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Role of vitamin D metabolites in the prevention of the osteopenia induced by ovariectomy in the axial and appendicular skeleton of the rat

R. G. Erben[1], B. Kohn[1], H. Weiser[2], F. Sinowatz[3], and W. A. Rambeck[1]

[1]Institute of Physiology, Physiological Chemistry and Nutrition Physiology, and [3]Institute of Veterinary Anatomy, Veterinary Faculty, Ludwig-Maximilians-University, Munich, Federal Republic of Germany
[2] Research Department, Hoffmann-La Roche Ltd., Basel, Switzerland

Abbreviation index

$1,25(OH)_2D_3$ = 1α,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$ = 24R,25-dihydroxyvitamin D_3; $1,24,25(OH)_3D_3$ = 1α,24R,25-trihydroxyvitamin D_3; $1\alpha(OH)D_3$ = 1α-hydroxyvitamin D_3; PTH = parathyroid hormone; OVX = ovariectomized; SHAM = sham-operated; Gp = group.

Summary: Forty Fischer-344 rats (10 weeks old, 130 g BW) were either bilaterally ovariectomized (OVX) or sham-operated (SHAM). The rats were allocated to the following groups: SHAM; OVX; OVX + 15 ng 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]/rat/d; OVX + 30 ng 1α,24R,25-trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$]/rat/d; OVX + 15 ng $1,25(OH)_2D_3$/rat/d + 30 ng $1,24,25(OH)_3D_3$/rat/d. The vitamin D metabolites were fed orally starting 4 weeks after surgery. Urine and blood samples were taken at several time points during the experiment. Twenty-one weeks after surgery all rats were sacrificed, and the proximal tibiae and the first lumbar vertebrae were processed undecalcified for static bone histomorphometry.

Ovariectomy induced a 40% reduction in vertebral cancellous bone area, and a 69% reduction in tibial cancellous bone area. This bone loss in OVX rats was associated with moderately increased biochemical and histomorphometric indices of bone formation and resorption as compared to values in sham-operated animals. Through inhibition of bone resorption, treatment of OVX rats with $1,25(OH)_2D_3$, $1,24,25(OH)_3D_3$, and the metabolite combination prevented the ovariectomy-induced osteopenia in the lumbar vertebra, and partially prevented cancellous bone osteopenia in the tibial metaphysis. However, OVX rats receiving $1,25(OH)_2D_3$ alone or in combination with $1,24,25(OH)_3D_3$ exhibited hypercalcemia, hyperphosphatemia, hypercalciuria, and impaired bone mineralization. Treatment of OVX rats with $1,24,25(OH)_3D_3$ alone, on the other hand, only slightly increased serum calcium levels and did not impair bone mineralization. Furthermore, the inclusion of $1,24,25(OH)_3D_3$ with $1,25(OH)_2D_3$ partially antagonized the untoward effects of $1,25(OH)_2D_3$ on bone mineralization.

These data suggest that the actions of $1,24,25(OH)_3D_3$ on bone metabolism might differ from that of $1,25(OH)_2D_3$, and that $1,25(OH)_2D_3$ and, particularly, $1,24,25(OH)_3D_3$ may be potentially effective agents for the prophylaxis of postmenopausal osteoporosis.

* In memoriam Prof. Dr. Hermann Zucker

Zusammenfassung: 40 Fischer-344-Ratten (10 Wochen alt, 130 g KG) wurden entweder beidseitig ovariektomiert (OVX) oder scheinoperiert (SHAM). Die Ratten wurden in folgende Gruppen eingeteilt: SHAM; OVX; OVX + 15 ng 1α,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]/Tier/Tag (d); OVX + 30 ng 1α,24R,25-Trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$]/Tier/d; OVX + 15 ng $1,25(OH)_2D_3$/Tier/d + 30 ng $1,24,25(OH)_3D_3$/Tier/d. 4 Wochen post operationem wurde mit der oralen Verabreichung der Vitamin-D-Metaboliten begonnen. Urin- und Blutproben wurden mehrfach während des Experiments entnommen. 21 Wochen post operationem wurden alle Ratten getötet und die proximale Tibia sowie der erste Lendenwirbelkörper für eine statische histomorphometrische Auswertung unentkalkt eingebettet.

Die Ovariektomie verursachte eine Abnahme der trabekulären Knochenmasse um 40 % im Lendenwirbelkörper und um 69 % in der Tibiametaphyse. Verglichen mit den scheinoperierten Tieren, ging der Knochenverlust bei OVX-Ratten mit mäßig erhöhten biochemischen und histomorphometrischen Parametern der Knochenformation und -resorption einher. Die Behandlung der OVX-Ratten mit $1,25(OH)_2D_3$, $1,24,25(OH)_3D_3$ oder der Metabolitkombination verhinderte die durch die Ovariektomie induzierte Osteopenie im trabekulären Knochen des Lendenwirbels und teilweise auch der Tibiametaphyse, wobei diese Wirkung durch eine Hemmung der Knochenresorption zustande kam. Die mit $1,25(OH)_2D_3$ allein oder in Kombination mit $1,24,25(OH)_3D_3$ behandelten Ratten zeigten jedoch eine Hyperkalzämie, Hyperphosphatämie, Hyperkalzurie und eine gestörte Knochenmineralisation. Andererseits führte die Behandlung von OVX-Ratten mit $1,24,25(OH)_3D_3$ allein nur zu einer leichten Zunahme des Serumkalziumspiegels und erzeugte keine Störung der Knochenmineralisation. Weiterhin wirkte $1,24,25(OH)_3D_3$ in Kombination mit $1,25(OH)_2D_3$ den ungünstigen Effekten von $1,25(OH)_2D_3$ auf die Knochenmineralisation teilweise entgegen.

Diese Ergebnisse deuten darauf hin, daß sich die Wirkungen von $1,24,25(OH)_3D_3$ auf den Knochenstoffwechsel möglicherweise von denen des $1,25(OH)_2D_3$ unterscheiden und daß $1,25(OH)_2D_3$ und insbesondere $1,24,25(OH)_3D_3$ eventuell auch für eine wirksame Prophylaxe der postmenopausalen Osteoporose geeignet wären.

Key words: ovariectomy; quantitative bone histomorphometry; bone mineralization; osteopenia; vitamin D

Schlüsselwörter: Ovariektomie; quantitative Knochenhistomorphometrie; Knochenmineralisation; Osteopenie; Vitamin D

Introduction

Osteoporosis is a bone disease that is characterized by a reduction in bone mass per unit volume and an increased incidence of fracture, particularly fractures of the vertebrae, distal radius, and proximal femur (6). The disease is very common among elderly persons in Western countries (22), and the socioeconomic impact of osteoporosis and associated fractures will grow in the near future as the number of elderly steadily increase in Western societies (21). It has been suggested that involutional osteoporosis is an etiologically heterogeneous disorder that can be subdivided into type I (postmenopausal) and type II (senile) osteoporosis (42). Early postmenopausal women experience a transient phase of increased bone turnover and accelerated cancellous bone loss (16, 18, 37). There is good evidence that this phenomenon is caused by estrogen deficiency due to ovarian insufficiency after menopause (28). However, the pathophysiology

of estrogen-deficiency-induced bone loss is still unclear. Recently, estrogen receptors have been identified in rat and human osteoblast-like cells (11, 26). Thus, it is possible that estrogens exert their bone-preserving effects through a direct receptor-mediated action on osteoblasts.

