



Short-term intermittent administration of parathyroid hormone facilitates osteogenesis by different mechanisms in cancellous and cortical bone



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ABSTRACT

Intermittent administration of human parathyroid hormone (1–34)[hPTH(1–34)] induces anabolic action on the bones. To understand the mechanism underlying the early phase of hPTH(1–34)-induced anabolic action, we investigated the expression profiles of osterix and sclerostin after short-term intermittent administration of hPTH(1–34) using immunohistochemistry in adult rats. In the cancellous bone, hPTH(1–34) administration greatly increased the number of osterix-positive cells in the bone marrow on day 1, but the cells gradually decreased on days 3 and 5. Injections of hPTH(1–34) induced no significant changes in the number of sclerostin-positive osteocytes in the cancellous bone. In the cortical bone, intermittent administration of hPTH(1–34) significantly reduced the number of sclerostin-positive osteocytes. The serum sclerostin level was downregulated and the osteocalcin level was upregulated on day 5 after intermittent administration of hPTH(1–34). Intermittent hPTH(1–34) injections increased osteoblast surface, osteoid thickness, and osteoid surface in cancellous bone, but not in cortical bone. This study suggested that the increase in osterix-positive osteoprogenitors in cancellous bone and the decrease in sclerostin-positive osteocytes in cortical bone play important roles in anabolic action on osteogenesis induced by short-term administration of hPTH(1–34).

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1. Introduction

Various hormones regulate bone metabolism. Among these, parathyroid hormone (PTH) has dual effects on bone formation and resorption (Silva and Bilezikian, 2015). In various animal experimental models and human cases (Reeve et al., 1976; Tam et al., 1982; Dempster et al., 1993; Wronski et al., 1993; Dobnig and Turner, 1995; Recker et al., 2009; Hanyu et al., 2012; Jilka, 2007), as well as cell culture experiments (Ishizuya et al., 1997; Nishida et al., 1994), intermittent administration of human PTH(1–34) [hPTH(1–34)] (Reeve et al., 1976; Tam et al., 1982; Dobnig and Turner, 1995; Hanyu et al., 2012; Jilka, 2007) or human PTH(1–84) [hPTH(1–84)] (Tam et al., 1982; Recker et al., 2009) has been shown to have an anabolic effect, such as increasing the bone mass by stimulating bone formation more than bone resorption. In contrast, continuous treatment with PTH results in decreased bone

mass due to enhanced bone resorption than formation, as demonstrated in various animal experimental models (Silva and Bilezikian, 2015). However, the precise mechanism of how PTH affects bone metabolism, especially its anabolic action at early phase, remains unclear.

We along with other researchers have shown that hPTH(1–34) injections increased the number of colony forming unit-fibroblastic (CFU-F) and CFU-alkaline phosphatase-positive (CFU-ALP), which presumably are osteoprogenitors (Nishida et al., 1994; He et al., 2013). These results suggest that hPTH(1–34) stimulates the proliferation and differentiation of osteoprogenitor cells in the bone marrow. Osteoblast differentiation is regulated by specific transcriptional factors, including Runx2 (Komori et al., 1997) and osterix (Nakashima et al., 2002). Research has indicated that osterix works as a downstream transcription factor of Runx2 during osteoblast differentiation (Komori et al., 1997; Nakashima et al., 2002; Yamaguchi et al., 2000; Komori, 2011). Mizoguchi et al. (2014) found that osterix marks distinct waves of primitive and definite stromal progenitors during bone marrow development. In addition, they showed that osterix labeling is restricted in the osteolineage cells of the adult mouse bone marrow (Mizoguchi et al., 2014). These results led us to explore the distribution of osterix-

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positive cells in adult bone marrow with or without hPTH(1–34) administration at the early phase.

Sclerostin is encoded by the *SOST* gene, which is primarily produced by osteocytes (Kousteni and Bilezikian, 2008; Kramer et al., 2010; Ke et al., 2012; Weivoda and Oursler, 2014). Sclerostin binds to low-density lipoprotein receptor-related proteins (LRP) 4, 5, and 6 to inhibit Wnt signaling (Kousteni and Bilezikian, 2008; Kramer et al., 2010; Ke et al., 2012; Weivoda and Oursler, 2014; Tu et al., 2015). Several lines of evidence indicate that PTH is a suppressor of *Sost* expression (Bellido et al., 2005; Keller and Kneissel, 2005; Silvestrini et al., 2007; Drake et al., 2010; Leupin et al., 2007; Ardawi et al., 2012). In rodent models, however, the effects of hPTH(1–34) on sclerostin expression vary. Bellido et al. showed that in lumbar vertebrae of mice, continuous administration of hPTH(1–34) markedly downregulated *Sost* mRNA and the sclerostin protein, but intermittent administration transiently suppressed *Sost* mRNA after hPTH(1–34) administration, which then turned to control levels (Bellido et al., 2005). Keller et al., however, reported that intermittent administration of hPTH(1–34) induced sustained suppression of *Sost* mRNA in the femoral cortical bone of estrogen-deprived rats (Keller and Kneissel, 2005). These results prompted us to investigate whether intermittent hPTH(1–34) administration exerts different effects on the suppression of sclerostin in cancellous and cortical bone. In previous

research, we found that cortical bone retained extremely greater number of sclerostin-positive osteocytes than cancellous bone using immunofluorescent imaging in the femurs of 3-day-old and 2- and 4-week-old rats (Watanabe et al., 2012). Since the effects of hPTH(1–34) on sclerostin expression in cancellous and cortical bone have not been well established, these studies may contribute to a better understanding of the regulatory mechanism of sclerostin by hPTH(1–34).

