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FIGURE LEGENDS

Figure 1 Linear relationship of fasting blood glucose and C-peptide levels

Seven cases of islet transplantation of 4 patients (A-D) were plotted. Data from the first transplantation were shown in filled circles and solid lines and data from the second transplantation (A, B and C) were shown in open circles and broken lines. Coefficient of determination (R^2) and P-value (P) are shown.

Figure 2 The SUIT index in a patient receiving exogenous insulin therapy

The clinical course of the SUIT index in a patient after islet transplantation was shown. Amount of exogenous insulin injection and the SUIT index were plotted against post-operative day.

Figure 3 Validation of the SUIT index as pancreatic β-cell function

Correlation of the SUIT index and the acute insulin response to glucagon were shown in islet-transplanted patients (A) and type 2 diabetic patients (B). Serum C-peptide levels were measured 6 min after intravenous injection of glucagon (1 mg). Coefficient of determination (R^2) and P-value (P) are

shown.

Figure 4 The SUIT index as early predictor of islet transplantation efficacy

The SUIT indexes were plotted against post-operative day. Data from the first transplantation were shown in filled circle and data from the second transplantation were shown in open circles.

Fig 1

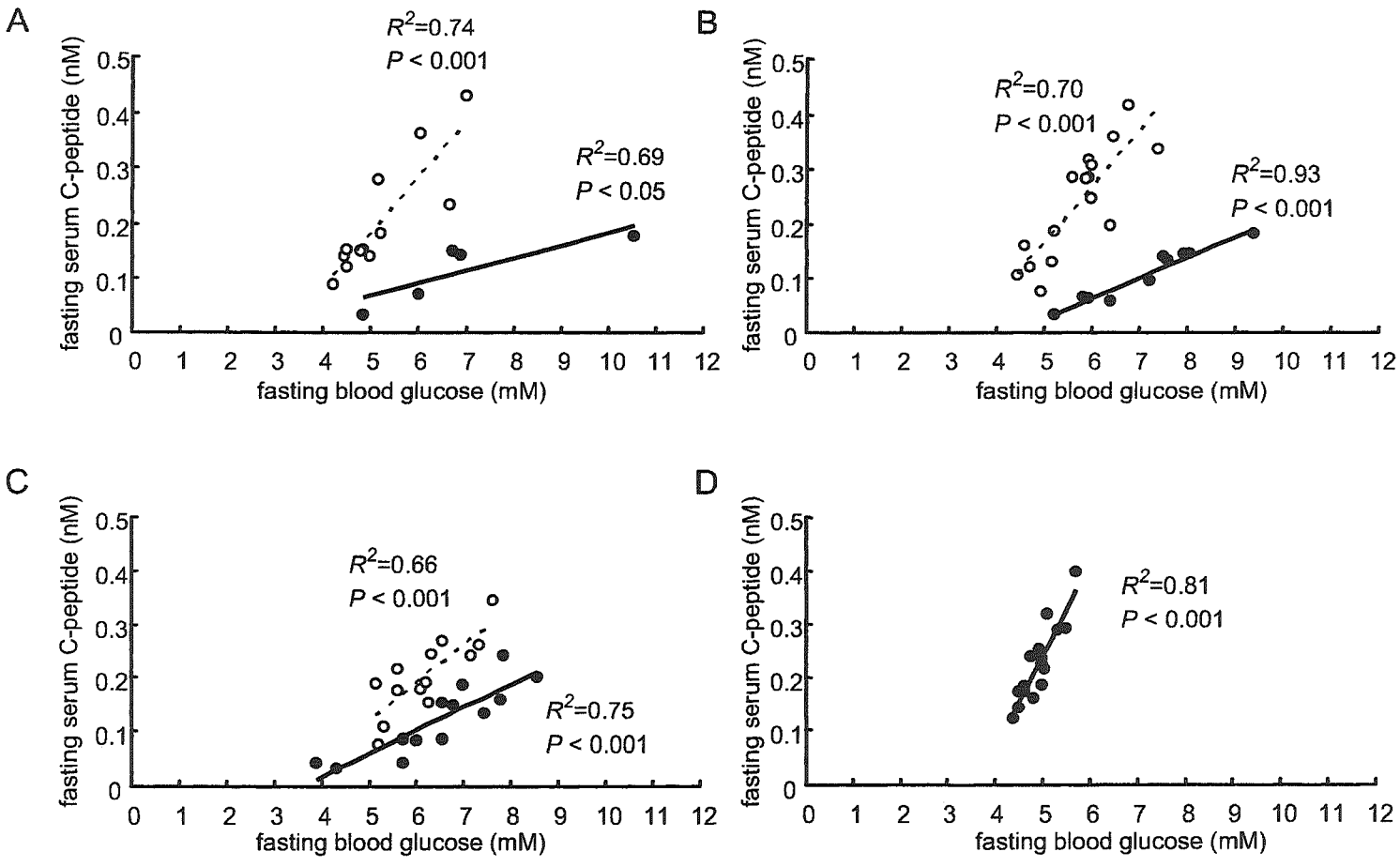

A
R^2=0.74
P < 0.001
R^2=0.69
P < 0.05
B
R^2=0.70
P < 0.001
R^2=0.93
P < 0.001
C
R^2=0.66
P < 0.001
R^2=0.75
P < 0.001
D
R^2=0.81
P < 0.001
fasting serum C-peptide (nM)
fasting blood glucose (mM)
0 0.1 0.2 0.3 0.4 0.5
0 1 2 3 4 5 6 7 8 9 10 11 12

Fig 2

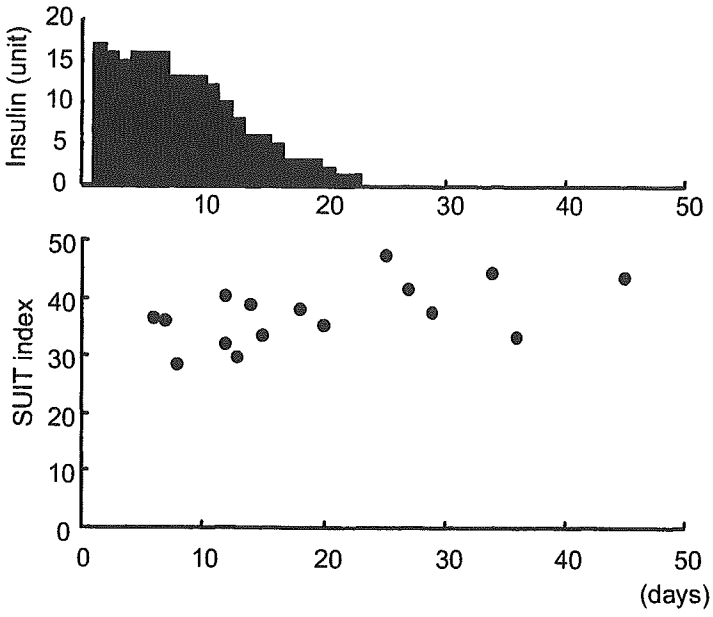
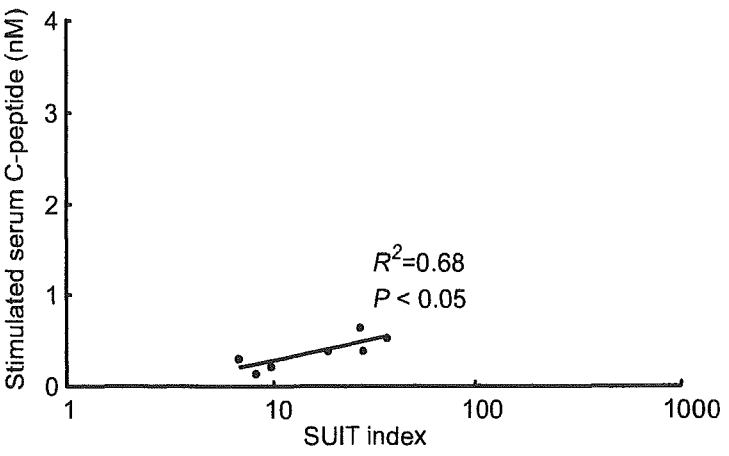

Insulin (unit)
20
15
10
5
0
10
20
30
40
50
SUIT index
50
40
30
20
10
0
0
10
20
30
40
50
(days)

Fig 3

A


Stimulated serum C-peptide (nM)
4
3
2
1
0
R^2=0.68
P < 0.05
1
10
100
1000
SUIT index

