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Tetraspanin CD9 regulates osteoclastogenesis via regulation of p44/42 MAPK activity

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Abstract

Tetraspanin CD9 has been shown to regulate cell–cell fusion in sperm-egg fusion and myotube formation. However, the role of CD9 in osteoclast, another multinucleated cell type, is not still clear. Therefore, we investigated the role of CD9 in osteoclast differentiation. CD9 was expressed in osteoclast lineage cells and its expression level increased during the progression of RANKL-induced osteoclastogenesis. KMC8, a neutralizing antibody specific to CD9, significantly suppressed RANKL-induced multinucleated osteoclast formation and the mRNA expression of osteoclast differentiation marker genes. To define CD9-regulated osteoclastogenic signaling pathway, MAPK pathways were examined. KMC8 induced long-term phosphorylation of p44/42 MAPK, but not of p38 MAPK. Constitutive activation of p44/42 MAPK by overexpressing constitutive-active mutant of MEK1 almost completely blocked osteoclast differentiation. Taken together, these results suggest that CD9 expressed on osteoclast lineage cells might positively regulate osteoclastogenesis via the regulation of p44/42 MAPK activity.

Keywords: Osteoclastogenesis; Multinuclear osteoclast; CD9; KMC8; p44/42 MAPK

Osteoclasts arise from hematopoietic cells of monocyte/macrophage lineage. Macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B ligand (RANKL) were identified as the key regulatory factors for osteoclast differentiation [1–3]. Although M-CSF and RANKL are essential for osteoclast differentiation, other factors also appear to be actively involved in the regulation of osteoclastogenesis. A recent study showed that ITAM motif-containing receptors critically regulate osteoclastogenesis as co-receptors of RANK in osteoclasts [4]. This report has revealed that M-CSF and RANKL alone are not sufficient to produce signals for osteoclastogenesis but other co-stimulatory signals are also required. Therefore, unveiling the regulatory molecules which are closely associated with osteoclast differentiation would be important for thorough understanding of osteoclast differentiation mechanism.

Previously, we reported that dexamethasone significantly suppressed the formation of multinucleated osteoclasts but not that of pre-fusion mononuclear osteoclasts in co-culture of bone marrow cells and calvarial osteoblasts [5]. Among the genes differentially regulated by dexamethasone, CD9 was shown to be down-regulated by dexamethasone. CD9 belongs to tetraspanin protein family which shares a characteristic four membrane-spanning structure [6,7]. In the tetraspanin web, CD9 is physically associated with integrins, G proteins, and other tetraspanin members such as CD63, CD81, CD82, and CD151 [8–10]. CD9 is widely expressed in nearly all tissues and involved in diverse biological processes including cell proliferation, adhesion, motility, and apoptosis [7,10]. Another physiological function of CD9 has been associated with cellular

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fusion. Oocytes from CD9-null mice showed severely impaired sperm-egg fusion [11]. Consistent with this, CD9 was concentrated in the oocyte microvilli surrounding the sperm head and sperm-egg fusion was strongly inhibited by anti-CD9 antibodies [12]. Moreover, CD9 appears to play an important role in the fusion of myoblasts during muscle cell syncytia formation [13]. Although CD9 has been reported to be implicated in cell–cell fusion process, it is not clear whether it functions similarly in other multinucleated cell types such as osteoclast. Very limited studies have been done to elucidate the biological role of CD9 in osteoclastogenesis. A study reported that CD9 expressed on stromal cells but not on osteoclasts is involved in osteoclast differentiation [14]. However, another study suggested a negative regulatory role of CD9 in the fusion of monocytes and alveolar macrophages [15].

