SUBSTRATA PREPARED FROM BONE MATRIX FOR CHONDROGENESIS IN TISSUE CULTURE

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INTRODUCTION

The literature on chondrogenesis in vitro is rich in knowledge of the products of differentiation but presents little information about agents which may initiate cartilage cell differentiation or conditions which control cartilage tissue morphogenesis. Previous investigations by our research group (18, 19) demonstrate that the character of the substratum may control differentiation. For example, an outgrowth of mesenchymal cells from muscle on to nonbiologic substrata (plastic or millipore membrane) produces fibrous connective tissue cell differentiation, while outgrowths on to a special preparation of undenatured, demineralized rat bone matrix differentiates into cartilage (30). If the substratum consists of bone matrix denatured by demineralization in a solution of 0.6 N HCl in 70% alcohol, the mesenchymal cells differentiate only into fibrous connective tissue (30). This communication demonstrates experimental modifications of a bone matrix substratum which either permit, retard, or prevent differentiation of cartilage.

MATERIALS AND METHODS

The femora of adult Sprague-Dawley strain rats were excised, trimmed, and washed to produce mid-diaphyseal marrow-free cylinders of cortical bone. Each cylinder was demineralized, extracted, prepared by one of the procedures listed in Table I, and then lyophilized. In preparation for the assembly of cultures, the products were washed and reconstituted for 30 min at room temperature in the culture medium and were finally wetted with chicken plasma (Grand Island Biological Co., Grand Island, N. Y.) in a petri dish for 10 min at room temperature. The pieces of reconstituted matrix were split into four parts and reassembled as shown in Fig. 1. The hemicylinders were cut halfway through at 1–2-mm intervals transversely with a no. 11 surgical blade to provide spaces for cell aggregation and additional surfaces for ingrowth of proliferating muscle mesenchyma, and to increase the supply of medium to minced muscle. Mesenchymal cells were cultured from muscle tissue of near-term fetal rats (21 days).

Samples of muscles were excised from the belly of the triceps humerus and minced in a drop of culture medium to obtain pieces approximately 0.5 mm in size. In some cultures, minced muscle was substituted with trypsinized muscle. The medium was CMRL-1066 with 15% heat-inactivated newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Grand Island Biological Co.). Approximately 0.75% of the medium was changed every 48 h and maintained for 3–14 days (and extended to 30 days for special experiments) on the reversed grid in a Falcon organ culture dish (Falcon Plastics, Div. of B-D Laboratories, Los Angeles, Calif.) at 37°C in a CO_2 incubator (5% CO_2 in air, model 3221, National Applicance Co., Portland, Ore.) as described previously (19). The initial pH of the medium was 7.3.

Cultures prepared as described above were grown on matrix denatured by demineralization at 2°C in 0.6 N HCl in 70% alcohol for 24 h. Cultures of muscle mesenchyma were also made on millipore membranes (8-μm pore size) or sandwiched between millipore membranes in order to simulate the condition of a crevice in the matrix.

Outgrowths of bone marrow cells, buffy coat cells, lymph node, spleen, thymus, or skin were also cultured on bone matrix substrata. Bone marrow cells were obtained from the femur of 2-wk old rat and buffy coat cells were obtained by means of heart puncture of 3-mo old rat.

^{35}S-*Labeled Explanted Mesenchymal Cells*

Explants on a substratum of insoluble bone gelatin were incubated with 10 μCi of ^{35}S (specific activity 694 mCi/mmol, New England Nuclear, Boston, Mass.) per ml of sulfur-free Krebs-Ringer solutions for 3 h at intervals of 3, 4, and 7 days in culture, and the explants were fixed for autoradiography.

The explants were examined by microradiographic, histologic, and autoradiographic methods. Autoradiographs of serial sections of 5-μm thickness were prepared by dipping slides in Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) and were developed with Dektol for 2 min at 17°C after a 3-wk exposure at 4°C, then stained with alcian blue and nuclear fast red.

Electron Microscopy of Cartilage Differentiation

The area of the explant growing into a crevice in insoluble bone gelatin at intervals of 3–7 days was excised

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in ice-cold cacodylate-buffered (pH 7.4) 3% glutaraldehyde and fixed in solutions of two parts cacodylate-buffered glutaraldehyde and one part ruthenium red (Sigma Chemical Co., St. Louis, Mo.) at 4°C for 2 h. Ruthenium red stain was prepared according to Laros and Cooper (12). Postfixation was carried out in equal parts 2% osmium tetroxide in cacodylate buffer and ruthenium red for 3 h, dehydrated through an ethanol series and acetone, and embedded in Epon 812. Gray sections were cut with a Dupont diamond knife (E. I. DuPont de Nemours & Co., Wilmington, Del.), stained with uranyl-acetate, and examined with a Siemens IA electron microscope. Sections cut 1 μm thick were stained with 0.5% toluidine blue for light microscope comparison.

