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The tyrosine kinase inhibitor GNF-2 suppresses osteoclast formation and activity

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RECEIVED JULY 2, 2013; REVISED SEPTEMBER 23, 2013; ACCEPTED OCTOBER 2, 2013. DOI: 10.1189/jlb.0713356

ABSTRACT

GNF-2, a tyrosine kinase inhibitor, was developed to overcome imatinib-resistant mutations found in CML patients. Osteoclasts are the principal bone-resorbing cells that are responsible for bone diseases, such as osteoporosis, tumor-induced osteolysis, and metastatic cancers. In this study, we investigated the effect of GNF-2 on osteoclast development induced by RANKL and M-CSF. We found that GNF-2 inhibited osteoclast differentiation from BMMs. GNF-2 suppressed RANKL-induced NF-κB transcriptional activity and the induction of c-Fos and NFATc1, which are two key transcription factors in osteoclastogenesis. We also observed that GNF-2 dose-dependently inhibited the proliferation of osteoclast precursors through the suppression of the M-CSFR c-Fms. In addition, GNF-2 accelerated osteoclast apoptosis by inducing caspase-3 and Bim expression. Furthermore, GNF-2 interfered with actin cytoskeletal organization and subsequently blocked the bone-resorbing activity of mature osteoclasts. In agreement with its in vitro effects, GNF-2 reduced osteoclast number and bone loss in a mouse model of LPS-induced bone destruction. Taken together, our data reveal that GNF-2 possesses anti-bone-resorptive properties, suggesting that GNF-2 may have therapeutic value for the treatment of bone-destructive disorders that can occur as a result of excessive osteoclastic bone resorption. *J. Leukoc. Biol.* 95: 337–345; 2014.

Introduction

The adult skeleton constantly undergoes bone remodeling, an event involving bone resorption by osteoclasts and bone formation by osteoblasts [1–5]. Excessive osteoclast formation and/or activity cause pathologic bone disorders, including osteoporosis and osteolytic bone metastasis [6–8]. Osteoclasts are specialized bone-resorbing cells that arise from hematopoietic precursors of the monocyte/macrophage lineage. Two cytokines, RANKL and M-CSF, are essential for osteoclast development [9–11].

The binding of RANKL to its receptor RANK promotes osteoclast differentiation through the activation of MAPKs and NF-κB signaling pathways [10, 12, 13]. The crucial role of NF-κB in osteoclastogenesis has been well-documented by in vivo genetic studies. Double-knockout mice deficient in the NF-κB subunits p50 and p52 exhibit an osteopetrotic phenotype as a result of impaired osteoclast differentiation [14, 15]. Activation of the NF-κB signaling cascade ultimately leads to the induction of c-Fos and NFATc1, which are important transcription factors in osteoclastogenesis [16–18].

Whereas RANKL regulates osteoclast differentiation, M-CSF is crucial for the proliferation and survival of osteoclasts and their precursors [6]. The critical role of M-CSF in osteoclast development has been well-demonstrated in M-CSF-mutated *op/op* mice. These mice produce dysfunctional M-CSF and therefore, exhibit an osteopetrotic phenotype, which is a result of a decrease in osteoclast numbers, resulting from defective proliferation of macrophages and differentiation of osteoclasts [19, 20]. M-CSF promotes cellular responses through its receptor tyrosine kinase, c-Fms. The binding of M-CSF triggers receptor dimerization and autophosphorylation, thereby inducing the activation of downstream signaling pathways, including ERK and PI3K/Akt [21, 22].

Protein tyrosine kinases have been pursued as potential anticancer targets. Imatinib is a tyrosine kinase inhibitor used for the treatment of CML, where it targets the oncogenic tyrosine kinase Bcr-Abl [23], and gastrointestinal stromal tumor, where it targets the PDGFR or stem cell factor receptor (c-kit) [24].

Abbreviations: A=absorbance, Bim=B cell lymphoma 2-interacting mediator of cell death, BMM=bone marrow macrophage, BV/TV=bone volume/tissue volume, ES/BS=eroded surface/bone surface, MNC=multinucleated cell, MTS=3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, NFATc1=NF of activated T cells c1, N.Oc/B.Pm=osteoclast number/bone perimeter, Oc.S/BS=osteoclast surface/bone surface, TRAP=tartrate-resistant acid phosphatase

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Interestingly, imatinib can also target c-Fms and suppress osteoclast formation [25]. In addition, imatinib has been shown to possess anti-inflammatory activity in an animal model of collagen-induced arthritis [26]; however, its influence on inflammation-induced bone loss remains unclear thus far.

GNF-2 is an allosteric, non-ATP-competitive tyrosine kinase inhibitor that was developed to overcome imatinib-resistant Bcr-Abl mutations found in patients with CML [27]. It has been reported that GNF-2 inhibits the proliferation of Bcr-Abl-transformed cells with a potency similar to that of imatinib [27]. However, its effect on osteoclast development and its in vivo effect on pathological bone destruction have not been elucidated. In the present study, we report the inhibitory effect of GNF-2 on the proliferation, survival, and function of osteoclasts. We also provide in vitro data to elucidate additional mechanisms of its suppressive effect on osteoclast differentiation. GNF-2 inhibits osteoclastogenesis through the suppression of NF-κB transcriptional activity, thereby attenuating c-Fos and NFATc1 induction. We further show the protective effects of two tyrosine kinase inhibitors, GNF-2 and imatinib, on inflammatory bone destruction in mice.

MATERIALS AND METHODS

Reagents and antibodies

rRANKL and rM-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against phospho-c-Fms phospho-IκBα, phospho-JNK, phospho-p38, phospho-ERK, phospho-Akt, IκBα, JNK, p38, ERK, Akt, cleaved caspase-3, and cleaved caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for c-Fms and c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-Bim antibody was obtained from BD Biosciences (San Jose, CA, USA). The anti-NFATc1 antibody was obtained from BD PharMingen (San Diego, CA, USA).