Studies in healthy women in their early natural menopause have demonstrated that daily administration of 0.25 μg $1,25(OH)_2D_3$ or 0.25 μg $1\alpha(OH)D_3$ has no prophylactic effect with regard to early postmenopausal bone loss (7, 8). On the other hand, there is accumulating evidence that daily doses of 0.5 to 1.0 μg $1,25(OH)_2D_3$ have a beneficial effect in preventing bone loss and reducing fracture rate in patients with established postmenopausal osteoporosis (1, 5, 14).

It is well established that ovariectomy results in cancellous bone loss in the appendicular and axial skeleton of the rat (49–51). The bone loss observed in OVX rats is accompanied by increased biochemical and histomorphometric indices of bone resorption and bone formation (19, 31, 49–51), and shows some similarities to postmenopausal bone loss (47, 50). Several studies have demonstrated that vitamin D metabolites can increase bone mass and can, at least partially, antagonize the development of osteopenia in OVX rats (10, 12, 20, 24, 25, 27, 30).

Previous investigations in our laboratory have indicated that in rachitic rats, chicks, and quails the bioactivity of $1,25(OH)_2D_3$ in various vitamin D bio-assays is enhanced synergistically by simultaneous administration of $24,25(OH)_2D_3$ or $1,24,25(OH)_3D_3$ (39–41). It is not known whether these overadditive effects can also occur in vitamin D-replete animals. Nevertheless, the potentially reinforcing effects of 24-hydroxylated vitamin D metabolites on $1,25(OH)_2D_3$ bioactivity might permit to reduce the dosage of $1,25(OH)_2D_3$ without changing its therapeutic effectiveness, thus possibly also reducing the risks associated with $1,25(OH)_2D_3$ treatment, i.e., the occurrence of hypercalcemia and hypercalciuria.

The current study employed bone histomorphometry together with analysis of biochemical markers of bone turnover to evaluate the ability of $1,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, alone or in combination, to prevent the development of ovariectomy-induced cancellous bone osteopenia in the axial and appendicular skeleton of the rat.

Materials and methods

Animal procedures

Forty female 10-week-old Fischer-344 rats (Institute for Biological and Medical Research, Switzerland) weighing about 130 g were used for this experiment. Thirty-two rats were bilaterally ovariectomized by dorsal approach under xylazine/ketamine anesthesia, and the remaining eight rats were sham-operated (SHAM). The animals were kept in individual cages at 24 °C with a 12 h each light/dark cycle and were fed a standard laboratory diet (Altromin, FRG) containing 0.9 % calcium, 0.75 % phosphorus, and 600 IU/kg vitamin D_3. Food and tap water were available ad libitum to all rats. The rats were allocated by weight to the following, weight-matched groups:

Group 1: SHAM (n = 8); Group 2: OVX (n = 8); Group 3: OVX + 15 ng $1,25(OH)_2D_3$/rat/d (n = 8); Group 4: OVX + 30 ng $1,24,25(OH)_3D_3$/rat/d (n = 8); Group 5: OVX + 15 ng $1,25(OH)_2D_3$/rat/d + 30 ng $1,24,25(OH)_3D_3$/rat/d (n = 8).

The vitamin D metabolites were dissolved in ethanol/1,2 propandiol (1:10) and added to the diet, starting 4 weeks after surgery. During the experimental period the rats were weighed every 2 weeks. Urine was collected in metabolic cages 5, 11, and 17 weeks postovariectomy. Blood samples were obtained by orbital sinus puncture under ether anesthesia 2, 8, and 14 weeks after ovariectomy, and at the end of the trial. The serum and urine samples were stored at –40 °C until assayed. Twenty-one weeks postovariectomy, all rats were sacrificed by an ether overdose. Success of ovariectomy was confirmed by failure to detect ovarian tissue and observation of marked atrophy of the uterine horns.

Blood and urine analysis

Total calcium in serum and urine was determined by flame photometry (ELEX 6361, Eppendorf Co., FRG). Serum inorganic phosphate and total serum alkaline phosphatase activity were measured with commercially available testkits (Boehringer Mannheim Co., FRG, and Sigma Chemical Co., FRG, respectively). Osteocalcin was determined by a rat-specific radioimmunoassay (kindly provided by Dr. P. v. Hauschka, Children's Hospital, Boston, Mass.) according to the method by Price and Nishimoto (38). Urinary hydroxyproline was analyzed according to a modified micromethod by Stegemann (45). Fasting urinary excretion of calcium and hydroxyproline was expressed as a ratio to creatinine excretion.

Histology

At autopsy, the first lumbar vertebrae and the right tibiae were carefully defleshed, and the proximal part of the tibia (about 10 mm long) was separated fom the shaft with a fine saw. The bones were fixed immediately in 40 % ethanol at 4 °C for 48 h (3), and embedded undecalcified in methyl-methacrylate, as described previously (9). Five-µm-thick undecalcified sections were prepared with a Jung Polycut E sledge microtome (Reichert-Jung, FRG). The sections were sampled in the median plane of the vertebrae and the midsagittal plane of the tibiae, and stained with toluidine blue at acid pH (3) and with von Kossa/toluidine blue (9).

Histomorphometry

All measurements were performed on the cancellous bone of the first lumbar vertebral body and the proximal tibial metaphysis.

First lumbar vertebra: With a semiautomatic system (Videoplan, C. Zeiss, FRG), 30 fields (4.4 mm^2) were evaluated in each section at ×250 magnification (×25 plan objective). The area within 0.8 mm from the cranial and caudal growth plates was excluded from the measurements (23). One section stained with toluidine blue was analyzed per animal.

The following parameters were determined: Total tissue area (Tt.T.Ar), total bone area (Tt.B.Ar), total bone perimeter (Tt.B.Pm), total osteoid area (Tt.O.Ar), total osteoid perimeter (Tt.O.Pm), total osteoblast perimeter (Tt.Ob.Pm), and the number of osteoclasts (N.Oc). Osteoblasts were defined as mononuclear, basophilic, cuboidal cells with a prominent Golgi apparatus and in contact with osteoid. Osteoclasts were defined as large, irregularly shaped cells with one or more nuclei and a foamy, slightly metachromatic cytoplasma. Typical osteoclast profiles without a nucleus were also regarded as "osteoclasts".

