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Importance of Geometry of the Extracellular Matrix in Endochondral Bone Differentiation

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ABSTRACT Subcutaneous implantation of coarse powders (74–420 μm) of demineralized diaphyseal bone matrix resulted in the local differentiation of endochondral bone. However, implantation of matrix with particle size of 44–74 μm (Fine matrix) did not induce bone. We have recently reported that the dissociative extraction of coarse matrix with 4 M guanidine HCl resulted in a complete loss of the ability of matrix to induce endochondral bone; the total loss of biological activity could be restored by reconstitution of extracted soluble components with inactive residue. To determine the possible biochemical potential of fine matrix to induce bone, the matrix was extracted in 4 M guanidine HCl and the extract was reconstituted with biologically inactive 4 M guanidine HCl-treated coarse bone matrix residue. There was a complete restoration of the biological activity by the extract of fine matrix upon reconstitution with extracted coarse matrix. Polyacrylamide gel electrophoresis of the extract of fine matrix revealed similar protein profiles as seen for the extract of coarse matrix. Gel filtration of the 4 M guanidine HCl extract of fine powder on Sepharose CL-6B and the subsequent reconstitution of various column fractions with inactive coarse residue showed that fractions with proteins of 20,000–50,000 mol wt induced new bone formation. These observations demonstrate that although fine bone matrix contains osteoinductive proteins, matrix geometry (size) is a critical factor in triggering the biochemical cascade of endochondral bone differentiation. Mixing of coarse matrix with Fine results in partial response and it was confined to areas in contact with coarse particles. The results imply a role for geometry of extracellular bone matrix in anchorage-dependent proliferation and differentiation of cells.

It is well known that most normal cells in vitro must attach to a solid substratum to spread and proliferate. This property is termed "anchorage dependence" by Stoker et al. (13). Transformed cells lose this property and become anchorage independent with respect to growth. The in vivo significance of these findings is not known although collagenous matrices are the substratum in all likelihood. The role of extracellular matrix in vitro in the control of cell shape and growth has been described (1–3). There is a paucity of information concerning the role of extracellular matrix in vivo. Subcutaneous implantation of coarse powders (74–420 μm) of demineralized collagenous bone matrix into allogeneic recipients induces a sequence of events resulting in de novo endochondral bone formation (6–8). In response to implantation, a cascade of events ensues starting with proliferation of mesenchymal precursor cells at 3 d after implantation, maximum chondrogenesis on day 7, followed by hypertrophy of chondrocytes with calcification of cartilage and osteogenesis by day 11, and finally hematopoiesis by day 21 (9). Extraction of demineralized coarse matrix with 4 M guanidine (Gdn)[1] HCl resulted in the loss of the bone inductive property. The reconstitution of such an extract with biologically inactive 4 M Gdn HCl treated matrix resulted in restoration of bone induction (12). This provides an experimental method to bioassay soluble components for their ability to induce endochondral bone differentiation in vivo.

Implantation of bone matrix of particle size 44–74 μm does not induce endonchondral bone. It was therefore interesting to determine whether 4 M Gdn HCl extracts of fine bone matrix contain endochondral bone differentiation activity, or if, as previously suggested, the geometry of the implanted matrix particles influences bone development (10). The results showed the complete restoration of biological activity in in-

[1] *Abbreviations used in this paper:* Gdn, guanidine.

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active coarse matrix residues by the addition of the extract of fine matrix indicating that fine matrix has proteins that can induce endochondral bone differentiation. They also demonstrate that the geometry of the matrix is a critical factor in triggering the biochemical cascade of endochondral bone differentiation.

MATERIALS AND METHODS

Preparation of Demineralized Rat Bone Matrix: Dehydrated diaphyseal shafts of rat femur and tibia were pulverized in a CRC micromill (Techni Laboratories, Vineland, NJ). Bone shafts were frozen in liquid nitrogen prior to and during pulverization to avoid possible heat denaturation of matrix components. Pulverized bone particles were sieved to two discrete sizes: (*a*) between 74–420 μm (Coarse); and (*b*) between 44–74 μm (Fine). Coarse and fine matrix were demineralized with 0.5 M HCl, extracted with water, ethanol, and ether, and prepared as described earlier (6). Demineralization was accomplished either at 4°C or at room temperature. In some experiments fine and coarse matrices were mixed thoroughly in various ratios and implanted subcutaneously.

