Increased bone mass in adult prostacyclin-deficient mice

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Abstract

Prostaglandins (PGs) are key regulatory factors that affect bone metabolism. Prostaglandin E₂ (PGE₂) regulates bone resorption and bone formation. Prostacyclin (PGI₂) is one of the major products derived from arachidonic acid by the action of cyclooxygenase and PGI₂ synthase (PGIS). Unlike PGE₂, there are few reports about the role of PGI₂ in bone regulation. Therefore, we investigated the potential effect of PGI₂ on bone metabolism. We used PGIS knockout (PGIS^{-/-}), PGIS heterozygous (PGIS^{+/-}), and wild-type mice to investigate the role of PGI₂. Notably, PGIS^{-/-} mice gradually displayed an increase in trabecular bone mass in adolescence. Adult PGIS^{-/-} mice showed an increase in trabecular bone volume/tissue volume. Histomorphometric analysis showed that PGIS^{-/-} mice displayed increases in

both bone formation and bone resorption parameters. Levels of serum osteocalcin and C-telopeptides were increased in adult PGIS^{-/-} mice. Furthermore, the increased bone mass patterns were rescued in PGIS^{-/-} mice. In conclusion, adult PGIS^{-/-} mice displayed an overall increase in the levels of both bone formation and bone resorption parameters, which suggests that PGI₂ deficiency accelerates high bone turnover activity with a greater increase in bone mass in aging. These results indicated that PGI₂ may contribute to the maintenance of normal bone mass and micro-architecture in mice in age-dependent manner. Our findings demonstrate for the first time that PGI₂ is involved in bone metabolism *in vivo*.

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Introduction

Prostaglandins (PGs) are one of the regulatory factors that affect bone metabolism (Raisz 1995, 1999, Harada & Rodan 2003). They are produced by the sequential oxygenation of arachidonic acid by cyclooxygenases (COXs) and terminal PG synthases (Simmons *et al.* 2004). The metabolites of COXs are involved in skeletal development. PGE₂ regulates bone remodeling by mediating osteoblast differentiation (Yoshida *et al.* 2002, Arikawa *et al.* 2004) and regulates bone resorption by increasing cAMP levels through EP₂ and EP₄ receptor activation (Li *et al.* 2000, Miyaura *et al.* 2000, Yoshida *et al.* 2002, Sugimoto & Narumiya 2007). Prostacyclin (PGI₂), a product converted by COXs and PGI₂ synthase (PGIS), also up-regulates cAMP levels through the activation of a PGI₂ receptor (IP; Namba *et al.* 1994). Resident bone cells; osteoblasts and osteocytes, can produce both PGE₂ and PGI₂ under mechanical loading (Rawlinson *et al.* 1991, 2000). Osteoblasts also produce PGI₂ in response to growth factors such as vascular endothelial growth factor (Clarkin *et al.* 2008).

Genetic manipulation in mice has been a powerful tool for a better understanding of mammalian physiology. COX-2 gene deletion in mice induced a negative effect on bone formation by reducing osteoblastogenesis and delaying bone healing (Zhang *et al.* 2002, Naik *et al.* 2009), while skeletal abnormalities have not yet been reported in membrane-associated PGE synthase 1 null mice (Uematsu *et al.* 2002, Kamei *et al.* 2004).

Bone regulation during development processes has been widely studied (Harada & Rodan 2003). In contrast, information about controlling bone remodeling and maintaining bone mass in adults, which could contribute to therapeutic applications, remains limited. Although the critical role of PGE₂ in bone has been widely demonstrated, the role of PGI₂ is poorly understood. In a recent paper, it has been demonstrated that the injected iloprost, a stable PGI₂ analog, stimulated calvarial bone formation in postnatal rats (Tuncbilek *et al.* 2008). Hence, the focus of the present study was to investigate the potential roles of PGI₂ in bone metabolism.

