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Bromodeoxyuridine-Inhibition of Substratum-Controlled Chondrogenesis[1,2] (38205)

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In tissue culture, cartilage differentiates from mesenchymal cell outgrowths of muscle as a consequence of interaction with a substratum of bone matrix (1, 2). Mesenchymal cell outgrowths onto plastic, millipore glass and other nonbiologic substrata differentiate only into fibroblasts (2). Differing from embryonic somite or limb bud mesenchymal cells which differentiate into cartilage either spontaneously or in response to enrichment of the media (3) or to covering the culture with paraffin to change the percentages of CO_2 and O_2 in the atmosphere (4), muscle mesenchymal cells require a morphogenetic substratum. The components of the substratum responsible for differentiation of cartilage are eliminated by autolytic digestion by a tissue-specific neutral protease, bone morphogenetic proteinase (BMPase) (5), tryptic digestion of noncollagenous proteins (5), lathyrism and other conditions (6–8). Neutral sulfhydryl group enzyme inhibitors preserve the morphogenetic property (9). Reversible inhibition occurs from interaction with a bone hydrophobic glycopeptide (10). Transformation of bone matrix collagen to insoluble bone gelatin enhances resorption and morphogenetic matrix cell interactions (11).

In the experiments reported in the communication, the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) is introduced into the culture media periodically to determine the time interval of cartilage differentiation. The maximum period of irreversible BrdU inhibition corresponds to the time of mitotic divisions of embryonic precursor cells. Holtzer *et al.* (12) postulate that BrdU inhibits quantal but not proliferative cell cycles. Quantal cell cycles are cycles that yield 1 or 2 daughter cells with competence different from that of the mother cells, while proliferative cell cycles yield cells with the same competence. The literature on BrdU in developing systems has been discussed in detail in recent articles (13–15). Levitt and Dorfman (16) observed that BrdU interferes with synthesis of the core protein of cartilage matrix protein polysaccharides.

Methods. Hemicylinders of insoluble bone matrix gelatin (BMG) were prepared from the femora of adult Sprague-Dawley rats by methods described previously (11). The BMG was the substratum for more than 100 muscle tissue culture assemblies constructed as follows. The BMG was washed for 30 min in the culture medium and coated with chicken plasma (GIBCO) at room temperature. Crevices were cut in hemicylinders transversely halfway through at 1–2 mm intervals with a No. 11 surgical blade to increase the surface areas for cell contact. Muscle tissue was excised from the triceps humerus of 21 day fetal rats, and minced in a drop of culture medium. The medium was CMRL-1066 (GIBCO) supplemented with 15% heat-inactivated newborn calf serum

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(GIBCO), penicillin (100 units/ml) and streptomycin (100 μg/ml). Muscle fragments, approximately 0.5 mm^3 in size, were placed on BMG and suspended on grids in Falcon organ culture dishes. The cultures were exposed to an atmosphere of 5% CO_2 in the air. Under these conditions, cartilage developed almost invariably on the seventh and was mature by the 14th day.

Various concentrations of BrdU (Sigma), 2 m*M*, 200 μM, 20 μM, and 2 μM were added to the culture medium for periods limited to 48 hr beginning at 0, 1, 2, 3, 4, 5, 6, and 7 days of culture. The BrdU was removed from the media after 48 hr by replacement 2 times with fresh media not containing BrdU. Except for the stage of BrdU exposure, approximately ¾ of the medium without BrdU was changed every 48 hr. All cultures were maintained for 14 days, with the exception of 6 control cultures examined at intervals of 2–6 days. On the 14th day, the cultures were fixed with 10% formalin. Paraffin-embedded sections were cut and stained with haematoxylin-eosin and azure II for histological examination.

Results. By the second day of culture, amoeboid mesenchymal cells migrated and proliferated radially from the muscle explant. Few if any cells were in close contact with the substratum. On the second day, crevices in the substratum were filled with an amorphous coagulum. Between the third and fourth days, the coagulum was replaced by spindle-shaped connective tissue cells. Between the fifth and sixth days, the spindle-shaped cells were enveloped in metachromatic-staining extracellular substance. The metachromatic substance increased in quantity in the interval between seventh and fourteenth days and many cells became encapsulated and separated by deeply azurophilic hyaline cartilage matrix.