Dissociative Extraction: The demineralized bone matrix (both coarse and fine) was extracted (30 ml/g matrix) with constant stirring at 4°C for 16 h in 4 M Gdn HCl/50 mM Tris, pH 7.4, containing a mixture of protease inhibitors: 5 mM benzamidine HCl/0.1 M 6-aminohexanoic acid/0.5 mM phenylmethylsulfonyl fluoride/5 mM *N*-ethylmaleimide (12). The extracts were centrifuged (40,000 *g*, 30 min, 4°C) and the supernatants were dialyzed against water at 4°C in spectropor 3 tubing (molecular weight ~3,500 cut off) and lyophilized; the residues (insoluble demineralized bone matrix remaining after extraction) were washed three times in distilled water before lyophilization. Each residue and each extract for both coarse and fine matrix were bioassayed for their potential to induce endochondral bone differentiation.

Reconstitution: Portions of the extract from coarse demineralized bone matrix were reconstituted with samples of extracted residue from both coarse and fine matrix. Similarly, portions of the extract from fine demineralized bone matrix were reconstituted with samples from both residue fractions. Reconstitution was accomplished by alcohol precipitation: 1 part (wt/wt) of lyophilized extract was dissolved in 2 ml of 4 M Gdn HCl. 10 parts (wt/wt) of insoluble demineralized coarse or fine bone matrix residue was added. The mixture was stirred for 2 h at 4°C. Cold absolute ethanol (8.5 ml) was added to the mixture which was then stirred for 30 min at 4°C. After centrifugation (10,000/*g* 10 min, 4°C), the supernatant was discarded. The reconstituted matrix was washed three times with 85% ethanol in water and then lyophilized. Column fractions obtained by gel filtration were also reconstituted with residues in a similar fashion. In some experiments the Gdn HCl residue was substituted by agarose beads (Sepharose 6B of size <70 μm and Sepharose 6MB of size ~300 μm) from Pharmacia Fine Chemicals, Piscataway, NJ) to test the potential of noncollagenous substrata for the role of geometry in bone induction.

Gel filtration: Aliquots of 4 M Gdn HCl extracts obtained either from demineralized coarse bone matrix or fine matrix were applied (150 mg/2 ml) to two columns in tandem (2.6 cm × 100 cm) of Sepharose CL-6B, equilibrated in 4 M Gdn HCl/50 mM Tris, pH 7.0, and eluted with the same eluent at a flow rate of 15 ml/h; 5-ml fractions were collected. The eluent was continuously monitored at 230 nm. Appropriate fractions were pooled (see Fig. 2), dialyzed against distilled water at 4°C, and lyophilized. Fractions were characterized by $NaDodSO_4$ polyacrylamide gel electrophoresis and amino acid analysis, and bioassayed for endochondral bone differentiation activity after reconstitution with coarse matrix residue.

Bioassay: Demineralized bone matrix (fine and coarse) and variously reconstituted bone matrix preparations were bioassayed for their ability to induce endochondral bone by subcutaneous implantation into ether anesthetized male Long-Evans rats (28–35 d) at bilateral sites located over the thorax. The day of implantation was designated as day 0 of the experiment. On day 12, $^{45}CaCl_2$ (17 mCi/μg Ca) in isotonic saline was injected intraperitoneally at a dose of 1 μCi/g of body weight 2 h before the rats were killed. The subcutaneous buttonlike plaques (implants) were dissected out and cleaned of adherent tissue. The implants were weighed and homogenized in 2 ml of ice-cold 0.15 M NaCl/3 mM $NaHCO_3$. Alkaline phosphatase activity of the supernatants, ^{45}Ca incorporation into the insoluble sediment and calcium content in the acid soluble fraction of the sediment (by atomic absorption) were determined as indices for bone formation as described (12). The histological appearance of the implants was also monitored by fixing the implants in Bouin's fixative and embedding in JB4 plastic medium (Polysciences, Inc., Warrington, PA). 1-μm sections were cut and stained with toluidine blue.