B

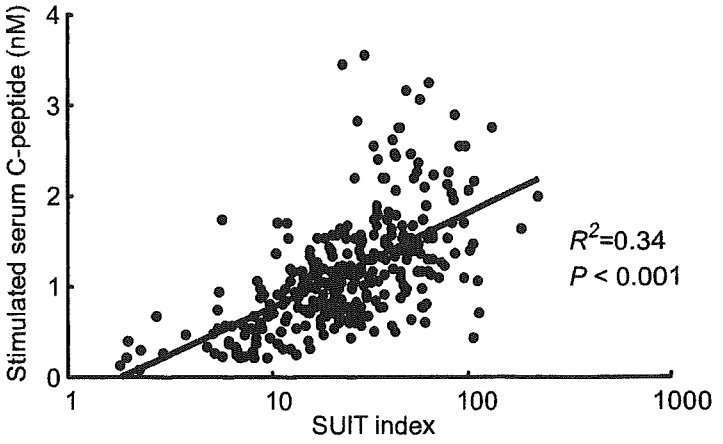
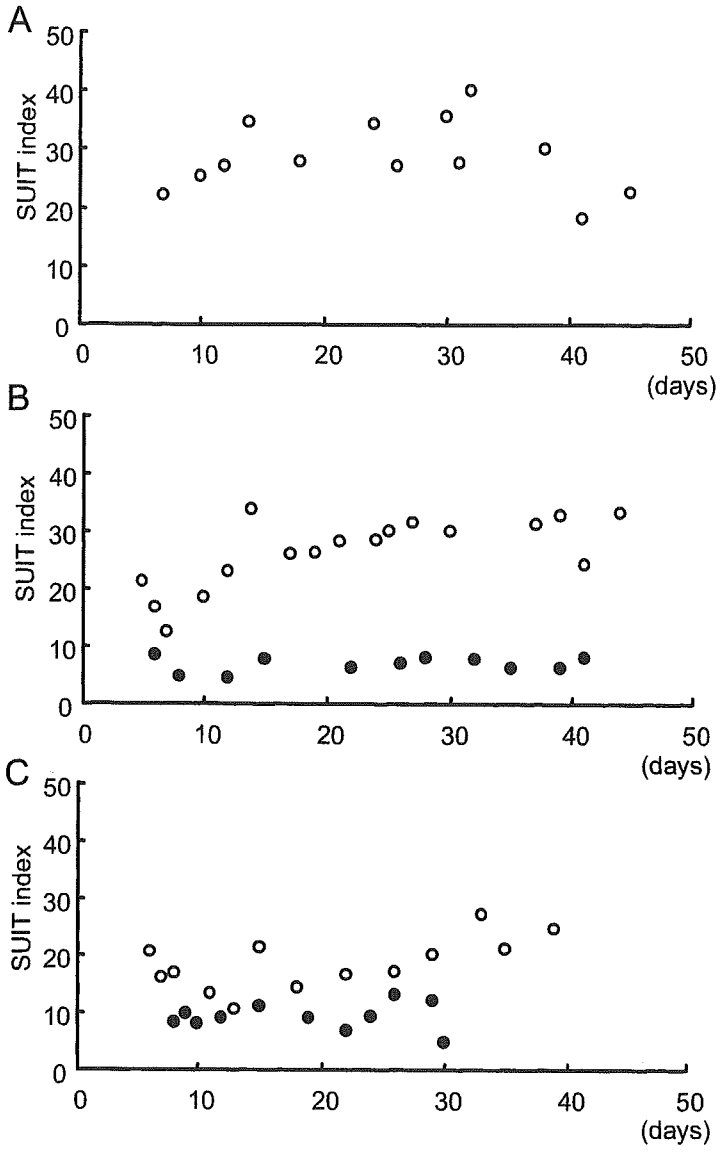

Stimulated serum C-peptide (nM)
4
3
2
1
0
R^2=0.34
P < 0.001
1
10
100
1000
SUIT index

Fig 4


A
SUIT index
50
40
30
20
10
0
0
10
20
30
40
50
(days)
B
SUIT index
50
40
30
20
10
0
0
10
20
30
40
50
(days)
C
SUIT index
50
40
30
20
10
0
0
10
20
30
40
50
(days)

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Gastric inhibitory polypeptide as an endogenous factor promoting new bone formation following food ingestion

