Journal of Biochemistry and Molecular Biology, Vol. 34, No. 5, September 2001, pp. 421-427

JOURNAL OF
Biochemistry
and
Molecular Biology

Osteoclast Differentiation Factor Engages the PI 3-kinase, p38, and ERK pathways for Avian Osteoclast Differentiation

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Received 31 May 2001, Accepted 25 June 2001

Osteoclasts, cells primarily involved in bone resorption, originate from the hematopoietic precursor cells of the monocyte/macrophage lineage and differentiate into multinucleated mature forms. We developed an *in vitro* osteoclast culture system using embryonic chicken bone marrow cells. This culture system can be utilized in studies on the differentiation and function of osteoclasts. Phosphatidylinositol 3-kinase (PI 3-kinase) and mitogen-activated protein kinases (MAPKs) have been implicated in diverse cellular functions including proliferation, migration, and survival. Using the developed avian osteoclast culture system, we examined the involvement of these kinases in osteoclast differentiation by employing specific inhibitors of the kinases. We found that the inhibition of the PI 3-kinase, p38, or ERK interfered with osteoclast formation, suggesting that the signaling pathways that involve these molecules participate in the process of chicken osteoclast differentiation.

Keywords: Osteoclast differentiation factor, PI 3-kinase, p38, ERK

Introduction

Overall bone homeostasis is regulated through bone remodeling. Osteoblasts that form bones, and osteoclasts that resorb bones, mediate this process. Osteoclasts originate from the hematopoietic cells and differentiate via the monocyte/macrophage linage. The differentiation from hematopoietic precursor cells into mature multinuclear osteoclasts, competent for bone resorption, involves a cell-to-cell interaction between osteoblastic/stromal cells and hematopoietic cells (Udagawa *et al.*, 1990; Suda *et al.*, 1992). Recently, several research groups cloned the tumor necrosis factor (TNF) and its receptor family genes that are important for osteoclast differentiation: ODF, RANK, and OPG (Lacey *et al.*, 1998; Yasuda *et al.*, 1998b; Hsu *et al.*, 1999; Kwon and Kwon, 1999). The osteoclast differentiation factor (ODF) is produced by osteoblast/stromal cells as a cell surface molecule (Yasuda *et al.*, 1998b). ODF was found to be identical to RANKL (receptor activator of NF-κB ligand), TRANCE (TNF-related activation-induced cytokine), and OPGL (osteoprotegerin ligand) (Anderson *et al.*, 1997; Wong *et al.*, 1997; Kong *et al.*, 1999). RANK, a receptor for ODF, is expressed on the membrane of osteoclast progenitor cells (Nakagawa *et al.*, 1998; Hsu *et al.*, 1999). The interaction between RANK and ODF in the presence of M-CSF results in osteoclast differentiation and activation (Nakagawa *et al.*, 1998; Jimi *et al.*, 1999; Takahashi *et al.*, 1999). Finally, osteoprotegerin (OPG), which is also called the osteoclastogenesis inhibitory factor (OCIF), is a decoy receptor for ODF. It inhibits osteoclastogenesis by interfering with the binding of ODF to RANK (Akatsu *et al.*, 1998; Hakeda *et al.*, 1998; Yasuda *et al.*, 1998a; Kanzawa *et al.*, 2000).

The major obstacle in the study of osteoclasts is that only a few osteoclast cell lines have been available. Since osteoclasts have a hematopoietic origin, and the bone marrow contains hematopoietic stem cells, a bone marrow culture is the most common way to get osteoclasts. Many investigators have reported bone marrow culture systems from various animal species. These include rabbits, canines, baboons, mice, hens, and humans (Hakeda and Kumegawa, 1996). In this study, we developed an osteoclast culture system using embryonic chicken bones. This method allows a rapid and convenient means to obtain mature functional osteoclasts.

