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High-phosphorus diet stimulates receptor activator of nuclear factor-κB ligand mRNA expression by increasing parathyroid hormone secretion in rats

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The purpose of the present study was to clarify the manner by which the supplementation of high-P diet induces bone loss. Eighteen 4-week-old male Wistar-strain rats were assigned randomly to three groups and fed diets containing three P levels (0·3, 0·9, and 1·5 %) for 21 d. A lower serum Ca concentration was observed in the rats fed on the 1·5 % P diet than in the other two groups. Serum P and parathyroid hormone concentrations and urinary excretion of C-terminal telopeptide of type I collagen were elevated with increasing dietary P levels. Serum osteocalcin concentration was increased in the rats fed on the 1·5 % P diet than in the other two groups. Bone formation rate of the lumbar vertebra was significantly increased in the two high-P groups than in the 0·3 % P group. Osteoclast number was significantly increased with increasing dietary P levels. Bone mineral content and bone mineral density of the femur and lumbar vertebra and ultimate compression load of the lumbar vertebra were decreased with increasing dietary P levels. Additionally, ultimate bending load of the femur was decreased in the rats fed on the 1·5 % P diet than in the other two groups. Receptor activator of NF-κB ligand (RANKL) mRNA expression in the femur was significantly higher with increasing dietary P levels. These results suggest that secondary hyperparathyroidism due to a high-P diet leads to bone loss via an increase in bone turnover. Furthermore, an increase in osteoclast number was caused by increased RANKL mRNA expression.

High-phosphorus diet: Parathyroid hormone: Receptor activator of nuclear factor-κB ligand mRNA expression: Bone loss

To maintain bone metabolism, adequate P intake, in addition to adequate Ca intake, is an important dietary factor. The adequate intakes of Ca and P for adults in Japan are 650–900 and 1050 mg/d, respectively. However, the total P consumption is increasing day by day and is presumably more than 1500 mg/d, considering the consumption of P-containing imported food products and food additives in Japan (Takeda *et al.* 2002). There are some reports regarding the relationship between high P intake and bone metabolism in animals and human subjects. In human adults, consumption of food containing P additives increased urinary hydroxyproline excretion, a bone resorption marker (Bell *et al.* 1977). In young female rats, a low-Ca and high-P diet clearly decreased the bone density (Bauer & Griminger, 1983). In ageing rats, a high-P diet elevated urinary hydroxyproline excretion (Draper *et al.* 1972). Thus, high P consumption resulted in bone loss as shown by an increase in the bone resorption marker and a decline in the densitometric quantity. Therefore, dietary P supplementation is considered as one of the risk factors of osteoporosis.

A characteristic phenomenon caused by the supplementation of a high-P diet is the increase in parathyroid hormone (PTH) secretion. Many studies have reported that the consumption of a high-P diet elevates serum PTH concentration in animals and human subjects. For example, Reiss *et al.* (1970) reported that the oral administration of P increased the serum concentration of PTH in human subjects. Calvo *et al.* (1988) reported that consuming high-P low-Ca diets increased the secretion of serum PTH in young adults. In addition, we have reported that a high-P diet enhanced PTH secretion in rats (Masuyama *et al.* 1995, 2000*a*; Katsumata *et al.* 2004*a*,*b*). Previous reports demonstrated that the elevation in PTH secretion in response to a high-P diet was due to changes in the serum concentrations of Ca and P (Masuyama *et al.* 1995, 2000*a*,*b*; Almaden *et al.* 1996; Katsumata *et al.* 2004*a*,*b*).

PTH is a major hormone that regulates Ca and P metabolism through the two principal target organs, kidneys and bones. In kidneys, PTH enhances Ca reabsorption and P excretion and activates 1α, 25(OH)D. In bones, it is well known that chronic PTH stimulation induces osteoclastogenesis. An indirect method is proposed as the major mechanism for the action of PTH on osteoclasts. In this model, PTH acts on the osteoblast, which produces mediators such as macrophage colony-stimulating factor, IL-6, or receptor activator of NF-κB (RANK) ligand (RANKL) that stimulate osteoclast-mediated bone resorption (Martin & Ng, 1994; Weir *et al.* 1996; Tsukii *et al.* 1998; Grey *et al.* 1999; Lee & Lorenzo, 1999; Ma *et al.* 2001).

Although it is accepted that high-P diet-induced bone loss is caused by the acceleration of bone resorption via elevated PTH

Abbreviations: BMC, bone mineral content; BMD, bone mineral density; CTx, C-terminal telopeptide of type I collagen; OPG, osteoprotegerin; PTH, parathyroid hormone; PTHrP, PTH-related peptide; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase.

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secretion, the details of this mechanism are not fully understood. Furthermore, the dose–response effects of high dietary P intake on bone metabolism have not yet been examined. The purpose of the present study was to clarify the mechanism by which the supplementation of a high-P diet induces bone loss. We determined the detailed changes in bone metabolism in rats fed on diets containing three different P levels using bone turnover markers, bone histomorphometry, bone marrow cell culture, and mRNA expressions of mediators for bone loss by the RT-PCR technique.

Materials and methods

Experimental design

Eighteen 4-week-old male Wistar strain rats were purchased from Clea Japan (Tokyo, Japan) and housed individually in metabolism cages in a room maintained at 22°C with a 12 h light–dark cycle. The present study was approved by The Tokyo University of Agriculture Animal Use Committee, and the animals were maintained in accordance with the guidelines of the university for the care and use of laboratory animals. Experimental diets were based on the AIN-93G diet (Reeves *et al.* 1993). Three experimental diets containing 0·3, 0·9, and 1·5 % P were prepared. All three experimental diets contained 0·5 % Ca (Table 1). All rats were fed on the 0·3 % P diet for the 7 d acclimatisation period. After 7 d, all rats were randomly divided into three experimental groups (C, 3P, and 5P) of six rats each and fed on three different P levels diets (diets containing 0·3, 0·9, and 1·5 % P) for 21 d, respectively. They were given free access to the diets and distilled water. Their faeces were collected from 18 to 21 d for a balance study. Their urine was collected on the last day for analysing the bone resorption marker. For bone histomorphometry, the rats were labelled with calcein (30 mg/kg body weight) at 7 and 3 d before killing. Calcein was injected intraperitoneally. At the end of the experimental period, all rats were killed, and the blood and bone samples were collected for analysis.