In the present study, we investigated skeletal abnormalities in both young and adult PGI₂-deficient mice. Bone abnormalities were assessed by radiography, histomorphometric analysis, and the measurement of biological markers. We found an increase in the bone mass of adult PGI₂-deficient mice. Based on our results, the high bone mass phenotype in aged PGIS-null mice was a result from net favor of bone formation and mineralization. This phenotype was rescued in the offspring of PGIS-knockout mice crossbred with PGIS-transgenic mice. This evidence suggests that PGI₂ is involved in bone metabolism and contributes to the lifelong maintenance of normal bone mass and micro-architecture in mice.

Materials and Methods

Animals

Female PGIS-null mice in a C57BL/6 strain (Yokoyama *et al.* 2002) were used in our study. PGIS^{+/-} animals have no abnormal phenotype. PGIS-transgenic mice were generated in a C57BL/6 strain by the microinjection method. Mouse PGIS cDNA was inserted into the multicloning site of the modified pCI-neo mammalian expression vector (Promega), in which the CMV immediate early enhancer/promoter was replaced with the CMV early enhancer/chicken β -actin (CAG) promoter. The linearized plasmid was microinjected in fertilized eggs from C57BL/6J mice (CLEA Japan Inc., Osaka, Japan). The PGIS^{tg/tg} mice were crossbred with PGIS^{-/-} mice to generate PGIS^{-/-}/PGIS^{tg/tg} (PGIS⁻/^{tg}) littermates. PGIS gene expression levels in the PGIS⁻/^{tg} mice were confirmed by quantitative real-time PCR by using the DyNAmo probe qPCR kit (Finnzymes, Espoo, Finland). Mice were allowed food (CE-2; CLEA Japan Inc.) and distilled water *ad libitum*. Mice were maintained in a 12 h light:12 h darkness cycle. The experimental procedures were reviewed and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

Radiographic assessment of bone

Peripheral quantitative computed tomography analysis For the longitudinal assessment of bone, 5- to 34-week-old mice were kept alive during the bone density measurement by peripheral quantitative computed tomography (pQCT) (XCT Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany) (Aoki *et al.* 2006, Alles *et al.* 2009). To conduct high-resolution scanning mode pQCT, mice were anesthetized with injections of medetomidine hydrochloride (0.5 mg/kg; Meijiseika, Tokyo, Japan) and ketamine hydrochloride (50 mg/kg; Sankyo, Tokyo, Japan; Saito *et al.* 2007).

Soft X-ray, dual X-ray absorptiometry and micro-focal-computed tomography analysis The mice were killed by using ether anesthesia. Their hind limbs (tibias and

femurs) were harvested. Muscles and soft tissue were roughly removed from the bones, and then the bones were fixed in phosphate-buffered glutaraldehyde (2.5%)-formalin (4%) fixative (pH 7.4) for 2 days. Then, the samples were rinsed and kept in PBS for 2 days and water for 1 day. Afterward, the prepared bone samples were used for the soft X-ray radiographic analysis (SRO-M50; Sofron, Tokyo, Japan).

Trabecular and cortical regions of long bones were assessed by conducting a longitudinal study with dual X-ray absorptiometry (DXA; DCS-600R; Aloka, Tokyo, Japan; Saito *et al.* 2007, Hussain Mian *et al.* 2008, Alles *et al.* 2009) and determining the trabecular bone volume/tissue volume (%) (BV/TV) by microfocal CT (micro-CT; SMX-90CT, Shimadzu, Kyoto, Japan) respectively. Trabecular bone in secondary spongiosa was measured at a 450–500 μ m distance from the growth plate of the tibia to avoid primary spongiosa.

Histological assessment of bone

Mice received s.c. injections of demeclocycline hydrochloride (8 mg/kg of body weight; Sigma) and calcein (10 mg/kg of body weight; Sigma) at 10 days and 3 days prior to killing respectively. After micro-CT analysis, the bone specimens were embedded as undecalcified sections in mixtures of methyl methacrylate and 2-hydroxyethylmethacrylate resins, as described previously (Saito *et al.* 2007). Polymerization was performed at 4 °C. Standard sections (3 μ m thick) were prepared with a Reichert-Jung microtome 2050 Supercut (Cambridge Instrument, Heidelberg, Switzerland). Undecalcified sections were stained with tartrate-resistant acid phosphatase (TRAP) and counterstained with toluidine blue. Some sections were stained with Von Kossa for visualizing mineralization. Standard histomorphometric analysis of the tibial metaphysis was performed by using an image analyzing system (KS400, Carl Zeiss, Jena, Germany), as described elsewhere (Parfitt *et al.* 1987, Aoki *et al.* 2006).