Polyacrylamide Slab Gel Electrophoresis: Dissociatively extracted components of fine matrix were characterized by polyacrylamide slab gel electrophoresis as described earlier (11, 12). Gradient gels (5–20%) were used as the separating gel and a spacer gel of 3% acrylamide/2 M urea was added to all the gels. Electrophoresis was carried out in 50 mM Tris-glycine, pH 8.3/0.1% $NaDodSO_4$ at 15 mA per slab and 20°C for 12 h. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R 250 in 50% methanol/10% acetic acid for 45 min and then destained in 10% methanol/7.5% acetic acid.

Analytical Procedures: Samples for amino acid analyses were hydrolyzed in 6 M HCl at 106°C for 24 h in a sealed tube in an atmosphere of nitrogen. The analyses were performed with a Durrum D-500 analyzer. Protein was determined by the method of Lowry et al. (5).

RESULTS

Influence of Geometry of Matrix

Subcutaneous implantation of coarse demineralized bone matrix (74–420 μm) into allogeneic rats resulted in the local differentiation of bone whereas implantation of fine matrix (44–74 μm) did not, as indicated by the specific activity of alkaline phosphatase, incorporation of ^{45}Ca, and calcium content in day 12 plaques (Table I). Cartilage and bone were absent histologically when fine matrix was implanted (Fig. 1). Fine matrix that had been kept cold during pulverization and demineralization to protect against heat denaturation also did not induce bone. As subcutaneous implantation of fine matrix did not result in new bone formation it was interesting to add coarse matrix in various ratios ranging from 25 to 75% (wt/wt) to the fine mixture. The results revealed a progress increase in bone induction and was directly proportional to the amount of coarse matrix (Table II). It was noteworthy that new cartilage and bone formation was entirely confined to the close vicinity of the coarse particles in the conglomerate of the matrix and responding cells (Fig. 2). These observations rule out the possibility that the fine particles may elicit a phagocytic response that, in turn, may prematurely degrade the matrix components critical for bone induction. Furthermore, mixing experiments clearly prove that the coarse matrix is active even when surrounded by fine particles.

TABLE I

4 M Guanidine HCl Extraction of Fine Matrix and Reconstitution with Coarse Residue for Bone Induction

Group	Extract: residue	Alkaline phosphatase	^{45}Ca-incorporation	Calcium content	Bone histology*
		U/mg protein	*cpm/mg tissue*	*μg/mg tissue*	
Coarse matrix		2.85 ± 0.99	5,431 ± 1,858	26.40 ± 14.29	+++
Fine matrix		0.11 ± 0.13*	152 ± 204*	0.61 ± 0.58*	—
GE (coarse) + GR (fine)	1:10	0.08 ± 0.07*	60 ± 45*	0.65 ± 0.43*	—
GE (fine) + GR (coarse)	1:10	1.29 ± 0.61	2,428 ± 1,657	11.03 ± 11.29	+++

Values are mean ± SEM of eight observations from four rats. *GE*, guanidine HCl extract; *GR*, guanidine HCl residue. —, absent; +++, extensive bone.
* For difference from coarse, $p < 0.01$.