In this study, we demonstrated the effects of intermittent administration of hPTH(1–34) on the expression of osterix and sclerostin in the period shortly following the treatment. Osterix expression is preferentially upregulated by hPTH(1–34) in bone marrow cells in cancellous bone, while sclerostin expression is preferentially downregulated in cortical bone. These findings indicate that intermittent hPTH(1–34) administration exerts its anabolic action through different mechanisms in cancellous and cortical bone during the early phase after hPTH(1–34) administration.

2. Materials and methods

2.1. Experimental animals

Twelve-week-old female Sprague–Dawley rats (Charles River, Kanagawa, Japan) were used for this experiment. The rats were

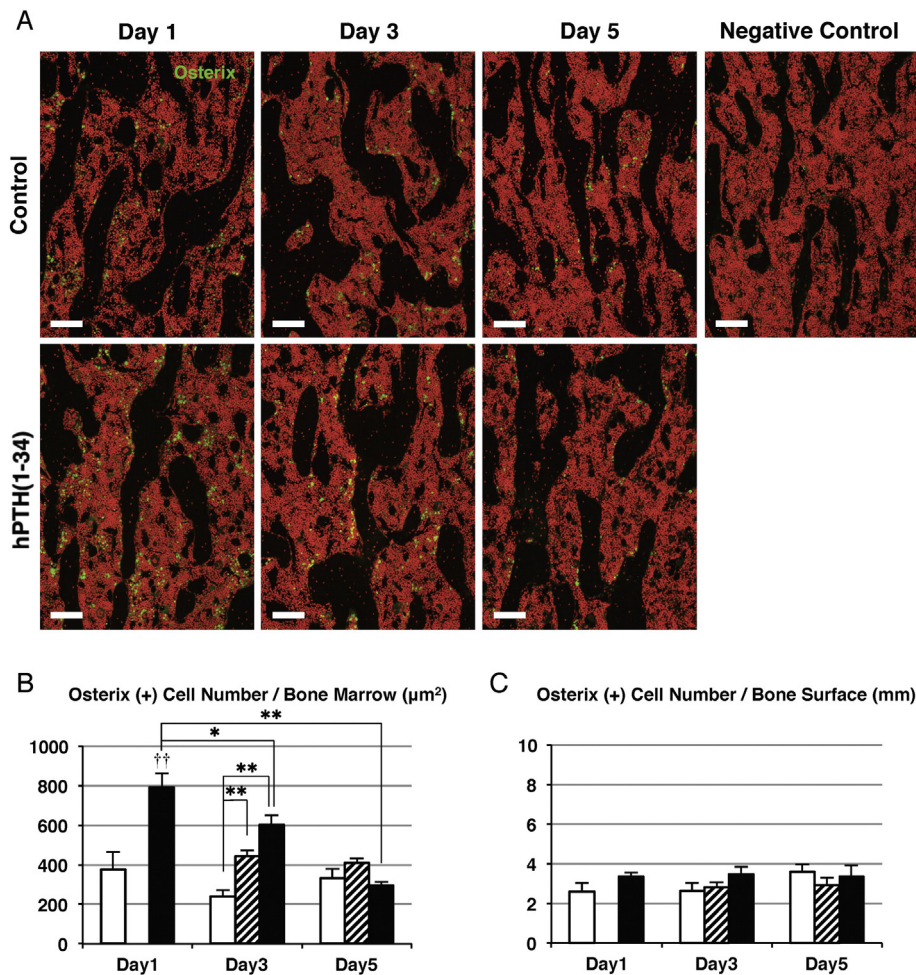


Fig. 1. Effects of PTH administration on the distribution of osterix-positive cells in cancellous bone. (A) Immunofluorescence of osterix-positive cells in the cancellous bone of the control group (upper panels) and daily hPTH(1–34) injection group (lower panels). The sections were immunostained for osterix (green) and counterstained with nuclear propidium iodide (red). Day 1, 3, and 5 indicate the days after administration of saline or hPTH(1–34). The black area represents cancellous bone and the red area is bone marrow. Negative control shows the section incubated with nonimmunized rabbit IgG as a substitute for the primary antibody. Scale bars: 100 µm. Time course of the changes in the number of osterix-positive cells in the bone marrow (B) and the number of osterix-positive osteoblasts on the cancellous bone surface (C). Open bars: control group, hatched bars: single hPTH(1–34) injection group, black bars: daily hPTH(1–34) injection group. Statistical analyses for two groups were performed by Student's *t*-test [control group versus daily hPTH(1–34) injection group on day 1, and single hPTH(1–34) injection group versus daily hPTH(1–34) injection group on day 3]. ††*p* < 0.01. Statistical analyses for data sets with more than two groups were compared by Dunnett's multiple comparisons test. **p* < 0.05, ***p* < 0.01. *n* = 7–8 for each group.

