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β-Glycerophosphate accelerates RANKL-induced osteoclast formation in the presence of ascorbic acid

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Despite numerous reports of the synergistic effects of β-glycerophosphate and ascorbic acid in inducing the differentiation of osteoblasts, little is known about their roles in osteoclastic differentiation. Therefore, we investigated the effect of β-glycerophosphate on osteoclastogenesis in the presence of ascorbic acid using primary mouse bone marrow cultures treated with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)-κB ligand (RANKL). β-Glycerophosphate dose-dependently increased RANKL-induced osteoclast formation in the presence of ascorbic acid. This stimulatory effect was apparent when β-glycerophosphate and ascorbic acid were only added during the late stages of the culture period, indicating that they influence later events in osteoclastic differentiation. While the combination of β-glycerophosphate and ascorbic acid inhibited RANKL-stimulated activation of ERK and p38, and degradation of IκB, it increased the induction of c-Fos and NFATc1. In addition, β-glycerophosphate and ascorbic acid together enhanced the induction of COX-2 following RANKL stimulation. Taken together, our data suggest that β-glycerophosphate and ascorbic acid have synergistic effects on osteoclast formation, increasing RANKL-mediated induction of c-Fos, NFATc1 and COX-2 in osteoclast precursors.

1. Introduction

Bone homeostasis requires a delicate balance between bone-forming osteoblasts and bone-resorbing osteoclasts (Rodan and Martin 2000). Excessive osteoclastogenesis results in the breakdown of bone, as occurs in osteoporosis, metastatic osteolytic lesions, Paget's disease, and rheumatoid arthritis. In contrast, reduced osteoclastogenesis causes osteopetrosis, a disorder characterized by significantly increased bone mass and lack of a marrow space (Rodan and Martin 2000). Therefore, defining the molecular mechanisms underlying osteoclastogenesis is essential for advancing our understanding of the molecular basis of the pathogenesis of bone diseases characterized by altered osteoclast activity.

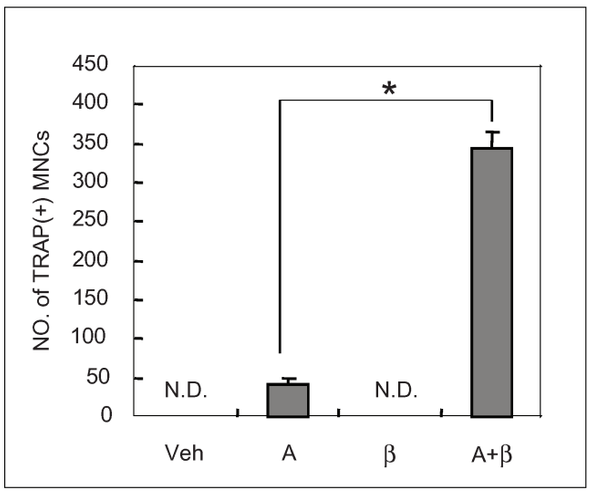
Osteoclasts are multinucleated cells formed from hematopoietic precursors (Boyle et al. 2003). Key factors regulating osteoclast formation are receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which are produced by bone-forming osteoblasts (Takahashi et al. 1988). A member of the TNF superfamily, RANKL binds to RANK on osteoclast precursors and recruits tumor necrosis factor receptor-associated factor 6 (TRAF6), resulting in the activation of multiple signaling pathways including those involving the MAP kinases ERK1/2, p38, and JNK (Walsh and Choi 2003). This triggers activation of the transcription factors NF-κB and c-Fos, which in turn induce the initial expression of the key transcription factor, nuclear factor of activated T cells c1 (NFATc1, also referred to as NFAT2 or NFATc), which is also activated by the Ca^{2+}-/calmodulin-regulated phosphatase calcineurin (Takayanagi et al. 2002; Sato et al. 2006). Ultimately, the activation of calcium signaling activates pre-existing NFATc1, which triggers the NFATc1 auto-amplification that is required for further osteoclast differentiation (Negishi-Koga and Takayanagi 2009).

