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Vitamin C-deficiency stimulates osteoclastogenesis with an increase in RANK expression

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Abstract

The effects of vitamin C (VC) on osteoclastogenesis were studied in vivo using ascorbate-requiring Osteogenic Disorder Shionogi (ODS) rats and in vitro using bone marrow-derived monocyte/macrophage cells (BMMs). The results confirmed previous findings of increases in the number of osteoclasts and in bone resorption at 2 and 3 weeks, but not 1 week, of VC-deficiency in ODS rats. The mRNA and protein levels of receptor activator nuclear factor kappaB (RANK) ligand and osteoprotegerin, and the mRNA level of macrophage-colony stimulating factor (M-CSF) in the proximal tibia of VC-deficient rats did not differ from those in VC-supplemented control rats. However, the mRNA levels of RANK, c-fos and c-jun were significantly increased at as early as 1 week of VC-deficiency. These results suggested that VC-deficiency stimulated osteoclastogenesis by increasing RANK expression. The osteoclastic differentiation of BMMs was suppressed in the presence of VC. The suppressed osteoclastogenesis was associated with decreased levels of RANK, c-fos and c-jun. The pretreatment of BMMs with VC or PD 98059, a specific inhibitor of extracellular signal regulated kinase (ERK)-activating MEK1, decreased the expression of RANK induced by M-CSF. VC inhibited the M-CSF-induced activation of ERK. These results suggested that VC-deficiency increased osteoclastogenesis by increasing RANK expression mediated through the activation of ERK.

Keywords: Vitamin C; Osteoclastogenesis; M-CSF signaling; Receptor activator nuclear factor kappaB; Extracellular signal regulated kinase

1. Introduction

Vitamin C (VC) is an essential nutrient for humans that lack the enzyme L-gulono-γ-lactone oxidase, the final enzyme in the biosynthetic pathway for VC [1]. It functions as a reducing agent and a cofactor for collagen synthesis, and the lack of Asc results in scurvy. In bone, generally, a view that VC is required for osteoclastic differentiation prevails through the observation that the depletion of ascorbic acid (Asc) from culture medium in a system using ST2 cells as mesenchymal support cells prevented differentiation [2,3]. In this co-culture system, VC is essential for promoting the expression of receptor activator nuclear factor kappaB ligand (RANKL), which is absolutely required for osteoclastic differentiation, after stimulating the synthesis and subsequent excretion of type I collagen in osteoblasts [4]. Provided permissive levels of macrophage-colony stimulating factor (M-CSF) are present, RANKL is both necessary and sufficient for osteoclastogenesis [5]. Although these previous findings clearly imply an indirect role for VC in osteoclastogenesis in vitro, little attention has been paid to the effect of VC-deficiency on osteoclastogenesis in vivo.

The intraperitoneal administration of VC was shown to significantly prevent increases in the number of osteoclasts on the surface of bone in ovariectomized mice [6]. Indeed, we observed an increase in osteoclasts in VC-deficient mature Osteogenic Disorder Shionogi (ODS) rats which already had fully differentiated osteoblasts [7]. Like humans, ODS rats have a hereditary defect in the synthesis of Asc due to a lack of L-gulonolactone oxidase, although normal rats can synthesize Asc [8,9]. Neither a loss of body weight nor a reduction in food intake was observed in mature ODS rats fed a VC-free diet during 3 weeks (3 weeks of VC-deficiency: D3) [7,10]. This study was designed to examine whether, and if so, how, VC-deficiency increases osteoclastogenesis in ODS rats at an early stage.

One possible explanation for the increased number of osteoclasts in VC-deficient rats involves increased expression of RANKL. RANKL in combination with M-CSF plays a critical role in the differentiation of osteoclast precursors into mature osteoclasts. Osteoprotegerin (OPG) is a decoy receptor that acts by binding to and neutralizing

Abbreviations: ALP, alkaline phosphatase; AP-1, activator protein-1; Asc, ascorbic acid; BMMs, bone marrow-derived monocyte/macrophage precursor cells; DHA, dehydroascorbic acid; ERK, extracellular signal regulated kinase; FCS, fetal calf serum; Hyp, hydroxyproline; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage-colony stimulating factor; MNCs, multinucleated cells; ODS, Osteogenic Disorder Shionogi; OPG, Osteoprotegerin; RANK, receptor activator nuclear factor kappaB; RANKL, receptor activator nuclear factor kappaB ligand; TRAP, tartrate-resistant acid phosphatase; VC, vitamin C.

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RANKL [11,12]. Osteoclastic differentiation is principally stimulated by an increase in the ratio of RANKL to OPG in bone [13].

Alternatively, a possible mechanism for accelerated osteoclastogenesis under VC-deficient conditions involves increased numbers of osteoclast precursors and/or the stimulation of osteoclastogenic signal transduction. Osteoclasts are generated from hematopoietic cells of the monocyte/macrophage lineage [14,15]. The hematopoietic precursor cells express c-Fms, the receptor of M-CSF. The binding of M-CSF to c-Fms on osteoclast precursors provides signals required for survival, proliferation and RANK expression [16]. RANKL acts on late-stage precursor cells that express its receptor, RANK and transmit osteoclastogenic signals through the activation of osteoclastogenesis-specific transcription factors, nuclear factor κB and activator protein-1 (AP-1) [17]. A series of experiments in vitro have suggested that mitogen-activated protein kinases (MAPKs) play an important role in osteoclastogenesis [18–20]. VC deficiency may stimulate osteoclastogenesis by modulating the signal transduction.

