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The Cell Substratum Modulates Skeletal Muscle Differentiation

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During chick embryogenesis, massive alterations occur in the migrating cell's substratum, or extracellular matrix. The possibility that some of the components of this milieu play a regulatory role in cell differentiation was explored in a cell-culture system derived from embryonic chick skeletal muscle tissue. In particular, the effects of collagen and the glycosaminoglycans were studied. Collagen is required for muscle cell attachment and spreading onto plastic and glass tissue-culture dishes. A major constituent of the early embryonic extracellular space, hyaluronate (HA), while having no significant effect on collagen-stimulated cell attachment and spreading, was found to inhibit myogenesis. The muscle-specific M subunit of creatine kinase was preferentially inhibited. Control experiments indicated that the inhibition was specifically caused by HA and not by other glycosaminoglycans. A general metabolic inhibition of the cultures was not observed. Muscle cells could bind to HA-coated beads at all stages of differentiation but were inhibited only when HA was added within the first 24 h of culture. Endogenous GAG in the culture is normally degraded during the first 24 h after plating as well; this may parallel the massive degradation of HA that occurs in the early embryo in vivo. These findings suggest a regulatory role for HA in modulating skeletal muscle differentiation, with degradation of an inhibitory component of the cell substratum a requirement for myogenesis.

Key words: skeletal muscle, myogenesis, chick embryo, hyaluronic acid, glycosaminoglycan, extracellular matrix

During early embryogenesis, cells of predetermined tissue types often undergo extensive migrations before they express their full developmental potential. Often, it is not until they arrive at their final destination that they turn on the synthesis of tissue-specific

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enzymes and other macromolecules. Since cells migrate through extracellular matrix, it seemed possible that some of the components of the matrix could play a role in modulating the developmental programs [1].

Of particular interest is hyaluronate, a glycosaminoglycan that is a major component in the large interstitial spaces of the early embryo [2–4]. As differentiation proceeds, the hyaluronate is degraded [5–8]. We explore here the possibility that when cells are migrating through hyaluronate, their tissue-specific differentiation is repressed, and when the hyaluronate is degraded, differentiation can proceed.

Cartilage tissue and the chondrogenic cell-culture system provided examples of this response when it was shown that cartilage formation by chondrocytes was inhibited by hyaluronate and stimulated by chondroitin sulfates [9–14]. Other developing systems have also been shown to be responsive to glycosaminoglycans. Work by Lash and his coworkers [15–18] indicated that glycosaminoglycans promoted chick somite morphogenesis and cell differentiation. Similarly, the development of the chick cornea can be affected by glycosaminoglycans [19] and collagen [20].

During the development of skeletal muscle, mononuclear myogenic precursor cells migrate and form aggregates, which then fuse to form muscle fibers. Many aspects of the differentiation process were clarified in studies in cell culture [21–23]. One of the extracellular matrix materials, collagen, has been shown to be required for myogenesis [24–26]. Nameroff and Holtzer showed that myogenesis was inhibited when muscle cells were plated on killed confluent cultures of chondrocytes and liver cells [27]. They concluded that a nondiffusible polysaccharide was the inhibitor. Postmitotic myoblasts were not inhibited, and the inhibition could be reversed with conditioned medium.

This research has indicated that the extracellular matrix may regulate processes in differentiating cells. There is still a need to collect biochemical data on a specific extracellular component's effect on well-defined differentiating systems. This paper describes the effect of one glycosaminoglycan, hyaluronate, on muscle differentiation, using as quantitative indicators of myogenesis, creatine kinase and fusion index.

MATERIALS AND METHODS

Cultures

Primary cultures of trypsin-dissociated skeletal muscle cells were prepared from 10-day chick embryo breast tissue by a modification of the method of Bischoff and Holtzer [28]. Breast muscle was freed of bone, minced, and incubated in 0.25% trypsin in Earle's balanced salt solution, Ca^{++} and Mg^{++}-free, at 37°C for 40 min. The tissue was washed 3 × with growth medium by pelleting, triturated in a Pasteur pipet, and filtered through two pieces of washed lens paper. The cells were plated in 0.5 or 1.0 ml medium 8:1:0.25 at an initial density of 1×10^5 cells on 3 cm diameter Petri dishes (Nunclon) coated with calf skin collagen (Sigma). The growth medium contained 84% modified Eagle's medium (MEM) with Earle's salts, 10% horse serum, 2.5% embryo extract, 1 mM glutamine, and penicillin/streptomycin (125 units/ml each). The medium was changed daily. All medium components were purchased from Gibco. Embryo extract was prepared by extruding whole 11- or 12-day chick embryos through a 50 cc syringe, diluting with an equal volume of balanced salt solution (BSS), and freezing. Upon thawing, the slurry was spun 40 min

at 1,500g and the supernatant aliquoted and frozen. Each aliquot was respun upon thawing before use. Under the conditions employed here, mononuclear myoblasts underwent one or two rounds of cell division and then fused to form multinucleate myotubes. Fusion began 1 1/2 days after plating and was complete by 2 1/2 days.

The percent fusion is the percent of the total nuclei present in myotubes. Several random fields in a dish were scored. All nuclei in mononuclear cells, including those in fibroblasts, were counted in the unfused population. The total number of nuclei assayed per datum always exceeded 500. The microscope resolution was sufficiently good to distinguish two overlapping myoblasts from a myotube with two nuclei, and "dimers" were consequently included in the estimate of nuclei in myotubes. A slight underestimate of fused cells may have occurred at very early fusion stages [29, 30].

Assays

Hyaluronic acid (HA), sodium salt from hog skin, chondroitin sulfates (ChS; chondroitin-4-sulfate, super special grade, sodium salt from whale cartilage, and chondroitin-6-sulfate, super special grade, sodium salt from shark cartilage) were purchased from Miles Laboratories, Inc. Several preparations of HA and ChS were used during these experiments. The HA ran as a single band on strip paper electrophoresis [31], and this band was completely destroyed by testicular hyaluronidase.

