Cell Death and Differentiation (2006) 13, 1879–1891

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Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-κB transactivation by RANKL

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Received 01.7.05; revised 15.12.05; accepted 18.1.06; published online 24.2.06
Edited by P Mehlen

Abstract

Osteoclast (Oc) differentiation is fundamentally controlled by receptor activator of nuclear factor kappaB ligand (RANKL). RANKL signalling targets include mitogen-activated protein kinases (MAPKs), nuclear factor kappaB (NF-κB), and nuclear factor of activated T cells (NFAT)c1. In this study, we found that p38 MAPK upstream components transforming growth factor-beta-activated kinase 1 (TAK1), MKK3, and MKK6 increased by RANKL in an early stage of osteoclastogenesis from primary bone marrow cells, which led to enhanced p38 activation. Retroviral transduction of dominant-negative (DN) forms of TAK1 and MKK6, but not that of MKK3, reduced Oc differentiation. Transduction of TAK1-DN and MKK6-DN and treatment with the p38 inhibitor SB203580 attenuated NFATc1 induction by RANKL. TAK1-DN, MKK6-DN, and SB203580, but not MKK3-DN, also suppressed RANKL stimulation of NF-κB transcription activity in a manner dependent on p65 phosphorylation on Ser-536. These results indicate that TAK1 and MKK6 constitute the p38 signalling pathway to participate to Oc differentiation by RANKL through p65 phosphorylation and NFATc1 induction, and that MKK6 and MKK3 have differential roles in osteoclastogenesis from bone marrow precursors.

Cell Death and Differentiation (2006) **13,** 1879–1891.
doi:10.1038/sj.cdd.4401882; published online 24 February 2006

Keywords: osteoclast differentiation; TAK1; MKK6; p38; NFATc1; NF-κB transactivation

Abbreviations: RANKL, receptor activator of nuclear factor kappaB ligand; M-CSF, macrophage colony-stimulating factor; BMM, bone marrow-derived macrophage; TRAP, tartrate-resistant acid phosphatase; TAK1, transforming growth factor-beta-activated kinase 1; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappaB; TRAF, tumor necrosis factor receptor-associated factor

Introduction

Physiological bone remodelling process requires an orchestrated action of osteoclast (Oc), cells specialized for bone resorption, and osteoblasts, cells that deposit and mineralize bone matrix. The differentiation and activity of Ocs are tightly regulated by complex mechanisms involving both soluble factors, such as hormones and cytokines, and cell surface molecules expressed on cells interacting with Oc precursors or differentiated Ocs, such as osteoblasts, bone marrow stromal cells, and immune cells. Disturbance in this regulation can result in pathologic bone resorption causing osteoporosis in postmenopausal women and bone erosion in patients with rheumatoid arthritis or periodontal diseases. A tumor necrosis factor (TNF) family cytokine named receptor activator of nuclear factor kappaB (NF-κB) ligand (RANKL) is expressed on the surface of Oc-interacting cells and plays a key role in driving the commitment of precursor cells to Ocs and further maturation to multinucleated cells, which are functionally competent for efficient bone resorption.[1,2] RANKL is also an important stimulator of the resorption activity and survival of differentiated Ocs.[3]

The precursors of Ocs are the monocyte/macrophage lineage of hematopoietic cells, of which proliferation and survival are dependent on macrophage colony-stimulating factor (M-CSF).[4] Thus, bone marrow-derived monocytes/macrophages (BMMs) generate functional Ocs, when cultured in the presence of RANKL and M-CSF *in vitro*. The macrophage cell line RAW264 or RAW264.7, when cultured with RANKL, can also differentiate to the Oc with bone-resorbing capability. Using BMMs, RAW264.7, and RANK-transfected 293 cells, we and others have revealed several aspects of RANKL signalling mechanisms. The binding of RANKL to its cognate receptor, RANK, invokes recruitment of TNF receptor-associated factor (TRAF) family proteins, which eventually results in the activation of transcription factors, NF-κB, AP-1, and nuclear factor of activated T cells (NFAT), to induce expression of genes associated with osteoclastogenesis and the survival of mature Ocs.[5,6]

The activation of mitogen-activated protein kinase (MAPK) pathways is one of the important signalling events downstream of TRAFs involved in Oc differentiation. All three MAPK family members, extracellular signal-regulated kinase (ERKs), c-Jun N-terminal kinase (JNKs), and p38 MAPKs, can be activated by RANKL and other osteoclastogenic

stimuli. However, certain selectivity and complexity in the contribution of these kinases to the regulation of different aspects of Oc responses may exist. For example, ERK was shown to have an antiapoptotic role but no resorption-activating effect in mature Ocs.[7] In the Oc differentiation process, the activity of ERKs was implicated for negative roles.[8] It was suggested that JNK participates to Oc differentiation by playing a role in RANKL induction of NFATc1 expression through c-Jun activation.[9] Specific activation of JNK1, but not JNK2, by RANKL in bone marrow monocytes (Oc precursors) and selective requirement of JNK1, but not JNK2, activity for efficient OC differentiation were reported.[10] The involvement of p38 in osteoclastogenesis has been demonstrated by the treatment of bone marrow precursor cells with pharmacological inhibitors of the kinase.[11–13] The expression of dominant-negative (DN) form of p38α into RAW264 cells partially inhibited RANKL-induced osteoclastic differentiation of these cells.[13] Interestingly, the activation of p38 MAPK by RANKL was observed in bone marrow precursor cells but not in fully differentiated Ocs.[12] The reason for this differentiation stage-dependent activation of p38 has not been revealed.

MAPKs are activated upon phosphorylation by the family of MAPK kinases (MAPKKs). In most cell types and in response to various stimuli, the MAPKKs MKK4 and MKK7 are responsible for JNK activation. Both MKK3 and MKK6 are the upstream kinases for p38 activation. The involvement of MKK7 in Oc differentiation from the precursors in spleen cells and from RAW264.7 cells was demonstrated by using a siRNA and DN form, respectively.[9,14] The expression of a DN form of MKK6 was shown to inhibit Oc differentiation form RAW264.7 cells.[13] Knowledge on the upstream kinases (MAPKKKs) that activate MKKs for Oc differentiation has remained limited. A study showed that transforming growth factor-beta-activated kinase 1 (TAK1), a MAPKKK, can form complexes with RANK, TRAF6, and TAB2 in RANK-over-expressing 293 cells.[15] The activity of TAK1 was stimulated by RANKL in the RANK-expressing 293 and RAW264.7 cells.[15] However, the effect of TAK1 activation on RANKL-induced Oc differentiation has not been demonstrated.

The activity of NFATc1 and NF-κB transcription factors is crucial for osteoclastogeneis. Recent studies have shown that the expression of NFATc1 is induced by RANKL treatment[16,17] and NFATc1-deficient embryonic stem cell lines failed to differentiate to Ocs.[17] It has been indicated in other cell types that MAPKs are involved in the regulation of NFAT family proteins.[18–21] JNK-mediated phosphorylation inhibits nuclear accumulation of NFATc3 in BHK cells while p38 activity counteracts nuclear accumulation of NFATc2 and NFATc4 in HeLa cells and adipocytes.[18–20] In T cells, p38 was shown to regulate NFATc1 through multiple mechanisms resulting in a net activation of this transcription factor.[21] Another transcription factor NF-κB is required for osteoclastogenesis.[5,6] The activation of NF-κB depends on its release from the inhibitory κB (IκB) subunits, mostly achieved by phosphorylation by IκB kinase α and β and subsequent proteasome-mediated degradation, translocation to the nucleus, and binding to DNA sites. However, recent studies have suggested that the regulation of transactivation ability is another important determinant of NF-κB activity.[22] The phosphorylation of p65 NF-κB subunit has been implicated in NF-κB activation by TNFα, LPS, and other stimuli.[22] There has been some evidence that MAPKs are involved in NF-κB regulation. A p38 inhibitor and MEK1 inhibitors suppressed p65 transactivation potential in response to TNFα.[23,24] In Oc differentiation, a potential connection between MAPKs and the NFAT and NF-κB transcription factors has not been explored.

In this study, we examined the expression levels of signalling molecules lying on the p38 pathway during Oc differentiation from bone marrow precursors. We also investigated participation of the p38 pathway signalling molecules to NFATc1 induction and NF-κB activation in RANKL-induced osteoclastogenesis. TAK1, MKK6, and MKK3 were found to be upregulated in early phase of the differentiation, which led to enhanced activation of p38 by RANKL. Like p38, TAK1 and MKK6 participated to RANKL-induced Oc formation by mediating NFATc1 expression and NF-κB transactivation through Ser-536 phosphorylation of p65 without significantly affecting DNA binding of NF-κB. However, MKK3 was not involved in osteoclastogenesis, NF-κB transactivation, and p65 phosphorylation. Therefore, TAK1 and MKK6 constitute the p38 signalling pathway that regulates NFATc1 and NF-κB transcription factors in RANKL-induced Oc differentiation.

