U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells.

Hitoshi Hotokezaka‡, Eiko Sakai§, Kazuhiro Kanaoka‡, Kan Saito¶†, Ken-ichiro Matsuo‡†, Hideki Kitaura‡, Noriaki Yoshida‡, and Koji Nakayama†

‡Division of Orthodontics and Biomedical Engineering, §Division of Oral Molecular Pharmacology, ¶Division of Pediatric Dentistry, and †Division of Microbiology and Oral Infection, Department of Developmental and Reconstructive Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8588, Japan

‡Corresponding Author: Hitoshi Hotokezaka, Ph.D.
Division of Orthodontics and Biomedical Engineering, Department of Developmental and Reconstructive Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki-city, Nagasaki 852-8588, Japan
Phone: +81-95-849-7669
Fax: +81-95-849-7670
e-mail: hotoke@net.nagasaki-u.ac.jp

Running Title: MEK inhibitors and osteoclast differentiation
SUMMARY

Osteoclasts are multinucleated cells that differentiate from hematopoietic cells, and possess characteristics responsible for bone resorption. To study the involvement of mitogen-activated protein kinases (MAPKs) in osteoclastogenesis of the murine monocytic cell line RAW264.7 which can differentiate into osteoclast-like cells in the presence of receptor activator of nuclear factor kappa B ligand (RANKL), we treated the cells with specific inhibitors of p38 MAPK, PD169316 and SB203580, and specific inhibitors of MAPK-extracellular signaling-regulated kinase (ERK) kinase (MEK), U0126 and PD98059. Each inhibitor blocked differentiation into osteoclast-like cells when the cells were plated at the standard cell density [2000-4000 cells per well (96-well)]. However, the effect of MEK inhibitors on osteoclastogenesis varied according to the initial cell density during culture because cell growth was clearly inhibited by them. When the cells were plated at more than 8000 cells per well, marked enhancement and acceleration of the differentiation were observed. In addition, immunoblot analysis revealed that phosphorylation of ERK was increased by treatment with the p38 inhibitors, whereas the MEK inhibitors increased phosphorylation of p38, which implies a seesaw-like balance between ERK and p38 phosphorylation. We suggest that osteoclastogenesis is regulated under a balance between ERK and p38 pathways, and that the MEK/ERK pathway negatively regulates osteoclastogenesis while the p38 pathway does so positively. This is the first report that an inhibitor of signal transduction enhanced osteoclastogenesis.
INTRODUCTION

Bone is continuously remodelled by bone formation and resorption, and the cooperative bone metabolism is tightly regulated to maintain homeostasis. Deviation from the normal conditions of bone resorption would result in bone diseases such as osteoporosis, osteopetrosis and periodontal disease. Osteoclasts that are responsible for bone resorption in bone metabolism are multinucleated cells originating from hematopoietic precursor cells of the monocyte/macrophage lineage. Osteoclast formation requires a cell-to-cell interaction of osteoclast precursor cells with osteoblasts and can be achieved by coculturing bone marrow precursor cells with osteoblasts/stromal cells (1). One of the key factors mediating the osteoblast-induced osteoclastogenesis is the tumor necrosis factor (TNF) family member receptor activator of nuclear factor kappa B ligand (RANKL) (2) also referred to as osteoclast differentiation factor (ODF) (3), TNF-related activation-induced cytokine (TRANCE) (4), and osteoprotegrin ligand (OPGL) (5). RANKL was found to be expressed on the surface of osteoblastic/stromal cells and essential for osteoclast differentiation (3, 6-8). RANK, one of TNF receptor family members, is expressed in osteoclasts and their precursor cells as the receptor of RANKL (6, 8, 9). An intracellular domain of RANK interacts with TNF receptor-associated factor (TRAF) 2 and TRAF 6 (10-14), which appear to be involved in the activation of downstream signals such as nuclear factor kappa B (NF-κB), phosphatidylinositol 3 (PI3), protein kinase B (PKB), and mitogen-activated protein kinases (MAPKs) (10, 13, 15, 16). MAPKs including two major subfamilies, extracellular signal-regulated kinase (ERK) and p38, have been well reviewed in the literature (17), ERK1/2 is linked to cell survival, whereas c-Jun N-terminal kinase (JNK) and p38
are linked to induction of apoptosis in PC-12 cells (18). The involvement of MAPKs in osteoclast differentiation has recently been studied using specific inhibitors of MAPK-ERK kinase (MEK) and p38 (15,16,19), which suggests that these MAPKs play important roles in osteoclastogenesis.

In the present study, we investigate the effects of specific inhibitors for MEK and p38 on differentiation of RAW264.7 cells into osteoclast-like cells, and find that MEK inhibitors enhance and accelerate the differentiation whereas p38 inhibitors suppress it. In addition, we observe a seesaw-like phosphorylation between ERK and p38 when the cells are treated with the inhibitors for MEK and p38.