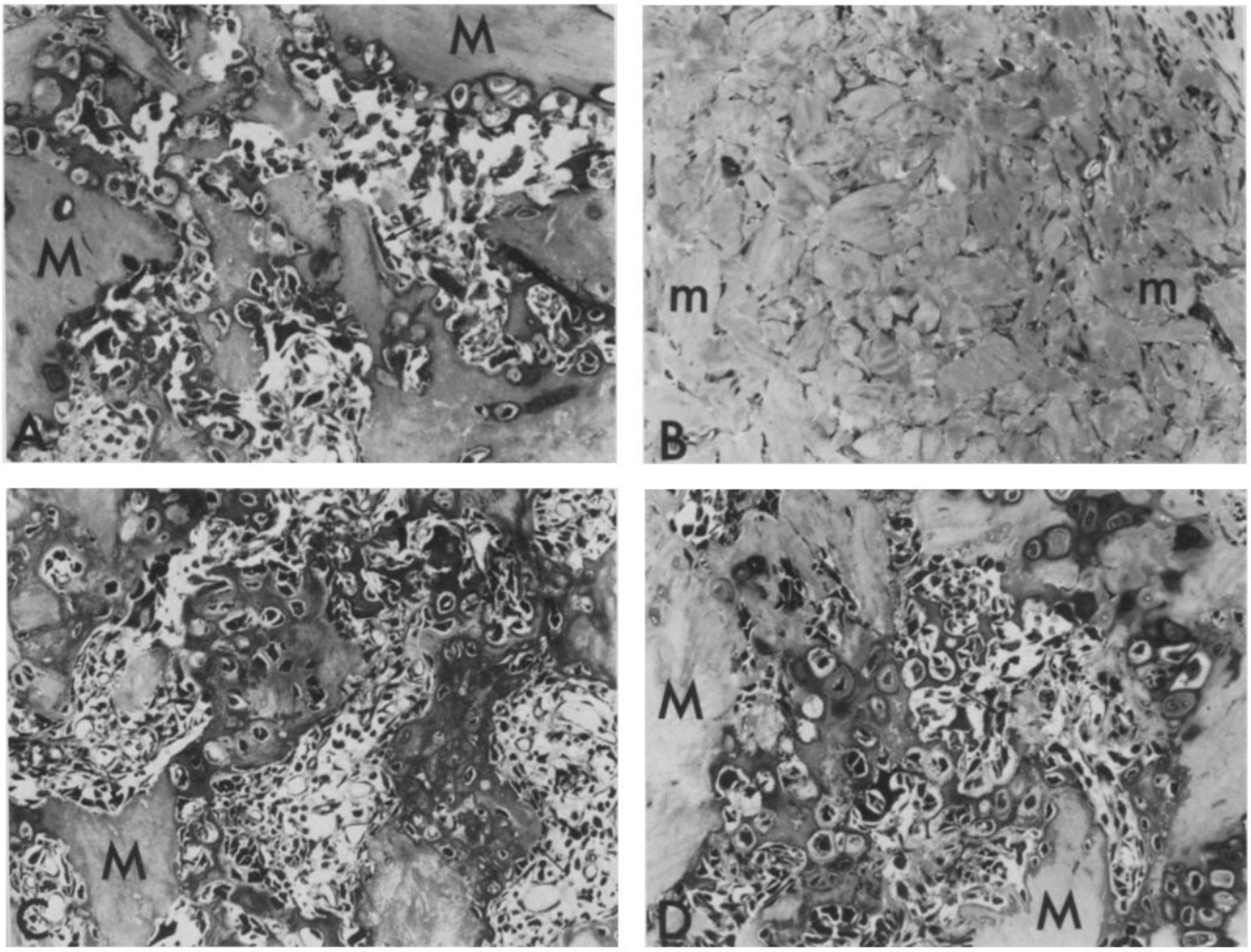

FIGURE 1 Photomicrographs of the implants on day 12. (*A*) Coarse matrix. Bone formation and vascular invasion are evident. *M*, implanted coarse demineralized bone matrix particles. The arrows indicate osteoblasts. (*B*) Fine matrix. Note the complete absence of new bone formation. *m*, implanted fine demineralized bone fine matrix particles. (*C*) Reconstitution of the Gdn-HCl residue (coarse) with fine Gdn-HCl extract. Note the complete restoration of bone formation. (*D*) Reconstitution of the Gdn-HCl residue (coarse) with fine IV peak. Complete restoration of bone induction is evident. × 200.

