EXPERIMENTAL STUDY

Involvement of osteoprotegerin/osteoclastogenesis inhibitory factor in the stimulation of osteoclast formation by parathyroid hormone in mouse bone cells

Michiko Kanzawa, Toshitsugu Sugimoto, Masanori Kanatani and Kazuo Chihara

Third Division, Department of Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

(Correspondence should be addressed to T Sugimoto; Fax: +81-78-3825899)

Abstract

Objective: Recently, osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) has been shown to inhibit osteoclast differentiation. On the other hand, we have reported that parathyroid hormone (PTH) stimulated osteoclast formation, presumably through a PTH-responsive cAMP-dependent protein kinase (PKA) pathway, in mouse bone cells.

Design and methods: The present study was performed to examine how OPG/OCIF expression is regulated by PTH and to further investigate the possible involvement of OPG/OCIF in the stimulation of osteoclast formation by PTH in mouse bone cells. OPG/OCIF mRNA expression was analyzed by Northern hybridization after 24 h treatments of mouse whole bone cells and mouse stromal cell line, ST2 cells with PTH or various second messenger analogs.

Results: Human (h) PTH(1–34) (10^{-10} and 10^{-8} mol/l) but not 10^{-8} mol/l hPTH(3–34) down-regulated OPG/OCIF mRNA expression in mouse bone cells. Dibutyryl cAMP, but not phorbol ester, an activator of protein kinase C, or A23187, a calcium ionophore, down-regulated it. The same was also observed in ST2 cells, suggesting that stromal cells are responsible for the inhibitory effect of PTH and cAMP analogs on OPG/OCIF mRNA expression in mouse bone cells.

Conclusions: The present study indicates that PTH down-regulates OPG/OCIF mRNA expression through the PKA pathway in stromal cells, which would result in the stimulation of osteoclast formation.

European Journal of Endocrinology 142 661–664

Introduction

Recently, osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) has been identified and reported to belong to the tumor necrosis factor (TNF) receptor superfamily and to be a soluble factor which inhibits osteoclast differentiation (1, 2). Indeed, *in vivo* evidence is available that OPG-knockout mice exhibit severe osteopenia (3) and that recombinant OPG increases bone density and protects against bone loss due to estrogen deficiency in the rat (1). There is also evidence about the effects of cytokines and hormones on OPG/OCIF levels in bone cells (4–10). Indeed, parathyroid hormone (PTH) inhibits OPG/OCIF expression in bone (9, 11, 12), but it still remains unclear how OPG/OCIF is regulated by PTH in bone cells. On the other hand, we previously reported that PTH stimulated osteoclast formation in mouse bone cells, partly through some unknown soluble factor released from osteoblasts/stromal cells (13). Therefore, we raised the possibility that OPG/OCIF might participate in the mechanism

through which PTH stimulated osteoclast formation. The present study was performed to examine how OPG/OCIF expression is regulated by PTH and to further investigate the possible involvement of OPG/OCIF in the stimulation of osteoclast formation by PTH in mouse bone cells.

Materials and methods

Materials

ICR mice were obtained from the Sizuoka Experimental Animal Center (Shizuoka, Japan). Dibutyryl cAMP (dbcAMP) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St Louis, MO, USA), human PTH (hPTH) (1–34) from Peptide Institute (Osaka, Japan), Sp-cAMPS from Biolog Life Science Institute (Bremen, Germany) and A23187 from Hoechst Japan (Tokyo, Japan). All other chemicals used were of analytical grade.

Cell culture

Mouse unfractionated bone cell culture was previously described in detail (14). In brief, femurs and tibias of 10- to 15-day-old ICR mice were aseptically removed. The bones from ten mice were dissected free of soft tissues and mechanically minced with a scalpel blade in phenol red-free α -MEM medium containing 5% charcoal-treated fetal calf serum (FCS). After removal of bone fragments by sedimentation under normal gravity, unfractionated bone cell suspensions were collected from the supernatant. These unfractionated bone cells included tartrate-resistant acid phosphatase-positive multinucleated cells, alkaline phosphatase-positive mononuclear cells (probably osteoblasts), stromal cells and other bone marrow cells. Unfractionated bone cells were cultured in α -MEM containing 5% FCS and mouse bone marrow stromal cell line ST2 cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in a humidified 10% CO₂–90% air atmosphere.

Probes

A 546 bp cDNA fragment corresponding to nucleotides 154–699 of the mouse OPG cDNA was amplified from mouse heart tissue total RNA by RT-PCR using the following primer, 5'-GAAACCCTTCCTCCAAAGTACTTGCAT-3', and 5'-TTCACACAGGGTGACATCATCTATTCAC-3'. The PCR product was cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The 1.2 kb β -actin cDNA was used as a reference.

Northern hybridization

The total RNA was extracted from mouse bone cells by the acid guanidinium–thiocyanate–phenol–chloroform extraction method (15). Twenty micrograms total RNA were denatured, and run on a 1% agarose gel containing 2% formaldehyde, then transferred to a nitrocellulose membrane, and hybridized to a ³²P-labeled DNA probe overnight at 42°C. After hybridization, the filter was washed twice with 2×SSC containing 0.5% SDS, and subsequently twice with 0.1×SSC containing 0.5% SDS at 60°C for 1 h.

