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Chondrogenesis in Tissue Cultures of Muscle Under the Influence of a Diffusible Component of Bone Matrix (39720)[1]

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When neonatal muscle is cultured on a nonbiologic substratum such as glass, plastic, or cellulose acetate, the fiber bundles disintegrate while the connective tissue disaggregates and becomes mesenchymal cells, which migrate and proliferate. On a biologic substratum such as bone matrix (1) or bone matrix gelatin (2), or on the residue of collagenase-digested bone matrix gelatin (3), the mesenchymal cells reaggregate and differentiate not into fibrous tissue but into cartilage (4–6). On a control biologic substratum of bone matrix either partially digested with trypsin or on gelatin prepared from bone autodigested by endogenous enzymes in a buffer solution (7), the cells differentiate into fibrous tissue only (3–4). Through contact of mesenchymal cells with a hypothetical bone morphogenetic protein (BMP) in bone matrix, the pathway of development is switched from fibrous tissue to cartilage (2). The following experiments demonstrate transfer of BMP to mesenchymal cells through the nutrient medium without direct contact with bone matrix.

Methods. Samples of muscle were excised from the middle of the belly of the triceps humeri of 3-week-old Lewis strain rat fetuses. The muscle tissue was minced in a drop of culture medium CMRL 1066 containing 15% heat-inactivated newborn calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co.). The initial pH of the medium was 7.3. The minced muscle tissue was placed on a chicken plasma clot and cellulose acetate membrane (Millipore Corp.), pore size 0.45 μm. The plasma-coated membrane was placed on a wire-grid platform in a Falcon organ culture dish (Falcon Plastics, Division of BD Laboratory, Los Angeles, Calif.). The culture was maintained at 37° in an incubator, 5% CO_2 in air, Model 3221 (National Appliance Co., Portland, Ore.).

Graduated quantities of the residue of bone matrix gelatin (BMG) (2) digested with purified collagenase (8) for only 4 hr were placed in the space beneath the grid, well *isolated from any possible contact* with either the cellulose acetate membrane or the cultured tissue. The assembly of the system is illustrated in Fig. 1; the dosages of BMG and the experimental results are summarized in Table I.

The cultures were conditioned with successively increasing quantities of BMG ranging from 0 to 50 mg (25 mg of BMG/ml of culture media). For controls, muscle tissue cultures were assembled: (a) without any BMG in the space beneath the grid; (b) with BMG prepared from autodigested bone (7); (c) with BMG in the system, but only for limited periods of time, ranging from 1 to 7 days; (d) with living calvarial bone. Duplicate cultures were transplanted on the 7th day into a muscle pouch in adult syngeneic recipients.

The explants and transplants were fixed in neutral formalin on the 21st day. Histological sections were prepared and stained with hematoxylin and eosin, azure II, and alcian blue.

Results. Mesenchymal-like fibrous connective tissue cells grew out of the explanted muscle tissue by the seventh day and spread out in a thin layer on the surface of the cellulose acetate membrane (Fig. 2). The muscle fibers atrophied, and myoblasts either formed multinuclear aggregates or

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disintegrated in pyknotic fragments. By the 14th day, in culture dishes containing 30 to 50 mg of BMG, cartilage cells differentiated in the space between the atrophied degenerated muscle tissue and the surface of the cellulose acetate membrane. By the 21st day, the cells were paired and enveloped in hyaline cartilage matrix. Azure II metachromatically stained and alcian blue extracellular substance extended into the pore openings for a distance of about 5 to 10 μm (Fig 3).

In control culture dishes containing less than 30 mg of BMG, or BMG prepared from autolysed bone, or even living calvarial bone, the connective tissue outgrowths differentiated into fibrous tissue only without any evidence of cartilage. When 50 mg of BMG were present for only limited periods of time from 1 to 6 days, cartilage developed on the 21st day (Table I). The yield of new cartilage at 21 days was somewhat less than when BMG was continuously present in the system. After 6 days in culture, the BMG disintegrated and could not be recovered. When the BMG was removed at earlier stages and transplanted into a muscle pouch in an adult rat, the matrix still had osteoinductive activity. The yield of new bone was much less than from unincubated BMG.