Creatine kinase (CK) (EC 2.7.3.2, ATP: creatine N-phosphotransferase) was measured by the coupled enzyme reaction as described by Rosalki [32]. Reagents were purchased from Calbiochem. To prepare lysates, cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), scraped into 2 ml of 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM 2-mercaptoethanol, and homogenized for 15 strokes in a ground-glass tissue grinder. Data presented are the averages of 4–6 determinations. Experiments were repeated 3 to 5 times. CK activity is expressed as units per milligram total lysate protein.

Control experiments to rule out an inadvertent effect of HA on the CK assay were done. When various concentrations of HA were added directly to aliquots of a cell lysate and the CK activity was then measured, little inhibition was seen. If the final lysate had a concentration as high as 0.2 mg/ml HA, the enzyme was inhibited by only 13% (Table I). It is not possible for the cells to have accumulated more than one-half of this amount, even assuming they could accumulate all of the HA ever provided in the medium: a 3-day culture was routinely given a total of 3 changes of 0.5 ml of medium containing 0.1 mg/ml HA, or 0.2 mg total, which was then diluted to 2 ml of lysate. If the cells had indeed taken up all the externally supplied HA, the lysate would contain 0.1 mg/ml HA, which would inhibit CK activity in the assay by only 10%. It is unlikely that the cells accumulated this amount of HA.

CK isoenzymes were determined by a method adapted from Klein et al [33]. Protein was determined by a modification of the method of Lowry et al [34]. Cells were generally harvested several hours after a feeding to avoid serum-starved stationary cells. Assays on each dish were performed in duplicate.

The amount of glycosaminoglycan (GAG) present in the culture was assayed by a modified procedure of Hamerman et al [35]. Cultures were grown in medium without phenol red, to avoid interference with the assay for hexuronic acid [36]; 2.7×10^6 cells were plated on 9 cm diameter Petri dishes in 5 ml 8:1:0.25. The medium was in contact with the cells for 24 h prior to collection for the assay, unless otherwise indicated, and the cells were given fresh medium daily. Twenty-five milliliters of combined media from 5

TABLE I. Effect of HA on CK Assay*

	HA (mg/ml)	CK (units/mg)	% Activity
Experiment 1	0	4.35	100 ± 5
	0.002	4.46	102 ± 2
	0.02	3.92	90 ± 2
	0.2	3.80	87 ± 0
Experiment 2	0	6.97	100 ± 1
	0.01	6.69	98 ± 3
	0.1	6.26	90 ± 4

*Lysates were prepared from 3-day (Experiment 1) or 6-day (Experiment 2) muscle cultures. HA was added to them at the final concentrations indicated, and CK was assayed.

dishes were dialysed against 3 liters of 5 mM sodium phosphate buffer, pH 7.5, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), at 4°C for 2 or more days in Spectropor 3 tubing. The buffer was changed 6 to 7 times. Samples were lyophilized and redissolved in 1.5 to 3.0 ml 0.1 N sodium chloride and 0.05 M potassium acetate, pH 4.8. Testicular hyaluronidase (Calbiochem, B grade, 702 units/mg) was added to give a final concentration of 250 units/ml, and the solution was incubated for 12 h at 37°C. Trichloroacetic acid, 50%, was added to each sample to give a final concentration of 5%. After incubating in ice for 20 min, the precipitate was spun for 15 min at 1,500g at 4°C, and the supernatant was assayed for uronic acid. Recoveries of HA added to the medium at a final concentration of 25 μg/ml are over 90%. A final concentration of PMSF of 1 mM or less did not interfere with the uronic acid assay. PMSF, at a concentration of 0.01 M, will also not interfere with hyaluronidase activity, as determined by electrophoresis of HA on cellulose acetate strips, nor will it interfere with the strip assay itself. When GAG in the cell layer was determined, the cells were scraped up in a total of 5 ml PBS, dialyzed, and treated in the same manner as the medium above. The basal level of GAG in the growth medium varied from 2.8 to 3.6 μg/ml. The same preparation of medium was used throughout an experiment.

HA was covalently bound to polyacrylamide gel beads by a modification of the methods of Hoare and Koshland [37] and Cuatrecasas [38]. All glassware was siliconized. Eighty-three milligrams of Aminoethyl Bio-Gel P-150 beads (Biorad, 1.2 mEq/g) were hydrated at room temperature for 4 h in 5 ml water with stirring and allowed to settle. The supernatant was decanted, leaving 2 ml of packed beads. Two milliters of HA, containing 50 mg and adjusted to pH 4 with HCl, were added. One milliliter of a solution of 200 mg/ml water-soluble carbodiimide, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDAC, Biorad), adjusted to pH 4.5, was added dropwise with stirring over 5 min. The pH was maintained with HCl at pH 4–5. The solution was stirred for 4 h at room temperature and an additional 14 h at 5°C. Unreacted groups on the beads were blocked by adding 1 mmole acetic acid and another 200 mg EDAC dropwise, incubating for 2 h at room temperature and another 2 h at 5°C. The beads were washed with 0.005 M sodium phosphate buffer, pH 7.5, containing 0.1 mM PMSF and stored at 4°C. Before binding cells, they were washed extensively in the binding medium. Assaying the HA bound to beads gave a yield of 24 μg HA bound per milligram of beads.

