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Journal of Cellular Biochemistry 109:755–763 (2010)

Journal of Cellular Biochemistry

Mechanisms Underlying Catabolic and Anabolic Functions of Parathyroid Hormone on Bone by Combination of Culture Systems of Mouse Cells

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ABSTRACT

Since bone resorption and formation by continuous and intermittent parathyroid hormone (PTH) treatments involve various types of cells in bone, this study examined the underlying mechanism by combining culture systems using mouse primary calvarial osteoblasts and bone marrow cells. The PTH/PTHrP receptor (PTH1R) expression and the cAMP accumulation in response to PTH were increased in accordance with the differentiation of osteoblasts. Osteoclast formation was strongly induced by continuous PTH treatment in the monolayer co-culture of osteoblasts and bone marrow cells, which was associated with RANKL expression in differentiated osteoblasts. Bone formation determined by ALP activity and the type I collagen mRNA expression was stimulated by intermittent PTH treatment in the monolayer co-culture and in the bone marrow cell layer of the separated co-culture in a double chamber dish, but not in the culture of bone marrow cells alone. The stimulation in the separated co-culture, accompanied by IGF-I production by osteoblasts, was abolished when bone marrow cells were derived from knockout mice of insulin-receptor substrate-1 (IRS-1−/−) or when osteoblasts were from PTH1R−/− mice. We conclude that differentiated osteoblasts are most likely the direct target of both continuous and intermittent PTH, while bone marrow cells are likely the effector cells. The osteoblasts stimulated by continuous PTH express RANKL which causes osteoclastogenesis from the precursors in bone marrow via cell-to-cell contact, leading to bone resorption; while the osteoblasts stimulated by intermittent PTH secrete IGF-I which activates IRS-1 in osteoblast precursors in bone marrow via a paracrine mechanism, leading to bone formation. J. Cell. Biochem. 109: 755–763, 2010.

KEY WORDS: PARATHYROID HORMONE (PTH); BONE; OSTEOBLAST; OSTEOCLAST; INSULIN-LIKE GROWTH FACTOR-I (IGF-I); INSULIN-RECEPTOR SUBSTRATE (IRS); RANKL

Continuous exposure to parathyroid hormone (PTH) is associated with a catabolic effect on bone, whereas intermittent exposure to PTH is associated with an anabolic effect [Ishizuya et al., 1997; Hodsman et al., 2005; Poole and Reeve, 2005]. Although several cell culture systems have been created to elucidate the mechanisms underlying the paradoxical functions of PTH, most of them failed to reproduce the in vivo findings. This may be because various types of cells in bone and bone marrow are involved in the functions [Locklin et al., 2003].

In bone, since the type 1 PTH/PTHrP receptor (PTH1R) is expressed in osteoblastic cells but not in osteoclastic cells, the direct target for the catabolic action of PTH is thought to be osteoblastic cells [Kousteni and Bilezikian, 2008]. In fact, the bone resorption caused by PTH is known to be due to receptor activator of nuclear factor-κB ligand (RANKL) expression by osteoblastic cells that induces osteoclastic differentiation from the hematopoietic precursors [Khosla, 2001; Ma et al., 2001]. However, which differentiation stage of osteoblasts predominantly expresses RANKL remains unclarified.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Japanese Ministry of Education, Culture, Sports, Science and Technology; Grant number: 17390413.

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Received 25 March 2009; Accepted 12 November 2009 • DOI 10.1002/jcb.22454 • © 2010 Wiley-Liss, Inc.
Published online 7 January 2010 in Wiley InterScience (www.interscience.wiley.com).

The osteoanabolic function of PTH has attracted considerable clinical attention and led to the approval of the recombinant human PTH (1–34) for osteoporosis treatment worldwide [Neer et al., 2001; Hodsman et al., 2005]; however, the underlying mechanism is still controversial and unclear. It has been proposed that PTH stimulates bone formation by inducing differentiation of osteoblast precursor cells or by activating quiescent osteoblasts [Nishida et al., 1994; Isogai et al., 1996]. Meanwhile, PTH is also reported to inhibit apoptosis of mature osteoblasts, thereby effectively prolonging the anabolic function [Jilka et al., 1999]. Evidence has emerged recently to indicate that insulin-like growth factor (IGF)-I is a mediator of the PTH anabolic function. PTH stimulates production of IGF-I by osteoblastic cells, which functions as an autocrine/paracrine factor and causes osteoblast proliferation, differentiation, and survival [McCarthy et al., 1989; Watson et al., 1995]. The PTH anabolic action is suppressed when administered to knockout mice of IGF-I or the adaptor molecule insulin-receptor substrate (IRS)-1 [Kadowaki et al., 1996], suggesting the importance of the IGF-I signal through the IRS-1 activation in vivo [Miyakoshi et al., 2001; Bikle et al., 2002; Yamaguchi et al., 2005]. However, the target cells of the PTH anabolic action or the IGF-I source remain unclear, as does the target of IGF-I.

In order to examine mechanisms whereby PTH regulates bone resorption and formation, the present study combined four types of culture systems using mouse primary calvarial osteoblasts and bone marrow cells: osteoblasts alone, bone marrow cells alone, the monolayer co-culture of osteoblasts and bone marrow cells in a single dish, and the separated co-culture in a double chamber dish divided by a porous membrane. Among these systems, we compared the effects of continuous and intermittent treatment of PTH. Furthermore, to know the direct target of PTH and IGF-I, we used cells derived not only from wild-type mice, but also from knockout mice of IRS-1 (IRS-1−/−) or PTH1R (PTH1R−/−).

