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Bone Morphogenesis in Implants of Insoluble Bone Gelatin

(cell differentiation/osteogenesis/noncollagenous proteins)

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ABSTRACT **Insoluble bone gelatin with inclusions of insoluble noncollagenous protein produces new bone when implanted in muscle in allogeneic rats. The implanted residue provides the milieu for expression of bone morphogenetic potential of migratory mesenchymal cells. Neutral buffer solutions activate endogenous enzymes that degrade components essential for cell interactions and differentiation of bone. Chloroform–methanol either denatures or extracts constituents responsible for degradation. Insoluble bone gelatin produces new bone after extraction at 2° with neutral salts, 0.5 M EDTA, 0.1 M Tris·HCl, 4 M urea, 0.5 M hydroxylamine, and 10 M KCNS, as well as after limited digestion with pepsin or collagenase, but not after extraction with 5 M guanidine, 7 M urea, water saturated with phenol, or after alkali hydrolysis with 0.1 N NaOH. The specific activity of cell populations interacting with insoluble bone gelatin suggests that a chemical bond between collagen and a noncollagenous protein or part of a protein, cleaved by a neutral proteinase, controls the bone morphogenetic reaction.**

When allogeneic demineralized bone matrix or insoluble bone gelatin is implanted in muscle in an adult rat (1), or used for a substratum for outgrowths of neonatal muscle in tissue culture (2), or inserted in subcutis in the form of a coarse powder (3), the product is cartilage and bone in 7–15 days and lamellar bone and bone marrow in 20–30 days. If demineralized under specified conditions (1), the matrix induces a bone morphogenetic response from migratory mesenchymal cells as consistently as any biological process presently known (3–12). This communication presents experiments on the biochemical properties of a consistently inductive preparation of insoluble bone gelatin.

MATERIALS AND METHODS

Long bones of adult Sprague–Dawley strain rats were cut into 3.0-mm segments and extracted in six solutions sequentially under conditions stated in Tables 1 and 2: (*1*) chloroform–methanol to extract lipids (13–16) and inhibit or extract endogenous enzymes (10–12); (*2*) HCl to demineralize the matrix and extract acid-soluble proteins (17); (*3*) $CaCl_2$ to extract low-molecular weight protein polysaccharides (4, 18); (*4*) EDTA to remove nonapatitic calcium and extract phosphoproteins (19), protein polysaccharides, and sialoproteins (20–22); (*5*) LiCl to shrink collagen fibrils (23) and extract high-molecular-weight protein polysaccharides (24); (*6*) water to extract the soluble fraction of bone gelatin. All extractant solutions except the first and last were at 2° to minimize thermal denaturation of protein.

Segments of rat diaphyseal cortical bone were transferred immediately after excision to 1:1 chloroform–methanol, 1:100 solid to liquid phase for 4 hr at 25°. The bone was either washed 3 times in cold distilled water and implanted in muscle in allogeneic rats, or similarly extracted in chloroform–methanol, demineralized in 0.6 N HCl at 2° for 24 hr and then implanted in muscle, or first demineralized, then extracted in chloroform–methanol, and implanted (Table 1).

Some segments were transferred to 2.0 M $CaCl_2$ for 24 hr, washed, and then implanted in muscle (Table 2, line 1). Other segments were transferred to 0.5 M EDTA for 4 hr, washed, and then implanted in muscle (Table 2, line 2). Still other treated segments were transferred to 8.0 M LiCl (pH 5.5) for 24 hr, washed, and then implanted in muscle (Table 2, line 3); 8.0 M LiCl lowered the shrinkage temperature of

TABLE 1. *Demonstration that in the cold, chloroform–methanol followed by 0.6 N HCl releases bone mineral and soluble proteins without eliminating the bone morphogenetic properties of implants of the residual matrix*

Treatment	% Reduction in weight of dry fresh bone	Protein, mg/g of dry fresh bone	Hydroxyproline, μg/g of dry fresh bone	Uronic acid, μmol/g of dry fresh bone	Yield, mg of new bone ash per g of preimplanted matrix
Chloroform–Methanol 1:1, 25°, 1 hr	—	0.16*	—	—	—
0.6 N HCl, 2°, 24 hr, after chloroform–methanol defatting	78.92 ± 0.62	1.30†	25.12†	0.18†	504 ± 32
0.6 N HCl without preliminary defatting in chloroform–methanol	79.09 ± 1.02	2.21†	3.0†	0.22†	486 ± 30

Experimental values are given ± the standard deviation.