Blood, urine and faeces analyses

The blood samples were centrifuged and the supernatant fractions were used as serum samples. Urine samples were diluted with water to adjust the volume to a constant amount. Serum and urine were stored at −80°C until further analysis. For the Ca and P measurements, faeces were dried, ashed, and then demineralized with an HCl solution (1 mol/l). Ca was analysed by atomic absorption spectrophotometry (Hitachi A-2000; Hitachi, Tokyo, Japan) according to the method of Gimblet *et al.* (1967). P in faeces was analysed colorimetrically according to the method of Gomori (1942). Serum P was analysed colorimetrically with P-test Wako (Wako Pure Chemical Industries, Osaka, Japan). Serum PTH was measured using an ELISA kit (Immutopics Inc., San Clemente, CA, USA). Serum osteocalcin was measured using an osteocalcin rat ELISA system (Amersham Pharmacia Biotech, Little Chalfont, UK). Urinary C-terminal telopeptide of type I collagen (CTx) was measured using RatLaps ELISA (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Urinary creatinine was measured using Creatinine-test Wako (Wako Pure Chemical Industries).

Table 1. Composition of the experimental diets (g/kg diet)

Group	C	3P	5P
Ca level (%)	0·5	0·5	0·5
P level (%)	0·3	0·9	1·5
Casein	200·0	200·0	200·0
Corn starch	529·486	503·120	476·755
Sucrose	100·0	100·0	100·0
Soyabean oil	70·0	70·0	70·0
Cellulose powder	50·0	50·0	50·0
AIN-93G mineral mixture*	35·0	35·0	35·0
AIN-93 vitamin mixture*	10·0	10·0	10·0
L-Cystine	3·0	3·0	3·0
Choline bitartrate	2·5	2·5	2·5
Tert-butylhydroquinone	0·014	0·014	0·014
KH_2PO_4	–	26·366	52·731

* Reeves *et al.* (1993).

Measurements of bone mineral content and bone mineral density

After killing, the left femur and 3rd lumbar vertebra were removed and cleansed of all soft tissues and were frozen at −80°C until further analysis. Bone mineral content (BMC) and bone mineral density (BMD) were measured by dual-energy X-ray absorptiometry adapted for use in the case of small animals (DCS-600; Aloka, Tokyo, Japan) (Masuyama *et al.* 2003; Tanaka *et al.* 2003). The mineralisation profiles of the specimens were stored as the monitoring images, and the BMC and BMD values for the left femur and 3rd lumbar vertebra were obtained.

Mechanical testing

A three-point bending test of the femur was performed as previously described (Tanaka *et al.* 2003) using a load tester (Tensilon UTA-1T; Orientec, Tokyo, Japan). The left femur was placed on a holding device with supports located at a distance of 10 mm, with the lesser trochanter proximal to and in contact with the proximal transverse bar. The midpoint served as the anterior loading point. A bending force was applied by the crosshead at a speed of 10 mm/min until fracture occurred. The ultimate bending load of the femur was directly obtained from the load-deformation curve, which was continually recorded by a computerised monitor linked to the load tester.

The 3rd lumbar vertebra was fixed with a clamp at the base of the transverse process in the holder of a diamond band saw (Exakt, Norderstedt, Germany). The planoparallel ends at a height of 4 mm were obtained by removing the cranial and caudal ends of the specimen. The cylinder samples were placed centrally on the smooth surface of a steel disk attached to the load tester (Tensilon UTA-1T). A craniocaudal compression force was applied to the specimen via a steel disk at a nominal deformation rate of 2 mm/min and the ultimate compression load was obtained.

Bone histomorphometry

Bone histomorphometry was performed as previously described (Masuyama *et al.* 2003; Tanaka *et al.* 2003). The left tibia and 4th lumbar vertebra were fixed with 4 % paraformaldehyde in PBS and embedded in methyl methacrylate after Villanueva's bone staining. From the middle portion of the specimen, 10 μm thick undecalcified sagittal sections were cut on a microtome (model 2050 Supercut; Reichert-Jung, Heidelberg, Germany).

After decalcification with 10 % buffered EDTA, the mid-sagittal section of the left tibia and 4th lumbar vertebra was embedded in paraffin. Specimens of the left tibia and 4th lumbar vertebra (5 μm thick) were cut on a microtome (RM 2125 RT; Leica, Nussloch, Germany) and then stained for tartrate-resistant acid phosphatase (TRAP). Histomorphometry of the specimens was performed using a semi-automatic image-analysing system linked to a light microscope (Cosmozone 1S; Nikon, Tokyo, Japan). The area of the secondary spongiosa was measured in each section. The regions within 0·75 mm of the growth plate and one cortical shell-width of the endocortical surface were not measured in order to exclude the primary spongiosa.

The trabecular bone volume (%) was obtained and used as the structural parameters of the proximal tibia and 4th lumbar vertebra. Mineralising bone surface (%), mineral apposition rate (μm/d), and surface referent bone formation rate ($\mu m^3/\mu m^2$/d) were obtained as parameters of bone formation. Osteoclast surface (%) and osteoclast number (per mm) were measured in the proximal tibia and 4th lumbar vertebra and used as bone resorption parameters. TRAP-positive cells that formed resorption lacunae at the surface of the trabeculae and contained two or more nuclei were identified as osteoclasts.

