

A Morphogenetic Matrix for Differentiation of Cartilage in Tissue Culture¹ (34829)

HIROSHI NOGAMI AND MARSHALL R. URIST
(With the technical assistance of Sandra Harris)

*Bone Research Laboratory, U.C.L.A. School of Medicine, Department of Surgery,
Division of Orthopaedics, Los Angeles, California 90024*

Up to the present time, skeletal tissues have been cultured *in vitro* as embryonic organ anlage which are predifferentiated or predestined to develop into a cartilage model, or as cartilage models with specialized surviving cells. Biggers (1) summarized the literature with a statement that the fate of a tissue in culture is generally determined at the stage of development which precedes explanation. For example, muscle mesenchyma is predetermined to differentiate *in vitro* into fibrous tissue. Changes in composition of the nutrient media (2, 3), or concentrations of oxygen and carbon dioxide (4, 5), influence metabolic processes but do not evoke differentiation of postnatal mesenchymal cells into chondrocytes. The experiments described in this communication demonstrate *in vitro* differentiation of postnatal muscle mesenchymal cells into cartilage with a calcifiable intercellular matrix. The culture system provides a morphogenetic substratum prepared from decalcified bone, and exposes cells to a series of chemically defined culture media designed to supply the essential minimum nutrient substances for cell proliferation of the initial phase and the differentiation, hypertrophy, and calcification of cartilage of the final stage of development.

Experimental Methods. More than 100 samples of minced muscle, approximately 2–3 mm³ in volume were excised from the thigh

muscles of newborn Sprague-Dawley rats, and washed in medium 199 (GIBCO) containing 15% fetal calf serum (GIBCO) to remove old blood. Special precautions were taken to exclude fibers of muscle origins and insertions. Each sample of washed muscle was placed in the medulla of a split segment of demineralized allogeneic diaphyseal cortical bone matrix, measuring 0.2 cm in length. The matrix had been demineralized (by microradiographic criteria) in 0.6 N HCl at 2° with a magnetic stirrer for 4 days, and lyophilized to preserve morphogenetic properties described in detail in previous publications (6, 7). The segment was split to provide maximum exposure to the culture medium. For control experiments, the bone matrix was prepared by demineralization in 0.6 N HCl in 70% alcohol at 2° for 4 days, a procedure known to inactivate the morphogenetic properties of the structure (7–9).

The above-described segments of bone matrix, loaded with fragments of muscle, were placed on wire grid platforms in organ culture dishes (Falcon) containing 2.0 ml of each of the following culture media and intervals of time: medium 199 (GIBCO) containing 15% fetal calf serum (GIBCO) for the first 5 days; the Fitton-Jackson modification of the BGJ (1) medium (GIBCO) containing 15% fetal calf serum between Day 6 and Day 10; and the modified BGJ medium alone during Days 11–40. The BGJ medium consists of an aqueous solution of approximately 50 ingredients including inorganic salts, glucose, amino acids, vitamins, and antibiotics. Impressions gained from exploratory experiments suggested that medium 199 alone produced rapid proliferation of fibroblastic cells but no further cell differentia-

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TABLE I. Products of Culture of Postnatal Muscle Mesenchymal Cells in Denatured-Decalcified and Decalcified Bone Matrix.

Group no.	I	II	III	IV
Substratum	Acid-alcohol-denatured bone matrix	0.6 N HCl-decalcified bone matrix		
Medium	199 + 15% FCS, then BGJ, + 15% FCS, then BGJ only		199 + FCS only	BGJ only
No. of explants	21	43	25	14
No. producing cartilage	0	23	4	3
No. producing calcified cartilage	—	2	0	0

tion. The BGJ alone produced relatively little cell proliferation but constitutes a more favorable medium for production of extracellular substances. When the muscle mesenchyma was exposed first to No. 199 and later to the BGJ, cell proliferation was followed by differentiation of cartilage in a high percentage of explants. Approximately three-fourths of the medium in use during each interval was changed every 48 hr. The culture dishes were incubated at 37° in a gaseous phase of 5% CO₂ in air. Cultures were examined at 5-

to 40-day intervals and fixed either in Bouin's fluid or 10% neutral formalin for histological sections. The formalin-fixed tissues were embedded in paraffin and sectioned undecalcified for staining by the von Kossa method for apatite mineral with counterstaining in hematoxylin and eosin. The Bouin's fluid-fixed tissues were fixed and decalcified simultaneously, similarly embedded, and stained in hematoxylin-eosin and azure II (HEA).