From these data, the following parameters were calculated (36):

Bone area (B.Ar/T.Ar) = Tt.B.Ar/Tt.T.Ar * 100	[%]
Bone perimeter (B.Pm/T.Ar) = Tt.B.Pm/Tt.T.Ar	[mm/mm^2]
Trabecular width (Tb.Wi) = Tt.B.Ar/Tt.B.Pm * 2000	[µm]
Osteoid area (O.Ar/B.Ar) = Tt.O.Ar/Tt.B.Ar * 100	[%]
Osteoid perimeter (O.Pm/B.Pm) = Tt.O.Pm/Tt.B.Pm * 100	[%]
Osteoblast perimeter (Ob.Pm/B.Pm) = Tt.Ob.Pm/Tt.B.Pm * 100	[%]

Table 1. Body weight, serum and urine biochemical parameters in sham-operated rats, OVX rats, and OVX rats orally treated with vitamin D metabolites, 21 weeks postovariectomy (17 weeks postovariectomy for urine parameters).

Variable \ Treatment	Group 1 (n = 7) SHAM	Group 2 (n = 8) OVX	Group 3 (n = 8) OVX + $1,25(OH)_2D$	Group 4 (n = 7) OVX + $1,24,25(OH)_3D$	Group 5 (n = 8) OVX + $1,25(OH)_2D$ + $1,24,25(OH)_3D$
Body weight [g]	$194^b \pm 7$	207 ± 12	$183^b \pm 8$	201 ± 8	$185^a \pm 15$
Serum calcium [mmol/liter]	2.64 ± 0.04	2.64 ± 0.03	$2.97^c \pm 0.11$	2.70 ± 0.10	$3.09^c \pm 0.06$
Serum phosphate [mmol/liter]	$1.24^a \pm 0.21$	1.05 ± 0.14	$1.56^b \pm 0.11$	$1.33^b \pm 0.17$	$1.69^b \pm 0.26$
Serum alkaline phosphatase [U/liter]	$128^b \pm 20$	157 ± 11	119*	119*	105*
Serum osteocalcin [ng/ml]	45.4 ± 3.2	53.8 ± 7.3	$71.3^b \pm 3.9$	53.1 ± 4.5	$68.2^b \pm 7.1$
Urinary calcium/creatinine [mmol/mmol]	0.39 ± 0.12	0.28 ± 0.10	$2.94^c \pm 0.57$	$1.69^c \pm 0.32$	$2.74^c \pm 0.43$
Urinary hydroxyproline/creatinine [µg/mg]	17.8 ± 2.3	20.1 ± 5.1	$12.0^a \pm 2.0$	$12.7^a \pm 1.8$	$11.0^b \pm 2.4$

All values are means ± SD.
$^a = p < 0.05$, $^b = p < 0.01$, $^c = p < 0.005$ vs. Gp 2 (OVX).
* Serum alkaline phosphatase activity was measured in pooled serum in Gps 3, 4, and 5.

Osteoid width (O.Wi) = Tt.O.Ar/Tt.O.Pm * 1000 [μm]
Osteoblast-osteoid ratio (Ob.Pm/O.Ar) = Tt.Ob.Pm/Tt.O.Ar [mm/mm^2]
Osteoclast number (N.Oc/Md.Pm) = N.Oc/Tt.Md.Pm [#/mm]

Since osteoclasts usually avoid osteoid, the parameter osteoclast number (N.Oc/Md.Pm) was related to the mineralized bone perimeter (Md.Pm), with Tt.Md.Pm = Tt.B.Pm – Tt.O.Pm. The parameter osteoblast-osteoid ratio (Ob.Pm/O.Ar) relates the osteoid surface covered by active osteoblasts to the total amount of osteoid present. The osteoblast-osteoid ratio is believed to be a sensitive static index of bone mineralization (43), and impaired bone mineralization is reflected by a reduction in this parameter.

Proximal tibial metaphysis: This measurement was made with an automatic image analysis system (IBAS, C. Zeiss, FRG) connected to a Zeiss Universal microscope (C. Zeiss, FRG) via a TV-camera (Bosch, FRG). All measurements with the automatic image analysis system were performed with a ×1.25 plan objective in sections stained with von Kossa/toluidine blue. The area within 1 mm from the growth plate was excluded from the measurements (23). The measuring field encompassed the whole secondary spongiosa of the proximal tibial metaphysis. The average measuring area was about 11 mm^2 in each section. One section was analyzed per animal. The image analysis system automatically determined the measuring area (= tissue area, T.Ar.), total bone area (Tt.B.Ar), total bone perimeter (Tt.B.Pm), and the number of trabeculae within the measuring area (N.Tb). From these data the following parameters were calculated (36):

Bone area (B.Ar/T.Ar) = Tt.B.Ar/T.Ar * 100 [%]
Bone perimeter (B.Pm/T.Ar) = Tt.B.Pm/T. Ar [mm/mm^2]
Trabecular width (Tb.Wi) = Tt.B.Ar/Tt.B.Pm * 2000 [μm]
Trabecular number (N.Tb/T.Ar) = N.Tb/T.Ar [#/mm^2]
Trabecular area (B.Ar/N.Tb) = Tt.B.Ar/N.Tb [mm^2]

Since the structural elements in the cancellous bone of both the lumbar vertebral body and the proximal tibial metaphysis show a markedly anisotropic distribution in the rat, we used only two-dimensional histomorphometric terms.

Statistical analysis

The data were analyzed using the Kruskal-Wallis H-test. When the Kruskal-Wallis H-test performed over all groups indicated a significant ($p < 0.05$) difference among the groups, statistical differences between two groups were evaluated with the two-tailed Wilcoxon-Mann-Whitney U-test. P values of less than 0.05 were considered significant. The data are presented as the mean ± SD. Relationships between variables were evaluated using linear regression analysis (least squares method).

Results

Body weight (Table 1)

At the end of the experiment, OVX rats weighed significantly more than sham-operated animals. The final body weights of OVX rats treated with 15 ng $1,25(OH)_2D_3$/rat/d alone or in combination with 30 ng $1,24,25(OH)_3D_3$/rat/d were reduced as compared to values in untreated OVX rats. The application of $1,24,25(OH)_3D_3$ alone did not result in a significantly decreased weight gain relative to untreated OVX rats.

Serum and urine biochemical findings (Table 1)

Effects of ovariectomy: Relative to sham-operated controls, OVX rats showed a significantly increased activity of serum alkaline phosphatase,

Table 2. Static histomorphometric data in the cancellous bone of the first lumbar vertebra in sham-operated rats, OVX rats, and OVX rats orally treated with vitamin D metabolites, 21 weeks postovariectomy.