Therefore, we investigated the functional role of CD9 in osteoclast differentiation by using KMC8, a neutralizing monoclonal anti-CD9 antibody, in the culture of bone marrow-derived monocytic/macrophage precursor cells. Here, we show that CD9 is expressed in differentiating osteoclast lineage cells and the blocking of CD9 by KMC8 significantly suppressed multinucleated osteoclast formation. KMC8 induced strong and sustained activation of p44/42 mitogen-activated protein kinase (MAPK), suggesting that CD9 might negatively regulate the intensity and duration of p44/42 MAPK activation to a certain level in order to support the progression of osteoclastogenesis.

Materials and methods

Reagents and antibodies. ICR mice were obtained from Samtaco (Sungnam, Korea). Recombinant human transforming growth factor β1 (TGF-β1), recombinant human M-CSF, and recombinant human soluble RANKL were purchased from Cytolab (Rehovot, Israel), easy-Blue™ and StarTaq™ from iNtRon Biotechnology (Sungnam, Korea), and AccuPower RT-Premix from Bioneer (Daejeon, Korea). PCR primers were synthesized by TaKaRa Korea (Seoul, Korea). Leukocyte Acid Phosphatase Stain kit was purchased from Sigma (St. Louis, MO, USA), rat anti-CD9 antibody (KMC8) and isotype matched $IgG_{2a\kappa}$ antibody from BD Biosciences Pharmingen (Chicago, IL, USA), anti-phospho-p44/42 MAPK antibody, anti-p44/42 MAPK antibody, anti-phospho-p38 MAPK, and anti-p38 MAPK antibody from Cell Signaling Technology (Bevery, MA, USA), anti-phospho-c-Jun N-terminal kinase (JNK) antibody and anti-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Alexa-fluor 488-conjugated goat anti-rabbit IgG antibody from Molecular Probe (Eugene, OR, USA).

In vitro osteoclastogenesis. Osteoclastogenesis from primary bone marrow cells was induced as the following. Briefly, nonadherent bone marrow cells from female ICR mice of 5 weeks old were seeded at a density of 8×10^6 cells per 60-mm culture dish or 4×10^5 cells per well in 48-well culture plate and cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) containing 40 ng/ml M-CSF and 1 ng/ml TGF-β1. One day after, the cells were fed with fresh medium containing the same cytokines and cultured for additional two days. These cells were used as bone marrow-derived monocytic/macrophage precursor (BMM) cells. BMM cells were cultured for additional four days in the differentiation medium containing 40 ng/ml M-CSF and 100 ng/ml soluble RANKL to generate multinucleated osteoclasts; during this culture period, culture medium was changed everyday.

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). To evaluate mRNA expression levels, semi-quantitative RT-PCR was performed in the range of linear amplification. Total RNAs were isolated using easy-Blue™ RNA Extraction Reagent. Complementary DNA was synthesized from 1 μg of total RNA by using AccuPower RT-PreMix and subsequently used for PCR amplification. PCR products were electrophoresed on 1% agarose gel and visualized under UV light by ethidium bromide staining. Mouse genes and their primer sequences for PCR are as the following; CD9-forward (f) 5′-TGCA GTGCTTGCTATTGGAC-3′, CD9-reverse (r) 5′-GCACAGGATCATG CTGAAGA-3′; CD81-f 5′-TTCCATGAGACGCTCAACTG-3′, CD81-r 5′-CTACAAAGCCTCTGGGCAAG-3′; osteoclast-associated receptor (OSCAR)-f 5′-ACTCCTGGGATCAACGTGAC-3′, OSCAR-r 5′-GA GACCATCAAAGGCAGAGC-3′; tartrate-resistant acid phosphatase (TRAP)-f 5-TGACAAGAGGTTCCAGGA-3′, TRAP-r 5′-AGCC AGGACAGCTGAGTG-3′; calcitonin receptor (CTR)-f 5′-TGAAA AGGCGGAATCT-3′, CTR-r 5′-AGGAACGTGTGCTTGTG-3′; vitronectin receptor (VNR)-f 5′-GCTCAGATGAGACTTTG-3′, VNR-r 5′-ATCAACAATGAGCTGGA-3′; RhoA-f 5′-CGCTTTTGGGTACATG GAGT-3′, RhoA-r 5′-TCTTTGAATTAGCGCCTGGT-3; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-f 5′-TCACCATCTTCCA GGAGCG-3′, and GAPDH-r 5′-CTGCTTACCACCTTCTTGA-3.