MATERIALS AND METHODS

CELL CULTURES

Mouse osteoblastic MC3T3-E1 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). Primary osteoblasts were isolated from calvariae of E18.5 wild-type and PTH1R−/− littermates as described earlier [Ogata et al., 2000]. Briefly, calvariae were digested for 10 min five times at 37°C in an enzyme solution containing 0.1% collagenase and 0.2% dispase and cells isolated by the last four digestions were combined as an osteoblast population. Bone marrow cells were collected from long bones of 8-week-old wild-type and IRS-1−/− male littermates [Kadowaki et al., 1996]. Primary osteoblasts and bone marrow cells were cultured in αMEM (Invitrogen, Carlsbad, CA) containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) and 1% penicillin/streptomycin (Invitrogen).

To gain several differentiation stages of osteoblasts, after MC3T3-E1 cells and primary calvarial osteoblasts became confluent in the αMEM/10% FBS medium above, the cells were pre-cultured with 10% FBS, 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO) and 10 mM β-glycerophosphate (Sigma-Aldrich) for the indicated days. The medium was then replaced, and cAMP accumulation, RANKL mRNA level, and medium IGF-I concentration were measured as described below after cultures with 10 nM recombinant rat PTH (1–34) (Sigma-Aldrich) or the vehicle for 15 min, 3 h, and 6 h, respectively.

CULTURE SYSTEMS

Twelve-well dishes were used for monolayer cultures, and FALCON™ CELL CULTURE INSERTS ([35]3103; Becton Dickinson Labware, Franklin Lakes, NJ) were used for separated co-cultures. For single layer primary osteoblast culture, cells were inoculated at a density of 5.0×10^4 cells/cm^2. For single layer bone marrow cell culture, cells were seeded at a density of 2.0×10^6 cells/cm^2. The same amount of cells was seeded in the single well for monolayer co-culture. For separated co-culture, primary osteoblasts were inoculated to the bottom layer and bone marrow cells were seeded to CELL CULTURE INSERT at the same density. Recombinant rat PTH (1–34) (10 nM) was added to each culture as follows. In the intermittent treatment group, the cells were exposed to PTH or the control vehicle for the first 6 h of each 48 h incubation cycle and then cultured in the absence of PTH or the vehicle during the remainder of the cycle. After three cycles of exposure to PTH or the vehicle, cells were evaluated. In the continuous treatment group, the cells were kept exposed to PTH or the vehicle for 6 days with medium change of every 48 h.

All of mice and experiments that we used above were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

REAL-TIME QUANTITATIVE RT-PCR OF OSTEOCALCIN, PTH1R, RANKL, AND COL I

For measuring RANKL mRNA level, MC3T3-E1 cells and primary osteoblasts that were pre-cultured with 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for indicated days were exposed to 10 nM recombinant rat PTH or the vehicle for 3 h, and cells were collected. For measuring type I collagen (COL I) mRNA level, primary osteoblasts and bone marrow cells were collected after three cycles of exposure to PTH or the vehicle. Total RNAs were reverse-transcribed with MultiScribe reverse transcriptase (Applied Biosystems, Inc., Foster City, CA). For RT-PCR, Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (ABI) using QuantiTect SYBR Green PCR Master Mix (QIAGEN, Tokyo, Japan), according to the manufacturer's instructions. A set of primers was designed using sequences obtained from the Genbank as follows: 5′-AAGCAGGAGGGCAATAAGGT-3′ and 5′-TTTGTAGGCGGTCTTCAAGC-3′ for osteocalcin mRNA (NM007541.1), 5′-GGGCACAAGAAGTGGATCAT-3′ and 5′-GGCCATGAAGACGGTGTAGT-3′ for PTH1R mRNA (NM011199.1), 5′-CCTGAGGCCCAGCCATTT-3′ and 5′-CTTGGCCCAGCCTCGAT-3′ for RANKL mRNA (AF019048), and 5′-ACGTCCTGGTGAAGTTGGTC-3′ and 5′-CAGGGAAGCCTCTTTCTCCT-3′ for COL I mRNA (U08020.1).

MEASUREMENTS OF INTRACELLULAR cAMP AND MEDIUM IGF-I CONCENTRATION

For measurement of cAMP accumulation, MC3T3-E1 cells and primary osteoblasts that were pre-cultured with 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for indicated days were exposed to 10 nM recombinant rat PTH or the vehicle with

1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) for 15 min. The amount of cAMP in the cell extracts was determined by cAMP Biotrak EIA System (Amersham Bioscience, Buckinghamshire, UK) according to the manufacturer's protocol. For measurement of IGF-I concentration, primary osteoblasts that were pre-cultured with 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for indicated days were exposed to 10 nM recombinant rat PTH or the vehicle for 6 h. The cultured medium was collected and IGF-I concentration was measured by Quantikine Mouse IGF-I kit (R&D Systems, Inc., Minneapolis, MN).

TARTRATE RESISTANT ACID PHOSPHATASE (TRAP) STAINING

The staining was performed at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate (Sigma-Aldrich) in *N*,*N*-dimethyl formamide as the substrate. Cells positively stained for TRAP containing more than three nuclei were counted as osteoclasts.

