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In vitro transformation of mesenchymal cells derived from embryonic muscle into cartilage in response to extracellular matrix components of bone

(bone differentiation/proteoglycans/alkaline phosphatase)

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ABSTRACT **Subcutaneous implantation of demineralized diaphyseal bone matrix into rats induces cartilage and bone formation *in vivo*. When minced skeletal muscle is cultured on hemicylinders of demineralized bone *in vitro*, mesenchymal cells are transformed into chondrocytes. In the present investigation, the potential of extracellular matrix components of bone to trigger cartilage differentiation *in vitro* was examined. Extraction of bone hemicylinders with 6 M guanidine·HCl resulted in the absence of chondrogenesis *in vitro* and endochondral bone formation *in vivo*. Biologically inactive hemicylinders of bone were then reconstituted with the guanidine extract and also with partially purified components extracted from bone matrix and bioassayed. Reconstitution completely restored the ability to elicit chondrogenesis *in vitro* and endochondral bone differentiation *in vivo*. Reconstitution of the whole guanidine extract on Millipore filters coated with gels of tendon collagen (type I) and subsequent culture with minced skeletal muscle also resulted in cartilage induction *in vitro*. These observations show that the extracellular matrix of bone is a repository of factors that govern local cartilage and bone differentiation.**

Extracellular matrix is necessary for adhesion, proliferation, and subsequent differentiation of cells (1–3). The extracellular matrix of bone (bone matrix) has been shown to elicit local differentiation of cartilage and bone when implanted subcutaneously in rats (4–6). A similar developmental sequence can be achieved in part, *in vitro*, by culturing skeletal muscle on a substratum of demineralized bone; under these conditions, muscle-derived cells form hyaline cartilage (7–10). The precise mechanisms underlying these phenotypic transformations are not well understood; however, surface charge and geometry of the matrix have been shown to be important in this regard (11, 12). When components of bone matrix were dissociatively extracted and reconstituted with the inactive residual collagenous matrix, full bone induction activity was restored (13). The putative transforming proteins, with an apparent molecular mass of <50,000 daltons, appear to be related in several species of mammals (14) and have been shown to stimulate fibroblast proliferation *in vitro* (15).

To gain further insights into the mechanism(s) of cell transformation *in vitro*, we have examined the action of extracellular components of bone matrix on embryonic skeletal muscle *in vitro*. The results reveal that fractions that elicit osteogenesis *in vivo* are also competent to transform mesenchymal cells derived from muscle into chondrocytes *in vitro*.

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MATERIALS AND METHODS

Tissue Preparation and Cultivation. The source tissue consisted of embryonic skeletal muscle excised from the thighs of 19- to 20-day fetal rats. Thigh muscle was aseptically isolated into an aliquot of ice-cold complete culture medium as described (16). During the isolation procedure, contaminant dermal, vascular, and nervous tissues were mechanically removed. Cultures consisted of aliquots of cleaned skeletal muscle grown on a substratum of 3- to 5-mm hemicylindrical segments of demineralized diaphyseal bone matrix. Gels of type I collagen (prepared as described below) served as a control substratum and were also used when extracts of bone were tested for the ability to confer bone-matrix-like activity on control cultures. Type I collagen was extracted from tail tendons of adult rats in 0.5 M acetic acid, and collagen gels were cast on Millipore filters (pore size, 0.45 μm; type HA; Millipore) (9, 10, 17) and sterilized prior to use by immersion in 70% ethanol for 1 hr. Two applications were sufficient to provide a gel that when re-hydrated, could be peeled intact from the filter. For each of the experiments described below, at least three parallel cultures were prepared and each experiment was performed twice.

Complete culture medium consisted of medium CMRL-1066 containing 15% fetal calf serum (pre-tested and heat-inactivated), 0.225% sodium bicarbonate, and gentamycin at 50 μg/ml (10). CMRL-1066 was added as a 10× concentrate, such that the final medium contained a 1× nutrient base. Explants onto bone matrix and collagen gels were fed on alternate days by changing one-half to three-fourths of the culture medium. Cultures were maintained at 37°C in a water-jacketed incubator (National Appliance, Portland, OR) in an atmosphere of 5% CO_2/95% air. The culture period for the present experiment was set at 18 days, to provide sufficient time for chondrogenesis to occur.