Western blot analysis. After various treatments, the cells were washed with phosphate-buffered saline (PBS), scraped into a lysis buffer consisting of 10 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail, and sonicated briefly. The protein concentrations were determined by a modified Bradford method. Each sample containing equal amounts of proteins was subjected to SDS–PAGE. The proteins separated in the gel were subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dried milk and incubated with each antibody followed by incubation with HRP-conjugated secondary IgG antibody. Luminescence was detected by LAS1000 (Fuji; Tokyo, Japan).

Immunofluorescence staining. In vitro osteoclast differentiation was induced in the chamber slide. At the end of culture, cells were rinsed briefly with PBS and fixed with 2% formaldehyde/PBS for 15 min at room temperature. After fixation, the cells were blocked with 5% normal serum for 1 h and incubated with rabbit anti-CD9 antibody (1:50 dilution) overnight at 4 °C. The cells were rinsed with PBS and incubated with Alexa-fluor 488-conjugated goat anti-rabbit antibody for 2 h at room temperature in dark. Subsequently, the cells were washed and subjected to confocal microscopy (Carl Zeiss LSM5 Pascal).

TRAP staining. TRAP staining was performed using Leucocyte Acid Phosphatase stain kit according to manufacturer's instruction. The number of TRAP-positive cells with three or more nuclei was scored under the light microscope. The graphical presentation of cell count was the average of three independent experiments and statistical difference was evaluated by unpaired Student's *t*-tests.

Adenoviral infection. Adenoviruses expressing constitutive-active mutant of MEK1 (Ad-caMEK) and control virus containing LacZ gene (Ad-LacZ) were kindly provided by Dr. Z.H. Lee (Seoul National University). After BMMs were prepared in a 48-well plate as described above, viral supernatants were added into osteoclast differentiation medium at 1d. After 24 h, the medium was removed and the cells were fed with fresh differentiation medium, cultured for additional four days, and TRAP staining was performed at the end of culture. We performed infection experiments three times independently and obtained the consistent results. To confirm that our adenoviral infection system worked well, whole-cell lysates were prepared at 48 h post-infection and subjected to immunoblot analysis for detecting total and phosphorylated p44/42 MAPK.

Results

CD9 expression increased during osteoclast differentiation

Since several reports showed the correlation between CD9 and cellular fusion [11,13,15], we examined whether

CD9 is involved in osteoclastogenesis. To confirm whether CD9 is expressed in osteoclast lineage cells, we used culture system in which BMM cells were induced to differentiate into osteoclasts by M-CSF and RANKL. The day at which RANKL was first added to cells was designated 1d. RT-PCR result showed that CD9 mRNA level increased during osteoclast differentiation whereas CD81, a closely correlated tetraspanin, did not (Fig. 1A). Immunoblot analysis showed that CD9 protein level was also elevated during the progression of osteoclast differentiation (Fig. 1B). CD9 expression in mature osteoclasts was also confirmed by immunofluorescence staining with anti-CD9 antibody (Fig. 1C). These results indicate that CD9 expression is associated with RANKL-induced osteoclast differentiation.