ALKALINE PHOSPHATASE (ALP) STAINING AND MEASUREMENT OF ALP ACTIVITY

For the ALP staining, cultured plates were rinsed with PBS, fixed in 100% ethanol, and stained with Tris–HCl-buffered solution (pH 9.0) containing naphthol AS-MX phosphate as a substrate and Fast Blue BB salt (Sigma-Aldrich) as a coupler. For ALP activity, cells were sonicated in 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and 0.5% Triton X-100. ALP activity in the lysate was measured using a Wako ALP kit (Wako Pure Chemical Industry, Ltd, Osaka, Japan). The protein content was determined using BCA protein assay reagent (Pierce, Rockford, IL).

STATISTICAL ANALYSIS

Means of groups were compared by ANOVA and significance of differences was determined by post hoc testing using Bonferroni's method.

RESULTS

DIFFERENTIATION STAGE OF OSTEOBLASTS AS THE TARGET OF PTH

To confirm the differentiation stages of osteoblasts as the target of PTH, we initially examined the mRNA expression of osteocalcin, the marker for osteoblast differentiation, and PTH1R in the cultures of MC3T3-E1 cells and mouse primary calvarial osteoblasts (POB) with the osteogenic medium in the presence of 10% FBS, ascorbic acid, and β-glycerophosphate (Fig. 1A,B) for the indicated days. The osteocalcin and PTH1R expression was increased as a function of the culture period in both cultures. When cAMP accumulation in response to 15 min treatment with PTH or the vehicle control was compared in osteoblasts that were pre-cultured with the osteogenic medium above for the indicated periods, the response to PTH was confirmed to be increased in accordance with an increase of mRNA expression of osteocalcin in both cells (Fig. 1A,C). PTH responsiveness defined by cAMP accumulation, rather than PTH1R mRNA expression, was more correlated with osteoblast differentiation, suggesting time lag for PTH1R to be active [Kondo et al., 1997]. This indicates that differentiated osteoblasts with abundant expression of PTH1R and high responsiveness to PTH are the most likely direct target of PTH.

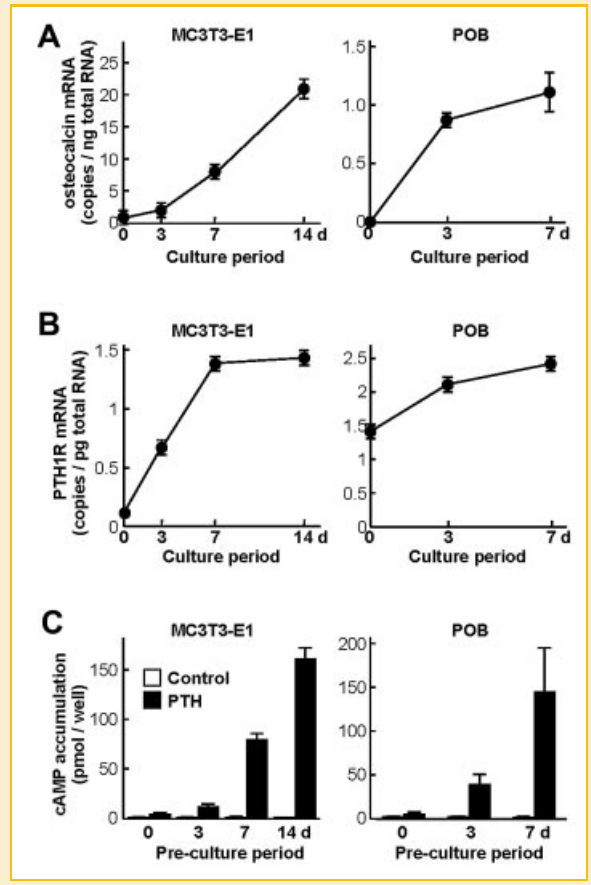

Fig. 1. Time courses of PTH1R expression and response to PTH in osteoblasts at several differentiation stages. A: Osteocalcin and (B) PTH1R mRNA levels determined by real-time quantitative RT-PCR in MC3T3-E1 cells and mouse primary calvarial osteoblasts (POB) cultured in the presence of 10% FBS, ascorbic acid and β-glycerophosphate for indicated days. C: Response to PTH determined by cAMP accumulation in several differentiation stages of MC3T3-E1 cells and POB. The cells were pre-cultured with 10% FBS, ascorbic acid and β-glycerophosphate for the indicated days, then the medium was replaced. cAMP accumulation was determined after 15 min exposure to 10 nM recombinant rat PTH (1–34) or the vehicle (control). Data are expressed as means (symbols or bars) ±SD (error bars) in respective culture systems from four independent experiments.

