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Research Article

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Rapid Publication

Resorption of Implanted Bone Prepared from Normal and Warfarin-treated Rats

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Abstract. Bone that was virtually depleted of the vitamin K-dependent protein, osteocalcin, and 93% reduced in the concentration of its characteristic amino acid, γ-carboxyglutamic acid, was obtained from rats treated with warfarin for 6 wk. Osteocalcin-deficient bone particles were resistant to resorption when implanted subcutaneously in normal rats. The relative resorption was 60% of control bone, as measured by histomorphometry as percent of bone particles in the field. Additionally, the number of multinucleated cells around the bone particles was reduced by 54%. These data suggest that osteocalcin is an essential component for bone matrix to elicit progenitor-cell recruitment and differentiation necessary for bone resorption.

Introduction

Osteocalcin (bone Gla protein), the most abundant noncollagenous protein in bone, is characterized by three residues of γ-carboxyglutamic acid (Gla)[1] per 5,700 molecule (1, 2). The protein is synthesized *de novo* in bone tissue dependent upon vitamin K and CO_2 and appears concomitant with the first formation of bone. The anticoagulant warfarin inhibits Gla formation in bone (3) and it also decreases the bone concentration of osteocalcin (4). Although many properties of the protein have been defined (1, 2), a physiologic role for osteocalcin has yet to be identified.

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1. *Abbreviations used in this paper:* BP, bone particles; Gla, γ-carboxyglutamic acid.

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Recently, it has been demonstrated that osteocalcin and specifically the amino acid Gla are chemoattractants for monocytes (5, 6). Therefore, the chemoattractant property of osteocalcin may be central to recruitment, differentiation, and regulation of bone resorbing cells, and as such this hypothesis was tested in vivo. Bone resorption can be quantitated by histomorphometric analysis of devitalized bone particles after implantation into subcutaneous pockets in rats (7, 8). This model provides an opportunity for assessing resorption of an altered bone matrix.

Methods

Animals. Seven pregnant Sprague Dawley rats were obtained at 19 d gestation and maintained on normal rodent chow (Purina 5009, Ralston Purina Co., Chicago, IL). Starting 1 d after birth, offspring were given daily subcutaneous injections of sodium warfarin (Endo Laboratories, Inc., Garden City, NY) and vitamin K (Sigma Chemical Co., St. Louis, MO) or saline (controls), precisely as described by Price and Williamson (4). At 44 d of age, the rats were exsanguinated by cardiac puncture under ether anesthesia. The limb bones were removed, stripped of soft tissue, and washed in 100% ethanol. The diaphyses of 50 experimental and control rats were pooled, lyophilized, pulverized in a liquid nitrogen mill, and sieved into 75–250-μm particles for implant studies.

Biochemical measurements. 5-mg aliquots of bone particles from six separate rats and the pooled bone from 50 rats were analyzed for Gla, hydroxyproline, calcium, phosphorus, and protein content as previously described (9). Rat bone osteocalcin was measured by a radioimmunoassay procedure that used purified rat osteocalcin as standard and tracer, with goat anti-rat osteocalcin antiserum, generously provided by Dr. Peter Hauschka (Children's Hospital, Boston). Antigen preparation and species-specific antisera were raised by following described procedures (10). The procedure for measurement of bone osteocalcin follows the assay described by Hauschka et al. (10) with the exception that the addition of 20,000 cpm of ^{125}I-osteocalcin was delayed 24 h. This increased the sensitivity with assays being linear between 0.5 and 25 ng/ml of osteocalcin with a limit of detectability of 0.2 ng/ml of osteocalcin. Antibodies to chick (10), bovine (11), and rat (12 and unpublished observations of this laboratory) osteocalcins do not distinguish native from thermally decarboxylated osteocalcins.