Abbreviated title: GIP as an endogenous osteogenic factor

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KEYWORD

GIP, incretin, PTH, osteoporosis, osteoblast

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Calcium plays a fundamental role as second messenger in intracellular signaling and bone serves as the body's calcium reserve to tightly maintain blood calcium levels. Calcium in ingested meal is the main supply and inadequate calcium intake causes osteoporosis and bone fracture. Here we describe a novel mechanism how ingested calcium is deposited on bone. Meal ingestion elicits secretion of the gut hormone gastric inhibitory polypeptide (GIP) from endocrine K cells in the duodenum. Bone histomorphometrical analyses revealed that bone formation parameters in the mice lacking GIP receptor (*GIPR*$^{-/-}$) were significantly lower than those of wild-type (*GIPR*$^{+/+}$) mice, and that the number of osteoclasts, especially multinuclear osteoclasts, was significantly increased in *GIPR*$^{-/-}$ mice, indicating that *GIPR*$^{-/-}$ mice have high-turnover osteoporosis. *In vitro* examination showed the percentage of osteoblastic cells undergoing apoptosis to be significantly decreased in the presence of GIP. Since *GIPR*$^{-/-}$ mice exhibited an increased plasma calcium concentration after meal ingestion, GIP directly links calcium contained in meal to calcium deposition on bone.

Introduction

Osteoporosis is the major cause of fractures in elderly men and women, and is a growing health-care problem in the world (1). In addition to its structural role, bone serves as the body's nutrient reserve of calcium to maintain blood calcium levels. Locally, this function is accomplished through continuous tissue renewal, called bone remodeling (2-4). Old bone is continuously resorbed by the haematopoietically-derived osteoclasts, and new bone is formed from the mesenchyme-derived osteoblasts. Such intercellular communication between osteoblasts and osteoclasts is crucial in bone remodeling.

Ingested calcium is crucial for bone health throughout life. Even in the satiate present, calcium is still insufficient and the elderly are much more likely to have insufficient calcium intake (5). Calcium alone is partially effective in preventing bone loss (6-8). However, besides the epidemiological data, little is known about the molecular mechanisms of calcium metabolism, especially the pathway from calcium derived from food to calcium deposition in bone.

Gastric inhibitory polypeptide (GIP) is a gastrointestinal peptide hormone of 42 amino acids that is released from duodenal endocrine K cells after absorption of glucose or fat (9,10). GIP was originally isolated from porcine intestine on the basis of its ability to inhibit gastric acid secretion (11) and subsequent studies of GIP revealed that GIP potentiates glucose-induced insulin secretion from pancreatic β-cells (12), so GIP also is referred to as glucose-dependent insulinotropic polypeptide. We have isolated a human cDNA and gene encoding the GIP precursor and the human GIP receptor (GIPR) (13-15), confirming that GIP belongs to the vasoactive intestinal peptide/glucagon/secretin family.

The GIPR has seven potential membrane-spanning domains, a feature characteristic of G protein-coupled receptors and is expressed in various cells including pancreatic β-cells, adipocytes, and osteoblasts (16). We have developed mice with a targeted mutation of the GIPR gene (*GIPR*$^{-/-}$) and revealed that insulin secretion from the pancreatic β-cells is regulated not only by glucose but also by GIP, especially in the early phase after glucose ingestion and that GIP is the major insulinotropic factor in response to oral glucose loading in ATP-sensitive potassium channel-deficient mice (17,18). Furthermore, GIP directly stimulates nutrients uptake into adipocytes and *GIPR*$^{-/-}$ mice fed a high-fat diet were clearly protected from obesity and insulin resistance, indicating that GIP promotes the efficient storage of ingested fat (19). Here we investigate the role of GIP on osteoblasts and revealed that GIP promotes the efficient storage of ingested calcium into bone. As intermittent administration of parathyroid hormone (PTH) can effectively prevent osteoporotic fractures (20,21), intermittently elevation of blood GIP levels elicited by meals plays a crucial role on preventing pathogenesis and development of osteoporosis.

Results

No effect of GIP signaling on endochondral ossification.

The longitudinal bone growth was determined by endochondral ossification in the cartilaginous growth plate. *GIPR*$^{-/-}$ mice have similar naso-anal length to wild-type (*GIPR*$^{+/+}$) mice (**Fig. 1A**). Soft X-ray analysis showed that longitudinal growth of limb bones is not affected in *GIPR*$^{-/-}$ mice (**Fig. 1B**). These observations indicate that GIP signaling does not affect endochondral ossification.