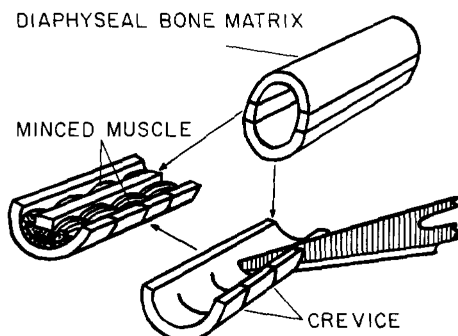

FIGURE 1 Assembly of muscle and segments of decalcified diaphyseal bone matrix. Crevices are cut in the hemicylindrical segments with a scalpel. Two explants are made up from one rat diaphysis.

RESULTS

Matrix (preparation no. 1, Table I) extracted in chloroform-methanol for 1 h and demineralized in 0.6 N HCl at 2°C within 24 h, served as a substratum for expression of chondrogenetic potential in 20 out of 20 cultures.

Matrix Degraded by Endogenous Enzyme

Outgrowths of muscle mesenchymal cells on to the substratum preparation no. 2 (preparation no. 1 incubated in phosphate buffer [pH 7.4] with penicillin [100 U/ml] and streptomycin [100 μg/ml] for 4 days at 37°C) produced limited expres-

TABLE I

Demonstration of Expression of Chondromorphogenetic Potential of Mesenchymal Cell Outgrowths on to a Substratum of Bone Matrix

Preparation no.	Procedures for preparations of substratum	No. of cultures	No. of cultures producing cartilage	Remarks
1	Chloroform-methanol-extracted, 0.6 N HCl demineralized bone matrix	20	20	High yield within deeply stained hyaline matrix on and below exposed surfaces of matrix
2	0.6 N HCl-demineralized matrix incubated at 37°C for 4 days	10	5	Low yield of defective cartilage
3	Preparation no. 1, preheated at 55°C	9	8	Thinly spread deposits of new cartilage
4	Bone matrix gelatin (BMG)	20	20	High yield and deposits of cartilage woven into the gelatinous substratum
5	BMG incubated at 37°C for 4 days	9	6	High yield in partially solubilized gelatinous substratum
6	BMG heated to 55°C for 4 days	12	12	Deposits of cartilage throughout thinly spread, solubilizing, gelatinous substratum
7	Pulverized BMG (particle size 500-800 μm).	4	2	Poor yield with small particles and only fair yield with larger particles
8	Acid-alcohol demineralized denatured bone matrix	16	2	Poor adherence of cells to the substratum and only a few chondrocytes developed in rarely developing cartilage tissue
9	Millipore membranes	8	0	Fibroblastic cells only
10	Undemineralized bone matrix	12	1	Encapsulated chondrocytes with little matrix aligned in a row in old vascular channel
11	Lathyritic bone matrix	10	2	Poorly stained and very few chondrocytes
12	Collagenase-digested BMG*	10	10	High yield on the surface and in the crevices
13	Trypsin-digested BMG‡§	4	4	No yield on the surface and in the crevices of the matrix. Poorly stained and a few chondrocytes in deep area of the matrix

* 43 μg of collagenase per ml of 50 mm Tris buffer containing 5 mm $CaCl_2$, pH 7.3, 2 matrices per ml of buffer. 37°C for 30 min of incubation. After incubation, matrices were washed in 0.1 N acetic acid for 1 h and then washed in distilled water for 1 h.

‡ 0.25% trypsin (Grand Island Biological Co., solution A) at 37°C for 3 h.

§ Only the surface area of the matrix, including crevices, was digested.

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sion of the chondrogenetic potential in 5 out of 10 explants. The chondrocytes were irregular in shape, size, and quantity of matrix. With preparation no. 3, consisting of matrix preheated to 55°C (heat-inactivated endogenous proteases), cartilage developed in eight out of nine explants.

Insoluble Bone Matrix Gelatin

Preparation no. 4, consisting of bone matrix gelatin (BMG) prepared by sequential extraction of soluble, noncollagenous proteins (29) produced cartilage in 20 out of 20 explants. The explants were examined by autoradiography and electron microscopy.