TABLE II

Influence of Geometry of Matrix on Endochondral Bone Differentiation

Group	Alkaline phosphatase	Calcium content	Bone histology
	U/mg protein	*μg/mg tissue*	
Coarse matrix	3.21 ± 0.56	11.52 ± 2.66	+++
Fine matrix	0.19 ± 0.11*	0.43 ± 0.32*	—
Coarse/Fine (25:75)	0.65 ± 0.30*	0.75 ± 0.52*	+
Coarse/Fine (50/50)	0.88 ± 0.36*	2.36 ± 0.96*	++
Coarse/Fine (75:25)	1.88 ± 0.72*	4.61 ± 1.60*	+++
Sepharose 6B (~70 μm)	0.05 ± 0.04*	ND	—
Sepharose 6B + GE (Coarse)	0.04 ± 0.05*	ND	—
Sepharose 6MB (~300 μm)	0.06 ± 0.04*	ND	—
Sepharose 6MB + GE (Coarse)	0.08 ± 0.07*	ND	—

Values are mean ± SEM of eight observations from four rats. *ND*, not detectable; *GE*, guanidine HCL extract; —, absent; +, small amounts; ++, moderate; +++, extensive bone.

* For difference from coarse matrix, $p < 0.01$.

Dissociative Extraction and Reconstitution

Dissociative extraction of coarse matrix with 4 M Gdn HCl resulted in a complete loss of the ability of the residual matrix to induce endochondral bone differentiation. Subcutaneous implantation of lyophilized extract alone was also without effect. The total loss of biological activity of coarse matrix could be restored by reconstitution of the inactive residue with the extracted soluble components either by dialysis (12) or by alcohol precipitation (Table III, Coarse Reconstitution) as measured by the specific activity of alkaline phosphatase, ^{45}Ca incorporation, calcium content, and histological appearance of bone in day 12 plaques. However, an identical dissociative extraction and reconstitution of fine matrix did not induce bone differentiation (Table III, Fine Reconstitution). The 4 M Gdn HCl solubilized extracellular components of coarse bone matrix were reconstituted with fine matrix residue by alcohol precipitation and bioassayed for endochondral bone differentiation activity. The results revealed that there was no biological activity in the reconstituted matrices. On the other hand, when 4 M Gdn HCl solubilized extracellular components of fine bone matrix were reconstituted with coarse residue by alcohol precipitation and bioassayed, there