Preparation of Bone Hemicylinders. Bone matrix was prepared as described (8). Extracellular matrix components were then extracted with 6.0 M guanidine·HCl/50 mM Tris buffer, pH 7.0 (10 segments per 100 ml), at 4°C, for 72 hr with constant stirring, in the presence of protease inhibitors (12). Extracts were collected, dialyzed against distilled water, at 4°C, in Spectropor 3 tubing (cut-off, $M_r \approx 3500$), and lyophilized. The insoluble bone matrix residue was washed three times with distilled water and lyophilized.

Reconstitution. The guanidine·HCl extract of bone matrix powder (particle size, 74–420 μm) was used (13) to reconstitute hemicylinders of guanidine-extracted bone residue. Aliquots of the guanidine·HCl extract were fractionated by chromatography on Sepharose CL-6B described (13) and the various column fractions were used to reconstitute the 6.0 M guanidine-extracted bone. Reconstitution was accomplished by alcohol precipitation as follows: bone segments and collagen gels were placed in a sterile glass Petri dish. To the sur-

face of each was added 20 μl of guanidine·HCl solution containing various concentrations of whole extract or CL-6B column fractions (usually 0.5–2 mg of protein/100 μl). The reconstituted segments and collagen gels were air dried in a laminar flow hood for 1 hr. Coating was repeated once more with the same concentration of extract. Protein was precipitated onto the bone segments or collagen gels by washing with 0.5 ml of cold 85% ethanol. Petri dishes were cooled to −10°C before and during alcohol precipitation. The guanidine·HCl/ethanol supernatant was then removed from the dishes and the entire procedure was repeated five times. Reconstituted bone segments and collagen gels were finally placed in cold 85% ethanol for 30 min and lyophilized.

Assay of Bone-Inductive Activity *in Vivo*. Diaphyseal rat bone was demineralized for *in vivo* bioassay of bone-inductive activity as described (4). Experiments involving inactivation and reconstitution were carried out as described above. Bone matrix (6.0 M guanidine·HCl extracted) and re-