In the current study, we corroborated previous findings that the osteoclastic activity and the number of osteoclasts increased in VC-deficient ODS rats. Next, we demonstrated that the deficiency did not significantly change the expression of RANKL, OPG and M-CSF, but increased the expression of RANK, c-fos and c-jun in the bone. Further, VC has been found to decrease osteoclastogenesis mediated by a decrease in RANK expression with the inhibition of M-CSF-induced activation of ERK in bone marrow-derived monocyte/macrophage cells.

2. Materials and methods

2.1. Materials

Recombinant murine M-CSF and recombinant soluble RANKL were purchased from Peprotech (London, UK). Ascorbic acid (Asc), dehydroascorbic acid (DHA), fetal calf serum (FCS) and the BCA protein assay kit were obtained from Nacalai (Kyoto, Japan), Gibco BRL (Gaithersburg, MD, USA) and Pierce (Rockford, IL, USA), respectively. The leukocyte acid phosphatase kit, minimal essential medium (MEM), PD98059, SP600125 and SB202190 were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for RANKL, OPG, RANK, c-Fos, c-Jun, ERK1/2, phospho-ERK1/2, Jun amino-terminal kinase (JNK), phospho-JNK, p38, phospho-p38 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. VC-deficient ODS rat

Homozygous ODS rats (*od*/*od*), 9-week-old females, were purchased from Japan Clea (Osaka, Japan) and deprived of VC as described previously [7]. Briefly, the control group (n=6) was given an Asc-supplemented diet [Asc-free food (CL-2, Japan clea, Osaka, Japan) and drinking water with Asc (1 mg/ml)] for 4 weeks and groups D1, D2 and D3 (n=6 for each group) were fed an Asc-free diet (Asc-free food and drinking water without Asc) for 1, 2 and 3 weeks after being on the Asc-supplemented diet for 3, 2 and 1 week, respectively. After 4 weeks of feeding, the femoral and tibial bones were collected under anesthesia with sodium pentobarbital (25 mg/kg) and used for biochemical and histological analyses, or preparing the bone marrow-derived monocyte/macrophage precursor cells (BMMs). Animal experiments were performed in accordance with the protocols approved by the Animal Care Research Committee of Nara Women's University.

2.3. Biochemical and histological analyses of proximal tibia

The bone of the proximal tibia (the quarter from the aspect of the knee of the tibia) was homogenized and the bone extract was prepared as described [7]. The activities of alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP) and cathepsin K and the amounts of Ca and hydroxyproline (Hyp) in proximal tibia were determined as reported previously [7,21]. The TRAP-stained cells in sections of tibia were measured on the metaphysis of proximal tibia at a magnification of 400× and shown as the number of osteoclasts per field [7].

2.4. Preparation of BMMs

Bone marrow cells were prepared from the femur and tibia of VC-deficient ODS (D2) or normal Wistar rats and cultured in MEM plus 10% fetal calf serum (FCS) for 16-24 h in the presence of M-CSF (5 ng/ml). After a Ficoll-Paque gradient of non-adherent cells, the cells at the gradient interface were used as BMMs.

2.5. Assay of osteoclastic differentiation

BMMs (1×10^4cells/well of a 96-well plate or 1.5×10^5cells/plate of a 35-mm plate) were cultured in MEM plus 10% FCS containing M-CSF (20 ng/ml) and RANKL (10 ng/ml) with or without VC (Asc or DHA). Cultures were maintained with a change of medium every 3 days. After 5 days, cells were used for TRAP staining and the assessment of cell viability. In the experiments for M-CSF signaling, BMMs were cultured in M-CSF-free medium for 30 min with or without Asc, DHA, PD98059, SP600125 or SB202190 before the addition of M-CSF.

2.6. TRAP staining and cell viability

Cells were fixed and subjected to TRAP staining using the leukocyte acid phosphatase kit (Sigma 387-A). The TRAP-positive multinucleated cells (MNCs) (three or more nuclei per cell) were counted manually by light microscopy.

Cell viability was assessed by colorimetric assay using WST-8 (Cell Counting Kit-8: Dojin, Japan) according to the manufacturer's instructions.

2.7. Western blot analysis

Bone extract or cell lysate was used for Western blot analysis. The protein concentrations were measured using the BCA protein assay kit. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes. Western blotting and reprobing were performed and chemiluminescent signals were quantified by a densitometer as reported previously [21].

2.8. Quantitative real-time PCR analysis

Total RNA from the proximal tibia or cultured cells was prepared using a commercial kit (NucleoSpin RNA II kit, Macherey-Nagel, France). In the case of bone, bone marrow was removed and the bone was homogenized in the presence of 0.1M EDTA. Real-time polymerase chain reaction (PCR) was performed using cDNA, or total RNA for the negative control, with SYBR-Green Real-time PCR Master Mix plus (Toyobo, Tokyo, Japan) and specific primers (RANKL, 5′-AGCGCAGATGGATCCTAACA-3′ and 5′-TCGAGTCCTGCAAACCTGTA-3′ ; OPG, 5′-TGTTCTGGTGGACAGTTTGC-3′ and 5′-GCTGGAAAGTTTGCTCTTGC-3′; M-CSF, 5′-CCTGATTGCAACTGCCTGTA-3′ and 5′-TTGGTTGCTCTGTTGACTCG-3′; c-fms, 5′-GTGGCTGTGAAGATGCTCAA-3′ and 5′-GCAG-CAGTATTCGGTGATGA-3′; RANK, 5′- TTAAGCCAGTGCTTCACGGG-3′ and 5′ GAGTC-CAATCAGTCCTGCAC-3′; c-fos, 5′-TCAAAGGGTTCAGCCTTCAG-3′ and 5′-