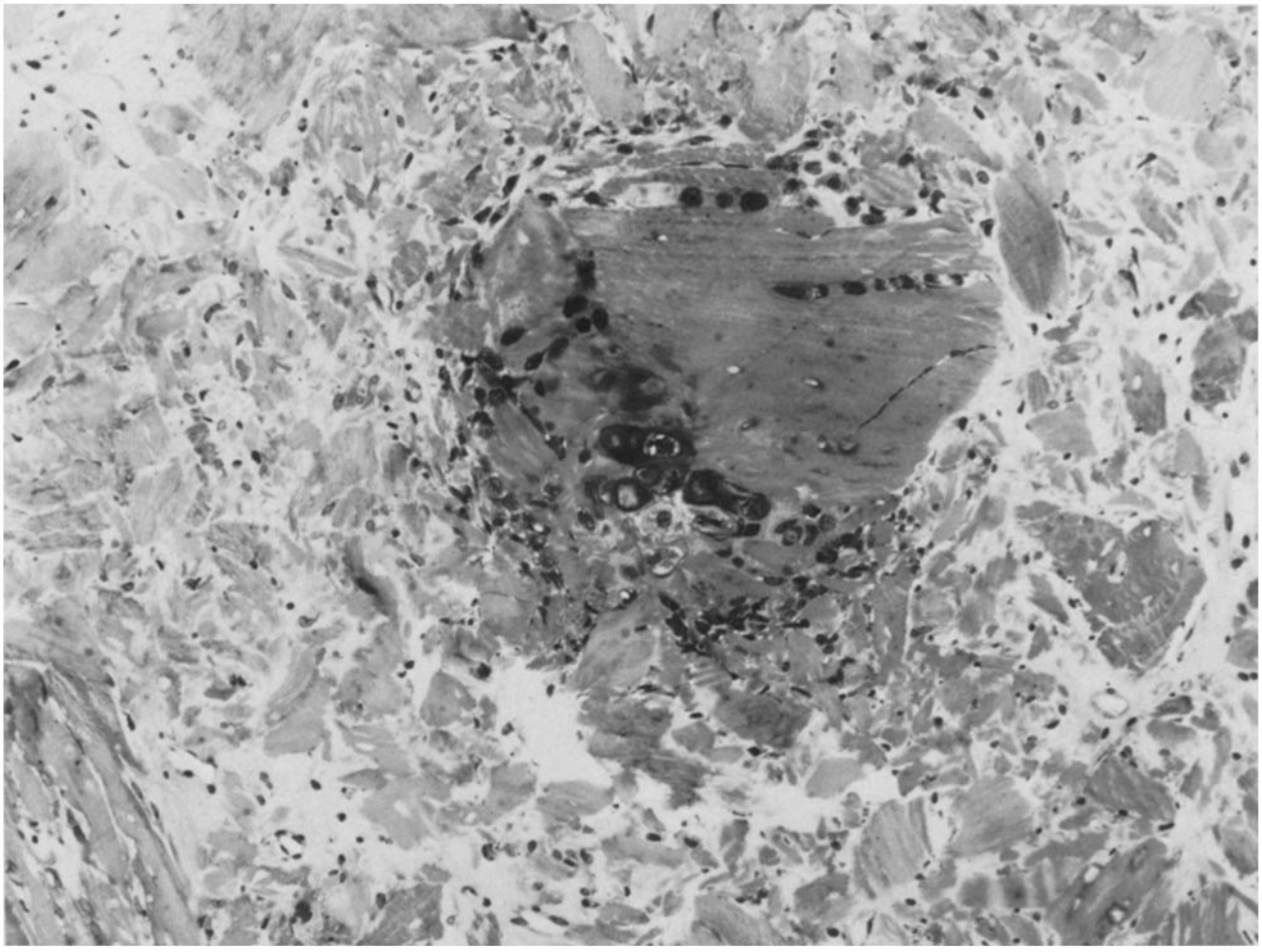

FIGURE 2 Influence of mixing coarse matrix (25%) with fine matrix (75%). Histology of the implants on day 12. Note the induction of cartilage on the coarse particle but not on the fine matrix. × 170.

was complete restoration of biological activity as measured by biochemical markers and histology in day 12 plaques (Table I; Fig. 1).

It was interesting to examine the chemically defined non-collagenous substrata such as agarose beads of two different sizes comparable with the dimensions of coarse and fine bone matrix for the role of geometry in bone induction. The results revealed that agarose beads uniformly elicited multinucleate giant cells and there was no evidence of cartilage and bone formation irrespective of the particle sizes (Table II).

Fractionation of 4 M Gdn HCl Solubilized Extracellular Components of Fine Bone Matrix

A gel filtration analysis of 4 M Gdn HCl solubilized components on Sepharose CL-6B is shown in Fig. 3. Fractions assigned I-V were pooled, respectively, dialyzed against water at 4°C and lyophilized. Lyophilized components of each fraction were reconstituted with 4 M Gdn HCl coarse residue and bioassayed. The results showed that only fraction IV restored the activity to matrix (Table III; Fig. 1). The other fractions did not show significant biological activity. Reconstitution of fraction IV of fine extract in two different ratios (1:10 and 1:20) with coarse residue revealed that the endochondral bone differentiation was concentration dependent as measured by biochemical markers (Table III). Histological appearance of bone was evident in both cases (Fig. 1 *D*, reconstituted with coarse residue at 1:10).