Results

p38 MAPK is activated in an early phase of Oc differentiation, but not in mature Ocs

RANKL induces Oc differentiation from M-CSF-dependent bone marrow precursor cells. Culturing nonadherent bone marrow cells with M-CSF enhances the proliferation and enrichment of Oc precursor cells (BMMs). These BMM cells differentiated into tartrate-resistant acid phosphatase (TRAP)-positive mononuclear prefusion Ocs (pOc) about 3 days after RANKL treatment (Figure 1a). TRAP-positive multinuclear Oc generated by cell fusion of pOc were observed between 5 and 6 days after treatment with RANKL (Figure 1a). Studies with pharmacological inhibitors and gene-deficient mice provided evidence that the activity of p38 and JNK1 is required for osteoclastogenesis.[10,11,13] In this context, the activation of p38 and JNK were detected during osteoclastogenesis (Figure 1b). The p38 and JNK activity was highest 1 day after RANKL treatment and reduced as cells become mature (Figure 1b). The changes in the activation of these MAPKs during differentiation could not be ascribed to the expression levels of the kinases (Figure 1b). When the enzymatic activity of p38 was examined by immune complex kinase assays, again the highest activity was detected at day 1 (Figure 1c). The activation of NF-κB reflected as the degradation of IκB was highest at day 1, but was also substantial at days 3 and 6 (Figure 1b). The examination of RANKL signalling at different stages of Oc differentiation has led to the finding that p38 activity is stimulated in BMMs, but not in mature OCs.[12] This differentiation stage-dependent activation of p38 by RANKL was confirmed in our experiments, and stimulation with TNFα also displayed the same type of differential response in p38 activation (Figure 1d).

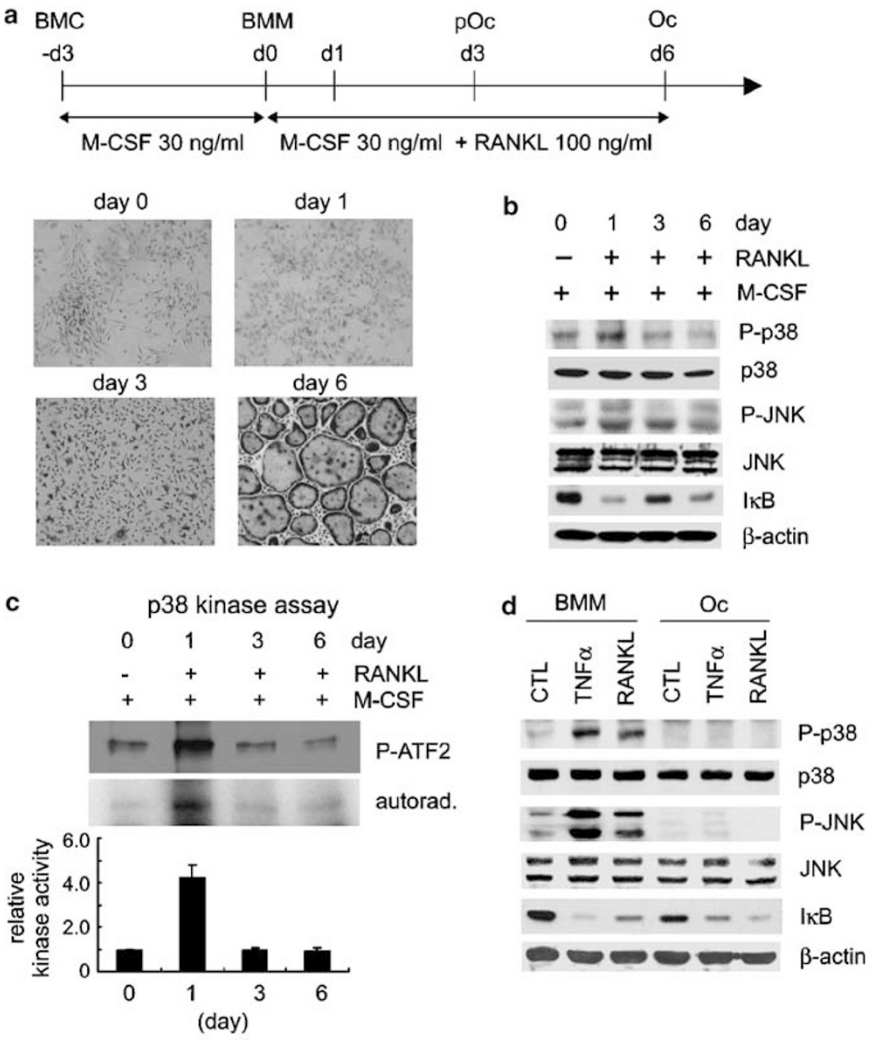

Figure 1 p38 and JNK activation is differentiation stage-dependent in osteoclastogenesis. (**a**) Osteoclast precursors (BMM) were generated by culturing nonadherent bone marrow cells (BMC) in the presence of 30 ng/ml M-CSF for 3 days. Osteoclast differentiation was induced by treating the BMM cells with 30 ng/ml M-CSF and 100 ng/ml RANKL. Cells were fixed and stained for TRAP activity at days 0, 1, 3, and 6. (**b**) BMMs treated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 0, 1, 3, and 6 days were lysed. Cell lysates were subjected to Western blotting for the detection of p38 and JNK activation with anti-phospho-p38 and anti-phospho-JNK antibodies. The activity of p38 and JNK increased at day 1 and returned to the basal level during further culture. Anti-p38 and anti-JNK blots show similar expression levels of the total p38 and JNK proteins during differentiation. Anti-β-actin was used to confirm equal loading. (**c**) BMMs were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 0, 1, 3, and 6 days. Cells were lysed and p38 kinase activity assays were performed with p38 immunoprecipitates using GST-ATF2 as the substrate. The kinase activity was detected either by Western blotting with anti-phospho-ATF2 or by autoradiography. The relative kinase activity is shown as a histogram. Error bars represent S.D. of mean values. (**d**) BMM and mature osteoclasts (Oc) were serum-starved in serum free α-MEM for 5 h and stimulated with 20 ng/ml TNFα or 500 ng/ml RANKL for 15 min. Cell lysates were subjected to Western blotting as in (**b**). Both TNFα and RANKL stimulated p38 and JNK activity in BMMs but not in Oc

JNK1/2 activation was also observed in BMMs but not in mature Ocs in response to either RANKL or TNFα (Figure 1d). In mature Ocs as well as in BMMs, both RANKL and TNFα stimulated NF-κB activation, although the extent of stimulation was lower than that in BMMs, indicating that not all signalling responses were muffled in mature Ocs (Figure 1d). Therefore, the differentiation stage-specific activation of p38 and JNK suggest that a signalling component(s) residing downstream of RANK and TNF receptor and upstream of p38 and JNK is regulated to mediate the differential responses during Oc differentiation.

TAK1, MKK3, and MKK6 are upregulated in an early phase of Oc differentiation

To find the potential component responsible for the p38 activation in early but not in later stage of Oc differentiation, we examined expression levels of signalling molecules involved in the p38 MAPK activation cascade. The protein and mRNA levels of p38 upstream MAPKKs, MKK3 and MKK6, and an MKK-activating kinase, TAK1, were detected during differentiation of BMMs to Ocs. The expression level of TAK1 protein increased in cells treated with RANKL and