Variable \ Treatment	Group 1 (n = 6) SHAM	Group 2 (n = 8) OVX	Group 3 (n = 5) OVX + $1,25(OH)_2D$	Group 4 (n = 5) OVX + $1,24,25(OH)_3D$	Group 5 (n = 5) OVX + $1,25(OH)_2D$ + $1,24,25(OH)_3D$
B.Ar/T.Ar, Bone area [%]	$26.1^{d} \pm 3.1$	15.7 ± 2.7	$31.4^{c} \pm 5.9$	$23.9^{c} \pm 4.2$	$30.0^{c} \pm 5.3$
B.Pm/T.Ar, Bone perimeter [mm/mm^2]	$5.27^{d} \pm 0.21$	3.54 ± 0.27	$5.06^{c} \pm 0.30$	$4.78^{c} \pm 0.32$	$5.08^{c} \pm 0.52$
Tb.Wi, Trabecular width [μm]	$99.1^{d} \pm 12.8$	88.3 ± 11.0	$125^{b} \pm 24$	100 ± 15	$117^{b} \pm 11$
O.Ar/B.Ar, Osteoid area [%]	$0.87^{d} \pm 0.24$	2.00 ± 0.58	2.63 ± 1.80	$0.54^{c} \pm 0.23$	2.25 ± 0.99
O.Pm/B.Pm, Osteoid perimeter [%]	$7.28^{d} \pm 1.65$	14.9 ± 2.8	21.1 ± 13.2	$5.43^{c} \pm 1.72$	21.2 ± 6.6
Ob.Pm/B.Pm, Osteoblast perimeter [%]	$5.58^{d} \pm 1.57$	11.5 ± 2.0	13.9 ± 10.3	$4.16^{c} \pm 1.02$	14.7 ± 5.2
O.Wi, Osteoid width [μm]	5.83 ± 0.59	5.79 ± 0.77	$7.64^{a} \pm 1.44$	$4.82^{a} \pm 0.43$	$6.01^{e} \pm 0.47$
Ob.Pm/O.Ar, Osteoblast-osteoid ratio [mm/mm^2]	133 ± 26	137 ± 26	$83.6^{c} \pm 12.9$	163 ± 22	$115^{e} \pm 12$
N.Oc/Md.Pm, Osteoclast number [#/mm]	1.08 ± 0.34	1.59 ± 0.46	$0.38^{c} \pm 0.17$	$0.75^{c} \pm 0.56$	$0.36^{c} \pm 0.31$

All values are means ± SD.
^{a} = $p < 0.05$, ^{b} = $p < 0.01$, ^{c} = $p < 0.005$, ^{d} = < 0.001 vs. Gp 2 (OVX).
^{e} = $p < 0.05$ vs. Gp 3 (OVX + $1,25(OH)_2D_3$).

and significantly reduced levels of serum phosphate, 21 weeks post-ovariectomy. Values for serum calcium, osteocalcin, and urinary excretion of hydroxyproline and calcium did not differ significantly in OVX and sham-operated rats. However, when compared with sham-operated controls, there was a non-significant trend towards increased values for serum osteocalcin and fasting urinary hydroxyproline/creatinine excretion in OVX rats at all time points during the study (data only partially shown).

Effects of vitamin D metabolites in OVX rats: Treatment of OVX rats with $1,25(OH)_2D_3$ alone or in combination with $1,24,25(OH)_3D_3$ significantly raised the serum levels of calcium, phosphate, and osteocalcin, and caused an about 10-fold increase in urinary excretion of calcium relative to

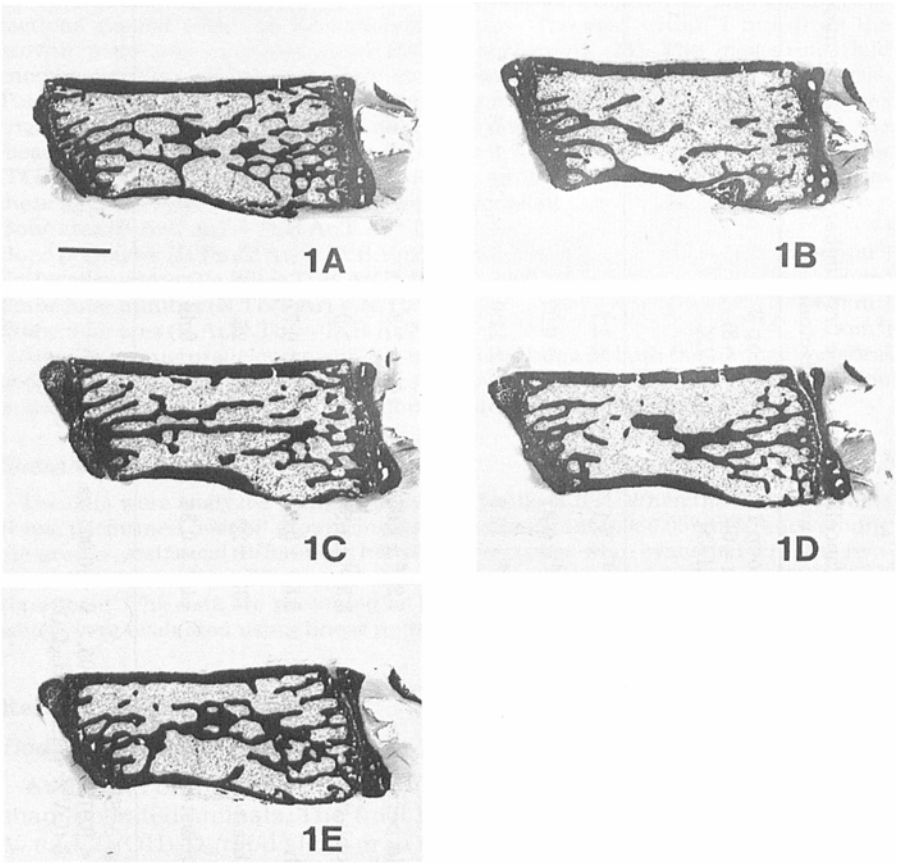

Fig. 1. Representative, undecalcified, median sections (5 μm thick) of the first lumbar vertebral bodies from sham-operated rats (Fig. 1A), OVX rats (Fig. 1B), OVX rats treated with $1,25(OH)_2D_3$ (Fig. 1C), OVX rats treated with $1,24,25(OH)_3D_3$ (Fig. 1D), and from OVX rats treated with $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$ (Fig. 1E). Note the loss of cancellous bone mass in the OVX animal (Fig. 1B). Also note that treatment with vitamin D metabolites prevented cancellous bone osteopenia in the lumbar vertebral body of OVX rats. Von Kossa/toluidine blue stain. Bar = 1 mm.

untreated OVX rats. Sole administration of $1,24,25(OH)_3D_3$ to OVX rats significantly augmented urinary excretion of calcium and increased serum phosphate, whereas the serum levels of calcium and osteocalcin remained nearly unchanged, as compared to untreated OVX rats. Relative to Gp 2 (OVX), all three OVX groups receiving vitamin D metabolites (Gps 3–5) demonstrated a significantly diminished urinary excretion of hydroxyproline, and serum alkaline phosphatase activity tended to be lower in these groups.