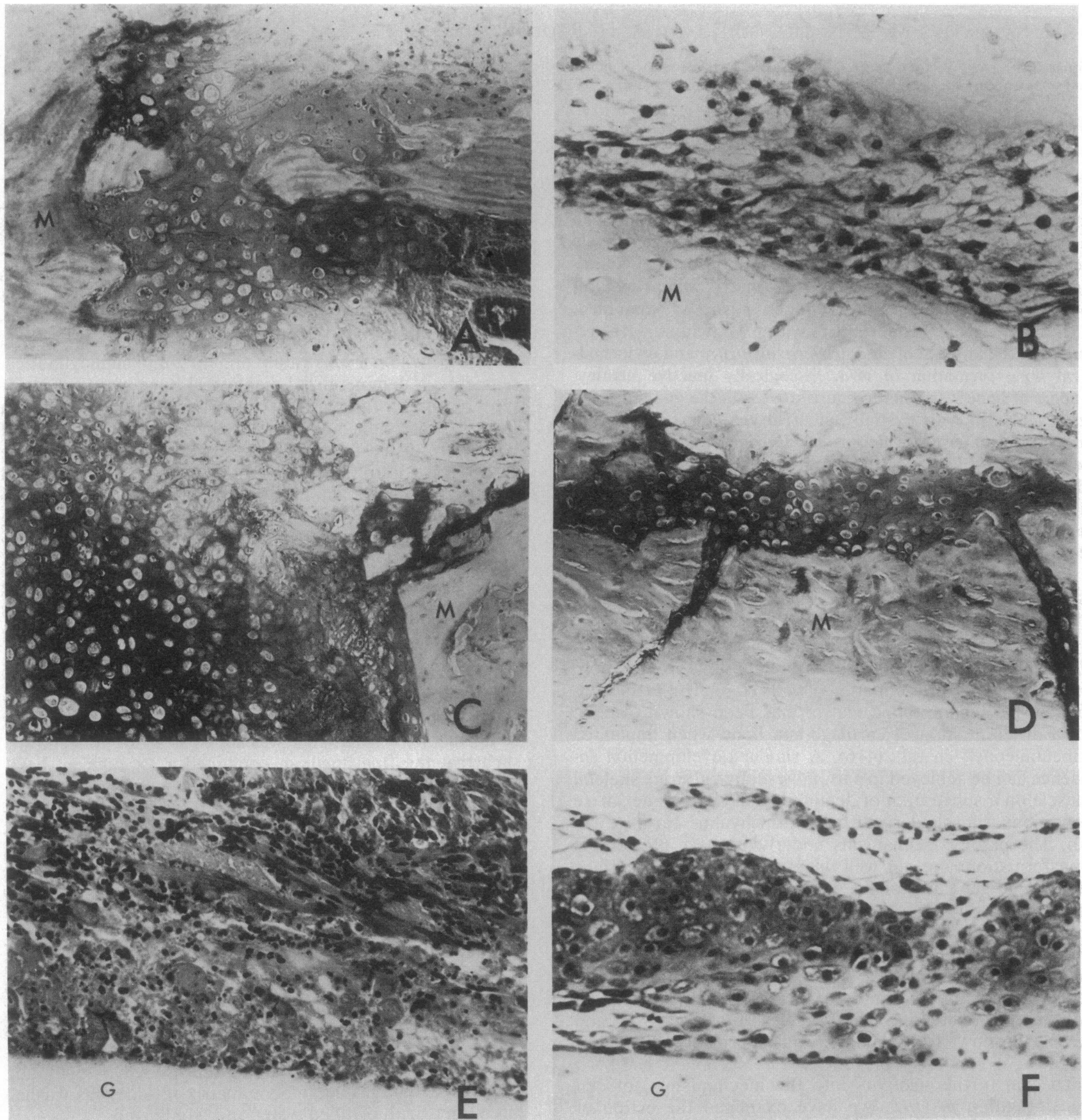

FIG. 1. Photomicrographs of histological sections of skeletal muscle cultured on bone matrix (M) and collagen-coated control substrata (G). *A*, unextracted bone matrix; *B*, bone matrix extracted with 6.0 M guanidine·HCl for 48 hr; *C*, bone matrix treated as in *B* and reconstituted with a guanidine extract of active bone matrix; *D*, bone matrix treated as in *B* but reconstituted with peak IV (see text for a description of this nomenclature); *E*, control substratum (gels of type I collagen in place of bone matrix); *F*, control substratum reconstituted with guanidine extracts of bone matrix. In each experiment, either bone matrix or collagen gels served as the substratum for minced 19- to 20-day embryonic skeletal muscle. The bone matrix substratum reproducibly elicited differentiation of chondrocytes, whereas guanidine-extracted bone matrix was inactive. Reconstitution of inactive bone matrix with guanidine extract was found to restore its activity. Reconstitution with peak IV resulted in restoration of biological activity *in vitro*. When collagen substrata were reconstituted with guanidine extract of active bone matrix, masses of chondrocytes formed. (×290.)

constituted bone matrix were implanted into ether-anesthetized, male, Long–Evans rats (28–35 days old) at bilateral subcutaneous sites located over the thorax. The day of implantation was designated day 0. On day 12, the implants were recovered, cleaned of adherent tissue, weighed, and homogenized in 2 ml of ice-cold 0.15 M NaCl/3 mM $NaHCO_3$. Homogenates were clarified by centrifugation at 20,000 × *g* for 15 min at 4°C. Alkaline phosphatase activity of the supernatants and calcium content of the acid-soluble fraction of the sediment were determined (by atomic absorption) as indices of bone formation (13, 14).