Mice

All animal experiments were approved by the Institutional Review Board of Kyungpook National University School of Medicine.

Macrophage isolation and osteoclast culture

Mouse BMMs were obtained as described previously [28]. Briefly, BMMs were isolated from 6- to 8-week-old C57/BL6 mice and cultured with α-MEM containing 10% FBS and M-CSF. After 3 days of culture, the adherent cells were used further as BMMs. For osteoclast generation, BMMs were cultured with RANKL (20 ng/ml) and M-CSF (10 ng/ml) for 4 days in α-MEM containing 10% FBS. The media were changed every 2 days.

TRAP staining

Cells were fixed in 4% PFA for 20 min and subsequently stained for TRAP activity with a 0.1 M acetate solution (pH 5.0) containing 6.76 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 0.5 mg/ml Fast Red Violet. Osteoclasts were identified as TRAP-expressing MNCs.

Cell viability assay

Cell viability was determined by MTS assay. After treatment with various concentrations of GNF-2 for 3 days, the cultured cells were incubated in CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA) for 4 h at 37°C. A_{490nm} was measured using a microplate reader.

Real-time PCR

Total RNA (1 μg) was extracted from cultured cells and used as a template for cDNA synthesis. PCR was performed on an ABI 7500 Real-Time PCR System using SYBR Green dye (Applied Biosystems, Foster City, CA, USA). The amplification reaction was performed as described previously [28]. Primers were synthesized on the basis of the reported mouse cDNA sequence. The following primers were used: c-Fos, 5′-AGGCCCAGTGGCTCAGAGA-3′ and 5′-GCTCCCAGTCTGCTGCATAGA-3′; NFATc1, 5′-ACCACCTTTCCGCAACCA-3′ and 5′-TTCCGTTTCCCGTTGCA-3′; TRAP, 5′-TCCCCAATGCCCCATTC-3′ and 5′-CGGTTCTGGCGATCTCTTTG-3′; cathepsin K, 5′-GGCTGTGGAGGCGGCTAT-3′ and 5′-AGAGTCAATGCCTCCGTTCTG-3′; RANK, 5′-TCTGCAGCTCTTCCATGACACT-3′ and 5′-GAAGAGGAGCAGAACGATGAGACT-3′.

Western blotting

Cultured cells were lysed in a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and protease and phosphatase inhibitors for 30 min on ice. Protein concentrations of the cell lysates were determined using a BCA kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). The extracted proteins were subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membranes were incubated with the indicated primary antibodies at 4°C overnight, followed by probing with secondary antibodies. The blots were visualized using ECL reagents (ECL Plus; Amersham, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Luciferase reporter assays

Raw264.7 cells were transiently transfected with a NF-κB luciferase reporter construct. The transfected cells were incubated with GNF-2 for 24 h and subsequently stimulated with RANKL for 24 h. The cells were lysed in Reporter Lysis Buffer (Promega), and the luciferase activity was measured using a luminometer.

Cell proliferation and apoptosis assays

BMMs were cultured with M-CSF (30 ng/ml) in the presence of various concentrations of GNF-2 for 3 days. Subsequently, BrdU was added to the culture medium and incubated for 4 h. The BrdU ELISA was conducted using the cell-proliferation Biotrak ELISA system (Amersham, GE Healthcare Life Sciences). A_{450nm} was measured using the Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA). The cell death assay was performed with the Cell Death Detection ELISA Kit (Roche, Mannheim, Germany), which detects cytoplasmic histone-associated DNA fragmentation. A_{405nm} was measured using a Bio-Rad microplate reader.

Actin-ring staining

BMMs were seeded on bone slices (IDS Nordic Bioscience, Herlev, Denmark) and cultured with M-CSF (10 ng/ml) and RANKL (20 ng/ml) to commit them to an osteoclast phenotype. The cells were then incubated with GNF-2 for 16 h in the presence of M-CSF (10 ng/ml) and RANKL (20 ng/ml). For the actin-ring reformation assay, mature osteoclasts generated on bone slices were washed twice with cytokine-free cold media for 5 min, followed by incubation at 37°C for 20 min. These washed cells were then incubated with or without GNF-2 (2 μM) in the presence of RANKL and M-CSF. F-actin and nuclei were stained with TRITC-labeled phalloidin (Sigma, St. Louis, MO, USA) and Hoechst 33258 (Sigma), respectively.

Resorption pit assay

For the staining of resorption pits, cells were removed from bone slices, which were incubated with peroxidase-conjugated wheat-germ agglutinin (Sigma) for 1 h and stained with 3,3′-diaminobenzidine (Sigma). To quantify osteoclast bone resorption, the pit areas were analyzed using image editing software (paint.net; http://www.getpaint.net/index.html).

In vivo experiments

Eight-week-old C57/BL6 mice were divided into four groups (n=5/group). The mice were administered i.p. injections of LPS (5 mg/kg) or PBS (control) on Days 1 and 5. GNF-2 (10 mg/kg), imatinib (10 mg/kg), or vehicle was injected i.p., 1 day before and every day after the LPS injection. All mice were sacrificed on Day 8. Femurs were fixed in 4% PFA, decalcified in 10% EDTA, and subsequently embedded in paraffin. Histological sections were prepared, stained for TRAP, and counterstained using hematoxylin. Osteoclastic perimeters were measured and analyzed using the OSTEO II program (Bioquant Image Analysis, Nashville, TN, USA).

Statistical analysis

All experiments were performed in triplicate. The data are presented as the means ± SD. Statistical significance was determined using a two-tailed Student's t-test.