Results. Table I presents results of 103 cultures of muscle mesenchyme divided into

MESENCHYMAL CELL PROLIFERATION
AND DIFFERENTIATION INTO CARTILAGE AND BONE
UPON A SUBSTRATUM OF COMPACT BONE MATRIX
IN RESPONSE TO SEQUENTIAL CHANGES IN CULTURE MEDIA

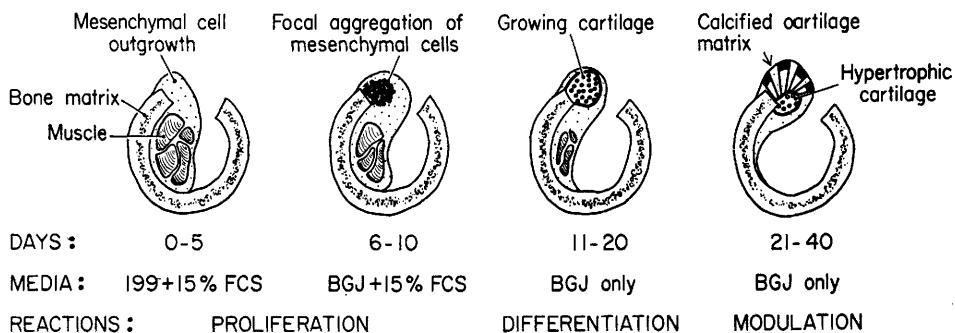


FIG. 1. Diagrammatic illustration of a tissue culture system consisting of a fragment of neonatal rat quadriceps muscle, in the interior of a split segment of 0.6 N HCl-decalcified bone matrix. The bone matrix is presumed to provide surfaces for cell aggregation, intercommunication, and mutual interaction leading to differentiation of mesenchymal cells into cartilage. The time intervals and serial changes in composition of the culture media noted above produced optimum cell proliferation, differentiation, and modulation.



FIG. 2. Photomicrograph of new cartilage developed from explant of the mesenchymal cell outgrowth of neonatal rat muscle tissue on surface of decalcified bone matrix in BGJ media for 20 days. Old decalcified matrix (D) and encapsulated new cartilage cells (arrow).

four groups corresponding to four different media and two different substrata. Group I was grown in a series of media designed to provide optimum conditions for growth and

development of cartilage, but the substratum of the culture consisted of acid-alcohol-inactivated bone matrix, and the results were consistently negative. Cultures in Group II

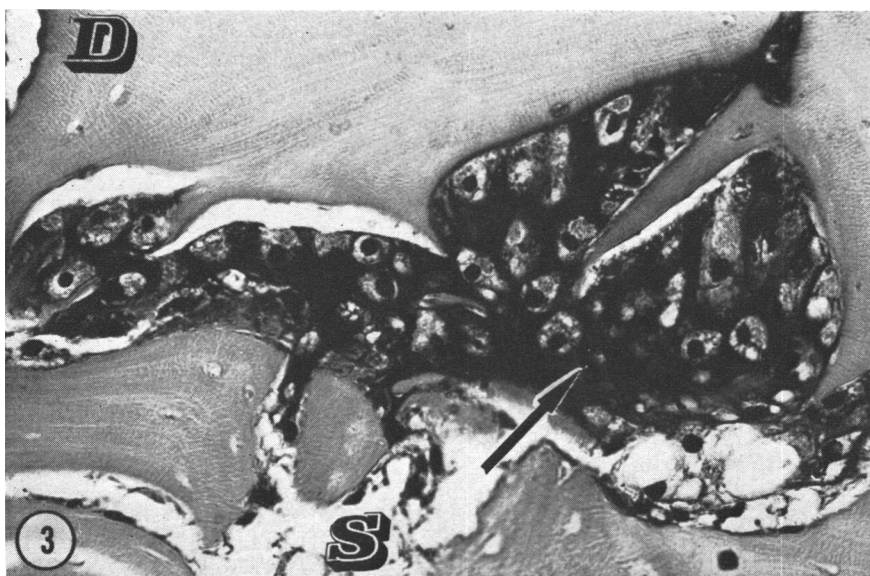


FIG. 3. Photomicrograph of mature cartilage cells with abundant hyaline intercellular matrix, developed from explant of mesenchymal cell outgrowth of neonatal rat muscle onto the surface of decalcified bone matrix in BGJ media for 30 days. Old decalcified matrix (D), mesenchymal cells (S), and cartilage (arrow).