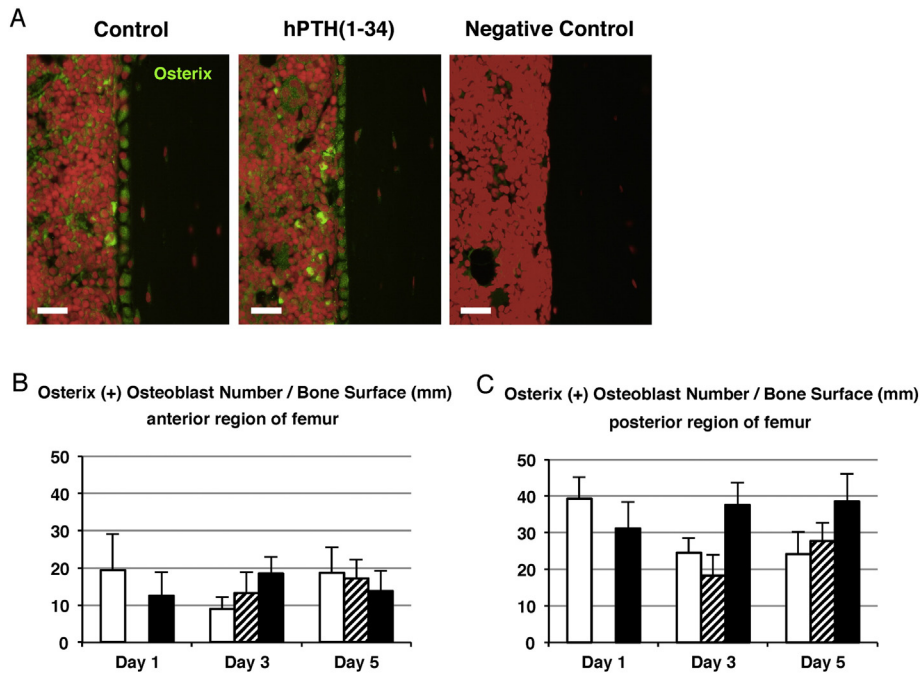


Fig. 2. Effects of PTH administration on the distribution of osterix-positive osteoblasts on the endosteal bone surface of the diaphysis of the femur. (A) Immunofluorescence of osterix in the cortical bone area in the control (control) and daily hPTH(1–34) injection group (PTH) on day 5. Green cells are osterix-positive cells. The black area in the right half represents cortical bone, and the red area in the left half shows bone marrow. Negative control shows the section incubated with nonimmunized rabbit IgG as a substitute for the primary antibody. Scale bars: 50 μ m. Time course of the changes in the number of osterix-positive osteoblasts on the endosteal surface per mm in the anterior region (B) and posterior region (C) of the cortical bone. Open bars: control group, hatched bars: single hPTH(1–34) injection group, black bars: daily hPTH(1–34) injection group. n = 7–8 for each group.

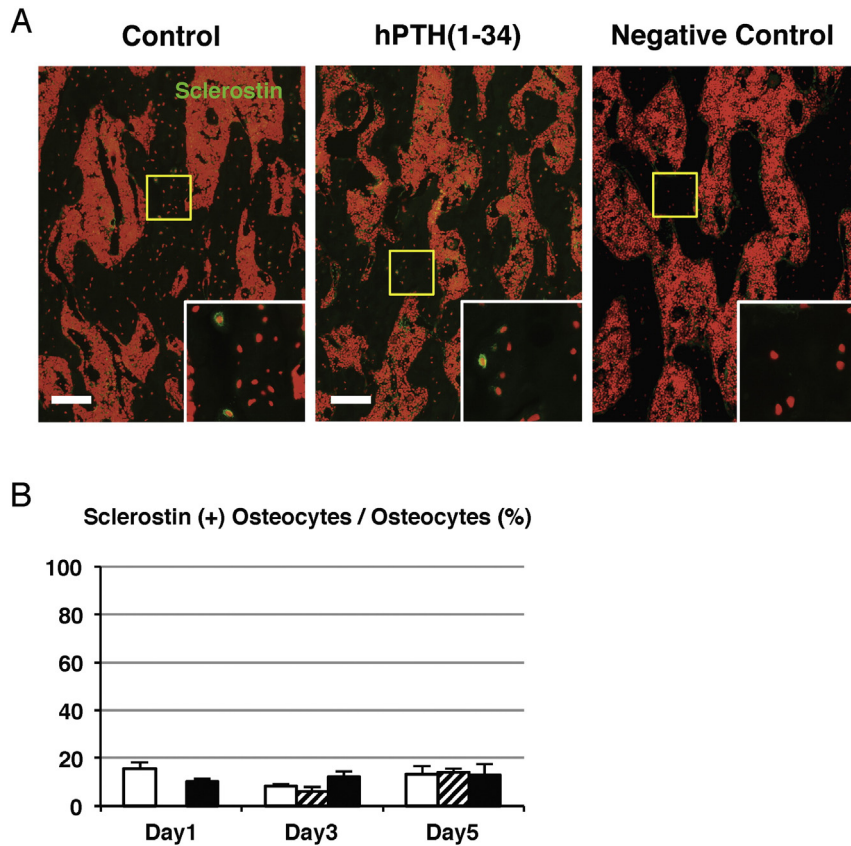


Fig. 3. Effects of hPTH(1–34) administration on the distribution of sclerostin-positive osteocytes in cancellous bone. (A) Distribution of sclerostin-positive osteocytes in the cancellous bone with or without hPTH(1–34) treatment on day 5. Sclerostin-positive cells are shown in green. The nucleus is red. The black area represents cancellous bone and the red area is bone marrow. The area surrounded by the square at the right bottom of the hPTH(1–34) injected group shows high magnification of the area demarcated by the yellow square in the center of the picture. Negative control shows the section incubated with nonimmunized goat IgG as a substitute for the primary antibody. Scale bars: 50 μ m. (B) Time course of the changes in the percentage of sclerostin-positive osteocytes in total osteocytes in cancellous bone. Open bars: control group, hatched bars: single hPTH(1–34) injection group, black bars: daily hPTH(1–34) injected group. n = 7–8 for each group.