Isolation of total RNA and reverse transcription-polymerase chain reaction

Total RNA was isolated from homogenised right femur using TRIzol (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's specifications. The amount and purity of the RNA were assessed by spectrophotometry. Reverse transcription of 1 μg total RNA was performed to give a 50 μl sample of first-strand cDNA using oligo$(dT)_{12-18}$ primer and Superscript™ RT II (Invitrogen Corp.), and cDNA samples were subjected to PCR using the Takara PCR amplification kit. Specific primers (Urena *et al.* 1993; Caputi *et al.* 1995; Myers *et al.* 1999) were used for the analysis of PTH–PTH-related peptide (PTHrP) receptor (sense, 5′-CCGGCTGTCTTCGTGGCTGTG-3′; antisense, 5′-CCCTGGAAGGAGTTGAAGAG-3′), RANK (sense, 5′-TTAAGCCAGTGCTTCACGGG-3′; antisense, 5′-ACGTAGACCACGATGATGTCGC-3′), RANKL (sense, 5′-ACGCAGATTTGCAGGACTCGAC-3′; antisense, 5′-TTCGTGCTCCCTCCTTTCATC-3′), osteoprotegerin (OPG) (sense, 5′-TGGCACACGAGTGATGAATGCG-3′; antisense, 5′-GCTGGAAAGTTTGCTCTTGCG-3′), and glyceraldehyde 3-phosphate dehydrogenase (sense, 5′-CCAGTATGATTCTACCCACGGC-3′; antisense, 5′-GAAGGCCATGCCAGTGAGCTTC-3′). PCR products were separated on a 1·0 % agarose gel and stained with ethidium bromide. The gel was photographed under UV illumination. Images were scanned and the Scion image program was used to quantify the ethidium bromide-stained bands. Data were normalised to an internal control, glyceraldehyde 3-phosphate dehydrogenase.

Rat bone marrow cell culture

Bone marrow cell culture was performed according to the method of previous reports (Takahashi *et al.* 1988; Seto *et al.* 1999). Bone samples were aseptically taken from the right tibia and cleansed of all soft tissues. The bone marrow cavity was flushed out using 5 ml α-MEM (Cosmo Bio, Tokyo, Japan) expelled from a syringe through a 23-gauge needle. Bone marrow cells obtained from the right tibia were inoculated into a twenty-four-well plate at a density of $1{\cdot}5 \times 10^6$ cells/well. The medium was α-MEM supplemented with 10 % fetal bovine serum, penicillin (10 000 U/l), streptomycin (10 000 μg/l), $NaHCO_3$ (2·4 g/l), sodium β-glycerophosphate (10 mmol/l), L-glutamine (2 mmol/l), and 1α,25-dihydroxyvitamin D_3 (10 nmol/l). These cells were cultured for 8 d at 37°C in a humidified atmosphere consisting of 5 % CO_2 and 95 % air. The medium was changed on alternate days. At the end of the culture, the culture medium was removed, fixed in 10 % formalin for 10 min, and refixed in an ethanol–acetone (1:1, v/v) solution for 1 min. After the plate dried up, the samples were treated with 3 mg naphthol AS-MX phosphate dissolved in 0·3 ml N, N-dimethylformamide containing sodium tartrate (50 mmol/l) and 18 mg fast red violet LB salt in 30 ml sodium acetate buffer (0·1 mol/l; pH 5·0) for 15 min at room temperature. Each sample was washed with water, and the number of osteoclast-like TRAP-positive multinucleated cells was counted under a light microscope.

Statistical analysis

Results are expressed as mean values with their standard errors for each group of six rats. All data were evaluated by covariate analysis using final body weight as a factor of covariance, because a decrease in body weight in the 5P group might influence BMD and biomechanical properties. The covariate analysis showed no significant effects of the decrease in body weight ($P = 0{\cdot}10$–$0{\cdot}89$) on all results except for food intake ($P < 0{\cdot}0001$). Therefore, after one-way ANOVA, Fisher's protected least significant difference test was used to determine significant differences between the groups. Differences were considered to be significant when the P value was less than 0·05. Statistical analysis was performed by StatView 5 software (SAS Institute, Cary, NC, USA).

Results

Body weight and food intake

A lower final body weight, weight gain, food intake, and Ca intake were observed in the 5P group than in the C and 3P groups (Table 2). P intake was significantly increased with increasing dietary P levels.

Serum calcium, phosphorus, and parathyroid hormone concentrations and markers of bone turnover

A lower serum concentration of Ca was observed in the 5P group than in the C and 3P groups (Fig. 1 (A)). Serum P concentration was significantly increased with increasing dietary P levels (Fig. 1 (B)). Serum PTH concentration was significantly increased in the 5P group in comparison with the C and 3P groups, and it tended to increase in the 3P group in comparison with the C group (Fig. 1 (C)). Serum osteocalcin was significantly increased in the 5P group in comparison with the C and 3P groups (Fig. 2 (A)). Urinary excretion of CTx was significantly increased with increasing dietary P levels (Fig. 2 (B)).

Apparent absorption of calcium and phosphorus

Apparent Ca absorption was significantly decreased in the 5P group in comparison with the C and 3P groups. The rate of apparent Ca absorption did not differ significantly among the three

Table 2. Body weight and food intake in rats fed diets containing 0·3 % (C), 0·9 % (3P) and 1·5 % (5P) phosphorus (six rats per group)*
(Mean values with their standard errors)

		Initial body weight (g)		Final body weight (g)		Weight gain (g/21 d)		Food intake (g/21 d)		Ca intake (mmol/21 d)		P intake (mmol/21 d)	
Group	P content (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	0·3	110·6	2·0	262·4[a]	4·2	151·9[a]	3·1	358·1[a]	6·6	42·2[a]	0·8	35·2[c]	0·6
3P	0·9	110·5	1·3	258·0[a]	4·7	147·4[a]	3·6	352·2[a]	4·0	42·9[a]	0·5	107·0[b]	1·2
5P	1·5	110·4	1·0	197·0[b]	6·6	86·6[b]	6·6	251·7[b]	5·1	30·9[b]	0·6	123·1[a]	2·5

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
*For details of diets, see Table 1.

groups. The amount and rate of apparent P absorption were significantly increased with increasing dietary P levels (Table 3).