Table 1
VC-deficient ODS rat

	Control	D1	D2	D3
Body weight (g)				
Start	182.9±2.5	182.7±3.9	185.3±4.0	185.4±3.7
Final	208.4±3.0	208.8±3.4	208.6±3.2	196.8±3.4
Length of tibia (mm)	31.8±0.4	31.9±0.3	31.8±0.4	31.4±0.8
Weight of tibia (g)	0.409±0.020	0.395±0.007	0.393±0.011	0.358±0.012
Weight of proximal tibia (g)	0.170±0.010	0.169±0.008	0.158±0.004	0.156±0.010
ALP activity (U/g proximal tibia)	23.6±1.64	23.6±0.98	25.6±1.01	23.9±1.80
TRAP activity (U/g proximal tibia)	0.908±0.054	1.105±0.029	1.557±0.044*	1.734±0.087*
Ca (mg/g proximal tibia)	98.5±3.7	99.0±3.5	81.7±2.9*	74.2±1.8*
Hyp (μmol/g proximal tibia)	91.0±2.4	91.7±3.2	80.2±1.9*	73.9±0.7*
The number of TRAP-positive cells (cells per field)	16±3	17±4	34±2*	38±3*

Values are the mean±S.E.M.

* Significantly different from the control value (P<.05).