**EXPERIMENTAL PROCEDURES**

*Reagents* - Polyclonal anti-phospho ERK (E-4) and anti-c-Src were purchased from Santa Cruz (Santa Cruz, CA). Polyclonal anti-ERK, anti-MEK1, anti-phospho-MEK1, monoclonal anti-p38 MAPK (5F11) and anti-phospho-p38 MAPK (28B10) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-C-terminal peptide of cathepsin K was prepared as described previously (20). RANKL was from Peprotech EC Ltd. (London, United Kingdom). PD169316, SB203580, U0126 and PD98059 were purchased from Calbiochem Corp. (La Jolla, CA). Minimal essential medium alpha modification (α-MEM)[glutamine (Gln) free] and all other chemicals were obtained from Sigma (St. Louis, MO).

*Cell Culture* - The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured in a 5% CO₂ in air atmosphere humidified incubator at 37°C, and maintained on 9 cm diameter uncoated plastic dishes in α-MEM containing 10% (v/v) heat-inactivated fetal calf
serum (FCS) with 2 mM Gln, 100 U/ml penicillin, and 100 µg/ml streptomycin. For osteoclastogenesis experiments, the indicated number of cells was seeded on a 96-well tissue culture plate with 2 mM Gln [α-MEM Gln (+)] or without it [α-MEM Gln (-)], in the presence or absence of RANKL and chemical reagents.

**Tartrate-resistant acid phosphatase (TRAP) staining and immunostaining**- Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for more than 60 min. The cells were then treated with 0.2% Triton X-100 in PBS at room temperature for 5 min, followed by rinsing twice with PBS. Finally, the fixed cells were incubated with 0.01% naphthol AS-MX phosphate (Sigma) and 0.05% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH5.0) for TRAP staining, and rinsed twice with PBS. For immunostaining, the cells fixed on the plate were incubated overnight at 4°C with a primary antibody that was diluted with the blocking buffer (5% skim milk, and 0.2% Triton X-100 in PBS). After washing with PBS, the plates were treated with 0.3% H₂O₂ in PBS to inhibit the endogenous peroxidase activity. The cells were then stained by the avidin-biotin-peroxidase complex (ABC) method using the Histofine SAB-PO kit (Nichirei Co. Ltd., Tokyo, Japan) according to the manufacture’s instructions. Finally, the plates were incubated with 0.5 mM 3,3’ -diaminobenzidine tetrahydrochloride and 0.005% H₂O₂ in PBS. Immunoreactive sites were visualized as brown benzidine precipitates. The counterstaining for nuclei was performed with methyl green.

**Measurement of TRAP intensity**- Following TRAP staining, the plates were scanned by a transparent light scanner, and the red color image was extracted from the scanned image using the
Photoshop (Adobe Systems Inc., San Jose, CA) computer program. The intensity of the red color image was measured using NIH image (National Institutes of Health), and was represented as TRAP intensity in this paper.

Cell proliferation assays- Cell proliferation was measured using Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Similar to the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide] assay, this kit measures intracellular mitochondrial dehydrogenase activity in living but not in dead cells by forming water-soluble formazan dye with the tetrazolium compound, WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Immunoblotting- Cells were seeded at 5 X 10⁶ cells/well on a 24-well plate and kept in a 5% CO₂ in air atmosphere humidified incubator at 37°C for 4 h. The cells were stimulated with RANKL or/and other chemical reagents and incubated for the indicated time. After the incubation time, the cells in the 24-well plate were rinsed twice with ice-cold PBS containing 1 mM sodium orthovanadate, followed by addition of 50 µl of sodium dodecyl sulfate (SDS)-sample buffer [1% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, 0.0025% bromophenol blue, 62.5 mM Tris/Cl pH6.8] containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml chymostatin, and 1 µg/ml leupeptin. The whole cell lysate was then treated in ice-cold water by sonication (15 s pulse length X 2 with 60 s interval) (Bioruptor UDC-200T, Cosmo Bio, Tokyo, Japan). After boiling for 5 min, 4-7 µl of the lysate (20 µg of protein) were applied to SDS-12.5% polyacrylamide gel electrophoresis (PAGE), and the proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membrane. The
membrane was incubated in 5% skim milk, 25 mM Tris/HCl pH 7.6, 150 mM NaCl, and 0.1% Tween-20 (TBST) at room temperature for 1 h, and washed twice with TBST for 5 min, then incubated with an antibody at 2000-4000 X dilution in TBST at 4°C for 16 h. The membrane was washed 3 times with TBST for 10 min, incubated with a horse radish peroxidase (HRP)-conjugated second antibody at 4000-8000 X dilution in TBST at room temperature for 1 h, washed vigorously 5 times for 10 min, and subjected to chemiluminescence (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ) to visualize HRP. In some experiments for reprobing, the membrane was treated with 1% SDS and 200 mM mercaptoethanol in 50 mM Tris/HCl (pH 6.7) at 55°C for 30 min, followed by washing 3 times with TBST.