Insoluble and Soluble BMG

Preparation no. 5, consisting of BMG incubated at 37°C for 4 days in distilled water to solubilize gelatin, produced cartilage in six out of nine implants. In preparation no. 6, in which BMG was preheated at 55°C, cartilage developed in 12 out of 12 explants. The deposits developed in relatively deep recesses in the insoluble residue.

Pulverized BMG

In preparation no. 7, in which the matrix was pulverized, cartilage developed in only two out of four explants, and only in juxtaposition to a few of the large particles. The volume of new cartilage was also very low.

Denatured Demineralized Bone Matrix

When the substratum (preparation no. 8) consisted of matrix (denatured by demineralization in a solution of 0.6 N HCl in 70% alcohol, mesenchymal cells grew out of the minced muscle but failed to grow into the depths of the crevices and did not adhere to the substratum. In 2 out of the 16 explants, a few bizarrely shaped chondrocytes with pale, irregularly staining matrix developed in one old vascular channel.

Millipore Membrane Control

No cartilage developed in outgrowths of muscle on to or between sheets of millipore membrane (preparation no. 9). The end-product was always an outgrowth of a loosely woven network of fibroblastic cells.

Undemineralized Bond

Only 1 out of 12 cultures of mesenchymal cell outgrowths on to undemineralized bone (preparation no. 10) produced cartilage. In serial sections, the new deposits were only about 8–15 cells in volume and aligned within the lumen of an old vascular channel (Fig. 2). These deposits appeared to be in close apposition to osteoid seams of building sites in the original cortical bone structure.

Lathyritic Bone Matrix

Only 2 out of 10 cultures of muscle mesenchymal cells developed in very small patches of new

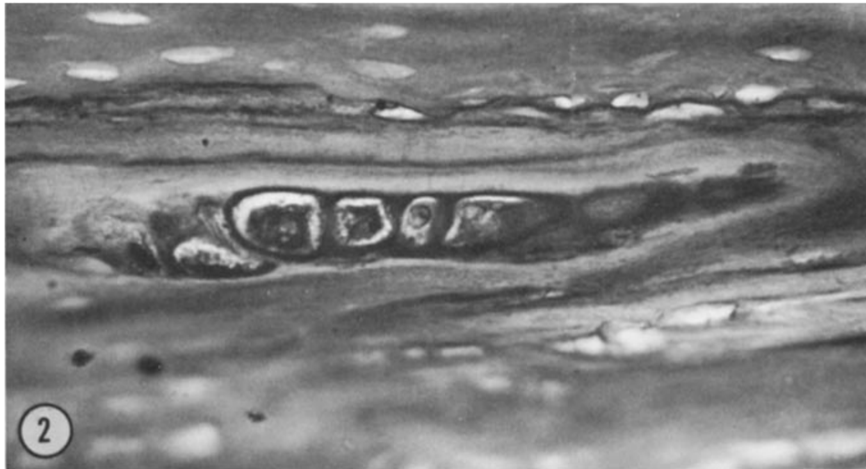

FIGURE 2 Photomicrograph of five chondrocytes in the lumen of an old vascular channel in the undermineralized bone. Hematoxylin-eosin Azure II stain. × 250.

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cartilage growing out of a substratum of demineralized lathyritic bone matrix (preparation no. 10). The deposits were deeply embedded in the interior of the old matrix in close apposition to areas of bone remnants which might have formed in the period of time before the donor was placed on a lathyritic diet.

Limited Digestion with Exogenous Enzymes

By methods described previously (29), BMG was prepared by limited digestion either with trypsin or with purified collagenase (Worthington Biochemical Corp., Freehold, N. J.) and used as a substratum for growth of mesenchymal cells (Table I, lines 12 and 13). Growth on to trypsin-digested BMG produced only fibrous tissue while growth on to residues of collagenase-digested BMG produced large deposits of new cartilage.

Oxygen Tension and pH

Cultures of mesenchymal cell outgrowth on to matrix preparation no. 4 were made in an atmosphere of 20% or of 60% oxygen (Fig. 3 *a*, *b*). The chondrocytes became more hypertrophic and vesiculated. Pyknotic and deeply stained degenerating fibrous connective tissue cells were numerous.

^{35}S-labeled Cells in Crevices in Insoluble Bone Gelatin

Cells growing in the depth of a crevice were long and spindle shaped and ^{35}S autoradiogram showed low uptake on the 4th day (Fig. 4 *a*). By the 7th day, the spindle cells were slightly swollen and had secreted alcian blue-staining extracellular subtance, and the ^{35}S autoradiographic grain density was markedly increased (Fig. 4 *b*). By the 14th day, when the cells were surrounded by alcian blue deep-staining hyaline-intercellular matrix, the crevice increased in both width and depth. The expanding volume of new cartilage distended the walls of the crevice and grew in secondary nodules out on to the surface of the substratum (Fig. 4 *d*).