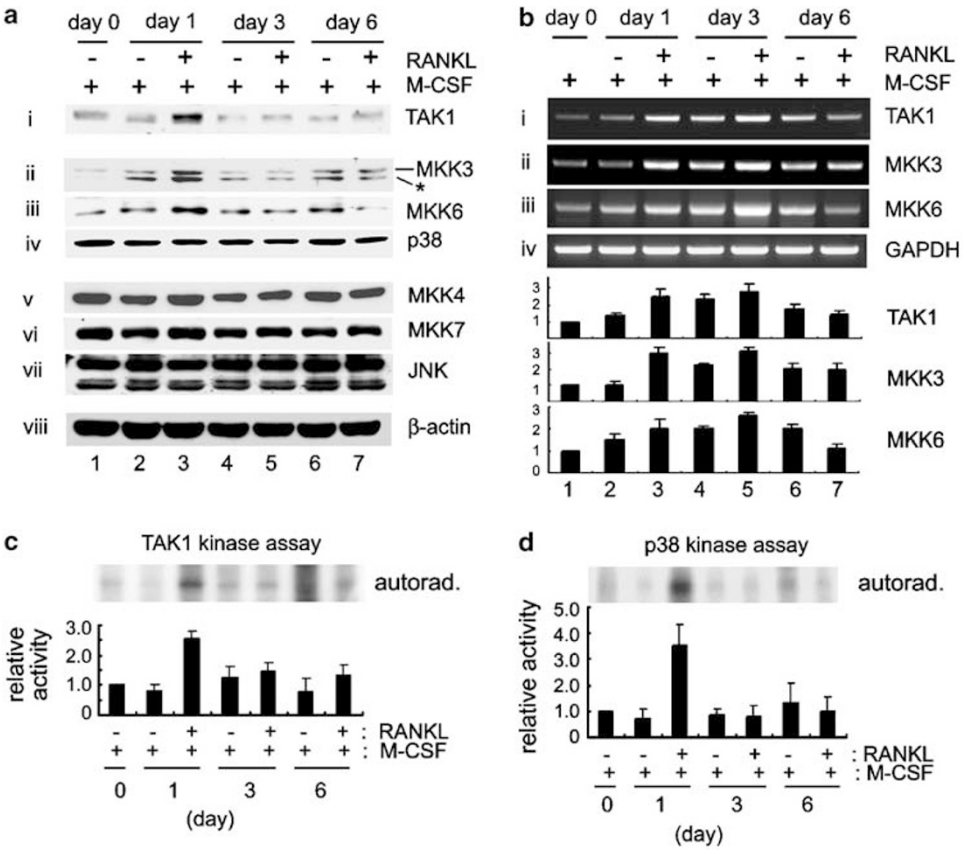

Figure 2 TAK1, MKK3, and MKK6 levels are elevated in an early stage of osteoclast differentiation. (**a**) BMMs were cultured in the presence of 30 ng/ml M-CSF together with or without 100 ng/ml RANKL for 1, 3, and 6 days. The expression levels of indicated proteins were analyzed by Western blotting. TAK1, MKK3, and MKK6 levels were regulated while other protein levels were similar during the culture. Anti-β-actin blot was used as loading control. *MKK6 protein crossreacted with the MKK3 antibody. (**b**) BMMs were cultured in the presence of 30 ng/ml M-CSF together with or without 100 ng/ml RANKL for 1, 3, and 6 days. The mRNA levels of TAK1, MKK3, and MKK6 were analyzed by RT-PCR. GAPDH mRNA levels are shown as internal control. The relative levels of TAK1, MKK3, and MKK6 are presented as histograms. Error bars represent S.D. of mean values. (**c**) BMMs were treated as in (**a**) and lysed. TAK1 was immunoprecipitated and kinase reaction was carried out with GST-MKK6-KD as the substrate. The kinase activity was detected by autoradiography. Error bars represent S.D. of mean values. (**d**) BMMs treated as in (**a**) were lysed. p38 was immunoprecipitated from the lysates and kinase reaction was performed with GST-ATF2. The kinase activity was detected by autoradiography. Error bars represent S.D. of mean values

M-CSF for 1 day but not in cells treated with M-CSF only (Figure 2a, panel i, lanes 2 and 3). TAK1 protein level returned to basal in mature Ocs (Figure 2a, panel i, lane 7). The protein levels of MKK3 and MKK6 were also elevated by treatment with RANKL plus M-CSF for 1 day (Figure 2a, panels ii and iii, lane 3). The p38 protein level did not change during the culture period (Figure 2a, panel iv). In contrast to the elevation in MKK3 and MKK6 levels in early differentiation stage, protein levels of MKK4 and MKK7, JNK-activating MAPKKs, were rather constant during the differentiation (Figure 2a, panels v and vi). Like p38, expression levels of JNK1/2 did not change during the culture period (Figure 2a, panel vii). These results suggest that the increase in TAK1, MKK3, and MKK6 may explain the p38 activation detected only in an early stage of Oc differentiation.

Next, we investigated whether the changes in protein levels of TAK1, MKK3, and MKK6 could be attributed to changes in mRNA levels. The mRNA levels of TAK1 increased by treatment with RANKL plus M-CSF from day 1, which was maximum at day 3 and reduced at day 6 (Figure 2b, panel i, lanes 3, 5, and 7). Treatment with M-CSF alone also increased TAK1 mRNA at day 3, and this level was sustained till day 6 (Figure 2b, panel i, lanes 4 and 6). The mRNA levels of MKK3 showed similar pattern as TAK1 in response to both RANKL/M-CSF and M-CSF alone (Figure 2b, panel ii). The expression of MKK6 mRNA was weakly increased at day 1 upon M-CSF treatment and the effect of RANKL addition to M-CSF for 1 day was more prominent on TAK1 mRNA (Figure 2b, panels i and iii, lanes 1–3). The induction pattern of MKK6 mRNA at days 3 and 6 was similar to that of TAK1 (Figure 2b, panel iii, lanes 4–7).

The Western blotting and RT-PCR results showed that protein levels of TAK1, MKK3, and MKK6 were maximal in day 1 cells treated with RANKL plus M-CSF, whereas their mRNA levels were highest at day 3 (Figure 2a and b). This discordance between protein and mRNA levels was also observed in M-CSF-treated cells (Figure 2a and b). Similarly, effects of pharmacological inhibitors on the induction of protein and mRNA of TAK1, MKK3, and MKK6 were incongruous with each other (data not shown). These results

indicate that the protein levels of TAK1, MKK3, and MKK6 are regulated by a complex mechanism involving both transcriptional and translational controls. Nevertheless, it is clear that the protein levels of TAK1, MKK3, and MKK6 increase in an early stage of Oc differentiation from bone marrow precursors.

We next investigated whether the increased protein levels of TAK1, MKK3, and MKK6 might lead to enhanced TAK1 and p38 activities during Oc differentiation. The kinases were immunoprecipitated from cells at different stages of differentiation and subjected to *in vitro* enzyme activity assays. As shown in Figure 2c and d, both TAK1 and p38 activities increased at day 1 in cells cultured with both M-CSF and RANKL, but not in cells cultured with M-CSF alone. TAK1 and p38 activities returned to basal levels thereafter. These results imply that the increase in TAK1 protein level led to an enhanced TAK1 activity and, together with increased MKK3 and MKK6 proteins, resulted in the subsequent activation of the downstream signalling target p38.

TAK1 upregulation is RANKL-dependent and leads to enhanced p38 activation in Oc precursor cells

We next investigated whether M-CSF is required for the induction of TAK1 in the early differentiation stage of Ocs. Cells were treated with M-CSF, RANKL, or RANKL plus M-CSF for 1 day and TAK1 level was determined by Western blotting. M-CSF did not cause induction of TAK1 (Figure 3a (i), lane 2) nor exhibited any synergistic effect on RANKL-induced TAK1 expression (Figure 3a (i), lanes 3 and 4). The increased expression of TAK1 protein was dependent on RANKL doses (data not shown). In line with the increased protein level, the kinase activity of TAK1 was elevated in cells treated with RANKL for 1 day (Figure 3a (ii), lanes 3 and 4). These results indicate that RANKL was the sole trigger of TAK1 induction under this culture condition.

The p38 activity is one of the important determinants in Oc differentiation.[11,12] To investigate whether the increase in TAK1 protein level can consequently cause an enhanced p38 activation, we incubated the Oc precursor cells with RANKL or vehicle for 1 day, deprived of serum and RANKL for 5 h, and re-stimulated with RANKL or TNFα for 15 min. TNFα, in the absence of RANKL, can stimulate Oc generation from RANKL-primed cells.[25] The elevated expression of TAK1 by 24 h RANKL treatment was confirmed (Figure 3b(i) and c(i), lanes 4 and 5). The activation of p38 was determined by Western blotting with a phopho-p38-specific antibody. Re-stimulation with RANKL or TNFα for 15 min caused p38 activation in cells not pretreated with RANKL (Figure 3b(i) and c (i), lane 3). However, the extent of p38 activation was significantly higher in RANKL-pretreated cells (Figure 3b(i)