RESULTS

In an in vitro culture system using osteoclast precursor cells purified from various tissues such as bone marrow, spleen, and liver, it is difficult to avoid contamination with other cell lineages such as T-cells and mesenchymal stromal cells. In this study, we took advantage of RAW264.7, a murine macrophage cell line that can differentiate into osteoclast-like cells in the presence of RANKL (6). We first examined the osteoclastogenic ability of the cells. As described previously, RAW264.7 cells could differentiate into multinuclear TRAP-positive osteoclast-like cells in the presence of RANKL (Fig. 1). When cultured in a dish coated with calcium phosphate, the cells formed resorption pits. The numbers of multinuclear TRAP-positive cells and resorption pits were increased by treatment with dexamethasone (Fig. 1) (21). Cathepsin K (22, 23) and c-Src (24, 25), which are known markers of osteoclasts, were also induced in the multinuclear cells.
These results showed that the multinuclear TRAP-positive cells from RAW264.7 cells possessed major characteristics of osteoclasts. [Fig. 1 should be here]

**Cell density and growth are important for the differentiation into osteoclasts**- Since the initial plating cell density is important for differentiation into chondrocytes (26), we tested whether the cell density of RAW264.7 cells at plating affects the differentiation into osteoclast-like cells. The cells were plated in wells at various cell densities and cultured in the presence of RANKL for five days. The TRAP intensity of the wells varied according to cell densities (Fig. 2A and B). The intensity peaked at 1000-2000 cells/well in α-MEM Gln (+). Next, we examined whether cell growth could affect the differentiation into osteoclast-like cells. We used α-MEM Gln (-) to down-regulate growth speed. Since Gln concentration in serum is 0.5 mM (27), the final concentration of Gln in α-MEM Gln (-) containing 10% FCS could be estimated at 0.05 mM. As shown in Fig. 2C, the cell doubling time was 16 h in α-MEM Gln (+), and 24 h in α-MEM Gln (-) during the day 2-3, resulting in a three fold difference in cell number between Gln (+) and Gln (-) cultures after five days. When cells were plated in wells at various cell densities and cultured in α-MEM Gln (-) supplemented with RANKL, the TRAP intensity of the wells peaked at 8000 cells/well. These results indicated that differentiation into osteoclast-like cells might be affected by initial cell density and growth speed. [Fig. 2 should be here]

**Effects of MAPKs inhibitors on osteoclastogenesis**- MAPK signaling pathways have been suggested to be critical for osteoclast differentiation. In order to determine the involvement of the pathways in osteoclastogenesis in this culture system, the cells were treated with specific inhibitors of p38 MAPK, PD169316 and SB203580, and specific inhibitors of MEK, U0126 and
PD98059. These inhibitors suppressed the formation of TRAP-positive cells in a dose-dependent fashion, which was consistent with several previous studies (Fig. 3A) (15, 16, 19). Interestingly, the inhibitory effects of these two types of inhibitors on RAW264.7 cells were different. Ten µM PD169316 and SB203580 completely inhibited formation of TRAP-positive cells. On the other hand, a high dose of U0126 and PD98059 failed to completely inhibit formation of TRAP-positive cells, where upon most of the remaining cells were TRAP-positive and multinucleated although their size was much smaller than the TRAP-positive multinuclear cells cultured in the absence of inhibitors. Furthermore, cell proliferation in the culture containing RANKL was inhibited with U0126 and PD98059 in a dose-dependent manner, whereas such inhibition of cell growth was not seen in the culture with SB203580 and PD169316 (Fig. 3B). Similar effects of these inhibitors on cell proliferation were observed in the absence of RANKL (data not shown). [Fig. 3 should be here] From these findings, we hypothesized that U0126 and PD98059 suppress cell growth, resulting in an apparent decrease of TRAP-positive cells. To test this hypothesis, cells were seeded at various densities and treated with U0126 and PD98059 in the presence of RANKL. Without inhibitors, TRAP-positive mononuclear cells appeared when seeded at an initial cell density of 8000 cells/well, and no multinuclear cells were observed (Fig. 4A and B). Surprisingly, with inhibitors, a marked increase of TRAP-positive cells were observed at cell densities of more than 8000 cells/well, compared to those without inhibitors. In several wells, gigantic TRAP-positive multinuclear cells with numerous numbers of nuclei, occupying the entire well bottom, were observed (Fig. 4C; panels PD60/32000, PD20/32000, and U3/128000). The increase of the number of TRAP-positive cells by the MEK inhibitors took place at 6.7-60
µM for PD98059 and 0.3-3 µM for U0126. The difference in effective concentration among these inhibitors might be explained by the difference in the affinity of the inhibitors to the substrate: U0126 was reported to have approximately 100-fold higher affinity for MEK than does PD98059 (28). [Fig. 4 should be here] The cells were proliferated and fused to form multinuclear osteoclast-like cells during 3 days of this experiment. Measuring by the Cell Counting Kit, cell density for the most effective multinuclear osteoclastogenesis after 3 days culture was equivalent to 128000-256000 cells/well (96-well) of freshly seeded mononuclear cells that completely covers the well bottom (data not shown).