KMC8, a CD9-blocking antibody, suppressed osteoclast differentiation

To examine whether CD9 is involved in the regulation of osteoclastogenesis, we observed the phenotypic changes induced by KMC8, a widely used CD9-specific antagonistic monoclonal antibody [14]. BMM cells were treated with KMC8 or isotype-matched control $IgG_{2a\kappa}$ antibody at a concentration of 1 μg/ml from 1d. KMC8 significantly suppressed TRAP-positive multinucleated cell formation (Fig. 2A). KMC8 treatment also decreased the formation of TRAP-positive mononuclear cells though the inhibitory effect was not as conspicuous as that on multinuclear osteoclast formation (data not shown). This result indicates that CD9 is functionally involved in osteoclastogenesis as a

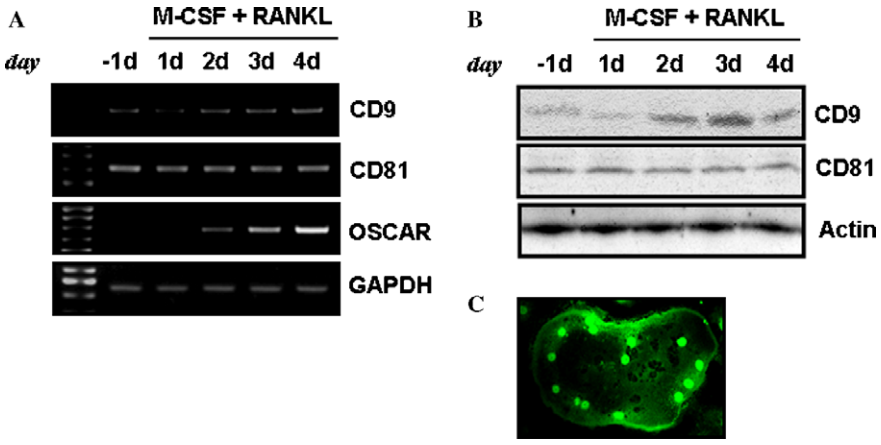

Fig. 1. CD9 expression increased during osteoclast differentiation. BMM cells were induced to differentiate into osteoclasts by RANKL and M-CSF treatment. (A) Semiquantitative RT-PCR showed that slight increase in mRNA expression of CD9 was observed during osteoclast differentiation. (B) Immunoblot analysis also showed that protein level of CD9 increased during osteoclast differentiation. (C) Confocal microscopy showed that CD9 was also expressed in multinucleated osteoclast.

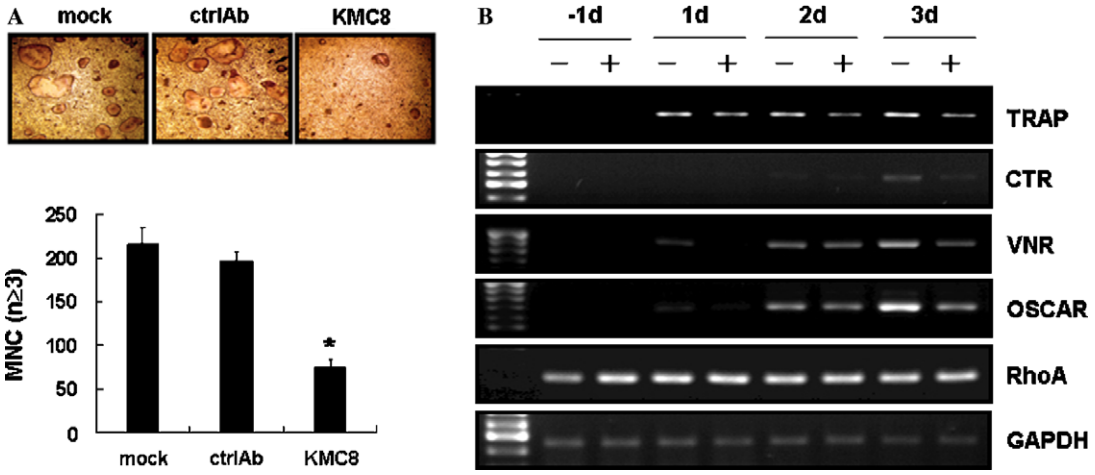

Fig. 2. KMC8, a neutralizing anti-CD9 antibody, suppressed RANKL-induced osteoclastogenesis. (A) TRAP-staining showed that KMC8 treatment significantly suppressed the formation of TRAP-positive multinuclear cells. TRAP-positive cells containing three or more nuclei were counted as multinucleated osteoclasts (MNCs). MNCs counting data represent average from three independent experiments and statistical probability of $p < 0.01$ was considered significant (*). (B) Inhibitory effect of KMC8 was further supported by decreased mRNA levels of osteoclast marker genes including TRAP, CTR, VNR, and OSCAR. RhoA was shown as a negative control that is not an osteoclast differentiation marker gene.