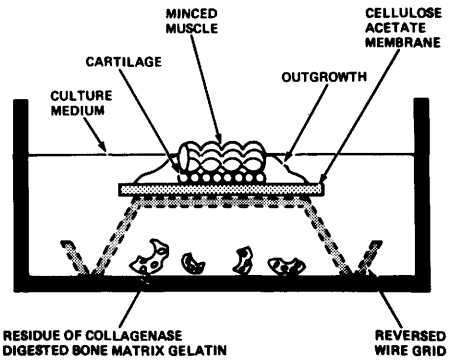

FIG. 1. Diagrammatic representation of tissue culture assembly for outgrowth of mesenchymal cells of rat neonatal muscle onto a cellulose acetate membrane (pore size, 0.45 μm).

On the 7th day, with 50-mg doses of BMG beneath the grid, and spindle-shaped cells growing out on the membrane above the grid, the membrane and tissue culture attached to it were transplanted into a muscle pouch in an adult syngeneic recipient. Fourteen days later the membranes were covered with new cartilage and deposits of endochondral bone (Fig. 4).

Discussion. In the culture assembly described above, muscle connective tissue outgrowths differentiated into cartilage on a cellulose acetate membrane without any possible contact with BMG. The muscle explant was separated from BMG not only by a membrane but also by a wire grid and 2 ml of culture medium. Under these conditions, the medium would have had to transfer a soluble rapidly diffusible chondrogenetic molecule from the BMG to the explant. The

TABLE I. DEMONSTRATION OF CHONDROGENESIS AND OSTEOGENESIS EVOCATED IN EXPLANTS OF MUSCLE TISSUE BY DIFFUSIBLE COMPONENTS OF BONE MATRIX GELATIN (BMG).

BMG (mg)	Number of cultures with cartilage/total number of explants	Day BMG was withdrawn from culture	Product	Product of transplantation of 7-day-old cultures into isogeneic rats for 2 weeks
0	0/9	0	Fibrous tissue only	Fibrous tissue
20	0/6	0	Fibrous tissue only	Fibrous tissue
30	3/12	0	Cartilage	Bone
40	6/12	0	Cartilage	Bone
50	8/12	0	Cartilage	Bone
50	2/10	1	Cartilage	Bone
50	1/6	2	Cartilage	Bone
50	1/4	3	Cartilage	Bone
50	2/10	4	Cartilage	Bone
50	7/18	6	Cartilage	Bone
50[a]	0/6	0	Fibrous tissue only	Fibrous tissue
60[a]	0/6	0	Fibrous tissue only	Fibrous tissue
90[a]	0/6	0	Fibrous tissue only	Fibrous tissue

[a] BMG prepared from autodigested bone.