Electron Micrographs

The conversion of bone matrix to insoluble bone gelatin produced an amorphous substratum with hardly any detectable fibrillar structure. Proliferating mesenchymal cells were elongated in proportion to the length and shape of the crevice. By the 3rd and 4th day of culture, mesenchymal cells had well-developed cytoplasmic polysomes and plasma membranes arranged in irregular folds to form filopods and lamellipods filled with microfilaments (Fig. 6). By the 7th day, the elongated, spindle-shaped cells developed coarse ER and were separated by an extracellular network of ruthenium red-staining granules. Later the same cells developed an electron-dense cortex characteristic of young cartilage cells (Fig. 7).

Outgrowths of Other Tissues

Fetal tissue mesenchymal cell outgrowths produced cartilage in two out of six cultures of bone marrow, one out of eight of spleen and two out of eight of thymus. The quantity of cartilage was always much lower than from muscle but similarly developed in deep recesses of a crevice. Mesenchymal cells did not produce cartilage in eight cultures of skin, or in five of lymph node, or in four of blood buffy coat cells. Trypsinized muscle connective tissue cells produced only fibrous tissues.

DISCUSSION

In tissue culture, mesenchymal cells migrate from fragments of rat triceps muscle and differentiate into cartilage in response to a substratum consisting of whole bone matrix or BMG, or BMG digested with collagenase, but not denatured bone matrix, or trypsin-digested BMG, or lathyritic bone matrix. These observations are entirely comparable to previously reported observations in implants of allogeneic matrix in muscle in the intact animal (25). The experiments are the basis of the hypothesis that differentiation is initiated by an insoluble, noncollagenous bone morphogenetic protein (BMP), or polypeptide or part of a protein firmly bound to collagen (29, 31). Whether muscle mesenchymal cells inherit determination for chondrogenesis expressed under the influence of BMP, or whether BMP institutes transdetermination is not clear. Transdetermination is a term adopted by Ephrussi (3) to indicate the change of an adult cell population from one normal to a different normal (non-neoplastic) pathway of development. The more general terminology, including discussions of the unsolved problem of extracellular controls of embryonic and postfetal cell development, are reviewed in detail in the literature (2, 5-7, 20, 27).

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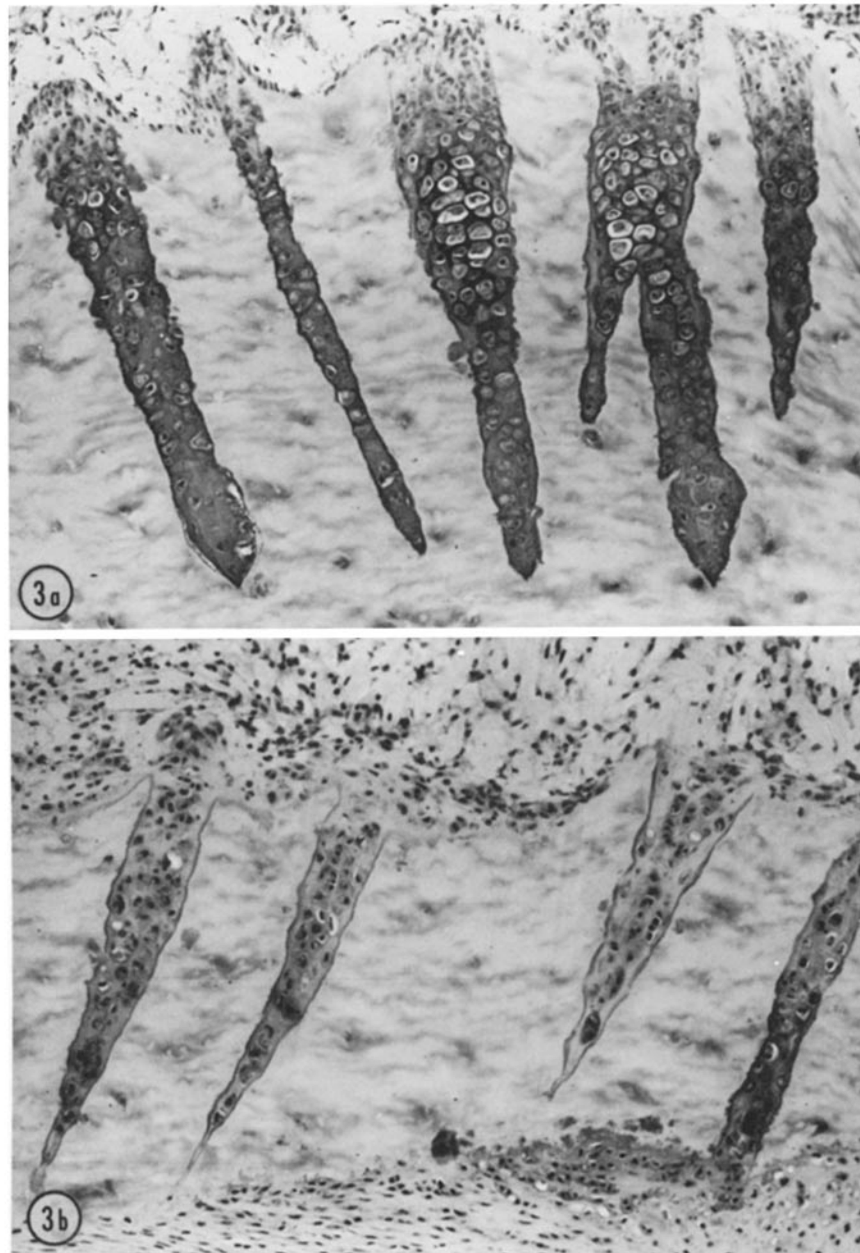