differentiation-regulatory molecule but the role of CD9 is not absolutely specific to cell–cell fusion process.

To confirm the inhibitory effect of KMC8, mRNA expression levels of several osteoclast differentiation marker genes were examined. RT-PCR data showed that KMC8 down-regulated the expression levels of TRAP, CTR, VNR, and OSCAR (Fig. 2B). However, mRNA expression of RhoA, a nonmarker gene, was not changed by KMC8, indicating that KMC8-induced down-regulation was limited to osteoclast differentiation marker genes. These data imply that regulation of osteoclastogenesis via CD9 is possibly related to osteoclast differentiation signals which lead to the expression of osteoclast differentiation marker genes.

KMC8 induced strong, sustained activation of p44/42 MAPK

We were then to examine whether KMC8 treatment leads to change in signaling pathways in osteoclastogenesis. Previously, it was shown that KMC8 induced tyrosine-phosphorylation of Syk, a nonreceptor tyrosine kinase [16]. So we examined whole cell tyrosine-phosphorylation state of BMM cells at 1d by immunoblot analysis. KMC8 treatment increased dramatically the number and level of tyrosine-phosphorylated proteins within 10–30 min (data not shown), suggesting that KMC8 binding to CD9 might cause significant changes in signaling pathways involved in osteoclast differentiation.

It has been shown that the activation of MAPKs such as p44/42 MAPK, p38 MAPK, and JNK is necessary for osteoclastogenesis [17–19]. Tyrosine-phosphorylation of these MAPKs is important to their functional activities together with threonine-phosphorylation [20,21]. Thus, we examined whether KMC8 could cause change in MAPK activation. Interestingly, p44/42 MAPK activation was strongly induced by KMC8 (Fig. 3A). It was notable that KMC8-induced activation of p44/42 MAPK was much stronger than RANKL-induced phosphorylation which normally transmits osteoclast differentiation signal. Also observed was phosphorylation of $p90^{RSK}$, a well-known downstream target of p44/42 MAPK. However, phosphorylation of p38 MAPK was not affected by KMC8. JNK phosphorylation was also induced by KMC8.

To make clear the difference between KMC8-induced and RANKL-induced p44/42 MAPK activation, long-term phosphorylation state up to 24 h was examined. KMC8-induced activation of p44/42 MAPK lasted up to 24 h whereas RANKL-induced activation was extinguished before 24 h (Fig. 3B), suggesting that KMC8-induced MAPK activation state might be different from RANKL-activated normal MAPK pathway leading to osteoclastogenesis. To minimize the influence of serum and/or exogenous cytokines, BMM cells were pre-incubated in differentiation medium containing M-CSF and RANKL for 1 h, washed with serum-free medium, and subsequently treated with 1 μg/ml KMC8 or control antibody in serum-free medium. KMC8 clearly activated p44/42 MAPK and $p90^{RSK}$ but not p38 MAPK (Fig. 3C). These patterns of MAPK activation were reproduced even when KMC8 was added at 2d or 3d of culture (data not shown), suggesting that p44/42 MAPK activation status might be sustained throughout the entire culture period when KMC8 was added to culture everyday. This result indicates that CD9 might negatively regulate the intensity and