Table I summarizes observations on cultures with BrdU added to the medium for periods limited to 48 hr. Cultures with high concentrations of BrdU (200 μM and 2 m*M*) added to the media at 5 days and 6 days respectively, differentiated into cartilage but the product was not the same as in control cultures. High concentrations of BrdU produced bizarre-shaped chondrocytes with disorganized irregular staining matrix. Cultures exposed to the minimum concentration of BrdU (2 μM) from the first day of culture produced normal hyaline cartilage, the same as controls; in fact, the total cell population was even greater than in control cultures. Reversible effects were noted in yields of about half the normal volume of cartilage on the fourteenth day from cultures exposed to 20 μM of BrdU between or after the third to the fifth day (Fig. 1). Exposed to the same concentration before 3 days, cultures produced only fibrous tissue and no cartilage whatsoever. The 200 μM concentrations had inhibitory effects which were similarly reversible but only after the fifth to seventh days of culture. Thus, the BrdU suppression of chondrogenesis was reversible after, and never before the period of 72 hr in which migra-

TABLE I. The Effect of BrdU on Mesenchymal Cell Differentiation in Response to a Substratum of Insoluble Bone Gelatin.

Interval of exposure to BrdU in culture, days	No. of cultures developing cartilage/total no. of cultures			
	2 m*M*	200 μM	20 μM	2 μM
0–2	0/4	0/4	0/4	4/4
1–3	0/4	0/4	0/4	4/4
2–4	0/4	0/4	0/4	4/4
3–5	0/4	0/4	3/4	4/4
4–6	0/4	0/4	3/4	4/4
5–7	0/4	3/4	4/4	4/4
6–8	2/4	—	—	—
7–9	1/4	—	—	—