maintained under a 12-/12-h light/dark cycle with free access to water and food (CRF-1, standard diet for rats, Oriental Yeast, Tokyo, Japan). The animals were allowed to acclimate to their environment for 1 week before the start of the experiments. The experimental protocols were approved by the Experimental Animal Ethics Committee at Asahi Kasei Pharma Corp. and conducted in accordance with the guidelines concerning the management and handling of experimental animals.

2.2. Experimental design

Rats in Groups 1 and 2 were euthanized on day 1 after administration of saline (Group 1) or hPTH(1–34) 30 µg/kg (Group 2) on day 0. Rats in Groups 3–5 were euthanized on day 3 after daily administration of saline on days 0, 1, and 2 (Group 3), daily administration of hPTH(1–34) 30 µg/kg on days 0, 1, and 2 (Group 4), or single administration of hPTH(1–34) 30 µg/kg on day 0 followed by daily administration of saline on days 1 and 2 (Group 5). Rats in Groups 6–8 were euthanized on day 5 after daily administration of saline on days 0 to 4 (Group 6), daily administration of hPTH(1–34) 30 µg/kg on days 0 to 4 (Group 7), or single administration of hPTH(1–34) 30 µg/kg on day 0 followed by daily administration of saline on days 1 to 4 (Group 8). The hPTH(1–34) was administered by subcutaneous injection. The euthanasia was performed 24 h after the last administration. After euthanasia, the both femurs were dissected. One sample was fixed in 4% paraformaldehyde, decalcified in 20% ethylenediaminetetraacetic acid (EDTA) at 4 °C for 5 weeks, and then embedded in paraffin. Longitudinal 4 µm serial sections were sliced and subjected to hematoxylin and eosin (H&E) and immunofluorescent stains. Another sample was fixed in 70% ethanol, stained in Villanueva bone stain, dehydrated in a graded ethanol series, defatted in acetone, and embedded in methyl methacrylate (Wako Pure Chemical Industries, Osaka Japan). Thin sections (5 µm) were cut from sagittal sections of the femur and

these samples were then prepared for conventional bone histomorphometric analyses.

2.3. Immunofluorescence studies

For immunohistochemistry, the following antibodies were used as primary antibodies: a rabbit polyclonal antibody against Sp7/osterix (ab22552, Abcam, Cambridge, MA), and a goat polyclonal antibody against SOST/sclerostin (AF1589, R&D systems, Minneapolis, MN). Goat anti-Rabbit IgG AlexaFluor 488 and Rabbit anti-Goat IgG AlexaFluor 488 (Invitrogen, Carlsbad, CA) were used as secondary antibodies (1:1000). Sections were counterstained with propidium iodide (PI) (Invitrogen) to stain the nuclei. Tiling imaging of immunostained bone sections was performed using the Axioskop2 instrument (Carl Zeiss, Oberkochen, Germany). Histomorphometric measurements were performed in the 3 mm² region (2 mm × 1.5 mm) in the secondary spongiosa region, which was 2 mm away from the growth plate of the proximal femur, and in the diaphysis of the cortical bone region, which was 10 mm away from the growth plate of the proximal femur. For negative controls, nonimmunized rabbit IgG or goat IgG was used as a substitute for the primary antibody.

2.4. Measurement of bone metabolic markers and biochemical assay of serum samples

The bone metabolic markers and biochemical data were measured in serum samples obtained from Groups 6, 7, and 8. The blood samplings were performed before the daily administration of saline or hPTH(1–34) on days 0 to 3. It was also performed at 6 h after the first administration of saline or hPTH(1–34) on day 0. The sampling on day 5 was performed at 24 h after the day 4 administration. All rats were fasted for at least 6 h before blood collection. Serum was obtained by centrifugation of the collected blood samples from the subclavian vein according to the