Extraction and Fractionation of Proteoglycans. Explants grown on bone matrix and collagen gels were labeled with $Na_2{}^{35}SO_4$ (10) and minced with a scalpel prior to dissociative extraction in 4.0 M guanidine·HCl in 0.05 M sodium acetate buffer (pH 5.8) in the presence of protease inhibitors (10). Salts and unincorporated isotope were removed by dialysis in the presence of protease inhibitors. After dialysis, an aliquot of the clear extract was assayed for radioactivity and the remainder was frozen at −80°C and lyophilized. Residual bone matrix and collagen fragments were digested with a quaternary amine solvent, Solusol (National Diagnostics, Somerville, NJ), and the radioactivity was determined in a specially designed counting solution, Soluscint-O (National Diagnostics, Somerville, NJ), to measure the amount of unextractable isotope.

Proteoglycans were fractionated by molecular sieve chromatography on columns of Sepharose CL-2B under associative conditions (10). Lyophilized proteoglycans were dissolved in 0.5 ml of the dissociation solvent, lacking benzamidine. Proteoglycans were then permitted to reassociate during dialysis against an association buffer consisting of 0.5 M ammonium acetate in 20% ethanol. Dialysis was carried out at 4°C against 2 liters of association buffer for 4 hr, followed by a second 2 liters overnight. Five microliters of aqueous phenol red and 100 μg of carrier proteoglycan monomer from rat chondrosarcoma (10) were added to the dialyzed extracts, which were then clarified by centrifugation at 20,000 × *g* and 4°C for 15 min. Following sample application, the analytical columns were eluted at 4°C at a constant pressure of 30 cm.

Histology. Samples grown *in vitro* were fixed for histological examination in 10% formalin in neutral phosphate buffer and embedded in Paraplast Plus. Seven-micrometer serial sections were stained with toluidine blue. *In vivo* samples were fixed in Bouin's fluid and embedded in JB4 plastic medium (Polysciences, Warrington, PA). One-micrometer sections were stained with toluidine blue.

RESULTS

Cartilage Differentiation on Bone Matrix *in Vitro*. Samples of demineralized bone matrix not subjected to dissociative extraction transformed mesenchymal cells derived from minced muscle to chondrocytes (Fig. 1*A*). The cells were surrounded by a metachromatic hyaline matrix. Chondrocytes were found in apposition to exposed bone matrix surfaces and in many cases were also found to penetrate into the crevices of the substratum. In contrast, 6.0 M guanidine·HCl-extracted bone matrix lacked this chondrogenic potential (Fig. 1*B*). Cultivation of minced muscle on this latter substratum resulted in the appearance of fibroblast-like cells that covered all exposed surfaces. Additional samples of guanidine-extracted bone matrix were subsequently reconstituted with the guanidine extract of rat bone matrix, which resulted in restoration of chondrogenic potential similar to that of demineralized bone matrix described above (Fig. 1*C*).

Guanidine·HCl extracts of bone matrix can be fractionated by chromatography on Sepharose CL-6B. Five major fractions have been previously identified; fraction IV contains components of <50,000 daltons with the osteoinductive activity of bone matrix (13). This fraction was reconstituted onto guanidine-extracted bone matrix and cultivated *in vitro* with minced skeletal muscle and resulted in chondrogenesis (Fig. 1*D*). Other fractions, containing proteins of >50,000 daltons, did not elicit chondrogenesis from skeletal muscle when reconstituted and cultivated *in vitro* (data not shown).

Cartilage Differentiation on Collagen Gels *in Vitro*. When gels of type I collagen were used as a control substratum (9, 10), regeneration of multinucleate myotubes was observed. It is noteworthy that explanted cells did not invade the colla-