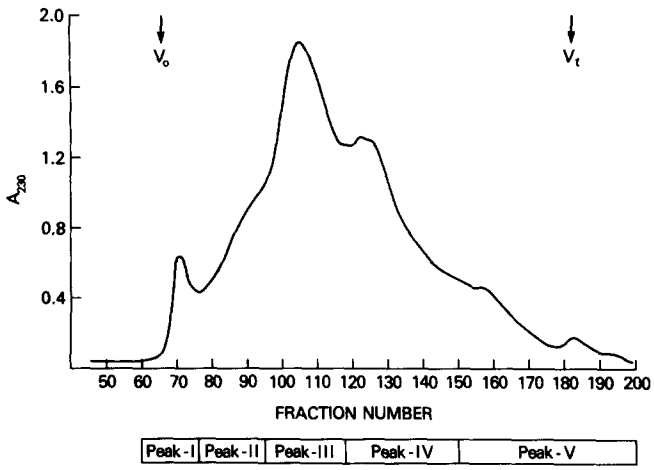

FIGURE 3 Gel filtration of the 4 M Gdn-HCl extract of demineralized diaphyseal bone matrix of <74 μm (fine matrix) on Sepharose GL-6B.

Characterization of Dissociatively Extracted Fine Matrix

The amino acid compositions of coarse and fine demineralized matrix, coarse and fine residue, and 4 M Gdn HCl solubilized coarse and fine extracellular bone matrix components showed no significant difference. However, 4 M Gdn

TABLE III

Reconstitution of Various Fractions Obtained on Sepharose CL-6B Gel Filtration with Coarse Residue for Bone Induction

Group	Extract:residue	Alkaline phosphatase	^{45}Ca incorporation	Calcium content	Bone histology
		U/mg protein	*cpm/mg tissue*	*μg/mg tissue*	
Coarse matrix		2.38 ± 0.30	4,895 ± 673	18.24 ± 2.29	+++
Coarse reconstitution	1:10	1.64 ± 0.22	3,700 ± 533	12.65 ± 2.45	+++
Fine reconstitution	1:10	0.20 ± 0.20*	27 ± 17*	0.14 ± 0.11*	—
GR (coarse) + P-I (fine)	1:10	0.06 ± 0.01*	64 ± 20*	0.28 ± 0.14*	—
GR (coarse) + P-II (fine)	1:10	0.09 ± 0.02*	358 ± 237*	0.44 ± 0.18*	—
GR (coarse) + P-III (fine)	1:10	0.07 ± 0.01*	38 ± 02*	0.29 ± 0.18*	—
GR (coarse) + P-IV (fine)	1:10	2.17 ± 0.39	4,218 ± 606	12.71 ± 3.06	+++
	1:20	1.32 ± 0.39	1,934 ± 859	6.02 ± 2.21	+++
GR (coarse) + P-V (fine)	1:10	0.14 ± 0.04*	173 ± 73*	0.34 ± 0.10*	—

Values are mean ± SEM of eight observations from four rats. *GR*, guanidine HCl residue; —, absent; +++, extensive bone.

TABLE IV

Amino Acid Composition

	Residues/1,000 residues										
	A	B	C	D	E	F	G	H	I	J	K
4HYD	82	81	87	94	29	50	79	71	38	14	2
ASP	53	53	48	52	88	66	52	58	79	94	85
THR	23	23	20	22	46	32	24	28	39	45	36
SER	47	43	39	42	56	47	46	46	53	60	79
GLU	79	79	74	79	109	89	82	87	104	118	126
PRO	113	110	107	77	81	87	103	101	86	66	205
GLY	308	299	309	329	164	222	295	263	187	128	117
ALA	102	101	102	98	82	86	101	97	89	74	60
VAL	25	26	27	25	43	33	29	15	45	50	42
MET	10	14	15	10	15	16	15	5	11	14	3
ILEU	13	15	14	12	26	24	17	19	25	30	23
LEU	30	33	28	29	64	60	35	41	59	71	49
TYR	7	7	5	6	26	15	8	9	19	29	39
PHE	16	16	14	15	33	23	17	20	27	34	27
HIS	5	16	17	19	31	48	21	26	36	48	39
HLYS	10	10	10	10	4	17	9	12	7	15	4
LYS	29	26	25	30	54	40	16	29	40	54	11
ARG	50	50	58	51	49	48	51	56	56	56	53