**MEK inhibitors enhance and accelerate the differentiation into TRAP-positive cells**- Since the differentiation into osteoclasts includes multi-step phases including cell adhesion, proliferation, fusion, and differentiation, the culture period needed for differentiation is relatively long. It takes at least 3 days and sometimes more than 10 days to differentiate into TRAP-positive multinuclear cells. We investigated whether the MEK inhibitors could accelerate differentiation into TRAP-positive multinuclear cells. To address this issue, cells were cultured with or without the MEK inhibitors in the presence of RANKL for 1-5 days (Fig. 5). Without the MEK inhibitors, a very small number of TRAP-positive mononuclear cells appeared on day 2, and multinuclear cells were observed from day 4. When the MEK inhibitor U0126 was added, a large number of TRAP-positive multinuclear cells with higher TRAP intensity than the cells without the inhibitor appeared on day 2. The TRAP intensity of RAW264.7 cells treated with U0126 was higher than that of the cells without U0126 all through the incubation period; however, the TRAP intensity of the cells without U0126 finally reached almost the same level as that of the cells with U0126.
These results indicated that the MEK inhibitors accelerated and enhanced differentiation into TRAP-positive multinuclear cells in RAW264.7 cells. [Fig. 5 should be here]

**Phosphorylation of ERK and p38 in the presence of MAPK inhibitors**- RANKL belongs to the TNF superfamily, and is known to activate MAPKs, which is believed to be one of pivotal pathways for osteoclastogenesis. In order to determine the effects of the MAPK inhibitors on signaling pathways, we performed immunoblot analysis with antibodies binding to phosphorylated MAPK molecules. The phosphorylation of ERK and p38 was increased within 10 min after RANKL stimulation, reached maximum after 30-60 min, and thereafter gradually decreased (Fig. 6, upper panel). The phosphorylation of ERK and p38 depended on the concentration of RANKL (Fig. 6, lower panel). [Fig. 6 should be here] The phosphorylation of ERK stimulated with RANKL was completely inhibited by U0126 (Fig. 7, upper panel, comparing lanes 2 to 4), and that of p38 was inhibited with SB203580 (Fig. 7, lower panel, comparing lanes 2 to 6). Interestingly, phosphorylation of ERK by treatment with RANKL was up-regulated in the presence of SB203580 (Fig. 7, upper panel, comparing lanes 2 to 6), whereas SB203580 treatment had no effect on ERK phosphorylation in the absence of RANKL (Fig. 7, upper panel, comparing lanes 1 to 5), suggesting that inhibition of p38 might induce phosphorylation of ERK. [Fig. 7 should be here]

**Crosstalk between ERK and p38**- New et al. (29) has recently reported a similar result, in which they observed the enhancement of ERK phosphorylation by treatment with SB203580 in PC12 cells in the presence of epidermal growth factor (EGF). Their finding prompted us to investigate the possibility of crosstalk between ERK and p38. RAW264.7 cells were incubated
with combinations of various concentrations of U0126 and SB203580 in the presence of RANKL, and subjected to immunoblot analyses with anti-phospho-ERK and anti-phospho-p38. Phosphorylation of ERK was markedly decreased with U0126, and was clearly increased in the presence of SB203580 (Fig. 8A). On the other hand, phosphorylation of p38 was increased with U0126 in a dose-dependent manner in the presence of SB203580, whereas the increase by treatment with U0126 did not occur in the absence of SB203580 (Fig. 8B). Taken together, the p38 inhibitor increased the phosphorylation of ERK, and the MEK inhibitor did that of p38. Phosphorylation of ERK peaked after 30 min in the absence of SB203580, and the presence of SB203580 delayed the peak of ERK phosphorylation in a dose-dependent manner (Fig. 8A). Such a delay of phosphorylation was not obvious in p38 (Fig. 8B). [Fig. 8 should be here]

The inhibitory effect of p38 inhibitor on ERK-mediated osteoclastogenesis- Since the above results suggested that a crosstalk might take place between MEK- and ERK-mediated pathways, we investigated the combined effect of MEK and p38 inhibitors on osteoclastogenesis of RAW264.7 cells. As described before (Figs. 3 and 4), RANKL-induced formation of TRAP-positive cells was enhanced by addition of U0126. The enhanced formation of TRAP-positive cells was suppressed by addition of SB203580 (Fig. 9). [Fig. 9 should be here]