Bone mineral content, bone mineral density, and ultimate load of the femur and lumbar vertebra

There were significant decreases in the BMC and BMD of the femur and lumbar vertebra and ultimate compression load of the lumbar vertebra with increasing dietary P levels (Fig. 3 (A) and (B); Fig. 4 (A–C)). The ultimate bending load of the femur was significantly decreased in the 5P group in comparison with the C and 3P groups (Fig. 3 (C)).

Bone histomorphometry

Proximal tibia. Trabecular bone volume was significantly decreased with increasing dietary P levels. Mineralising bone surface was significantly decreased and mineral apposition rate was significantly increased in the 5P group in comparison with the C and 3P groups. Surface referent bone formation rate did not differ significantly among the three groups. Osteoclast surface and osteoclast number were significantly increased with increasing dietary P levels (Table 4).

Lumbar vertebra. Trabecular bone volume was significantly decreased with increasing dietary P levels. Mineralising bone surface was significantly decreased in the 5P group in comparison with the C and 3P groups. Mineral apposition rate was significantly increased with increasing dietary P levels. Surface referent bone formation rate was significantly increased in the 3P and 5P groups in comparison with the C group. Osteoclast surface and osteoclast number were significantly increased with increasing dietary P levels (Table 4).

Receptor activator of nuclear factor-κB (RANK), RANK ligand, osteoprotegerin and parathyroid hormone–parathyroid hormone-related peptide receptor mRNA expression in the femur

RANK and RANKL mRNA expression were significantly higher with increasing dietary P levels (Fig. 5; Table 5). OPG and PTH–PTHrP receptor mRNA expression did not significantly differ among the three groups.

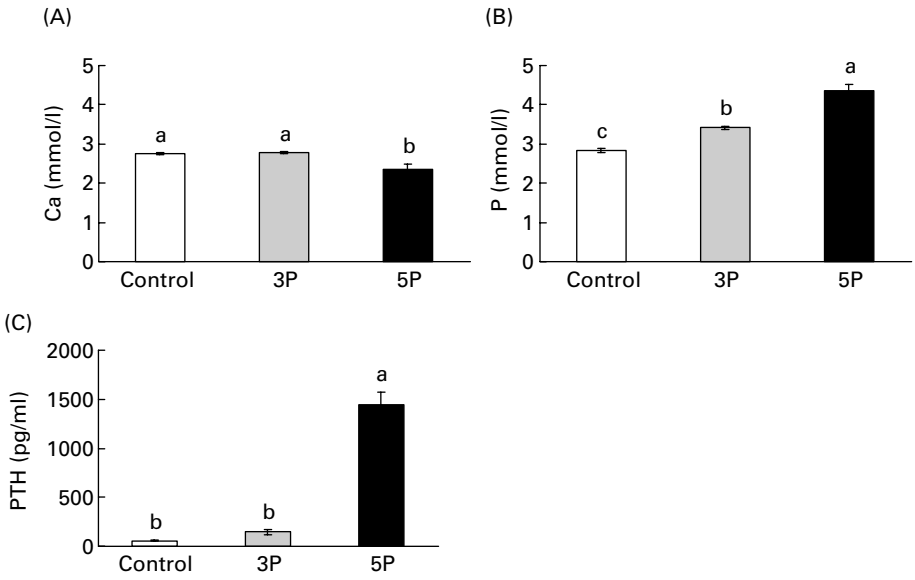

Fig. 1. Serum Ca (A), P (B) and parathyroid hormone (PTH; C) concentrations in rats fed diets containing 0·3 % (Control), 0·9 % (3P) and 1·5 % (5P) P. Mean values for six rats per group are shown, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

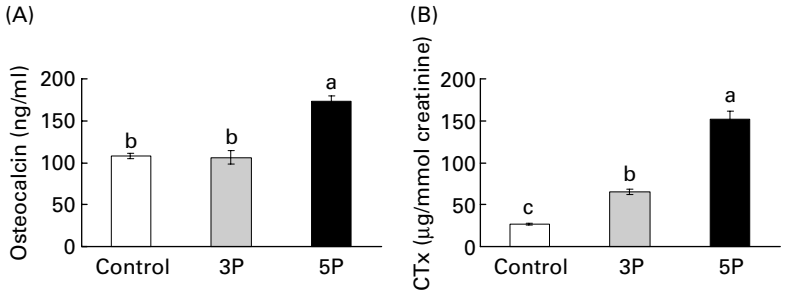

Fig. 2. Serum osteocalcin concentration (A) and urinary C-terminal telopeptide of type I collagen (CTx) excretion (B) in rats fed diets containing 0·3 % (Control), 0·9 % (3P), and 1·5 % (5P) P. Mean values for six rats per group are shown, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

Bone marrow cell culture

The number of osteoclast-like TRAP-positive multinucleated cells was significantly increased with increasing dietary P levels (Fig. 6).

Discussion

Previous reports explained that the elevated PTH as a result of a high-P diet was due to changes in the serum Ca and P concentrations (Masuyama *et al.* 1995, 2000*a*,*b*; Almaden *et al.* 1996; Katsumata *et al.* 2004*a*,*b*). In the present study, by increasing dietary P levels to 1·5 %, remarkably high PTH secretion was induced along with a decrease in serum Ca concentration and an increase in serum P concentration. Recently, we demonstrated that high-P diets (1·2 % or greater) caused secondary hyperparathyroidism with a decrease in serum Ca concentration, and decreased PTH action in the kidney, due to PTH–PTHrP receptor mRNA down regulation (Katsumata *et al.* 2004*b*). In the present study, high-P diets that resulted in an increased PTH secretion did not change the PTH–PTHrP receptor mRNA expression in the femur. These results suggest that PTH has different actions on the two target organs, kidneys and bones, when rats were fed on a high-P diet. PTH action on the kidneys was decreased whereas that on the bone was maintained at normal in spite of PTH hypersecretion induced by a high-P diet. This discovery is an important key for the understanding of Ca and P metabolism and further studies will be required to investigate the details.