TABLE II. Time Course for Accumulation of CK Activity in the Presence and Absence of HA*

Days after plating	Creatine kinase (units/mg)	
	−HA	+HA
1	0.66 ± 0.26	0.49 ± 0.14
2	0.88 ± 0.23	0.76 ± 0.25
3	4.54 ± 0.87	2.27 ± 0.45
4	4.73 ± 0.41	3.93 ± 0.35

*Cells were plated in medium 8:1:0.25 with or without 0.1 mg/ml HA and fed daily. Duplicate cultures were harvested at 24 h intervals by rinsing the dishes in PBS and storing them at −70°C. All the lysates were prepared and assayed simultaneously for CK and protein. Values given are means and one standard deviation.

To prepare untrypsinized cells for binding to beads coated with HA, minced muscle tissue was incubated in collagenase (Sigma, Fraction A, from Clostridium histolyticum, 200–500 units per mg), 0.2 mg/ml for 30 min at 37°C in BSS.

Incorporation of thymidine was assayed with thymidine (methyl-^{3}H-) (New England Nuclear, 6.7 Ci/mmole) at a final concentration of 5 μCi/ml. Included in the incubation was 20 μM unradioactive thymidine. Incorporation of amino acids was measured with a tritiated L-amino acid mixture (New England Nuclear, 1.0 mCi/ml) also at 5 μCi/ml. Cells were maintained in growth medium. Incorporations were assayed by rinsing cells 3 times in cold PBS, scraping them up twice into a total of 1.0 ml 0.01 N NaOH per dish, and incubating at 37°C for 15 min. One milliliter of 10% trichloroacetic acid containing 1 mg/ml bovine serum albumin (BSA) was added, and macromolecules were precipitated after 30 min at 0°C. Precipitates were collected on glass fiber filters (Whatman GF/C), dried and counted in 3 ml of scintillation fluid containing 2 parts toluene, one part Triton X-100, and 5 g 2,5-diphenyloxazole (PPO) per liter. The counting efficiency was 28%.

RESULTS

HA Modulates Differentiation Indicators of Muscle Cell Cultures

Time course of CK activity. One of the enzymes that increases following fusion of myoblasts is creatine kinase (CK) [39, 40]. The increase in culture has been shown to be due primarily to the muscle-specific isoenzyme, the MM form; thus, the increase in total CK is a valid indicator of myogenesis after 2 days in culture [41–44].

A time course of CK activity is shown in Table II. The activity increases 10-fold from day 2 to day 3. The addition of 0.1 mg/ml HA to the cultures diminishes the rate of the increase about twofold (Table II). In the presence of HA, the CK activity on day 3 is reduced to 50% of the control activity. By day 4, however, the activity is reduced by only 22%. Eventually, full activity is reached. It appears that HA delays, but does not permanently repress, the appearance of CK under these culture conditions.

Dose response curve and specificity. When cells were given various concentrations of HA at the beginning of the culture period, there was a substantial depression in the CK specific activity on the third day (72 h) of culture (Fig. 1). At 0.001 mg/ml HA there was

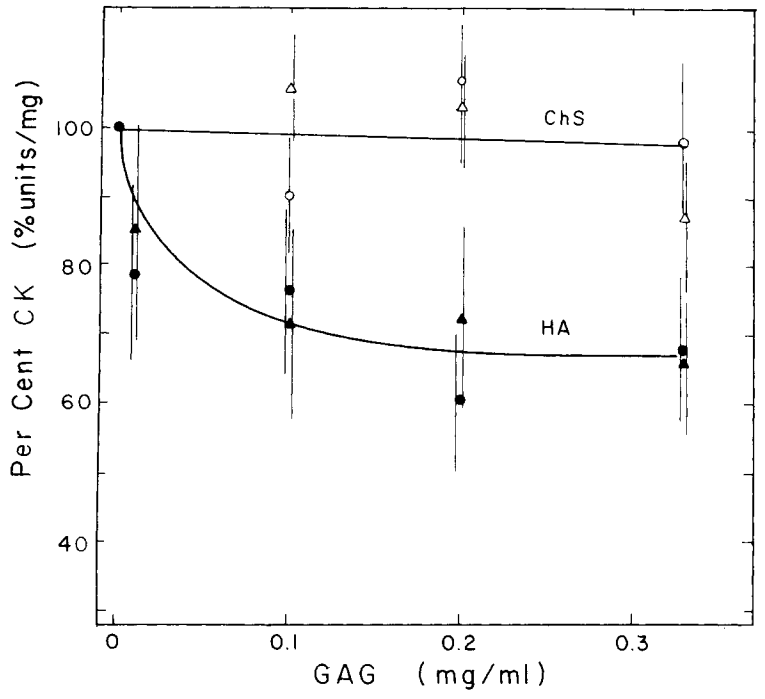

Fig. 1. Percent CK specific activity as a function of HA and ChS concentrations in medium containing 2.5% (8:1:0.25) or 10% (8:1:1) embryo extract. Muscle cells from 10-day-old chick embryos were plated in the indicated medium and incubated for 4 h to allow complete attachment. The medium was replaced with similar medium containing the indicated concentrations of HA or an equimolar mixture of chondroitin-4-sulfate and chondroitin-6-sulfate. The cells were harvested after 3 days and assayed in duplicate for CK and protein. Average results are reported in terms of percent of activity of cells incubated in the absence of glycosaminoglycans. Error bars indicate one standard deviation. (—○—), ChS in 8:1:1; (—△—), ChS in 8:1:0.25; (—●—), HA in 8:1:1; (—▲—, HA in 8:1:0.25.

little loss in CK activity, whereas significant loss occurs at 0.01 mg/ml HA. The maximum inhibition at 72 h varied between 40% and 50% at 0.1 mg/ml HA, with no further inhibition even at 0.33 mg/ml.