Figure 1 PGI₂-deficient mice displayed skeletal changes in a time-course analysis. A longitudinal assessment of the bone density of mouse tibia was performed by pQCT. Trabecular bone density (A) and cortical bone density (B) in PGIS^{-/-} mice tibia from 5- to 33-week-old anesthetized mice were measured. PGIS^{+/-} mice were used as a control. Values are mean $\pm$ s.d., $n=3$, except 33-week-old PGIS^{-/-} mice, $n=2$, * $P<0.05$.

Measurement of bone biological markers

After blood samples were collected, serum osteocalcin levels were measured by using a Mouse osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA; Tomomatsu *et al.* 2009). The quantity of C-terminal telopeptides (CTX), which are generated by osteoclasts as a degradation product of type I collagen, was measured by RatLaps EIA (Immunodiagnostic system, Boldon, UK; Garner *et al.* 2003).

Statistical analyses

Statistical analysis was performed by using the Student's *t*-test and one-way ANOVA. When a significant *F* ratio was identified, groups were compared by using Tukey's honestly significant differences (HSD) protected least-significant difference *post hoc* test.

Results

Skeletal phenotypic change was detected in $PGIS^{-/-}$ mice

$PGIS^{-/-}$ mice were born at the expected Mendelian ratio with grossly normal bone shape and size. Body weights of $PGIS^{-/-}$ mice were similar to those of $PGIS^{+/-}$ and wild-type (WT) littermates. $PGIS^{+/-}$ mice showed no obvious phenotype change. We examined the change in bone content in 5-week-old anesthetized $PGIS$ -null mice by pQCT measurement, and the $PGIS^{-/-}$ mice showed a significant decrease in trabecular bone density. The pQCT measurement was repeated periodically. Over time, $PGIS^{-/-}$ mice developed an increase in trabecular bone density that became significantly different as compared to $PGIS^{+/-}$ littermates (by week 27; Fig. 1A). Cortical bone density in $PGIS^{-/-}$ mice at the later period of life was not significantly different (Fig. 1B).

Figure 2 Decreased bone mass in 5-week-old $PGIS^{-/-}$ mice. Five-week-old $PGIS^{-/-}$ mice had lower trabecular bone density, as determined by soft X-ray (A) (scale bar=5 mm) and micro-CT analysis of distal tibia metaphysis (B) (scale bar=1 mm). From DXA results, the tibial bone mineral density (BMD, mg/cm²) in $PGIS^{-/-}$ mice was lower than in wild-type (WT) and $PGIS^{+/-}$ mice (C). Micro-CT analysis demonstrated a lower trabecular bone volume/tissue volume (BV/TV, %) in $PGIS^{-/-}$ mice (D). Values are mean $\pm$ s.d., *n*=7, 4, and 6 respectively, **P*<0.05, ***P*<0.01.

Figure 3 Trabecular bone was increased in adult PGIS^{-/-} mice. The 34-week-old PGIS^{-/-} mice showed an increased bone mass phenotype at the distal metaphysis of the tibia and femur, as determined by soft tissue X-ray (A) and micro-CT analysis (B) respectively. Trabecular BV/TV (%) of the tibia metaphysis in PGIS^{-/-} mice was increased (C); values are mean $\pm$ s.d., $n=8$ for each group, ** $P<0.01$. Histology of the metaphysis of an undecalcified section of tibia of aged PGIS^{-/-} mice stained by Von Kossa staining (D) (scale bar = 0.5 mm) showed an increase in the mineralized bone matrix. Counterstaining with toluidine blue and TRAP specimens (E, scale bar = 400 μ m and F, scale bar = 200 μ m) showed an increase in the trabecular bone along the marrow space. An increase in TRAP-positive osteoclast cells occurred along the bone surface.