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Various extracellular stimuli, such as growth factors and cellular stresses, activate protein kinases that convey the signal to targets in the cytoplasm and the nucleus. Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that become activated by phosphorylation on threonine and tyrosine residues upon extracellular stimuli (Schaeffer *et al.*, 1999). Three families of MAP kinases (MAPK/ERK, JNK/SAPK, and p38) have been identified (Schaeffer *et al.*, 1999). PI 3-kinase appears to play a role in the macrophage colony stimulating factor (M-CSF)-induced motility of osteoclasts (Pilkington *et al.*, 1998). PI 3-kinase leads to the production of PIP_3 that binds selectively to the PH domains of protein kinases PDK1 and Akt/PKB, inducing their membrane association and further signaling (Toker *et al.*, 2000). Akt/PKB is activated by ODF in mature osteoclasts. It is important for ODF-mediated osteoclast survival (Wong *et al.*, 1999).

In the present study, we examined the possible involvement of MAPKs and PI 3-kinase in the ODF-induced osteoclast differentiation using specific inhibitors of MEK1 (the upstream kinase of ERK), p38, and PI 3-kinase in the embryonic chicken bone marrow culture system. In this culture system, multinuclear mature forms of osteoclasts were observed within 36 h of culture and the osteoclast differentiation was accelerated by adding ODF. Osteoclast differentiation was determined by counting the TRAP-positive cells and by measuring the resorption activity on calcium phosphate-coated resorption plates. The osteoclast differentiation was inhibited by the employed inhibitors of MEK1, p38, and PI 3-kinase.

Materials and Methods

Reagents LY294002 and wortmannin were purchased from Sigma (St. Louis, MO). SB202190 and PD98059 were from Calbiochem (La Jolla, CA). M-CSF, and a soluble form of the extracellular domain of human ODF, was obtained from Peprotech EC (London, England).

Embryonic Chicken Bone Marrow Cell Culture To isolate embryonic chicken bone marrow cells, the chicken eggs were incubated at 38°C, 80-82% humidity for 14 days with rocking every two h. The bone marrow cells were isolated from the tibiae of 14-day old chicken embryos. After removal of the soft tissues and cartilage, the tibiae were submerged into an ice-cold α-minimum essential medium (MEM) and chilled. The bone marrow cells were flushed out from the chilled bones with α-MEM. The cells were resuspended in an α-MEM/10% fetal bovine serum (FBS), placed at 1×10^6/well in 24-well plates, and cultured at 37°C in a humidified atmosphere of 5% CO_2. After 4 h of culture, the non-adherent cells were removed. The remaining adherent cells were further cultured with the indicated concentrations of the inhibitors for 48 h.

TRAP Assay Cytochemical staining of TRAP-positive cells was performed using the Leukocyte Acid Phosphatase Assay Kit (Sigma) following the manufacturer's procedure.

Resorption Assay For resorption assays, the cells were plated on OAAS™ culture plates (Oscotech, Cheonan, Korea) that were coated with calcium phosphate crystals. After the culture period, the attached cells were completely removed from the plates by a sequential treatment with 1.2% sodium hypochloride and distilled water. After drying the plates, photographs were taken under a light microscope at 40× magnification. The total areas of resorption lacunae were analyzed by the Image Pro-Plus program version 4.1 (Media Cybernetics, Silver Spring, MD). Alternatively, the total number of resorption lacunae was counted. The results are expressed as means ± SD (standard deviation) of the triplicate samples. To confirm the bone-resorbing ability of osteoclasts, the cells were placed on dentine slices and further cultured in the same way for 72 h. Resorption pits on the dentine slices were observed by staining with the hematoxylin solution (Sigma).

Statistical Analysis Data were analyzed by students' t-test. Every experiment was performed in triplicate. The results are representative of the three independent experiments.

