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Hyaluronate in Morphogenesis: Inhibition of Chondrogenesis *In Vitro*

(chick somites/chick limb buds/cartilage nodule formation)

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ABSTRACT **Purified hyaluronate, at a concentration as low as 1 ng/ml, blocks the formation of colonies and cartilage nodules in stationary cultures of cells, isolated by treatment with trypsin, from embryonic chick somites and limb buds. This phenomenon *in vitro* is correlated with sequences of hyaluronate production and hyaluronidase activity during chondrogenesis in embryonic and regenerating tissues *in vivo*. An hypothesis is proposed in which hyaluronate acts as a regulator or inhibitor of mesenchymal cell aggregation in embryogenesis, its synthesis and removal being part of the mechanism of timing of migration, aggregation, and subsequent differentiation.**

Chondrogenesis in the regeneration-blastema of the newt limb and in embryo limb buds and the axial skeleton of the chick is preceded by accumulation of mesenchymal or blastemal cells and synthesis of hyaluronate (see ref. 1). Removal of hyaluronate by endogenous hyaluronidase accompanies differentiation of the accumulated cells and the formation of cartilage (1). An analogous series of events accompanies the migration of mesenchymal cells into the early embryonic cornea, their differentiation to corneal fibroblasts, and the development of transparency (2). The close correlation of initiation of hyaluronidase activity with the first appearance of overt differentiation led us to investigate the possibility that hyaluronate inhibits chondrogenesis and that its enzymatic removal permits the process to proceed.

Embryonic chick cartilage cells will form aggregates or nodules with the morphologic and chemical characteristics of cartilage if cultured under suitable conditions (3–7). This paper describes the inhibition of such cartilage nodule formation by the addition to the culture medium of very small amounts of hyaluronate.

MATERIALS AND METHODS

Ham's F 12 nutrient medium containing 10% fetal calf serum, 1% bovine serum albumin, 100 units of penicillin/ml, and 100 μg of streptomycin/ml was used in the cultures. Highly-purified hyaluronate preparations from human umbilical cord and rooster comb were obtained from Biotrics Inc. (Arlington, Mass.) (Lots nos. 1106 and 1112) through the courtesy of Dr. Endre Balazs. Umbilical cord hyaluronate was also purchased from Sigma Chemical Co. (Type III-S or III-P). The Biotrics preparations were used in most experiments and were similar in properties and composition to those described by Darzynkiewicz and Balazs (8). Crude trypsin was obtained from Sigma Chemical Co. and [^{3}H]thymidine (0.36 Ci/mmol), from Schwarz BioResearch Inc.

Cells were freshly liberated from somites of chick embryos, stages 17 and 26, or from limb buds of stage 26 by incubation in 0.1% trypsin at 37° for 45 min. They were then dropped through a 1:1 mixture of fetal-calf serum and Tyrode's solution, dispersed by vibration in 1 ml of medium, counted, and plated on 60-mm Falcon plastic tissue culture dishes at a density of 2.5 or 5.0 $\times$ 10^6 cells per dish. Incubation was performed in an atmosphere of 5% CO_2 in air for 4–7 days, unless otherwise indicated.

Cultures were stained with 2% toluidine blue at pH 7.0 for 18 hr at room temperature after the medium was decanted and the attached cells were fixed with 2.5% glutaraldehyde in 50% Tyrode's solution.

[^{3}H]Thymidine (25 μCi/dish) was added to each of four control and four hyaluronate-treated dishes in each experiment. The medium was decanted and the cells were harvested after an 18-hr incubation in the presence of the isotope. The cells were dissolved in 0.1 M NaOH at 40° for 15 min, and the DNA that precipitated on standing overnight in 0.5 M perchloric acid at 4° was collected by centrifugation and dissolved again in 1.0 ml of 0.5 M acid at 70°. Duplicate aliquots were used for measurement of radioactivity and for DNA assay. Radioactivity was measured in a liquid scintillation counter with Bray's solution (9), and DNA was measured by the method of Burton (10).