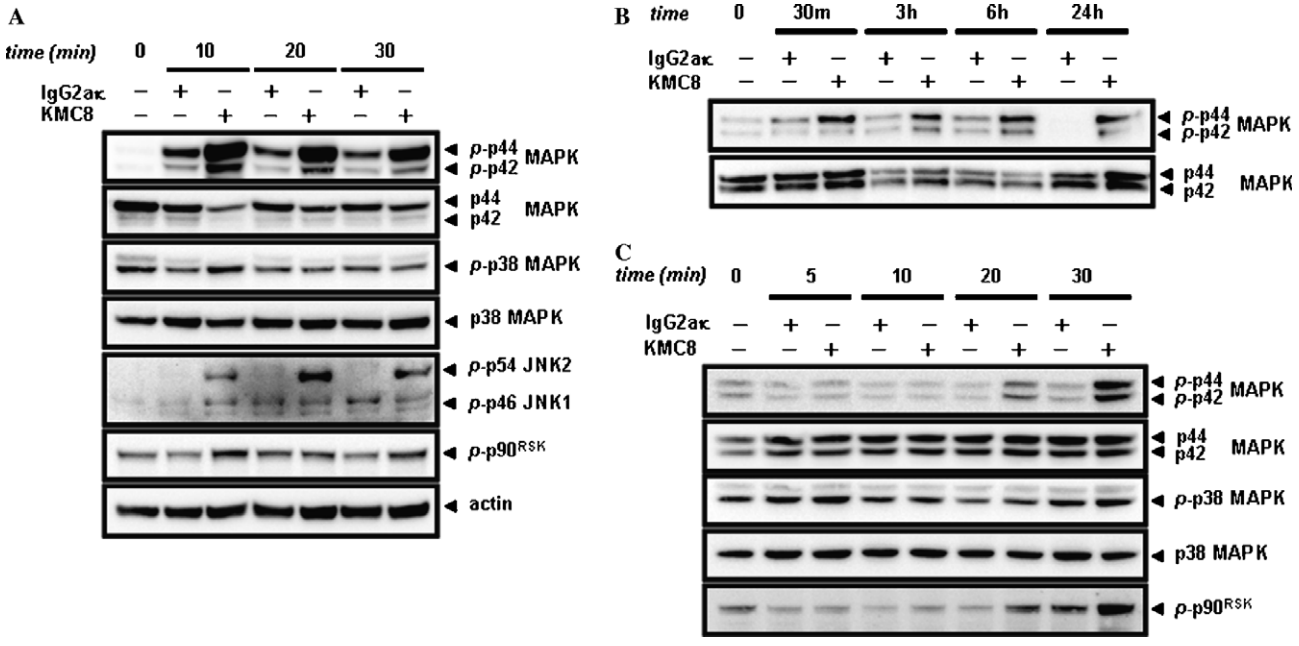

Fig. 3. KMC8 induced strong and sustained activation of p44/42 MAPK. BMM cells at 1d were stimulated with 1 μg/ml KMC8 or isotype-matched control $IgG_{2a\kappa}$ antibody in the presence (A,B) or absence (C) of M-CSF, RANKL, and serum. Note that KMC-induced p44/42 MAPK phosphorylation level was much higher than that normally induced by the M-CSF and RANKL.