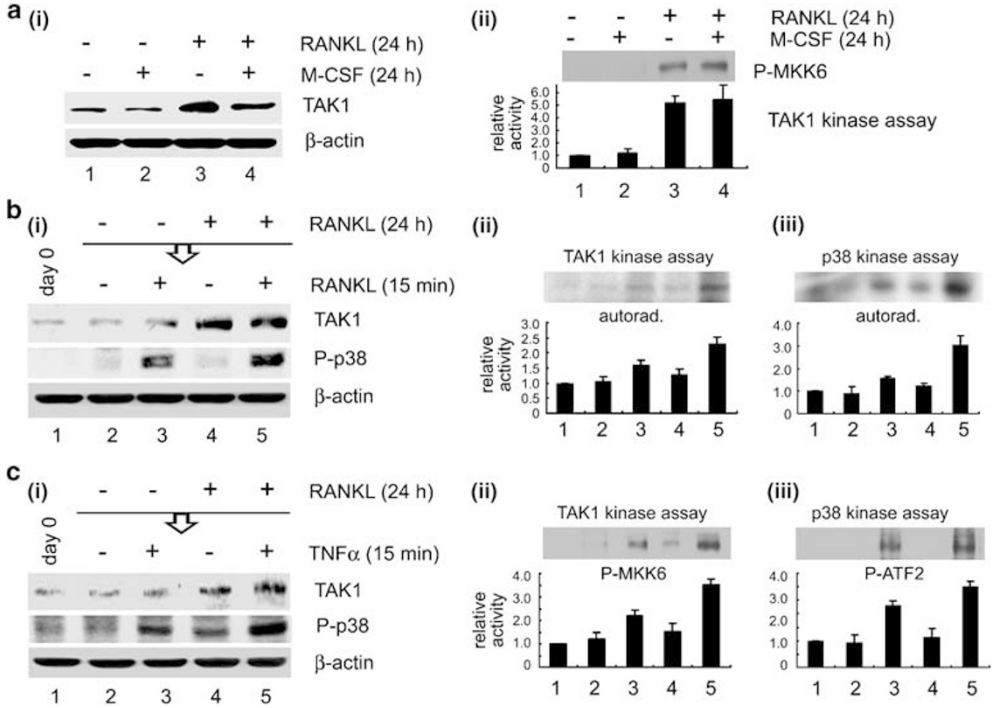

Figure 3 Increase in TAK1 expression is dependent on RANKL and leads to enhanced p38 activation. (**a**) BMMs were treated with 30 ng/ml M-CSF and/or 100 ng/ml RANKL for 24 h. (i) The expression of TAK1 protein was analyzed by Western blotting. TAK1 expression was elevated by RANKL, but not by M-CSF. β-Actin levels were analyzed to ensure equal loading. (ii) The TAK1 kinase activity was determined after immunoprecipitation with the GST-MKK6-KD substrate. Error bars represent S.D. of mean values. (**b**) BMMs were treated with or without 100 ng/ml RANKL for 24 h, serum-starved for 5 h, and stimulated with 1 μg/ml RANKL for 15 min. (i) The protein levels of TAK1 and phosphorylated p38 were examined by Western blotting. Anti-β-actin blot was used as loading control. P38 activation by RANKL was higher in RANKL-pretreated cells, which showed elevated TAK1 expression. (ii and iii) The TAK1 and p38 kinase activities were measured after immunoprecipitation as described in Materials and Methods. Error bars represent S.D. of mean values. (**c**) BMMs were treated with or without 100 ng/ml RANKL for 24 h, serum-starved for 5 h, and stimulated with 20 ng/ml TNFα for 15 min. (i) The protein levels of TAK1 and phosphorylated p38 were examined by Western blotting. P38 activation by TNFα was higher in RANKL-pretreated cells, which had increased TAK1 expression. (ii and iii) The TAK1 and p38 kinase activities were measured with immunoprecipitates as described in Materials and Methods

and c(i), lane 5). Similar results were obtained when the activation of p38 was assessed by kinase assays (Figure 3b(iii) and c(iii)). A stronger stimulation of TAK1 activity upon restimulation with RANKL or TNFα in the RANKL-primed than in unprimed cells was observed (Figure 3b(ii) and c(ii)). These responses were not due to increased expression levels of respective receptors, RANK and TNFR (data not shown). These results suggest that the elevation of TAK1 protein level by RANKL reinforces p38 activation during Oc differentiation.

Suppression of TAK1 activity reduces Oc differentiation

We next investigated the significance of TAK1 expression during Oc differentiation by examining effects of kinase-inactive DN TAK1 on Oc differentiation. A wild-type (WT) or DN form of TAK1 was introduced to BMMs by retroviral transduction. The overexpression of TAK1-WT and TAK1-DN was confirmed by Western blotting analyses (Figure 4a). The transduction efficiency of the retroviral system was about 70–90% (data not shown). These cells were cultured in the presence of RANKL and M-CSF for 5 days to induce osteoclastogenesis. The number of Ocs generated increased by overexpression of TAK1-WT, whereas it was suppressed by transduction of TAK1-DN (Figure 4b and c). Similar effects of TAK1-WT and TAK1-DN were observed when osteoclastogenesis was induced by TNFα (Figure 4d). The enhanced stimulation of p38 by RANKL in TAK1-WT-infected cells compared to the control virus infected cells was confirmed (Figure 4e, lane 6). The contrary was observed in TAK1-DN-transduced cells (Figure 4e, lane 4). TNFα stimulation also led to an enhanced and attenuated extent of p38 activation in TAK1-WT and TAK1-DN-infected cells, respectively, compared to the control virus-infected cells (Figure 4f).

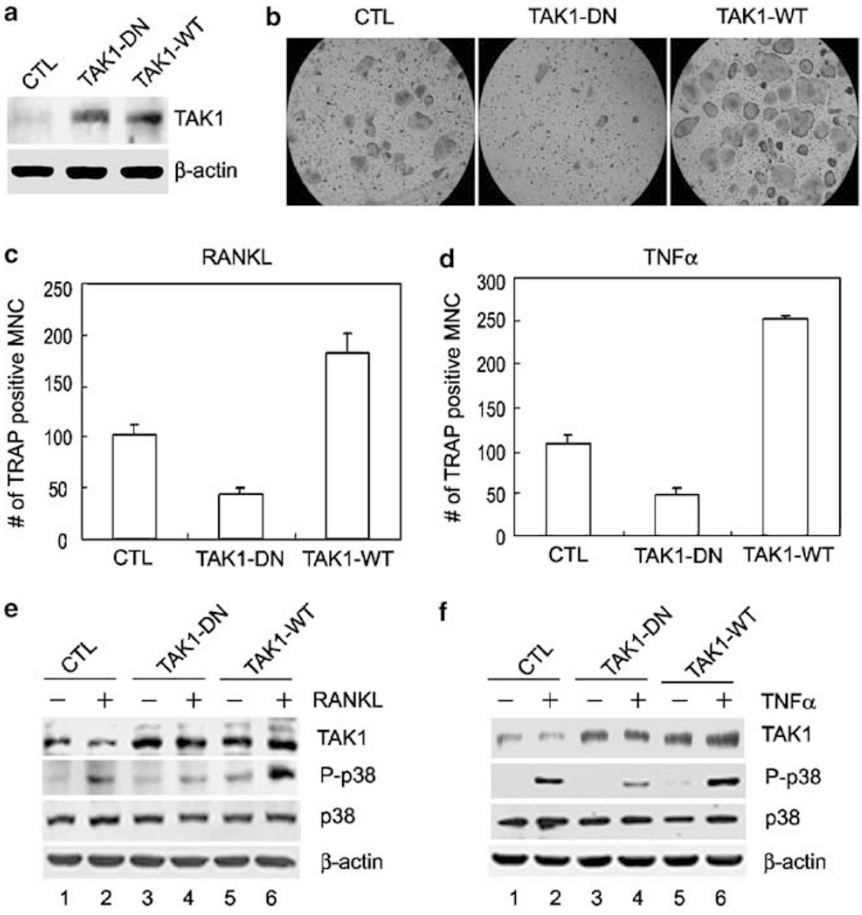

Figure 4 DN TAK1 decreases osteoclast differentiation from BMMs. (**a**) BMMs were infected with the retroviruses containing a DN or WT form of TAK1 or the control viruses. At 2 days after infection, the expression level of TAK1 was assessed by Western blotting. Expression of both TAK1-DN and TAK1-WT was greatly increased by the retroviral gene transfer. (**b** and **c**) BMMs infected with indicated retroviruses were cultured in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL for 5 days. Cells stained for TRAP activity are shown in (**b**). The number of TRAP-positive multinuclear cells (MNC) was scored under a light microscope (**c**). Error bars represent S.D. of mean values. TAK1-DN decreased and TAK1-WT increased osteoclast formation. (**d**) BMMs infected with indicated retroviruses were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 1 day and further cultured in the presence of 30 ng/ml M-CSF and 20 ng/ml TNFα for 4 days. Error bars represent S.D. of mean values. Osteoclast generation was decreased by TAK1-DN and increased by TAK1-WT. (**e**) At 2 days after retroviral gene transfer, BMMs were serum-starved for 5 h and then stimulated with RANKL (1 μg/ml) for 15 min. The activation of p38 was examined by Western blotting with anti-P-p38 antibody. TAK1-DN decreased and TAK1-WT increased RANKL-stimulated p38 activation. (**f**) Infected BMMs were serum-starved and then stimulated with TNFα (20 ng/ml) for 15 min. The activation of p38 by TNFα was decreased by TAK1-DN and increased by TAK1-WT

Inhibition of MKK3 and MKK6 has differential effects on Oc differentiation

As MKK3 and MKK6 can be activated by TAK1 to mediate p38 activation, we next examined the participation of these MAPKKs to osteoclastogenesis. Recombinant retroviruses harboring a DN form of MKK3 or MKK6 were infected into BMMs, and osteoclastogenesis was induced by culturing the cells in the presence of RANKL. The expression of transduced MKK3-DN and MKK6-DN was confirmed by Western blotting (Figure 5a). Under these conditions, both MKK3-DN and MKK6-DN suppressed RANKL-stimulated p38 activation (Figure 5b). However, their effects on Oc differentiation were in striking contrast. MKK6-DN reduced TRAP-positive MNC formation to 25% of the control level, whereas MKK3-DN did not show any inhibitory effect on Oc generation (Figure 5c and d). In fact, MKK3-DN transduction mildly increased Oc formation. These results indicate that although both MKK3 and MKK6 mediate p38 activation in response to RANKL in Oc precursor cells, they have distinct roles in modulation of RANKL-induced osteoclastogenesis.