OSTEOCLAST FORMATION BY INTERMITTENT OR CONTINUOUS PTH TREATMENT

To know the mechanism underlying the PTH action on bone resorption, we compared the effects of intermittent (6/48; first 6 h of 48 h exposure) and continuous (48/48; 48 h exposure) PTH treatments on TRAP-positive multinucleated osteoclast formation in the four types of culture systems using mouse primary calvarial osteoblasts and bone marrow cells: osteoblasts alone, bone marrow cells alone, the monolayer co-culture, and the separated co-culture in which the osteoblast layer and the bone marrow cells layer were separated in a double chamber dish. No osteoclast was formed in the culture of osteoblasts alone or in the osteoblast layer of the

separated co-culture either with or without PTH (data not shown), confirming that osteoclasts are originated not from osteoblasts but from the precursor cells in bone marrow. PTH treatment indeed stimulated osteoclast formation from bone marrow cells as compared to the vehicle treatment in bone marrow cell culture, the monolayer co-culture, and the bone marrow layer of the separated co-culture; however, the effect was different between intermittent and continuous treatments, as well as among the culture systems (Fig. 2A). Continuous PTH treatment induced osteoclast formation more potently than intermittent treatment in the bone marrow cells alone, the monolayer co-culture, and the bone marrow layer of the separated co-culture, and the effect was the strongest in the monolayer co-culture. These findings are consistent with previous reports that PTH induces osteoclast formation via the direct action on osteoblasts that stimulate osteoclastic differentiation of the bone marrow precursors through cell-to-cell contact [Ma et al., 2001].

Hence, to know the differentiation stages of osteoblasts as the target of this PTH catabolic function, we compared RANKL expression in MC3T3-E1 cells and primary calvarial osteoblasts at several differentiation stages. The RANKL mRNA level in response to 3 h PTH treatment was higher in the differentiated osteoblasts in

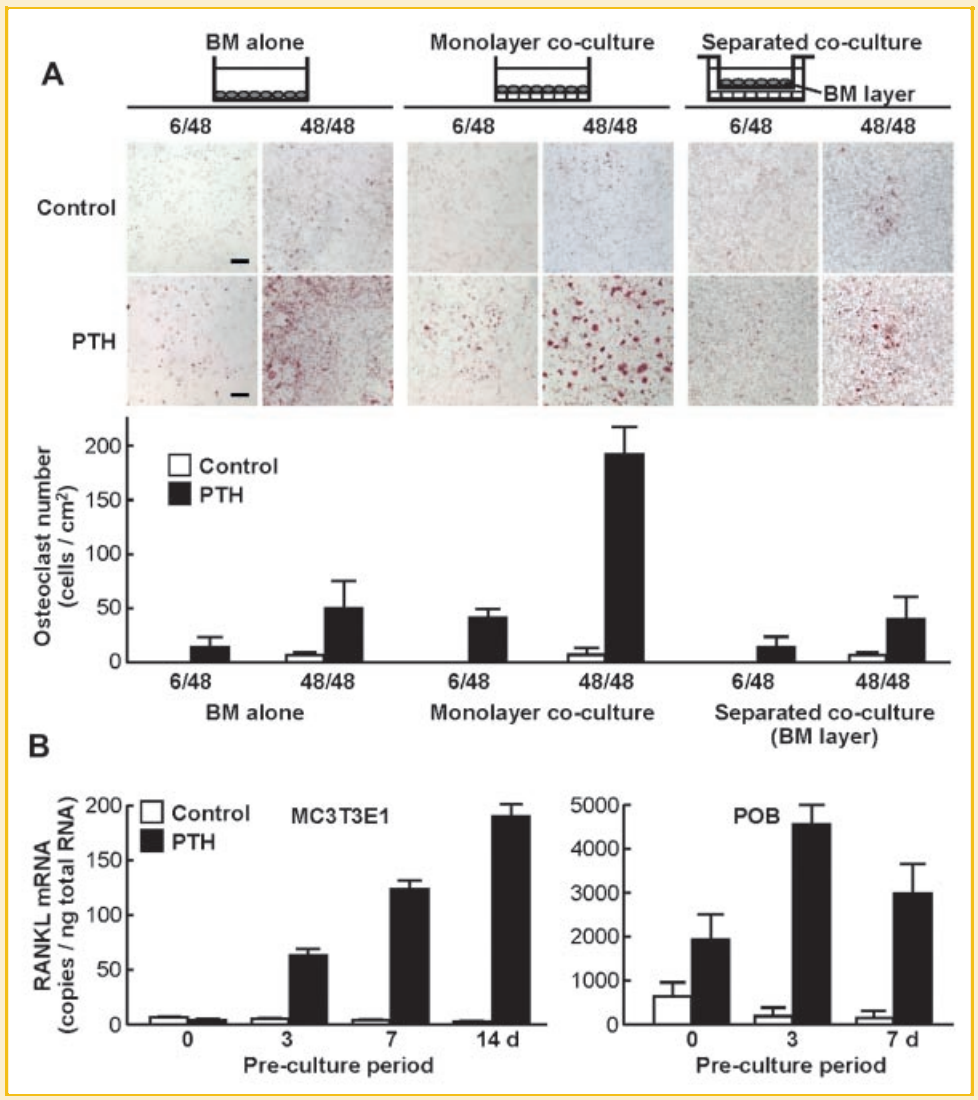

Fig. 2. Effects of PTH treatment on osteoclast formation and RANKL expression. A: Osteoclast formation by intermittent (6/48; first 6 h of 48 h exposure) or continuous (48/48; 48 h exposure) treatment with 10 nM recombinant rat PTH (1–34) or the vehicle (control) for 6 days (three cycles) in culture systems: mouse bone marrow cells (BM) alone, the monolayer co-culture of mouse primary calvarial osteoblasts and BM, and the separated co-culture in a double chamber dish. Cells with positive TRAP staining and more than three nuclei were counted as osteoclasts. Scale bars, 100 μm. Data are expressed as means (bars) ±SD (error bars) for 4 wells/group from four independent experiments. No osteoclast was formed in the culture of osteoblasts alone or in the osteoblast layer of the separated co-culture (data not shown). B: RANKL mRNA levels determined by real-time quantitative RT-PCR in several differentiation stages of MC3T3-E1 cells and primary calvarial osteoblasts (POB). The cells were pre-cultured with 10% FBS, ascorbic acid and β-glycerophosphate for the indicated days, then the medium was replaced. RANKL mRNA level was determined after 3 h treatment with 10 nM PTH or the vehicle (control). Data are expressed as means (bars) ±SD (error bars) for 4 wells/group from four independent experiments.