* Total, (dialyzable and nondialyzable) chloroform–methanol solution was completely evaporated, dissolved with NaOH, and analyzed by the Lowry method (25).

† Nondialyzable only.

TABLE 2. *Demonstration of progressive reduction of dry weight of bone matrix by removal in sequence of soluble noncollagenous proteins, and of conversion of collagen to insoluble bone gelatin without loss of bone morphogenetic properties*

Sequentially used extraction procedure	% Reduction in dry fat-free weight of bone matrix	mg of Non dialyzable protein extracted per g of dry weight	mg of Non-dialyzable hydroxyproline extracted per g of dry weight	μmol of Non-dialyzable uronic acid extracted per g of dry weight	Bone yield, mg of bone ash per g of preimplanted matrix
2 M $CaCl_2$, 2°	3.87 ± 0.91	2.52	0.059	0.65	499 ± 36
0.5 M EDTA, 2°	2.32 ± 0.20	2.41	0.006	0.36	401 ± 54
8 M LiCl, 2°	2.39 ± 0.26	5.91	0.064	0.35	502 ± 47
H_2O, 55°	11.30 ± 1.36	17.16	2.236	0.98	409 ± 36
Total	19.88 ± 2.73	28.00	2.365	2.34	

native collagen from 65° to 2°. LiCl-shrunken matrix was transferred to distilled water at 55° for 4 hr and then implanted in muscle (Table 2, line 4).

To determine the bone yield from residues of each step in the process of sequential extraction, 120 segments of diaphyseal bone matrix were divided into two groups, each containing 10 subgroups of six segments each. In Group I, the 10 subgroups were treated by sequential extraction of noncollagenous proteins as described in Fig. 2, washing in distilled water between.

TABLE 3. *Alkali hydrolysis of noncollagenous proteins and reduction in bone yield at 2°*

Preparation	Concentration of NaOH, N	Total protein*	Non-dialyzable protein*	5% Trichloroacetic acid precipitated protein*	Non-dialyzable uronic acid extracted, μmol/g	Bone yield, mg ash/g preimplanted residue	% reduced weight after 72 hr NaOH treatment
Demineralized bone matrix	0.02	6.55	4.39	4.33	1.11	326 ± 10	5.47
	0.05	4.35	1.57	1.99	1.36	180 ± 18	4.11
	0.1	6.70†	2.27	2.33	1.20	0	8.90
	0.5	18.64	7.57‡	4.52	0.76	0	13.19
Residue after 2 M $CaCl_2$, 0.5 M EDTA, 8 M LiCl 2° H_2O at 55°	0.02	4.29	2.14	3.69	1.85	464 ± 13	4.48
	0.05	5.08	2.43	2.77	1.44	250 ± 15	4.85
	0.1	9.87†	4.59†	3.20	1.15	0	5.88
	0.5	74.89	23.85‡	6.07	0.77	0	51.11

* mg/g of dry weight residue.
† included no significant quantities of hydroxyproline.
‡ included large quantities of hydroxyproline.

Extraction conditions were: 1:1 chloroform–methanol for 1 hr, 25°; 0.6 N HCl, 24 hr, 2°; 2 M $CaCl_2$, 1 hr. 2°; 0.5 M EDTA, 1 hr, 2°; 8 M LiCl, 1 hr, 2°. In Group II, the 10 subgroups, after extraction, were incubated in 0.1 M phosphate buffer (pH 7.4), at 37° for 48 hr. After lyophilization and weighing, the segments in Groups I and II were implanted intramuscularly for 3 weeks for histological and bone ash weight analyses.