DISCUSSION

In this paper, the effects of MEK and p38 inhibitors on differentiation of RAW264.7 into osteoclast-like cells were investigated. MEK inhibitors markedly enhanced osteoclastogenesis in the presence of RANKL, while p38 inhibitors suppressed it. Since chemical inhibitors against
signal transduction are powerful tools to explore signaling pathways, use of chemical inhibitors has become more widespread to investigate what pathways are involved in cell responses such as growth, differentiation, and apoptosis. Osteoclasts are differentiated from hematopoietic precursor cells, and the signal transduction pathways for osteoclast differentiation have been studied. Several research groups have investigated the involvement of MAPKs in this signaling pathway (15, 16, 19). They concluded that MEK inhibitors suppress or do not affect differentiation into osteoclasts from bone marrow cells. Although the cells used here are different from the cells they used, we obtained similar results when RAW264.7 cells were cultured at a particular cell density for a defined period. For example, osteoclastogenesis was inhibited at the initial cell density of 2000 cells/well in the presence of MEK inhibitor (Figs. 3 and 5), while such inhibition was not observed at 4000 cells/well (Fig. 5). In this study, we found that the MEK inhibitors did enhance differentiation into osteoclast-like cells at initial cell densities of more than 8000 cells/well. Differentiation into osteoclasts requires multiple steps; cell attachment to the plate as a scaffold, cell proliferation, differentiation to mononuclear TRAP-positive cells, and fusion of the TRAP-positive mononuclear cells to yield multinuclear TRAP-positive cells. Since each step is indispensable for the differentiation, impairment of each step could result in failure of osteoclast differentiation. MEK inhibitors clearly inhibited growth in RAW264.7 cells, which may explain the apparent inhibition of osteoclastogenesis with MEK inhibitors at the cell density commonly used because we found enhancement and acceleration of osteoclastogenesis by treatment with MEK inhibitors at higher cell densities (Fig. 4 and 5) that could allow the cells to skip the cell proliferation step and immediately differentiate into osteoclasts.
The duration of ERK activation has been suggested to be critical for the fate of signals (29-41). For example, EGF transiently stimulates the ERK pathway in PC12 cells leading to proliferation, whereas nerve growth factor causes a sustained response that evokes neuronal differentiation (29, 34). In this study, the greater the amount of SB203580 added to the culture system, the larger the delay of ERK phosphorylation observed (Fig. 8, upper panel), with the result that SB203580 inhibited osteoclastogenesis (Fig. 9). Not only the inhibition of p38 phosphorylation with SB203580, but also the delayed up-regulation and duration of ERK phosphorylation with the same inhibitor may be involved in the inhibition of differentiation of RAW264.7 cells into osteoclast-like cells.

Immunoblot analyses using antibodies against phosphorylated MAPKs revealed a “seesaw crosstalk” between ERK and p38: p38 inhibitors caused decrease of phosphorylated p38 with increase of phosphorylated ERK, while MEK inhibitors caused decrease of phosphorylated ERK with increase of phosphorylated p38. A similar “seesaw crosstalk” was previously reported, in which SB203580 by itself did not affect the activity of ERK1/2 but significantly extended EGF-induced ERK activation in PC12 cells (42). How can the “seesaw crosstalk” occur? It is unlikely that the secondary products derived from transcripts by transcription factors downstream of a MAPK pathway affected the other MAPK pathway because the enhancement of phosphorylation by MAPK inhibitors took place after only 30 min. Does the crosstalk take place upstream of MAPKs? Although MEK has been suggested to be a substrate of ERK for feedback signaling (43), we did not detect any obvious increase or decrease of phosphorylated MEK by treatment with p38 inhibitors (data not shown), suggesting that the increment of phosphorylation of ERK
by the p38 inhibitor may have occurred downstream from MEK. However, MEKK1, an upstream mediator of the MEK pathway, has been reported to act as an E3 ligase and mediate ubiquitination and degradation of ERK1/2 (44), which suggested that a signal transduction molecule can directly suppress other signaling molecules, and implies that the “seesaw crosstalk” may occur upstream of MEK. Phosphatases or scaffold proteins for MAPKs pathways can also be the candidates involved in the above events. Alternatively, the chemical inhibitors of MAPKs may affect other unknown molecules. Recently, SB203580 has been reported to inhibit a nucleoside transporter, which leads to the inhibition of uridine-uptake followed by the inhibition of differentiation of K562 erythroleukemia cells (45). Their finding may not be directly linked to the results in this study, but suggests the possibility that the chemical inhibitors may affect more than one molecule. The stream of signal transduction may be comparable to that of a river with downstream branches in the natural scenery: when a branch point of signaling pathways is activated, the signal should flow to both downstream pathways. If one downstream pathway is blocked by treatment with inhibitors, the signal may flow more along the other pathway. In order to resolve the complexity of signal transduction, mathematical models have been studied (39, 46), in which the feedback mechanisms and signal propagation have been analysed. Further studies are needed to clarify the reciprocal effects of MAPK inhibitors on osteoclastogenesis and to elucidate the mechanism by which the inhibition of ERK phosphorylation by MEK inhibitors results in p38 phosphorylation, and conversely does the inhibition of p38 phosphorylation in ERK phosphorylation. [Fig. 10 should be here]
ACKNOWLEDGEMENT

We thank Dr. Yukio Okada, Dr. Shigemasa Hanazawa, and members of Division of Microbiology and Oral Infection for helpful comments and discussions, Dr. Takayuki Nemoto, Dr. Kiyotaka Shiba, Dr. Hidenori Ichijo, Dr. Dennis J. Templeton, Dr. Shigeru Amano, Dr. Mitsue Shibata, and Dr. Akira Kitamura for material supports, and Dr. Yuka Hotokezaka for reviewing the manuscript. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J. and Suda, T. (1990) Proc. Natl. Acad. Sci. USA 87, 7260-7264.

2. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D. and Galibert, L. (1997) Nature (Lond) 390, 175-195.

3. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochi-zuki, S. I., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N. and Suda, T. (1998) Proc. Natl. Acad. Sci. USA 95, 3579-3602.

4. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S., Frankel, W. N., Lee, S. Y. and Choi, Y. (1997) J. Biol. Chem. 272, 25190-25194.
5. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R.,
Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E.,
Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo,
J., Delaney, J. and Boyle, W. J. (1998) Cell 93, 165-176.

6. Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Tan, H. L.,
Elliott, G., Kelley, M. J., Sarosi, I., Wang, L., Xia, X. Z., Elliott, R., Chiu, L., Black, T.,
Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M. B. and Boyle, W. J.
(1999) Proc. Natl. Acad. Sci. USA 96, 3540-3545.

7. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S.,
Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R.,
Lacey, D. L., Mak, T. W., Boyle, W. J. and Penninger, J. M. (1999) Nature (Lond) 397,
315-323.

8. Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Yano, K., Morinaga,
T. and Higashio, K. (1998) Biochem. Biophys. Res. Commun. 253, 395-400.

9. Li, J., Sarosi, I., Yan, X. Q., Morony, S., Capparelli, C., Tan, H. L., McCabe, S., Juan, S.
C., Sun, Y., Tarpley, J., Martin, L., Christensen, K., McCabe, J., Kostenuik, P., Hsu, H.,
Fletcher, F., Dunstan, C. R., Lacey, D. L. and Boyle, W. J. (2000) Proc. Natl. Acad. Sci.
USA 97, 1566-1571.

10. Wong, B., Besser, D., Kim, N., Arron, J., Vologodskaya, M., Hanafusa, H. and Choi, Y.
(1999) Mol. Cell 4, 1041-1049.

11. Kim, H., Lee, D., Shin, J., Lee, Y., Jeon, Y., Chung, C., Ni, J., Kwon, B. and Lee, Z.
(1999) FEBS Lett. 443, 297-302.

12. Galibert, L., Tometsko, M., Anderson, D., Cosman, D. and Dougall, W. (1998) J. Biol. Chem. 273, 34120-7.

13. Wong, B., Josien, R., Lee, S., Vologodsgkaia, M., RM, S. and Choi, Y. (1998) J. Biol. Chem. 273, 28355-28359.

14. Darnay, B. G., Ni, J., Moore, P. A. and Aggarwal, B. B. (1999) J. Biol. Chem. 274, 7724-7731.

15. Lee, S. E., Woo, K. M., Kim, S. Y., Kim, H.-M., Wack, K. K., Lee, Z. H. and Kim, H.-H. (2002) Bone 30, 71-77.

16. Wei, S., Wang, M., Teitelbaum, S. and Ross, F. (2002) J. Biol. Chem. 277, 6622-30.

17. Chang, L. and Karin, M. (2001) Nature 410, 37-40.

18. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Science 270, 1326-1331.

19. Matsumoto, M., Sudo, T., Saito, T., Osada, H. and Tsujimoto, M. (2000) J. Biol. Chem. 275, 31155-31161.

20. Kamiya, T., Kobayashi, Y., Kanaoka, K., Nakashima, T., Kato, Y., Mizuno, A. and Sakai, H. (1998) J. Biochem. 123, 752-9.

21. Kaji, H., Sugimoto, T., Kanatani, M., Nishiyama, K. and Chihara, K. (1997) J. Bone Miner. Res. 12, 734-741.

22. Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M. and Kokubo, T. (1995) Biochem. Biophys. Res. Commun. 206, 89-96.
23. Drake, F., Dodds, R., James, I., Connor, J., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G. and Gowen, M. (1996) J. Biol. Chem. 271, 12511-12516.

24. Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Cell 64, 693-702.

25. Horne, W., Neff, L., Chatterjee, D., Lomri, A., Levy, J. and Baron, R. (1992) J. Cell Biol. 119, 1003-10013.

26. Seghatoleslami, M. and Tuan, R. (2002) J. Cell. Biochem. 84, 237-48.

27. Rohde, T., MacLean, D. A., Hartkopp, A. and Pedersen, B. K. (1996) Eur. J. Appl. Physiol. 74, 428-434.

28. Favata, M., Horiuchi, K., Manos, E., Daulerio, A., Stradley, D., Feeser, W., Van Dyk, D., Pitts, W., Earl, R., Hobbs, F., Copeland, R., Magolda, R., Scherle, P. and Trzaskos, J. (1998) J. Biol. Chem. 273, 18623-18632.

29. Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Biochem. J. 288, 351-355.