Resorption assay: implantation of devitalized bone particles BP. In each experiment, ~50 mg of pooled BP were inserted into bilateral, subcutaneous pockets in two or three normal rats (Charles River CD strain, male, 28 d old) providing four to six specimens per group. 12 d later, BP and surrounding tissues were harvested as encapsulated buttons and prepared for nondecalcified histomorphometric analysis (7). The percent area in contiguous fields representing bone particles was calculated with a Zeiss-Kontron digital image analyzer. Values are expressed as means of 80–120 fields (±SEM). The *t* test for unpaired data was used to estimate significance in each experiment.

Results

After 6 wk of sodium warfarin treatment, mean weight of the experimental group was 135±27 g, compared with the control group, which was 160±22 g. Bone parameters of total ash, calcium, phosphorus, total protein, and collagen were not significantly different in control and experimental groups (Table I). In warfarin-treated animals, Gla content of the bone pool used for implants was 0.046±0.02 residues/10^3 amino acids, which is only 7.1% of the control value (0.65±0.05 residues/10^3 amino acids). Osteocalcin was virtually absent in warfarin bone, 0.05±0.0003 mg/g bone, which is 0.6% of the control concentration of 2.075±0.28 mg/g. The only difference observed in our results from the results described by Price and Williamson (4) was a 15% decreased growth rate in the warfarin-treated animals.

Implants removed on day 12 showed that bone particles from the warfarin-treated animals were resorbed to a much lesser extent than were BP from control animals. Fig. 1 represents typical fields of specimens of BP from control (*A*) and warfarin-treated (*B*) animals, which show the particles and surrounding connective tissue. The osteocalcin-deficient BP were larger and showed much less evidence of surface resorptive lacunae than was seen in control specimens. Histomorphometric analysis (Table II) of three experiments showed a consistent defect in the resorption of BP from warfarin-treated rats ($P < 0.001$). For example, in experiment one, the BP from control bone accounted for 27.9% of the microscopic field, which is equivalent to 44.2% resorption of the original BP volume. In contrast, 39.9% of the field was occupied by osteocalcin-deficient bone particles, which is equivalent to 20.2% resorption. Thus, only 46% of osteocalcin-deficient bone particles was resorbed relative to the control group. Multinucleated bone resorbing cells were located in lacunae in the controls (Fig. 1 *C*) with only occasional multinucleated cells evident around the osteocalcin-deficient particles (Fig. 1 *D*). The number of multinucleated cells per field was significantly lower in all experimental specimens (5.5±0.7 cells per field) as compared with the controls (11.9±1.1, $P < 0.001$; Table III).

Table I. Analysis of Warfarin-treated and Control Cortical Bone

	Controls		Warfarin-treated	
	x_6±SEM*	Pool‡	x_6±SEM*	Pool‡
Percent by dry weight:				
Ash	66.5±1.7	66	65.9±1.3	67.1
Calcium	23.8±0.9	23.2	23.3±0.4	23.8
Phosphorous	10.7±0.5	10.9	10.7±0.4	10.9
Protein	20.4±0.9	19.0	18.8±0.6	19.0
Collagen	15.4±0.9	16.2	16.1±0.5	16.0
Gla, res 10^3 amino acid	0.65±0.05	0.65	0.059±0.02	0.046
Osteocalcin (*mg/g*)	2.07±0.15	2.05	0.015±0.004	0.013

* Mean value from six individual rats.
‡ Value obtained from pooled bone of 50 rats used for implants.

Discussion

The subcutaneous implantation of devitalized BP in normal rats elicits the recruitment and activation of blood-derived bone resorbing cells. This model provides an experimental system for observing the rate of resorption related only to an alteration in the matrix composition of bone. These experiments demonstrate that particles of bone prepared from animals treated with warfarin are not resorbed to the same extent as are particles of bone from normal animals. The only bone parameters known to be altered by the warfarin protocol after 6 wk are a 93% decreased Gla content and a 99.4% depletion of osteocalcin. This deficiency in bone occurs because osteocalcin that is synthesized during warfarin treatment is not carboxylated. This form is found in the circulation because it does not bind to hydroxyapatite and, therefore, does not accumulate in bone (13). Our results demonstrate a skeletal abnormality resulting from Gla and osteocalcin deficiency in bone matrix; the model is not complicated by metabolic events in live bone or the net increase of bone synthesis over bone resorption in rapidly growing animals.