Vertebral cancellous bone histomorphometry (Table 2)

Effects of ovariectomy: Ovariectomy induced a highly significant loss of cancellous bone mass in the first lumbar vertebral body, indicated by a 40 % reduction in bone area in OVX rats relative to sham-operated controls, 21 weeks postovariectomy (Fig. 1). Furthermore, bone perimeter and trabecular width were significantly decreased in OVX rats. The bone formation parameters osteoid area, osteoid perimeter, and osteoblast perimeter were about twofold higher in OVX animals than in sham-operated rats. Ovariectomy did not induce changes in the parameters osteoid width and osteoblast-osteoid ratio, indicating that mineralization of osteoid was normal in OVX rats. Values for osteoclast number were about 50 % higher in OVX rats compared to sham-operated controls. This difference did not reach statistical significance, however.

Effects of vitamin D metabolites in OVX rats: Ovariectomy-induced vertebral cancellous bone loss was prevented in all three OVX groups receiving vitamin D metabolites (Gps 3–5) (Fig. 1). Significant findings in OVX rats treated with $1,25(OH)_2D_3$ (Gp 3), $1,24,25(OH)_3D_3$ (Gp 4), or the metabolite combination (Gp 5) were increased values for bone area, bone perimeter, and trabecular width (NS for Gp 4 vs. OVX) relative to Gp 2 (OVX). Bone area and trabecular width were increased in Gps 3 and 5 to levels beyond that of the control rats, whereas the values for these parameters in $1,24,25(OH)_3D_3$-treated OVX rats were similar to those in Gp 1 (SHAM). Compared to untreated OVX rats, the osteoclast number was markedly reduced in all three OVX groups receiving vitamin D metabolites. Values for osteoid area, osteoid perimeter, and osteoblast perimeter in Gps 3 and 5 were not significantly different from that in untreated OVX rats. Administration of $1,25(OH)_2D_3$ alone significantly increased osteoid width and reduced osteoblast-osteoid ratio relative to Gp 2 (OVX), indicating impaired bone mineralization in $1,25(OH)_2D_3$-treated OVX rats. Moreover, several animals in Gp 3 (OVX + $1,25(OH)_2D_3$) showed histologically apparent signs of hyperosteoidosis (Fig. 2). In contrast to that, application of $1,24,25(OH)_3D_3$ alone (Gp 4) resulted in significant decreases in osteoid area, osteoid perimeter, osteoblast perimeter, and osteoid width compared to untreated OVX rats. In OVX rats treated with the metabolite combination, the osteoid width was only slightly increased (NS) and the osteoblast-osteoid ratio was moderately decreased (NS) relative to untreated OVX animals, and the difference in these parameters between Gp 3 (OVX + $1,25(OH)_2D_3$) and Gp 5 (OVX + $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$) reached statistical significance ($p < 0.05$). A very typical finding in OVX rats treated with $1,25(OH)_2D_3$ alone or in combination with

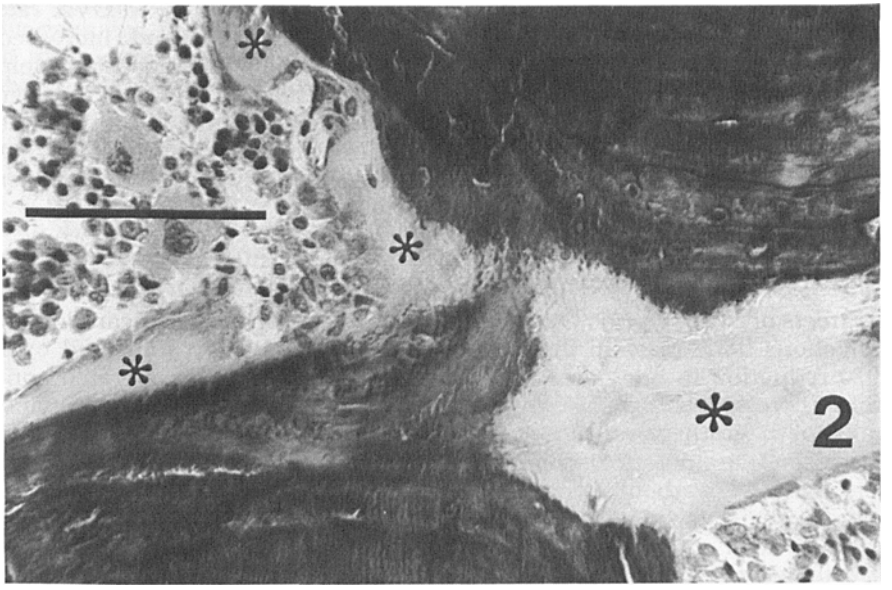

Fig. 2. Excessive amounts of osteoid (*) in the cancellous bone of the lumbar vertebral body of an OVX rat treated with 15 ng $1,25(OH)_2D_3$/rat/d. Mineralized bone appears dark. Toluidine blue stain. Bar = 100 µm.

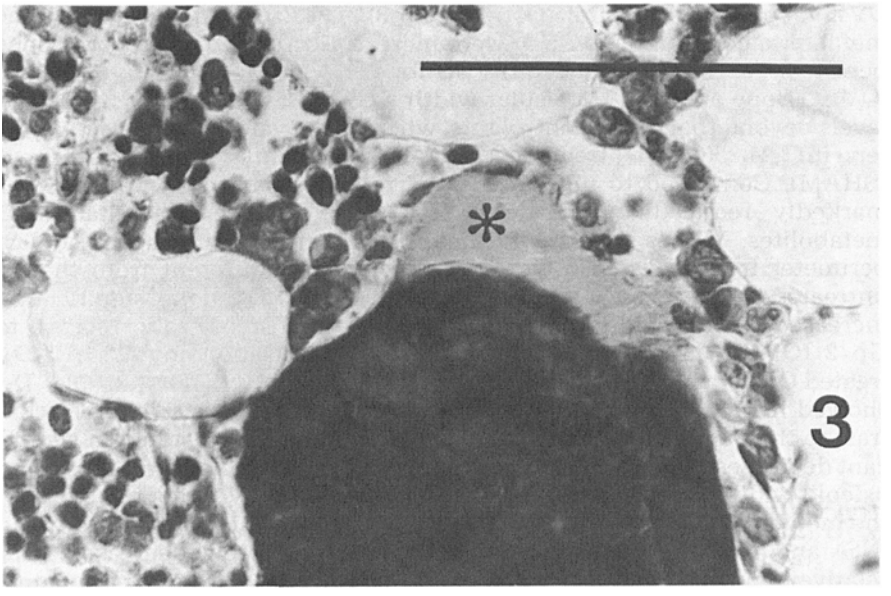

Fig. 3. Typical "osteoid button" (*) protruding over the endosteal bone surface in the cancellous bone of the lumbar vertebral body of an OVX rat treated with 15 ng $1,25(OH)_2D_3$/rat/d. Mineralized bone appears dark. Toluidine blue stain. Bar = 100 µm.