Results

Osteoclast differentiation from embryonic chicken bone marrow cells We developed a method to culture avian osteoclasts from bone marrow cells of chicken embryos. Fertilized chicken eggs were incubated for 14 days and the bone marrow cells were obtained by flushing the tibiae of the embryos. The cells were allowed to attach on culture dishes for 4 h and the nonadherent cells were removed. The attached cells were further cultured for 48-72 h. Fig. 1A shows the typical morphology of mature osteoclasts under a phase-contrast microscope. Mature osteoclasts were multinucleated, some contained more than 20 nuclei and showed membrane ruffles (Fig. 1A). The avian osteoclasts could be identified by the TRAP-reactivity that was detected within 48 h of culture (Fig. 1B). About 60-120 TRAP-positive cells were detected on each well of the 24-well plates and ~10% of the TRAP-positive cells contained more than three nuclei (Fig. 1B). The generation of TRAP-positive osteoclasts was increased by 3-5 fold in the presence of soluble ODF (Fig. 3, A vs. B "Con" lanes).

To confirm the resorption ability of generated osteoclasts, the chicken bone marrow cells were cultured, either on dentine slices or on culture plates, that were coated with calcium phosphate crystals. The avian mature osteoclasts could form pits on the dentine slices (Fig. 2, A and B). The resorption activity was increased in the presence of ODF (Fig. 2, B vs. A). The resorption activity of the chicken osteoclasts was also confirmed with calcium phosphate-coated plates, as evidenced by the formation of clear zones (Fig. 2C). These results showed that embryonic chicken bone marrow cells could be differentiated into functional osteoclasts in this culture system without any additional supporting cells or factors. However, the addition of ODF could further stimulate the avian osteoclast differentiation.

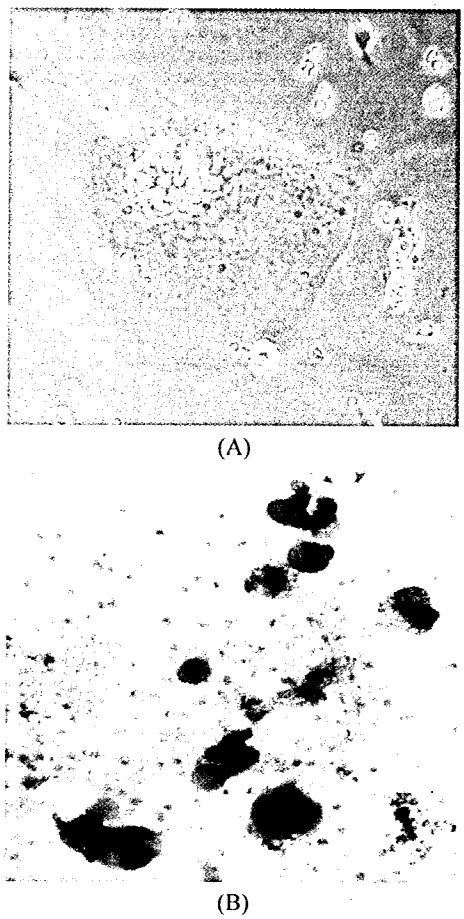

Fig. 1. Morphology of chicken osteoclasts. Chicken bone marrow cells prepared from the day 14 chicken embryos were seeded at 1×10^6 cells per well in 24-well plates (described in *Materials and Methods*) and incubated for 48 h. (A) A phase contrast photographs taken at 400-fold magnification. (B) Typical TRAP-stained chicken osteoclasts.