This in vivo model permits a direct examination of the recruitment of progenitor cells (Table II) but does not rule out additional effects on cell attachment, migration, or lytic activity. Our results suggest that the vitamin K-dependent bone protein participates in the induction of bone resorption. This hypothesis is consistent with other data. For example, documentation of the chemotactic attractant property of osteocalcin and free γ-carboxylated amino acid (5, 6) suggested that osteocalcin in the matrix may play a role in the initiation of resorption. The results of this present study are also consistent with observations of Price et al. (14) who showed closure of the growth plate along with greater bone mass in warfarin-treated rats. However, that study also demonstrated an identical apposition rate in their control and warfarin-treated rats. Their observations are consistent with a decrease in resorption of osteocalcin-deficient bone. A potential function for osteocalcin as a regulatory protein in resorptive processes of bone is also suggested by observations

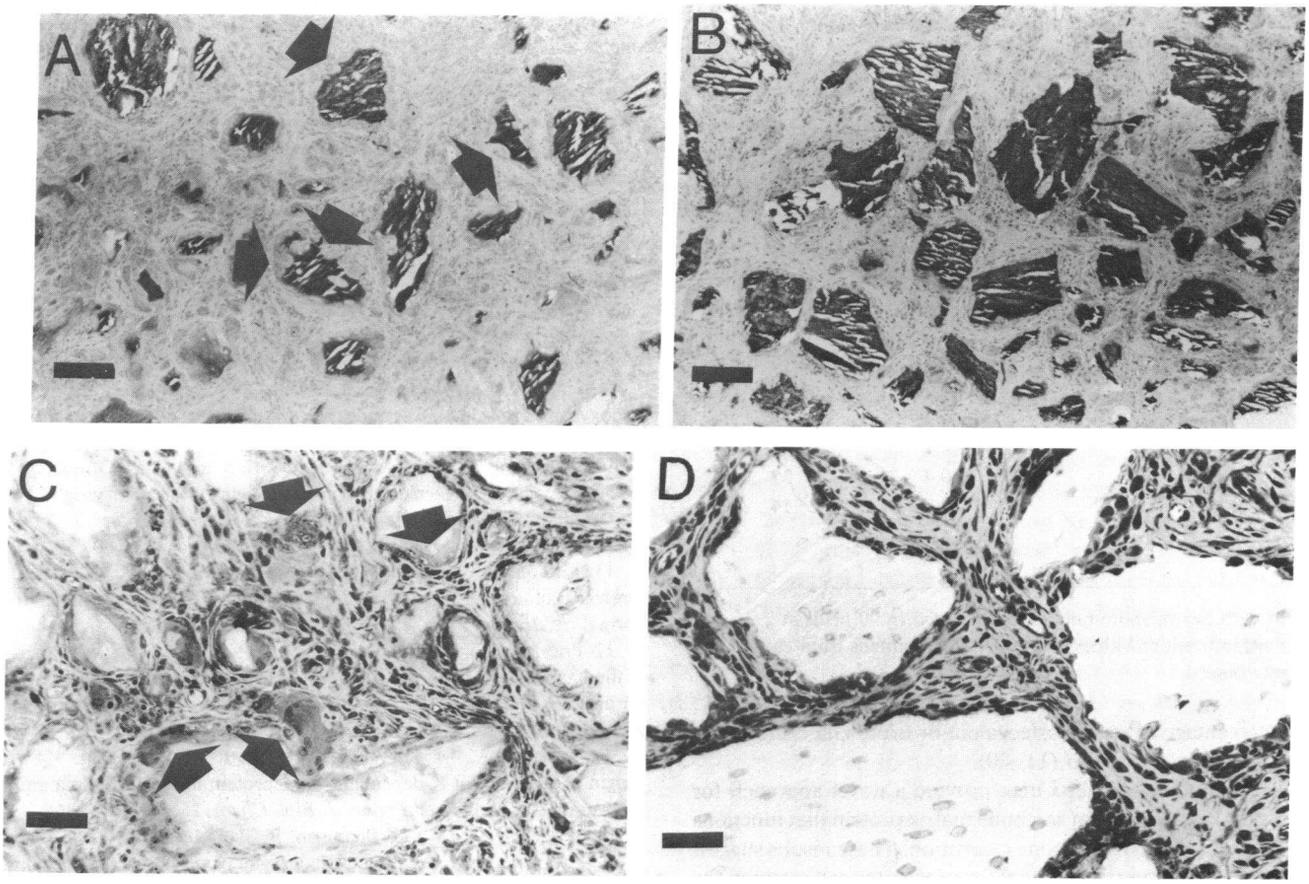

Figure 1. Resorption of control and osteocalcin-deficient bone. Subcutaneous bone powder implants removed after 12 d. Arrows indicate resorptive cavities in control bone particles (*A*). Note larger size of particles and their smooth surface with few resorption lacunae in osteocalcin-deficient bone (*B*). (*A* and *B:* Von Kossa hematoxylin and eosin, × 2,220; bars, 50 μm.) Multinucleated cells indicated by arrows in (*C*) surround control BP. Normal fibroblasts and vascularity are found in the tissue surrounding the osteocalcin-deficient BP (*D*). (*C* and *D:* Toluidine Blue, × 560; bars, 20 μm.)

Table II. Resorption of BP Prepared from Warfarin-treated and Control Rats

Experiment	Bone	BP	BP resorbed	Relative resorption*
		% of field±SEM	%	%
1	Control	27.9±0.47	44.2	100
	Warfarin	39.9±0.58	20.2	46
2	Control	22.2±0.31	55.6	100
	Warfarin	31.1±0.45	37.8	68
3	Control	27.6±0.65	44.8	100
	Warfarin	35.3±0.73	29.4	66

* Relative resorption is defined as the amount of BP resorbed in the experimental group relative to the control.

