Vitamin D deficiency causes a selective reduction in deposition of transforming growth factor β in rat bone: Possible mechanism for impaired osteoinduction

(bone repair/insulin-like growth factor I/insulin-like growth factor II/osteocalcin)

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ABSTRACT We demonstrated previously that implants of bone matrix prepared from vitamin D-deficient (–D) rats were less osteoinductive and contained less extractable mitogenic activity compared with control implants prepared from vitamin D-replete (+D) rats and proposed that bone from –D rats is deficient in one or more specific growth factors. To test this hypothesis, bones from rats that were fed either +D or –D diets and kept in the dark for 8 wk were extracted and assayed for insulin-like growth factors I and II (IGF-I and IGF-II) and transforming growth factor β (TGF- β), the three most abundant growth factors in rat bone, and osteocalcin. Serum calcium, 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] were determined at sacrifice. In –D rats, there were significant reductions in serum calcium, 25-hydroxyvitamin D₃, and 1,25(OH)₂D₃, and skeletal TGF- β but no differences in extractable skeletal protein, IGF-I, IGF-II, or osteocalcin compared with +D rats. To determine whether 1,25(OH)₂D₃ increased TGF- β production by bone cells, we treated mouse calvaria for 6 days and mouse osteoblasts for 2 days with 10 nM 1,25(OH)₂D₃. Production of TGF- β was increased almost 100% by 1,25(OH)₂D₃. We conclude that vitamin D deficiency reduces deposition of TGF- β in rat bone and that diminished skeletal TGF- β could contribute to the previously observed decrease in osteoinduction in implants from –D rat bone. The findings support the possibility that vitamin D and bone-derived TGF- β are required for normal repair of the skeleton.

Demineralized bone matrix is osteoinductive, can locally regulate bone formation, and can induce the differentiation of mesenchymal cells into cartilage *in vitro* (1–3) and into cartilage and bone *in vivo* (3–5). This induction is thought to be mediated by the release of one or more mitogenic agents from demineralized bone matrix, and investigations to characterize further the inductive effects of demineralized bone have identified a large number of polypeptide growth factors present in bone matrix (6). In addition, other osteoinductive factors (7) and bone morphogenetic proteins (BMPs) (8, 9) have been described. These factors may act to stimulate the proliferation or function of bone cells and may act locally to regulate bone formation (10, 11).

There is evidence that vitamin D deficiency alters bone. Collagen from vitamin D-deficient (–D) bone has increased hydroxylation of lysine residues (12) and changes in the amount and types of cross-linking (12–15) compared with collagen from vitamin D-replete (+D) bone. Matrix from –D bone has less phosphoprotein (16) and altered glycosamino-

glycans, proteoglycans, and phospholipids (17–19) compared with matrix from +D bone.

A role for vitamin D in osteoinduction has not been established. We demonstrated previously that demineralized bone matrix from –D rats was less osteoinductive *in vivo* and contained less extractable mitogenic activity *in vitro* than bone matrix from +D rats (20). Furthermore, fewer osteoinductive proteins were found in rachitic bone (21). Based on these findings, we have proposed that –D bone matrix may be deficient in one or more specific growth factors (20). To test this hypothesis, we extracted bone from –D and +D rats and assayed the extracts for insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), and transforming growth factor β (TGF- β), the three most abundant growth factors in rat bone. In addition, we determined the effect of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on TGF- β production by mouse calvaria and mouse osteoblasts.

MATERIALS AND METHODS

Animals. For *in vivo* studies, male Sprague–Dawley rats (Holtzman, Madison, WI), 60–64 days old and weighing 300–325 g, were used. They were fed ad libitum a semisynthetic diet containing 1.2% calcium and 0.6% phosphorus either with or without supplementary dietary vitamin D₃ (2 international units per g of diet) and were kept in the dark for 8 wk (20, 22). At the end of this time, blood was drawn for serum assays, and the animals were sacrificed. There were 15 animals in each group.

For *in vitro* studies, Swiss–Webster timed-pregnant mice were obtained from Bantin and Kingman (Fremont, CA) 1 wk before the projected date of parturition.

Preparation of Bone Extracts. After sacrifice, tibial and femoral diaphyses were removed, cleaned of soft tissue, and crushed. Bone pieces were washed successively in distilled water (20 min), 95% ethanol (20 min; two changes), 100% ethanol (20 min), and chloroform/methanol (1:1) (1 hr; three changes). Bone pieces were dried overnight at 30°C and crushed in a Wiley mill (Fisher); particles 90–850 μ m in diameter were frozen at –70°C until further use. Bone particles were washed for 1 hr in 4 M guanidine hydrochloride (Gdn-HCl) and 30 mM Tris-HCl (pH 7.4) containing protease inhibitors (5 mM benzamidine/100 mM ϵ -aminocaproic

Abbreviations: –D, vitamin D deficient; +D, vitamin D replete; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; TGF- β , transforming growth factor β ; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; BMP, bone morphogenetic protein; Gdn-HCl, guanidine hydrochloride.

