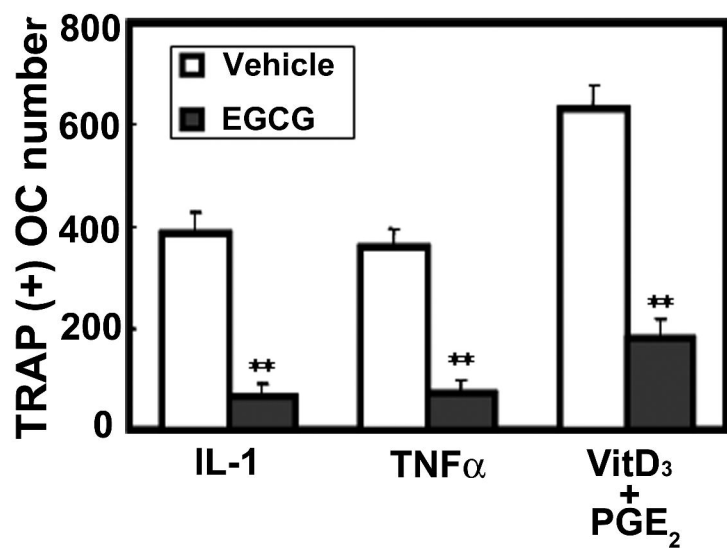
293T cells were cotransfected with RANK and NF- κ B-luciferase plasmid. At 12 h after transfection, the cells were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h and were then stimulated with RANKL (100 ng/ml) for the indicated times. Cells were lysed and the luciferase activity was determined by using a luciferase reporter assay system (*, $P < 0.05$ versus untreated control). B, BMMs were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) in the presence of M-CSF for 