accordance with an increase of osteocalcin mRNA level (Figs. 1A and 2B). Taken together, these data indicate that PTH, especially continuous treatment, directly induces RANKL expression predominantly in differentiated osteoblasts, which causes osteoclast formation from bone marrow precursors via the cell-to-cell contact, leading to bone resorption.

BONE FORMATION BY INTERMITTENT OR CONTINUOUS PTH TREATMENT

Next, to learn the mechanism underlying the PTH action on bone formation, we compared the effects of intermittent (6/48) and continuous (48/48) PTH treatments on ALP activity and COL I mRNA expression in the four types of culture systems above (Fig. 3). Proliferation rates of osteoblasts after intermittent or continuous treatment were similar in all of the culture systems (data not shown). In none of the cultures, did the continuous PTH treatment affect the ALP activity or COL I mRNA expression. Meanwhile, the intermittent PTH treatment significantly stimulated ALP activity and COL I mRNA expression in the monolayer co-culture and in the bone marrow cell layer of the separated co-culture. The intermittent treatment did not affect the culture of osteoblasts alone or the osteoblast layer of the separated co-culture, indicating that the most of the effector of PTH is not osteoblasts, but bone marrow cells. Interestingly, however, the treatment did not affect the culture of bone marrow cells alone in the absence of osteoblasts. These lines of results indicate that PTH acts on osteoblasts, which secrete humoral factors that induce osteoblastic differentiation of osteoprogenitor cells in bone marrow via a paracrine mechanism.

MEDIATION OF IGF-I/IRS-1 AND PTH1R IN THE PTH ANABOLIC FUNCTION

Since IGF-I is the most probable candidate for the paracrine factor as reported previously [McCarthy et al., 1989; Watson et al., 1995; Miyakoshi et al., 2001; Bikle et al., 2002; Yamaguchi et al., 2005], we next measured IGF-I concentration in the culture medium of mouse primary calvarial osteoblasts at several differentiation stages (Fig. 4A). Although the IGF-I concentration was increased in accordance with the differentiation depending on the pre-culture period with the osteogenic medium in both PTH and the vehicle-treated cultures, the response to PTH treatment as compared to the