A, demineralized bone matrix (coarse); *B*, demineralized bone matrix (fine); *C*, 4 M Gdn-HCl residue (coarse); *D*, 4 M Gdn-HCl residue (fine); *E*, 4 M Gdn-HCl extract (coarse), *F*, 4 M Gdn-HCl extract (fine); *G*, Fine-Sepharose CL-6B Peak-I; *H*, Peak-II; *I*, peak-III; *J*, peak-IV; *K*, peak-V.

HCl-solubilized fine matrix components had more collagenous peptides as revealed by hydroxyproline and hydroxylysine residues (Table IV). Amino acid compositions of Sepharose CL-6B fractions of 4 M Gdn HCl solubilized extract showed that fraction IV, which induced endochondral bone differentiation, also had a composition indicative of the presence of collagenous fragments. Components of coarse and fine bone matrix solubilized by 4 M Gdn HCl revealed similar protein profiles when electrophoresed on 5–20% polyacrylamide gels. Electrophoresis of Sepharose CL-6B fractions of fine dissociative extracts showed protein profiles similar to those seen for coarse matrix.

DISCUSSION

The influence of geometry of bone matrix on matrix-induced bone formation has been mentioned earlier (10). The importance of anchorage dependence (the ability of normal cells to grow only when attached to a solid substratum) for cell proliferation in vitro (1, 2, 13) and the role of collagenous matrix to serve as a substratum in vivo has been documented (11). We previously reported that endochondral bone differentiation activity could be extracted dissociatively by 4 M Gdn HCl from the coarse matrix, thus providing an experimental method to bioassay soluble proteins for osteoinductive potential in a reproducible and quantitative manner (12). In the present study, failure to demonstrate the biological activity of fine matrix particles or 4 M Gdn HCl-treated fine matrix residue reconstituted with the endochondral differentiation activity that had been obtained from coarse matrix revealed the critical role of matrix geometry in cellular differentiation. However, dissociative extraction of fine extracellular bone matrix components and reconstitution with biologically inactive coarse residue resulted in complete restoration of biological activity. These observations demonstrate that although fine matrix has osteoinductive potential, the geometry of the bone matrix is a crucial factor in determining its suitability as a substratum for anchorage-dependent cell proliferation and differentiation of endochondral bone in vivo. Although the geometry of the matrix is critical other possible explanations such as rapid diffusion of factors from fine matrix and the lack of adequate space between the implanted particles should be kept in mind.

The importance of collagenous matrix as a substratum was examined by using chemically defined substrates such as agarose beads of sizes comparable with coarse and fine matrices. The results revealed agarose beads with or without Gdn HCl extracts consistantly elicited a multinucleate foreign body giant cell response. It is therefore likely that collagenous

substratum is more favorable for anchorage dependent proliferation and differentiation of cells in vivo. These observations are consonant with the well known fact that most extracellular matrices, especially in bone are predominantly collagenous in nature. In view of this it is possible that collagenous matrix in the bone may be critical for local matrix-cell interactions.

Gel filtration of 4 M Gdn HCl solubilized fine extracellular bone matrix components and further reconstitution of different fractions with biologically inactive coarse residue support the earlier finding (12, 14) that osteoinductive proteins are smaller than 50,000 daltons. Reconstitution of fine peak IV in two different ratios with coarse residue indicates that the bone differentiation activity in response to osteoinductive proteins is dose dependent. In summary, the results imply that both coarse and fine matrix contain components necessary for induction of endochondral bone differentiation. However, the biological potential of bone induction is restricted to coarse matrix. The inability of fine matrix to induce bone is explained by the crucial role of matrix geometry in triggering the biochemical cascade of endochondral bone differentiation in vivo.

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