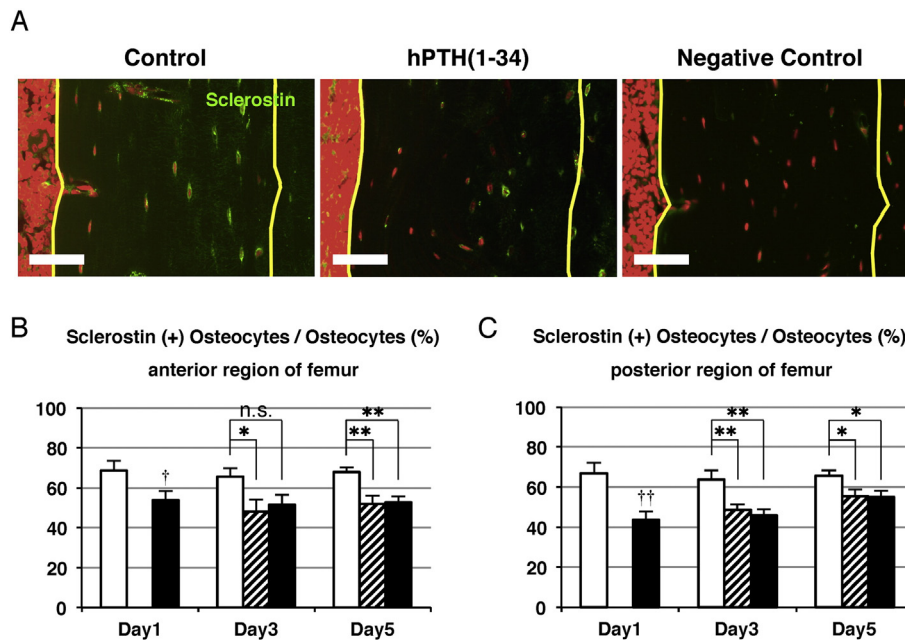


Fig. 4. Effects of hPTH(1–34) administration on the distribution of sclerostin-positive osteocytes at the endosteal surface of cortical bone in the femur diaphysis. (A) Distribution of sclerostin-positive osteocytes in the control and hPTH(1–34) administration groups (1 day after the injection). The black area in the right half represents cortical bone and the red area in the left half denotes bone marrow. Sclerostin-positive cells are shown in green. The nucleus is red. In each panel, the left hand yellow line demarcates the endosteal surface and the right hand yellow line delineates a distance 200 µm away. Negative control shows the section incubated with nonimmunized goat IgG as a substitute for the primary antibody. Scale bars: 50 µm. Time course of the changes in the number of sclerostin-positive osteocytes at the endosteal surface per mm in the anterior region (B) and posterior region (C) of the cortical bone. Open bars: control group, hatched bars: single hPTH(1–34) injection group, black bars: daily hPTH(1–34) injection group. Statistical analyses for two groups were performed by Student's *t*-test [control group versus daily hPTH(1–34) injection group on day 1]. †*p* < 0.05, ††*p* < 0.01. Statistical analyses for data sets with more than two groups were compared by Dunnett's multiple comparisons test. **p* < 0.05, ***p* < 0.01. n.s.: not significant. n = 7–8 for each group.

time schedules as mentioned above. The serum samples were aliquoted and stored at -80°C until analysis.

The serum level of osteocalcin (OC) was determined using an osteocalcin rat ELISA system (GE Healthcare, Piscataway, NJ, USA). The serum level of sclerostin (SOST) was measured using Mouse/Rat SOST Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). The following serum biochemical assays were performed: inorganic phosphorus (Pi) using L-type Wako Pi (Wako Pure Chemical Industries, Osaka, Japan), calcium (Ca) using Calcium E-HA test Wako (Wako Pure Chemical Industries), alkaline phosphatase (ALP) activity using L-type Wako ALP-J (Wako Pure Chemical Industries). All assays were performed according to the manufacturer's instructions.

2.5. Bone histomorphometric analyses

Histomorphometric measurements were performed in the cancellous bone tissue in the secondary spongiosa region (2 ± 1 mm from the growth plate) and the cortical bone tissue at the diaphyseal region (10 ± 0.5 mm from the growth plate) of the femur. The following parameters were measured in the cancellous bone: bone volume [BV/TV (%)], osteoid thickness [O.Th (μm)], osteoid surface [OS/BS (%)], and osteoblast surface [Ob.S (μm) and Ob.S/BS (%)]. In the cortical bone, osteoid thickness [O.Th (μm)] and osteoid surface [OS/BS (%)] were measured (Parfitt et al., 1987). These parameters were assessed in the undecalcified sections. We also measured osteoblast number [N.Ob/BS (N/mm)] in the decalcified paraffin sections used for immunohistochemical studies.

2.6. Statistical analysis

The data are presented as mean \pm standard errors of mean. Data sets with two groups were compared by Student's t-test. Data sets with more than two groups were compared by one-way analysis of variance followed by Dunnett's multiple comparisons test. p values less than 0.05 were considered significant.

3. Results

3.1. The effects of hPTH(1–34) on the localization of osterix-expressing cells in cancellous bone

In the control group, osterix-positive cells were sparsely distributed in the bone marrow (Fig. 1A, B). Administration of hPTH(1–34) significantly increased the number of osterix-positive cells in the bone marrow on day 1 after the injection (Group 2) (Fig. 1A, B). The osterix-positive cells tended to distribute in the vicinity of bone surface, and some cells formed clusters. On day 3 after daily administration of hPTH(1–34) (Group 4), the number of osterix-positive cells in the bone marrow significantly decreased compared to that on day 1 (Group 2), but it was still significantly greater than that of the control group on day 3 (Group 3) (Fig. 1A, B). On day 5 after daily injections of hPTH(1–34) (Group 7), the number of osterix-positive cells in bone marrow significantly decreased compared to that of day 1 (Group 2), and its level went down to that in control group (Group 6) (Fig. 1A, B). A single injection of hPTH(1–34) on day 0 increased the number of