FIGURES 3 *a* and *b* Chrondrocytes in the artifactual crevices in the substratum on day no. 14 of culture. The cultures were exposed to 60% (3 *a*) and 20% (3 *b*) oxygen. Note hypertrophic cartilage (3 *a*) in culture with high O_2-saturated atmosphere. Hematoxylin eosin and Azure II stain. × 60.

Triceps muscle mesenchymal cells are descendents of embryonic limb bud mesenchyma but normally differentiate only into fibrous tissue. Mesenchyma of the chick differentiates by the third day (10) of incubation and procedures cartilage spontaneously within 48 h in culture (4). Rat muscle mesenchymal cells do not differentiate into cartilage spontaneously, but are comparable to embryonic cells (undifferentiated with respect to chondrogenesis) insofar as bromodeoxyuridine

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(Terashima and Urist, unpublished observations) irreversibly inhibits BMG-controlled differentiation.

The physicochemical structure of bone matrix somehow substitutes for inductive extracellular substances developed in the 3-day chick embryo (covert differentiation) for cartilage development (overt differentiation) on the 6th day (1, 13, 29, 32). Enriched media, anoxia under paraffin or vitelline membranes, or a critical cell mass (9, 10, 13) provide conditions favorable for cartilage development (21) but do not initiate differentiation of muscle mesenchymal cells (19). Medium no. 199 is sufficient for cell proliferation (14, 15, 21) while another, BGJ, promotes cell differentiation (18, 19), while still another, CMRL-1066, provides the essential nutriments for both proliferation and differentiation. In the CMRL-1066 medium, mesenchymal cell outgrowths from different organs on to BMG produce variable quantities of cartilage. Bone marrow, spleen, and thymus produced relatively low yields, while skin produced no yields of cartilage. Disaggregation and digestion of intercellular substances with trypsin inhibited a chondromorphogenetic response to the substratum.

Bone matrix is approximately 90% collagen and 10% soluble and insoluble organic noncollagenous substances. The component of the matrix essential for chondrogenesis is a collagenase-resistant, trypsin-labile, noncollagenous fraction referred to previously as BMP (29). A trypsin-like hypothetical neutral proteinase (BMPase) (16, 27, 28) in bone