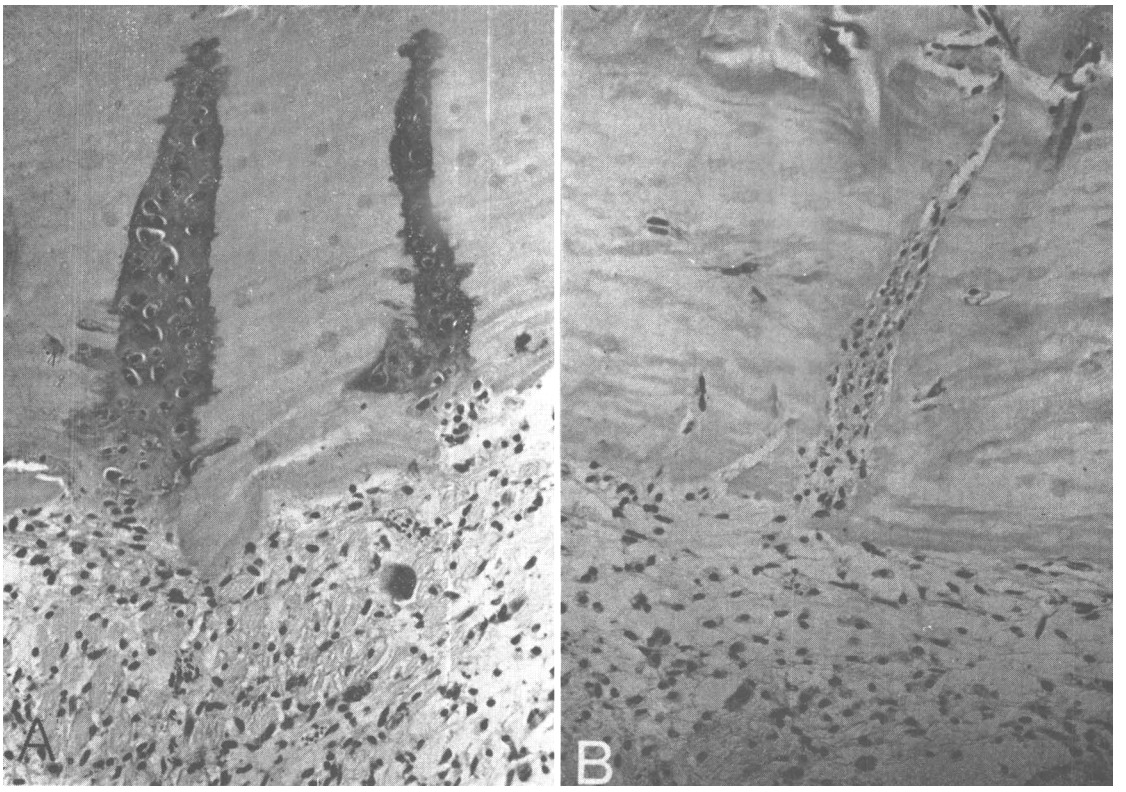

FIG. 1A. In a culture exposed to same concentration to BrdU as in Fig. 1B except that the period of exposure was from 3 to 5 days. Note cartilage derived from muscle mesenchymal cells in crevices cut in the surface bone morphogenetic gelatin after 14 days of culture. Hematoxylin-eosin and azure stain. ×64.

FIG. 1B. Photomicrograph of a culture exposed to 20 μg BrdU from 2 days to 4 days. Note spindle-shaped fibroblasts but no chondrogenetic differentiation at 14 days of culture.

tion and mitosis occur, and contact-interaction between the mesenchymal cells and the substratum ensues.

Discussion. Rat muscle mesenchymal cell 48 hr outgrowths are like embryonic somite and limb bud cartilage precursor cells (13–16) in that they are irreversibly inhibited from differentiation by BrdU. This observation suggests that the influence of the substratum on differentiation of mesenchymal cells may occur some time between the second and third day of culture. Migration–contact-migration (17) upon the substratum appears to be the same for BrdU-inhibited cells as for normal cells. But biosynthesis of core proteins of such chondroitin sulfate complexes as are characteristic of cartilage matrix does not follow if BrdU replaces thymidine in 10% of the DNA in the predifferentiated genome. Once differential gene activation for synthesis of cartilage matrix is established, the inhibitory effects of differentiation of cartilage cells are reversible simply by washing BrdU out of the culture medium. In this respect, the effects of BrdU on 3 day and older cultures are the same as on stabilized or differentiated cell populations.

Previously published quantitative determinations of hyaluronate accumulation *in vivo* in undenatured compared with denatured implants of bone matrix in muscle (18), support the assumption that first 48–72 hr is the critical period of development of a mesenchymal cell population and switching the cell biosynthetic machinery

from a fibrous to a cartilage tissue pathway of development. The reaction of mesenchymal cells to the substratum is not elucidated by BrdU inhibition but it conclusively demonstrates that the maximum time for cell differentiation is 72 hr. Since the matrix crevices are cell free on the second day and occupied only between the second and third days, 24 hr or less may be sufficient for transfer of the signal from matrix to plasma membrane located possibly in mesenchymal cell filopods (8). Isolation of the components of BMG, receptor sites in mesenchymal cell membranes and enzymic regulatory factors, including tissue-specific inhibitors are required before it is possible to define initiative agent or agents of differentiation. The hypothesis under investigation by our research group is that an insoluble noncollagenous bone morphogenetic protein (BMP) is transferred from bone matrix to mesenchymal cell membranes by means of an enzyme BMPase (8, 9).

Summary. Upon a substratum of insoluble bone matrix gelatin (BMG) in tissue culture, mesenchymal cells migrate out of muscle, proliferate, reaggregate on the second day and develop into cartilage on the eighth day. Mesenchymal cells proliferating in culture media containing BrdU during the first 2–3 days are irreversibly inhibited from differentiation and development of cartilage. In media containing BrdU during any interval between 3 and 8 days of culture, inhibition is reversible and cartilage develops almost invariably. Hence acquisition and stabilization of the differentiated state occurs in less than 3 days and within the 24 hr interval between 2 and 3 days coinciding with the time of interaction of mesenchymal cell filopods with surface substances of BMG.

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