Inhibition of GIP signaling causes thinner bone trabeculae

Histological analyses of 8-week-old male mice showed thinner trabeculae in the bones of *GIPR*$^{-/-}$ mice compared to those of *GIPR*$^{+/+}$ mice (**Fig. 2A and 2B**), and the phenotype was observed in both sexes and was also observed in femurs (data not shown).

Inhibition of GIP signaling causes decreased osteoblastic bone formation and increased osteoclastic bone resorption

Histochemical analyses showed that the number of osteoblasts in *GIPR*$^{-/-}$ mice was similar to that in *GIPR*$^{+/+}$ mice (**Fig. 3A**), and the number of osteoclasts was increased in *GIPR*$^{-/-}$ mice (**Fig. 3B**), which is consistent with the increased urinary elimination of deoxypyridinoline crosslink, a biochemical marker of bone resorption, in *GIPR*$^{-/-}$ mice (**Fig. 4A**). These phenotypes were observed in both sexes and were also observed in femurs (data not shown). We performed the bone histomorphometrical analyses of younger *GIPR*$^{+/+}$ and *GIPR*$^{-/-}$ mice (6-week-old) and quantified the skeletal phenotype (**Table 1**). The trabeculae tended to be thinner in *GIPR*$^{-/-}$ mice, and cellular activity parameters about bone formation revealed that bone formation parameters in *GIPR*$^{-/-}$ mice were significantly lower than those of *GIPR*$^{+/+}$ mice (**Table 1**). On the other hand,

cellular activity parameters about bone resorption, such as the number of osteoclast, especially multinuclear osteoclast which are the fully-differentiated cells and are responsible for active bone resorption, were significantly increased in *GIPR*$^{-/-}$ mice (**Table 1**). These changes observed in *GIPR*$^{-/-}$ mice indicate decreased osteoblastic bone formation and increased osteoclastic bone resorption.

Effect of GIP signaling on calcium homeostasis

We compared the calcium concentrations before and after meal ingestion. *GIPR*$^{+/+}$ mice showed no significant difference in the fasting and fed plasma calcium concentration (**Fig. 4B**). In contrast, *GIPR*$^{-/-}$ mice had similar levels of plasma calcium before meal ingestion, but had higher levels after meal ingestion (**Fig. 4B**). These findings and the results of cellular activity parameters about bone formation of *GIPR*$^{-/-}$ mice indicated that GIP directly links calcium contained in meal to calcium deposition on bone.

No direct effect of GIP on osteoclasts

To determine if the effects of GIP are the results of direct action of the hormone on the osteoclasts or indirect action mediated by osteoblasts, we investigated the effects of GIP using cell culture. First, we examined the effects of GIP on survival and pit-forming activity of mature osteoclasts (2). When crude osteoclast preparation was placed on dentine slices, many resorption pits were formed. Although calcitonin strongly inhibited pit-formation by osteoclasts, GIP showed no inhibitory effect on the pit-forming activity of osteoclasts (**Fig. 5A, 5B**). These results indicate that GIP does not directly inhibit osteoclasts function.

Anti-apoptotic effect of GIP on osteoblasts

Since osteoclast differentiation and function are regulated by osteoblasts (2), we examined the direct effects of GIP on osteoblasts. Treatment of Saos-2 cells, a human osteoblastic cell line, with GIP increased the intracellular concentration of cAMP, indicating functional expression of GIP receptors on Saos-2 osteoblastic cells (**Fig. 6A**). Exposure to etoposide, one of the representative pro-apoptotic reagents acting on osteoblasts, for 6 hours induced apoptosis of Saos-2 cells, as indicated by the appearance of pyknotic nuclei (**Fig. 6B**). The percentage of cells undergoing apoptosis was significantly decreased by pretreatment of GIP or PTH (**Fig. 6C**). The anti-apoptotic effect of GIP was dose-dependent and still evident at 1 nM. The results of the same experiments using primary mouse osteoblasts confirmed that GIP prevents etoposide-induced apoptosis (**Fig. 6D**).