It is known that an elevated circulating PTH stimulates bone resorption. In contrast, intermittent PTH administration increases bone formation (Kroll, 2000). Because the high-P diet causes chronic PTH hypersecretion, PTH should enhance bone resorption in the present study. We measured markers of bone resorption and formation, urinary excretion of CTx and serum osteocalcin concentration. CTx is excreted into the body fluids when bone tissue is broken down during bone remodelling, and it is useful for showing acute changes in bone resorption (Bonde *et al.* 1994, 1995). Osteocalcin is one of the major non-collagenous proteins of the bone matrix and is synthesised and released from osteoblasts (Brown *et al.* 1984). Urinary excretion of CTx was significantly increased with increasing dietary P levels, and the serum osteocalcin concentration was significantly increased in rats fed on the 1·5 % P diet in comparison with the other two groups. These results suggested that secondary hyperparathyroidism induced by a high-P diet increased bone turnover, which has been implicated as a significant aetiological factor for bone loss (Ravn *et al.* 1997). Bone histomorphometric parameters also indicated a high turnover bone metabolism in rats fed on the high-P diets. In lumbar vertebra, surface referent bone formation rate was significantly increased in the rats fed on the 0·9 % and 1·5 % P diets in comparison with the rats fed on the 0·3 % P diet, and osteoclast surface and osteoclast number were also significantly increased with increasing dietary P levels. Consequently, BMC and BMD of the femur and lumbar vertebra and ultimate compression load of the lumbar vertebra were decreased with increasing dietary P levels. In addition, the ultimate bending load of the femur was decreased in the rats fed on the 1·5 % P diet in comparison with the other two groups. These results showed that a high-P diet weakened bone with increasing dietary P level, which was related to the serum PTH concentration. In parathyroidectomised rats under the condition of maintaining the serum PTH concentration, we previously reported that PTH-independent regulation of bone resorption with a high-P diet existed (Katsumata *et al.* 2004*a*). In the

Table 3. Apparent absorption of calcium and phosphorus in rats fed diets containing 0·3 % (C), 0·9 % (3P) and 1·5 % (5P) phosphorus (six rats per group)*

(Mean values with their standard errors)

		Ca absorption				P absorption			
		mmol/d		%		mmol/d		%	
Group	P content (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	0·3	1·51[a]	0·02	71·75	1·69	1·41[c]	0·03	77·01[c]	2·02
3P	0·9	1·52[a]	0·06	68·67	2·51	4·61[b]	0·10	84·24[b]	1·45
5P	1·5	1·12[b]	0·07	71·70	1·69	6·21[a]	0·25	92·88[a]	0·72

[a,b,c]Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
*For details of diets, see Table 1.

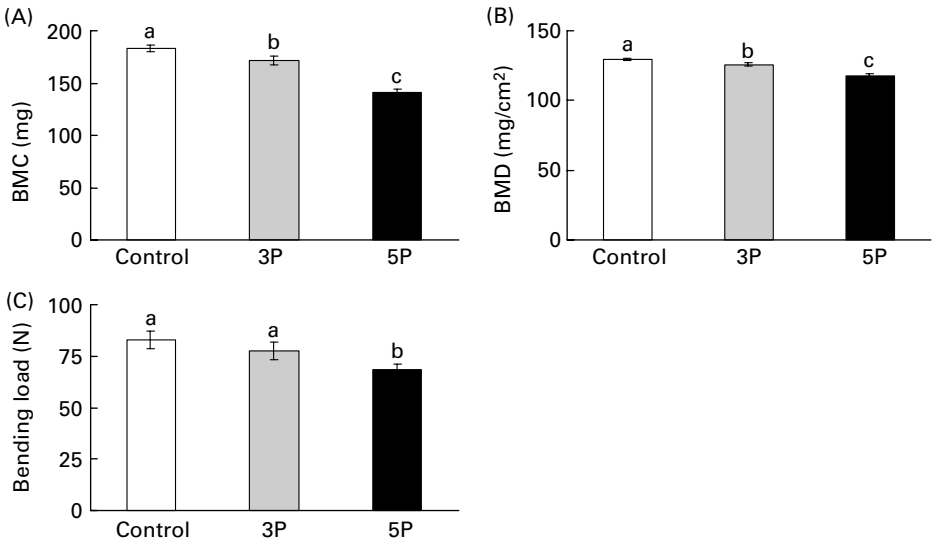

Fig. 3. Bone mineral content (BMC; A), bone mineral density (BMD; B) and bending load (C) of the femur in rats fed diets containing 0·3 % (Control), 0·9 % (3P) and 1·5 % (5P) P. Mean values for six rats per group are shown, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

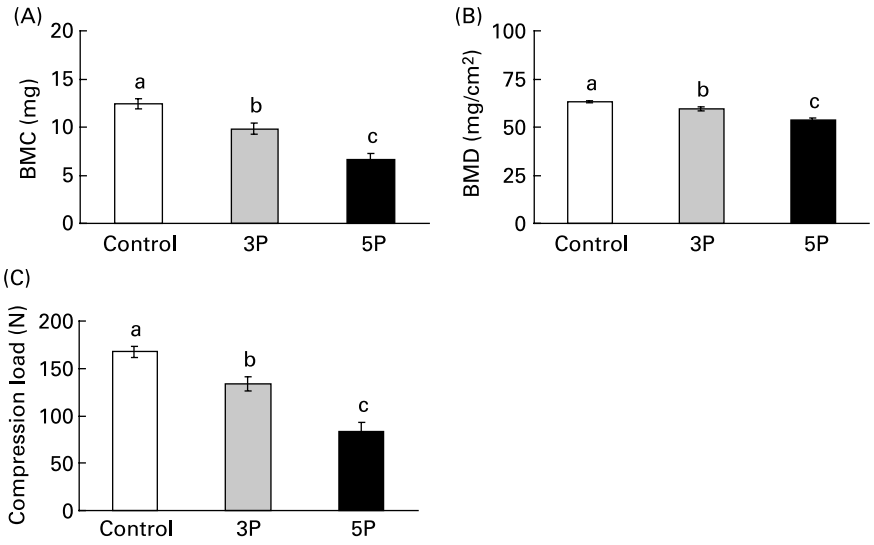

Fig. 4. Bone mineral content (BMC; A), bone mineral density (BMD; B) and compression load (C) of the lumbar vertebra in rats fed diets containing 0·3 % (Control), 0·9 % (3P), and 1·5 % (5P) P. Mean values for six rats per group are shown, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