At 5 weeks of age, lower bone mineral density (BMD) was observed in $PGIS^{-/-}$ mice by soft X-ray photographs (Fig. 2A), micro-CT (Fig. 2B), and DXA analysis (Fig. 2C). To determine the skeletal change in more detail, we performed bone analysis by using micro-CT analysis. Fractional trabecular bone volume BV/TV (%) in $PGIS^{-/-}$ mice was decreased as compared to that in the WT mice (Fig. 2D).

Adult $PGIS^{-/-}$ mice have increased trabecular bone mass

The increasing bone mass phenotype was observed in $PGIS^{-/-}$ mice (Fig. 3A and B). Corresponding to the time-dependent study by pQCT measurement, 34-week-old $PGIS^{-/-}$ mice showed an increase in the trabecular BV/TV as compared to $PGIS^{+/+}$ mice (Fig. 3C) (each group, $n=8$). This increase can be visualized in two-dimensional micro-CT images at the dense trabeculation in metaphyseal regions along the midshaft area. Although female mice were used in our study, these bone phenotypes were observed in both females and males (Supplementary Figure 1A and B, see section on supplementary data given at the end of this article).

Based on histochemical sections of tibia examined by Von Kossa staining, the $PGIS^{-/-}$ mice showed a greater mass of trabecular bone along the marrow space of the tibia distal metaphyseal area, as compared with the $PGIS^{+/+}$ mice. The results of Von Kossa staining indicated that the trabecular bones filling in the marrow space were well mineralized in adult $PGIS^{-/-}$ mice (Fig. 3D). Toluidine blue and TRAP staining showed an increase in the number of osteoclast-like cells along the newly formed bone surface (Fig. 3E and F). In a histomorphometric analysis of adult $PGIS^{-/-}$ mice, both bone formation and bone resorption parameters were increased (Fig. 4A–G). To determine whether the increase in trabecular bone mass was due to an acceleration in bone formation, a dynamic histomorphometric analysis was performed by using double-fluorochrome labeling at a 7-day interval with demeclocycline and calcein, which are markers of the amount of newly formed bone (Fig. 5A). The results demonstrated increases in the bone formation parameters of mineral surface per bone surface, bone formation rate, and mineral appositional rate (Fig. 5B–D). The serum osteocalcin level, which is a biochemical parameter for bone formation, was significantly increased in $PGIS^{-/-}$ mice (Fig. 5E). Serum CTX, which represents bone resorption activity, was also increased in $PGIS^{-/-}$ mice (Fig. 5F).

Bone phenotype was rescued in crossbred $PGIS^{-/-}$ $PGIS^{tg/tg}$ mice

The $PGIS$ gene was abundantly expressed in $PGIS^{tg/tg}$ mice. The mRNA expression in $PGIS^{tg/tg}$ mice was increased 2.5- to 3-fold compared to that in WT mice (data not shown). $PGIS^{tg/tg}$ mice displayed no skeletal phenotypic change. We next examined whether changes in skeletal phenotypes occurred in the offspring of overexpressed $PGIS$ mice crossbred with $PGIS$ -null mice. The $PGIS^{-/-}$ $PGIS^{tg/tg}$ mice showed

the rescuing of the bone phenotype, as confirmed by micro-CT analysis (Fig. 6A). The trabecular BV/TV value was recovered by $\sim 50\%$ as compared to $PGIS^{-/-}$ mice (Fig. 6B). To confirm that the morphological changes corresponded to the biochemical aspect, we measured serum osteocalcin and CTX. Similar to the radiographic results, the serum osteocalcin level in $PGIS^{-/-}$ $PGIS^{tg/tg}$ mice tended to be recovered to the level in $PGIS^{+/+}$ mice (Fig. 6C); the serum CTX level in $PGIS^{-/-}$ $PGIS^{tg/tg}$ mice was significantly lower than that in $PGIS^{-/-}$ mice and was similar to the level in $PGIS^{+/+}$ mice (Fig. 6D). Notably, $PGIS^{-/-}$ $PGIS^{tg/tg}$ mice were not different from the $PGIS^{+/+}$ group by micro-CT and histological analysis (Supplementary Figure 2A–C, see section on supplementary data given at the end of this article).