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acid/1 mM phenylmethylsulfonyl fluoride), placed in dialysis tubing (Spectra/Por 3; M_r cut-off, 3500; Spectrum Medical Industries), and extracted by dialysis against 4 M Gdn-HCl and 30 mM Tris-HCl (pH 7.4) containing 10% EDTA and protease inhibitors (Gdn-HCl/EDTA) at 4°C (28 days; five changes) (23). Samples were desalted by dialysis against 20 mM acetic acid at 4°C (7 days; three changes) and clarified by centrifugation. Supernatants and residues were dried by lyophilization.

Analytical Procedures and Assays. Serum calcium (24) and phosphorus (25) were measured by automated colorimetric procedures. Serum 25-hydroxyvitamin D₃ was measured in duplicate at two concentrations by competitive protein binding with -D rat serum (26) after extraction and chromatography with acetonitrile, washing with phosphate buffer, chromatography on C₁₈ Sep-Pak, and elution with acetonitrile (27). 25-Hydroxyvitamin D₃ was separated from other vitamin D metabolites before the binding assay by chromatography on silica Sep-Pak and elution with hexane/propanol (94:6). Serum 1,25(OH)₂D₃ was measured by the method of Reinhardt *et al.* (27).

Undemineralized bone or Gdn-HCl/EDTA residues were decalcified or hydrolyzed in 6 M hydrochloric acid for 6 days at 4°C (23) and assayed directly for calcium by atomic absorption spectroscopy (28). Hydroxyproline was determined by measuring hydroxyproline concentrations of hydrolyzates by an automated method (29). Gdn-HCl/EDTA extracts were reconstituted in 20 mM acetic acid (1 ml per g of original bone) for the following assays. (i) IGF-I: IGF-I was measured by RIA with recombinant human IGF-I (CIBA-Geigy) as tracer and standard and rabbit polyclonal antiserum UB 286 (kindly provided by Louis Underwood and Judson Van Wyk, University of North Carolina) at a dilution of 1:6000 as described (30). IGF binding protein artifacts (31) in the IGF-I RIA or in the IGF-II radioreceptor assay (see below) were removed by preincubating samples with excess IGF-I or IGF-II as described (32). (ii) IGF-II: IGF-II was measured by radioreceptor assay with H-35 rat hepatoma cells (American Type Culture Collection, CRL 1548) as the receptor source (33). Human IGF-II purified from bone matrix extract (34) was used as standard and tracer, and the assay was performed as described (30). (iii) TGF- β : TGF- β was measured by bioassay by measuring the inhibition of DNA synthesis by mink lung epithelial cells (Mv 1 Lu; American Type Culture Collection, CCL 64) as described (35). Although it is known that TGF- β describes a multigene family of related peptides (36), our assay measures total TGF- β activity and does not distinguish among the different forms of TGF- β . It has been shown, however, that six other growth factors—epidermal growth factor, platelet-derived growth factor, acidic and basic fibroblast growth factor, IGF-I, and IGF-II—do not cross-react in the mink lung cell assay (35). In addition, to verify that our assay measured authentic TGF- β in bone extracts, aliquots of samples were incubated with an anti-TGF- β neutralizing antibody (R & D Systems, Minneapolis, MN) before assay to block TGF- β activity. (iv) Osteocalcin: Osteocalcin was measured with an RIA kit (Biomedical Technologies, Stoughton, MA) based on the method of Gundberg *et al.* (37). (v) Protein: Protein was estimated by a dye binding assay (Bio-Rad) using bovine immunoglobulin as standard (38).

In Vitro Studies. Calvaria from newborn 1-day-old mice were removed aseptically (39) and incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) containing 1 mg of bovine serum albumin per ml (Sigma), penicillin (100 units/ml)/streptomycin (100 μ g/ml) (Irvine Scientific), and either 10 nM 1,25(OH)₂D₃ (generously provided by Hoffmann-LaRoche) or vehicle (0.01% ethanol) in 24-well plates (Corning) for 6 days with one medium change on day 3. Conditioned media were collected

Table 1. Effects of vitamin D deficiency on body weight and serum values

Treat- ment	Body wt, g (15)	Serum Ca, mg/dl (15)	Serum P, mg/dl (15)	Serum 25-OHD ₃ , ng/ml (7)	Serum 1,25(OH) ₂ D ₃ , pg/ml (7)
+D	477 $\pm$ 9	10.1 $\pm$ 0.1	7.0 $\pm$ 0.2	9.1 $\pm$ 0.9	31.9 $\pm$ 4.2
-D	448 $\pm$ 2	7.0 $\pm$ 0.3	7.4 $\pm$ 0.3	0.6 $\pm$ 0.4	7.8 $\pm$ 2.1
P value	NS	<0.001	NS	<0.001	<0.001