present study, serum PTH concentration was slightly, but not significantly, increased in rats fed the 0·9 % P diet compared with those fed the 0·3 % P diet. Moreover, the 0·9 % P diet did not change serum Ca concentration and increased serum P concentration in comparison with the 0·3 % P diet. These results suggested that high P status influenced bone loss regardless of changes in Ca metabolism and PTH hypersecretion, and the direct effects of dietary high P on bone loss might exist.

In the present study, final body weight and BMD of the femur were decreased in the rats fed the 1·5 % P diet compared with those fed the 0·3 % P diet. Therefore, it was considered that the decrease in body weight contributed to the decrease in BMD. However, covariate analysis showed that the body weight did not influence BMD of the femur ($P=0·48$). Furthermore, when pair-fed control rats were used as the control to rats fed a 1·5 % P diet, we previously demonstrated that the 1·5 % P diet caused PTH hypersecretion (pair-fed control, 232·79 (SEM 14·91) pg/ml; 1·5 % P diet group, 1559·59 (SEM 40·78) pg/ml), and increased serum osteocalcin concentration (pair-fed control, 85·74 (SEM 2·39) ng/ml; 1·5 % P diet group, 162·95 (SEM 2·73) ng/ml) and urinary excretion of CTx (pair-fed control, 22·29 (SEM 1·72) μg/mmol creatinine; 1·5 % P diet group, 184·67 (SEM 4·55) μg/mmol creatinine), although BMD was not determined (Katsumata S, unpublished results). In the present study, the 0·9 % P diet caused an increase in bone turnover and bone loss with no change of body weight gain in rats compared with the rats fed the 0·3 % P diet. Thus, these results suggested

Table 4. Bone histomorphometric parameters of the proximal tibia and lumbar vertebra in rats fed diets containing 0·3 % (C), 0·9 % (3P) and 1·5 % (5P) phosphorus (six rats per group)*

(Mean values with their standard errors)

		BV/TV (%)		MS/BS (%)		MAR (μm/d)		BFR/BS ($\mu m^3/\mu m^2$ per d $\times 10^{-2}$)		Oc.S/BS (%)		Oc.N/BS (no./mm)	
Group	P content (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Proximal tibia													
C	0·3 %	18·67[a]	0·54	18·65[a]	0·37	1·668[b]	0·058	30·87	1·13	2·46[c]	0·09	1·12[c]	0·10
3P	0·9 %	12·75[b]	0·80	17·71[a]	1·08	1·813[b]	0·070	28·00	2·15	5·53[b]	0·36	2·61[b]	0·17
5P	1·5 %	8·79[c]	0·37	13·41[b]	0·75	2·408[a]	0·160	27·57	2·58	9·59[a]	0·81	4·54[a]	0·32
Lumbar vertebra													
C	0·3 %	24·65[a]	0·32	20·39[a]	0·81	1·899[c]	0·039	36·85[b]	1·06	2·51[c]	0·35	1·24[c]	0·13
3P	0·9 %	17·06[b]	0·98	22·75[a]	0·65	2·403[b]	0·069	54·70[a]	2·30	5·13[b]	0·26	2·33[b]	0·13
5P	1·5 %	11·43[c]	0·47	17·69[b]	1·08	3·116[a]	0·097	55·22[a]	4·75	10·14[a]	0·59	4·35[a]	0·24

BV/TV, trabecular bone volume; MS/BS, mineralising bone surface; MAR, mineral apposition rate; BFR/BS, surface referent bone formation rate; Oc.S/BS, osteoclast surface; Oc.N/BS, osteoclast number.

[a,b,c]Mean values within a column for proximal tibia or lumbar vertebra with unlike superscript letters were significantly different ($P<0·05$).

*For details of diets, see Table 1.

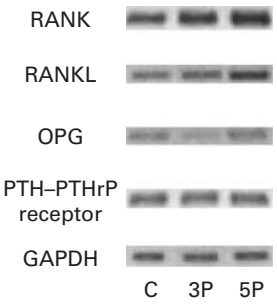

Fig. 5. Receptor activator of NF-κB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG), parathyroid hormone–parathyroid hormone-related peptide (PTH–PTHrP) receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in the femur of rats fed diets containing 0·3 % (C), 0·9 % (3P) and 1·5 % (5P) P by RT-PCR.

that a high-P diet might be a dominant cause of bone loss in either status with or without decreasing both body weights and food intakes.

Bone resorption is mediated by osteoclasts. In the present study, the osteoclast number in the proximal tibia and lumbar vertebra was significantly increased with increasing dietary P levels. It is known that various cytokines and hormones regulate osteoclast differentiation and activation. Recently, some researchers discovered that osteoclastogenesis was mediated by RANK–RANKL–OPG signalling (Lacy *et al.* 1998; Yasuda *et al.* 1998*a*,*b*; Hsu *et al.* 1999). RANKL is expressed on stromal cells and osteoblasts and regulates osteoclast differentiation and activation by binding to its receptor RANK, which is expressed on pre-osteoclasts or mature osteoclasts. Osteoblasts secrete OPG, which acts as a decoy receptor by blocking RANKL binding to its receptor RANK. Lee & Lorenzo (1999) reported that PTH stimulated RANKL and inhibited OPG mRNA expression, resulting in increasing osteoclast-like cell formation in murine bone marrow cultures. Tsukii *et al.* (1998) showed that RANKL mediated an essential signal for bone resorption induced by PTH in the microenvironment of bone. In the present study, RANK and RANKL mRNA expressions were significantly increased with increasing dietary P levels, and OPG mRNA expression did not significantly differ among the three groups. These results demonstrated that increased serum PTH concentration resulting from a high-P diet acted on the osteoblast PTH–PTHrP receptor and stimulated RANKL mRNA expression, which accelerated bone resorption. Moreover, a high-P diet did not result in a difference in OPG mRNA expression in comparison with the change of RANKL mRNA expression; this was the reason for the increase in the osteoclast number that caused bone loss.