Figure 4 The increase in trabecular bone in $PGIS^{-/-}$ mice was the net result of increased bone forming and bone resorption parameters. Histomorphometric analysis of undecalcified tibia metaphysis sections of adult $PGIS^{-/-}$ mice was measured by KS400 analyzer. Aged $PGIS^{-/-}$ mice displayed a significant increase in BV/TV (%) (A) and trabecular number (TbN, no/mm) (B), and a decrease in trabecular separation (TbSp, μm) (C) and $PGIS^{-/-}$ mice showed normal trabecular thickness (TbTh, μm) (D). A significant increase in the osteoblast surface/bone surface (ObS/BS %) was observed in $PGIS^{-/-}$ mice (G). Osteoclast number per bone surface (NOc/BS, %) and osteoclast surface per bone surface (OcS/BS, %) were also increased in $PGIS^{-/-}$ animals (E and F). Values are mean $\pm$ s.d., $n=5$, * $P<0.05$, ** $P<0.01$.

Discussion

PGs usually have a biphasic effect on bone formation, which is mediated by cAMP regulation (Raisz & Fall 1990, Raisz 1999). The roles of endogenous PGs in skeletal growth are suggested by studies of fracture repair and heterotopic ossification, which probably involve the production of both PGE₂ and PGI₂ (Rawlinson *et al.* 1991, 2000, Raisz 1999). PGs have important effects on the skeleton, and it is strongly

suggested that these effects are moderated by different receptors and specific pathways, which still remain unidentified. A large number of specific PG receptor gene-deleted animals have been cloned (Sugimoto & Narumiya 2007). PGE₂ stimulates bone formation and bone resorption via the EP4 receptor. EP4 agonist is an effective bone anabolic agent in rats (Hagino *et al.* 2005, Ito *et al.* 2006); however, the efficacy of the anabolic effect in mice is limited (Kato *et al.* 2007). In regards to the bone phenotype in EP4-knockout animals,

Figure 5 Adult PGIS^{-/-} mice have increased bone turnover activity. Dynamic histomorphometry of trabecular bone in undecalcified sections of the metaphysis of tibia was studied by double-fluorochrome labeling. Demeclocycline (yellow) and calcein (green) were injected in 7-day intervals (A) (scale bars; red = 40 μm and yellow = 5 μm). PGIS^{-/-} mice displayed a significant increase in the bone formation rate per bone surface (BFR, μm³/μm² per d) (B), mineral apposition rate (MAR, μm/d) (C), and mineral surface per bone surface (MS/BS, %) (D); values are mean ± s.d., 34-week-old PGIS^{-/-} mice, *n* = 5 for each group. Serum osteocalcin (OCN) and CTX levels were measured by ELISA (E and F). Values are mean ± s.d., *n* = 6 for each group, **P* < 0.05, ***P* < 0.01.

Figure 6 Enhancement of PGIS gene expression recovered the high bone mass phenomenon. PGIS^{-/-tg} littermates showed a 50% recovery of the high bone mass phenotype, as determined by micro-CT image analysis (A and B) (values are mean $\pm$ s.d., $n=8$, except PGIS^{-/-tg} mice, $n=6$, * $P<0.05$). Two serum bone markers, OCN (C) and CTX (D), were quantified by ELISA. Values are mean $\pm$ s.d., $n=6$, * $P<0.05$, ** $P<0.01$.

mice with a global deletion of the EP4 receptor developed osteopenia and impaired fracture healing with age (Li *et al.* 2005), while a recent study showed that targeted deletion of the EP4 receptor showed no skeletal change in aged mice (Gao *et al.* 2009). Thus, the roles of PGI₂ in bone metabolism may be associated with different underlying mechanisms. It has been demonstrated that the injected iloprost, a stable PGI₂ analog, stimulated calvarial bone formation in postnatal rats (Tuncbilek *et al.* 2008). Their data may support our present observation in which at 5 weeks of age, the BMD was lower in PGIS^{-/-} mice.