HA specificity was tested by assaying CK activity in the presence of other glycosaminoglycans. Since HA is a polyanion, it was possible that its effect was an electrostatic one and could be mimicked by other polyelectrolytes. The chondroitin sulfates are also polyanions found in the extracellular matrix. However, an equimolar mixture of chondroitin-4-sulfate and chondroitin-6-sulfate had no effect on the specific activity of CK after 3 days of culture (Fig. 1). Therefore, the inhibition by HA was not likely to have been an electrostatic effect alone. Nor is HA, as a polyanion, sequestering divalent cations necessary for myogenesis [56, 57]. At 0.1 mg/ml HA, there are approximately 0.2 mEq/liter negative charges. MEM contains 1.8 mM Ca^{++} or 3.6 mEq/liter positive charges. At most, only 5% of the Ca^{++} could be sequestered by the HA.

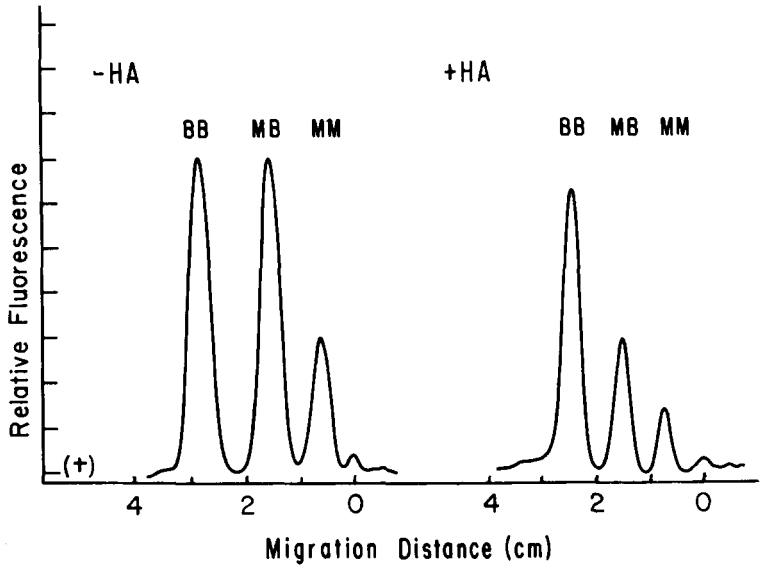

Fig. 2. A comparison of isoenzymes of CK from cultures treated with hyaluronate to those from untreated cultures. Peaks representing the 3 dimeric forms of CK are labeled, from left to right, BB, MB, and MM. The scan on the left shows the distribution in the control culture (–HA), and the scan on the right is the distribution from the treated culture (+HA).

Also indicated in Figure 1 are the dose-response curves for HA and ChS at two different embryo extract concentrations. Medium 8:1:1 contained 10% embryo extract, and medium 8:1:0.25 contained 2.5%. The CK specific activity was about 30% higher in 8:1:1. In both, the same relative dose-response curves for HA-induced inhibition were obtained. In both, ChS gave no inhibition. Thus, it is not likely that the HA effect is mediated or prevented by a component of embryo extract.

CK isoenzymes. Creatine kinase is a dimeric protein containing two subunits, B or M, in 3 isoenzymes, BB, MB, and MM. During early myogenesis, the B-subunit predominates; it is later supplanted by an M-subunit [39–44]. An isoenzyme analysis of CK is shown in Figure 2. In 3-day cultures, the predominant isoenzymes are the BB and MB dimers. HA reduced the MB and MM isoenzymes. In several experiments, the M-subunit accounted for 33–36% of the total control activity. In the presence of hyaluronate, this M-subunit activity decreased to 23–30% of its control level. The muscle-specific subunit thus appears to be more inhibited than total CK activity.

Time course of fusion. The effect of HA on the muscle cells' fusion index was also tested. The rate of fusion of cultures treated with HA parallels that of untreated cultures, although fusion was delayed by 3 h by HA (Fig. 3). A 3 h lag was found each time this experiment was performed. The slope of the fusion curve was 3.02% per hour in the control and 3.21% per hour in the HA-treated culture (as determined by a least-squares analysis).

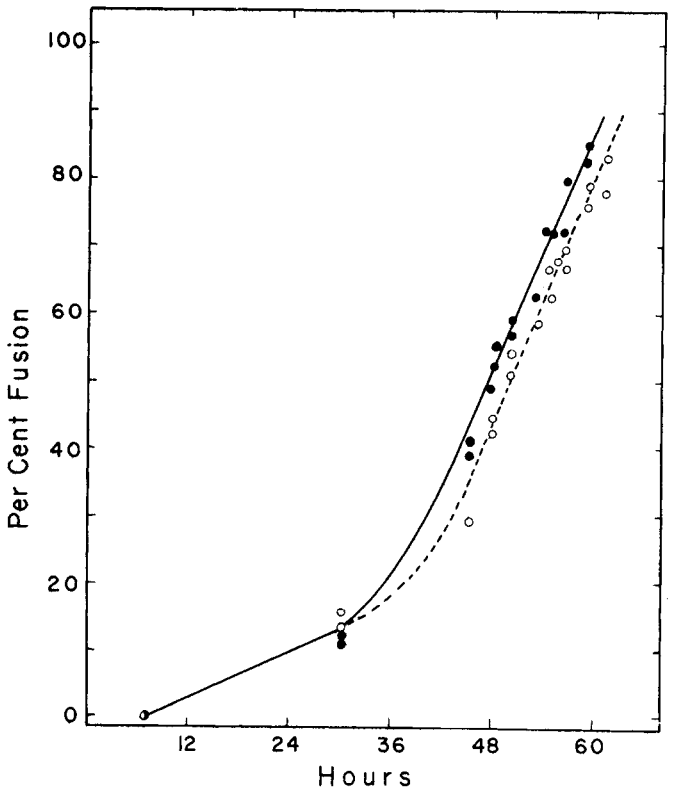

Fig. 3. Percent fusion of muscle cultures with and without HA. The percent of the nuclei in myotubes was determined as described in Methods. Fourteen dishes in all were followed. Two or 3 dishes were averaged per datum. (—•—), control; (---o---), 0.1 mg/ml HA.