It is generally known that the bone marrow contains progenitor cells of both osteoclasts and osteoblasts. In the present study, a

Table 5. Receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG) and parathyroid hormone–parathyroid hormone-related peptide (PTH–PTHrP) receptor mRNA expression in rats fed diets containing 0·3 % (C), 0·9 % (3P) and 1·5 % (5P) phosphorus (six rats per group)*

(Mean values with their standard errors)

		RANK		RANKL		OPG		PTH–PTHrP receptor	
Group	P content (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C	0·3	0·998[c]	0·035	0·792[c]	0·022	0·755	0·016	0·894	0·048
3P	0·9	1·116[b]	0·020	0·952[b]	0·013	0·794	0·019	0·957	0·031
5P	1·5	1·247[a]	0·041	1·159[a]	0·055	0·762	0·020	0·958	0·080

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

*For details of diets, see Table 1.

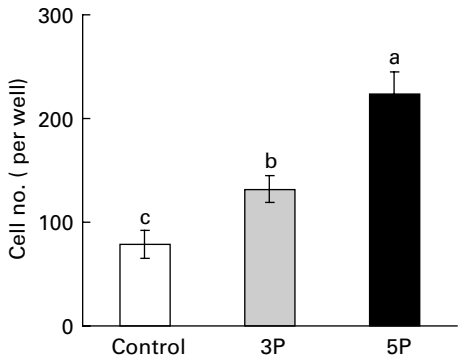

Fig. 6. Number of tartrate-resistant acid phosphatase-positive multinucleated cells by bone marrow cell culture in rats fed diets containing 0·3 % (Control), 0·9 % (3P) and 1·5 % (5P) P. Mean values for six rats per group are shown, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

bone marrow cell culture system was used for measurement of the change in the population of osteoclast progenitor cells in the bone marrow cavity. The result showed that the number of osteoclast-like TRAP-positive multinucleated cells was significantly increased with increasing dietary P levels. Since *in vivo* changes in bone marrow cells are maintained when the cells are placed *in vitro* (Sakai *et al.* 1998), the number of osteoclast progenitor cells in the bone marrow cavity was increased in the rats fed on the high-P diets. These results suggested that a high-P diet enhanced osteoclast differentiation, and increased osteoclast progenitor cells were associated with an increase in RANKL mRNA expression.

We previously observed that ultimate compression load of the lumbar vertebra was declined in rats fed on the 0·6 % P diet (124·50 (SEM 9·21) N) in comparison with rats fed on the 0·3 % P diet (145·60 (SEM 4·58) N) ($P<0·05$ Katsumata S, unpublished results), following the elevation of serum PTH concentration (0·6 % P diet, 117·14 (SEM 8·46) pg/ml; 0·3 % P diet, 58·96 (SEM 5·16) pg/ml; $P<0·05$ (Katsumata *et al.* 2004*b*)). These results showed that a small increase in dietary P levels causes bone loss, and this is a very serious problem with regard to human bone metabolism. Total P consumption is increasing day by day and is more than the adequate intake in Japan. Therefore, if chronic P intake, that is, more than the adequate intake continues, it may have adverse effects on human bone health.

In conclusion, secondary hyperparathyroidism induced by a high-P diet leads to bone loss via an increase in bone turnover with increasing dietary P levels. Furthermore, it is suggested that a high-P diet increases RANKL mRNA expression, which causes osteoclast differentiation and an increase in the osteoclast number.

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References

Almaden Y, Canalejo A, Hernandez A, Ballesteros E, Garcia NS, Torres A & Rodriguez M (1996) Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands *in vitro*. *J Bone Miner Res* **11**, 970–976.

Bauer KD & Griminger P (1983) Long-term effects of activity and of calcium and phosphorus intake on bones and kidneys of female rats. *J Nutr* **113**, 2011–2021.

Bell RR, Draper HH, Tzeng DY, Shin HK & Schmidt GR (1977) Physiological responses of human adults to foods containing phosphate additives. *J Nutr* **107**, 42–50.

Bonde M, Qvist P, Fledelius C, Riis BJ & Christiansen C (1994) Immunoassay for quantifying type I collagen degradation products in urine evaluated. *Clin Chem* **40**, 2022–2025.

Bonde M, Qvist P, Fledelius C, Riis BJ & Christiansen C (1995) Applications of an enzyme immunoassay for a new marker of bone resorption (CrossLaps): follow-up on hormone replacement therapy and osteoporosis risk assessment. *J Clin Endocrinol Metab* **80**, 864–868.

Brown JP, Delmas PD, Malaval L, Chapuy MC, Delmas PD, Edouard C & Meunier PJ (1984) Serum bone gla protein: a specific marker for bone formation in post-menopausal osteoporosis. *Lancet* **i**, 1091–1093.

Calvo MS, Kumar R & Heath H III (1988) Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. *J Clin Endocrinol Metab* **66**, 823–829.

Caputi M, Melo CA & Baralle FE (1995) Regulation of fibronectin expression in rat regenerating liver. *Nucleic Acids Res* **23**, 238–243.

Draper HH, Sie TL & Bergan JG (1972) Osteoporosis in aging rats induced by high phosphorus diets. *J Nutr* **102**, 1133–1142.