RESULTS

GNF-2 inhibits osteoclast formation

To investigate the effect of GNF-2 on osteoclast formation, primary BMMs were cultured in the presence of RANKL, M-CSF, and various concentrations of GNF-2 or imatinib. After 4 days, the cells were stained for TRAP, which is a cytochemical marker of osteoclasts. Whereas vehicle-treated BMMs effectively differentiated into TRAP-positive multinuclear osteoclasts, GNF-2, like imatinib, inhibited osteoclast formation in a dose-dependent manner (**Fig. 1A**). We observed that GNF-2 completely inhibited osteoclast generation at a concentration of 2 μM with a potency similar to that of imatinib (Fig. 1B). To evaluate the cytotoxicity of this compound, we performed the MTS assay on BMMs, Raw264.7 macrophages, and MC3T3-E1 osteoblastic cells. GNF-2 did not show any cytotoxicity at the concentrations used in this study (Fig. 1C), indicating that GNF-2-mediated suppression of osteoclast formation was not a result of toxic effects on the precursor cells.

GNF-2 attenuates RANKL-induced c-Fos and NFATc1 expression

To examine further its inhibitory effect on osteoclast formation, we evaluated the impact of GNF-2 on the expression of c-Fos and NFATc1, which are important modulators of osteoclast differentiation. As shown in **Fig. 2A**, RANKL stimulation of control cells increased the mRNA levels of c-Fos and NFATc1. Pretreatment with GNF-2 significantly reduced the induction of these two transcription factors (Fig. 2A). The inhibitory effect of GNF-2 on the protein expression of c-Fos and NFATc1 was analyzed further by immunoblotting. GNF-2 caused a strong decrease in the protein levels of c-Fos (Fig. 2B) and NFATc1 (Fig. 2C) in response to RANKL. Reflecting the decrease in NFATc1 expression, the expression of osteoclast-specific NFATc1 target genes, such as TRAP and cathepsin K, was also attenuated in the presence of GNF-2 (Fig. 2A and C).

A previous study has demonstrated that imatinib inhibits RANK expression [25]. Therefore, we next examined whether

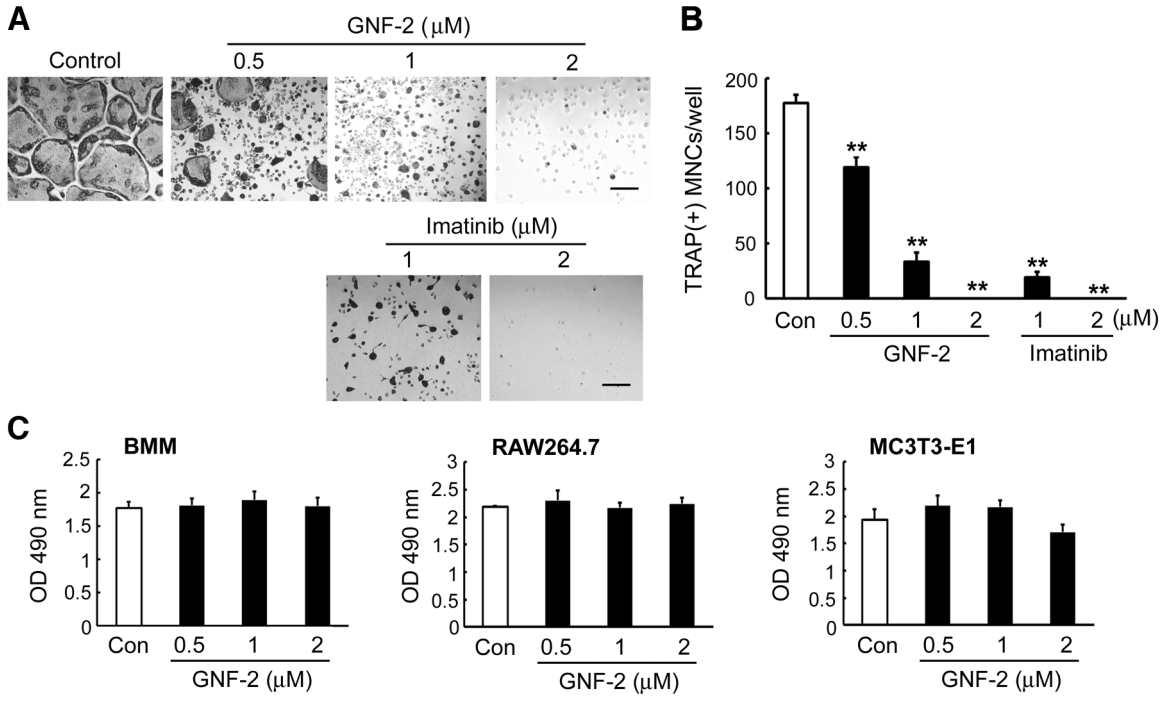

Figure 1. GNF-2 inhibits osteoclast formation. BMMs were cultured with M-CSF (10 ng/ml), RANKL (20 ng/ml), and the indicated doses of GNF-2 or imatinib. (A) After 4 days, the cells were fixed and stained for TRAP. Original scale bar: 100 μm. (B) TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. The data are expressed as the means ± SD. **$P < 0.001$ versus vehicle-treated control (Con). (C) BMMs, Raw264.7, or MC3T3-E1 cells were cultured with the indicated concentrations of GNF-2. Cell viability was evaluated by MTS assay, as described in Materials and Methods.