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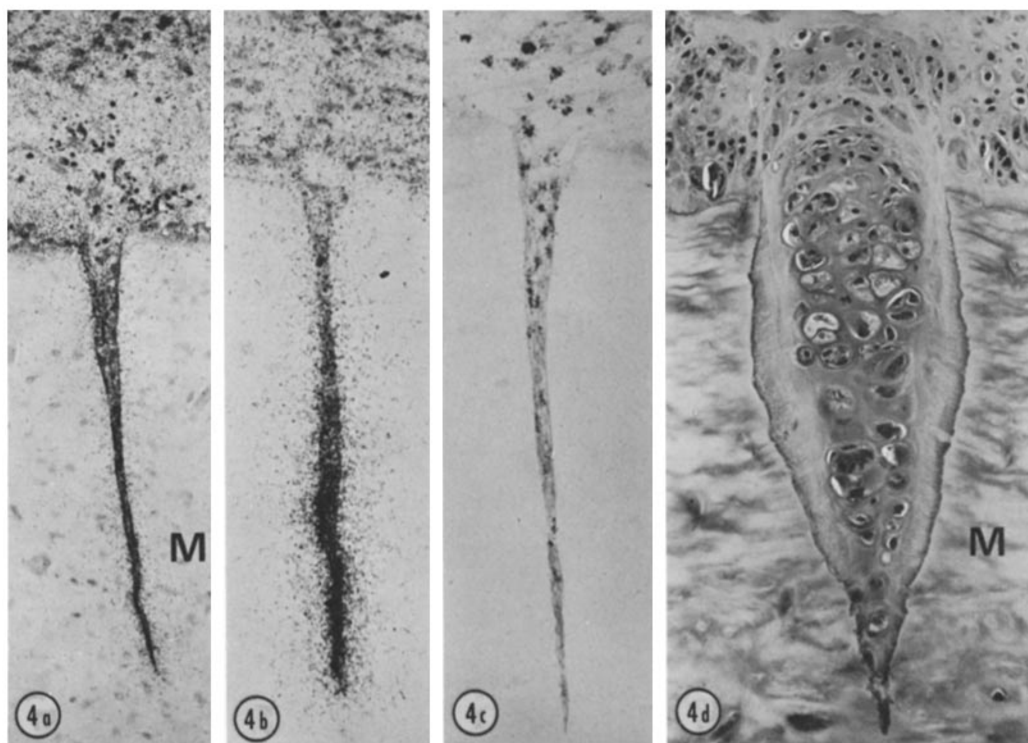

FIGURE 4 *a* ^{35}S Autoradiograph on the 4th day of culture. No difference was observed in grain density of cells growing inside and outside of a crevice (M). Alcian blue stain. × 100.

FIGURE 4 *b* ^{35}S Autoradiograph on the 7th day of culture, showing heavy labeling of cells inside the crevice. Alcian blue stain. × 100.

FIGURE 4 *c* [^{3}H]thymidine autoradiograph on the 7th day of culture, showing cells inside of the crevice but derived from muscle tissue prelabeled in vitro before culture on the substratum of bone matrix. Alcian blue stain. × 100.

FIGURE 4 *d* Hypertrophic chondrocytes inside and immature chondrocytes outside of the crevice of the bone matrix (M) on 30th day of culture. Hematoxylin eosin Azure II stain. × 10.

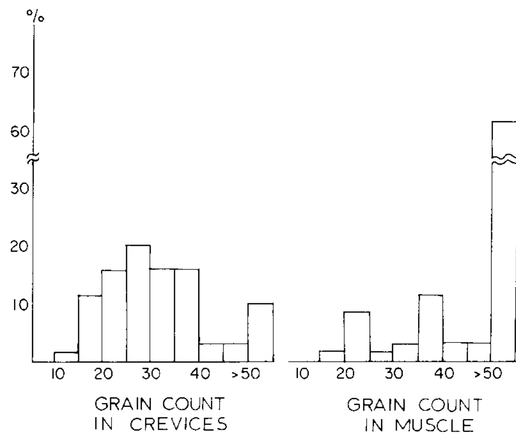

FIGURE 5 Grain counts of [^{3}H]thymidine of cells inside and outside of the crevice on the 3rd day of culture.

matrix digests BMP. Although it is just as insoluble as the highly cross-linked bone collagen itself, BMP is specifically cleaved, or digested, or degraded by BMPase by incubating bone matrix in phosphate buffer, pH 7.4, at 37°C (26, 28). Preheating the matrix at 55°C inactivates BMPase without denaturation of BMP (28). Bone matrix is the carrier of BMP, possibly through a covalent bond, and retains morphogenetic property even after the bone collagen structure is converted at 2°C by 8 M LiCl to insoluble bone gelatin.