Gimblet EG, Marney AF & Bonsnes RW (1967) Determination of calcium and magnesium in serum, urine, diets and stool by atomic absorption spectrophotometry. *Clin Chem* **13**, 204–214.

Gomori G (1942) A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J Lab Clin Med* **27**, 955–960.

Grey A, Mitnick M, Masiukiewicz U, Sun B, Rudikoff S, Jilka RL, Manolagas SC & Insobna K (1999) A role for interleukin-6 in parathyroid hormone-induced bone resorption *in vivo*. *Endocrinology* **140**, 4683–4690.

Hsu H, Lacey DL, Dunstan CR, *et al.* (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* **96**, 3540–3545.

Katsumata S, Masuyama R, Koshihara M, Matsuzaki H, Uehara M & Suzuki K (2004*a*) High phosphorus diet changes phosphorus metabolism regardless of PTH action in rats. *Biosci Biotechnol Biochem* **68**, 243–246.

Katsumata S, Masuyama R, Uehara M & Suzuki K (2004*b*) Decreased mRNA expression of the PTH/PTHrP receptor and type II sodium-dependent phosphate transporter in the kidney of rats fed a high phosphorus diet accompanied with a decrease in serum calcium concentration. *Biosci Biotechnol Biochem* **68**, 2484–2489.

Kroll MH (2000) Parathyroid hormone temporal effects on bone formation and resorption. *Bull Math Biol* **62**, 163–188.

Lacy DL, Timms E, Tan H, *et al.* (1998) Osteoprotegerin (OPG) ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176.

Lee S & Lorenzo JA (1999) Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology* **140**, 3552–3561.

Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ & Onyia JE (2001) Catabolic effects of continuous human PTH (1-38) *in vivo* is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. *Endocrinology* **142**, 4047–4054.

Martin TJ & Ng KW (1994) Mechanisms by which cells of the osteoblast lineage control osteoclast formation and activity. *J Cell Biochem* **56**, 357–366.

Masuyama R, Kajita Y, Odachi J, Uehara M, Shigematsu T, Suzuki K & Goto S (2000*a*) Chronic phosphorus supplementation decreases the

expression of renal PTH/PTHrP receptor mRNA in rats. *Am J Nephrol* **20**, 491–495.

Masuyama R, Nakaya Y, Katsumata S, Kajita Y, Uehara M, Tanaka S, Sakai A, Kato S, Nakamura T & Suzuki K (2003) Dietary calcium and phosphorus ratio regulates bone mineralisation and turnover in vitamin D receptor knockout mice by affecting intestinal calcium and phosphorus absorption. *J Bone Miner Res* **18**, 1217–1226.

Masuyama R, Uehara M & Suzuki K (2000*b*) High P diet induces acute secretion of parathyroid hormone without alteration of serum calcium levels in rats. *Biosci Biotechnol Biochem* **64**, 2316–2319.

Masuyama R, Uehara M, Suzuki K & Goto S (1995) The action of magnesium in reducing renal calcification in rats receiving high phosphorus supplemented diet. *Nutr Res* **15**, 1673–1682.

Myers DE, Collier FM, Minkin C, Wang H, Holloway WR, Malakellis M & Nicholson GC (1999) Expression of functional RANK on mature rat and human osteoclasts. *FEBS Lett* **463**, 295–300.

Ravn P, Rix M, Andreassen H, Clemmesen B, Bidstrup M & Gunnes M (1997) High bone turnover is associated with low bone mass and spinal fracture in postmenopausal women. *Calcif Tissue Int* **60**, 255–260.

Reeves PG, Nielsen FH & Fahey GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.

Reiss E, Canterbury JM, Bercovitz MA & Kaplan EL (1970) The role of phosphate in the secretion of parathyroid hormone in man. *J Clin Invest* **49**, 2146–2149.

Sakai A, Nishida S, Okimoto N, Okazaki Y, Hirano T, Norimura T, Suda T & Nakamura T (1998) Bone marrow cell development and trabecular bone dynamics after ovariectomy in ddy mice. *Bone* **23**, 443–451.

Seto H, Aoki K, Kasugai S & Ohya K (1999) Trabecular bone turnover, bone marrow cell development, and gene expression of bone matrix proteins after low calcium feeding in rats. *Bone* **25**, 687–695.

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A & Suda T (1988) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* **122**, 1373–1382.

Takeda E, Sakamoto K, Yokota K, Shinohara M, Taketani Y, Morita K, Yamamoto H, Miyamoto K & Shibayama M (2002) Phosphorus supply per capita from food in Japan between 1960 and 1995. *J Nutr Sci Vitaminol* **48**, 102–108.

Tanaka S, Tsurukami H, Sakai A, Okimoto N, Ikeda S, Otomo H & Nakamura T (2003) Effects of $1,25(OH)_2D_3$ on turnover, mineralization, and strength of bone in growing rats with liver cirrhosis induced by administration of carbon tetrachloride. *Bone* **32**, 275–283.

Tsukii K, Shima N, Mochizuki S, *et al.* (1998) Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, or parathyroid hormone in the microenvironment of bone. *Biochem Biophys Res Commun* **246**, 337–341.

Urena P, Kong XF, Abou-Samra AB, Juppner H, Kronenberg HM, Potts JT Jr & Segre GV (1993) Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. *Endocrinology* **133**, 617–623.

Weir EC, Lowik CWGM, Paliwal I & Insogna KL (1996) Colony stimulating factor-1 plays a role in osteoclast formation and function in bone resorption induced by parathyroid hormone and parathyroid hormone-related protein. *J Bone Miner Res* **11**, 1474–1481.

Yasuda H, Shima N, Nakagawa N, *et al.* (1998*a*) Identity of osteoclastgenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastgenesis in vitro. *Endocrinology* **39**, 1329–1337.

Yasuda H, Shima N, Nakagawa N, *et al.* (1998*b*) Osteoclast differentiation factor is a ligand for osteoprotegerine/osteoclastgenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* **95**, 3597–3602.