TAK1-MKK6-p38 signalling is necessary for NFATc1 induction for osteoclastogenesis

The NFATc1 transcription factor has been implicated for Oc differentiation.[9,17] The expression of this transcription factor was shown to be induced by RANKL during osteoclastogenesis.[16,17] As the TAK1-MKK6-p38 pathway positively regulated Oc differentiation, we investigated whether this MAPK signalling pathway is linked to NFATc1 induction by RANKL. BMMs were infected with TAK1-DN or the control viruses and cultured for 24 h in the presence of RANKL and NFATc1 expression was assessed by Western analyses. The NFATc1 protein level increased in cells infected with control viruses in response to RANKL treatment (Figure 6a, lane 2). This RANKL-dependent induction of NFATc1 was strongly attenuated by TAK1-DN expression (Figure 6a, lane 4). In addition, MKK6-DN attenuated the NFATc1 induction by RANKL (Figure 6b, lane 4). Interestingly, MKK3-DN overexpression, which had no inhibitory effect on Oc differentiation (Figure 5), also blocked RANKL induction of NFATc1 (Figure 6b, lane 6). These results suggest that MKK6 and MKK3 are likely to work in different modes in signalling mechanisms involved in Oc differentiation other than NFATc1

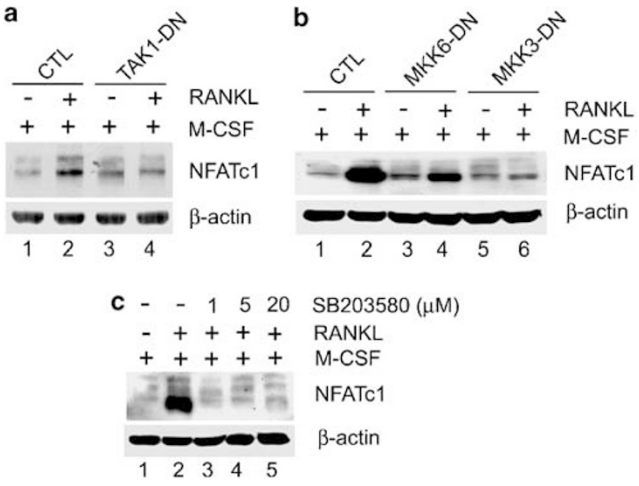

Figure 6 RANKL-stimulated NFATc1 induction is attenuated by TAK1-DN, MKK6-DN, and p38 inhibitor. (**a**) BMMs were infected with the control or TAK1-DN retroviruses. Cells were then treated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 24 h. The expression levels of NFATc1 were determined by Western blotting. TAK1-DN infection diminished the extent of NFATc1 induction by RANKL. (**b**) BMMs infected with the control, MKK6-DN, or MKK3-DN retroviruses were treated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 24 h. The expression level of NFATc1 was assessed by Western blotting. Both MKK6-DN and MKK3-DN suppressed the NFATc1 induction by RANKL. (**c**) Uninfected BMMs were treated with indicated concentrations of SB203580, M-CSF (30 ng/ml), and RANKL (100 ng/ml) for 24 h. NFATc1 expression was examined by Western blotting. SB203580 treatment abolished the RANKL induction of NFATc1

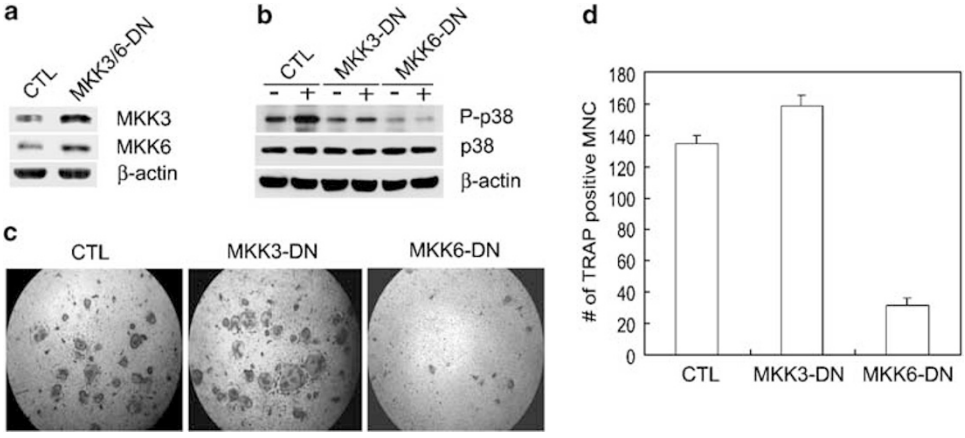

Figure 5 DN form of MKK6, but not that of MKK3, suppresses osteoclast differentiation. (**a**) BMMs were infected with the control, MKK3-DN, or MKK6-DN retroviruses. The expression levels of MKK3 and MKK6 were examined by Western blotting. Anti-β-actin blot shows loading control. Overexpression of both MKK3-DN and MKK6-DN was observed in infected BMMs. (**b**) BMMs infected with the control, MKK3-DN, or MKK6-DN retroviruses were serum-starved for 5 h. Cells were stimulated with 1 μg/ml RANKL for 15 min and activation of p38 was assessed by Western blotting with anti-P-p38 antibody. Both MKK3-DN and MKK6-DN reduced p38 activation. (**c** and **d**) Infected BMMs were cultured for 5 days in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL. Cells were stained for TRAP and photographed. The number of TRAP-positive MNC was quantified. Error bars represent S.D. of mean values

induction by RANKL. We next examined the effect of SB203580, a specific p38 inhibitor, on RANKL induction of NFATc1. Treatment of BMMs with the p38 inhibitor blocked the RANKL-stimulated increase in NFATc1 (Figure 6c). Together, these data indicate that TAK1-MKK6-p38 signalling pathway is required for NFATc1 induction by RANKL in osteoclastogenesis.

TAK1-MKK6-p38 pathway regulates NF-κB transcription activity for osteoclastogenesis

NF-κB is strongly activated by RANKL and is essential for osteoclastogenesis.[26] TAK1 has been suggested to contribute to NF-κB activation by TNFα and RANKL.[15,27] Therefore, we investigated whether the TAK1-MKK6-p38 pathway is relevant to NF-κB activation by RANKL. We first examined the effect of TAK1-DN on IκB phosphorylation and degradation, steps required for release and nuclear translocation of NF-κB, by RANKL. RANKL caused IκB phosphorylation and degradation in both control and TAK1-DN retrovirus-infected BMMs (Figure 7a). In line with this observation, the comparison of DNA-binding activity of NF-κB by electrophoretic mobility shift assays revealed only very minor, if not insignificant, difference in RANKL- and TNFα-induced NF-κB DNA binding between control and TAK1-DN-transduced cells (Figure 7b and c). The specificity of the NF-κB–DNA complex was verified by competition by unlabelled probe and supershift by p65 and p50 NF-κB antibodies (Figure 7b and c). However, when the transcription activity of NF-κB was measured in BMMs using adenoviruses harboring NF-κB-dependent luciferase reporter, TAK1-DN potently suppressed NF-κB activity (Figure 7d). The NF-κB transcriptional activity was also inhibited by MKK6-DN (Figure 7d). Interestingly, MKK3-DN could not suppress NF-κB transcriptional activity (Figure 7d). We next assessed the potential link between p38 and NF-κB activity by using the p38 inhibitor SB203580. SB203580 inhibited RANKL-stimulated NF-κB activity in a dose-dependent manner (Figure 7e). These data indicate that the TAK1-MKK6-p38 signalling pathway can mediate RANKL-induced NF-κB activation in a manner independent on the regulation of NF-κB DNA binding and that MKK3 is not involved in this response.