Ascorbic acid is a key regulator of various types of cell differentiation. In skeletal development, ascorbic acid plays an important role in the development of the osteoblast lineage (Franceschi et al. 1994; Otsuka et al. 1999 and 2000; zur Nieden et al. 2003). Exposure to ascorbic acid enhances the expression of markers of osteoblast differentiation and the formation of a mineralized extracellular matrix. β-Glycerophosphate enhances this process, partly by increasing the availability of phosphate ions (Franceschi et al. 1994; Chung et al. 1992; Franceschi and Iyer 1992). In addition, ascorbic acid may influence the osteoclast lineage. The trophic role of ascorbic acid in osteoclastogenesis, realized through the regulation of RANKL expression in osteoblasts, is well established (Otsuka et al. 2000). Furthermore, ascorbic acid alters the dynamics of osteoclastogenesis, stimulating both the generation of new osteoclasts and the removal of mature cells (Le Nihouannen et al. 2010).

Although several studies have demonstrated synergistic effects of β-glycerophosphate and ascorbic acid on osteoblast differentiation, the potential role of β-glycerophosphate in the regulation of osteoclastic differentiation has not previously been addressed. In the present study, we examined the effects of β-glycerophosphate on the formation of osteoclasts from mouse bone marrow precursors cultured in the presence of ascorbic acid.

2. Investigations, results, and discussion

Multinucleated, TRAP-positive osteoclasts were efficiently formed from bone marrow-derived monocyte/macrophage



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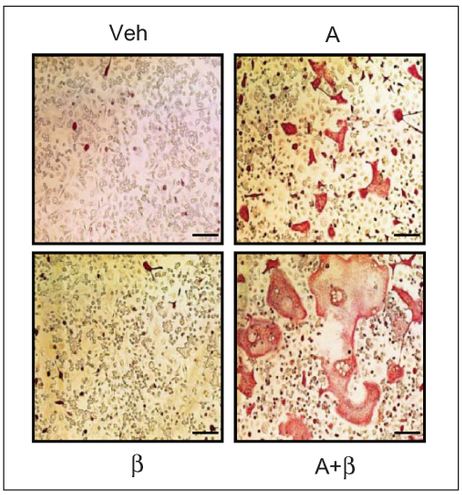

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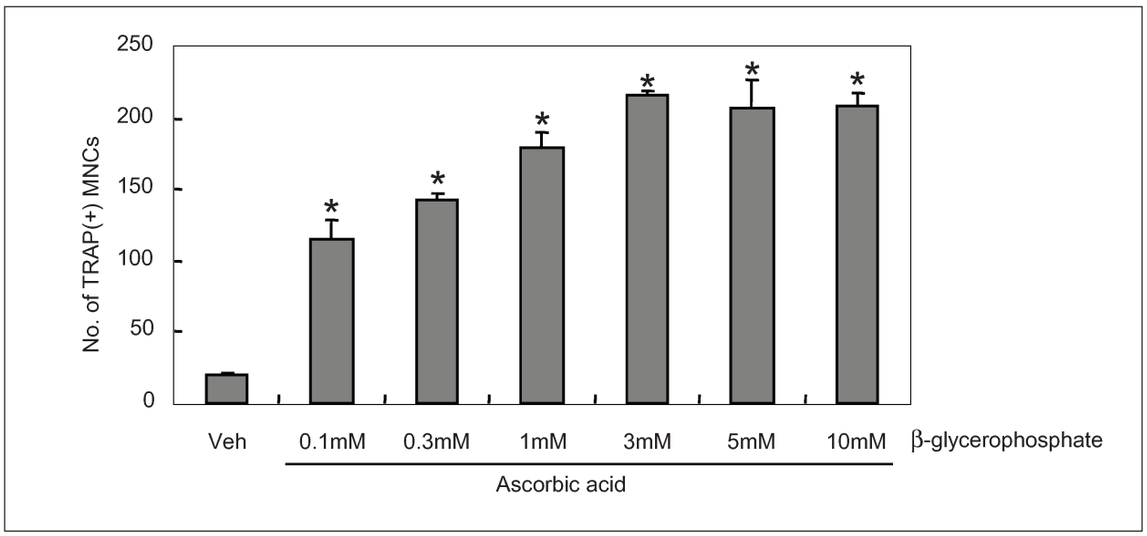