Discussion

Calcium plays a fundamental role as second messenger in intracellular signaling. Ca^{2+} information is processed by sensor proteins, like calmodulin and Ca^{2+}-sensitive enzymes, and transmitted to target molecules to exert a lot of biological effects. Therefore, blood calcium levels should be tightly controlled by proper balance between calcium intake from ingested meal and calcium excretion to urine and stool and bone serves as the body's calcium reserve. Even in the satiate present, calcium is still insufficient and osteoporosis is a growing health-care problem in the world. However, the molecular mechanism underlying the deposition of calcium from meal is known little.

Cyclic activation of cell surface receptors often leads to a different biological response than sustained activation. Continuous activation of PTH receptors by endogenous PTH functions to maintain normal extracellular calcium levels by enhancing osteoclastic bone resorption through activation of osteoblasts and liberation of calcium from the adult skeleton. In contrast, cyclic activation of PTH receptors by exogenous PTH when administered intermittently as a pharmacologic agent is known to exert an anabolic effect on bones (20-21). Protecting osteoblasts from apoptosis has been reported to contribute (22-24). However, the physiological role of anabolic effects of PTH has not yet been determined. Considering that plasma GIP levels are greatly increased after meal ingestion (10), and that GIP, like PTH, protects osteoblasts from apoptosis, we hypothesized that GIP plays a physiological role in calcium metabolism *in vivo*.

The results of resorption pit assay indicated that GIP has no direct effects on mature osteoclasts. Therefore, primary site of GIP action is osteoblasts. We are supposing that,

in *GIPR*$^{-/-}$ mice, osteoblasts are stimulated continuously by PTH and intermittently by GIP in vivo, and in *GIPR*$^{-/-}$ mice, osteoblasts are stimulated only by PTH. Continuous stimulation of PTH has been shown to induce increased bone resorption rather than decreased bone formation, consistent with our results. The differential responses of skeletal bone to intermittent elevation of endogenous GIP versus continuous elevation of endogenous PTH, both of which increase the intracellular cAMP concentration, represent a systemic regulator of bone remodeling with important clinical and therapeutic implications (**Figure 6**). As GIP receptors are expressed in small intestine and administration of GIP inhibits gastrointestinal motility, it is possible that GIP inhibits intestinal calcium absorption. However, there have been no reports examining the effects of GIP on calcium absorption. Further examinations are required to elucidate the role of GIP on extra-bone tissues.

The metabolically thrifty GIP gene promotes not only the efficient storage of ingested fat (19) but also of ingested calcium, and the GIP signal represents a novel therapeutic strategy for anti-osteoporosis drugs.

Methods

Animals. The generation of *GIPR*$^{-/-}$ mice was previously described (17-19). The Animal Care Committee of Kyoto University Graduate School of Medicine approved animal care and procedures.

Bone Histomorphometry. To assess the parameters for bone histomorphometry, 6-week-old male mice were double labeled with subcutaneous injections of 30 mg/kg of tetracycline hydrochloride (Sigma, St. Louis, MO) at 4 days before sacrifice, and 10 mg/kg of calcein (Dojindo Co., Kumamoto, Japan) at 2 days before sacrifice. Tibiae were removed from each mouse, and fixed with 70% ethanol. They were trimmed to remove the muscle, stained with Villanueva bone stain for 7 days, dehydrated in graded concentrations of ethanol, and embedded in methyll-methacrylate (Wako Chemicals, Kanagawa, Japan) without decalcification. Frontal plane sections (5-μm-thick) of the proximal tibia were cut using a Microtome (LIECA, Germany). The cancellous bone was measured in the secondary spongiosa located 500 μm from the epiphyseal growth plate and 160 μm from the endocortical surface. Bone histomorphometric measurements of the tibiae were made using a semiautomatic image analyzing system (System Supply, Nagano, Japan) and a fluorescent microscope (Optiphot; Nikon, Tokyo, Japan) set at a magnification of 400×. Standard bone histomorphometrical nomenclatures, symbols, and units were used as described in the report of the ASBMR Histomophometry Nomenclature Committee (25). Statistical analysis was done using Student's t test.

Histochemistry. The tibiae of 8-week-old male mice were dissected free and immersed in the fixative at 4 °C for 8 hours. The specimens were then decalcified with 4.13 % EDTA at 4 °C for 2 weeks, dehydrated with an increasing concentration of ethanol, and