In the present study, we primarily observed the skeletal changes in PGIS^{-/-} mice by pQCT and micro-CT analysis. Lower bone mass was observed in 5-week-old PGIS^{-/-} mice and was corrected at puberty period at 6–8 weeks old, which may have occurred from adapting process or changes in endogenous hormone levels. Unexpectedly, PGIS^{-/-} mice showed a gradual increase in trabecular density and BV/TV values in age-dependent manner, while changes in cortical component in adult period of life were limited.

By histology and histomorphometry, we found that adult PGIS^{-/-} mice displayed increases in bone formation and bone resorption parameters. This phenomenon was confirmed by measurements of serum bone markers,

osteocalcin and CTX, which were significantly increased. The total net result in increased bone mass may be caused by the favoring of bone formation over bone resorption. Therefore, we performed a mineralized nodule formation assay, osteoclast formation, and pit assay with bone marrow cells derived from these mice. However, these *in vitro* assays did not show any differences between PGIS^{-/-} and PGIS^{+/-} mice (Supplementary Figure 3A–D, see section on supplementary data given at the end of this article). Thus, our *in vitro* data suggest that the lack of PGI₂ does not affect the ability of bone precursor cells to differentiate.

While PGIS^{tg/tg} mice showed no distinct skeletal changes, bone mass and serum bone markers of the crossbred PGIS^{-/-tg} mice were rescued back to normal, as compared with those of knockout animals. These data confirmed that the lack of PGI₂ in mice caused the accelerated bone turnover and that PGI₂ may play a role as a negative regulator of bone metabolism, although it is not clear how the mechanism of PGI₂ is involved.

Bone is a highly vascular structure. Blood supply in bone is important for bone modulation and remodeling. PGI₂ is mainly released by vascular endothelial cells. The signaling of PGI₂ via its cell surface receptor, the IP receptor, is well documented in vascular biology and inflammatory responses. IP receptor serves the critical roles with the vasodilatation, mediating platelet aggregation and controlling vascular smooth muscle cell proliferation (Namba *et al.* 1994, Murata *et al.* 1997, Stitham *et al.* 2007). It is demonstrated that there is a communication between endothelial and bone cells, which is important for controlling vascular supply during bone growth, remodeling, and repair (Deckers *et al.* 2000, Clarkin *et al.* 2008). However, there is no report of any changes in the bone phenotype of IP receptor knockout mice. Meanwhile, PGI₂ action via nuclear receptors in other physiological responses is gradually being more appreciated. PGI₂ exerts its effects by interacting with a nuclear hormone receptor, the peroxisome proliferator-activated receptor (PPAR). Therefore, the response to PGI₂ should be considered by both IP and PPAR in certain cell types (Namba *et al.* 1994, Hertz *et al.* 1996, Hatae *et al.* 2001). One of the three subtypes of PPARs, the PPAR δ uses PGI₂ as an endogenous ligand to modulate specific cellular and metabolic functions (Lim *et al.* 1999, Gupta *et al.* 2000, Hatae *et al.* 2001). Further studies are needed to determine whether PGI₂ is involved with PPAR δ signaling in bone tissues.

In conclusion, we demonstrated that the bone mass in adult PGI₂-deficient mice is increased. This increase in bone mass was associated with an increase in both bone formation and bone resorption, which suggests that PGI₂ deficiency accelerates high bone turnover activity with a greater increase in bone mass associated with aging. Furthermore, this imbalance was reversed in knockout animals by the insertion of PGIS overexpression. Our finding suggests that PGI₂ may play a role as a regulator in maintaining normal bone mass and micro-architecture.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-09-0376>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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