Sequentially extracted matrix was treated in solutions of 0.02–0.5 N NaOH for 24–72 hr at 2° (Table 3) to hydrolyze noncollagenous proteins. Sequentially extracted matrix was also extracted in 5 M quanidine·HCl, 7 M urea, 10 M KCNS, 2.5 M guanidine·CNS, and digested with pepsin and collagenase under conditions specified in Fig. 3. Extractant solutions were analyzed by standard methods for protein (25), uronic acid (26), and hydroxyproline (27). Preparations of bone matrix denatured by demineralization in 0.6 N HCl in 70% alcohol (1, 4) were used for controls. Experimental and control residues were lyophilized, weighed, and implanted on opposite sides of the anterior abdominal wall in allogeneic recipients.

RESULTS

Table 1 summarizes data demonstrating that undemineralized bone defatted with chloroform–methanol produced only

FIG. 1 (*Upper*) Photomicrograph of a 4-week implant of insoluble bone gelatin (*arrows*) prepared by sequential extraction of bone matrix described in Table 2. Note: appositional deposits of new bone (*N*); inclusions of new bone marrow (*R*); fibrous connective tissue envelope (*E*). (Approximately × 85.)

(*Lower*) Photomicrograph of control implant of 2°, 24 hr, 0.6 N HCl demineralized bone matrix, 4 weeks after implantation in an allogeneic rat. Note the high proportion of unresorbed old matrix (*I*) to deposits of new bone (*B*), bone marrow (*R*), fibrous tissue envelope (*F*), and surrounding muscle (*S*). (Approximately × 77.)

fibrous tissue after implantation in muscle, but the same preparation demineralized in the cold produced high yields of new bone. Chloroform–methanol alone extracted only about 0.16 mg of protein and little or no hydroxyproline or uronic acid, and caused hardly any reduction in dry weight of the bone. Demineralization in cold HCl, either after or before chloroform–methanol extraction, reduced the total bone dry weight 78–79%. Chloroform–methanol extraction increased to a slight extent the efflux of nondialyzable protein, uronic acid, and hydroxyproline derived from the very small quantities of acid–soluble collagen released by demineralization of bone.

Sequential extraction was done to determine how much of the chemically soluble component of bone matrix can be released without loss of bone yield from the residual matrix. Table 2, line 1, shows that at 2° 2 M $CaCl_2$ reduced the dry weight of the matrix 3.87% by extracting nondialyzable protein and uronic acid, but very little hydroxyproline. The yield of new bone from implantation of the residue was almost as high as from whole matrix. This residue (Table 2, line 2) extracted with EDTA lost additional dry weight, including nondialyzable protein and uronic acid, but hardly any hydroxyproline.

The effects of 8 M LiCl solutions (pH 5.5) upon the $CaCl_2$- and EDTA-extracted-residue were striking. By preliminary tests on diaphyseal segments (0.6 mm long) of demineralized bone in solutions of LiCl ranging from 0.5 to 10.0 M, concentrations above 6.0 M were found to lower the shrinkage temperature from 65° to 2°. Conforming to the multidirectional orientation of layers, the collagen fibrils in cortical bone shrank about 40% in three dimensions. Electron micrographs of the collagen fibrils cut in longitudinal sections showed loss or reduction of the 640-Å interval of the characteristic cross-bands. In cross-section, the space occupied by interfibrillar