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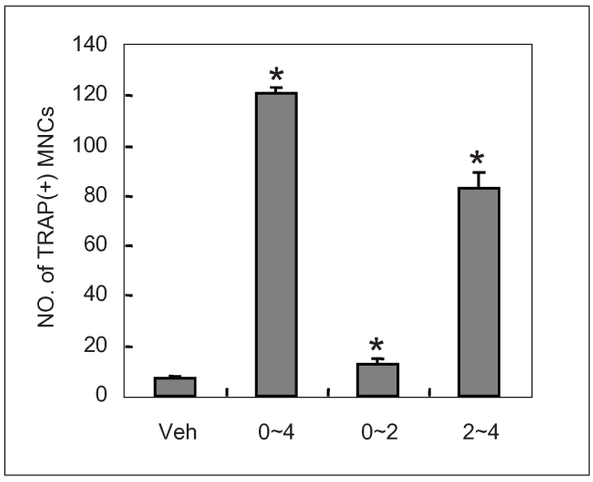
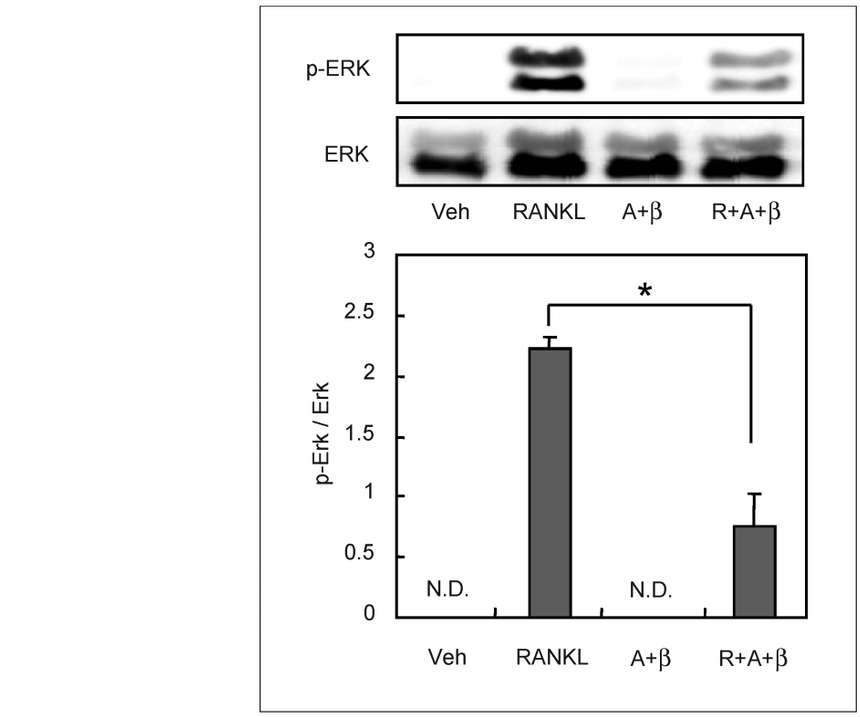


(D)

Fig. 1: β-Glycerophosphate stimulates RANKL-induced osteoclast formation in the presence of ascorbic acid. BMMs were cultured in 96-well plates for 4 days in medium containing M-CSF (20 ng/ml) and RANKL (100 ng/ml). During the whole culture period, β-glycerophosphate was applied to the growth medium at a concentration of 5 mM (A and B) or various concentrations (C) in the presence or absence of ascorbic acid (50 μg/ml). (D) β-glycerophosphate (5 mM) and ascorbic acid (50 μg/ml) were applied to the culture medium during the indicated periods of the culture. Then cells were fixed and stained for TRAP. TRAP-positive (+) multinucleated cells were counted. *p<0.05. Data are expressed as the mean ± SD of three independent experiments