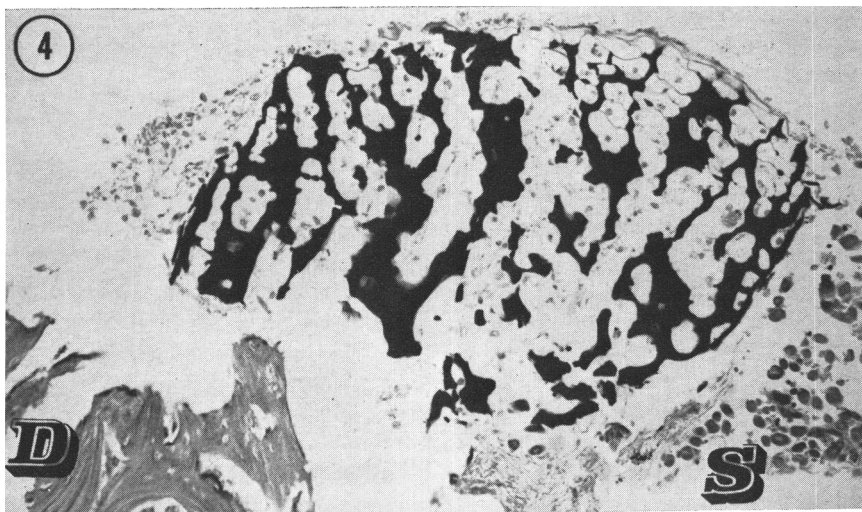


FIG. 4. Photomicrograph of hypertrophic calcified cartilage developed from mesenchymal cell outgrowth of explant of neonatal rat muscle on a surface of decalcified bone matrix. The explant placed in medium No. 199 including 15% fetal calf serum for 5 days; BGJ + No. 199 15% fetal calf serum for 10 days; and BGJ media alone for 20 days—a total of 35 days in tissue culture. Old decalcified matrix (D), old muscle and mesenchymal cells (S).

were grown in the same series of media, but on a substratum of cold 0.6 *N* HCl-decalcified bone matrix, and produced cartilage and chondroosteoid in 23 instances—two of which included hypertrophic cells with calcified intercellular matrix (Figs. 1–3). The sequence of events is illustrated in Fig. 1. Medium 199 alone, Group II, produced a lower percentage of cultures with cartilage presumable because the components were favorable for proliferation but not differentiation. Biggers medium alone, Group III, was equally unsatisfactory possibly because the composition was less than optimum for the initial phase of cell proliferation. When the two media were employed, with a change from 199 to BGJ from Day 6 to Day 10 in culture, chondrocytes differentiated on Day 11 on the surface of the old matrix and in the interior of every available old vascular channel (Figs. 2 and 3). Immediately after exposure to BGJ medium some of the mesenchymal cells fused, and formed giant cells containing five or more nuclei. These cells had some of the morphologic and staining properties of osteoclasts, but did not produce resorption or replacement of the old bone matrix. Between Days 30 and 40, in two cultures, cartilage cell hypertrophy was asso-

ciated with dissolution of some part and calcification of other residual parts of intercellular matrix. The structure of the hypertrophic calcified cartilage resembled that of the zone of preparatory calcification of normal growing epiphyseal plates (Fig. 4). There were also deposits of chondroosteoid matrix in isolated areas between calcified and uncalcified lobules of the cartilage.