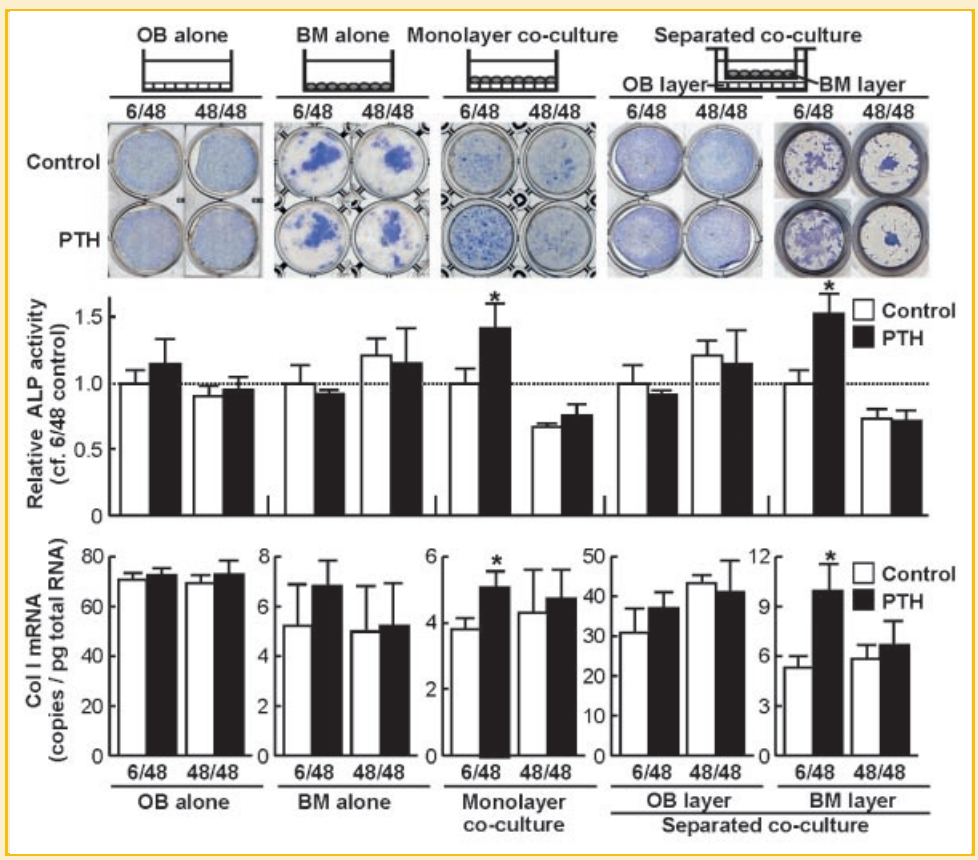

Fig. 3. Effect of intermittent or continuous PTH treatment on ALP activity and COL I mRNA expression in four culture systems. ALP staining and relative ALP activity, and COL I mRNA expression by intermittent (6/48; first 6 h of 48 h exposure) or continuous (48/48; 48 h exposure) treatment with 10 nM recombinant rat PTH (1–34) or the vehicle (control) for 6 days (three cycles) in culture systems: mouse primary calvarial osteoblasts (OB) alone, bone marrow cells (BM) alone, the monolayer co-culture, and the separated co-culture. In the separated co-culture, OB layer and BM layer were separately assessed. Relative ALP activity was normalized against the 6/48 control in each culture system. Absolute ALP activity of 6/48 control culture (IU/g); OB alone: 278.6, BM alone: 75.4, monolayer co-culture: 260.1, OB layer of separated co-culture: 163.4, BM layer of separated co-culture: 66.0. Data are expressed as means (bars) ±SD (error bars) of the ratio compared to the 6/48 control culture in respective culture systems for 4 wells/group from four independent experiments. *Significant effect versus control, $P < 0.01$.