We obtained cell counts for assessment of cell numbers during the course of culture by scraping the cells from the dishes, suspending them by trypsinization for 10 min at 37°, and counting in a hemocytometer. At least four control and four hyaluronate-treated dishes were counted in each experiment.

RESULTS

The cells from the earlier stage-17 embryos did not form cartilage, but gave rise in culture to large nonmetachromatic colonies of fibroblast-like cells, whereas the stage-26 cells organized into numerous metachromatic cartilage-like aggregates or nodules. One comparative experiment was also performed with stage-26 limb bud cells, which formed small numbers of metachromatic cartilage nodules. When the colonies were counted, only those greater than 0.2 mm in size were considered.

Effect of Hyaluronate on Cell Aggregation. The formation of colonies in culture by chick embryo cells from the three different sources—stage-17 somites, stage-26 somites, and stage-26 limb buds—was inhibited by addition of hyaluronate to the medium at incubation time zero (Table 1). Human umbilical cord hyaluronate from Biotrics or Sigma and rooster comb hyaluronate from Biotrics were active. Concentrations

TABLE 1. *Hyaluronate inhibition of aggregation of embryonic cells from three different sources*

Cells	No. of dishes	No. of exps.	Colonies per dish: Controls Mean	Controls Range	+ Hyaluronate* Mean	+ Hyaluronate* Range
Stage-17 somites	60	6	13	8–24	1.6	0–3
Stage-17 somites†	30	3	25	20–30	2.8	1–4
Stage-26 somites	80	10	24	12–37	3.3	0–10
Stage-26 limb buds	6	1	6	4–12	0	0

* Concentrations of hyaluronate used in these experiments ranged from 100–500 μg/ml.

† This set of experiments was performed with Sigma hyaluronate; all other series were with hyaluronate from Biotrics.

of hyaluronate in the range 1 ng–500 μg/ml were equally effective in blocking aggregation of stage-26 somite cells (Table 2). Concentrations between 1 and 100 pg/ml were partially inhibitory, with the number of colonies usually increasing with decreasing hyaluronate concentration. An experiment with stage-17 somites that used concentrations of hyaluronate from 10 pg–500 μg/ml gave similar results. Enrichment of the medium with 50 μg/ml of sodium ascorbate greatly increases the number of cartilage nodules, as was also observed by others (11), and does not influence the inhibitory effect of hyaluronate (Table 3).

Effect of Hyaluronate on Cell Proliferation. In order to assess the possibility that a simple diminution in cell number was responsible for failure of colony formation, either by increased cell death or decreased multiplication, we measured cell number, DNA content, and incorporation of [^{3}H]thymidine into DNA. Cultures of stage-26 somite cells in the presence and absence of a range of concentrations of hyaluronate yielded similar results by all three measurements (Table 4). No significant differences were obtained at 1, 3, or 5 days of incubation. For stage-17 somite cells, a 25–30% decrease in cell number resulted from the addition of 250 μg/ml hyaluronate (Table 4). Reduction by initial seeding of 50% of the number of cells used for control cultures had little effect

TABLE 2. *Effect of various concentrations of hyaluronate on aggregation of stage-26 somite cells*

Concentration of hyaluronate* (μg/ml)	No. of dishes	No. of exps.	Colonies per dish: Mean	Range
100–500	80	10	3.3	0–10
1–50	80	6	4.6	0–10
10^{-3}–0.5	65	6	3.5	0–9
10^{-4}	18	3	9.1	5–15
10^{-5}	12	2	12.0	6–21
10^{-6}	12	2	15.5	10–30
0	80	10	23.5	12–37

* Biotrics hyaluronate.

TABLE 3. *Effect of ascorbate on hyaluronate inhibition**

Additions to medium	No. of dishes	Colonies per Dish: Mean	Range
None	10	23	10–35
Ascorbate (50 μg/ml)	10	74	39–140
Ascorbate (50 μg/ml) + Hyaluronate (0.1 μg/ml)	10	1.8	0–10

* Stage-26 somite cells and Biotrics hyaluronate were used.

on the number of colonies formed from somite cells, stage 17 or 26.