$1,24,25(OH)_3D_3$ was the appearance of "osteoid buttons" protruding over the endosteal bone surface (Fig. 3). These "osteoid buttons" were exclusively observed in Gps 3 and 5.

An inverse relationship was found between vertebral bone area and osteoclast number ($r = -0.64$, $p < 0.001$, $n = 29$; Fig. 4), as well as between vertebral bone area and urinary hydroxyproline/creatinine excretion ($r = -0.58$, $p < 0.01$, $n = 27$). Furthermore, urinary hydroxyproline/creatinine excretion increased in highly significant positive correlation with osteoclast number ($r = 0.59$, $p < 0.001$, $n = 27$). Serum osteocalcin was significantly correlated with bone area ($r = 0.48$, $p < 0.05$, $n = 25$), osteoid perimeter ($r = 0.49$, $p < 0.01$, $n = 25$), osteoblast perimeter ($r = 0.38$, $p < 0.05$, $n = 25$), and osteoblast-osteoid ratio ($r = -0.56$, $p < 0.01$, $n = 25$; Fig. 5).

Tibial cancellous bone histomorphometry (Table 3)

Effects of ovariectomy: OVX rats exhibited a pronounced reduction in bone area, bone perimeter, trabecular number, and trabecular area in the cancellous bone of the proximal tibial metaphysis relative to sham-operated controls (Fig. 6). The parameter trabecular width was only non-significantly decreased in OVX rats.

Effects of vitamin D metabolites in OVX rats: Treatment of OVX rats with $1,25(OH)_2D_3$ alone (Gp 3) or in combination with $1,24,25(OH)_3D_3$ (Gp 5) partially prevented the ovariectomy-induced bone loss in the tibial metaphysis (Fig. 6), and significantly increased the values for bone area, bone perimeter, and trabecular number relative to Gp 2 (OVX). However, the animals in Gps 3 and 5 did not reach the values of the sham-operated group in these parameters. Particularly the treatment with the metabolite combination resulted in a coarsening of tibial cancellous bone structure (Fig. 6E) with concomitant (non-significant) increases in trabecular width and trabecular area relative to untreated OVX rats. Administration of $1,24,25(OH)_3D_3$ alone to OVX rats induced small, but significant increments in bone area and bone perimeter, when compared to untreated OVX rats (Fig. 6D). However, these effects were distinctly less pronounced than in the $1,25(OH)_2D_3$-treated Gps 3 and 5 (Figs. 6C and 6E).

Discussion

In agreement with previous findings of ovariectomy-induced cancellous bone loss accompanied by increased bone turnover in the axial and appendicular skeleton of the rat (47, 49–51), the present study demonstrates a 40 % reduction in cancellous bone area in the first lumbar vertebral body and a 69 % reduction in cancellous bone area in the proximal tibial metaphysis of OVX rats, 21 weeks postovariectomy. The bone loss in OVX rats was associated with raised serum levels of alkaline phosphatase and an about twofold increase in static histomorphometric indices of bone formation, whereas histomorphometric and biochemical indices of bone resorption were only non-significantly enhanced. Our finding of a merely moderate increment in bone turnover in OVX rats 21 weeks postovariectomy is in keeping with the serial histomorphometric studies by Wronski

Table 3. Static histomorphometric data in the cancellous bone of the proximal tibial metaphysis in sham-operated rats, OVX rats, and OVX rats orally treated with vitamin D metabolites, 21 weeks postovariectomy.

Variable \ Treatment	Group 1 (n = 7) SHAM	Group 2 (n = 8) OVX	Group 3 (n = 5) OVX + $1,25(OH)_2D$	Group 4 (n = 5) OVX + $1,24,25(OH)_3D$	Group 5 (n = 5) OVX + $1,25(OH)_2D$ + $1,24,25(OH)_3D$
B.Ar/T.Ar, Bone area [%]	$21.3^{d} \pm 8,4$	6.78 ± 1.99	$14.5^{c} \pm 2.3$	$10.3^{a} \pm 1.5$	$16.4^{c} \pm 4.8$
B.Pm/T.Ar, Bone perimeter [mm/mm^2]	$5.06^{d} \pm 1.47$	1.92 ± 0.43	$3.68^{c} \pm 0.47$	$2.80^{b} \pm 0.38$	$3.87^{c} \pm 0.79$
Tb.Wi, Trabecular width [µm]	82.7 ± 11.5	$70.2 \pm 8,4$	78.7 ± 6.7	73.9 ± 8.2	86.5 ± 14.3
N.Tb/T.Ar, Trabecular number [#/mm^2]	$3.16^{c} \pm 0.71$	1.74 ± 0.44	$2.65^{a} \pm 0.38$	2.05 ± 0.59	$2.18^{a} \pm 0.59$
B.Ar/N.Tb, Trabecular area [mm^2 * 10^{-3}]	$71.7^{a} \pm 37.9$	42.2 ± 23.0	56.2 ± 14.3	53.8 ± 19.1	84.1 ± 47.9

All values are means ± SD.
^{a} = $p < 0.05$, ^{b} = $p < 0.01$, ^{c} = $p < 0.005$, ^{d} = $p < 0.001$ vs. Gp 2 (OVX).

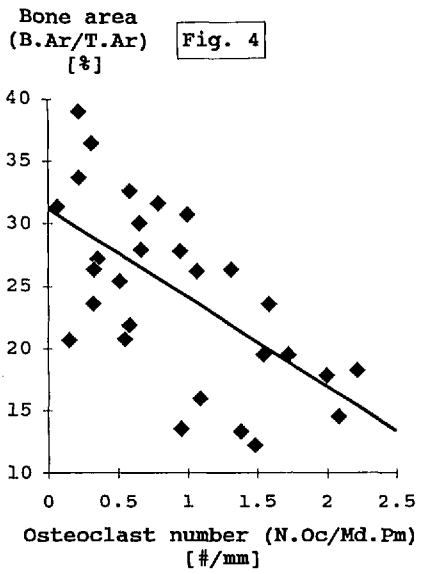

Fig. 4. Linear plot of vertebral cancellous bone area (B.Ar/T.Ar) on osteoclast number (N.Oc/Md.Pm) ($y = 31.24 - 7.42 * x$; $r = -0.64$; $p < 0.001$; $n = 29$).