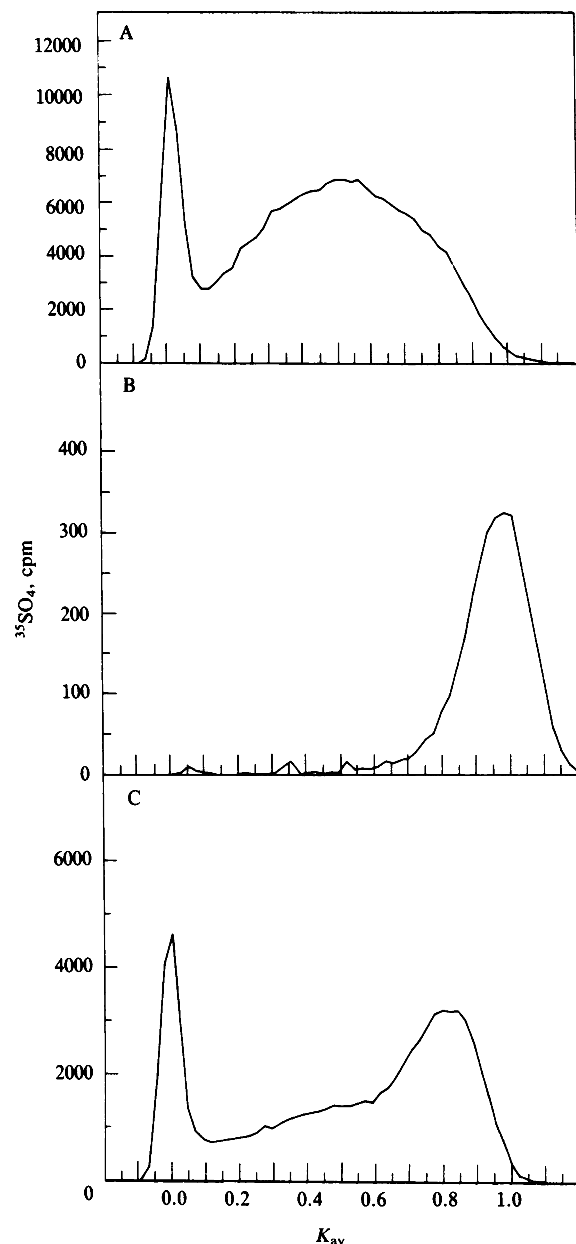

FIG. 2. Sepharose CL-2B chromatograms of ^{35}S-labeled material extracted from skeletal muscle grown on bone matrix (*A*), control collagen gels (*B*), and collagen gels reconstituted with a 4.0 M guanidine extract of bone matrix (*C*). Bone hemicylinders elicit synthesis of proteoglycan aggregate (K_{av} = 0) and monomer (K_{av} = 0.49). Material of lower molecular size (K_{av} = 0.98) is the sole species synthesized by skeletal muscle on collagen gels. Collagen gels reconstituted with an extract of bone matrix support synthesis of sulfated species that migrate as proteoglycan aggregate and a polydisperse material that occupies the remainder of included volume. On reconstituted gels, lower molecular size material migrates at K_{av} = 0.80 and appears larger than species detected on unreconstituted gels.

Table 1. Dissociative extraction and reconstitution of biological activity of bone matrix *in vivo* (day 12).

Sample	Alkaline phosphatase, units/mg of protein	Calcium content, μg/mg of tissue	Bone histology
Untreated bone matrix	0.75 ± 0.26	0.50 ± 0.25	++
6.0 M guanidine·HCl-treated bone matrix	0.25 ± 0.20	0.42 ± 0.26	−
Reconstituted bone matrix	2.23 ± 0.49	4.45 ± 0.98	+++

Values are mean ± SEM for four samples from each group. −, Absent; ++, moderate; +++, extensive.

gen gels but remained on their surface (Fig. 1*E*). The chondrogenic response was restored when a similar collagen gel was reconstituted with an unfractionated guanidine·HCl extract (Fig. 1*F*). The reconstituted gels contained nodules of hyaline cartilage. However, in collagen gels reconstituted with fraction IV no cartilage formed (data not shown); also, the muscle tissue did not regenerate and the explanted cells remained fibroblast-like.