The segment of the cell population proliferating in artifactual crevices and old vascular channels in bone matrix develops into mature cartilage with large quantities of deeply stained hyaline matrix. Compressive forces of growth in a semirigid space within a crevice may account for structural modifi-

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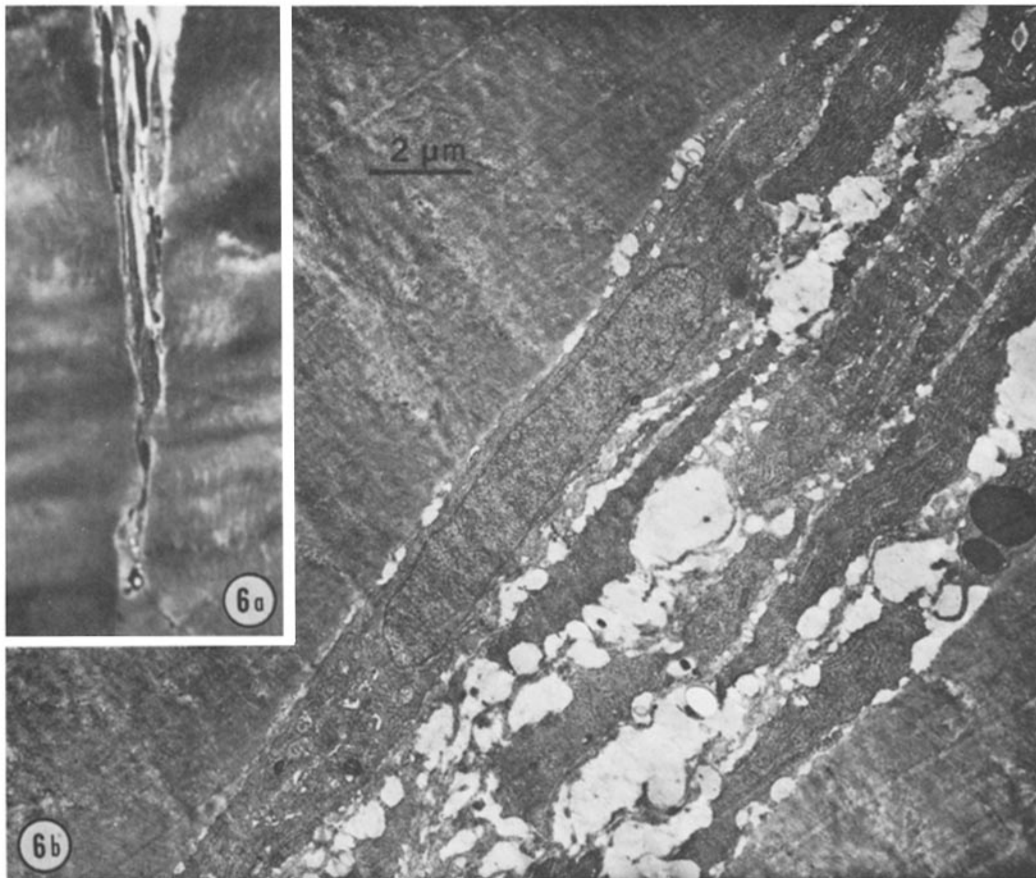

FIGURE 6 *a* Photomicrograph showing elongated mesenchymal cells inside of the crevice on the 3rd day of culture. Toluidine blue stain. × 400.

FIGURE 6 *b* Electron micrograph of migratory mesenchymal cells shown in Fig. 6 *a*, inside of the crevice on the 3rd day of culture. Note convolutions in plasma membranes. Ruthenium red and uranyl acetate stains. × 3,000.