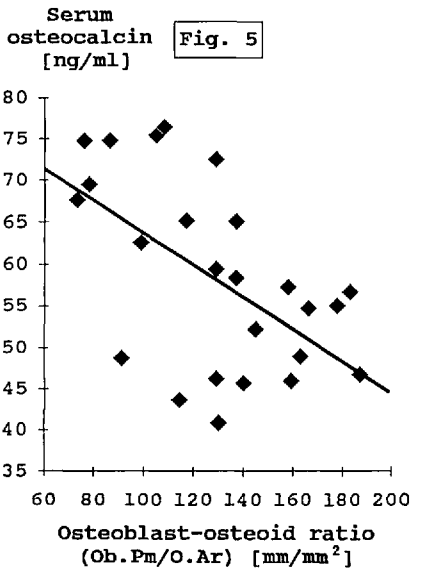

Fig. 5. Linear plot of serum osteocalcin on osteoblast-osteoid ratio (Ob.Pm/O.Ar) ($y = 82.41 - 0.185 * x$; $r = -0.56$; $p < 0.01$; $n = 25$).

et al. (49, 51) in OVX rats which have shown that tibial and vertebral cancellous bone turnover is maximally increased within the first 10 weeks after ovariectomy, and gradually declines towards control levels subsequently.

The development of vertebral osteopenia was prevented by daily administration of 15 ng $1,25(OH)_2D_3$, 30 ng $1,24,25(OH)_3D_3$, or the combination of both metabolite dosages to OVX rats in this experiment. On the other hand, tibial cancellous bone was only partially preserved in OVX rats in all three treatment groups, and the protective effect of $1,24,25-(OH)_3D_3$ alone against tibial cancellous bone loss in OVX rats was comparatively weak. This finding can probably be explained by the fact that the administration of vitamin D metabolites to OVX rats was commenced not before 4 weeks after ovariectomy in this experiment. Ovariectomy-induced bone loss in the rat is more rapid in the proximal tibia than in the vertebral body, probably due to a greater initial increase in bone turnover after ovariectomy in the long bones of the appendicular skeleton (51). Within 35 days postovariectomy, OVX rats loose about 30 % of tibial cancellous bone (49), whereas only about 10 % of vertebral cancellous bone is lost within the same time (51). Thus, the substantial loss of tibial cancellous bone mass that can be expected to have developed by 4 weeks postovariectomy in the current study could apparently not be compensated for by treatment with vitamin D metabolites at dosages that increased bone area in the lumbar vertebral body of OVX rats over that of

control animals. This finding suggests that with regard to the prophylaxis of the ovariectomy-induced osteopenia in the appendicular skeleton of the young adult rat, most benefit can be derived from a treatment regimen starting immediately after ovariectomy, i.e., before major bone loss has developed. A previous study conducted in our laboratory demonstrated that the application of vitamin D metabolites can completely prevent tibial cancellous bone osteopenia in OVX rats, when the treatment is started immediately postovariectomy (10). The combination of $1,25(OH)_2D_3$ with $1,24,25(OH)_3D_3$ did not result in additive or over-additive, synergistic effects in the present study.

The mechanism of action of vitamin D metabolites very likely consists in inhibition of bone resorption, which is demonstrated in the current study by significant reductions in osteoclast number and urinary hydroxyproline excretion in OVX rats treated with vitamin D metabolites. Furthermore, this concept is corroborated by the negative correlations of osteoclast number and urinary hydroxyproline excretion with vertebral bone area observed in this experiment (Fig. 4). Although $1,25(OH)_2D_3$ is one of the most powerful stimulators of bone resorption in vitro (29), several studies have shown that in vivo administration of $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ diminishes bone resorption in humans and rats (1, 34, 35, 48). The depressive effect of vitamin D metabolites on bone resorption in vivo most likely involves suppression of PTH secretion due to augmented intestinal absorption of calcium and phosphate, and possibly also due to direct inhibitory actions of 1-hydroxylated vitamin D metabolites on PTH secretion (2, 33). PTH is one of the main regulators of osteoclastic bone resorption in vivo (32). Another possible mechanism that could indirectly decrease osteoclastic bone resorption in OVX rats treated with vitamin D metabolites is stimulation of calcitonin secretion due to hypercalcemia. Calcitonin directly inhibits osteoclast activity (32).

The bone-preserving effects of the treatment with $1,25(OH)_2D_3$ alone or in combination with $1,24,25(OH)_3D_3$ were accompanied by a variety of negative side effects in OVX rats in the present study. The OVX rats in Gps 3 and 5 receiving 15 ng $1,25(OH)_2D_3$ were hypercalcemic, hyperphosphatemic, hypercalciuric, and exhibited – particularly in Gp 3 (OVX + $1,25(OH)_2D_3$) – impaired bone mineralization. The body weights in these groups were reduced to a level below that of the control group, indicating that for the long-term treatment of Fischer-344 rats a dose of 15 ng $1,25(OH)_2D_3$/rat/d may be too high and not devoid of toxic side effects. Our finding of impaired bone mineralization at the dose of 0.08–0.11 µg $1,25(OH)_2D_3$/kg BW/d used in the present study is in agreement with previous studies which have provided evidence that $1,25(OH)_2D_3$ inhibits bone mineralization and suppresses fluorochrome-based dynamic histomorphometric indices of bone formation in the rat at doses higher than about 0.10 to 0.20 µg/kg BW/d (13, 15, 17, 48).

Thus, the finding that the static histomorphometric parameters of bone formation (osteoid area, osteoid perimeter, and osteoblast perimeter) were not reduced in the $1,25(OH)_2D_3$-treated Gps 3 and 5 relative to untreated OVX rats most likely reflects impaired bone mineralization and not a stimulating action of $1,25(OH)_2D_3$ on bone formation. The mechanism of the inhibitory action of $1,25(OH)_2D_3$ on bone mineralization at higher

dosages is still unclear. Since PTH is known to stimulate bone mineralization (46), suppression of PTH secretion could be a possible explanation for the disturbance of mineralization in $1,25(OH)_2D_3$-treated rats. Another possibility for the $1.25(OH)_2D_3$-induced impairment of bone mineralization is increased osteoblastic synthesis of osteocalcin (13), a concept that may be supported by the negative correlation of serum osteocalcin with osteoblast-osteoid ratio ($r = -0.56$, $p < 0.01$; Fig. 5) observed in this experiment. Osteocalcin is a non-collagenous bone matrix protein exclusively synthesized by osteoblasts. Osteocalcin contains calcium-binding residues of gamma carboxyglutamic acid, and is a very effective inhibitor of bone