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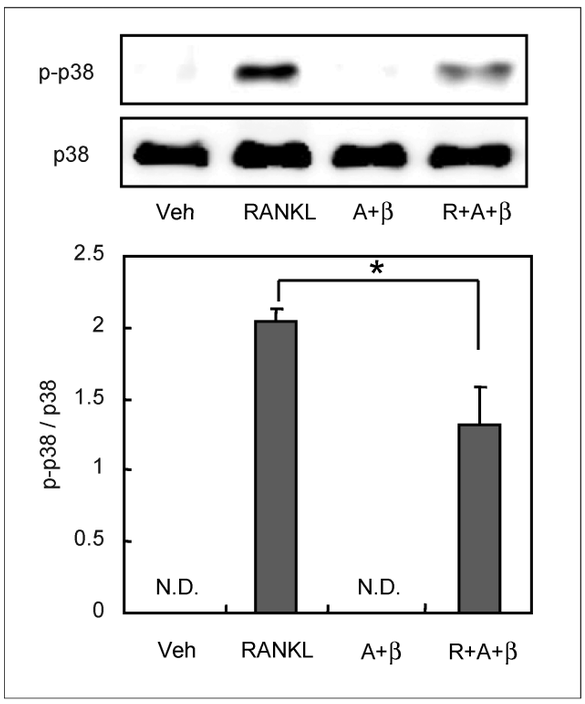

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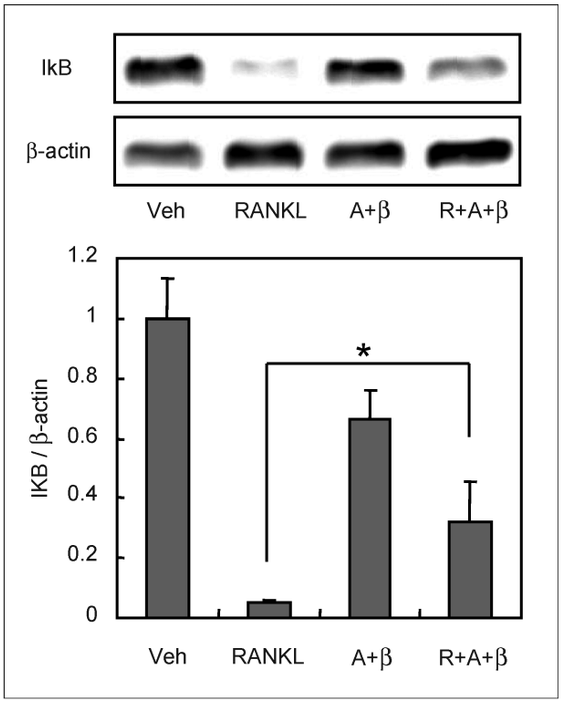

(C)

Fig. 2: The combination of β-glycerophosphate and ascorbic acid stimulates osteoclast formation independently of ERK, p38 MAPK, and NF-κB BMMs were pretreated with 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid and then stimulated with RANKL (100 ng/ml) for 15-30 min. Western blotting was performed using the indicated antibodies. β-actin served as an internal control. $*p < 0.05$. Data are expressed as the mean ± SD of three independent experiments

precursors (BMMs) cultured for 4 days in the presence of M-CSF and RANKL. We found that ascorbic acid on its own increased RANKL-induced osteoclast formation, in accordance with the findings of a previous report (Le Nihouannen et al. 2010). To assess the individual and combined effects of β-glycerophosphate and ascorbic acid on osteoclast formation, osteoclastogenic medium was supplemented with 5 mM β-glycerophosphate and/not 50 μg/ml ascorbic acid (Fig. 1). β-Glycerophosphate synergized with ascorbic acid to increase the number of differentiated osteoclasts formed. Treatment with β-glycerophosphate alone did not induce osteoclast formation (Fig. 1A). The joint presence of β-glycerophosphate and ascorbic acid appeared to increase osteoclast size and the number of nuclei per osteoclast, as estimated using a microscope (Fig. 1B).