Results

Our previous study revealed that PTH stimulated osteoclast-like cell formation in mouse bone cells (13, 14). In the present study using the same culture system as well as mouse stromal cell line ST-2 cells, we examined the effect of PTH on OPG/OCIF mRNA expression. The time-course study revealed that 10^{−8} mol/l hPTH(1–34) decreased OPG/OCIF mRNA levels within 6 h and the inhibitory effect of 24 h treatment was more prominent than that of 6 h treatment (data not shown). As shown in Fig. 1,

Figure 1 Northern blot analysis of OPG/OCIF mRNA after treatment of mouse bone cells and ST2 cells with the indicated concentrations of hPTH(1–34) or hPTH(3–34) for 24 h. Data shown are representative of several separate cell preparations and similar results were obtained from other preparations.

treatment with hPTH(1–34) (10^{−10} and 10^{−8} mol/l) for 24 h concentration-dependently decreased the OPG/OCIF mRNA level, although 10^{−8} mol/l hPTH(3–34), which did not possess the ability to increase cAMP production, did not obviously affect it. Our previous study revealed that PTH stimulated osteoclast formation in mouse bone cells partly through some unknown soluble factor released from osteoblasts/stromal cells. Therefore, we examined the effect of PTH on OPG/OCIF mRNA levels using mouse stromal cells, ST2. Treatment with hPTH(1–34) also concentration-dependently down-regulated OPG/OCIF mRNA expression in these cells (Fig. 1, right). PTH acts on bone through cAMP-dependent protein kinase (PKA) and phospholipase C-coupled calcium/protein kinase C (Ca²⁺/PKC) pathways (16). The next experiment was performed to examine the effect of PTH-responsive second messenger analogs on OPG/OCIF mRNA levels. As shown in Fig. 2, dbcAMP at 10^{−4} mol/l down-regulated the OPG/OCIF mRNA level in mouse bone cells, but PMA, an activator of PKC, and A23187, a Ca ionophore, at 10^{−7} mol/l did not. In ST-2 cells, dbcAMP as well as Sp-cAMPS, a

Figure 2 Northern blot analysis of OPG/OCIF mRNA after treatment of mouse bone cells and ST2 cells with several second messenger analogs for 24 h. Data shown are representative of several separate cell preparations and similar results were obtained from other preparations.

PKA activator, at 10^{-4} mol/l, also down-regulated it, while PMA and A23187 at 10^{-7} mol/l did not (Fig. 2, right).

Discussion

In the present study, PTH down-regulated OPG/OCIF mRNA expression in mouse bone cells as well as in ST-2 cells. The present findings are compatible with recent data from mouse bone marrow cells and osteoblastic/stromal cells (9, 11, 12), although there is also recent evidence that PTH does not affect OPG/OCIF levels in some osteoblastic cells such as MC3T3 E-1 cells (11) and SaOS-2 cells (17, 18).

PTH possesses dual signal transduction systems (PKA and Ca^{2+} /PKC) (14). In the present study using mouse bone cells as well as mouse stromal cells, PTH(1–34) and cAMP analogs down-regulated OPG/OCIF mRNA levels, although PTH(3–34), PMA and A23187 did not. The present findings first indicated that PTH down-regulated OPG/OCIF mRNA expression presumably through the PKA pathway. Since the same findings were obtained in mouse bone cells and stromal cells, it seems likely that stromal cells are responsible for the inhibitory effect of PTH and cAMP analogs on OPG/OCIF mRNA expression in mouse bone cells.

Recently, OPG-ligand (OPGL)/osteoclast differentiation factor (ODF) expressed on the membrane of osteoblasts/stromal cells has been identified and shown to mediate osteoclastogenesis through binding to osteoclast precursor cells (19, 20) and activation of mature osteoclast (17, 21). OPGL/ODF is the ligand for OPG/OCIF, which belongs to the TNF receptor superfamily, and the stimulatory effect of OPGL/ODF on osteoclast differentiation are blocked by OPG/OCIF (17, 19–21). Taken together, OPG is regarded as a soluble type of decoy receptor for OPGL/ODF, namely OPG/OCIF acts as a soluble inhibitory factor in osteoclastogenesis. Our previous (13, 14) and present studies revealed that PTH as well as cAMP analogs down-regulated OPG/OCIF mRNA expression and stimulated osteoclast differentiation in mouse bone cells. These findings suggest the involvement of OPG/OCIF in the stimulation of osteoclast formation by PTH. It has been generally accepted that osteoblasts/stromal cells play a crucial role in the regulation of osteoclast differentiation (17, 22). It has been reported that PTH stimulates osteoclast differentiation at least in part through an increase in ODF expression in osteoblasts/stromal cells (9, 11, 12). On the other hand, in our previous study, conditioned medium from osteoblasts/stromal cells pretreated with PTH and cAMP analogs stimulated osteoclast formation from osteoclast precursor cells, although soluble factors responsible for this phenomenon were not identified. The reduction in OPG/OCIF in conditioned medium of cells would not directly promote osteoclast formation, but it is possible that its reduction promotes osteoclastogenesis indirectly, through

augmenting ODF action. The present study revealed that PTH and cAMP analogs down-regulated OPG/OCIF mRNA expression in ST2 stromal cells as well as in mouse bone cells. Taken together, it seems likely that PTH acts on stromal cells to inhibit OPG/OCIF production and release via the PKA pathway, which would result in the stimulation of osteoclast formation.