30. Heasley, L. E. and Johnson, G. L. (1992) Mol. Biol. Cell 3, 545-553.

31. Vouret-Craviari, V., Van Obberghen-Schilling, E., Scimeca, J. C., Van Obberghen, E., and Pouyssegur, J. (1993) Biochem. J. 289, 209-214.

32. Nguyen, T. T., Scimeca, J. C., Filloux, C., Peraldi, P., Carpentier, J. L., Van Obberghen, E., (1993) J. Biol. Chem. 268, 9803-9810.

33. Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P., and Ullrich, A. (1994) Curr. Biol. 4, 694-701.
34. Marshall, C. (1995) Cell 80, 179-185.
35. Cook, S. J. and McCormick, F. (1996) Biochem. J. 320, 237-245.
36. Weber, J. D., Raben, D. M., Phillips, P. J., and Baldassare, J. J. (1997) Biochem J 326, 61-68.
37. Balmanno, K. and Cook, S. J. (1999) Oncogene 18, 3085-3097.
38. Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E., and Assoian, R. K. (1999) Mol. Biol. Cell 10, 3197-3204.
39. Heinrich, R., Neel, B. and Rapoport, T. (2002) Mol. Cell 9, 957-970.
40. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C., and Blenis, J. (2002) Nat. Cell. Biol. 4, 556-564.
41. Assoian, R. K. (2002) Nat. Cell Biol. 4, E187-188.
42. New, L., Li, Y., Ge, B., Zhong, H., Mansbridge, J., Liu, K. and Han, J. (2001) J. Cell. Biochem. 83, 585-596.
43. Brunet, A., Pages, G. and Poussegur, J. (1994) FEBS Lett. 346, 299-303.
44. Lu, Z., Xu, S., Joazeiro, C., and Cobb, M.H., Hunter, T. (2002) Mol. Cell 9, 945-956.
45. Huang, M., Wang, Y., Collins, M., Gu, J., Mitchell, B. and Graves, L. (2002) J. Biol. Chem. 277, 28364-28367.
46. Asthagiri, A. and Lauffenburger, D. (2001) Biotechnol. Prog. 17, 227-239.
FOOTNOTES

The abbreviations used are: MAPK, mitogen-activated protein kinase; RANKL, receptor activator of nuclear factor kappa B ligand; MEK, MAPK-ERK kinase; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; NF-κB, nuclear factor kappa B; Gln, glutamine; FCS, fetal calf serum; TRAP, tartrate-resistant acid phosphatase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, 150 mM NaCl and 0.1% Tween-20 in 25 mM Tris/HCl pH7.6; HRP, horse radish peroxidase.
FIGURE LEGENDS

Fig. 1. Differentiation of RAW264.7 cells into osteoclast-like cells.
2 X 10³ RAW264.7 cells in 250 µl of α-MEM Gln (+) were cultured in either a 96-well tissue culture plate or a plate coated with calcium phosphate (BD BioCoat™Osteologic™Bone Cell Culture System, Nippon BD, Tokyo, Japan) in the presence of 50 ng/ml RANKL and 10 nM dexamethasone (DX) for 5 days. TRAP activity was visualized by TRAP staining. For observation of resorption pits, cells were removed from wells. Immunostaining with anti-cathepsin K and anti-c-Src was shown in the lower panels. TRAP+MG indicates double staining for TRAP and nuclei.

Fig. 2. Effect of cell density and growth speed on osteoclastogenesis.
The indicated number of RAW264.7 cells was cultured for 5 days in 250 µl of α-MEM containing 50 ng/ml RANKL with or without 2 mM Gln in a 96-well tissue culture plate. The cells were stained for TRAP (A), and the TRAP color intensity was determined as described in EXPERIMENTAL PROCEDURES (B). RAW264.7 cells (1.2 X 10⁵) were seeded in α-MEM Gln (+) or α-MEM Gln (-) and cultured for 1-5 days. The number of cells collected from the plate was counted by microscopy, and the mean of three different determinations was plotted (C).

Fig. 3. Effects of MAP Kinase inhibitors on osteoclastogenesis and cell proliferation in standard culture conditions. (A) 2 X 10³ RAW264.7 cells were seeded and cultured in 250 µl of α-MEM Gln (+) for five days in the presence of 50 ng/ml RANKL and the indicated concentration of MAPK inhibitors in a 96-well tissue culture plate. The cells were fixed and stained for TRAP. (B) 2 X 10³ RAW264.7 cells were seeded in 250 µl of α-MEM Gln (+) in the
presence of 50 ng/ml RANKL and various concentrations of MAPK inhibitors in a 96-well tissue culture plate. After 3 days incubation, the cell number was measured using the Cell Counting Kit. The optical density value was converted to cell number by a calibration curve that was obtained from freshly seeded cells. The mean of three different determinations was plotted. The inhibitor concentrations were 0, 0.1, 0.3, 1, 3, and 10 μM for SB203580, PD169316, and U0126; and 0, 0.7, 2, 6, 20, and 60 μM for PD98059, respectively. Value of cell numbers in the graph reflects the intracellular mitochondrial dehydrogenase activity, and does not always reflect the exact cell number, because subpopulation of cells fused to form multinuclear cells during the culture period.