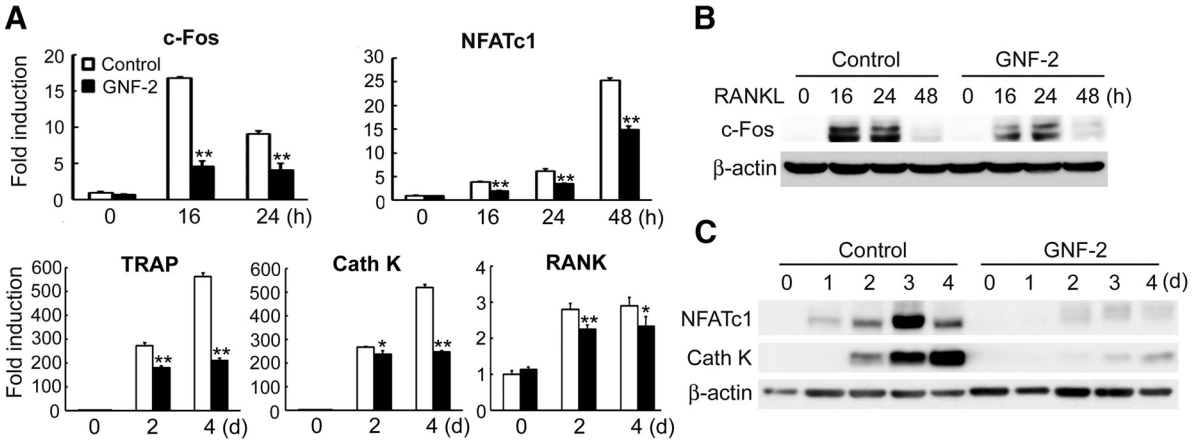

Figure 2. GNF-2 attenuates the expression of c-Fos and NFATc1 induced by RANKL. BMMs were cultured with M-CSF and RANKL for the indicated times in the presence or absence of GNF-2 (2 μM). Real-time PCR (A) or immunoblotting (B and C) was performed to detect the indicated genes. GAPDH (A) or β-actin (B and C) served as loading controls. Cath K, cathepsin K. The data are expressed as the means ± SD. $^{*}P < 0.05$; $^{**}P < 0.001$ versus control.

the same holds for GNF-2. We observed that GNF-2 at 2 μM reduced mRNA levels of RANK by 20%, relative to controls on Days 2 and 4 of culture (Fig. 2A).

GNF-2 inhibits NF-κB transcriptional activity induced by RANKL

To investigate the molecular mechanism for the suppressive effect of GNF-2 on RANKL-induced c-Fos expression and osteoclastogenesis, we evaluated its impact on the RANKL signaling pathway. Serum-starved BMMs were pretreated with GNF-2 and analyzed for the activation of MAPKs and NF-κB after exposure to RANKL. We found that GNF-2 did not affect the phosphorylation of JNK, p38, or IκBα in response to RANKL (**Fig. 3A**).

As RANKL induces the transcriptional activity of NF-κB, we next assessed the effect of GNF-2 on NF-κB activity following RANKL stimulation in Raw264.7 cells. Whereas RANKL exposure increased NF-κB transcription activity, GNF-2 reduced this induction significantly in a dose-dependent manner (Fig. 3B). These results demonstrate that GNF-2 inhibits RANKL-induced NF-κB transcriptional activity without affecting the phosphorylation of IκBα.

GNF-2 suppresses precursor proliferation and osteoclast survival by targeting c-Fms

The number of osteoclasts is influenced by the rate of precursor proliferation and the apoptosis of osteoclasts, as well as osteoclast differentiation. To investigate the effect of GNF-2 on the proliferation of osteoclast precursors, we cultured BMMs with M-CSF for 72 h in the absence or presence of 1–3 μM GNF-2. As expected, M-CSF induced the proliferation of vehicle-treated BMMs (**Fig. 4A**). The addition of GNF-2 significantly suppressed the proliferation of osteoclast precursor cells in a dose-dependent manner (Fig. 4A). We next assessed the effect of GNF-2 on osteoclast apoptosis. Fully differentiated osteoclasts were cultured in the presence or absence of GNF-2 for 16 h and stained for TRAP. We observed that treatment with GNF-2 accelerated the apoptosis of mature osteoclasts (Fig. 4B and C). The apoptotic effect of GNF-2 was confirmed further by assessment of DNA fragmentation using ELISA. As shown in Fig. 4D, the addition of GNF-2 increased the rate of apoptosis significantly in mature osteoclasts. Consistent with its proapoptotic effect, GNF-2 activated caspase-3 and caspase-9,

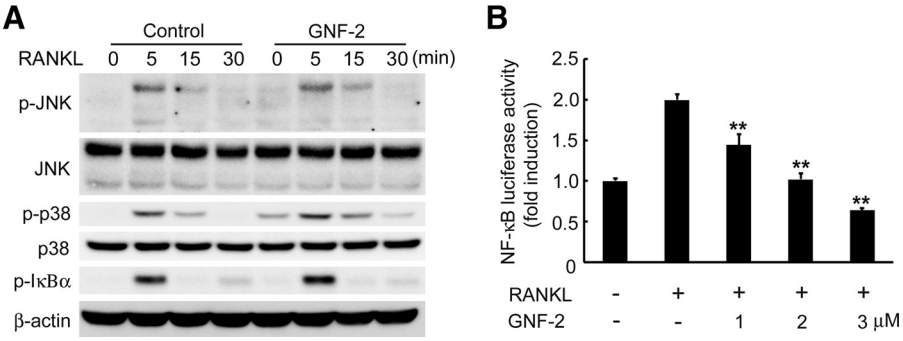

Figure 3. GNF-2 suppresses RANKL-induced NF-κB transcriptional activity. (A) BMMs were pretreated with GNF-2 (2 μM) or vehicle (Control) for 2 h and subsequently exposed to RANKL (50 ng/ml) for the indicated times. Total protein extracts were analyzed by immunoblotting with the indicated antibodies. The total JNK, p38, and β-actin levels served as loading controls. p, Phosphorylated. (B) Raw264.7 cells were transiently transfected with a NF-κB luciferase construct for 24 h. The cells were pretreated with the indicated concentrations of GNF-2 for 24 h and subsequently stimulated with RANKL (200 ng/ml) for 24 h. The cells were lysed, and the luciferase activity was measured using a luciferase reporter assay system. The data are expressed as the means ± SD. $^{**}P < 0.001$ versus RANKL alone.