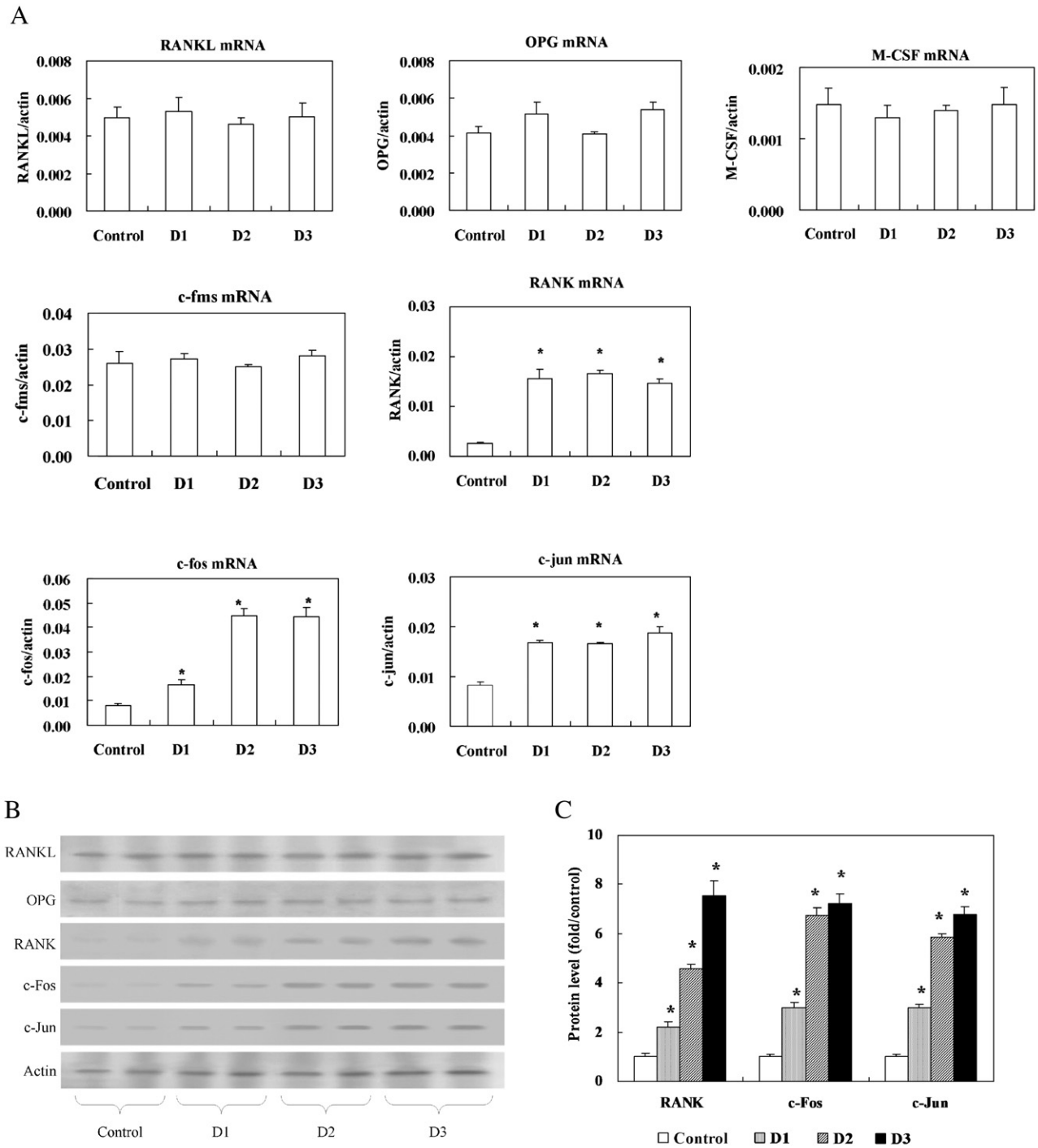

Fig. 1. Expression of RANKL, OPG, M-CSF, c-fms, RANK, c-fos and c-jun in proximal tibia. (A) The mRNA levels of RANKL, OPG, M-CSF, c-fms, RANK, c-fos and c-jun. Total RNA was extracted from the proximal tibias of control or VC-deficient (D1, D2 or D3) rats. The mRNA levels were determined by real-time RT-PCR and are expressed relative to an internal standard, actin. (B) Western blot analysis of RANKL, OPG, RANK, c-Fos and c-Jun. (C) The protein levels of RANK, c-Fos and c-Jun were quantified by densitometry and are represented graphically. Values represent the mean±S.E.M. Asterisks indicate significant differences from the control value ($P<.05$).

CTTCACCCTGCCTCTTCTCA-3′; c-jun, 5′-TGAAGCAGAGCATGACCTTG-3′ and 5′-TAGTGGTGATGTGCCCATTG-3′; actin, 5′-AGCCATGTACGTAGCCATCCA-3′ and 5′-TCTCCGGAGTCCATCACAATG-3′), and the levels of gene expression are shown relative to the internal standard (actin), as previously described [22].

2.9. Statistical analysis

All statistical analyses were performed with a one-way analysis of variance with pairwise comparison by the Bonferroni method using the Microsoft Excel data analysis program. Values of $P<.05$ were considered statistically significant.

3. Results

3.1. VC-deficient ODS rat

As shown in Table 1, the body weight and length and weight of tibias of VC-deficient rats were not significantly different from the control values. The ALP activity was unchanged during 3 weeks of VC-deficiency. However, the activities of TRAP and cathepsin K significantly increased in D2 and D3 rats. The amounts of Ca and Hyp

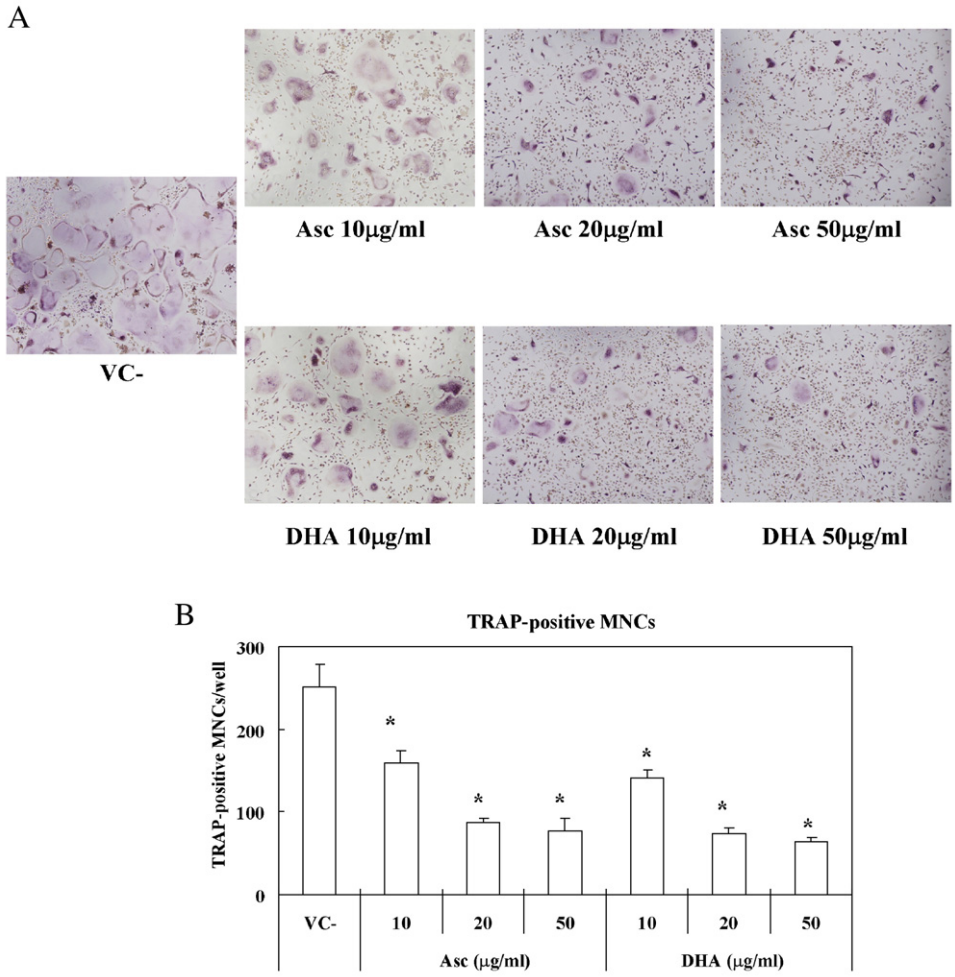

Fig. 2. Effects of VC on osteoclastogenesis of BMMs. BMMs were cultured with M-CSF (20ng/ml) and RANKL (10 ng/ml) for 5 days in the absence or presence of Asc or DHA (10, 20 or 50 μg/ml). The cultured cells were used to determine the number of TRAP-positive MNCs and cell viability. (A) The cells were fixed and stained for TRAP. The photographs are representative of 6 experiments. (B) After the staining, the TRAP-positive MNCs were counted. Values are means±S.D. Asterisks indicate significant differences from the value for VC− (P<.05).

decreased in D2 and D3 rats. The number of TRAP-positive cells significantly increased in D2 and D3 rats compared with the controls, and was proportional to the TRAP activity. These results indicated that bone resorption and the number of osteoclasts in proximal tibias increased in D2 and D3 but not D1 rats. This pattern was similar to that for the distal femur reported previously [7].