In conclusion, the present study first indicated that PTH down-regulates OPG/OCIF mRNA expression via the PKA pathway in stromal cells and that OPG/OCIF would be partly involved in the stimulation of osteoclast formation by PTH.

Acknowledgements

This work was supported in part by Smoking Research Foundation.

References

- 1 Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997 **89** 309–319.
- 2 Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T *et al.* Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochemical and Biophysical Research Communications* 1997 **234** 137–142.
- 3 Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T *et al.* Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochemical and Biophysical Research Communications* 1998 **247** 610–615.
- 4 Brandstrom H, Jonsson KB, Ohlsson C, Vidal O, Ljunghall S & Ljunggren Ö. Regulation of osteoprotegerin mRNA levels by prostaglandin E_2 in human bone marrow stroma cells. *Biochemical and Biophysical Research Communications* 1998 **247** 338–341.
- 5 Hofbauer LC, Dunstan CR, Spelsberg TC, Riggs BL & Khosla S. Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenic protein-2, and cytokines. *Biochemical and Biophysical Research Communications* 1998 **250** 776–781.
- 6 Vidal NOA, Sjogren K, Eriksson BI, Ljunggren Ö & Ohlsson C. Osteoprotegerin mRNA is increased by interleukin- 1α in the human osteosarcoma cell line MG-63 and in human osteoblast-like cells. *Biochemical and Biophysical Research Communications* 1998 **248** 696–700.
- 7 Brändström H, Jonsson KB, Vidal O, Ljunghall S, Ohlsson C & Ljunggren Ö. Tumor necrosis factor- α and - β upregulate the levels of osteoprotegerin mRNA in human osteosarcoma MG-63 cells. *Biochemical and Biophysical Research Communications* 1998 **248** 454–457.
- 8 Vidal NOA, Brandstrom H, Jonsson KB & Ohlsson C. Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. *Journal of Endocrinology* 1998 **159** 191–195.
- 9 Horwood NJ, Elliott J, Martin J & Gillespie MT. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. *Endocrinology* 1998 **139** 4743–4746.
- 10 Takai H, Kanematsu M, Yano K, Tsuda E, Higashio K, Ikeda K *et al.* Transforming growth factor- β stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. *Journal of Biological Chemistry* 1998 **273** 27091–27096.
- 11 Lee S-K & Lorenzo JA. Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic

- acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology* 1999 **140** 3552–3561.
- 12 Murakami T, Yamamoto M, Yamamoto M, Ono K, Nishikawa M, Nagata N *et al.* Transforming growth factor- β 1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells. *Biochemical and Biophysical Research Communications* 1998 **252** 747–752.
- 13 Kaji H, Sugimoto T, Kanatani M, Fukase M & Chihara K. Involvement of dual signal transduction systems in the stimulation of osteoclast-like cell formation by parathyroid hormone and parathyroid hormone-related peptide. *Biochemical and Biophysical Research Communications* 1993 **194** 157–162.
- 14 Kaji H, Sugimoto T, Kanatani M, Nasu M & Chihara K. Estrogen blocks parathyroid hormone (PTH)-stimulated osteoclast-like cell formation by selectively affecting PTH-responsive cyclic adenosine monophosphate pathway. *Endocrinology* 1996 **137** 2217–2224.
- 15 Chomczynski P & Sacchi N. Single-step method of RNA isolation by acid guanidinium–thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* 1987 **162** 156–159.
- 16 Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E *et al.* Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. *Proceedings of the National Academy of Sciences of the USA* 1992 **89** 2732–2736.
- 17 Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT & Martin TJ. Modulation of osteoclast differentiation and function by the new member of the tumor necrosis factor receptor and ligand families. *Endocrine Reviews* 1999 **20** 345–357.
- 18 Itoh K, Shinki T, Udagawa N, Ueno Y, Matsuzaki K, Yakahashi N *et al.* The mechanism of osteoclastogenesis induced by PTH. *Journal of Bone Mineral Research* 1999 **14** (Suppl 1) SU205.
- 19 Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998 **93** 165–176.
- 20 Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinoshita M, Mochizuki S *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Sciences of the USA* 1998 **95** 3597–3602.
- 21 Fuller K, Wong B, Fox S, Choi Y & Chambers TJ. TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *Journal of Experimental Medicine* 1998 **188** 997–1001.
- 22 Suda T, Takahashi N & Martin TJ. Modulation of osteoclast differentiation. *Endocrine Reviews* 1992 **13** 66–80.

Received 14 September 1999

Accepted 22 February 2000