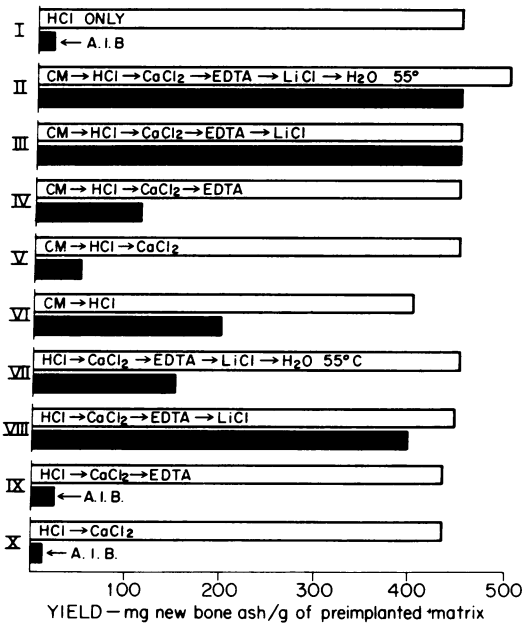

FIG. 2. Yields of new bone from bone matrix that was extracted sequentially with various solvents. Extraction conditions were described in *Methods*. *CM* indicates chloroform–methanol. Extracted matrix was implanted before (*unfilled bars*) or after incubation in buffer (*A.I.B., filled bars*). Incubation in the phosphate buffer allowed degradation of bone morphogenetic material by endogenous enzyme.

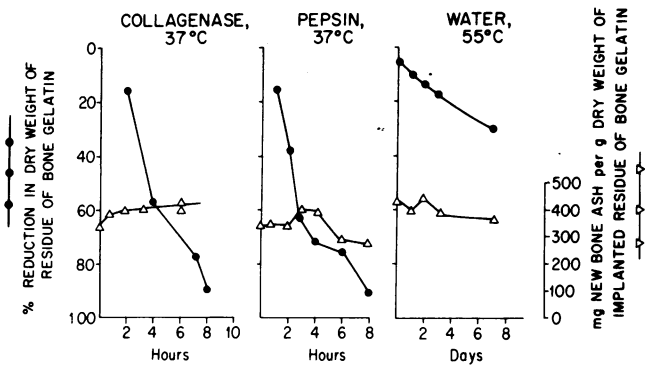

FIG. 3. The relationship between reduction in dry weight of bone gelatin by collagenase, pepsin, and water (55°), and bone yield.

substances was irreversibly lost and the collagen fibrils came into direct contact.

The 8.0 M solution of LiCl further reduced the weight of the matrix (Table 2) and extracted more nondialyzable protein uronic acid, but removed relatively little hydroxyproline. However, the relative yield of new bone was high.

The effects of extraction with distilled water at 55° of the $CaCl_2$–EDTA–LiCl–matrix residue were most remarkable (Table 2, line 4). The dry weight of the matrix was further reduced 11.3% for a total of almost 20% less than the control matrix. The reduction was attributable mainly to a reduction of nondialyzable protein. There is a reduction of 0.98 μmol of uronic acid, and 2.24 mg of nondialyzable hydroxyproline per g dry weight of the whole matrix. After LiCl shrinkage (or denaturation), a significant percentage of the matrix proteins, including gelatin, became soluble in 55° water.

Histologically, insoluble bone gelatin residue was less well permeated by mesenchymal cells than whole matrix. Old microvascular channels normally infiltrated with mesenchymal cells and differentiating cartilage cells were obliterated by shrinkage of the matrix. Despite the low penetrability of bone gelatin and the smaller than normal quantity of new cartilage, resorption was rapid, and mesenchymal cells in contact with every available surface first proliferated and then differentiated into bone. Overt differentiation occurred as early as 10 days, and by the 29th day post-implantation, about 75% of the implanted matrix was resorbed and replaced by new bone and bone marrow (Fig. 1).

Chemical Separation from Bone Matrix of an Enzyme That Degrades Bone Morphogenetic Properties. Fig. 2 summarizes observations on 10 different residues of bone matrix prepared by sequential extraction of various constituents of bone matrix, and implanted without and with preincubation in phosphate buffer (pH 7.4), 37° for 48 hr. Matrix demineralized in HCl produces no bone after incubation in neutral phosphate buffer solutions (*I*). Matrix extracted sequentially, as outlined in Table 2, produces higher than normal yield, and does not lose this capacity after incubation in buffer solution (*II*). Extraction of water-soluble gelatin lowers yield slightly but has no significant effect on bone yield after incubation in buffer (III); omission of LiCl causes loss of 80% after incubation (*IV*). Omission of EDTA (*V*) causes 90% loss, but omission of the $CaCl_3$ only 60% (*VI*). Chloroform–methanol extraction-fixation (before demineralization in HCl and incubation)

alone produces yields 40% of normal (*VI*), but without chloroform–methanol, all the extractant solutions together do not prevent loss of over 70% of bone yield (*VII*). Omitting water at 55° has equivocal effects (*VIII*); omitting LiCl produces yields with only 10% of unincubated normal controls (*IX*); omitting EDTA decreases yield to 5% or nil (*X*).