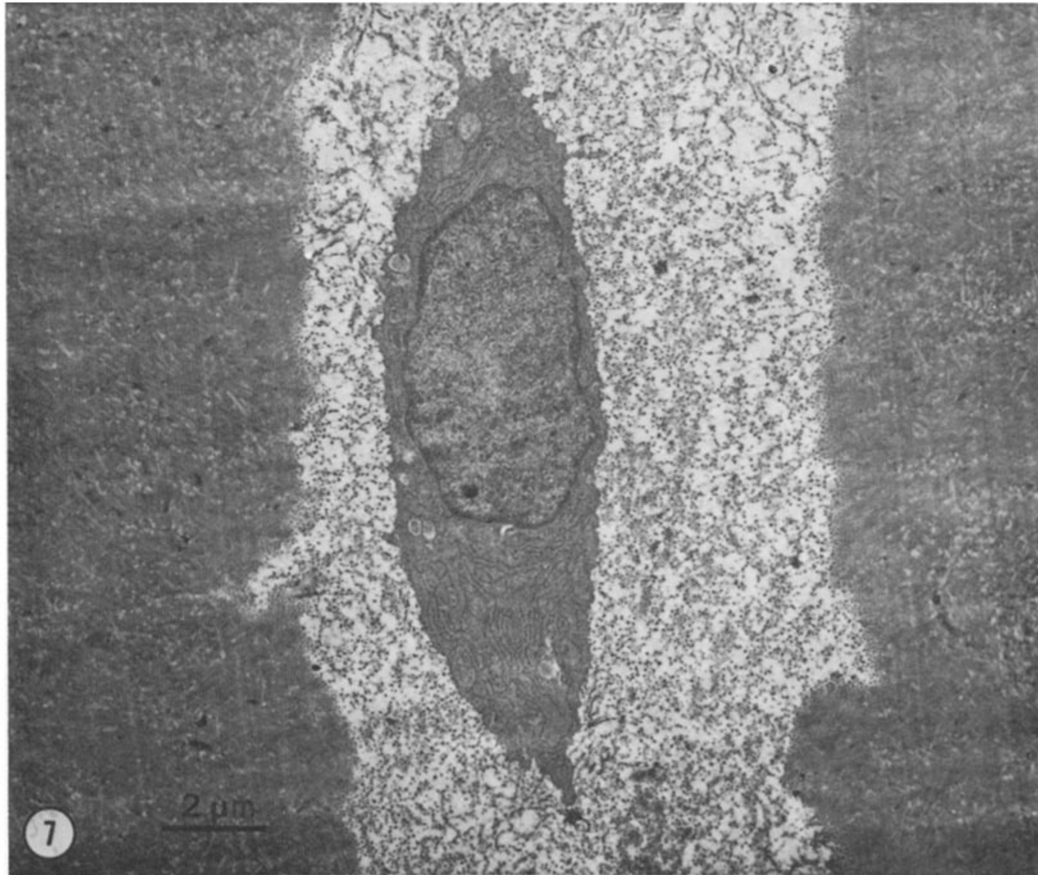

FIGURE 7 Electron micrograph of chondrocyte inside the crevice on the 7th day of culture. Note ruthenium red-stained protein polysaccharide of the matrix. Ruthenium red and uranyl acetate stains. × 3,000.

cations but not differentiation. Cell populations proliferating in closed spaces in preparations of undemineralized, denatured, or degraded matrices do not differentiate into cartilage. Pressure and avascularity, however, favor development of the cartilage only from a proliferating differentiated cell population.

Neonatal mesenchymal cells proliferating in matrix crevices may synthesize ruthenium red- and alcian blue-staining chondromucoproteins in response to a local, progressively developing anoxia. 20% hyperoxia produces hypertrophy and vesiculation of mature chondrocytes while 60% is toxic to cells in culture. For unknown reasons, this cartilage does not produce matrix vesicles, hypertrophy, synthesize enzymes for degradation of protein polysaccharide inhibitors of apatite crystal growth, or calcify in tissue culture. However, cartilage that is developed and labeled with [^{3}H]thymidine in tissue culture and then transplanted into syngenic recipients can calcify and even produce new bone with labeled osteocyte nuclei (unpublished experiments). This observation supports experimental evidence in the literature on metaplasia of hypertrophic cartilage cells into bone cells (8, 11, 16, 22–24).

SUMMARY

Cartilage developed from muscle mesenchymal cell outgrowths into old vascular channels and artifactual crevices cut in the substratum of undenatured bone matrix. Overt expression of chondrogenetic determination, observed in ^{35}S autoradiographs and electron micrographs, occurred on about the 7th day of culture in medium CMRL-1066. Large deposits of cartilage developed upon a substratum of whole bone matrix, or

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of insoluble bone gelatin (BMG), or BMG partially digested with collagenase. Little or no cartilage differentiated after autolytic digestion of matrix incubated in phosphate buffer pH 7.4 at 37°C for 4 days. Matrix heated to 55°C before incubation in buffer was less autolyzed and promoted cartilage differentiation. Small isolated patches of cartilage differentiated from outgrowths on to pulverized matrix but little or no cartilage grew on lathyritic demineralized or undemineralized normal bone, or on BMG partially digested with trypsin. No cartilage was ever obtained from muscle mesenchymal cell outgrowths on to millipore membrane. 20% O_2 saturation produced cartilage-cell hypertrophy and vesiculation. 60% O_2 was toxic for mesenchymal cells in culture. The number of cells or the yields of new cartilage were greater from mesenchymal cell outgrowths from muscle than from bone marrow, thymus, spleen, or skin.

The authors are indebted to Mrs. Susan Kirkpatrick, Mrs. Carmel Fox and Gillian Molson for excellent technical assistance.

These investigations were supported by grants-in-aid from the John A. Hartford Foundation and the United States Public Health Service, National Institute of Dental Research (DE-02103-01) and the Leo Hulseman Foundation. Dr. H. Nogami received a research fellowship from the Solo Cup Co., Chicago, Ill.

Received for publication 26 October 1973, and in revised form 25 February 1974.

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