12 h and then stimulated with RANKL (100 ng/ml) for the indicated times. Expression of mRNA for TNF α , Nfkb2, and ICAM-1 was analyzed by real time PCR using HPRT mRNA as an endogenous control. C, BMMs were pretreated with EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h in the presence of M-CSF (30 ng/ml) and then stimulated with RANKL (100 ng/ml) for the indicated time points. The phosphorylation of I κ B α and p65 (Ser276 and Ser536) were detected with specific antibodies. D, BMMs were pretreated EGCG (20 μ M) or vehicle (dimethylsulfoxide) for 12 h in the presence of M-CSF (30 ng/ml) and then stimulated with RANKL (100 ng/ml) for 15 or 30min. Cells were harvested and nuclear extracts were prepared. DNA binding activity of NF- κ B was assessed by EMSA. N.S., nonspecific band.

Fig. 5. Therapeutic value of EGCG on IL-1-induced bone destruction in vivo. A collagen sponge treated with vehicle (PBS) or IL-1 (2 μ g) was implanted over 6-week-old mouse calvaria. EGCG (15 μ g/g of body weight) or vehicle (dimethylsulfoxide) was intraperitoneally administered daily. The mice were sacrificed at 7 days after implantation. TRAP staining of whole calvaria (A). Three-dimensional images of calvarial bone by micro-CT analysis were presented (B) and BMC measured (C; **, $P < 0.01$ versus untreated control; ##, $P < 0.01$ versus group treated with IL-1 only). Histological sections of calvarial bone were TRAP stained with hematoxylin counterstaining (D), and osteoclast number (left)/surface (right) was analyzed (E, left; **, $P < 0.01$ versus untreated