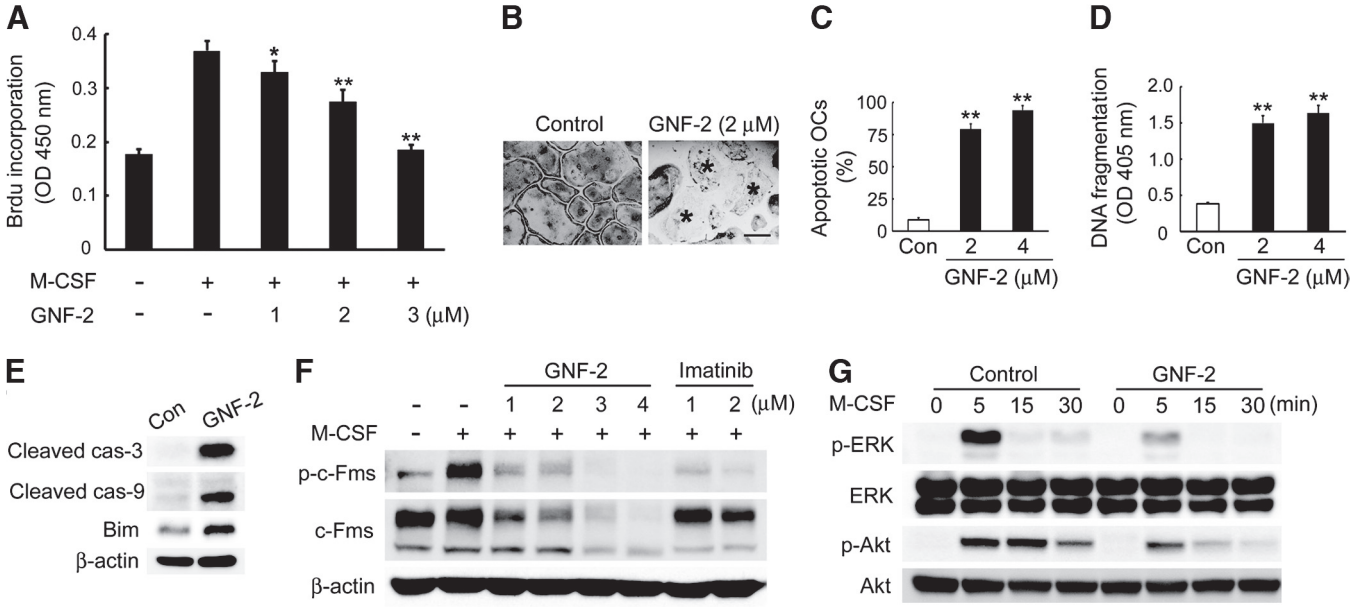

Figure 4. GNF-2 inhibits precursor proliferation and survival of osteoclasts by suppressing c-Fms signaling. (A) BMMs were cultured with or without M-CSF (30 ng/ml) in the presence of the indicated concentrations of GNF-2 for 3 days. Incorporation of BrdU during the last 4 h of culture was determined. The data are expressed as the means ± SD. $*P < 0.05$; $**P < 0.001$ versus M-CSF alone. (B) Osteoclasts were cultured for 16 h with vehicle (Control) or GNF-2. The cells were fixed and stained for TRAP. Apoptotic osteoclasts are indicated with asterisks. Original scale bar: 100 μm. (C) The percentage of apoptotic osteoclasts (OCs) shown in B. The data are expressed as the means ± SD. $**P < 0.001$ versus control. (D) The magnitude of apoptosis was measured by ELISA. $**P < 0.001$ versus control. (E) Osteoclasts were cultured for 16 h with vehicle (Con) or GNF-2 (4 μM). The contents of cleaved caspase (cas)-3, caspase-9, and Bim were determined by immunoblotting. β-Actin served as a loading control. (F) BMMs were serum-starved for 4 h, pretreated with the indicated concentrations of GNF-2 or imatinib for 2 h, and subsequently exposed to M-CSF (50 ng/ml) for 5 min. Phosphorylated and total c-Fms were detected by immunoblotting. β-Actin served as a loading control. (G) BMMs were pretreated with GNF-2 (2 μM) or vehicle for 2 h and subsequently exposed to M-CSF (50 ng/ml) over time. The phosphorylation of ERK and Akt was determined by immunoblotting. Total ERK and Akt levels served as loading controls.

in addition to increasing the expression of Bim, found in osteoclasts (Fig. 4E).

M-CSF binds to c-Fms, its receptor tyrosine kinase, and regulates cell proliferation and apoptosis. As GNF-2 suppressed osteoclast formation by inhibiting the proliferation of osteoclast precursors and enhancing the apoptosis of osteoclasts, we investigated the effect of GNF-2 on c-Fms signaling. As reported previously, imatinib inhibited the phosphorylation of c-Fms in response to M-CSF (Fig. 4F). Pretreatment of cultures with GNF-2 suppressed M-CSF-induced c-Fms phosphorylation with a similar potency to that of imatinib. Furthermore, in contrast to imatinib, GNF-2 attenuated the expression of c-Fms in a dose-dependent manner (Fig. 4F). We next assessed the activation of several MAPKs that function as downstream signaling molecules of M-CSF/c-Fms. As expected, GNF-2 significantly attenuated the phosphorylation of ERK and Akt in response to M-CSF (Fig. 4G).

GNF-2 blocks cytoskeletal organization and bone-resorptive activity

Osteoclastic bone resorption depends on the formation of actin rings, cytoskeletal structures referred to as the sealing zone. To determine whether GNF-2 influences cytoskeletal organization in osteoclasts, we first examined the effect of GNF-2 on osteoclast morphology. To this end, we cultured BMMs with M-CSF and RANKL for 3 days and committed them to the osteoclast phenotype. The cells were then incubated with GNF-2 or vehicle (as a control) for 16 h in osteoclastogenic media and stained for TRAP activity. As shown in **Fig. 5A**, vehicle-treated control cells generated characteristic well-spread osteoclasts. In contrast, GNF-2-treated cells formed small and irregular shapes of TRAP-expressing MNCs (Fig. 5A and B), suggesting a cytoskeletal defect.