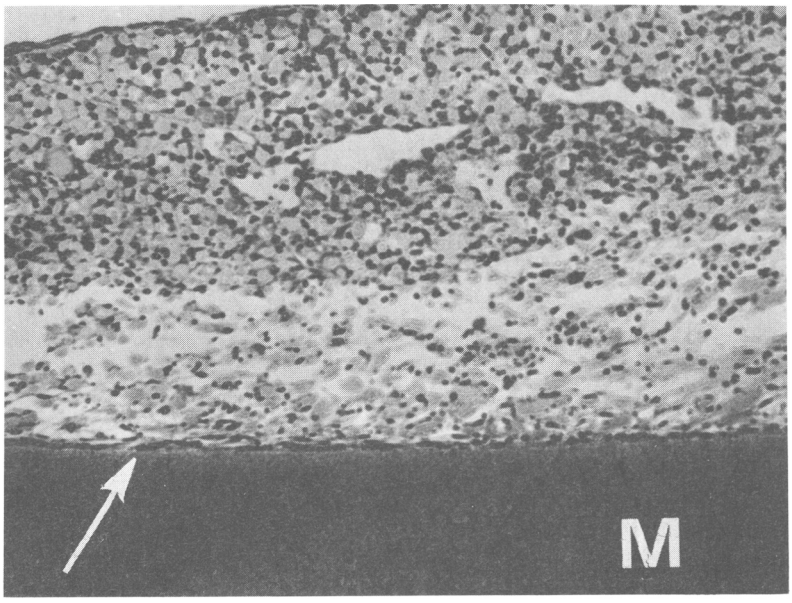

FIG. 2. Photomicrograph of a 7-day-old explant in the tissue culture assembly shown in Fig. 1. The arrow indicates monolayer of mesenchymal cells derived from interfibrous connective tissue and spread out on the surface of the membrane (M).

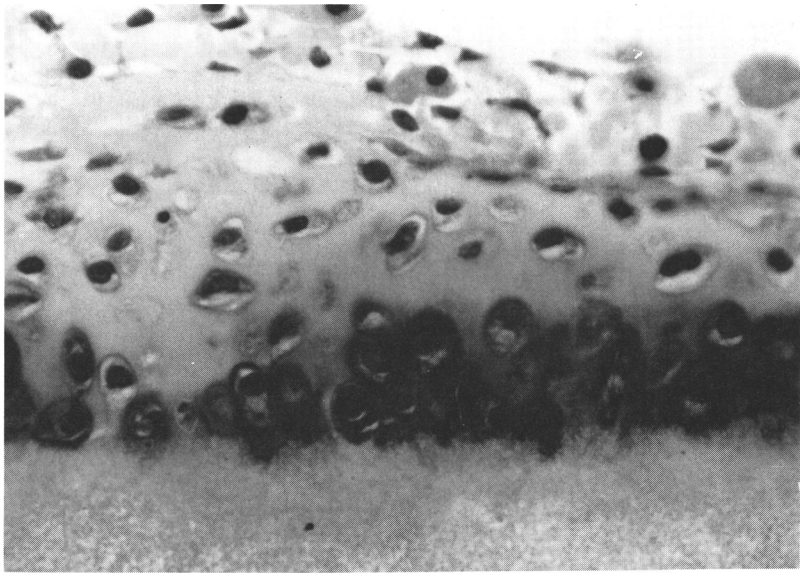

FIG. 3. Photomicrograph of a 21-day-old explant with hyaline cartilage from the outgrowth of muscle interfibrillar connective tissue cells shown in Fig. 2.

transfer occurred even when the BMG was in the system only for the first 24 hr. The effect was bone morphogenetic as well as chondrogenic because, when the culture was transplanted into a syngeneic recipient, it differentiated into bone. Control cultures containing autolysed bone matrix or denatured or living fully mineralized calvarial bone produced fibrous tissue only.

While close contact between cells and matrix is characteristic of bone morphogenetic systems under normal conditions (9–10),