Cells could be secreting factors that might be interacting with HA to either produce or diminish the inhibition by HA. Diluting these factors should diminish their effect. An experiment in which the cells' growth medium was changed every 12 h was performed to see what these semi-perfusion conditions would do to the HA effect. Control cells that were fed frequently showed delayed fusion and accumulation of CK activity (not shown), which is consistent with the observations of Konigsberg [45], who grew cells in perfused medium and found that fusion was delayed. HA under these frequent feeding conditions further delayed fusion by about 11 h and similarly delayed the appearance of CK activity (not shown). Changing the growth medium frequently, which would dilute conditioning factors, thus increased the length of the delay in CK activity and fusion caused by HA, suggesting that something in the medium may ordinarily overcome the HA-inhibition.

In these and other experiments, the maximum level of CK specific activity and fusion is eventually obtained in the presence of HA. It is therefore unlikely that HA has an effect on cell viability or on relative populations of myoblasts and fibroblasts. Furthermore, the overall appearance of the cells grown in HA is normal. It would thus appear that HA is affecting just the rate of onset of myogenesis.

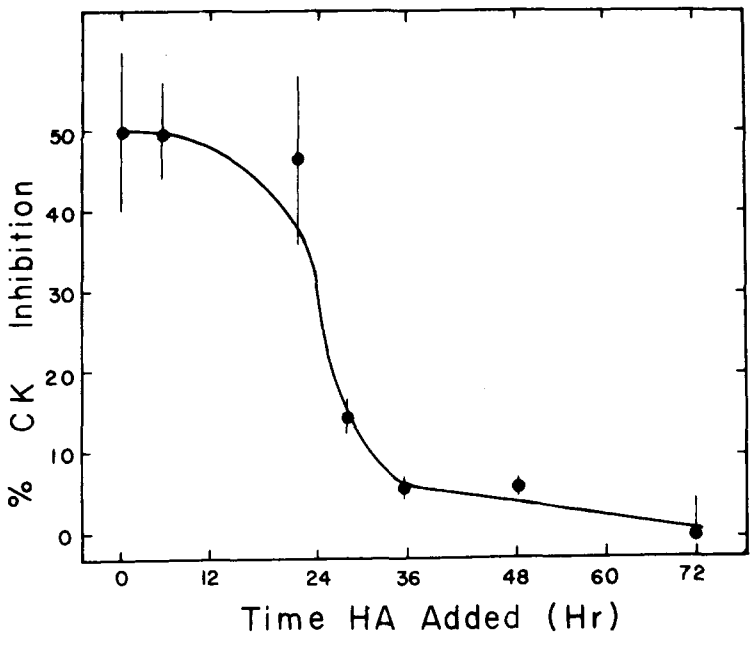

Fig. 4. Effect of time of addition of HA on later CK activity. Cells were plated in 8:1:0.25, and medium was changed daily. HA, 0.1 mg/ml, was added at the various times (hours) indicated on the abscissa. CK specific activity was assayed at 72 h after plating. The data indicate the means and one standard deviation.

Mechanism of the HA Effect

Critical time. The transient nature of the HA effect suggested that something was occurring in the culture to overcome it. This conclusion was supported by the experiment indicated in Figure 4. Exogenous HA had to be added within the first 20 h after plating in order to produce a delay in CK activity. Thereafter, it had no effect.

Effect on attachment. The inhibition of HA on attachment and spreading of cells on their collagen substratum was ruled out in the experiment illustrated in Figure 5. Freshly plated cells on the bottom of a Petri dish were randomly photographed, and the fraction of cells that were non-spherical was counted as spread. While collagen is required for efficient spreading of cells to Petri dishes, HA has no significant effect on collagen-mediated attachment and spreading in full growth medium by 1 h after plating.

Metabolic effects. Since the first day in culture is one during which there is cell growth and mitosis [46], the effect of HA on protein and DNA synthesis was examined. The incorporation of ^{3}H-amino acids or ^{3}H-thymidine was, however, not inhibited by HA (not shown). There is a negligible difference in the rate of uptake of these precursors in 1- or 4-day cultures. The total increase in cell culture protein over a week in culture is identical with and without HA, suggesting that there is no general metabolic inhibition.

GAG is degraded in early myogenic cell cultures. Freshly prepared trypsinized cells were found to contain a small amount of glycosaminoglycan (GAG) bound to them. During cell culture, the amount of hexuronic acid-containing GAG found in the cell layer declined