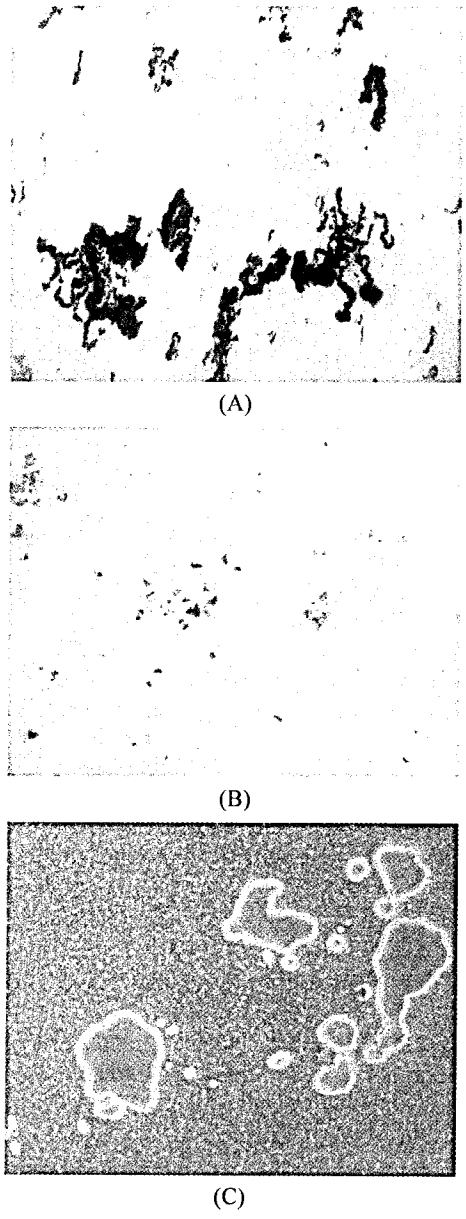

Fig. 2. Resorption pit formation by chicken osteoclasts. Embryonic chicken bone marrow cells were seeded at 5×10^5 cells on a dentine slice (A, B) or in a well of 48-well OAAS™ plates coated with calcium phosphate crystals (C) in the presence (A, C) or absence (B) of ODF (50 ng/ml).

Effects of kinase inhibitors on the avian osteoclast differentiation Mitogen-activated protein (MAP) kinases are involved in cell growth, differentiation, and survival in various cell types. PI 3-kinase is involved in osteoclastic bone resorption. The activation of PI 3-kinase is required for the M-CSF-induced spreading of osteoclasts (Grey *et al.*, 2000). To investigate the possible involvement of MAP kinase signaling pathways in the differentiation of embryonic chicken bone marrow cells into TRAP-positive osteoclasts, the effects of PD98059 (a specific inhibitor of MEK1, an upstream activating kinase of ERKs) and those of a specific p38 inhibitor, SB202190, were examined. In addition, whether PI 3-kinase is necessary for the avian osteoclast differentiation was determined by using two different types of specific PI 3-kinase inhibitors, LY294002 and wortmannin.

In the culture without ODF, PD980059, SB202190, and LY294002 inhibited the formation of the TRAP-positive cells (Fig. 3A). However, in the culture with ODF, the extent of inhibition by these inhibitors was stronger (Fig. 3B). This implies that the activation of ERK and p38 MAPKs, as well as PI 3-kinase is required for the differentiation of avian osteoclast cells. Also, ODF stimulates the differentiation through signaling pathways mediated by ERK, p38, and PI 3-kinase.

Effects of the inhibitors on the chicken osteoclast differentiation were also assessed by measuring the resorbing activity of osteoclasts. The bone marrow cell preparations were cultured on plates coated with calcium phosphate crystals in the presence of the inhibitors. This embryonic

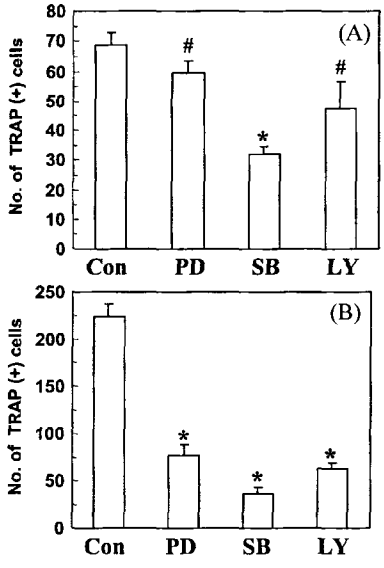

Fig. 3. Effect of MAPK and PI 3-kinase inhibitors on the generation of TRAP-positive osteoclasts from embryonic chicken bone marrow cells. Chicken bone marrow cells prepared from the day 14 chick embryos were seeded at $1x10^6$ cells per well in 24-well plates (described in *Materials and Methods*). They were then incubated for 48 h in the presence of 0.2% DMSO control vehicle, 30 mM PD98059, 10 mM SB202190, and 15 mM LY294002 without ODF (A), or in the presence of 50 ng/ml ODF (B). The cells were then subjected to TRAP staining and TRAP-positive cells were counted. #, $p<0.05$ versus control. *, $p<0.01$ versus control.