control; ##, $P < 0.01$ versus group treated with IL-1 only, right; **, $P < 0.01$ versus untreated

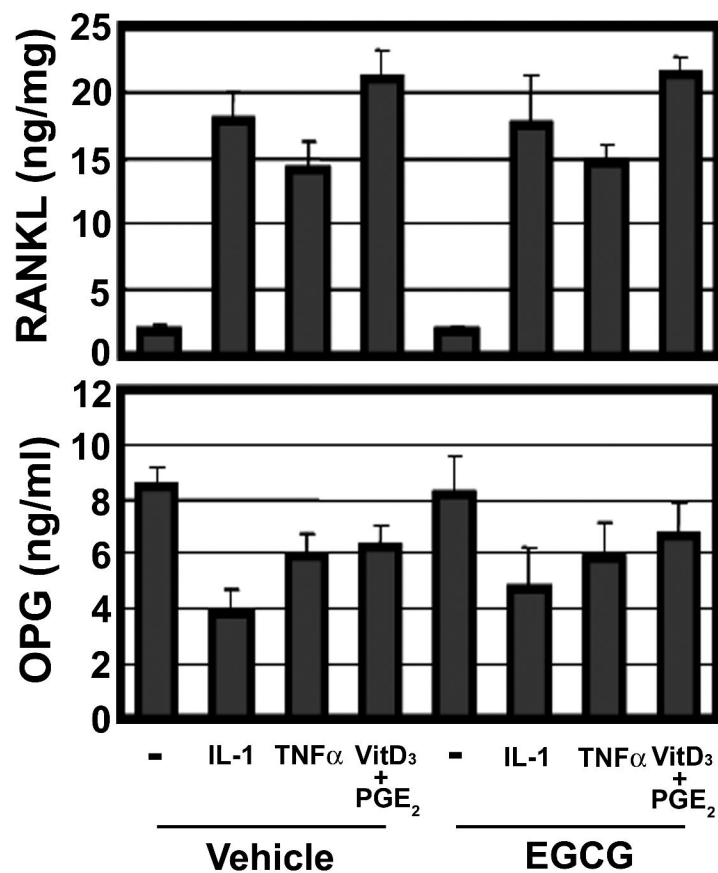
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control; #, $P < 0.05$ versus group treated with IL-1 only).

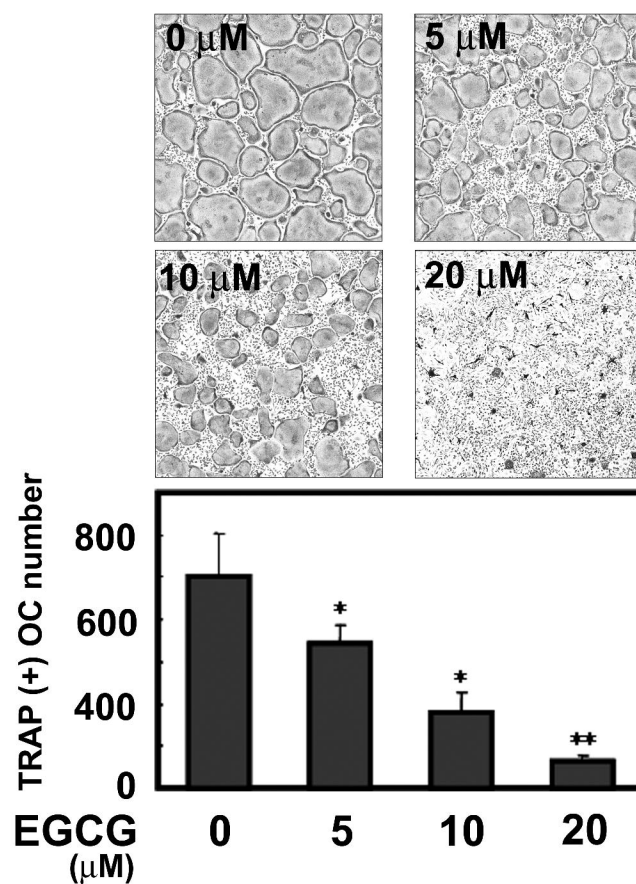
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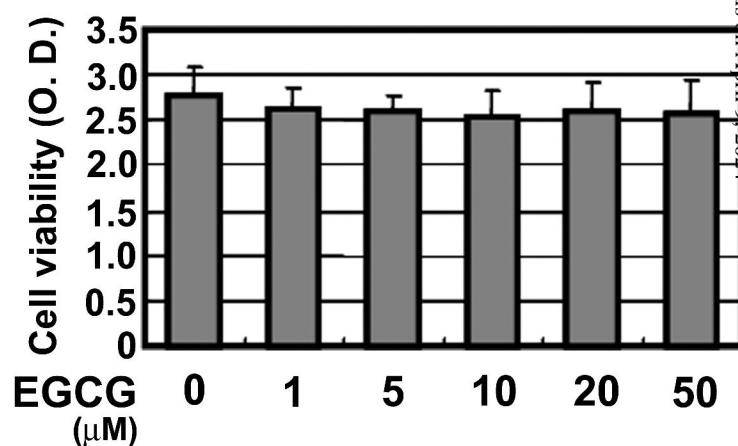


Fig. 2

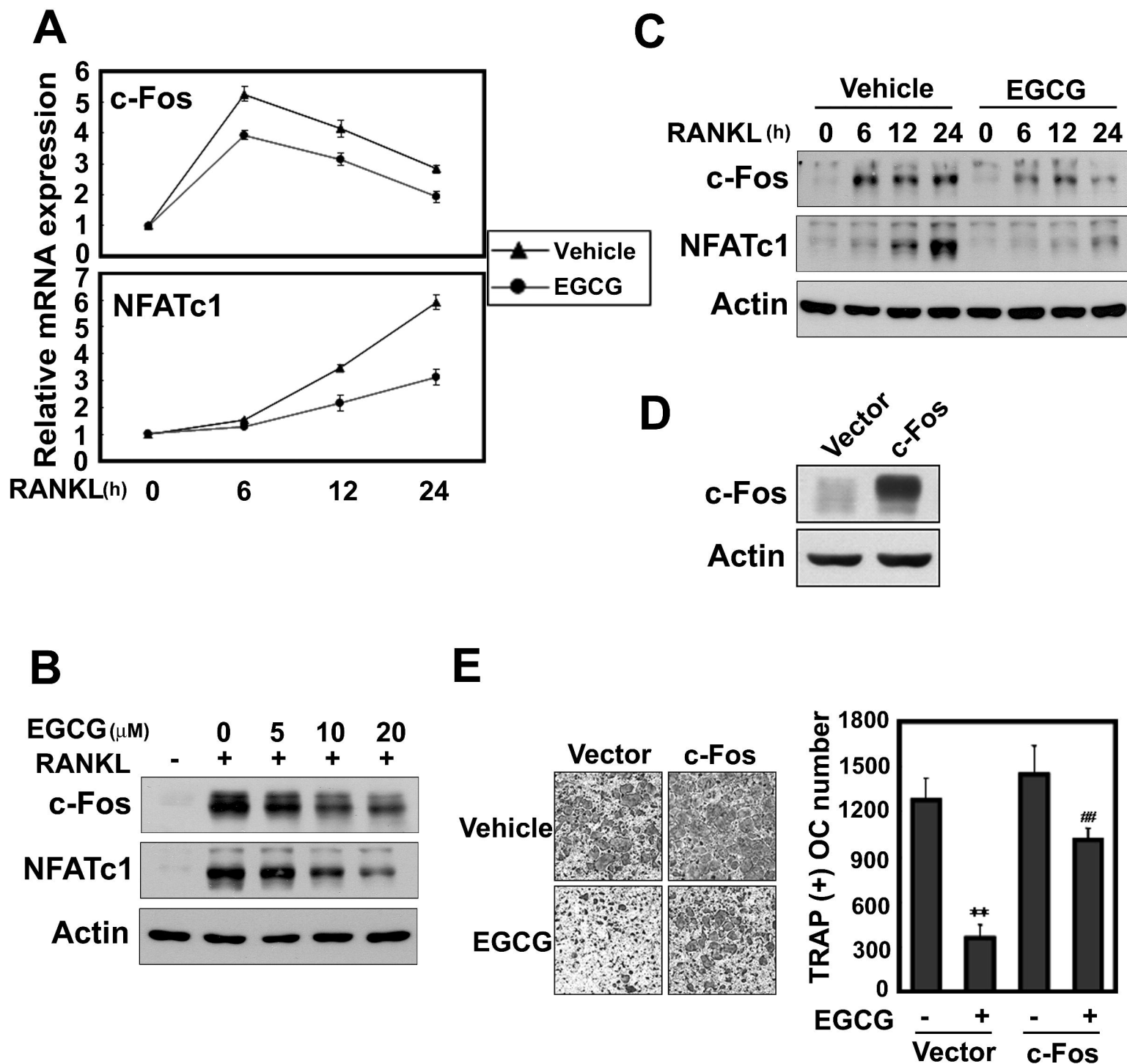
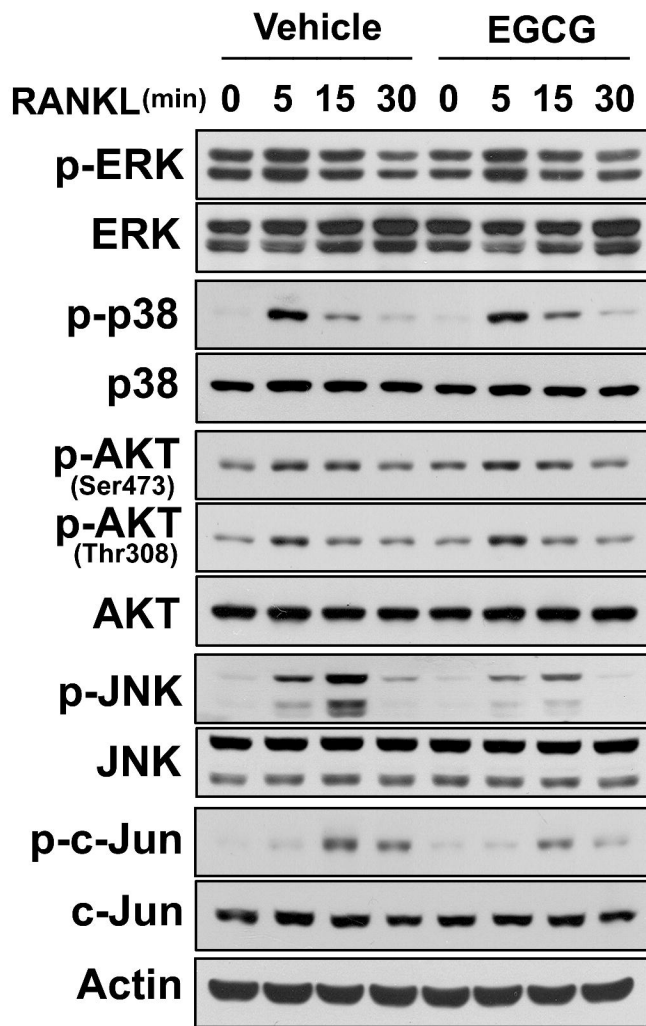
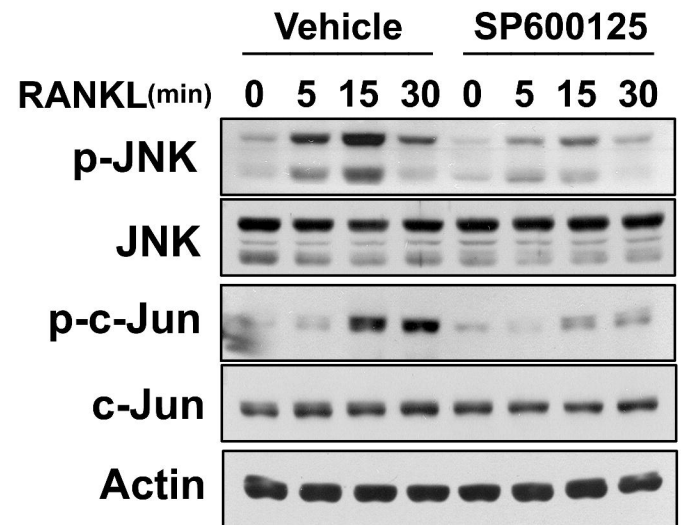