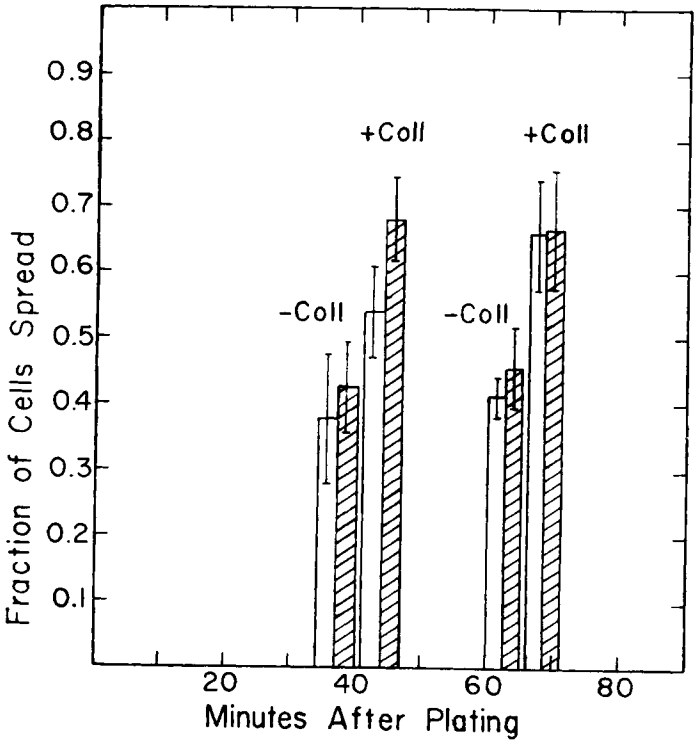

Fig. 5. Effect of HA on attachment and spreading. Trypinized cells were plated at a density of 2.5×10^5 cells per 35 mm dish, which contained 8:1:0.25 prewarmed to 37°C. The dishes were either coated with collagen or not, as indicated. At about one-half hour after plating, 4–5 random fields in 3 dishes were photographed. This was repeated 1 h after plating. The fraction of cells spread is the ratio of spread cells (nonspherical or cells with processes coming out) to the total cells counted on the bottom of the dish in the field. The 4–5 fields were averaged, and one standard deviation is shown. The total number of cells counted per field ranged from 19 to 129 at 1/2 h after plating, with an average of 67, and 45–109, with an average of 73, at 1 h after plating. Open bars, minus HA; hatched bars, plus HA.

for the first 12 h in full growth medium and then slowly increased (Fig. 6a). In particular, the specific activity of the GAG declined dramatically during that time (Fig. 6b). GAG in the medium increased rapidly for the first 4 h, accounting for the loss from cells in the first 4 h, and then also declined during the next 20 h in culture. Subsequently, the amount of GAG found on both the cell layer and in the medium slowly increased. In other experiments, it was found that the specific activity of GAG in the cell layer continued to decline slowly, but the specific activity of the amount found in the medium over cells, produced within 24 h, remained constant for up to 5 days in culture.

It appears from these results that some of the GAG bound in vivo to cells survives trypsinization and is either given up or secreted into the culture medium over the first 4 h. In the first 24 h after plating, however, there is a decline in the total amont of these present. Thereafter, they accumulate steadily.

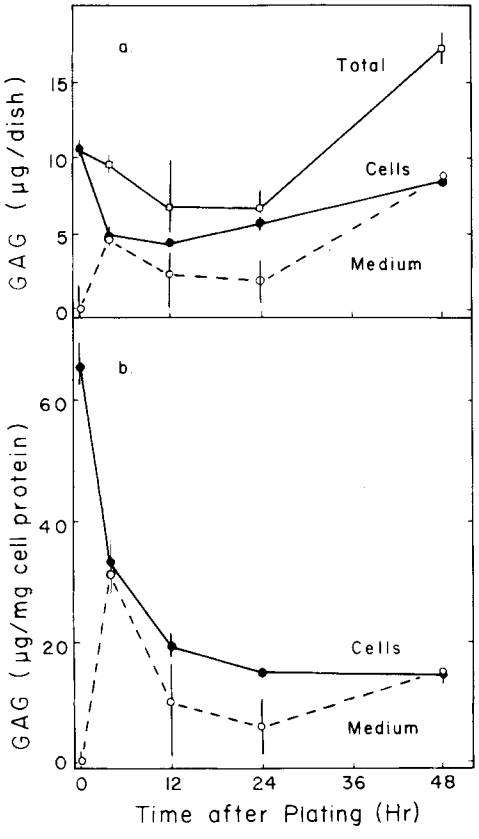

Fig. 6. GAG content of muscle cell cultures. The ordinate represents the uronic acid-containing GAGs and was obtained by multiplying the uronic acid content by 2.5. The abscissa is the hours after plating. (a) GAG associated with the cells and in the medium was assayed as described in Methods. In this experiment, the medium was not changed over the 48 h indicated. The curve of total GAG is the sum of the other two curves. The background in the medium was 2.80 μg/ml and was subtracted from the GAG found in the medium. Error bars are one standard deviation. (b) Specific activity of GAG is μg GAG/dish divided by the amount of cell-layer associated protein per dish. The protein content stayed constant for the first 4 h and then increased linearly for the next 44 h. Error bars indicate one standard deviation.

Cells bind to HA at all stages. Freshly prepared muscle cells, isolated with either trypsin or protease-free collagenase, will bind to beads that contain covalently bound HA (Fig. 7). Binding occurred in PBS, MEM, BSS, or 8:1:0.25, and within 15 min at 22°C or 5 min at 37°C. The binding can be partially competed with free HA (2 mg/ml) or 2–4 mM each of glucuronic acid and N-acetyl-glucosamine, the alternating subunits of HA. Binding is not competed by these concentrations of sialic acid and galacturonic acid. Increasing amounts of EDTA (0.1–4 mM) caused aggregation of collagenized cells and did not inhibit their binding to HA beads.