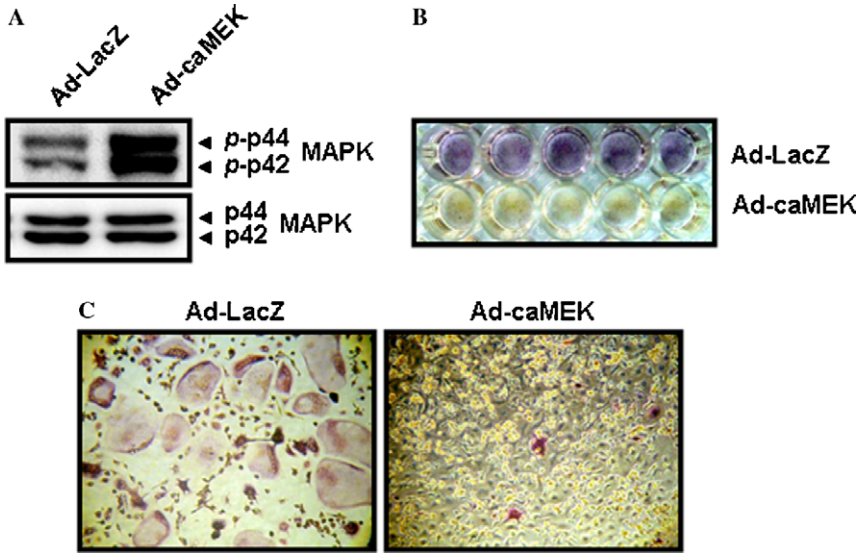

Fig. 4. Constitutive activation of p44/42 MAPK inhibited osteoclast differentiation. BMM cells were infected by adenovirus expressing constitutive-active mutant of MEK1 (Ad-caMEK) or control virus containing LacZ gene at 1d. (A) Immunoblot analysis showed that activation of p44/42 MAPK was efficiently induced by Ad-caMEK infection. (B,C) Infected BMMs were induced to differentiate into osteoclasts followed by TRAP staining. Cells infected with Ad-LacZ were normally differentiated into TRAP-positive multinuclear cells. However, osteoclast formation was almost completely blocked in cells infected with Ad-caMEK.

duration of p44/42 MAPK activation to a certain level to permit the progression of RANKL-induced osteoclast differentiation.

Constitutive activation of p44/42 MAPK negatively regulated osteoclastogenesis

To make clear the functional role of KMC8-mediated activation of p44/42 MAPK in osteoclastogenesis, we examined whether constitutive activation of p44/42 MAPK could suppress osteoclast differentiation. To activate p44/42 MAPK constitutively, we infected BMMs with Ad-caMEK and induced to differentiate into osteoclasts. Immunoblot result showed that p44/42 MAPK activation was highly induced by Ad-caMEK infection (Fig. 4A). TRAP staining showed that Ad-caMEK almost completely blocked osteoclast differentiation while Ad-LacZ-infected cells were normally differentiated into TRAP-positive multinucleated osteoclasts (Fig. 4B and C). We could have hardly observed TRAP-positive mononuclear cells as well in Ad-caMEK-infected cells. This result suggests that excessive activation of p44/42 MAPK negatively regulates osteoclastogenesis. Taken together, our results present evidence that CD9 molecules expressed on BMM cells and differentiating osteoclasts contribute to osteoclastogenesis at least by negatively regulating p44/42 MAPK signaling pathway.

Discussion

In this study, we showed that CD9 expressed on BMM cells and differentiating osteoclasts was functionally associated with osteoclastogenesis. A previous study suggested that CD9 molecules expressed on stromal cells were involved in osteoclastogenesis but those on bone marrow cells/osteoclasts were not [14]. However, using the culture system that minimizes the presence of stromal cells, we showed that CD9 proteins expressed on osteoclast lineage cells were responsible for the regulation of osteoclastogenesis. In this culture system, blocking of CD9 by KMC8 suppressed TRAP-positive multinuclear osteoclast formation and decreased the expression levels of osteoclast differentiation marker genes.

KMC8 was reported to prevent egg-sperm fusion [11] and to inhibit the fusion of the myoblasts [13]. However, KMC8 was also shown to facilitate the fusion of alveolar macrophages [15]. During the preparation of this manuscript, one paper was published that showed CD9 expression was induced by RANKL and blocking of CD9 by KMC8 or by RNA interference suppressed the fusion of RAW264.7 cells [22]. They showed that CD9 is distributed on the surface of the cells that were in contact with each other before fusion, suggesting that role of CD9 was specific for cell–cell contact and cell fusion. These results were more or less similar to ours. In our study, however, although KMC8 treatment significantly inhibited multinucleated osteoclast formation from BMM cells, this inhibitory effect was not absolutely specific to fusion events because the number of TRAP-positive mononuclear cells was also diminished by KMC8. These results suggest that CD9 plays another role in addition to the regulation of fusion in the osteoclastogenesis. The suppressive effect of KMC8 would occur specifically through CD9 and not through the interaction between Fc portion of KMC8 and Fc receptors, since isotype-matched control $IgG_{2a\kappa}$ antibody did not show suppressive effect on osteoclastogenesis.