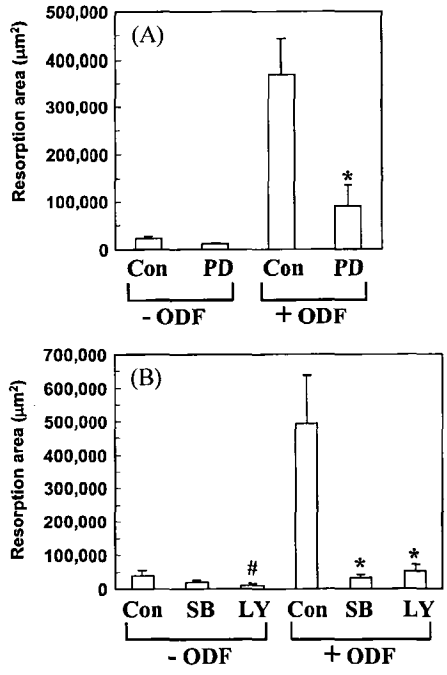

Fig. 4. Inhibition of resorption lacuna formation by ERK, p38, and PI 3-kinase inhibitors in chicken bone marrow cell cultures. Embryonic chick bone marrow cells were seeded at 5×10^5 cells per well in 48-well OAAS™ plates coated with calcium phosphate crystals and treated with 0.2% DMSO control vehicle, 30 mM PD98059 (A), 10 mM SB202190 (B), and 15 mM LY294002 (B) without or with 50 ng/ml ODF. After removing adhered cells from the plates, resorption pits were identified under a light microscope with 40-fold magnification. The total resorbed areas in plates were calculated using an image analysis program. #, $p<0.05$ versus control. *, $p<0.01$ versus control..

chicken culture system generated functional osteoclasts that were competent for resorption (Fig. 4, A and B). Both LY294002 and SB202190 completely abolished the ODF-induced resorption activity (Fig. 4B). PD98059 also inhibited resorption, but to a lesser extent than LY294002 and SB202190 (Fig. 4A).

Effects of another PI 3-kinase inhibitor, wortmannin, on osteoclast differentiation were also assessed by counting the number of resorption lacunae that were formed by osteoclasts, which were generated in the presence or absence of M-CSF and ODF. M-CSF is often used for osteoclast cultures from bone marrow cells (Hakeda and Kumegawa, 1996). The number of resorption lacunae, formed in the absence of any added factor and in the presence of only M-CSF, was about 100120 per well (Fig. 5, A and B). The addition of ODF increased the number to about 450500 per well. Wortmannin inhibited the effect of ODF in a dose dependent manner, regardless of the presence of M-CSF (Fig. 5, C and D).

Discussion

Bone remodeling is a coordinated process between bone formation and resorption. If this process is in an unbalanced state, pathologic disorders occur, such as osteoporosis and osteopetrosis. Osteoclasts are the primary cells responsible for bone resorption, thus studies on the function and regulatory mechanisms of osteoclasts will be very important to the understanding of the whole process of bone metabolism. However, the main limitations in the study of osteoclasts are the lack of osteoclastic cell lines, the difficulty of obtaining pure osteoclasts, and the requirement of supporting cells, such as osteoblasts or stromal cells for osteoclast differentiation. The recent discovery of ODF, the essential cytokine for osteoclast differentiation, made it possible to culture osteoclasts without supporting cells (Nakagawa *et al.*, 1998; Yasuda *et al.*, 1998b; Jimi *et al.*, 1999; Udagawa *et al.*, 1999; Tsurukai *et al.*, 2000). In this study we established an avian bone marrow culture system, and examined the role of the three signaling pathways in osteoclast differentiation by applying the specific pharmacological inhibitors of PI 3-kinase, p38 MAPK, and ERK.