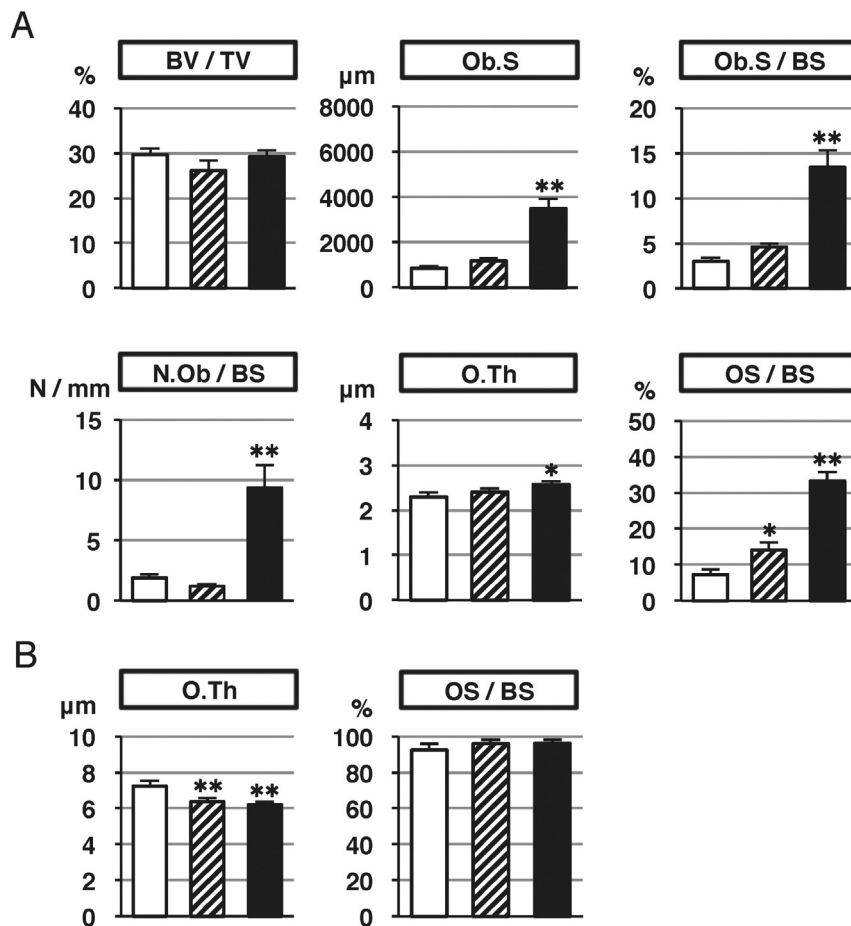


Fig. 5. Bone histomorphometric analyses of cancellous bone (A) and cortical bone (B). Bone histomorphometric analysis was conducted as described in Materials and Methods. BV/TV: bone volume, Ob.S: osteoblast surface, Ob.S/BS: osteoblast number: N.Ob/BS, osteoblast surface, O.Th: osteoid thickness, and OS/BS: osteoid surface. Open bars: control group, hatched bars: single hPTH(1–34) injection group, black bars: daily hPTH(1–34) injection group. Statistical analyses were performed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$. $n = 7-8$ for each group.

osterix-positive cells in the bone marrow on day 3 (Group 5) compared to that in control group (Group 3), though its stimulatory effect was significantly smaller compared to the daily injection on day 3 (Group 4) by Student's t-test ($p < 0.05$) (Fig. 1B). The stimulatory effect of a single injection of hPTH(1–34) diminished on day 5 (Group 8)(Fig. 1B).

The number of osterix-positive cells on the cancellous bone surface showed no significant difference among groups of control and hPTH (1–34) administration groups by single or daily injection during the experimental period (Fig. 1A, C).

Negative controls which were incubated with nonimmunized rabbit IgG as a substitute for the primary antibody exhibited no immunoreaction as shown in Fig. 1A.

3.2. The effects of hPTH(1–34) on the localization of osterix-expressing cells in cortical bone

Osterix-positive cells were more frequently distributed on the endosteal bone surface than the periosteal region in the cortical bone. Since the number of osterix-positive cells on the endosteal bone surface was greater at the posterior (30–40%) than that at the anterior (10–20%) region of the femur in the control group (Fig. 2B, C), we counted osterix-positive osteoblasts on the endosteal surface separately at anterior and posterior regions. We found no significant difference in the number of osterix-positive cells on these endosteal surfaces (Fig. 2A, B, C).

3.3. The effects of hPTH(1–34) on the localization of sclerostin-expressing cells in cancellous bone

In the control group, the expression of sclerostin was observed exclusively in osteocytes in the cancellous bone area, but the incidence of positive osteocytes was very small (10–15% of total osteocytes) (Fig. 3A, B). Treatment with hPTH(1–34) induced no significant change in the number of sclerostin-positive osteocytes in cancellous bones among the groups tested (Fig. 3A, B). Negative

controls which were incubated with nonimmunized goat IgG as a substitute for the primary antibody exhibited no immunoreaction as shown in Fig. 3A.

3.4. The effects of hPTH(1–34) on the localization of sclerostin-expressing cells in cortical bone

There were many sclerostin-positive osteocytes in both anterior and posterior regions of the in femoral cortical bone (~70% of total osteocytes) (Fig. 4A, B, C, and Supplemental Fig. 1A, B, C). We counted the number of sclerostin-positive osteocytes at the full thickness areas of the sagittal cortical bone sections from endosteal surface to periosteal surface (Supplemental Fig. 1A, B, C). Administration of hPTH(1–34) significantly decreased the number of sclerostin-positive osteocytes at both the anterior and posterior regions of the cortical bone on days 1 (Group 2) and 3 (Group 4) after daily hPTH(1–34) injection, but no significant changes on day 5 after daily injections (Group 7) (Supplemental Fig. 1A, B, C). A single injection of hPTH(1–34) significantly decreased the number of sclerostin-positive osteocytes in only the posterior region of the cortical bone on days 3 (Group 5) (Supplemental Fig. 1B, C).