Rats were fed a synthetic diet either with (+D) or without (-D) vitamin D₃ and were kept in the dark for 8 wk. The animals were then sacrificed, and blood was collected and assayed. The numbers in parentheses are the number of animals or determinations. NS, not significant; 25-OHD₃, 25-hydroxyvitamin D₃.

at 3 and 6 days and assayed for calcium (Calcette, Precision Systems, Natick, MA) and TGF- β . In addition, calvaria were extracted with 50% formic acid (500 μ l per half-calvaria), treated as described (40), and assayed for total TGF- β content.

Osteoblast-like mouse bone cells were obtained from collagenase digests of neonatal mouse calvaria and cultured as described (41). Cells were incubated in serum-free medium containing either vehicle (0.01% ethanol) or 10 nM 1,25(OH)₂D₃ for 48 hr. Conditioned media were removed and dialyzed (Spectra/Por 3) at 4°C against 1 M acetic acid (24 hr) and then against 20 mM acetic acid (24 hr). Dialyzates were lyophilized, reconstituted in 0.5 ml of 20 mM acetic acid, and assayed for TGF- β . Cells were extracted with 0.01% Triton X-100 and assayed for protein as described earlier.

Statistical Analysis. Data are presented as means $\pm$ SE. Significance of differences for *in vivo* studies and for *in vitro* studies of osteoblasts was determined by Student's *t* test and for *in vitro* studies of calvaria by analysis of variance and Fisher's least significant difference test.

RESULTS

Vitamin D Status. Rats maintained in the dark on a -D diet for 8 wk developed hypocalcemia and had significantly lower values for serum 25-hydroxyvitamin D₃ and 1,25(OH)₂D₃ compared with +D animals (Table 1). Mean body weight and serum phosphorus were not different from each other in the two groups.

Analysis of Bone Extracts. When mineralized bone samples were analyzed after demineralization with hydrochloric acid, there were no differences in content of either calcium (225.9 $\pm$ 7.9 versus 227.8 $\pm$ 2.9 mg per g of bone; not significant) or hydroxyproline (10.0 $\pm$ 0.3 versus 9.1 $\pm$ 0.1 mg per g of bone; not significant) of bone from -D rats compared with bone from +D rats, respectively. Analysis of samples after extraction with Gdn-HCl/EDTA showed no calcium (data not shown), indicating that demineralization was complete. The concentrations of protein, IGF-I, IGF-II, and osteocalcin were not different from each other in the extracts of bone

Table 2. Effects of vitamin D deficiency on extractable bone protein, growth factors, and osteocalcin

Treatment	Protein	IGF-I	IGF-II	TGF- β	Osteocalcin
+D (14)	1.7 $\pm$ 0.2	370 $\pm$ 45	49 $\pm$ 9	58 $\pm$ 13	226 $\pm$ 18
-D (15)	2.2 $\pm$ 0.3	366 $\pm$ 31	61 $\pm$ 7	23 $\pm$ 5	265 $\pm$ 26
P value	NS	NS	NS	0.014	NS

Rats were fed a synthetic diet with (+D) or without (-D) vitamin D₃ and were kept in the dark for 8 wk. The animals were then sacrificed; the tibial and femoral diaphyses were removed, cleaned, and extracted; and the extracts were assayed. Number of animals is given in parentheses. Protein levels are expressed as mg per g of bone; IGF-I, IGF-II, and TGF- β levels are expressed as ng per mg of protein; and osteocalcin levels are expressed as μ g per mg of protein.

Table 3. Inhibition of bone extract TGF- β by anti-TGF- β antibody

Addition	TGF- β activity, pg per well	
	– anti-TGF- β	+ anti-TGF- β
None	ND	ND
TGF- β 1 (50 pg)	53.2	ND
Sample 1	129.5	ND
Sample 2	61.7	ND
Sample 3	98.9	ND
Sample 4	56.4	ND
Sample 5	23.0	ND
Sample 6	83.3	ND

Rat bone extracts were pooled and assayed for TGF- β activity after a 1-hr preincubation (22°C) with (+) or without (–) anti-TGF- β neutralizing antibody (25 μ g). Samples represent pooled extracts from four or five bones. ND, not detected (i.e., <5 pg per well).

from the +D and –D animals (Table 2). In contrast, the concentration of TGF- β was significantly lower in extracts of bone from –D rats compared with extracts of bone from +D animals. As shown in Table 3, TGF- β activity in samples was completely abolished by preincubation with anti-TGF- β neutralizing antibody, indicating that our assay measured authentic TGF- β .