To elucidate the function and regulatory mechanisms of osteoclasts, it is necessary to establish an osteoclast culture system. However, it is very difficult to obtain osteoclasts, because the number of osteoclasts is much lower than that of other bone cells. Also, osteoclasts adhere tightly to bone

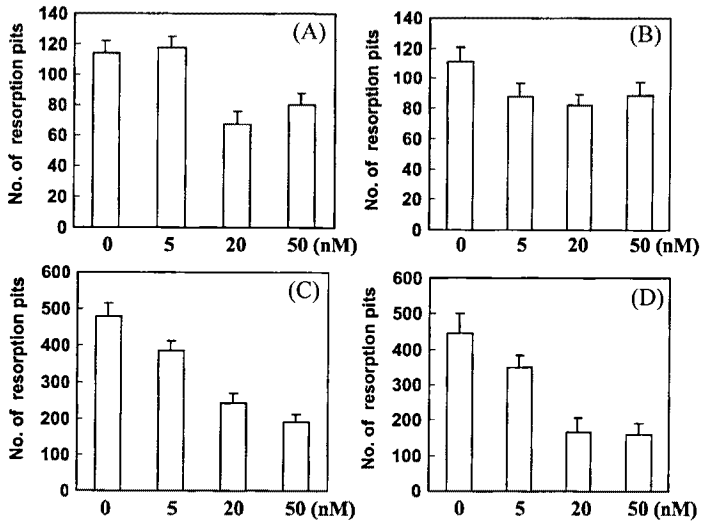

Fig. 5. Effect of PI 3-kinase inhibitor, wortmannin, on resorption lacuna formation in embryonic chicken bone marrow cell cultures. The bone marrow cells were grown in the culture medium alone (A), or the medium containing 30 ng/ml M-CSF only (B), 50 ng/ml ODF only (C), or M-CSF plus ODF (D) in the presence of indicated concentrations of wortmannin. The number of resorption lacunae was counted.

matrix. Neither of the cell lines that preserve the characteristics of osteoclasts, or their precursors, have been developed to date. Therefore, many investigators have tried to establish new methods for osteoclast culture systems. Since osteoclasts have a hematopoietic stem cell origin and bone marrow contains hematopoietic stem cells, bone marrow cultures are therefore widely used to study the osteoclast formation from various animal species (Hakeda and Kumegawa, 1996). The culture method to obtain avian osteoclasts was first developed by Collin-Osdoby *et al.* (1991) where bone marrow cells were flushed from femurs of chicks on a low-calcium diet. This isolation method is useful for many molecular studies and may also be applicable for the isolation of mammalian osteoclasts. However, the number of purified osteoclasts is low, which limits studies that require a large quantity of osteoclasts. Also, as this method involves immunomagnetic beads, the expense is high. We developed a new method for osteoclast cultures from embryonic chicken bone marrow cells. This method is very simple and efficient. The cold treatment was included during the isolation procedure from chicken bone marrow cells. The cultured cells showed a typical morphology of osteoclast-like cells under a light microscope and were TRAP-positive (Fig. 1, A and B). The formation of osteoclasts from bone marrow cells in the absence of additional exogenous ODF is unique to this embryonic chicken culture system. Cultures of bone marrow cells from other species require the addition of ODF (Lacey *et al.*, 1998). It is plausible that bone marrow cells from embryonic chicken contain stromal cells that are capable of supporting osteoclastogenesis, perhaps by constitutively expressing surface ODF.

Activated osteoclasts can resorb the bone matrix. To confirm the bone-resorbing activity of osteoclasts, several *in vitro* bone resorption assays were developed. They can provide a functional readout for osteoclasts that play a role in the removal of bone under physiological and pathological conditions. Many investigators from industry would be interested in testing the potential drugs for anti-resorptive activity. Isolated osteoclasts can make Howships lacunae-like pits in slices of devitalized dentine or bone (Boyde *et al.*, 1984; Chambers *et al.*, 1984; Jones *et al.*, 1984). The ability to make a pit in a dentine or bone slice has become a definitive characteristic of osteoclasts in systems that determine functional osteoclasts from precursors. TRAP-positive osteoclasts, isolated in our chicken bone marrow system, could form pits on dentine slices, especially in the presence of ODF (Fig. 2A). Without ODF, the isolated chicken osteoclasts could slightly resorb the dentine (Fig. 2B). Therefore, we confirmed that ODF is an important stimulator of mature osteoclasts. This system could be used to determine the bone-resorbing activity of osteoclasts.