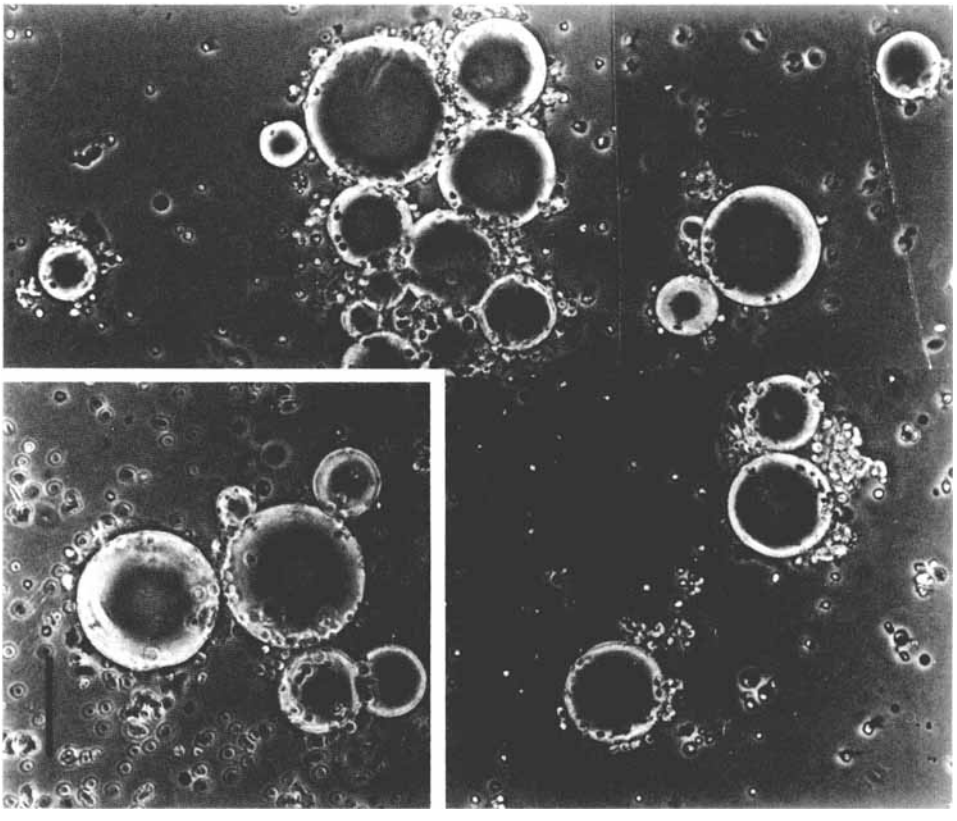

Fig. 7. Binding of fresh skeletal muscle cells to HA beads. Trypsinized cells were incubated with HA beads in 8:1:0.25 or (insert) PBS + 0.2 mg/ml BSA for 10 min at room temperature. Bar = 100 μm.

Chick erythrocytes do not bind to the beads at all. A population of white blood cells did bind, as well as what appeared to be platelets. Colchicine (5 μM) or PMSF (10 μM) do not prevent binding of muscle cells to HA beads.

P-200 beads to which no HA has been attached did not bind cells (Fig. 8). Two days after plating cells with P-200 beads, the beads remained in the medium over the differentiated cells. When HA beads were present, however, the cells attached to the beads and pulled them down within the muscle network, with myotubes extending up over the beads (Fig. 9). There were also cells on the free HA beads. Close inspection of the cells found on the floating beads showed a predominance of myotubes and cells with a myoblast morphology. Only one fibroblast-like cell was observed on the many beads examined. These observations indicate that muscle cells are capable of HA recognition and binding and that the ability to bind to HA is not lost following differentiation to myotubes.

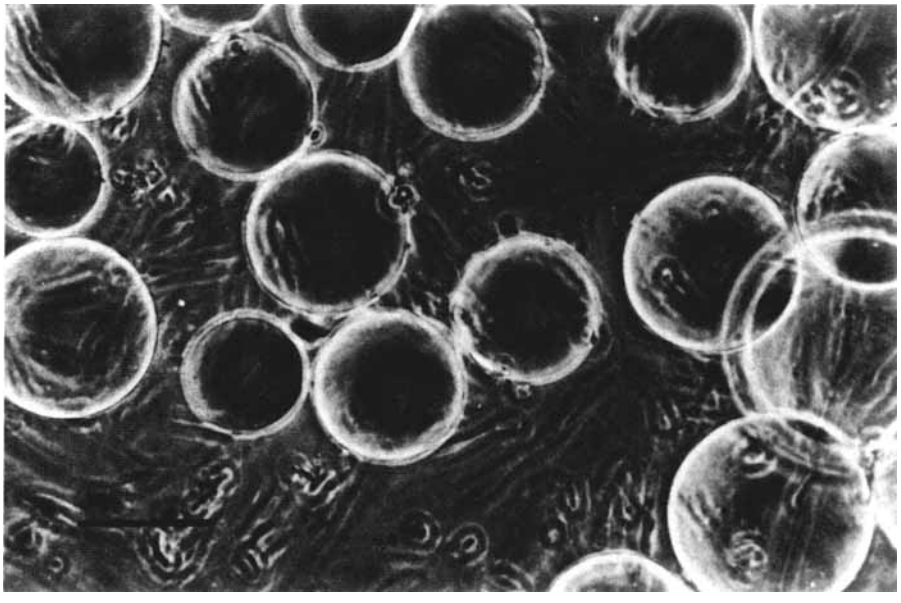

Fig. 8. Cells cultured with control beads. Cells dissociated with collagenase were mixed with P200 polyacrylamide beads (Biorad) that had not been coated with HA. The cells were plated and photographed after 2 days in culture. Bar = 100 μm.

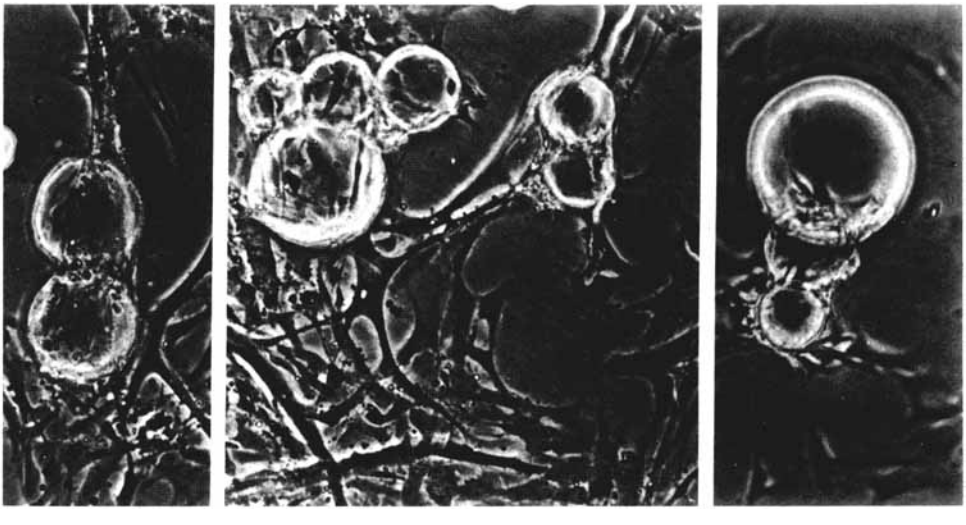

Fig. 9. Cells cultured with HA beads. Cells prepared as in Figure 8 were mixed with HA beads, plated, and photographed after 3 days in culture. Bar = 100 μm.