MAPK pathways have been shown to be induced during osteoclastogenesis [17–19]. p38 MAPK is involved in differentiation signals by RANKL and its activation leads to downstream activation of mi/Mitf which controls the expression of TRAP and cathepsin K [17,18]. p44/42 MAPK is also activated by RANKL and regulated by an upstream kinase MEK1/2 [18]. The regulatory aspects of osteoclastogenesis by p44/42 MAPK seem to be complicated and controversial. Nearly all studies trying to reveal the functional role of p44/42 MAPK pathway in osteoclastogenesis have made use of specific inhibitors. Among these, U0126 and PD98059 have been widely used as specific inhibitors of MEK1/2 to inactivate p44/42 MAPK signaling. One study showed that both inhibitors inhibited osteoclast differentiation, suggesting that p44/42 MAPK activation is required for osteoclastogenesis [18]. Other studies showing that p44/42 MAPK activation is induced by RANKL seem to support this notion [19,23]. In contrast, another work presented opposing evidence that both inhibitors enhanced osteoclast formation, indicating that the p44/42 MAPK pathway is involved in the negative regulation of osteoclastogenesis [24]. These results raise complexity in the role of p44/42 MAPK pathway in osteoclastogenesis. Although these MEK1/2 inhibitors are very potent and commonly used tools, experimental approach using these inhibitors has a limitation in defining all facets of the regulatory role of MEK-MAPK pathway since they basically act by abrogating MEK-MAPK signal completely for their acting duration regardless of a tightly regulated balance between activation and inactivation of MEK-MAPK pathway. In fact, RANKL-induced activation of p44/42 MAPK was reported to be transient in studies supporting the positive regulatory role of p44/42 MAPK in osteoclastogenesis [18,23]. However, our data showed that KMC8 induced p44/42 MAPK activation much stronger and for much longer time than RANKL did. In addition, constitutive activation of p44/42 MAPK by Ad-caMEK almost completely blocked osteoclastogenesis. Considering these results, it seems that the tight regulation of the intensity and the duration of p44/42 MAPK activation would be important to the progression of osteoclast differentiation. Another possible explanation might be the seasaw cross-talk model, as suggested by Hotokezaka et al. [24]. In this model, they suggested that a balance between negatively regulating p44/42 MAPK and positively regulating p38 MAPK regulates osteoclastogenesis. Since KMC8 affected p44/42 MAPK phosphorylation exclusively without affecting p38 MAPK, CD9 might be specifically involved in a line of p44/42 MAPK-regulating pathway.

On the other hand, KMC8 also induced JNK activation. JNK pathway is necessary for osteoclast differentiation [25]. Our result showed that only JNK2 was potently activated by KMC8. The correlation between JNK activation by KMC8 and KMC8-induced suppression of osteoclastogenesis was not defined in this study and needed is further study.

In conclusion, we here present tetraspanin CD9 molecule as a novel positive regulator of osteoclastogenesis. CD9 is expressed on differentiating osteoclast lineage cells and its expression level increases during osteoclast differentiation. Blocking CD9 causes strong and sustained activation of p44/42 MAPK, leading to suppression of multinucleated osteoclast formation. Although many aspects of the regulatory functions of CD9 in osteoclastogenesis should be further defined, understanding the role of CD9 would be another good step to unveil the mechanism of osteoclastogenesis and help to find therapy to bone disease such as osteoporosis.

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