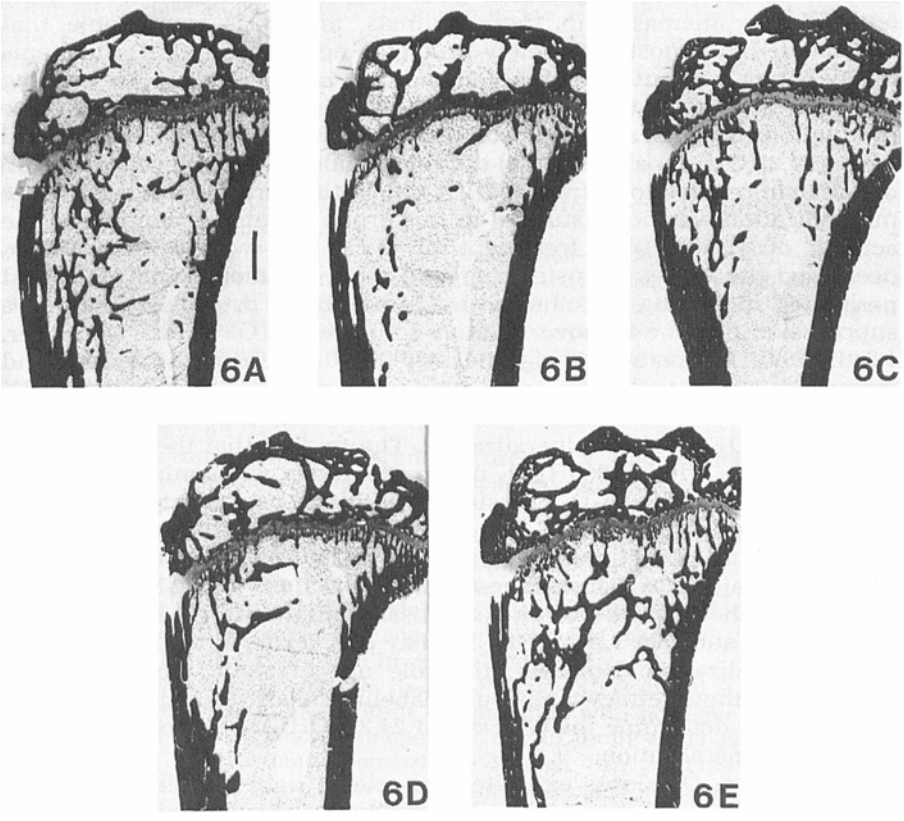

Fig. 6. Representative, undecalcified, midsagittal sections (5 μm thick) of the proximal tibiae from sham-operated rats (Fig. 6A), OVX rats (Fig. 6B), OVX rats treated with $1,25(OH)_2D_3$ (Fig. 6C), OVX rats treated with $1,24,25(OH)_3D_3$ (Fig. 6D), and from OVX rats treated with $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$ (Fig. 6E). Note that treatment with vitamin D metabolites provided partial protection against the pronounced loss of cancellous bone mass observed in the proximal tibial metaphysis of the untreated OVX rat. Also note the coarsening of cancellous bone structure in the OVX animal treated with the metabolite combination (Fig. 6E). Von Kossa/toluidine blue stain. Bar = 1 mm.

mineralization in vitro (4). However, the physiological role of osteocalcin in vivo is unclear. $1,25(OH)_2D_3$ is known to stimulate osteoblastic secretion of osteocalcin (44), a fact which is reflected in increased serum levels of osteocalcin in the $1,25(OH)_2D_3$-treated Gps 3 and 5 in the current experiment.

The characteristic "osteoid buttons" observed in $1,25(OH)_2D_3$-treated OVX rats in this study (Fig. 3) probably indicate that 1, $25(OH)_2D_3$ is able to directly transform a quiescent endosteal bone surface to a forming surface without intervening bone resorption, or, in other words, that $1,25(OH)_2D_3$ is able to induce a modeling activity in vertebral cancellous bone of OVX rats.

Although urinary excretion of calcium was enhanced by a factor of about 6 in $1,24,25(OH)_3D_3$-treated OVX rats, serum calcium levels were only slightly increased in these animals, and it is remarkable that $1,24,25(OH)_3D_3$ almost completely protected against vertebral osteopenia in OVX rats without inducing concomitant hypercalcemia. In keeping with the finding that low doses of $1,24,25(OH)_3D_3$ do not increase the production of osteocalcin by osteoblasts in vitro (44), OVX rats treated with $1,24,25(OH)_3D_3$ alone (Gp 4) did not exhibit elevated levels of serum osteocalcin relative to untreated OVX rats in the current study, and bone mineralization was not disturbed in this group of rats. In contrast to the actions of $1,25(OH)_2D_3$, treatment of OVX rats with $1,24,25(OH)_3D_3$ decreased the values for osteoid area, osteoid perimeter, and osteoblast perimeter, indicating (together with a reduction in osteoclast number) a suppression of bone turnover relative to untreated OVX rats. Moreover, $1,24,25(OH)_3D_3$ treatment significantly reduced osteoid width, and increased (NS) osteoblast-osteoid ratio, as compared to untreated OVX animals. The latter data are consistent with a promoting effect of $1,24,25(OH)_3D_3$ on bone mineralization. The finding that the inclusion of $1,24,25(OH)_3D_3$ with $1,25(OH)_2D_3$ in Gp 5 partially antagonized the untoward effects of $1,25(OH)_2D_3$ on osteoid width and osteoblast-osteoid ratio further supports this concept. In a similar fashion, the study by Tam et al. (46) demonstrated a stimulating effect of $24,25(OH)_2D_3$ on bone mineral apposition rate. Our data suggest that the actions of 24-hydroxylated vitamin D metabolites on skeletal tissue might differ from that of $1,25(OH)_2D_3$, and that $1,24,25(OH)_3D_3$ may play a role in the regulation of bone mineralization. However, the sole use of static bone histomorphometry without tetracycline double labeling in our study did not permit to accurately determine the effects of $1,24,25\text{-}(OH)_3D_3$ on bone formation and bone mineralization.

In summary, OVX rats exhibited cancellous bone loss in the lumbar vertebral body and the proximal tibial metaphysis associated with moderately increased biochemical and histomorphometric indices of bone turnover, 21 weeks postovariectomy. Prophylactic treatment of OVX rats with $1,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, alone or in combination, prevented the development of osteopenia in the lumbar vertebral body, and partially prevented cancellous bone loss in the proximal tibial metaphysis. The mechanism of action of vitamin D metabolites consists in inhibition of bone resorption. However, the administration of $1,25(OH)_2D_3$ alone or in combination with $1,24,25(OH)_3D_3$ to OVX rats was accompanied by hyper-

calcemia, hyperphosphatemia, hypercalciuria, and impaired bone mineralization. Although $1,24,25(OH)_3D_3$ alone had a pronounced protective effect against vertebral cancellous bone osteopenia in OVX rats, it neither induced hypercalcemia nor an impairment of bone mineralization. Since considerable similarities can be shown to exist between the skeletal effects of ovarian hormone deficiency in rats and humans (50), these data suggest that $1,25(OH)_2D_3$ and, particularly, $1,24,25(OH)_3D_3$ may be potentially effective agents in the prevention of early postmenopausal bone loss.

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Authors' address:

Dr. R. G. Erben, Institute of Animal Physiology, Veterinärstraße 13, 8000 München 22