Bone Cell Production of TGF- β . 1,25(OH) $_2$ D $_3$ caused a significant increase in TGF- β production by mouse calvaria (Table 4). Although an estimate of total TGF- β (data not shown) in the calvaria suggested that 1,25(OH) $_2$ D $_3$ stimulated synthesis as well as release of TGF- β , the conclusion that synthesis was actually increased was established by the finding that treatment of mouse osteoblasts directly with 1,25(OH) $_2$ D $_3$ significantly increased production of TGF- β (Table 5).

DISCUSSION

In this study, we found less extractable TGF- β in bone from –D rats than in bone from +D animals. There were no differences in the amount of extractable protein, IGF-I, IGF-II, or osteocalcin in the same samples. The data provide evidence that the difference resulted in part from deficiency of 1,25(OH) $_2$ D $_3$. This possibility was supported by the observation that TGF- β production by mouse calvaria and by mouse osteoblast-like bone cells increased in response to 1,25(OH) $_2$ D $_3$. Previous studies demonstrated that 1,25(OH) $_2$ D $_3$ increased the release of TGF- β by mouse calvaria (42) and rat bone cells (43).

Osteoinduction involves the specific proliferation and differentiation of precursor cells via a cellular cascade into functional bone cells, osteoblasts and osteocytes, with the elaboration of bone matrix and subsequent mineralization (44). Since demineralized bone implants from –D rats were less osteoinductive *in vivo* and contained less mitogenic activity *in vitro* than bone implants from +D rats, we

Table 4. Effects of 1,25(OH) $_2$ D $_3$ on TGF- β production by neonatal mouse calvaria

Treatment	Calcium, mM	TGF- β , ng per half-calvaria
Control (6)	1.66 $\pm$ 0.03	2.45 $\pm$ 0.47
1,25(OH) $_2$ D $_3$ (6)	2.26 $\pm$ 0.09	4.21 $\pm$ 0.47
<i>P</i> value	<0.001	<0.05

Calvaria from neonatal mice were treated with 1,25(OH) $_2$ D $_3$ or vehicle in serum-free DMEM for 6 days. Media were collected at 3 and 6 days and assayed for calcium and TGF- β . Calcium values are the average of 3- and 6-day medium samples; TGF- β values are the total of combined 3- and 6-day medium of individual samples. Number of observations is given in parentheses.

Table 5. Effects of 1,25(OH) $_2$ D $_3$ on production of TGF- β by mouse osteoblasts

Treatment	Cell protein, μ g per well	TGF- β , pg per μ g of protein
Control (12)	123.6 $\pm$ 2.1	14.0 $\pm$ 0.8
1,25(OH) $_2$ D $_3$ (12)	118.0 $\pm$ 2.2	22.9 $\pm$ 1.1
<i>P</i> value	NS	<0.001

Osteoblast-like cells derived from neonatal mouse calvaria were treated with 1,25(OH) $_2$ D $_3$ or vehicle for 48 hr, conditioned media were collected and assayed for TGF- β , and cells were extracted and assayed for protein. Number of wells is given in parentheses.

proposed that bone implants from –D rats were deficient in one or more factors that stimulate bone cells (20).

Rat bone cells produce a number of growth factors including TGF- β (45), IGF-I (46, 47), and IGF-II (47). Our finding that –D bone contains less TGF- β than +D bone is consistent with the possibility that the reduction could be wholly or partly responsible for the decreased osteoinductive or mitogenic activities in extracts of bone from –D rats and suggests a role for TGF- β in the osteoinductive mechanism. In keeping with this hypothesis was the demonstration that removal of TGF- β from bone extracts with osteoinductive activity caused a marked loss in activity and that addition of TGF- β restored that activity (48).

A number of BMPs were isolated from bone matrix and found to have osteoinductive properties (49–51). BMPs make up a family of related peptides, seven of which have been described to date (8, 9, 52). Six BMPs (BMP-2 through BMP-7) are structurally related to the TGF- β family (9), but, despite this similarity, these six were found to have no activity in the mink lung cell assay (Vicki Rosen, personal communication). These results provide evidence that our assay measures activity of TGF- β and not that of BMP. Our findings, however, do not eliminate the possibility that –D bone could have reduced amounts of other osteoinductive proteins such as BMP.

Our result that –D rat bone is deficient in TGF- β activity implicates TGF- β as an important factor in the osteoinductive mechanism. Others have also assigned TGF- β an important role both in stimulating bone formation (53, 54) and in bone induction or repair (7, 55). Our data suggest that –D bone matrix is less osteoinductive because of the reduction of TGF- β caused by vitamin D deficiency and support the hypothesis that sufficient bone TGF- β is necessary for normal bone repair processes.

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