3.2. Expression of RANKL, OPG, M-CSF, c-Fms, RANK, c-fos and c-jun in proximal tibias

To evaluate the effects of VC-deficiency on the mRNA levels of RANKL and OPG, the expression of each was determined in proximal tibia of the control, D1, D2 and D3 rats. As shown in Fig. 1A, VC-deficiency did not significantly stimulate RANKL mRNA expression during the 3 weeks. Similarly, OPG mRNA expression was not significantly altered. The expression levels of M-CSF and c-fms in VC-deficient rats (D1, D2 and D3) were also similar to the control values (Fig. 1A). However, the mRNA levels of RANK, c-fos and c-jun were significantly higher in D1, D2 and D3 rats than in the control rats. The protein levels of RANKL and OPG were unaltered during VC-deficiency (Fig. 1B), resulting in no substantial change in the RANKL/OPG ratio. However, the amounts of RANK protein in D1, D2 and D3 rats significantly increased to about 2.5-, four- and sixfold the control level, respectively (Fig. 1C). Similarly, the protein levels of c-Fos and c-Jun were also significantly higher in VC-deficient rats than in the control.

3.3. Effect of VC on osteoclastogenesis of BMMs in vitro

To clarify the direct effects of VC on the osteoclast precursor cells, the osteoclastogenesis of BMMs induced by M-CSF and RANKL was examined with or without VC. Asc or DHA inhibited the osteoclastic differentiation of BMMs as shown in Fig. 2A. The presence of Asc or DHA at 10, 20 or 50 μg/ml decreased the number of TRAP-positive MNCs to about 60%, 30% and 25% of the control level, respectively (Fig. 2B). Cell viability was not affected by the presence of Asc or DHA (data not shown). In the experiment using BMMs from Wistar (wild-type) rats, the presence of Asc or DHA similarly decreased the number of TRAP-positive MNCs.

3.4. Effects of VC on the expression of c-fms, RANK, c-fos and c-jun during the osteoclastogenesis of BMMs

The expression of c-fms, RANK, c-fos and c-jun in BMMs was examined during osteoclastogenesis induced by M-CSF and RANKL in the presence or absence of VC. As shown in Fig. 3, the levels of c-fms

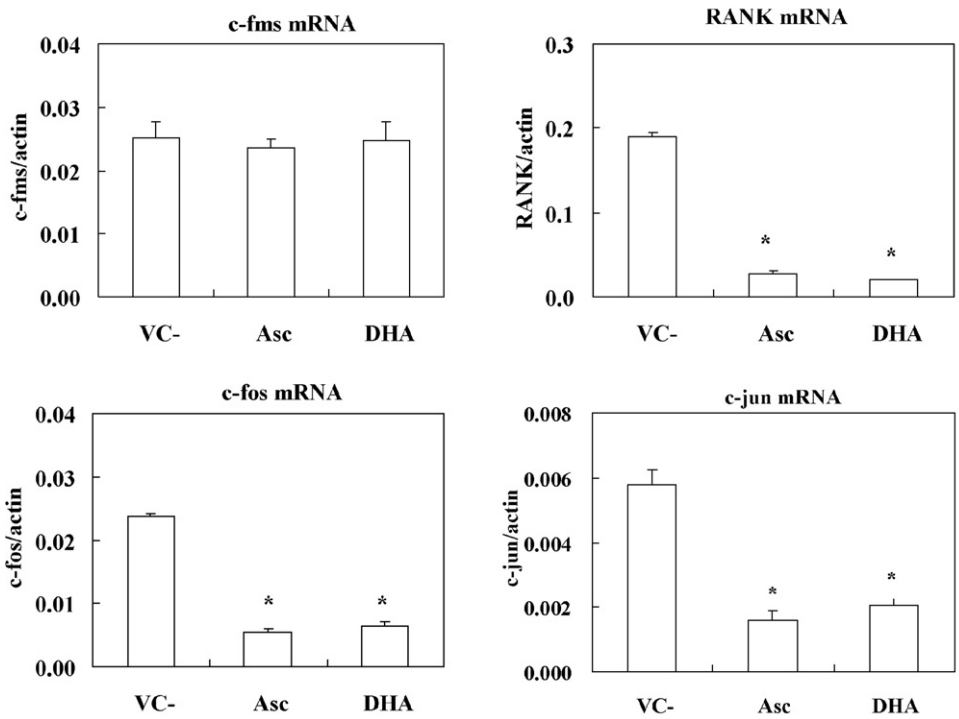

Fig. 3. Effects of VC on the expression of c-fms, RANK, c-fos and c-jun during osteoclastogenesis of BMMs. BMMs were cultured with M-CSF (20 ng/ml) and RANKL (10 ng/ml) for 24 h in the absence (VC−) or presence of Asc or DHA (50 μg/ml). Total RNA was extracted from the cultured cells and mRNA levels were determined by real-time RT-PCR and are expressed relative to an internal standard, actin. Values represent the mean±S.E.M. Asterisks indicate significant differences from the value for VC− ($P<.05$).

were similar in the three groups; the culture without VC (VC−), the culture with Asc, and the culture with DHA. However, the presence of Asc significantly decreased the level of RANK, c-fos and c-jun to about 15%, 25% and 25% of the value for VC−, respectively. The presence of DHA similarly decreased the expression of these genes.

3.5. Effects of VC and specific inhibitors for MAPK on the mRNA and protein expression of RANK induced by M-CSF in BMMs

BMMs were precultured with or without VC in the absence of M-CSF for 30min and then M-CSF was added. At 24 h after the addition of M-CSF, the mRNA levels of RANK in BMMs cultured in the presence of Asc and DHA (50 μg/ml) decreased to about 20% and 10% of the value for VC−, respectively (Fig. 4A). The amount of RANK protein in the VC-loaded cells significantly decreased to about 30% of that in VC− (Fig. 4B). However, no effect of VC on cell viability was observed.

To investigate whether MAPKs are involved in M-CSF signaling for osteoclastic differentiation in BMMs, the effects of PD98059, SP600125 and SB202190, specific inhibitors of the ERK-activating kinase MEK1, JNK and p38, respectively, on RANK expression and cell viability were examined (Fig. 4A and B). PD98059 significantly suppressed the mRNA and protein levels of RANK to about 10% and 5% of those in VC- at 20 μM, respectively, without any detectable cytotoxicity. The JNK inhibitor SP600125 had no inhibitory effect on the mRNA and protein levels of RANK. SB202190 almost completely inhibited the expression of RANK at 20 μM. However, SB202190 at 20 μM decreased cell viability to about 20% of the value of VC−, although no cytotoxicity by PD98059 or SP600125 was observed.

3.6. Effects of VC on ERK activation induced by M-CSF in BMMs

The phosphorylation of ERK 1/2 increased at 3 min after the addition of M-CSF in VC−, while much less phosphorylation was observed in the VC-loaded cells (Fig. 5A). The levels of phosphorylated ERK1/2 in the VC-loaded cells decreased to about 30% of the value for VC− (Fig. 5A). JNK1, but not JNK2, was phosphorylated 3 min after the addition of M-CSF (Fig. 5B). No significant difference was observed in the levels of phospho-JNK1 between VC− and the VC-loaded cells (Fig. 5B). No phosphorylation of p38MAPK was observed by M-CSF (Fig. 5C).

4. Discussion

This study confirmed that numbers of osteoclasts were increased in VC-deficient rats (D2 and D3) [7]. To examine the involvement of enhanced osteoclastogenesis in the increase, the effects of VC on osteoclastic differentiation were investigated in vivo using bone of VC-deficient ODS rats and in vitro using cultures of BMMs. The precursors of osteoclasts are hematopoietic cells of monocyte/macrophage lineage originating in the bone marrow. Their differentiation into osteoclasts, however, occurs on the bone surface [23,24]. Therefore, the gene expression of factors involved in osteoclastogenesis, RANKL, OPG, M-CSF, c-fms, RANK, c-fos and c-jun, was examined in bone. RANKL plays a critical role in osteoclastogenesis. To evaluate its importance in VC-deficient rats, the expressions of RANKL and its decoy receptor OPG were assessed. The mRNA and protein levels of RANKL in proximal tibial bone of the VC-deficient rats were not different from the control values. The expression of OPG did not change during 3 weeks of VC-deficiency. It seemed that the increased number of osteoclasts in VC deficiency was not associated with the up-regulation of RANKL expression and its biological availability in the bone of matured ODS rats. In addition to these functionally conflicting factors, RANKL and OPG, ALP were also expressed by osteoblasts [13]. ALP activity was maintained during 3 weeks of VC deficiency, consistent with previous observation [7]. In vitro studies have reported that VC induced the expression of ALP and osteoblastic differentiation and indicated that the formation of a collagen matrix by VC was essential for the induction of osteoblastic differentiation

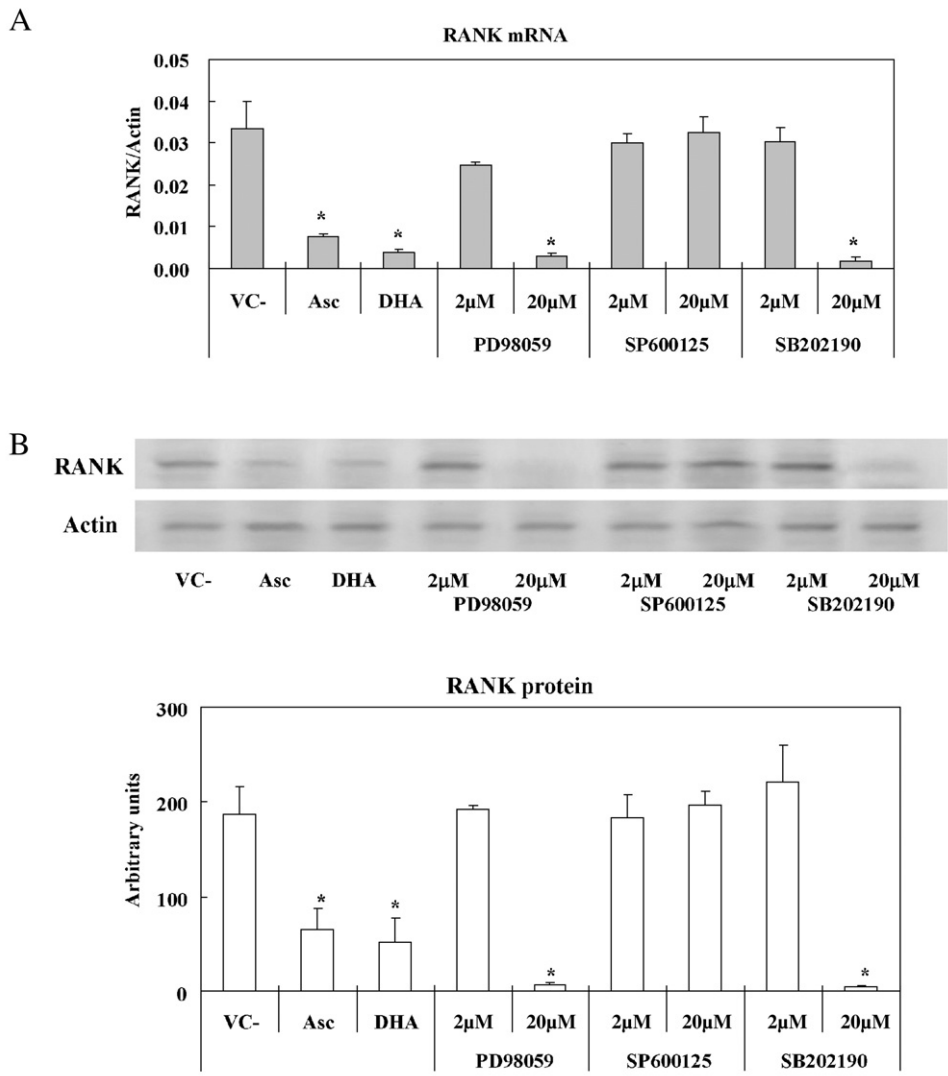

Fig. 4. Effects of vitamin C and specific inhibitors for MAPK on the M-CSF-induced RANK expression of BMMs. BMMs were pre-cultured for 30 min in the absence of M-CSF with or without Asc (50 μg/ml), DHA (50 μg/ml), PD98059 (2 or 20 μM), SP600125 (2 or 20 μM) or SB202190 (2 or 20 μM), and then M-CSF (20 ng/ml) was added. At 24 h after the addition of M-CSF, the cells were used for RT-PCR (A) and western blotting (B). The mRNA levels were expressed relative to an internal standard, actin. Protein levels were quantified by densitometry and are graphically represented. Data represent the mean±S.D. Asterisks indicate significant differences from the value for VC− (P<.05).

[25,26]. A collageneous matrix with which osteoblasts interact already exists at the onset of VC deficiency in the bone of mature rats. Degradation of the collagen matrix of bone may be necessary to decrease ALP activity and osteoblastic differentiation in the bone of mature animals [7]. Only about a 20% reduction was observed in the hydroxyproline content of bone with up to 3 weeks of deficiency in this study. These suggested that VC deficiency at an early stage did not affect osteoblasts in the bone of matured ODS, which already had a well-developed collagen matrix and fully differentiated osteoblasts.

In addition to RANKL, M-CSF is required for osteoclastic differentiation. M-CSF induces the proliferation of osteoclast precursor cells, supports their survival and up-regulates RANK expression, which is a prerequisite for osteoclastogenesis. c-Fms is a receptor of M-CSF, and the binding of M-CSF to c-fms induces the expression of RANK on the osteoclast precursor cells. The binding of RANKL to RANK activates the transcription factor AP-1, a heterodimer of c-fos and c-jun. The mRNA levels of M-CSF and c-fms in VC-deficient rats were similar to the control values, suggesting that the signal for RANK expression, M-CSF and the pool of osteoclast precursors which express c-fms and respond to M-CSF were not affected by the deficiency. However, the mRNA and protein levels of RANK significantly increased after 1 week of VC-deficiency. RANK was reported to be expressed not only in osteoclast precursors but also in osteoclasts [27]. The numbers of osteoclasts and levels of TRAP activity in D1 rats were similar to control values. These results suggested that the RANK-expressing precursor cells increased in the bone of D1 rats. This is the first report that VC-deficiency increased RANK expression in vivo. Further, the expression of c-fos and c-jun also increased at as early as 1 week of VC-deficiency. AP-1(c-fos and c-jun) is required in the RANK-RANKL signaling pathway for differentiation into osteoclasts [17]. These results suggested that VC-deficiency increased the expression of RANK followed by the up-regulation of c-fos and c-jun and stimulated osteoclastogenesis.

To examine whether VC directly acts on the monocyte/macrophage lineage cells and affects the expression of RANK, experiments were performed in vitro using BMMs. Pretreatment of BMMs with VC suppressed the osteoclastogenesis induced by M-CSF and RANKL. These results suggested that VC decreased the differentiation of BMMs into osteoclasts by directly acting on BMMs. Further, the inhibitory effects on osteoclastogenesis were observed with DHA as