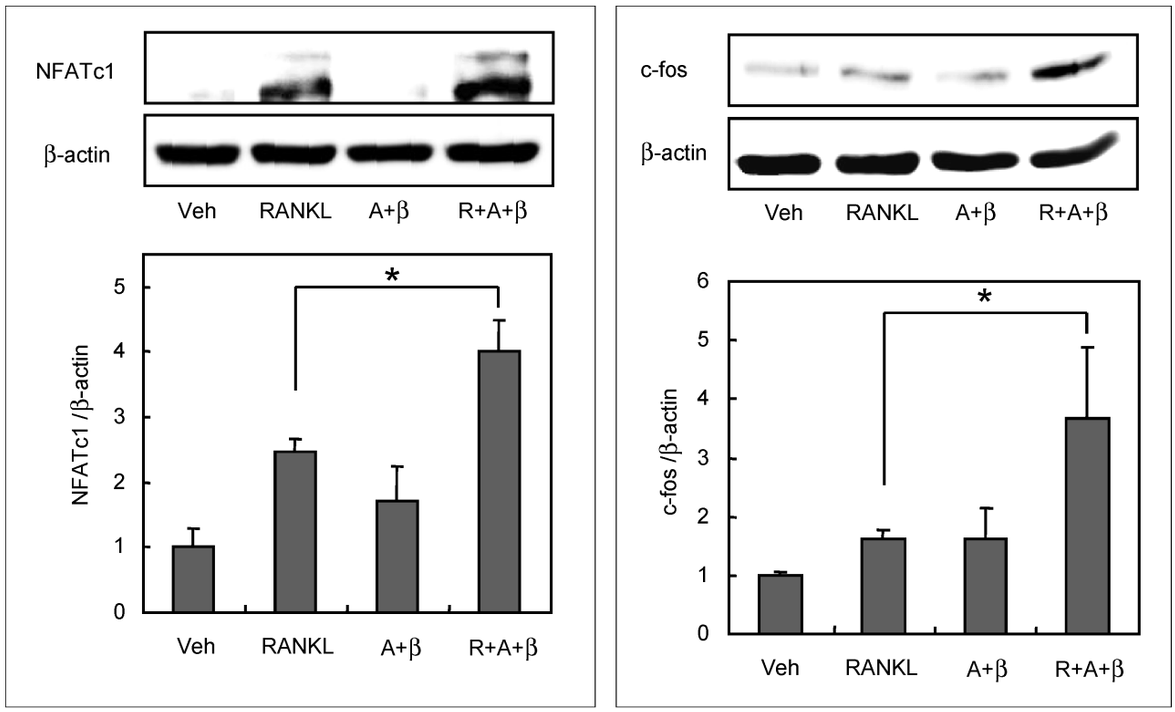

Fig. 3: The combination of β-glycerophosphate and ascorbic acid enhances RANKL-induced expression of NFATc1 and c-Fos expression BMMs were treated with 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid in the presence of M-CSF (20 ng/ml) and RANKL (100 ng/ml) for 3 days (A) or 24 h (B). Western blotting was performed using the indicated antibodies. β-actin served as an internal control. *$p < 0.05$. Data are expressed as the mean ± SD of three independent experiments

As shown in Fig. 1C, the response to β-glycerophosphate was dose-dependent, with the maximum effect occurring at a concentration of 10 mM. We conclude that β-glycerophosphate and ascorbic acid synergize to increase osteoclast formation.

To explore the stimulatory effect of β-glycerophosphate on osteoclast formation in further detail, we applied β-glycerophosphate and ascorbic acid to BMM cultures treated with RANKL and M-CSF in early (0–2 days) and late (2–4 days) stages of the differentiation process (Fig. 1D). Although maximal stimulation of osteoclast formation followed exposure of cells to both β-glycerophosphate and ascorbic acid for the entire culture period, similar effects were observed in cells treated with β-glycerophosphate with ascorbic acid between culture days 2 and 4. However, a stimulatory effect was not apparent when β-glycerophosphate and ascorbic acid were applied to cultures from day 0 to day 2. These observations suggest that β-glycerophosphate and ascorbic acid stimulate later events during osteoclastogenesis.

A key signaling event induced by the binding of RANKL to RANK is the activation of MAP kinases and NF-κB signaling (Tanaka et al. 2003). To elucidate the mechanism by which β-glycerophosphate and ascorbic acid stimulate osteoclastic differentiation, and the signaling pathways involved, BMMs were treated for 0–30 min with RANKL and/not β-glycerophosphate in the presence of ascorbic acid. Unexpectedly, RANKL-induced phosphorylation of ERK and p38 and degradation of IκB were inhibited by the combination of β-glycerophosphate and ascorbic acid (Fig. 2).