To confirm that the morphological abnormalities of GNF-2-treated osteoclasts reflect impaired osteoclast function, we next assessed the effect of GNF-2 on actin-ring formation. BMMs were plated on bone in the presence of M-CSF and RANKL, committed to the osteoclast phenotype, and treated with GNF-2. The actin cytoskeleton was stained with TRITC-phalloidin. Whereas control cells contained abundant actin rings, the addition of GNF-2 blocked the formation of actin rings in a dose-dependent manner (Fig. 5C and D). To confirm these findings further, we performed an actin-ring reformation assay [29]. Mature osteoclasts generated on bone were washed with cytokine-free cold media to disrupt the actin rings. The cells were then incubated for 3 h in the presence of osteoclastogenic cytokines, with or without GNF-2. Whereas the stimulation of RANKL and M-CSF restored actin-ring formation in the absence of GNF-2, GNF-2 blocked actin cytoskeletal organization in osteoclasts (Fig. 5F and G). To examine

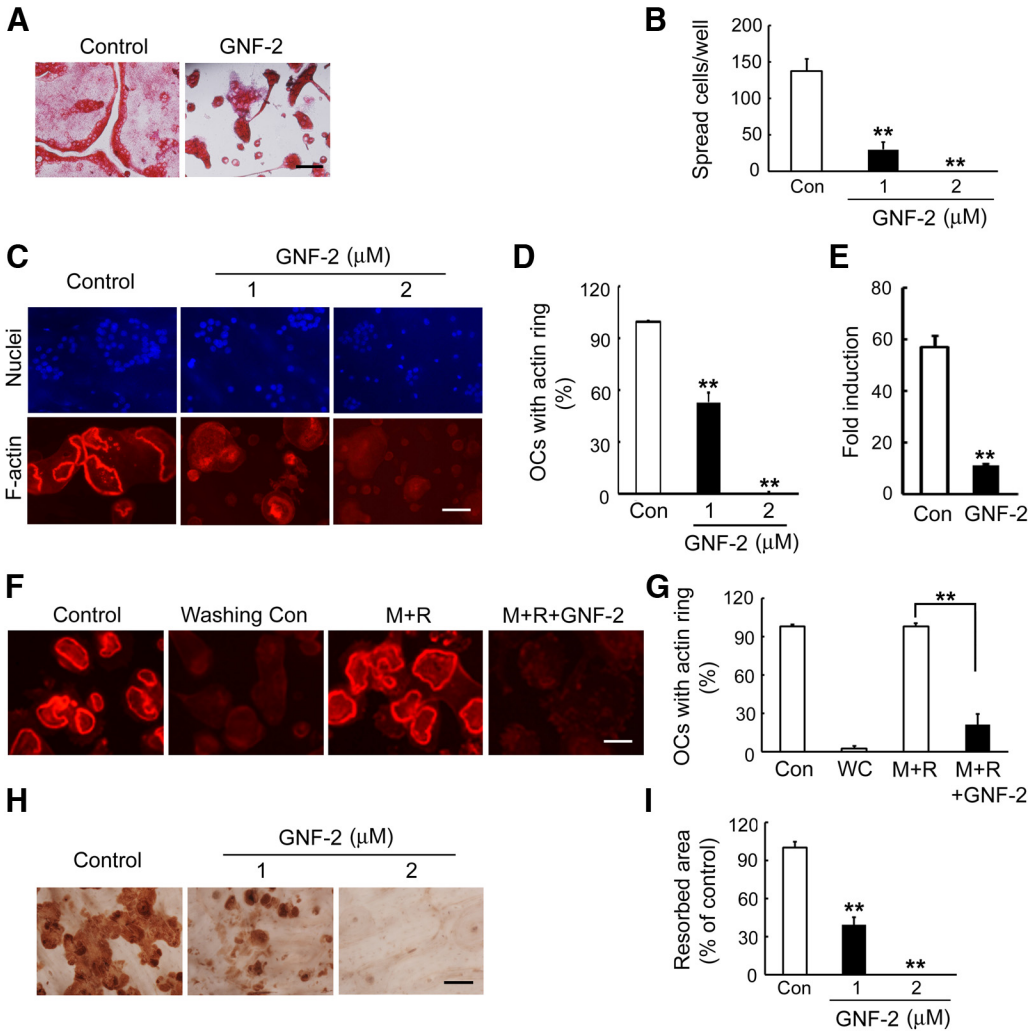

Figure 5. GNF-2 blocks actin ring and resorption pit formation. (A and B) BMMs were cultured with M-CSF and RANKL for 3 days to commit them to the osteoclast phenotype. The cells were then maintained with GNF-2 (1 or 2 μM) or vehicle for 16 h. (A) Cells were stained for TRAP. (B) Statistical analysis of spread TRAP-expressing MNCs/well. (C and D) BMMs were seeded onto bone slices and incubated with M-CSF and RANKL for 3 days to commit them to the osteoclast phenotype. The cells then were treated with or without GNF-2 for 16 h. (C) Cells were fixed and stained for F-actin. (D) Statistical analysis of the number of actin rings/bone slice. (E) BMMs were cultured in M-CSF and RANKL with or without GNF-2 (2 μM). The mRNA level of β3 integrin was analyzed by real-time PCR. (F and G) Mature osteoclasts, cultured on bone, were fixed before [control (Con)], after washing with cytokine-free cold media [washing control (WC)], or after washing and incubation with or without GNF-2 (2 μM) in the presence of 20 ng/ml RANKL (R) and 10 ng/ml M-CSF (M) for 3 h. (F) The cells were fixed, and F-actin was stained with TRITC-phalloidin. (G) Statistical analysis of the percentage of osteoclasts with actin rings. The data are expressed as the means ± SD. $^{**}P < 0.001$. (H and I) BMMs were cultured as in C. (H) Bone slices were stained with peroxidase-conjugated wheat-germ agglutinin after removal of cells. (I) Resorption pit areas were analyzed using the images. (B, D, E, and I) The data presented are expressed as the means ± SD. $^{**}P < 0.001$ versus control. Original scale bar: 50 μm (A, C, F, and H).

further the effect of GNF-2 on the cytoskeleton, we investigated its impact on the expression of β3 integrin, which is a key regulator of cytoskeletal organization and bone resorption [30]. Treatment with GNF-2 significantly attenuated the mRNA level of β3 integrin (Fig. 5E). In agreement with its inhibitory effect on the cytoskeleton, GNF-2 treatment suppressed resorption pit formation in a dose-dependent manner (Fig. 5H and I).