Loss of bone yield, determined by reduction in quantity of new bone ash per g of preimplanted residue, was minimal after sequential extraction with six solutions; each followed by a wash in cold water. These solutions, HCl, $CaCl_2$, EDTA, and LiCl at 2°, and water at 55°, under the conditions of time and concentration designed to extract soluble proteins, did not denature bone morphogenetic properties. Most of the loss of bone yield after incubation in buffer solutions was prevented by extraction with 1:1 chloroform–methanol at 25° for 1 hr 8 M LiCl at 2° for 1 hr. Each reagent, e.g., $CaCl_2$, EDTA, extracted some of the degradative factor, but together 1:1 chloroform–methanol and 8 M LiCl accounted for nearly all of the reductions in bone yield. Thus the constituent responsible for *degradation* of the bone morphogenetic properties is extractable by chemical solutions that separate noncollagenous proteins from either bone collagen or insoluble bone gelatin.

Alkali Hydrolysis. Whole bone matrix and insoluble bone gelatin were extracted with NaOH and analyzed at intervals of 24, 48, and 72 hr. A reduction in bone yield with time occurred in each solution; by 72 hr 0.1 N NaOH eliminated all morphogenetic properties while producing only about 5–9% loss of dry weight of insoluble bone gelatin but no significant release of hydroxyproline (Table 3). The small quantities of nondialyzable or 5% trichloroacetic acid-precipitable protein suggested that 0.02 N and 0.05 N NaOH released noncollagenous protein molecules; 0.5 N NaOH caused rapid reduction in bone yield and concurrent release of large quantities of total and nondialyzable proteins including hydroxyproline Reacidifying the matrix in cold 0.01 to 0.1 N HCl after alkali hydrolysis but before implantation did not restore bone yield.

Specific Morphogenetic Activity Measured in Terms of Bone Yield from Implants of Insoluble Bone Gelatin. Protein solvents or denaturants, and proteolytic enzymes changed the composition of bone gelatin and produced corresponding changes in bone yield. The total hydroxyproline content was 11.358 ± 12.2 mg/g of bone gelatin residue. 5 M Guanidine·HCl (pH 5.5), 2° solubilized about 12.3% of the residue, but released only about 5% of the total hydroxyproline, while reducing at least 95% of the bone morphogenetic activity. High yields were retained, however, after extraction of acetic acid-soluble-proteins at 2°, which included no measurable quantity of hydroxyproline. In the cold, water saturated 50% with phenol reduced bone yields about 50%; 5 M guanidine (pH 5.5), 5 M guanidine (pH 7.4), or 7 M urea also significantly reduced bone yield; 10 M KCNS (pH 6.2) or 2.5 M guanidine·CNS (pH 5.4), however, reduced bone yield relatively little.

Fig. 3 demonstrates the progressive changes in bone yield from insoluble bone gelatin digested over increasing periods of time with collagenase, or pepsin, or dissolved in water warmed to 55°. Pepsin digested bone gelatin within 8 hr but reduced relatively little of the bone yield measured per unit weight of undigested residue. Purified bacterial collagenase digested 78% of the dry weight of insoluble bone gelatin in 6 hr, but the undigested residue also produced as much new bone per unit weight as whole bone matrix; *collagenase-resistant* material (about 5% of the dry weight) remaining after 8 hr produced no new bone whatsoever. Water at 55° slowly reduced the dry weight of the bone gelatin about 30% over a period of 7 days, but the specific activity of the undissolved residue, measured in terms of bone yield, was hardly lower on the seventh than on the first day.