Discussion. The foregoing experiments demonstrate the effects of a morphogenetic substratum of demineralized bone matrix upon muscle connective tissue cells in culture media which promote proliferation and differentiation of cartilage, including extracellular calcification. Only fibrous tissue and no cartilage development appears in similarly prepared cultures of muscle if acid-alcohol-denatured decalcified bone is used as a substrate in place of native bone matrix. No growth whatsoever appears from cultures of decalcified lyophilized bone matrix alone in the same media without minced muscle. When muscle mesenchyma proliferates and cartilage does differentiate, the bone matrix promotes cell aggregation and retards growth in a monolayer onto the surface of the plastic culture vessel.

The mode of action of the bone matrix

upon competent mesenchymal cells is not known. Present interpretations, based on recent experiments with lathyritic rat bone matrix, suggests the morphogenetic property emanates from nonsolubilized components (9). For example, implants of lathyritic matrix, which are defective in cross-links in the structure of the bone collagen, produce neither cartilage nor bone. This leads one to speculate on the possibility that the weave pattern of the undenatured bone matrix could be imposed upon the plasma membranes of competent or preprogrammed cell populations, which then synthesize relatively large quantities of alkaline phosphatase, and other enzymes associated with deposition of calcifiable matrix (10).

Chondrogenesis occurs when the proper nutrients and humoral factors are presented in the proper sequence as well as adequate supply. Medium 199 plus 15% fetal calf serum promotes proliferation of mesenchymal cells, cell aggregation, and differentiation of cartilage, but the quantity of intercellular matrix is relatively small (11). The BGJ medium alone produces cartilage but the volume of the new tissue is relatively small. When the culture medium provides nutrients, minerals, and humoral factors in the proper sequence and in optimum quantities, the chondrocytes hypertrophy, calcify, and, in some places, modulate to produce chondroosteoid. BGJ medium, which supports cell differentiation, compared with medium 199, which does not, provides higher concentrations of glucose and proline by a factor of 10, glycine by 14, cysteine by 900, and ascorbic acid by 1000! Quantitative studies should be done to determine the critical concentrations and specific effects of each of these ingredients of these tissue culture media. The calcium, phosphate, and sulfate ion concentrations are not significantly different. Thus the modified BGJ medium is better designed than the 199 to supply building stones for biosynthesis of the large quantity of polysaccharide and collagen in cartilage. The ascorbate requirements are of particular interest; Kivirikko *et al.* (12) reported that embryonic bone anlage fails to incorporate ^{14}C proline into tropo-collagen *in vitro* in the absence of

ascorbate in the culture medium. Medium 199 contains a supplement of 0.10 mg of vitamin A per milliliter, which is sufficient to discharge lysosomal glycolytic enzymes (13–15) and promote calcification of cartilage matrix (16, 17). The BGJ medium does not contain the vitamin A additive, and cultures of cartilage in this medium alone do not calcify.

The influences of certain components of a culture medium on the end products of mesenchymal cell differentiation are clearly critical (18–22). One ingredient, namely, embryo extract, is often added to the culture medium for a growth accelerator, but the question of other more specific effects of this complex mixture on cell differentiation remains unanswered (1, 23, 24). Strudel (25) regards embryo extract as a source of inducers for cartilage morphogenesis by somatic mesenchyme. Chacko *et al.* (26) described as “permissive,” a chemically defined medium which includes embryo extract irrespective to whether it promotes chondrogenesis. Coon (27) designates a chemically defined medium as “permissive” if it supports chondrogenesis but does not contain embryo extract. Because of the chemically undefined character of embryonic tissue homogenates, and the possible inclusion of soluble and particulate derivatives of embryonic cartilage anlage (24), which possess morphogenetic action, our experiments are performed with media which exclude embryonic extracts. Under such conditions, it is reasonable to attribute to the substratum of bone matrix, a morphogenetic role similar to that of other substrata observed in embryonic systems (18, 19, 28, 29).

Summary. Under the influence of a substratum consisting of cold 0.6 *N* HCl-decalcified bone matrix, mesenchymal cells of muscle cultured in a series of chemically defined media aggregate and differentiate into chondrocytes which become enclosed in a calcifiable intercellular matrix. For reasons unknown, in nearly every other culture, mesenchymal cells do not gain contact with the bone substratum but grow radially out onto the surface of the plastic container to form monolayers of fibroblasts.

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