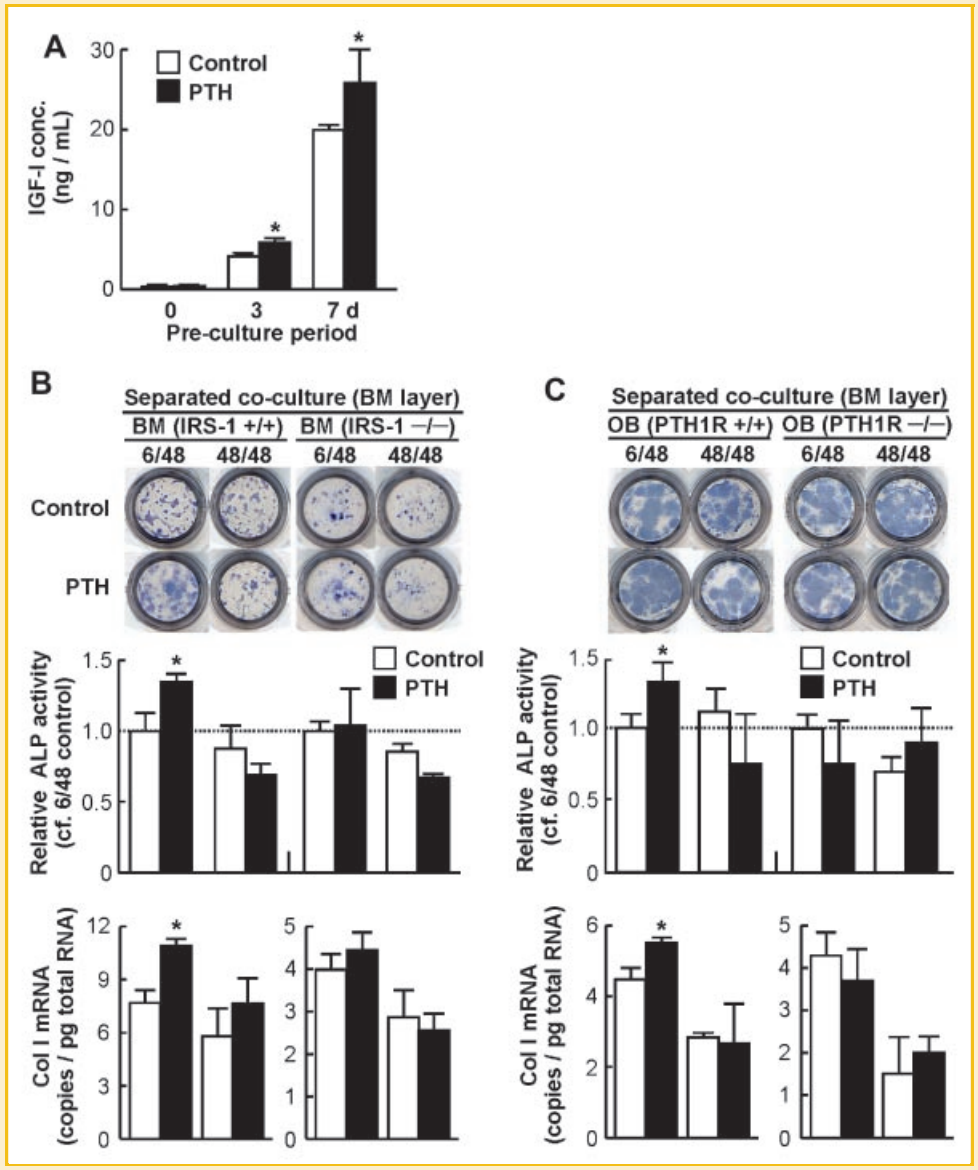

Fig. 4. Mediation of IGF-I/IRS-1 and PTH1R in the anabolic function of PTH. A: IGF-I concentration in the culture medium of mouse primary calvarial osteoblasts at several differentiation stages. Primary osteoblasts were pre-cultured with 10% FBS, ascorbic acid and β-glycerophosphate for the indicated days, then the medium was replaced. IGF-I concentration was measured after 6 h treatment with 10 nM recombinant rat PTH (1–34) or the vehicle (control). Data are expressed as means (bars) ±SD (error bars) for 4 wells/group from four independent experiments. *Significant effect versus control, $P < 0.05$. B,C: ALP staining and relative ALP activity, and COL I mRNA expression of bone marrow cells (BM) in the separated co-culture with primary calvarial osteoblasts (OB), by intermittent (6/48; first 6 h of 48 h exposure) or continuous (48/48; 48 h exposure) treatment with 10 nM PTH or the vehicle (control) for 6 days (three cycles). For (B), BM are derived from IRS-1+/+ mice [BM (IRS-1+/+)] or IRS-1−/− mice [BM (IRS-1−/−)], and OB are derived from wild-type mice. For (C), BM are derived from wild-type mice, and OB are derived from PTH1R+/+ mice [OB (PTH1R+/+)] or PTH1R−/− mice [OB (PTH1R−/−)]. Relative ALP activity was normalized against the 6/48 control in each culture system. Absolute ALP activity of 6/48 control culture (IU/g); (B) BM (IRS-1+/+): 66.4, BM (IRS-1−/−): 44.8, (C) OB (PTH1R+/+): 72.5, OB (PTH1R−/−): 84.2. Data are expressed as means (bars) ±SD (error bars) of the ratio compared to the 6/48 control culture in respective culture systems for 4 wells/group from four independent experiments. *Significant effect versus control, $P < 0.01$.

vehicle treatment became significant in differentiated cells cultured more than 3 days.

Next, to confirm whether or not the target of IGF-I produced by osteoblasts in response to PTH is bone marrow cells, we analyzed ALP activity and COL I mRNA expression as shown in Figure 3 in the separated co-culture using bone marrow cells derived from knockout mice of IRS-1 (IRS-1−/−), an essential adaptor molecule for the PTH/IGF-I signaling in bone [Ogata et al., 2000; Akune et al., 2002; Yamaguchi et al., 2005] (Fig. 4B). The IRS-1 deficiency in bone marrow cells abolished the stimulation of ALP activity and COL I mRNA expression by the intermittent PTH treatment in the bone marrow cell layer of the separated co-culture (Fig. 4B). Furthermore, when osteoblasts derived from the PTH1R−/− mice were used in the separated co-cultures, the anabolic stimulation by the intermittent PTH treatment in the bone marrow cell layer was also abrogated in the bone marrow cell layer of the separated co-culture (Fig. 4C).

These lines of results demonstrate that the target of the PTH anabolic function and the source of IGF-I are most likely differentiated osteoblasts, and the main target of the IGF-I action is bone marrow cells. It is therefore speculated that osteoblasts stimulated by the intermittent PTH treatment express IGF-I, which then activates IRS-1 signaling in osteoblast precursor cells in bone marrow, leading to bone formation.

DISCUSSION

In this study, we have established cell culture systems which reproduce PTH catabolic and anabolic functions on bone in vivo, by combining cultures of mouse primary calvarial osteoblasts and bone marrow cells in the mono-layer culture or the separated co-culture with a double chamber dish divided by a porous membrane. Using the systems, we found the mechanisms whereby continuous and intermittent PTH treatments stimulate bone resorption and formation as shown in Figure 5. One of the direct targets of both continuous and intermittent PTH seems to be differentiated osteoblasts, while the effector to be the respective precursors in bone marrow. The osteoblasts stimulated by continuous PTH express RANKL which causes osteoclastogenesis from the hematopoietic precursors in bone marrow via the cell-to-cell contact, leading to bone resorption; while the osteoblasts stimulated by intermittent PTH secrete IGF-I which activates IRS-1 in osteoblast precursors in bone marrow via the paracrine mechanism, leading to bone formation.

Although osteoanabolic function of the intermittent PTH treatment has been intensively studied, it is still controversial as to the target cells [Poole and Reeve, 2005; Kousteni and Bilezikian, 2008]. In the present study, PTH1R expression and cAMP accumulation in response to PTH were enhanced in accordance with osteoblast differentiation (Fig. 1), and the PTH anabolic function was abolished when PTH1R−/− osteoblasts were used in the co-cultures (Fig. 4C), indicating that the direct target, at least in part, is differentiated osteoblasts. In fact, previous in vitro studies using various types of osteoblastic cells have shown that the PTH1R expression increased in accordance with the differentiation [Bos et al., 1996; McCauley et al., 1996; Kondo et al., 1997]. cAMP accumulation was much higher in later stage of differentiation in spite of the receptor mRNA level being essentially the same. This may be due to time lag for PTH1R to be active after its mRNA expression since IGF-I level, which is reported to be mediated through cAMP signal by PTH [Canalis et al., 1989], were more correlated with osteoblast differentiation as well as cAMP accumulation. In vivo observations also showed that the intermittent PTH stimulated bone formation by actions on post-mitotic osteoblasts [Jilka et al., 2009]. Contrarily, there are some reports showing that the PTH anabolic function is mediated by the proliferation and differentiation of osteoprogenitor cells in bone marrow [Nishida et al., 1994], and that PTH regulates osteoblast differentiation positively or negatively depending on the differentiation stages [Isogai et al., 1996; Hollnagel et al., 1997; Schiller et al., 1999]. Hence, immature osteoblastic cells might possibly be involved as the direct target of the PTH action, despite the weaker PTH1R expression as compared to mature osteoblasts [Dobnig and Turner, 1995; Kondo et al., 1997]. In addition to osteoblasts at several differentiation stages, osteocytes and bone lining cells may possibly be the targets of the PTH anabolic function [Dobnig and Turner, 1995; Leaffer et al., 1995]. Osteocytes actually express abundant PTH1R mRNA [Divieti, 2005] and stimulate bone formation in response to PTH through suppression of sclerostin, a potent anti-osteogenic factor [Keller and Kneissel, 2005]. The widespread osteocytic production of sclerostin in human bone is reported to maintain bone lining cells in a quiescent state on bone surfaces, which is decreased in response to PTH [Poole et al., 2005]. Hence, although the present study showed the pivotal role of mature osteoblasts, there may be various cellular and molecular networks underlying the PTH anabolic function in bone.