DISCUSSION

The foregoing observations demonstrate that 8% of the dry weight of the bone matrix can be solubilized by $CaCl_2$–EDTA–LiCl sequential extraction without loss of the bone morphogenetic activity. An additional 11% of the dry weight (chiefly bone gelatin) can be solubilized, after LiCl-shrinkage of bone collagen in the cold, by extraction with water at 55°, with only slight reduction in the specific activity of the bone morphogenetic property. Bone collagen is resistant to swelling in dilute acids and bases but, like soft tissue collagens, shrinks at about 65° (21, 23, 28–31). In solutions of high ionic strength e.g., LiCl > 5 M (pH 5.5), the shrinkage temperature falls from 65° to 2°. Shrinkage and dehydration, extrusion of interfibrillar proteins, rupture of peptide linkages, and collapse of the helical structure convert bone collagen to insoluble bone gelatin. At 55°, water solubilizes LiCl-shrunken bone matrix and reduces the dry weight about 11%; bone yield obtained from implants of the undissolved residue is almost as high as from whole matrix. Similarly, collagenase, which digests 78% of the dry weight of bone gelatin within 6 hr, and pepsin, which digests 68% within 8 hr, reduce bone yield from implants of the undigested residue relatively little. Thus, bone yield is determined by the physicochemical structure of the insoluble residue. The bone morphogenetic property is lost or extracted by hydrolysis with 0.1 NaOH at 2°; at 2° NaOH releases hardly any hydroxyproline even from soft tissue collagens (32–39). Significant loss also occurs at 2° without a proportionate solubilization of bone gelatin by extraction with 5 M guanidine, 7 M urea, or 50% water saturated with phenol. Surprisingly little loss occurs at 2° by extraction with KCNS or dilute acetic acid.

Reduction in bone yield from bone matrix digested by either endogenous enzymes at 37° (10, 11) or trypsin at 15° (9), suggests that the bone morphogen, however tightly bound to the collagen polypeptide structure, is not collagen, but a separate difficultly soluble noncollagenous protein. Conceptually, bone yield is controlled by an enzyme extractable by neutral buffers, BMPase (10, 11), an insoluble noncollagenous protein or part of a protein (BMP), and a chloroform–methanol extractable bone antimorphogen. Mikulski *et al.* (unpublished) have recently isolated and characterized the antimorphogen as a hydrophobic glycopeptide, and the BMPase as a proteolytic enzyme of high specificity and affinity for bone matrix and insoluble bone gelatin. At this time, our research group is analyzing collagenase digests of bone matrix and gelatin for BMP, using BMPase and bone antimorphogen to identify it. A clue to the nature of the hypothetical BMPase-labile bond between BMP and collagen may be found in failure of lathyritic bone matrix, which is deficient in aldehyde groups, to produce bone (35), and the low bone yield from penicillaminated bone collagen, in which aldehyde groups are blocked by thiazolodinization (9). No bone forms in matrix exposed to glutaraldehyde, which competes with proteins for aldehyde groups. Bone does not form

in matrix exposed to high concentrations of acid or long periods of time at room temperature. Borohydride-reduced bone matrix produces high bone yields and may be slightly less acid labile than normal.

Circumstantial evidence points to collagen, possibly through lysinoaldehyde groups, as biologic carrier of a BMP. Collagen may also orient and store diverse tissue-specific insoluble morphogenetic molecules. Collagen-oriented morphogenesis occurs in bone (5, 6, 8), skin (26), salivary gland (37), muscle (38), and lung, ureter, and pancreas (39), and during wound healing (40). Bone morphogenesis may be initiated by a complex series of cell biochemical reactions but include biophysical phenomena (1), e.g., electromagnetic forces generated along solid surfaces (41), and serum-clotting electromagnetic forces (42). A better understanding of insoluble morphogenetic molecules and the cell membranes with which they interact should open new vistas onto developmental biology.

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