Fig. 3

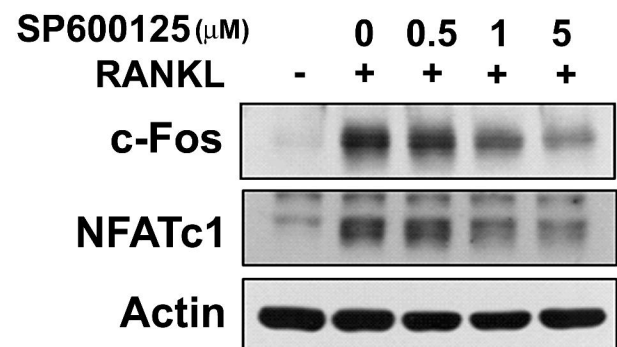
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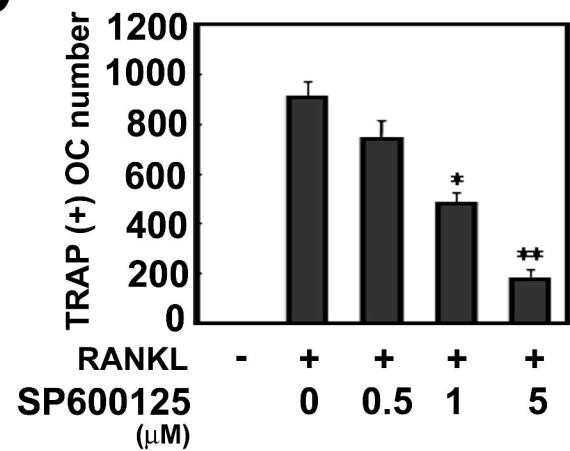
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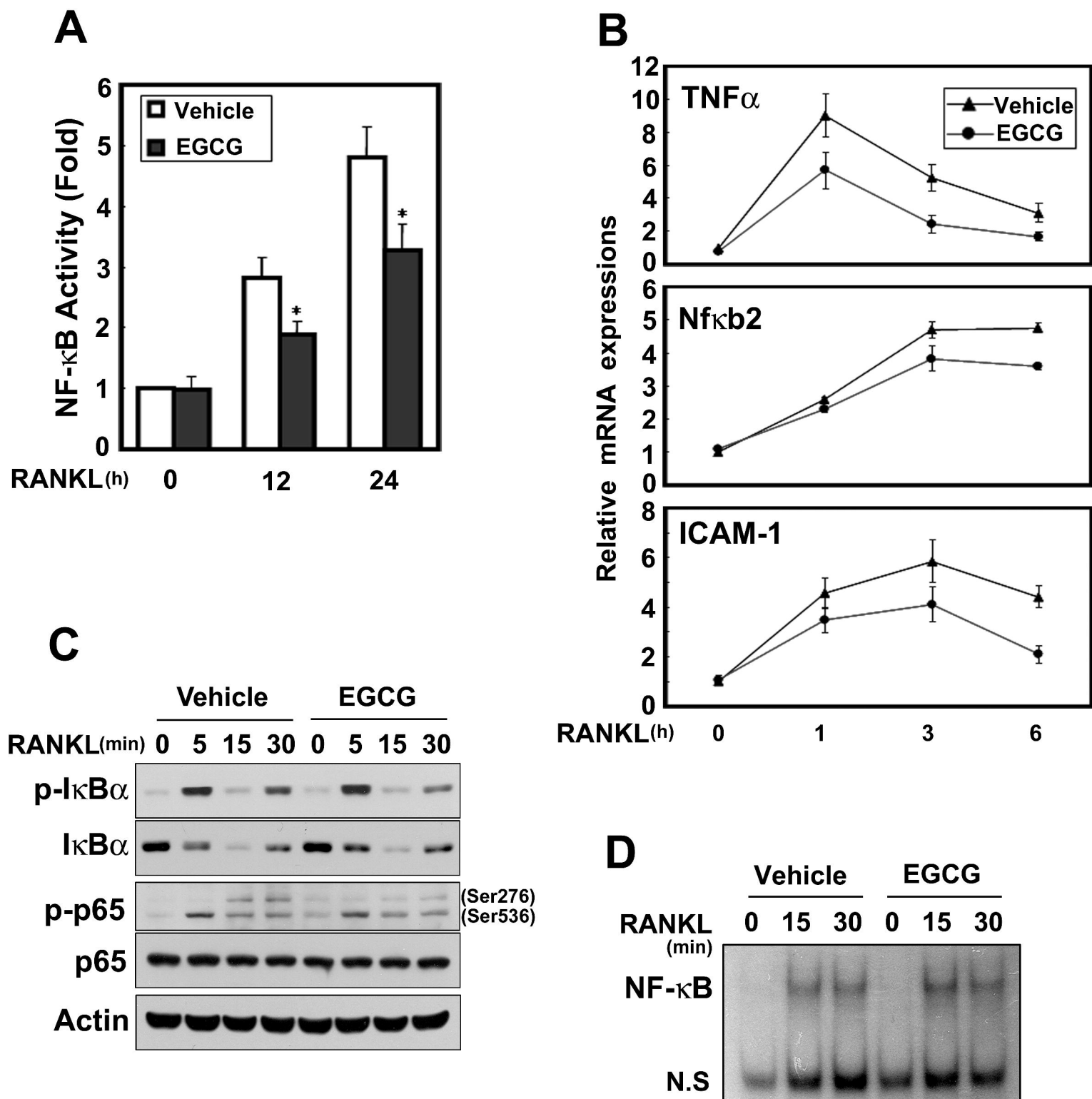
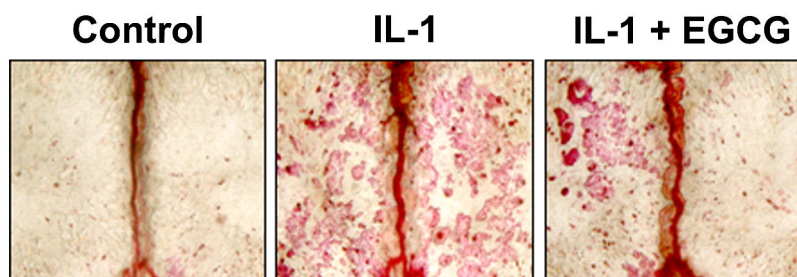
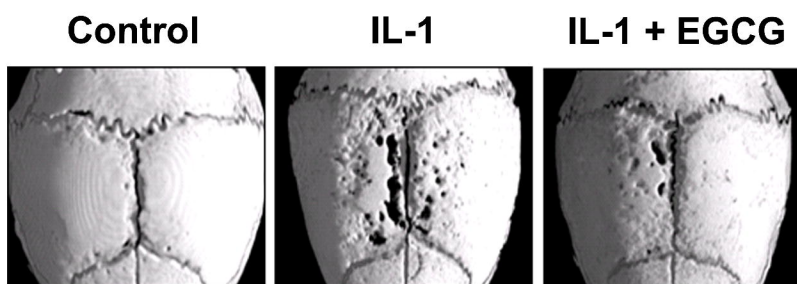


Fig. 5

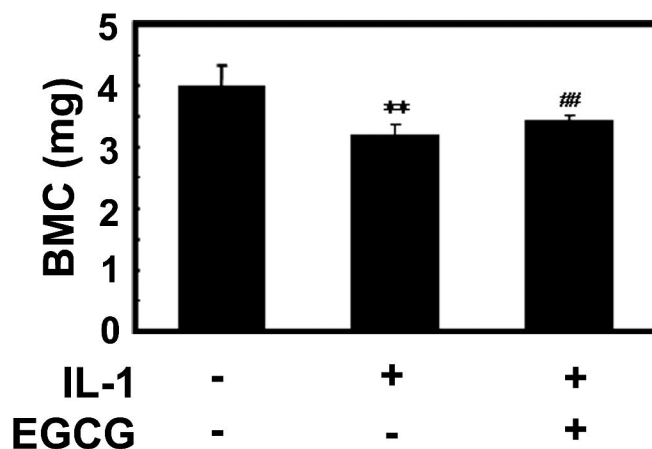
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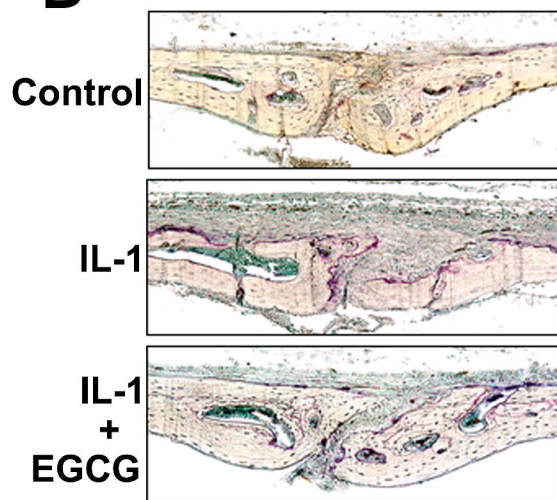
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