DISCUSSION

The synthesis of hyaluronate followed by its enzymatic removal in developmental sequences *in vivo* has been correlated with the accumulation and overt differentiation, respectively, of mesenchymal cells (1, 2). These observations, coupled with the effect of hyaluronate on chondrogenesis *in vitro*, described herein, lead us to postulate the following functions for this glycosaminoglycan in morphogenesis: (*a*) hyaluronate prevents aggregation of mesenchymal cells and consequently facilitates their migration, but interferes with expression of their differentiated state; (*b*) removal of hyaluronate by the appropriately timed and localized production, or activation, of hyaluronidase then permits aggregation and subsequent differentiation to proceed in the proper sequence for tissue organization; (*c*) this effect of hyaluronate on cell aggregation may lead to alteration in the rates of synthesis of specific differentiated cell products, e.g., chondroitin sulfate/keratan sulfate–protein (12) and cartilage collagen, [α1(II)]$_3$ (13). Studies are in progress to determine whether this last prediction is the case.

Several conditions have been described that interfere with chondrogenesis in chondrocyte cultures, e.g., heterotypic cell mixtures (3, 6, 14), addition of a high molecular weight fraction of chick embryo extract (4, 15), and use of conditioned medium (7). All these treatments could result in addition

TABLE 4. *Cell numbers and proliferation in hyaluronate*

Concentration of hyaluronate (μg/ml)	No. of exps.	Percentage (+HA/−HA)*: Cell counts	DNA	[^{3}H]Thymidine incorporation
Stage-26 somites:				
0.001	1	80	88	115
0.1	1	—	117	—
25	2	102	83	75
250–500†	4	80	94	83
Stage-17 somites:				
250	1	69	75	72

* Abbreviation: HA, hyaluronate (Biotrics). Each experiment used at least four control and four treated dishes. Mean results from treated dishes are expressed as percentages of the means from control dishes for all three measurements.

† At this concentration, experiments were performed after 1, 3, and 5 days in culture, and no significant differences were obtained. All other experiments were performed after 3 days.

of small amounts of hyaluronate, which would prevent cartilage nodule formation.

Since hyaluronate will prevent mesenchymal cell aggregation and chondrogenesis *in vitro* at a concentration as low as one nanogram per ml (about 500 molecules per cell), its mode of action is not likely to be based on steric hindrance to cell interactions due to molecular entanglement, as proposed for the inhibition of mitogen-induced stimulation of lymphocytes by hyaluronate (8). Past studies have indicated the possibility that hyaluronate may reduce rates of cell division (16, 17), but cell proliferation in the system described here was not significantly affected by hyaluronate (Table 4). Evidence has been obtained for the association of hyaluronate and other glycosaminoglycans with the surface of various cell-types in culture (16, 18), in embryonic tissues during morphogenesis (19, 20), and in neural tissues (19, 21).

Most current efforts to understand the biochemical mechanisms of cell interactions and migration in morphogenesis and differentiation are focused on identification of specific, interactive cell-surface substances (22, 23). However, we view the association of hyaluronate with the cell surface as a regulator or inhibitor, involved in control of timing of morphogenetic events requiring cell interactions. Perhaps its repetitive negative charge distribution, or its ability to extend its molecular "domain" through an enormous volume in solution (24), or its cation-binding capacity, especially with respect to calcium ion (25), causes hyaluronate to modify or inhibit a cell-surface activity, e.g., glycosyltransferase action (23) or cyclic AMP metabolism (26). The strategically timed removal of hyaluronate by hyaluronidase would result in cell interactions leading to communication, differentiation, and immobilization in the correct place and sequence. This permissive, rather than instructive, event might then be one of the control mechanisms involved in embryonic cartilage induction, and perhaps in other types of tissue organization.

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