Dentine, or bone slice-based assay, is labor-intensive in nature. It is very difficult to make dentine slices. The quantification of resorption pits is often tricky and requires staining. Many investigators have tried to find new methods to replace the use of bone or dentine slices. We used culture plates that were coated with calcium phosphate crystals to assess the resorbing activity of osteoclasts (Kim *et al.*, 2001). This method provided a convenient way to measure the activity of osteoclasts. After detaching the cells, the resorbed area could be observed simply under a light microscope with no special staining.

It has been reported that PI 3-kinase is involved in a multitude of cellular responses, including cell survival and death. Using pharmacological inhibitors, the PI 3-kinase signaling pathway was shown to mediate osteoclast

attachment, spreading, and chemotaxis (Nakamura *et al.*, 1995; Lakkakorpi *et al.*, 1997; Nakamura *et al.*, 1997). In our study, LY294002 and wortmannin, specific inhibitors of PI 3-kinase, showed an inhibitory effect on osteoclast differentiation. This inhibitory effect was prominent in the presence of ODF (Fig. 3A, 3B). This result suggests that PI 3-kinase is involved in the ODF-mediated osteoclast differentiation. MAP kinase has been reported to be the major signaling pathway in various cells, including neuronal and endothelial cells (Klint *et al.*, 1995; Kouhara *et al.*, 1997; Klint *et al.*, 1999; Kim *et al.*, 1999). The treatment of the p38 MAPK inhibitor, SB203580, and a dominant negative form of p38, or MAPK kinase (MKK) 6, blocked the ODF-induced osteoclast differentiation in murine osteoclast precursors (Matsumoto *et al.*, 2000). Dominant negative Ras that inhibits the ERK activity induced the apoptosis of murine osteoclast-like cells (Miyazaki *et al.*, 2000). Conversely, ERK activation by constitutively active MEK1 remarkably lengthened their survival by preventing spontaneous apoptosis (Miyazaki *et al.*, 2000). However, the roles of MAPK/ERK and p38 kinase for osteoclast differentiation have not been elucidated in detail. When the ERK inhibitor, PD98059, and p38 inhibitor, SB202190, were added in the chicken bone marrow culture, osteoclast differentiation was inhibited, especially in the presence of ODF (Fig. 3, A and B). The interference of osteoclast formation by the inhibitors in the absence of ODF may suggest the possibility of the inhibitors effect on signals from molecules other than ODF. However, it is also possible that this culture contained cells expressing ODF, supporting osteoclastogenesis. Further characterization of the osteoclastogenesis in the absence of ODF addition in this culture is needed for a clearer understanding. At the treated concentrations of PD98059 and SB202190, no cytotoxicity on HeLa and other cell lines was observed (data not shown). This suggests that the inhibitory effect on osteoclastogenesis is unlikely to be from general cytotoxicity of the reagents. These results suggested that, as in the case of PI 3-kinase, ERK and p38 signaling pathways are involved in ODF-mediated osteoclast differentiation. Taken together, PI 3-kinase, ERK, and p38 are likely to be involved in differentiation of osteoclasts by transducing signals from ODF.

In summary, we developed an embryonic chicken osteoclast culture system. This system can be applied to studies on the differentiation and activation of osteoclasts. Using this system, we found that the inhibition of PI 3-kinase, p38, or ERK interfered with the TRAP-positive cell formation, suggesting that the signaling pathways mediated by these kinases participate in the process of chicken osteoclast differentiation.

Acknowledgments This work was supported by NRL and ERC grants from the Ministry of Science and Technology, Korea and the Korea Science and Engineering Foundation, as well as by the BK21 Program from the Ministry of Education, Korea.

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