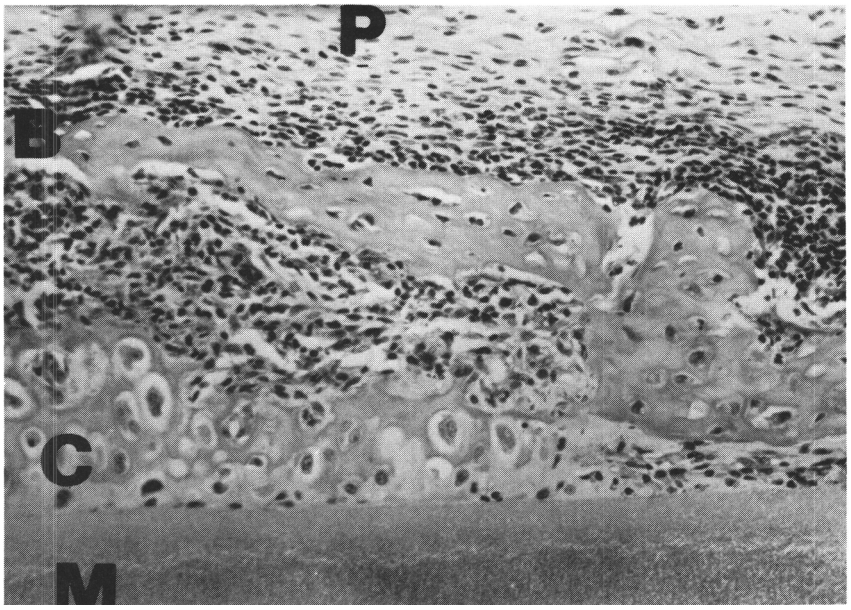

FIG. 4. Photomicrograph of a transplant of 1 week-old-muscle tissue culture, along with its substratum of cellulose acetate, 2 weeks after transplantation into a muscle pouch in an adult syngeneic rat. Recipient muscle pouch (P); cartilage (C); new bone (B); cellulose acetate substratum (M).

under unusual conditions, i.e., in diffusion chamber (11) and in the tissue culture system described above, cartilage differentiation occurs without contact. Cartilage and bone morphogenesis occurs without cell contact with matrix in BMG-filled diffusion chambers having walls 300 μm in thickness and pore sizes of 0.025 to 0.45 μm (11). Even more striking, chondroosteogenesis occurs on the outside of the empty part of duplex chambers consisting of one chamber loaded with the residue of the collagenase digest of the bone matrix gelatin beside another filled with *interstitial fluid* percolating through from the surrounding recipient bed (12). The appearance of cartilage in avascular and bone in vascular environments in response to a rapidly diffusible BMG (2) suggests a complete change in phenotypic expression of the same genome (13).

The evidence that BMP is a protein is that it is trypsin labile at 15°, cathespin resistant, and hydrolyzed in 0.1 *N* NaOH at 2° within 12 hr without release of hydroxyproline (2). Moreover, BMP is collagenase resistant. The specific activity of BMP is high in BMG which is prepared by sequential chemical extraction of proteoglygcans, sialoproteins, and various other EDTA-soluble noncollagenous proteins (2). BMG is insoluble at 2°, but slowly soluble at 37° (2), and incorporates BMP in the so-called collagenase-released fraction of noncollagenous protein. Bone morphogenesis at the openings of infinitely tortuous pores of multiple-walled cellulose acetate membranes, which contain no EM-demonstrable fibrils or parts of fibrils (11), excludes the idea that BMP is derived from collagen. Whether BMP is a bone collagen extension peptide or a scission product of the bone procollagen molecule is presently under investigation.

Kosher *et al.* (14) observed the effects of proteoglycans and Solursh *et al.* (15) observed the effects of a culture medium conditioned with a trypsin-labile, heat-inactivated, mercaptoethanol-sensitive molecule, 30 to 150,000 daltons in weight. In both instances expression of cartilage differentiation was enhanced either in somite or limb-bud axial mesenchyme. BMP resembles this molecule as it is diffusible in a culture medium and operated by a positive feedback mechanism, but differs insofar as its target tissue is not embryonic organic anlagen but postfetal migratory mesenchymal cells.

When penetration of mammalian cells by diffusible molecules can be measured and their functions explored (16), knowledge of the composition and mode of action of the BMP is bound to emerge.

Summary. Under the influence of a diffusible molecule in bone matrix gelatin (BMG), connective tissue-cell outgrowths of neonatal rat muscle in tissue culture differentiate into cartilage. The muscle explant was suspended on a cellulose acetate membrane and separated from the BMG by a wire-grid platform and 2 ml of culture medium. Cartilage differentiated even when the BMG was removed from the system, within the first 24 hr of culture. Cultures transplanted on the seventh day into a muscle pouch in syngeneic recipients for an additional 14 days produced deposits of cartilage and woven bone.

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