Since the effect of hPTH(1–34) on the reduction of sclerostin-positive osteocytes was more prominent at the endosteal and the periosteal regions than the central region in the cortical bone, we next investigated the distribution of the sclerostin-positive osteocytes at the endosteal region (200 μm area from the endosteal surface). In this study, daily hPTH(1–34) treatments significantly decreased the number of sclerostin-positive osteocytes on days 1, 3, and 5 after the injections in posterior region (Fig. 4A, C), and on days 1 and 5 at anterior region (Fig. 4B). A single hPTH(1–34) treatment also induced a reduction on days 3 and 5 after the injection in both anterior and posterior regions (Fig. 4B, C). Negative controls which were incubated with nonimmunized goat IgG as a substitute for the primary antibody exhibited no immunoreaction as shown in Fig. 4A.

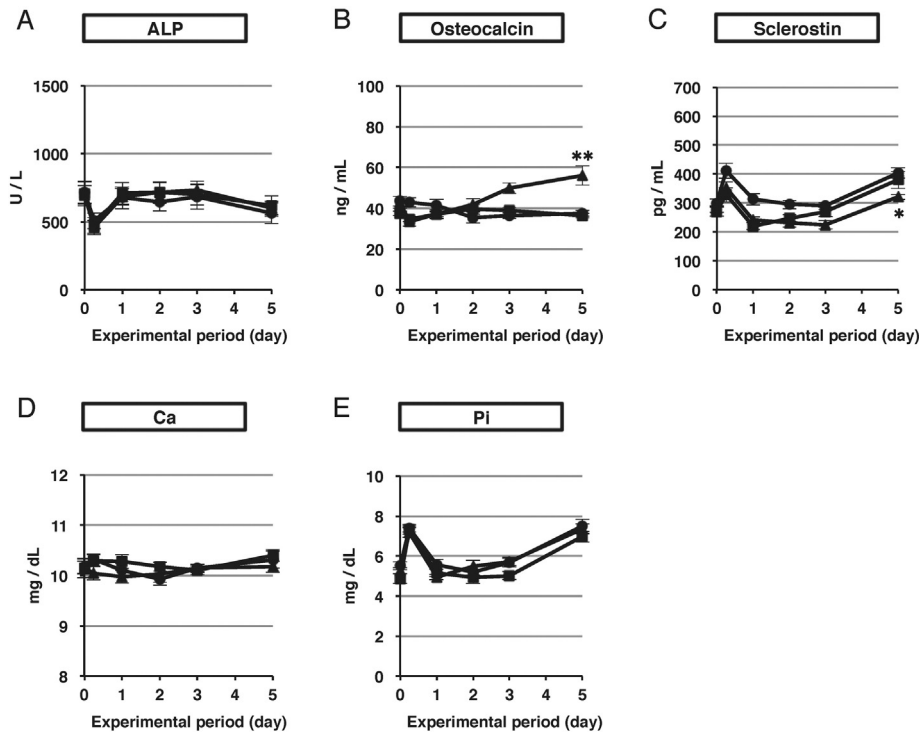


Fig. 6. Effects of hPTH(1–34) administration on serum markers. Time courses of the changes in alkaline phosphatase (A), osteocalcin (B), sclerostin (C), calcium (D), and inorganic phosphorus (E). Closed circles: control group, closed squares: single hPTH(1–34) injection group, closed triangles: daily hPTH(1–34) injection group. Statistical analyses compared to control group were performed by Dunnett's multiple comparisons test on day 5. * $p < 0.05$, ** $p < 0.01$. $n = 7$ –8 for each group.

3.5. Bone histomorphometric analyses

Fig. 5 summarizes the bone histomorphometric analyses of the cancellous bone area (A) and cortical bone area (B) on day 5 after hPTH(1–34) administration. In cancellous bone, hPTH(1–34) administration induced no significant change in bone volume (BV/TV) among the groups, but daily hPTH(1–34) administrations increased osteoblast surface (Ob.S), osteoblast surface (Ob.S/BS), osteoblast number (N.Ob/BS), osteoid thickness (O.Th), and osteoid surface (OS/BS) compared to those in the control group (Fig. 5A). A single injection of hPTH(1–34) significantly increased osteoid surface(OS/BS), but induced no significant change in osteoblast surface (Ob.S), osteoblast surface (Ob.S/BS), osteoblast number(N.Ob/BS), or osteoid thickness(O.Th) compared to that of the control group in cancellous bone area (Fig. 5A). In cortical bone, hPTH(1–34) administration increased osteoid thickness (O.Th), but no significant changes in osteoid surface (OS/BS) at the endosteal surface (Fig. 5B).

3.6. The effects of hPTH(1–34) on serum markers

Serum levels of osteocalcin, which is a bone formation marker, showed no significant difference among the control group, single hPTH(1–34) injection group, and daily hPTH(1–34) injection group until day 3 after the administration. The daily hPTH(1–34) injection group had a significantly increased serum osteocalcin level on day 5 compared to that of the control and single hPTH(1–34) injection group (Fig. 6B). Serum sclerostin levels also showed no significant changes among the control group, single hPTH(1–34) injection group, and daily hPTH(1–34) injection group until day 3 after the administration, while the daily hPTH(1–34) injection group had a significantly decreased serum sclerostin level on day 5 compared to that of the control and single hPTH(1–34) injection groups (Fig. 6C). There were no significant differences in serum levels of alkaline phosphatase activity (Fig. 6A), calcium (Fig. 6D), or inorganic phosphorus (Fig. 6E) among the control group, single hPTH(1–34) injection group and daily hPTH(1–34) injection group during the experiments.