DISCUSSION

We explore here the possibility that a component of the extracellular matrix can modulate the expression of a differentiating cell. The term "modulation" is used here in the specific sense defined by Weiss [47], in which a cell's environment can produce a reversible response in the cell's behavior or program. In the case presented here, modulation by hyaluronate may serve the purpose of giving cells time to finish morphogenetic movements and other early events before differentiating to terminal forms. Adding HA in large amounts to skeletal muscle cell cultures delays the onset of two myogenic indicators, fusion and accumulation of CK activity [48, 49]. The delay in the appearance of CK activity is striking and represents a good biochemical parameter for further molecular studies into the mechanism of the delay. Other glycosaminoglycans tested, chondroitin sulfates, do not inhibit CK activity, which suggests that the effect is specific for HA and not for just polyanions. The muscle-specific isoenzyme of CK (MM) is preferentially reduced compared to the embryonic CK isoenzyme (BB).

Ahrens et al analyzed the glycosaminoglycans accumulated in primary chick muscle cultures over a 24 h period 72 h after plating [50]. They found that muscle cultures synthesized hyaluronate, chondroitin, chondroitin sulfates, and heparin sulfates. Most of the material was found in the culture medium, and specifically, very little HA remained in the cell layer. Rubin [51] showed that secondary chick fibroblasts secrete HA, and it is possible that the fibroblasts are producing HA in primary skeletal muscle cell cultures as well. Other workers have also noted GAG synthesis by primary muscle cultures [52–54]. Analysis of endogenous hexuronic acid levels in these cultures confirmed that the cultured cells secrete glycosaminoglycans. The very early culture results, however, are quite startling. The basal level of HA and chondroitins in the growth medium, contributed by embryo extract and horse serum, is 2.80 μg/ml. This rapidly rises, but the increase can be accounted for by the loss from the cell layer. Over the first 12 h in culture, however, the total hexuronic acid-containing GAG content of the cell layer, as well as in the medium, declines. Thereafter, it increases concomitantly with cell protein accumulation. The simplest explanation for these observations is that there may be hyaluronidase activity in the culture whose effects are apparent within the first 24 h after plating the cells. The source of the endogenous hyaluronidase activity is unknown, although there is some evidence for hyaluronidase production by cultured cells. Chick fibroblasts obtained from skin have been shown to secrete hyaluronidase in culture [55]. The low pH optimum of this enzyme makes it unlikely that it could function in a standard growth medium unless it does so in association with other factors. It is also possible that GAG in the medium could be taken up by the cells to be degraded within lysosomes. If such hyaluronidase activity as seen in culture is related to the known degradation of HA that occurs in vivo, then these results suggest that part of the early developmental program in these cells may involve a degradation of the extracellular GAG in the matrix around myoblasts.

The fact that high concentrations of exogenous HA can only inhibit when added at early times suggests that HA is removed during an early step in myogenesis and that once muscle cells have gone beyond this step they can not be turned off by HA.

Feeding the cells frequently delayed overall myogenesis, possibly by diluting conditioning factors or inducers of differentiation. HA added in excess under these semi-perfusion conditions was found to exaggerate further the differentiation delay. Konigsberg [45] showed that continuous perfusion of myogenic cultures delayed fusion. He postu-

lated that myogenesis required depletion of medium components or accumulation of cell products in the medium, or a combination of the two. It is possible that the HA/hyaluronidase system postulated here was involved.

How HA, a major component of the early embryo extracellular matrix, can cause a delay in differentiation is unknown. It was shown not to affect significantly attachment and spreading, cell viability, or metabolism. A direct interaction between HA and the cells is indicated by the ability of cells to bind to HA-coated beads in serum-free buffers. The cells may have membrane receptors for HA, or the binding could be mediated by tightly bound trypsin- and collagenase-resistant peripheral molecules. One mechanism that might explain these results is that HA bound to the cell surface alters the cell's microenvironment. Local changes in ion distributions or charged lipid distributions could result in changes in membrane properties. For instance, myoblast membranes undergo large changes in membrane fluidity before they fuse [58–60]; if bound HA interfered with these membrane alterations, it could inhibit differentiation.

The inhibitory effect is not permanent; the cells' differentiation, as assayed by fusion and CK, has been delayed. The fact that the inhibition is reversible is not surprising. In vivo, the HA inhibition would eventually have to be overcome for myogenesis to proceed. The model of Toole [6] depicts HA as an inhibitor of differentiation during the cells' migratory phase, with a reversal of this inhibition when cells reach their final destination. If this model is correct, then it is possible that the cell culture system mimics this in vivo situation.

By extending the cell-culture observations presented here to the in vivo case, one could postulate that differentiation of muscle precursor cells, or myoblasts, is regulated by components of the cells' microenvironment, including the extracellular matrix. Hyaluronate can be added to the growing list of regulating components, which already contains collagen and fibronectin. These may modulate the behavior of cells until they are in their final positions in space and time. The cells' specific gene products can subsequently be generated by the removal of inhibitors or the appearance of inducers, or both, so that development can proceed.

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