The NFATc1 and c-Fos pathways play critical, fundamental roles in osteoclast formation. Indeed, the lack of either of these transcription factors arrests osteoclastogenesis (Teitelbaum 2004). We next examined the combined effects of β-glycerophosphate and ascorbic acid on the expression and activation of NFATc1 and c-Fos (Fig. 3). β-Glycerophosphate and ascorbic acid together increased RANKL-stimulated expression of both NFATc1 (Fig. 3A) and c-Fos (Fig. 3B). Taken together, these results suggest that β-glycerophosphate and ascorbic acid stimulate osteoclast formation by activating NFATc1 and c-Fos, but not the ERK, p38, or NF-κB signaling pathways.

It was previously reported that RANKL-dependent induction of COX-2 expression in osteoclast precursors is required for osteoclast differentiation (Han et al. 2005). Furthermore, it has been shown that COX-2 expression is regulated by both c-Fos and NFATc1 (Huang et al. 2010). Therefore, we investigated the combined effects of β-glycerophosphate and ascorbic acid on RANKL-induced COX-2 expression. In agreement with previous findings (Han et al. 2005), we found that RANKL induced COX-2 expression, a response that was augmented by treatment with a combination of β-glycerophosphate and ascorbic acid. These observations lead us to conclude that β-glycerophosphate and ascorbic acid may in part stimulate osteoclast formation by increasing COX-2 expression in osteoclast precursors.

It is well known that β-glycerophosphate and ascorbic acid synergize to promote the maturation of osteoblasts (Franceschi et al. 1994; Chung et al. 1992; Franceschi and Iyer 1992), with β-glycerophosphate accentuating the effects of ascorbic acid on both alkaline phosphatase activity and the accumulation of a collagen-rich matrix in mature cultures. The molecular mechanism by which β-glycerophosphate exerts its stimulatory effects remains unclear. Some believe that it merely functions as a source of inorganic phosphate, and that its presence in the growth medium of differentiating osteoblasts is necessary for hydroxyapatite-containing bone nodules to form (Chung et al. 1992). However, others demonstrated that β-glycerophosphate can induce the phosphorylation of Smad 1/5/8 phosphorylation and the expression of both Runx2 and Osx (Bear et al. 2008), suggesting that β-glycerophosphate plays a much more complex role in osteoblastic differentiation.

In the present study, we have shown that β-glycerophosphate augments the stimulatory effect of ascorbic acid on the differen-

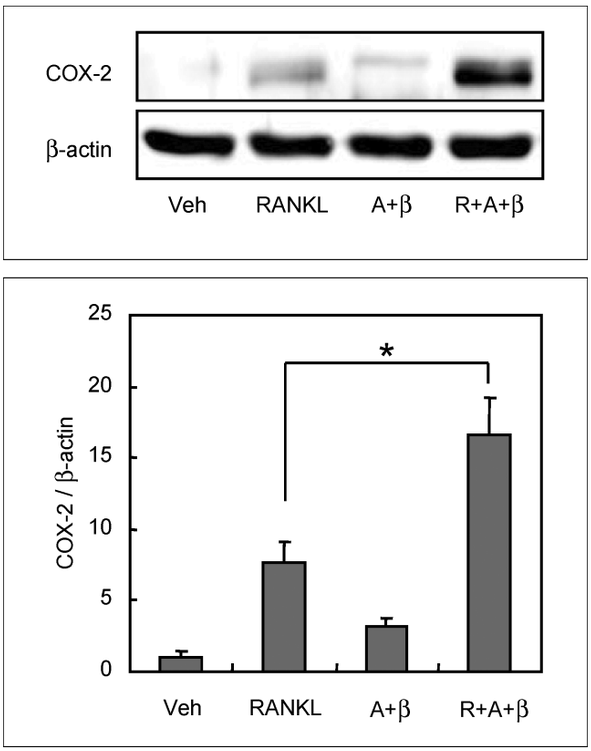

Fig. 4: β-Glycerophosphate and ascorbic acid act synergistically to increase RANKL-induced COX-2 expression BMMs were pretreated with 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid and then stimulated with RANKL (100 ng/ml) for 12 h. Western blotting was performed using the indicated antibodies. β-actin served as an internal control. $*p < 0.05$. Data are expressed as the mean ± SD of three independent experiments