Proteoglycan Synthesis *in Vitro*. Additional evidence for chondrocyte differentiation in response to bone matrix components was obtained by examination of synthesis of cartilate-specific proteoglycans. Explants onto untreated bone matrix were found to contain two prominent peaks of ^{35}S-labeled material after chromatography on Sepharose CL-2B (Fig. 2*A*). One peak at the column void volume migrated as a proteoglycan aggregate. The second eluted at a K_{av} of 0.49 and represented proteoglycan monomer. Aggregate and monomer comprise 17.2% and 82.8%, respectively, of the total ^{35}S label. Explants onto collagen gels contained a peak of low molecular size material that migrated close to the total volume of the column (Fig. 2*B*). The proteoglycan profile from collagen gels reconstituted with the guanidine·HCl extract is shown in Fig. 2*C*. Three fractions were detected in these latter cultures: one occurred at the void volume, comprised 18.7% of the total ^{35}S label, and represents proteoglycan aggregate; the second fraction consisted of a broad peak with a K_{av} of 0.1–0.65; and the third eluted at a K_{av} of 0.8. The second and third fractions comprised 28.2% and 53.1% of the ^{35}S label, respectively. These data demonstrate the ability of reconstituted components of bone matrix to elicit cartilage differentiation as indicated by proteoglycan synthesis.

Endochondral Bone Differentiation in Bone Hemicylinders *in Vivo*. Subcutaneous implantation of demineralized hemicylinders of bone matrix into rats induced cartilage differentiation on day 7 after implantation and bone formation on day 12 as measured biochemically (Table 1) and histologically. When 6.0 M guanidine-extracted bone matrix was implanted subcutaneously, no evidence of cartilage or bone formation was observed. However, implantation of reconstituted matrix resulted in complete restoration of biological activity (data not shown).

DISCUSSION

Growth of embryonic skeletal muscle on demineralized bone (bone matrix) results in transformation of mesenchymal cells derived from embryonic muscle into hyaline cartilage. Such skeletal muscle and its associated connective tissue would not form cartilage in the absence of bone matrix. Although the cellular and biochemical changes accompanying matrix-directed transformation of muscle into cartilage have been examined (6–10), the precise mechanisms remain unclear. The aim of the present study was to investigate whether components of the bone matrix, supplied exogenously, could induce phenotypic transformation of mesenchymal cells and connective tissue cells to chondroblasts *in vitro*. Previous work has shown that components of bone matrix, which are responsible for its biological activity, can be extracted with guanidine·HCl (13). The extracts can be reconstituted with an inactive residual matrix, resulting in restoration of *in vivo* bone-inductive activity. The results of the present study show that extraction of bone matrix with 6.0 M guanidine·HCl completely abolishes the *in vitro* transformation of embryonic muscle into cartilage and that this activity is restored following reconstitution of the inactive residue with the guanidine extracts. Previous studies have demonstrated the stimulatory effects of guanidine·HCl extracts on glycosaminoglycan synthesis (18) and the accumulation of cartilage proteoglycans and type II collagen by muscle-derived mesenchymal cells (19).

Histological analysis of skeletal muscle cultured on inactivated bone matrix revealed stellate fibroblasts as the sole surviving cell type after 18 days *in vitro*. These data support the hypothesis that extracellular matrices of defined composition are necessary to maintain the phenotypic expression of muscle. The effect of the matrix is not merely that of a nonspecific anchorage site, since control collagen gels were able to support muscle regeneration. Careful examination of explants on guanidine-extracted matrix also showed that the resultant cell densities were less than those from comparable explants onto active matrix. Previous experiments have shown that 4.0 M guanidine·HCl extracts of bone matrix contain a growth factor for mesenchymal cells (15). It is currently unclear whether failure of myogenesis was due to a failure of myogenic cells to attach and/or proliferate or whether these phenomena are dependent on a growth factor released from the matrix.

The specificity of the substratum was examined by substitution of rat tail tendon collagen, reconstituted with an extract of bone matrix. The results revealed that the unfractionated extract conferred bone matrix-like activity on the collagen gel. However, reconstitution with a partially purified extract of molecular mass <50,000 daltons (devoid of intact collagen chains) was ineffective in this regard. Since partially purified extracts are capable of eliciting cartilage and bone formation when reconstituted with inactive bone matrix, this suggests that a bone-specific collagenous substratum may be required for the anchorage-dependent attachment and differentiation of cells. Unfractionated guanidine·HCl extracts contain considerable amounts of bone collagen (13). In conclusion, guanidine extracts of bone matrix contain a component that is capable of transforming mesenchymal cells to cartilage *in vitro* as well as *in vivo*.

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