GNF-2 and imatinib prevent inflammatory bone destruction in vivo

We next investigated the in vivo effect of GNF-2 using an animal model of inflammatory bone destruction. Mice received i.p. administration of LPS, with or without GNF-2 or imatinib. A histological examination of femur sections stained with H&E illustrated the protective effect of GNF-2 and imatinib on bone loss induced by LPS (**Fig. 6A**). As shown in Fig. 6B, BV/TV was decreased significantly in the LPS-treated mice, and this decrease was abrogated by GNF-2 or imatinib treatment. To explore further whether GNF-2 prevents LPS-induced bone destruction by inhibiting osteoclast formation, the femur sections were stained for TRAP. LPS injection increased N.Oc/B.Pm, the percentage of Oc.S/BS, and the percentage of ES/BS, whereas simultaneous injection of GNF-2 or imatinib blocked LPS-induced increases in these parameters (Fig. 6B).

DISCUSSION

Increased activity of monocyte/macrophage lineage cells, including osteoclasts, which are derived from a common hematopoietic precursor cell, is associated with various diseases, such as osteoporosis, metastatic cancers, and osteolytic bone destruction. Therefore, the inhibition of osteoclast or macrophage activity may represent a useful therapeutic approach for the treatment of pathological bone disorders. In this study, we have shown that GNF-2, which was developed to overcome imatinib-resistant mutations, blocks osteoclast formation and function by suppressing RANKL-induced NF-κB activity and

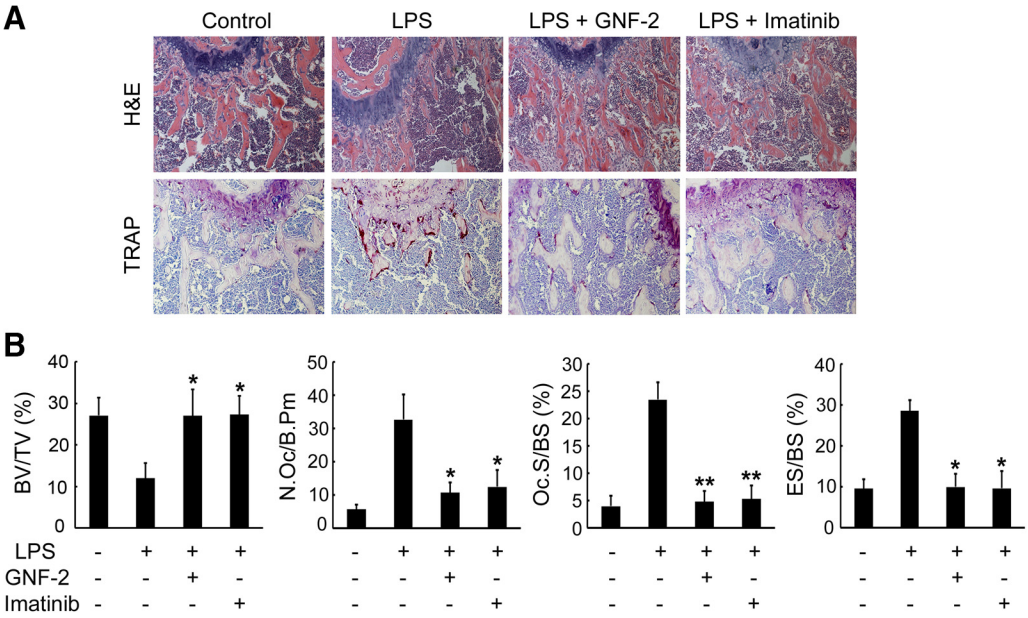

Figure 6. GNF-2 and imatinib protect LPS-induced bone erosion in mice. LPS (5 mg/kg) or PBS control were injected i.p. on Days 1 and 5. GNF-2 (10 mg/kg) or imatinib (10 mg/kg) was administered as described in Materials and Methods. (A) Femurs were decalcified, embedded, and sectioned. The sections were stained with H&E (upper). The sections were also stained for TRAP and counterstained with methyl green to identify osteoclasts (lower). (B) BV/TV, N.Oc/B.Pm, percentage of Oc.S/BS, and percentage of ES/BS were analyzed using the Bioquant OSTEO II program. The data are expressed as the means ± SD (n=5/group). *$P < 0.05$; **$P < 0.001$ versus LPS alone.

M-CSF/c-Fms signaling. Thus, GNF-2 may have therapeutic potential for the treatment of bone-destructive diseases.

c-Fos is a key transcription factor in RANKL-induced osteoclast differentiation [17, 18, 31]. The crucial role of c-Fos in osteoclastogenesis was revealed by genetic studies using c-Fos knockout mice [16, 18]. These mice are osteopetrotic as a result of a failure of osteoclast generation. In this study, we observed that GNF-2 attenuated c-Fos expression in response to RANKL. NFATc1 is also an important mediator of osteoclastogenesis, and its expression was abolished in c-Fos-deficient cells [17, 32], implying that NFATc1 is a downstream target gene of c-Fos during osteoclast differentiation. Mirroring the decreased expression level of c-Fos, GNF-2 attenuated the expression of NFATc1. In agreement with our observations, a recent study has demonstrated that another tyrosine kinase inhibitor, dasatinib, reduces RANKL-induced expression of c-Fos and NFATc1 [33]. In addition, PCI-32765, the Bruton tyrosine kinase inhibitor, has been shown to suppress osteoclastogenesis by inhibiting PLCγ2 activation [34], which is required for NFATc1 induction during osteoclastogenesis.