4. Discussions

One of the major actions of PTH-induced anabolic bone action is stimulation of osteoprogenitor cells in bone marrow (Silva and Bilezikian, 2015; Nishida et al., 1994; He et al., 2013), but characterization of such progenitors has not been well documented. In the present study, we showed that hPTH(1–34) administration increased the number of osterix-positive cells in the bone marrow as early as day 1 after hPTH(1–34) injection (Fig. 1A, B). Mizoguchi et al. (2014) recently reported that osterix-expressing cells in the bone marrow of adult mice are osteolineage-restricted cells that lack the differentiation potential to become other mesenchymal cells. These findings suggest that the osterix-positive cells in bone marrow increased by hPTH(1–34) injection are osteoprogenitors, and such increment is an integral part of PTH-induced anabolic bone action.

Sclerostin is one of the target molecules related to PTH anabolic action (Silva and Bilezikian, 2015; Jilka, 2007; Kousteni and Bilezikian, 2008; Kramer et al., 2010; Ke et al., 2012; Weivoda and Oursler, 2014; Tu et al., 2015; Bellido et al., 2005; Keller and Kneissel, 2005; Silvestrini et al., 2007; Drake et al., 2010; Leupin et al., 2007; Ardawi et al., 2012), but there has been limited information about the effects of PTH administration on the precise distribution of sclerostin-positive cells in bone. Bellido et al. (Bellido et al., 2005) reported that continuous infusion of hPTH(1–34) to mice for 4 days decreased the expression of *Sost* mRNA and sclerostin by Western blotting in vertebrate bone, but 4 daily administrations induced no apparent reduction of *Sost* mRNA and sclerostin protein. They also showed that continuous injection induced dramatic decrease in sclerostin-positive osteocytes in cancellous bones by

immunohistochemistry, but intermittent treatment showed minimal effect on decrease in sclerostin-positive osteocytes in cancellous bone without any specific description about its distribution in cortical bones (Bellido et al., 2005). Keller et al. reported that long-term intermittent hPTH(1–34) injections into estrogen-deprived rats induced a sustained downregulation of *Sost* mRNA in femoral cortical bone (Keller and Kneissel, 2005). Since these reports implied that the effects of intermittent administration of PTH on distribution of sclerostin-positive osteocytes differ depending on parts of bone, we compared the distribution of sclerostin-positive osteocytes between cancellous bone and cortical bone after intermittent administration of hPTH(1–34). This study revealed that intermittent administration of hPTH(1–34) induced significant reduction in the number of sclerostin-positive osteocytes in cortical bone but not in cancellous bone (Figs. 3 and 4). We also showed the reduction in serum level of sclerostin on day 5 after daily administration of hPTH(1–34) (Fig. 6C). Apparent decrease in the number of sclerostin-positive osteocytes in cortical bone might reflect the reduction of the serum sclerostin level at early phase of PTH-induced anabolic action. Collectively, the intermittent administration of hPTH(1–34) more preferentially reduced the number of sclerostin-positive osteocytes in cortical bone than cancellous bone, and thus the increase in the osterix-positive osteoprogenitors in bone marrow might play more important role in anabolic bone action induced by hPTH(1–34) at early phase of the administration.

Although bone histomorphometric analyses in this study revealed that hPTH(1–34) injections induced no significant change in the volume of cancellous bone on day 5 after hPTH(1–34) administration, daily injections of hPTH(1–34) significantly increased the parameters related to bone formation, including osteoblast surface, osteoid thickness, and osteoid surface in cancellous bone, and only osteoid thickness in cortical bone. Significant increase in serum osteocalcin level on day 5 after daily injections of hPTH(1–34) may be due to the stimulation of bone formation in cancellous bones. These findings imply that the anabolic action of hPTH(1–34) first appears in cancellous bone via elevated levels of osterix-positive osteoprogenitors and subsequent differentiation into mature osteoblasts, while hPTH(1–34) anabolic action on cortical bone may be delayed because several signaling pathways, including the Wnt pathway, are activated after sclerostin downregulation. Only a limited number of reports investigated the effects of hPTH(1–34) on cancellous and cortical bones within 5 days after the hPTH(1–34) injection. Toromanoff et al. (Toromanoff et al., 1998) demonstrated that daily hPTH(1–34) injections increased the bone mineral density (BMD) of femurs on days 10 and 15 after hPTH(1–34) administration, but there was no change on day 5. These results are consistent with our results. Interestingly, Kim et al. found that intermittent hPTH(1–34) administration converts quiescent lining cells into mature osteoblasts at the periosteal region of femurs by tracing osteoblast-lineage cells using ROSA26R reporter mice crossed with *Dmp1*-CreERT2 mice (SW et al., 2012). This finding may reflect an early phenomenon of the anabolic action of hPTH(1–34) at the periosteal region of cortical bone.

5. Conclusions

This study suggested that the increase in osterix-positive osteoprogenitors in cancellous bone and the decrease in sclerostin-positive cells in cortical bone play important roles in hPTH(1–34)-induced anabolic action on osteogenesis at early phase.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bonr.2016.01.002>.

Conflict of interest

None.

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