tiation of mouse bone marrow cells into osteoclasts. Osteoclastic differentiation comprises multiple steps, including the attachment of cells to a scaffold, cell proliferation, differentiation to mononuclear TRAP-positive cells, and the fusion of the TRAP-positive mononuclear cells to form multinuclear TRAP-positive cells (Yavropoulou and Yovos 2008). Because each step is indispensable, impairment of any one step may block differentiation. Our data show that treatment with the combination of β-glycerophosphate and ascorbic acid resulted in the formation of large, multinucleated osteoclasts. Furthermore, it was during the later stages of osteoclastic differentiation that β-glycerophosphate and ascorbic acid exerted their stimulatory effects. Therefore, it is possible that β-glycerophosphate and ascorbic acid influence the fusion of osteoclast precursors, although further experiments are required to confirm this.

In conflict with the generally held view that ascorbic acid is an outstanding antioxidant (Meister 1992), it was recently suggested that, depending on its concentration, it can act as an oxidant during osteoclastogenesis (Le Nihouannen et al. 2010). By increasing oxidative stress, ascorbic acid stimulated osteoclast formation, but also increased rates of osteoclast apoptosis. Oxidative stress stimulates several signaling pathways in osteoclasts, including those involving c-Fos and NFATc1 (Han et al. 2007). In agreement with this observation, our findings demonstrated that β-glycerophosphate and ascorbic acid stimulated osteoclast formation by activating NFATc1 and c-Fos. Because NFATc1 is the master transcription factor for osteoclastogenesis (Tanaka et al. 2003), its activation by the combination of β-glycerophosphate and ascorbic acid may have overcome the inhibitory effects of these two compounds on ERK, p38, and NF-κB.

In conclusion, we have demonstrated that β-glycerophosphate accelerates RANKL-induced osteoclast formation in the presence of ascorbic acid. The stimulatory effects of β-glycerophosphate and ascorbic acid were attributable to the induction of c-Fos, NFATc1 and COX-2.

3. Experimental

3.1. Reagents

Ascorbic acid and β-glycerophosphate were purchased from Sigma Aldrich (St. Louis, MO). Recombinant human soluble RANKL and macrophage colony stimulating factor (M-CSF) were purchased from PeproTech EC (London, United Kingdom). Specific antibodies against NFATc1 and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Specific antibodies against phospho-ERK, ERK, phospho-p38, p38, IκB, c-fos and COX-2 were purchased from Cell Signaling Technology (Beverly, MA). All other reagents were form Sigma Aldrich (St. Louis, MO).

3.2. Cell culture and in vitro osteoclastogenesis

Total bone marrow cells from 5- to 6-week-old ICR mice were cultured for 1 day in α-MEM containing 10% fetal bovine serum (FBS). Nonadherent cells were collected and further cultured with M-CSF (30 ng/ml) in α-MEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The nonadherent cells were collected and cultured with 30 ng/ml M-CSF for 3 days, and further adherent cells were used as bone marrow-derived macrophages (BMMs). BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of 50 μg/ml and/or 5 mM β-glycerophosphate for 4 days. After cell cultures, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline (−) for 20 min. After treatment with 95% ethanol for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 1 mg/ml fast red violet LB salt] for 30 min. Then, tartrate-resistant acid phosphatase positive (TRAP-(+)) multinucleated cells were counted as osteoclasts under a microscope.

3.3. Immunoblot analysis

Cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 270 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, 0.5 mM Pefabloc SC), and centrifuged at 12,000 rpm for 10 min. The supernatants were collected and their protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by 10% SDS–PAGE and transferred to a PVDF membrane (GE Healthcare, Piscataway, NJ). The membrane was then blocked with 5% skim-milk in TBS-T (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20) for 1 h and sequentially incubated with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody (GE Healthcare). Subsequently, the membranes were washed and developed using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Bukinghamshire, UK).

3.4. Statistical analysis

Data are presented as the mean ± SD from three experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A p value<0.05 was considered statistically significant.

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