of induced synthesis of osteocalcin by 1,25$(OH)_2$-vitamin D_3 (15, 16), a stimulator of bone resorption (17). Osteocalcin may be synthesized in response to 1,25$(OH)_2$-vitamin D_3 and/or subsequently released from bone for recruitment of cells in the mobilization of mineral.

As a result of well-defined properties of osteocalcin, early studies of osteocalcin in bone have addressed its potential as a modulator or inhibitor of mineralization. Despite numerous studies relating Gla and osteocalcin to bone formation and mineralization (1, 2), a physiologic role for this protein has not been defined. Several clinical (11, 18–21) studies have demonstrated that circulating osteocalcin levels are increased with increases in bone turnover; for example, in Paget's disease (19, 20), primary hyperparathyroidism (19), osteoporosis (19, 20), and calcitriol-treated x-linked hypophosphatemia and autosomal recessive vitamin D-dependent rickets patients (21). Although circulating osteocalcin is higher in children than adults, it is not clear in both normal subjects and patients with metabolic bone diseases

Table III. Induction of Multinucleated Resorbing Cells by BP from Control and Warfarin-treated Rats*

Bone	Rat	Multinucleated cells per field	n (No. fields)
Control	1	14.3±0.4	40
	2	14.1±0.6	40
	3	11.6±0.6	40
	4	9.5±0.5	40
	5	10.1±0.6	40
	Mean	11.9±1.1	
Warfarin	1	6.4±0.4	40
	2	4.4±0.2	40
	3	5.1±0.3	40
	4	6.9±0.4	35
	5	4.7±0.7	14
	Mean	5.5±0.7	

* Cells with two or more nuclei were counted in 20 fields at 450× magnification. Values from the two specimens from each rat were pooled.

what percentage of serum osteocalcin or urine Gla excretion is contributed by resorption (11, 20).

The methods described here provide a novel approach for understanding osteocalcin as a bone matrix protein that functions as a factor in induction of bone resorption. These results suggest that osteocalcin may be essential for progenitor cell interactions in the recruitment and differentiation of a functional bone-resorbing cell.

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