RANKL stimulates phosphorylation of p65 on Ser-536 via TAK1-MKK6-p38 pathway

Because the TAK1-MKK6-p38 signalling pathway played a role in the NF-κB activation by RANKL without much effect on IκB degradation and NF-κB DNA binding (Figure 7), we hypothesized that this pathway may promote NF-κB transactivation by phosphorylating the p65 subunit in response to RANKL. The phosphorylation of p65 on Ser-536 in the transactivation domain has been suggested to contribute to NF-κB transcription activity stimulated by TNFα and LPS in HeLa and HEK293 cells.[27,28] To explore the potential link between the TAK1-MKK6-p38 pathway and p65

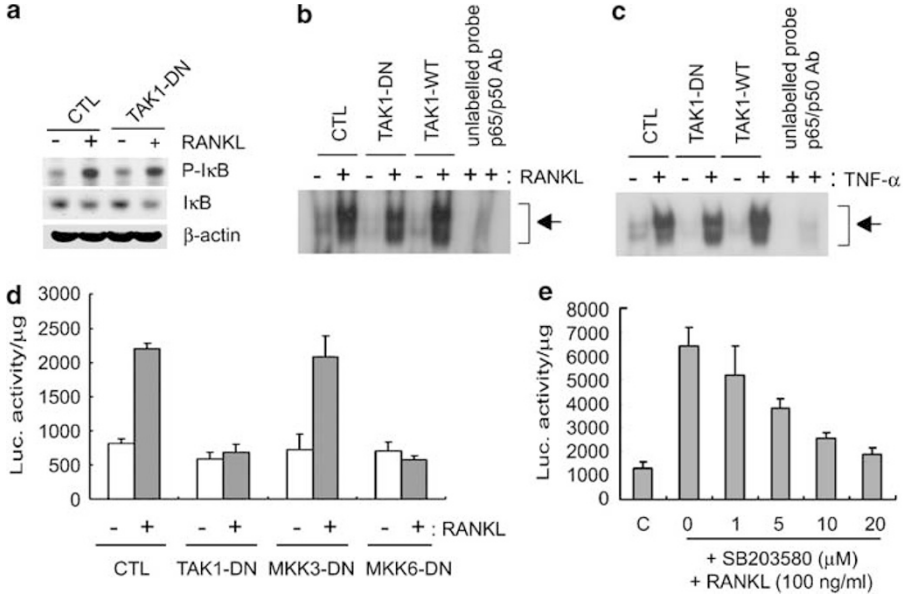

Figure 7 RANKL-stimulated NF-κB activation is suppressed by TAK1-DN, MKK6-DN, and SB203580. (**a**) BMMs were infected with the control or TAK1-DN retroviruses. At 2 days after infection, cells were serum-starved for 5 h and stimulated with 1 μg/ml RANKL for 10 min. The phosphorylation and degradation of IκB were assessed by Western blotting with anti-P-IκB and anti-IκB antibodies, respectively. Anti-β-actin blot was included as loading control. TAK1-DN did not affect IκB phosphorylation and degradation. (**b** and **c**) BMMs infected with the control or TAK1-DN retroviruses were serum-starved for 5 h and stimulated with 1 μg/ml RANKL or 20 ng/ml TNFα for 15 min. The DNA-binding activity of NF-κB was assessed by EMSA. TAK1-DN expression did not significantly reduce the RANKL- and TNFα-stimulated increases in DNA binding of NF-κB. (**d**) BMMs were infected with NF-κB reporter adenoviruses and subsequently with the TAK1-DN, MKK6-DN, MKK3-DN, or control retroviruses. At 24 h post-transfection, cells were incubated with 100 ng/ml RANKL for 8 h. The luciferase activity per μg cellular protein was determined. Error bars represent S.D. of mean values. Expression of TAK1-DN and MKK6-DN, but not MKK3-DN, inhibited NF-κB activity. (**e**) BMMs were infected with NF-κB reporter adenoviruses. At 24 h after transfection, cells were incubated with 100 ng/ml RANKL and indicated concentrations of SB203580 for 8 h. The luciferase activity per μg cellular protein is presented as mean ± S.D. Treatment with the p38 inhibitor suppressed NF-κB activation by RANKL

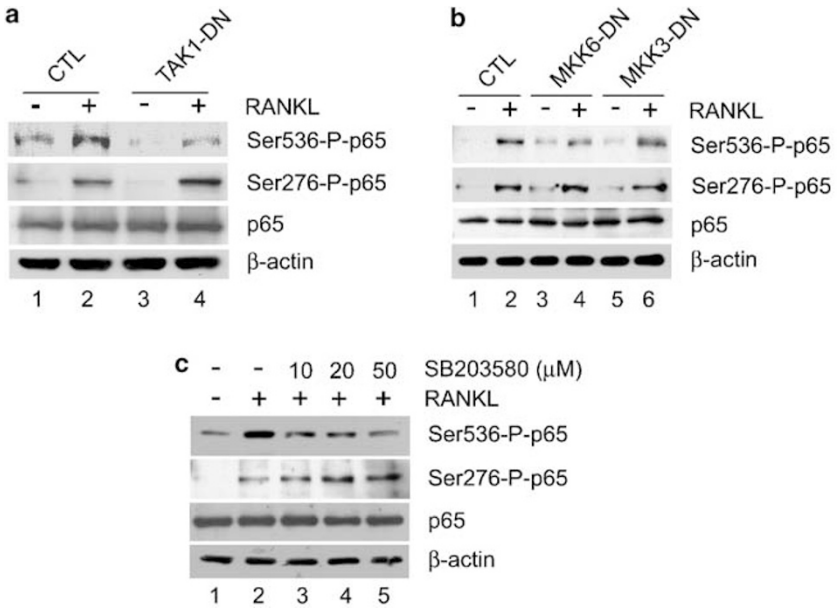

Figure 8 RANKL stimulates Ser-536 phosphorylation of p65 in a manner dependent on TAK1, MKK6, and p38. (**a**) BMMs were infected with the control or TAK1-DN retroviruses. At 2 days after infection, cells were serum-starved for 5 h and stimulated with 1 μg/ml RANKL for 15 min. The phosphorylation of p65 was assessed by Western blotting with an antibody that specifically recognizes p65 phosphorylated at Ser-536 or Ser-276. P65 and β-actin levels were analyzed as loading controls. TAK1-DN reduced Ser-536, but not Ser-276, phosphorylation of p65. (**b**) BMMs infected with the control, MKK6-DN, or MKK3-DN retroviruses were serum-starved and stimulated with 1 μg/ml RANKL for 15 min. The phosphorylation of p65 was assessed by Western blotting with the anti-phospho-p65 antibody. MKK6-DN, but not MKK3-DN, attenuated RANKL-induced Ser-536 phosphorylation of p65. Both MKK6-DN and MKK3-DN did not reduce RANKL-induced Ser-276 phosphorylation of p65. (**c**) BMMs were pretreated with SB203580 for 1 h and stimulated with 1 μg/ml RANKL for 15 min. The phosphorylation of p65 was analyzed by Western blotting. The p38 inhibitor suppressed RANKL stimulation of p65 phosphorylation on Ser-536, but not on Ser-276

phosphorylation in Oc precursor cells, we investigated whether RANKL causes p65 phosphorylation and, if it does, whether TAK1 is involved in this response. In control virus-infected BMMs, RANKL increased the Ser-536 phosphorylation of p65 (Figure 8a, lane 2). This RANKL-stimulated p65 phosphorylation was significantly attenuated in TAK1-DN-transduced cells (Figure 8a, lane 4). Similar to the results of NF-κB-dependent reporter assays (Figure 7d), MKK6-DN, but not MKK3-DN, suppressed the RANKL-stimulated Ser-536 phosphorylation of p65 (Figure 8b). Treatment with the p38 inhibitor SB203580 also blocked the p65 phosphorylation on Ser-536 by RANKL in Oc precursor cells (Figure 8c). Another reported phosphorylation site of p65 NF-κB, Ser-276, was also phosphorylated in response to RANKL (Figure 8). However, the Ser-276 phosphorylation of p65 was not reduced by TAK1-DN, MKK6-DN, or SB203580 (Figure 8). Taken together, our data demonstrate that the TAK1-MKK6-p38 signalling pathway participates to the RANKL stimulation of NF-κB transactivation in Oc precursor cells by mediating p65 phosphorylation on Ser-536, but not on Ser-276.