Activation of the NF-κB transcription factor is a critical signal for RANKL-induced osteoclast differentiation. Mice lacking p50 and p52 develop osteopetrosis as a result of defective osteoclastogenesis [14, 15]. In addition, NF-κB has been reported to function upstream of c-Fos [35] and NFATc1 [36] during RANKL-induced osteoclast differentiation. In this study, GNF-2 decreased the transcriptional activation of NF-κB without affecting the phosphorylation of IκBα. Thus, our results indicate that the defective transcriptional activity of NF-κB in the presence of GNF-2 contributes to its potent inhibition of c-Fos and NFATc1 induction. Consistent with our findings, a previous study has demonstrated that imatinib also inhibited NF-κB activation mediated by LPS [37]. Given that GNF-2 targets tyrosine kinases, further studies are needed to clarify the molecular mechanism by which GNF-2 suppresses NF-κB transcriptional activity. Taken together, our data demonstrate that GNF-2 inhibits osteoclast differentiation through the suppression of RANKL-induced NF-κB activity and c-Fos expression.

M-CSF/c-Fms is indispensable for the proliferation of osteoclast precursors, macrophages, as well as the survival of osteoclasts. The binding of M-CSF to c-Fms activates downstream signaling pathways, including MAPKs. It has been reported that imatinib inhibits the M-CSF-induced proliferation of macrophages through the inhibition of c-Fms [38]. In this study, we also observed that GNF-2 suppresses M-CSF-mediated proliferation of osteoclast precursors. GNF-2 significantly inhibited c-Fms phosphorylation in response to M-CSF and had a potency similar to that of imatinib. However, in contrast to imatinib, GNF-2 also attenuated the protein expression of c-Fms. Reflecting its inhibitory effect on c-Fms, GNF-2 reduced M-CSF-induced ERK and Akt activation. In addition to its antiproliferative activity, GNF-2 accelerated apoptosis in mature osteoclasts by inducing caspase-3 activation and Bim expression. Collectively, our data suggest that GNF-2-mediated suppression of proliferation and survival of osteoclast lineage cells are likely to contribute to its inhibitory effect on osteoclast formation.

Terminally differentiated osteoclasts attach to the bone matrix and organize their cytoskeleton, yielding the characteristic actin ring or sealing zone. These processes are crucial for the bone-resorbing activity of osteoclasts and require the engagement of integrins with ECM proteins. The $\alpha v\beta 3$ integrin is abundant in osteoclasts and plays an essential role in the organization of the actin cytoskeleton [30, 39]. Deletion of the $\beta 3$ integrin results in impaired bone resorption, as a result of defective cell spreading, and actin-ring formation and consequently, increases bone mass [30]. In this study, we observed that GNF-2-treated osteoclasts failed to spread and produced small TRAP-positive osteoclasts. Upon further examination of the inhibitory effect of GNF-2 on cytoskeletal organization, we found that GNF-2 blocked actin-ring formation. These findings are similar to our previous observations that demonstrated that

failure of osteoclasts to spread reflects cytoskeletal disorganization [29, 40]. Furthermore, we observed that GNF-2 attenuated the induction of β3 integrin expression. Therefore, the decreased expression level of β3 integrin would likely account for its inhibition of actin cytoskeletal organization, eventually resulting in impaired bone resorption.

LPS, an outer membrane component of gram-negative bacteria, is capable of inducing in vivo bone destruction by promoting the secretion of proinflammatory cytokines, such as TNF-α. These cytokines subsequently trigger osteoclast formation from hematopoietic precursors. Furthermore, a previous study reported that the blockade of M-CSF/c-Fms using anti-c-Fms antibody prevented TNF-α-induced inflammatory osteolysis in mice, indicating that M-CSF and its receptor c-Fms are candidate therapeutic targets in inflammatory bone erosion [41]. Based on these findings, we hypothesized that GNF-2 and imatinib could prevent bone destruction mediated by LPS. Indeed, we found that GNF-2 and imatinib blocked LPS-induced bone loss, confirming the in vivo efficacy of these tyrosine kinase inhibitors in inflammatory osteolytic bone diseases. Importantly, in another animal study, we have noted that overall appearance and eating were not altered in mice treated with 10 mg/kg GNF-2 for 5 weeks (unpublished data), indicating that there was no apparent toxicity in mice receiving GNF-2. However, the skeletal effects of long-term treatment of GNF-2 in healthy animals are not known. As bone mass is maintained by osteoclast-mediated bone resorption and osteoblast-mediated bone formation during bone remodeling, the influence of GNF-2 on osteoblast and bone turnover is another important issue that needs to be investigated in the future.

In conclusion, our findings provide evidence that GNF-2, a small molecule tyrosine kinase inhibitor, suppresses osteoclast formation and activity in vitro and in vivo. Importantly, our study highlights novel aspects of osteoclast inhibition by GNF-2 versus other tyrosine kinase inhibitors. Thus, GNF-2 inhibits osteoclast differentiation by suppressing RANKL-induced NF-κB activity and blocks osteoclast cytoskeletal organization mediated by M-CSF and RANKL. Therefore, GNF-2 may have potential for the treatment of inflammatory bone destruction characterized by increased osteoclast number and/or activity.

AUTHORSHIP

H-J.K. and S-Y.K. designed research. H-J.K. and H-J.Y. conducted experiments. H-J.K., H-J.Y., J-Y.C., I-K.L., and S-Y.K. performed data analysis. H-J.K. wrote the manuscript.

ACKNOWLEDGMENTS

This research was supported by the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111345), and the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2A10005515).

DISCLOSURES

The authors declare no conflict of interest.

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KEY WORDS:
osteoporosis · NF-κB · c-Fos · c-Fms · LPS