**Fig. 4. Enhancement of osteoclastogenesis of RAW264.7 cells by treatment with the specific inhibitors of MEK, U0126 and PD98059.**

The indicated number of cells was seeded and cultured for three days in 250 μl of α-MEM Gln (+) in the presence of 10 ng/ml RANKL and the indicated concentration of MEK inhibitors using 96-well tissue culture plates. The cells were fixed and stained for TRAP (A). The plates were directly scanned and the TRAP intensity of each well was determined (B). Cell shapes in the cultures with various concentrations (μM) of U0126 (U) and PD98059 (PD)/various initial cell numbers per well indicated were shown (C).

**Fig. 5. Acceleration of osteoclastogenesis of RAW264.7 cells by the MEK inhibitor U0126.**

The indicated number of cells was seeded and cultured for the indicated days in 250 μl of α-MEM Gln (+) in the presence of 10 ng/ml RANKL using 96-well tissue culture plates. The
cells were fixed and stained for TRAP. The red color image was extracted from the scanned plate (A) and the TRAP intensity of each well was determined (B).

**Fig. 6. Phosphorylation of ERK and p38 in RAW264.7 cells treated with RANKL.**

RAW264.7 cells were treated with 50 ng/ml RANKL and collected after the indicated time, followed by immunoblotting using anti-phospho-ERK or –p38 (A). Cells were treated with the various concentrations of RANKL for 30 min (B).

**Fig. 7. Phosphorylation of ERK and p38 in RAW264.7 cells treated with RANKL and MAPK inhibitors.**

Cells were treated with 1.25 µM U0126 or SB203580 in the presence of 10 ng/ml RANKL and collected after 30 min, followed by immunoblotting using anti-phospho-ERK or –p38.

**Fig. 8. A possible crosstalk between ERK and p38.**

RAW264.7 cells were treated with combinations of various concentrations of U0126 (0.4, 1.2, and 3.6 µM) or SB203580 (1, 3, 9, and 27 µM) in the presence of 10 ng/ml RANKL, and collected after the indicated time, followed by immunoblotting using anti-phospho-ERK (A) and –p38 (B).

**Fig. 9. Inhibition of U0126-induced osteoclastogenesis by SB203580.**

The indicated number of cells were seeded and cultured for three days in 250 µl α-MEM Gln (+) in the presence of 10 ng/ml RANKL and the indicated concentrations of SB203580 and U0126 in a 96-well tissue culture plate. The cells were fixed and stained for TRAP. The red color image was extracted from the scanned plate (A) and the TRAP intensity of each well was determined (B).
Fig. 10. A hypothetical model, “seesaw crosstalk” between MEK/ERK and p38 pathways in osteoclastogenesis of RAW264.7 cells.

RANKL activates both MEK/ERK and p38 pathways. The MEK/ERK pathway suppresses osteoclastogenesis, which is inhibited by U0126 or PD98059. In contrast, the p38 pathway enhances osteoclastogenesis, which is inhibited by SB203580 or PD169316. U0126 and PD98059 inhibit ERK and activate p38, whereas SB203580 and PD169316 inhibit p38 and activate ERK.
Fig. 1
Fig. 2
Fig. 3

Cell number/well

RANKL (-)

Inhibitor concentration

SB203580

PD169316

PD98059

U0126
Fig. 4
### Fig. 5

| Day   | Cell number / well | TRAP intensity | Cell number / well | U0126 (µM) |
|-------|--------------------|----------------|--------------------|------------|
| Day1  | 2000               | 1              | 2000               | 0          |
|       | 4000               | 1              | 4000               | 0          |
|       | 8000               | 1              | 8000               | 0          |
|       | 16000              | 1              | 16000              | 0          |
|       | 32000              | 1              | 32000              | 0          |
|       | 64000              | 1              | 64000              | 0          |
|       | 128000             | 1              | 128000             | 0          |
|       | 256000             | 1              | 256000             | 0          |

**Cell number / well**

- Day 1: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 2: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 3: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 4: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 5: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000

**TRAP intensity**

- U0126 (µM): 0, 1

**Cell number / well**

- 2000 (RANKL-): 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000

**U0126 (µM)**

- 1: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000

**Cell number / well**

- Day 1: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 2: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 3: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 4: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
- Day 5: 2000, 4000, 8000, 16000, 32000, 64000, 128000, 256000
Fig. 6
|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|----------|---|---|---|---|---|---|---|
| RANKL    |  |  | - | + | - | + | + |
| U0126    |  |  |  | - | + | - |  |
| SB203580 |  |  |  |  | - | + | + |

**Fig. 7**
Fig. 8
Fig. 10
U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells
Hitoshi Hotokezaka, Eiko Sakai, Kazuhiro Kanaoka, Kan Saito, Ken-ichiro Matsuo, Hideki Kitaura, Noriaki Yoshida and Koji Nakayama

J. Biol. Chem. published online September 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208284200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts