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INVOLVEMENT OF DUAL SIGNAL TRANSDUCTION SYSTEMS IN THE STIMULATION OF OSTEOCLAST-LIKE CELL FORMATION BY PARATHYROID HORMONE AND PARATHYROID HORMONE-RELATED PEPTIDE

Hiroshi Kaji*, Toshitsugu Sugimoto, Masanori Kanatani, Masaaki Fukase, and Kazuo Chihara

Third Division, Department of Medicine
Kobe University School of Medicine, Kobe 650, Japan

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The present study was performed to examine whether parathyroid hormone(PTH) and parathyroid hormone-related peptide(PTHrP) would stimulate osteoclast-like cell formation via soluble factor(s) released from osteoblasts and, if so, to characterize the involvement of PTH/PTHrP-responsive dual signal transduction systems [cAMP-dependent protein kinase(PKA) and calcium/protein kinase C(PKC)]. Osteoblasts-conditioned medium(CM) was obtained from rat osteoblastic osteosarcoma cells (UMR-106 cells), which had been cultured in serum free medium for 24 hrs after treatment with various kinds of reagents. The CM of osteoblasts treated with either 10^{-7}M human(h)PTH-(1-34) or 10^{-7}M hPTHrP-(1-34) equally stimulated osteoclast-like cell formation from hemopoietic blast cells derived from mouse spleen cells, although the CM treated with 10^{-8}M 1,25dihydroxyvitaminD_3 failed to affect it. The CM treated with both 10^{-4}M dibutyryl-cAMP and a direct PKA activator, 10^{-4}M Sp-cAMPS significantly increased osteoclast-like cell formation. The CM treated with a PKC activator, 10^{-7}M phorbol 12-myristate 13-acetate(PMA) and calcium ionophores(10^{-7}M A23187 and 10^{-7}M ionomycin) also significantly enhanced osteoclast-like cell formation. The present study first indicated the osteoblast-mediated stimulation of osteoclast-like cell formation by PTH and PTHrP, and the participation of PTH/PTHrP-responsive dual signal transduction systems of osteoblasts in the stimulation of osteoclast-like cell formation by PTH and PTHrP. © 1993 Academic Press, Inc.

There have been several lines of evidence that parathyroid hormone(PTH) receptor is present in osteoblasts but not in osteoclasts and that osteoblastic cells mediate the stimulation of osteoclastic bone resorption by PTH(1,2). Previous reports revealed that PTH stimulated bone resorption through soluble factor(s) released from osteoblasts(3,4). It is, however, unclear

*To whom correspondence and reprint requests should be addressed.
Abbreviation: cAMPS, adenosine cyclic 3',5'-phosphorothioate.

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whether PTH can stimulate osteoclast formation via soluble factor(s) released from osteoblasts, although osteoclastic bone resorption is considered to be promoted by development of new osteoclasts as well as activation of quiescent osteoclasts. On the other hand, parathyroid hormone-related peptide(PTHrP), a causative peptide associated with humoral hypercalcemia of malignancy, has been purified and its amino acid sequence analysis revealed high similarity with that of PTH at the amino terminal portion(5,6). Although it has been reported that PTHrP stimulated bone resorption through the release of a 9 k dalton bone-resorbing protein from osteoblasts(7), it is unknown whether PTHrP can stimulate osteoclast formation through soluble factor(s) released from osteoblasts. Successful cloning of complementary DNA(cDNA) of the PTH/PTHrP receptor demonstrated that a single cDNA clone, expressing rat bone PTH/PTHrP receptor, mediates the stimulation by PTH and PTHrP of both adenylate cyclase and phospholipase C, when expressed in COS cells(8). Our recent study also revealed that PTHrP as well as PTH possessed dual signal transduction systems[cAMP-dependent protein kinase(PKA) and calcium/protein kinase C(PKC)] in osteoblastic UMR-106 cells(9). Moreover, we have also demonstrated the participation of dual signal transduction systems in the regulation of osteoblast proliferation and collagen synthesis by PTH and PTHrP(10,11). The present study first indicated that PTHrP as well as PTH stimulated osteoclast-like cell formation through some soluble factor(s) released from osteoblasts, by the mechanisms in which dual signal transduction systems in osteoblasts are involved.

Materials and Methods

Materials

BDF_1 mice were purchased from Shizuoka Experimental Animal Center(Shizuoka, Japan). 5-fluorourasil(5-FU) was generously provided by Kyowa Hakko Co.(Tokyo, Japan). 1,25dihydroxyvitaminD_3[1,25$(OH)_2D_3$] and human recombinant interleukin-6(IL-6) were kind gifts from Chugai Pharma. Co. Ltd.(Shizuoka, Japan). Murine recombinant interleukin-3(IL-3) and human recombinant granulocyte-macrophage colony stimulating factor(GM-CSF) were purchased from Genzyme Co.(Boston, MA), human(h)PTH-(1-34) and hPTHrP-(1-34) from Peptide Institute Inc.(Osaka, Japan), Sp-cAMPS from Biolog Life Science Institute(Bremen, Germany), N_6,O_2'-dibutyryl adenosine 3'5'cyclic monophosphate(dbcAMP), phorbol 12-myristate 13-acetate(PMA), 4α–phorbol-12,13-didecanoate(4α–PDD) and ionomycin from Sigma Chemical. Co.(St.Louis, MO), and A23187 from Hoechst Japan Co.(Tokyo, Japan). Other materials used were commercial products of the highest grade available.

Preparation of osteoblasts-CM

Osteoblastic osteosarcoma cells(UMR-106) were maintained in Dulbecco's Modified Eagle Medium(DMEM) containing 10% fetal calf serum(FCS), as previously described in detail(12). For the preparation of osteoblasts-CM, cells were cultured in 24-well plates. After treatment of UMR-106 cells with various

chemicals for 24 hr, cells were washed several times with serum-free DMEM. Then, 300 μl serum free DMEM was added to each well. Another 24 hr later, CM was collected.

Formation of osteoclast-like cells

Osteoclast-like cell formation was measured according to the method from Kumegawa et al.(13). Briefly, 5-FU was administered to six-week-old female BDF_1 mice at a dosage of 150 mg/kg of body weight through a tail vein. Four days after injection, spleen cells were harvested and cell suspensions were prepared. Aliquots of $2.6x10^6$/ml spleen cells were plated into 35mm culture dishes(Falcon, Oxnard, CA) in 1 ml of α–minimum essential medium(αMEM) containing 1.2% methylcellulose, 50U/ml IL-3 , 10^{-8}M IL-6, 10mg/ml bovine serum albumin(BSA)(Sigma Chemical Co., St. Louis, MO) and 30% FCS. The colonies of hemopoietic blast cells appeared after approximately 7 days and were lifted from the dish with a 3 μl Eppendorf micropipette. For preparation of osteoclast precursors, 10^4/ml hemopoietic blast cells were cultured in 96-well microplates containing 100μl of αMEM supplemented with 5% FCS and 200 U/ml GM-CSF for 7 days. Then, 30% osteoblasts-CM was added to this medium, followed by 4 more days culture. Then, cells adherent to the plates were washed with phosphate-buffered saline, dried, and promptly stained for tartrate-resistant acid phosphatase(TRAP), a marker enzyme of osteoclasts. Cells were viewed under an inverted phase-contrast microscope and TRAP-positive cells containing three or more nuclei were counted as TRAP-positive MNC. These MNC had various characteristics of osteoclasts, including responsiveness to calcitonin and osteoclastic bone resorption as evidenced by co-cultureing with bone rudiments(13). Moreover, these TRAP-positive MNC were observed in each colony derived from each replated blast cell as well, indicating that these hemopoietic blast cells would be precursors for osteoclasts.

Significance of differences between comparable groups was determined by Student's t test or Duncan's multiple range test.

Results and Discussion

We examined the effect of the CM treated with PTH and PTHrP on osteoclast-like cell formation. As shown in figure1, the CM treated with 10^{-7}M hPTH-(1-34) significantly stimulated TRAP-positive MNC formation, compared to untreated CM. And the CM treated with 10^{-7}M hPTHrP-(1-34) also stimulated TRAP-positive MNC formation to a similar degree as the CM treated with 10^{-7}M hPTH-(1-34) did. On the other hand, the CM treated with 10^{-8}M $1,25(OH)_2D_3$ failed to affect TRAP-positive MNC formation, although 10^{-8}M $1,25(OH)_2D_3$ caused a direct stimulation of MNC formation. Our findings first indicated that both PTH and PTHrP stimulated osteoclast-like cell formation through soluble humoral factor(s) released from osteoblasts. There has been recent evidence that PTH, PTHrP, and $1,25(OH)_2D_3$ stimulated bone resorption by mature osteoclasts through soluble factor(s) released from osteoblasts(2, 7, 14). Taken together with them, our findings indicated that PTH stimulates bone resorption by accelerating osteoclast formation as well as mature osteoclastic activity through soluble factor(s) released from

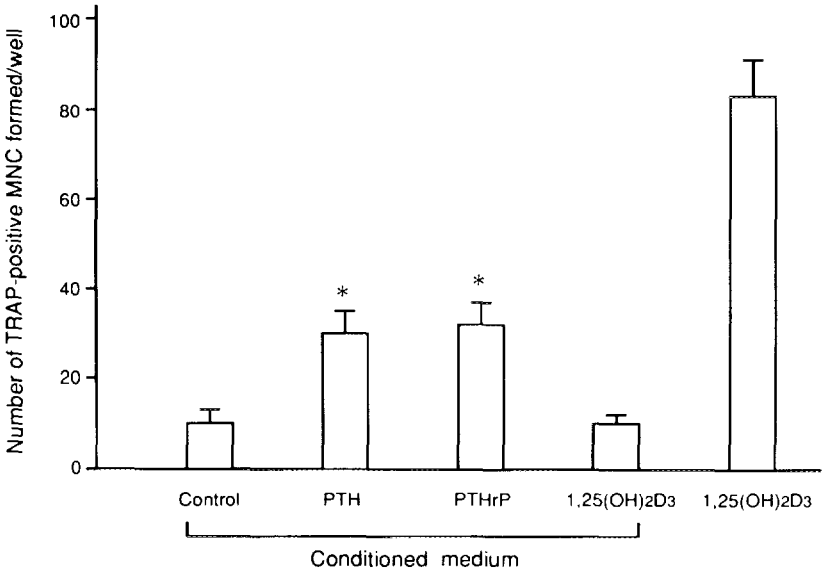

Figure 1. The effect of conditioned medium treated with PTH, PTHrP, and $1,25(OH)_2D_3$ on osteoclast-like cell formation.
Conditioned medium was obtained from UMR-106 cells treated with 10^{-7}M hPTH-(1-34), 10^{-7}M hPTHrP-(1-34), 10^{-8}M $1,25(OH)_2D_3$. After treatment of mouse hemopoietic blast cells with 30% of each conditioned medium or 10^{-8}M $1,25(OH)_2D_3$, TRAP-positive MNC were counted, as described in Materials and Methods. Each bar(□) represents the mean ± SEM of four determinations.
* $P<0.01$, compared to untreated CM.

osteoblasts, and also suggested that PTHrP stimulated bone resorption presumably through the same mechanism as PTH did.

In the next experiment we studied to characterize PTH/PTHrP-responsive dual signal transduction systems(PKA and Ca/PKC) in osteoblast-mediated stimulation of osteoclast-like cell formation by PTH and PTHrP. DbcAMP and Sp-cAMPS were employed as PKA activators. Useful tools for studying the two arms of the polyphosphoinositide system include calcium ionophores, which are thought to mimic myoinositol 1,4,5-triphosphate by increasing $[Ca^{2+}]_i$ levels and the tumor promoting phorbol ester, which can become a substrate for 1,2-diacylglycerol and activate PKC. As shown in figure2, the CM treated with 10^{-4}M dbcAMP and 10^{-4}M Sp-cAMPS significantly stimulated TRAP-positive MNC formation, suggesting that PKA activation of osteoblasts induced the release of soluble factor(s), resulting in an increase in osteoclast-like cell formation. The CM treated with 10^{-7}M A23187, 10^{-7}M ionomycin, and 10^{-7}M PMA also significantly stimulated TRAP-positive MNC formation, although the CM treated with 10^{-7}M 4α-PDD, incapable of activating PKC, failed to affect it. Our data suggested that an increase in $[Ca^{2+}]_i$ as well as PKC

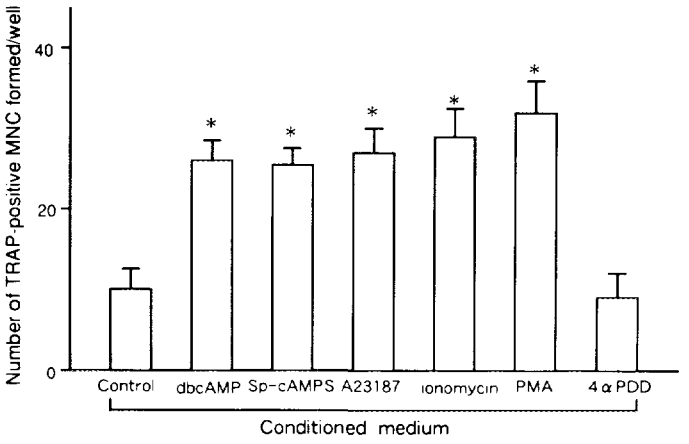

Figure 2. The effect of conditioned medium treated with second messenger analogues on osteoclast-like cell formation.

Conditioned medium was obtained from UMR-106 cells treated with 10^{-4}M dbcAMP, 10^{-4}M Sp-cAMPS, 10^{-7}M A23187, 10^{-7}M ionomycin, 10^{-7}M PMA, or 10^{-7}M 4αPDD. After treatment of mouse hemopoietic blast cells with 30% of each conditioned medium, TRAP-positive MNC were counted, as described in Materials and Methods. Each bar(□) represents the mean ± SEM of four determinations.

* $P<0.01$, compared to untreated CM.

activation of osteoblasts induced the release of soluble factor(s), resulting in an increase in osteoclast-like cell formation, although we could not completely rule out the possibility that calcium ionophores and PMA acted through some other mechanisms than an increase in $[Ca^{2+}]_i$ and PKC activation, respectively. There has been recent evidence that a single cDNA clone, expressing rat bone PTH/PTHrP receptor, mediates the stimulation by PTH and PTHrP of both adenylate cyclase and phospholipase C, when expressed in COS cells(8). Indeed, Civitelli et al. and we reported that PTHrP as well as PTH caused polyphosphoinositide breakdown, resulting in an increase in $[Ca^{2+}]_i$ and an activation of PKC in UMR-106 cells(9, 15-17). The present study, therefore, suggests that PTH/PTHrP-responsive dual signal transduction systems are involved in osteoblasts-mediated stimulation of osteoclast formation by PTH and PTHrP. The present study, however, did not clarify the precise nature of soluble factors involved in osteoblasts-mediated stimulation of osteoclast formation by PTH and PTHrP. Further study is necessary to clarify it.

In conclusion, PTH and PTHrP can cause osteoblasts-mediated stimulation of osteoclast-like cell formation and PTH/PTHrP-responsive dual signal transduction systems would be involved in the release of soluble factor(s) stimulating osteoclast-like cell formation by PTH and PTHrP.

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References

1. Chambers, T.J., McSheehy, P.M.J., Thomson, B.M., and Fuller, K. (1985)Endocrinology 116, 234-239.
2. McSheehy, P.M.J., and Chambers, T.J. (1986)Endocrinology 118, 824-828.
3. McSheehy, P.M.J., and Chambers, T.J. (1986)Endocrinology 119, 1654-1659.
4. Perry, H.M., Skogen, W., Chappel, J., Kahn, A.J., Wilner, G., and Teitelbaum, S.L. (1989)Endocrinology 125, 2075-2082.
5. Moseley, J.M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R.E.H., Kemp, B.E., Suva, L.J., Rodda, C.P., Ebeling, P.R., Hudoson, P.J., Zajac, J.D., and Martin, T.J. (1987) Proc. Natl. Acad. Sci. USA 84, 5048-5052.
6. Suva, L.J., Winslow,G.A.,Wettenhall, R.E.H., Hammonds,R.G., Moseley, J.M., Diefenbach-Jagger, H., Roddu, C.P., Kemp, B.E., Rodriguez, M., Chen,E.R., Hudson,P.J., Martin,T.J., and Wood,W.I. (1987) Science 237, 893-896.
7. Morris, C.A., Mitnick, M.E., Weir, E.C., Horowitz, M., Kreider, B.L., and Insogna, K.L. (1990) Endocrinology 126, 1783-1785.
8. Abou-Samra, A.B., Juppner, H., Force, T., Freeman, M.W., Kong, X.F., Schipani, E., Vrena, P., Richards, J., Bonventre, J.V., Potts, J.T., Kronenberg, H.M, and Segre, G.V. (1992) Proc. Natl. Acad. Sci. USA. 89, 2732-2736.
9. Sugimoto, T., Kano, J., Ikeda, K., Fukase, M., and Chihara, K. (1993) J. Bone Min. Res. 8, 451-458.
10. Kano, J., Sugimoto, T., Fukase, M., and Fujita,T (1991) Biochem. Biophys. Res. Comm. 179, 97-101.
11. Kano, J., Sugimoto, T., Fukase, M., and Chihara, K. (1992) Biochem. Biophys. Res. Comm. 184, 525-529.
12. Sugimoto, T., Nakada, M., Fukase, M., Imai, Y., Kinoshita, Y., and Fujita, T. (1986) Calcif. Tissue Int. 39, 171-174.
13. Kurihara, N., Suda, T., Miura, Y., Nakauchi, H., Kodama, H, Hiura, K., Hakeda, Y., and Kumegawa M. (1989) Blood 74, 1295-1302.
14. Mcsheehy, P.M.J., and Chambers, T.J. (1987) J. Clin. Invest. 80, 425-429.
15. Reid, I.R., Civitelli, R., Halstead, L.R., Avioli, L.V., and Hruska, K.A. (1987) Am. J. Physiol. 252, E45-E51.
16. Civitelli, R., Reid, I.R., Westbrook, S., Avioli, L.V., and Hruska, K.A. (1988) Am. J. Physiol. 255, E660-E667.
17. Civitelli, R., Martin, T.J., Faust, A., Gunaten, S.L., Hruska, K.A., and Avioli, L.V. (1989) Endocrinology 125, 1204-1210.