Discussion

Ocs are generated from monocyte/macrophage lineage of hematopoietic cells through a differentiation program, of which molecular mechanisms are yet to be unraveled in detail. It is evident, however, that the Oc differentiation program requires two essential factors, RANKL and M-CSF. While M-CSF stimulates survival and proliferation of Oc precursors, RANKL drives commitment to Ocs by signalling through its receptor, RANK, in the precursor cells. The downstream events of multiple signal-transduction pathways utilized by RANK include activation of transcription factor NF-κB, induction and activation of NFATc1, and stimulation of three MAPK families – ERKs, JNKs, and p38s.[5,6]

The p38 MAPK has been well characterized to play roles in cellular responses to inflammatory cytokines, environmental stresses, such as UV radiation and osmotic shock, and growth factors. This MAPK has also been implicated in the regulation of chondrocyte, epithelial cell, and skeletal muscle differentiation.[29,30] In this study, we show that expression levels of MKK3/6 and TAK1, upstream molecules in the p38 signalling pathway, are elevated by RANKL stimulation during the early stage of Oc differentiation from bone marrow-derived precursor cells (BMMs). This elevation augmented the p38 activation by RANKL or TNFα in RANKL-primed BMMs. Suppression of TAK1 and MKK6 activity in BMMs by retroviral transduction of DN forms decreased RANKL-induced p38 activation and osteoclastic differentiation. In addition, RANKL stimulation of NFATc1 expression, NF-κB transcription activity, and Ser-536 phosphorylation of p65 was attenuated by TAK1-DN and MKK6-DN in BMMs. All of these responses were also recapitulated in BMMs treated with the p38 inhibitor SB203580. Therefore, TAK1 and MKK6 constitute a canonical p38 MAPK signalling cascade that mediates RANKL-induced Oc differentiation from bone marrow precursor cells.

The NFATc1 transcription factor has been implicated in Oc differentiation from bone marrow cells and the RAW264.7 cell line, a model cell line for osteoclastogenesis.[16,17] Although the molecular mechanism is not clearly resolved, JNK1 has been suggested to be involved, through c-Jun phosphorylation, in NFATc1 expression during osteoclastogenesis.[9] In contrast, there has been no previous report indicating a link between the p38 MAPK and the NFAT transcription factor in Oc differentiation. However, in T cells, the p38 MAPK was shown to regulate multiple stages of NFATc1 activation: induction of NFATc1 mRNA expression; mild enhancement in NFATc1 mRNA stability; promotion of NFATc1 translation; and moderate stimulation of nuclear export of NFATc1 protein.[21] The sum of these effects of p38 MAPK in T cells was overall activation of NFATc1.[21] In our study, expression of DN forms of TAK1 and MKK6, and treatment with the p38 inhibitor SB203580 led to a decrease in the RANKL-dependent induction of NFATc1 protein levels in Oc precursor cells (Figure 6). This study is the first to show that TAK1 and MKK6 are involved in the RANKL induction of NFATc1. Our results also indicate that one of the roles of p38 signalling pathway in Oc differentiation program is to participate to the NFATc1 induction by RANKL. Whether the multiple mechanisms utilized by p38 to regulate NFATc1 activation in T cells are also involved in Oc differentiation remains to be investigated.

The activation of NF-κB transcription factors can be achieved by two distinct pathways. Phosphorylation and subsequent degradation of the inhibitory subunit IκB followed by translocation of the released NF-κB subunits to the nucleus and binding to DNA target sites constitutes the classical pathway. Alternatively, the processing of p100 (NF-κB2) to p52 results in the activation of p52:RelB dimmers of the NF-κB transcription factors.[22] Regardless of the differences in the activation pathways, the transcriptional activation domains contained in the Rel proteins – cRel, RelA (p65), and RelB – mediate the transcription activity of NF-κB. Several studies have shown that p65 can be phosphorylated at Ser-276, Ser-529, and Ser-536 by protein kinase A, casein kinase II, and IKK, respectively.[27,31,32] This phosphorylation was suggested to increase the transactivation potential of NF-κB. In our study, TAK1-DN did not reduce the RANKL-induced phosphorylation and degradation of IκB (Figure 7a), indicating that RANKL stimulation of IKK was not likely to be inhibited by this retroviral transduction. Consistently, the effect of TAK1-DN on NF-κB DNA binding seemed insignificant (Figure 7b and c). However, the transcription activity of NF-κB was very strongly suppressed by TAK1-DN, when determined by using a reporter system (Figure 7d). This suppression was also observed with either overexpression of MKK6-DN or SB203580 treatment (Figure 7d and e). These data are consistent with the hypothesis that the NF-κB regulatory mechanism involving p65 phosphorylation may be the target of TAK1-MKK6-p38 signalling pathway in RANKL-induced Oc differentiation. This hypothesis was supported by the results showing reduced RANKL-stimulated phosphorylation of p65 on Ser-536 by expression of TAK1-DN and MKK6-DN and treatment with SB203580 in BMMs (Figure 8). Therefore, our study indicates that the enhancement of NF-κB transcription activity through stimulation of p65 phosphorylation is an attribute of the TAK1-MKK6-p38 pathway, which contributes to Oc differentiation.

In our study, the TAK1-MKK6-p38 pathway was shown to mediate both NFATc1 induction and NF-κB activation in RANKL signalling. The NFATc1 induction by RANKL has been shown to involve c-Fos, c-Jun, and possibly NFATc2 transcription factors.[9,17] On the other hand, the relationship between NF-κB activity and NFAT expression in Oc differentiation has not been addressed, except a recent study where an NF-κB inhibitor, (−)-DHMEQ, was shown to suppress the RANKL induction of NFATc1 in BMMs.[33] Although it has been clearly demonstrated that the p38 MAPK is required for Oc differentiation, the understanding on the downstream targets of p38 for this response has been limited. A DN form of ATF2, a well-know downstream target of p38, was shown not to affect osteoclastogenesis.[9] CREB, another transcription factor shown to be activated by p38, neither did regulate Oc differentiation (ZH Lee, unpublished data). The MITF transcription factor was suggested to be a downstream target of the p38 pathway relevant to Oc differentiation as it was phosphorylated by p38 and increased the TRAP gene promoter activity.[34] Here, we clearly demonstrate that NFATc1 induction is another downstream event invoked by p38 activation in RANKL signalling. It may be postulated that this event involves the increase in the transactivation ability of p65 NFκB upon Ser-536 phosphorylation by the p38 MAPK.

Unexpectedly, the retroviral transduction of MKK3-DN did not elicit inhibitory effects on osteoclastogenesis (Figure 5d), NF-κB transcription activity (Figure 7d), and p65 phosphorylation on Ser-536 (Figure 8b) while provoking suppressive effects on p38 activation (Figure 5b) and NFATc1 induction (Figure 6b) by RANKL. These observations appear paradoxical to the effects of the p38 inhibitor SB203580 and MKK6-DN that compromised osteoclastogenesis, NFATc1 induction, NF-κB transactivation, and Ser-536 phosphorylation of p65. One explanation for this paradox might be the selective isoforms of the p38 MAPK that can be activated by MKK3 and MKK6. MKK3 activates p38α and p38γ isoforms, while MKK6 activates the p38β isoform in addition to p38α and p38γ.[35] SB203580 inhibits both p38α and p38β.[35] Therefore, it is a possibility that the effects of SB203580 and MKK6-DN on the RANKL-stimulated NF-κB transactivation and Ser-536 phosphorylation of p65 are mediated by p38β, while the induction of NFATc1 is mediated by p38α. Although it has been assumed that MKK3 and MKK6 have redundant function in general, some reports provided evidence for differential roles of these MAPKKs. In cardiac myocytes, expression of activated forms of MKK3 and MKK6 led to different responses; active MKK3 caused apoptosis while active MKK6 invoked hypertrophy.[36] Studies with MKK3$^{-/-}$ and MKK6$^{-/-}$ mice also revealed distinct functions of these MKKs in immune responses.[37]

The lack of inhibitory effect of MKK3-DN on Oc differentiation in spite of the decrease in NFATc1 induction by RANKL (Figures 5d and 6b) is puzzling in view of the reports that suggested NFATc1 as a key regulator of osteoclastogenesis.[9,17] In those reports, the TRAP promoter activity was regulated by NFATc1 and the Oc formation was defective in BMMs treated with NFAT inhibitors, cyclosporin A and FK506, which caused NFATc1 reduction by blocking

autoamplification.[9,17] However, in another study, cyclosporin A, even at a high dose, did not reduce TRAP expression.[16] This discrepancy was suggested to indicate a possibility that the NFAT transcription factor is not absolutely required when other transcription factors are present in sufficient amounts.[38] Thus, it might be possible that MKK3-DN expression causes an alteration in amounts or activities of other transcription factors to compensate for low NFATc1 levels. In this regard, an increased AP-1 activity was reported in NFATc2-deficient cells.[39] Alternatively, the requirement for NFAT may be minimal and the residual amount of NFATc1 in MKK3-DN-infected cells might have been sufficient to meet the requirement of this transcription factor for osteoclastogenesis. Under this condition, the activity of other transcription factor, such as NF-κB, may be a more critical determinant for Oc differentiation.