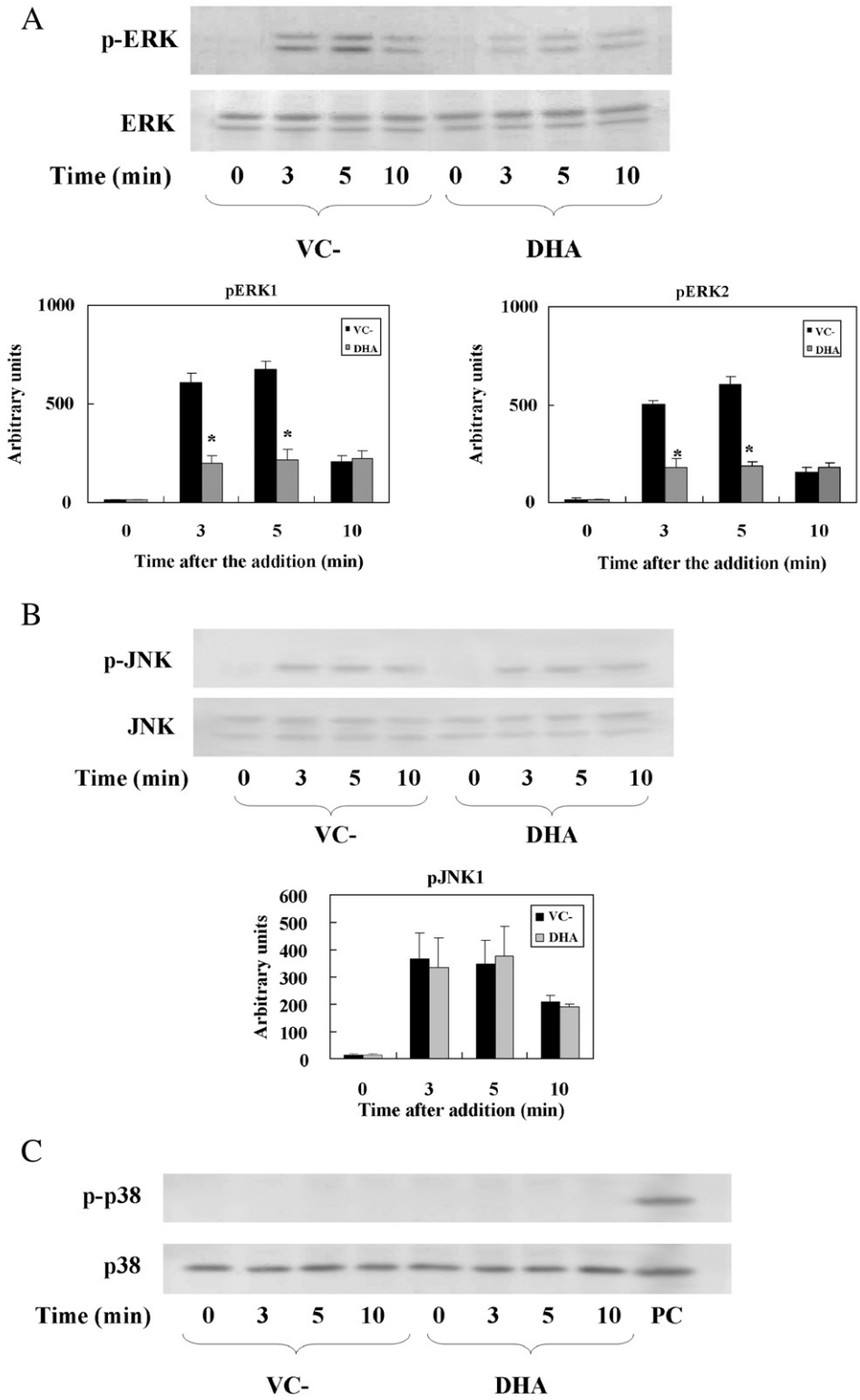

Fig. 5. Effects of VC on the M-CSF-induced activation of MAPK. BMMs were pre-cultured for 30 min in the absence of M-CSF with or without DHA (50 μg/ml), and then M-CSF (20 ng/ml) was added. The cells were harvested and used for western blotting for phospho-ERK (A), phospho-JNK (B) and pp38 (C). The membranes were stripped and reprobed with antibodies against ERK1/2, JNK and p38. Protein levels were quantified by densitometry and are graphically represented. Data represent the mean±S.E.M. PC (in Fig. 5C) indicates positive control. Asterisks indicate significant differences from the corresponding value for VC− ($P<.05$).

well as Asc. VC is transported into most cells including bone marrow cells in the oxidized form, DHA, via facilitative glucose transporters and accumulates as Asc inside the cells [28]. These results suggested that the inhibitory effect of VC on osteoclastogenesis could occur at intracellular rather than extracellular sites in BMMs. The pretreatment of BMMs with VC decreased the mRNA and protein levels of RANK. These in vitro experiments suggested that VC suppressed the generation of osteoclasts by reducing RANK expression in the

osteoclast precursor cells, reflecting the situation observed in VC-deficient ODS rats. Further PD98059, a specific inhibitor of the ERK-activating kinase MEK1, decreased the expression of RANK without cytotoxicity. The activation of ERK observed after the addition of M-CSF was inhibited in the presence of VC. These results suggested that the suppression of RANK expression was caused by inhibition of the M-CSF-induced activation of ERK. ERK was suggested to be involved in osteoclast survival [19], but this finding was made in mature osteoclasts not osteoclast precursors. Cell death was not observed in osteoclast precursors in our study. The inhibition of the activation of ERK by VC was also reported during granulocyte macrophage-colony-stimulating factor (GM-CSF)-induced signaling transduction in human monocytic U937 cells [29]. To date, the MAPKs have been shown to occur downstream of RANK-RANKL signaling in osteoclastogenesis. For the first time, ERK activation was suggested to be involved in M-CSF signal transduction and the expression of RANK in BMMs.

Taken together, our findings suggested that VC-deficiency increased numbers of osteoclasts through the up-regulation of RANK expression mediated by the activation of ERK.

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