RANKL and IGF-I levels were increased in accordance with the differentiation of primary osteoblasts and PTH-stimulated accumulation of cAMP as the central mediators of catabolic and anabolic actions of PTH, respectively; however, there was a difference between them. The induction of RANKL mRNA level was limited to the PTH-treated culture (Fig. 2B), while that of the IGF-I concentration was also seen in the control vehicle-treated culture (Fig. 4A). RANKL is reported to be directly induced by PTH through cAMP/protein kinase A (PKA) signal, the major downstream signal of PTH1R, predominantly in differentiated osteoblasts [Kondo et al., 2002], and actually, the pattern of increase is similar to that of cAMP accumulation (Fig. 1B). Contrarily, IGF-I induction by PTH is known to be mediated by both cAMP/PKA and phospholipase C/protein kinase C pathways [Kousteni and Bilezikian, 2008]. IGF-I is also known to be induced not only by PTH, but also by several factors like prostaglandin E_2 that may be produced endogenously by cultured

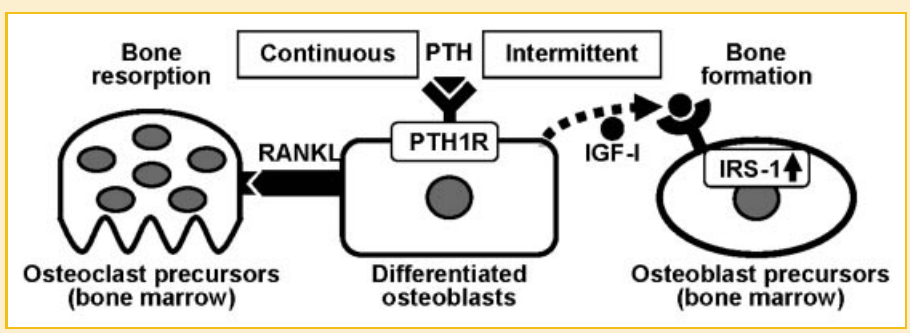

Fig. 5. Schematic mechanisms whereby continuous and intermittent PTH treatments regulate bone resorption and formation.

osteoblasts and function via an autocrine mechanism [McCarthy and Centrella, 2001]. In addition, PTH promotes the secretion of IGF binding proteins (IGFBPs) such as IGFBP-3, IGFBP-4, and IGFBP-5 [Schmid et al., 1994; Kudo et al., 1997; Watson et al., 1999], possibly causing the decrease in the concentration of free fraction of the IGF-I protein. Furthermore, IGF-I is reported to suppress the PTH1R gene expression via the MAP kinase pathway [Kawane and Horiuchi, 1999]. These varieties of mechanisms may cause the induction of IGF-I by PTH to be much more complicated than that of RANKL. In addition, PTH induces not only IGF-I but also other important potential mediators of PTH effects on various stromal-osteoblastic cells [Partridge et al., 2006; Jilka, 2007] and we cannot exclude those mediators. Further studies regarding involvement of other potential mediators are needed.

In addition to the mechanism underlying the PTH anabolic function via IGF-I as a paracrine factor, which was clarified in the present study, there used to be other potential explanations for the lack of PTH response in IRS-1−/− mice [Yamaguchi et al., 2005]: (1) The IRS-1 signaling affects the intracellular signaling of PTH1R after PTH binds to it; (2) The IRS-1 deficiency causes impairment of osteoblast differentiation, so that they are insensitive to PTH. Since the present study demonstrated that the direct target of PTH is differentiated osteoblasts while the effector is bone marrow progenitors, the former hypothesis seems to be unlikely. In addition, bone formation was impaired when bone marrow cells were derived from IRS-1−/− mice even in the presence of wild-type differentiated osteoblasts, the latter hypothesis may also be denied. As a most possible mechanism of PTH anabolic function, we propose that osteoblasts stimulated by PTH secrete IGF-I which activates IRS-1 in osteoblast precursors in bone marrow via the paracrine mechanism.

By combining cultures of mouse osteoblasts and bone marrow cells, we were able to get a clue to understanding of the cellular and molecular mechanisms underlying the paradoxical functions of PTH on bone. There remain, however, many issues to be elucidated, including the mechanism underlying the difference of the actions between the continuous and intermittent treatments. Further studies using the present systems and their modification will greatly help to elucidate the complex network of bone regulation by PTH.

ACKNOWLEDGMENTS

The IRS-1−/− mice and PTH1R−/− mice were generous gifts from Dr. Naoto Kubota (University of Tokyo, Tokyo, Japan) and Dr. Tatsuya Kobayashi (Massachusetts General Hospital, Boston, USA), respectively. We also thank Reiko Yamaguchi for her excellent technical assistance.

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