In conclusion, we demonstrated in this study that in an early stage of Oc differentiation from bone marrow precursor cells, the p38 signalling pathway molecules TAK1, MKK6, and MKK3 are induced by RANKL, leading to enhanced p38 signalling capacity. We also provide evidence that TAK1 and MKK6, but not MKK3, participates in transmitting the RANKL signal to elicit the dual responses of NF-κB transactivation and NFATc1 induction resulting in efficient Oc differentiation. Understanding on the molecular details for the differential effects of MKK6 and MKK3 on NF-κB transactivation and Oc differentiation requires further investigation.

Materials and Methods

Reagents and antibodies

RANKL was obtained from PeproTech (Rocky Hill, NJ, USA). TNFα and M-CSF were from R&D Systems (Minneapolis, MN, USA). Leukocyte Acid Phosphatase Assay Kit was purchased from Sigma (St Louis, MO, USA). Lipofectamine 2000 was from Invitrogen Life Technologies (Carlsbad, CA, USA). Anti-TAK1 and anti-MKK6 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and Stressgen Biotechnologies (Victoria, BC, Canada), respectively. Antibodies for MKK3, MKK4, MKK7, p38, JNK, IκB, phospho-p38, phospho-JNK, phospho-IκB, and phospho-p65 were from Cell Signalling Technology (Beverly, MA, USA). SB203580 was obtained from Calbiochem (San Diego, CA, USA).

Bone marrow macrophage culture and Oc differentiation

Bone marrow cells were obtained by flushing the marrow space of tibiae and femora from 5- to 6-week-old ICR mice with α-minimal essential medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were suspended in α-MEM supplemented with 10% fetal bovine serum (FBS), plated on 100-mm bacterial dishes, and cultured for 16–24 h with M-CSF (5 ng/ml) in 5% CO_2 at 37°C. Nonadherent cells were completely removed by aspiration and remaining cells further cultured for 3 days in the presence of 30 ng/ml M-CSF. The adherent cells were considered BMMs and used as Oc precursor cells. To achieve Oc differentiation, BMMs were seeded in 96-well plates at 1×10^5 cells/well or in six-well plates at 2×10^6 cells/well. Cells were cultured for 6 days with 30 ng/ml M-CSF and 100 ng/ml RANKL.

Tartrate-resistant acid phosphatase cytochemistry

Following the time course of Oc differentiation, cells were washed with PBS and fixed with 3.7% formaldehyde. Then, the cells were incubated with 0.1% Triton X-100 for 5 s and stained with the Leukocyte Acid Phosphatase Assay kit (Sigma) following the manufacturer's instruction. TRAP-positive multinucleated cells containing three or more nuclei were counted as Ocs under a light microscope. The data were expressed as means ± S.D. of triplicate samples.

Western blotting analysis

Cells were washed twice with ice-cold PBS and ruptured with a lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. Cell extracts were microcentrifuged for 20 min at 10 000 × *g* and the supernatants were collected. 20–35 μg of cellular proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 3% skim milk in Tris-buffered saline containing 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies. Membranes were washed, incubated for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase, and developed using a chemiluminescence system.

Reverse transcription-polymerase chain reaction analysis

Cells were washed with ice-cold PBS and lysed in TRI Reagent (Sigma). In all 2 μg of prepared RNA were reverse-transcribed with SuperScriptII reverse transcriptase (Invitrogen). A total of 10% of the reverse-transcribed cDNA was amplified by PCR. The PCR primer sequences used are as follows: TAK1, 5′-GATCACTACCTCAGGACCAA-3′ (forward) and 5′-TGTATTTTGCTGGTCCTTTT-3′ (reverse); MKK3, 5-AATC CAAAAGGAAAAAGGAC-3′ (forward) and 5′-TGACAGTGTAGAAG CAGTCG-3′ (reverse); MKK6, 5-TTATTGATAAAGGCCAAACAA-3′ (forward) and 5′-GCACTGTGAGGTAAAGTCAAC-3′ (reverse); and GAPDH, 5-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCAC CACCCTGTTGCTGTA-3′ (reverse). The amplification cycle was comprised of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 58–60°C, and an extension step for 30 s at 72°C. The number of cycles was determined to be in a linear range of amplification. 25 cycles were run for GAPDH and 30 cycles for TAK1, MKK3, and MKK6. PCR products were separated on 1.2% agarose gels and stained with ethidium bromide.

Immune complex kinase assay

Cells were washed twice with ice-cold PBS and lysed as above. Cell lysates (750–1500 μg) were immunoprecipitated with anti-TAK1 or anti-p38 antibody. The precipitates were suspended in a kinase assay buffer (25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate) containing ATP (20 μM), [γ-^{32}P]ATP (5 μCi), and substrate protein (2 μg). The GST fusion protein of kinase-inactive MKK6 (GST-MKK6-KD) and GST-ATF2 were used as substrates for TAK1 and p38 assays, respectively. The plasmids for GST-MKK6-KD and GST-ATF2 were generated by PCR amplication and cloning into pGEX vector. The fusion proteins were purified from culture lysates of transformed *Escherichia coli*. The kinase reaction was performed for 30 min at 30°C and stopped by adding sample buffer. The reaction products were separated by SDS-PAGE and subjected to autoradiography. Alternatively, the kinase reaction was performed with nonradioactive ATP only and the substrate

phosphorylation was detected by Western blotting with anti-phospho-MKK6 or anti-phospho-ATF2 antibody.

Retroviral gene transfer

Recombinant retroviral vectors harboring WT and DN forms of TAK1 were generated by subcloning TAK1-WT and TAK1-DN (provided by Dr. Tohru Ishitani, Nagoya University, Japan) into the retroviral vector pMx. MKK6-WT, MKK6-DN, MKK3-WT, and MKK3-DN (provided by Dr. Roger J Davis, Howard Hughes Medical Institute/University of Massachusetts Medical School, Worcester, MA, USA) were also subcloned into the pMx vector. Generated recombinant plasmids and the parental pMx vector were transfected into Plat-E cells. Plat-E cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 2×10^6 cells were seeded in a 60-mm dish. Cells were transfected for 6 h with 4 μg of plasmids and 10 μl Lipofectamine 2000 reagent following the manufacturer's instruction. The medium was replaced with DMEM/10% FBS and cells were further incubated. Next day, the medium was changed with 4 ml α-MEM/10% FBS and culture was continued for 48 h. Cell culture medium containing viral particles were collected and centrifuged for 10 min at $1500 \times g$. The supernatants were stored at -70°C until use. For infection with retroviruses, BMMs plated in six-well plates (1×10^6 cells/well) were incubated with the virus-containing supernatant (2 ml/well), polybrene (10 μg/ml), and M-CSF (30 ng/ml) for 1 day. Equal volume of α-MEM/10% FBS containing 30 ng/ml M-CSF was added and the incubation was extended for another 24 h.

Electrophoretic gel mobility shift assay

BMM Oc precursor cells were infected with TAK1-WT or TAK1-DN retroviruses as described above. Cells were serum-deprived for 5 h and stimulated with 1 μg/ml RANKL or 20 ng/ml TNFα for 15 min. Nuclear extracts were prepared and NF-κB-binding DNA gel mobility shift assays were performed as described previously.[40] The sequence of NF-κB-binding DNA was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. In some experiments, 100-fold excess amounts of unlabelled DNA or 0.5 μg of p50 and p65 antibodies were included during DNA-binding reaction.

Luciferase reporter assay

BMMs were plated at 5×10^5/well in six-well plates. Next day, cells were infected with adenoviruses harboring NF-κB-dependent luciferase gene (Gene Transfer Vector Core, University of Iowa, IA, USA) at 100 MOI. After incubation for 30 min, cells were washed and infected with the TAK1-WT, TAK1-DN, or control retroviruses as described above. After incubation for 24 h at 37°C in a CO_2 incubator, cells were stimulated with 100 ng/ml RANKL for 8 h. Cells were lysed in Reporter Lysis Buffer (Promega) and luciferase activity was measured using a luminometer. In experiments with SB203580, BMMs were infected with the NF-κB reporter adenoviruses. At 24 h after infection, cells were treated with RANKL and SB203580 for 8 h before luciferase activity measurement.

Acknowledgements

We thank Drs Tohru Ishitani and Roger J Davis for kindly providing plasmids. We are also grateful to Dr Paul McCray for providing an adenoviral NF-κB reporter system through the Gene Transfer Vector Core at the University of Iowa. This work was supported by the 21C Frontier Functional Proteomics Project Grant (FPR05C2-280) and the Molecular and Cellular BioDiscovery Research Program (M1-0311-00-0024) from the Ministry of Science and Technology, Korea.

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