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Extracellular Lectin and Its Glycosaminoglycan Inhibitor in Chick Muscle Cultures

Howard Ceri, Paula J. Shadle, David Kobiler, and Samuel H. Barondes

Department of Psychiatry, Unviersity of California, San Diego, La Jolla, California 92093

Embryonic chick muscle contains two developmentally regulated lectins, which may be involved in cell interactions. These endogenous lectins are assayed as agglutinins of appropriate test erythrocytes. One of these, called lectin-2, interacts with specific glycosaminoglycans, especially heparin and dermatan sulfate. Lectin-2 is present at constant levels in both chick fibroblast and chick muscle cells throughout 14 days of culture but is released into the medium of cultured embryonic muscle after 7–8 days of culture, soon after myoblast fusion. Lectin-2 interacts strongly with a component of substrate-attached material in embryonic muscle cultures which is extractable from the culture dishes with alkali after the cells have been removed with ethylediaminetetraacetic acid. The active component in the substrate-attached material appears to be a glycosaminoglycan that is a more potent inhibitor of lectin-2 agglutination activity than any of the known glycosaminoglycans that we have tested. The active material is degraded by chondroitinase ABC but not by chondrotinase AC, hyaluronidase, or proteolytic enzymes and thus appears to be similar to dermatan sulfate. The results of these studies raise the possibility that lectin-2 functions by interacting with glycosaminoglycans, either associated with the cell surface or with the extracellular matrix.

Key words: lectin, glycosaminoglycan, extracellular material, cell matrix, cellular interactions, myoblast development

Specific surface interactions of developing cells with each other and with extracellular materials are believed to play an important role in tissue formation. While changes in cell surface components have been found during development, little is presently known about molecules which mediate specific cell interactions.

We have previously reported the presence of two developmentally regulated lectins in embryonic chick muscle that are candidates for a role in cellular interactions during development. The first (here referred to as lectin-1) has been purified by affinity chromatography [1, 2] and is assayed as an agglutinin of fixed trypsinized rabbit erythrocytes (here referred to as type I cells). Hemagglutination activity of this dimeric protein is inhibited by a number of sugars including lactose. Lectin-1 activity is low in extracts of pectoral muscle from 8-day-old chick embryos, rises to a maximum at about 16 days,

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and then declines [3, 4]. The other lectin we have identified (here called lectin-2), also shows changes in activity in developing chick pectoral muscle [5] and is assayed as an agglutinin of modified erythrocytes (here called type II cells). These cells are type I cells that have been modified either by aging [5] or by alcohol washing [6]. Lectin-2 hemagglutination activity can be inhibited by low concentrations of several glycosaminoglycans [6]. It is also inhibited by several saccharides including N-acetyl-D-galactosamine [5, 6].

In the present report we show that lectin-2 is released into the medium of cultured chick embryo muscle cells after cell fusion has occurred. We also show marked inhibition of lectin-2 activity by a component of substrate-attached material (SAM) derived from cultured myoblasts. This inhibitor appears to be a glycosaminoglycan. Taken together our results suggest an extracellular function for lectin-2, perhaps a role in cell-matrix interactions.

MATERIALS AND METHODS

Cell Culture

Twelve-day chick embryo pectoral muscle was dissociated in 0.25% trypsin in Hanks' balanced salt solution and cultured on collagen surfaces at 4.5×10^4 cells per 1 cm^2 in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Lectin Extraction

Cell culture. Cells from 6–10 plates were lifted with phosphate-buffered saline (PBS) (0.075 M $NaKPO_4$, 0.075 M NaCl, pH 7.2) containing 2 mM ethylenediaminetetraacetic acid (EDTA). The cells were washed twice in PBS and homogenized in MEPBS (PBS containing 4 mM β-mercaptoethanol + 2 mM EDTA) containing 0.1 M lactose and 0.1 M N-acetyl-D-galactosamine.

Tissue. Lectin-1 and lectin-2 were extracted from 15-day chick embryo pectoral muscle in nine volumes of MEPBS containing 0.1 M lactose and 0.1 M N-acetyl-D-galactosamine [1, 5]. The extract was spun 1 h at 100,000g to remove debris and the supernatant was then centrifuged for 12 h at 100,000g [6]. The supernatant was found to contain all of the lectin-1 activity. The pellet was resuspended in 1 M NaCl + 4 mM β-mercaptoethanol and contained all of the lectin-2 activity [6].

Media. Media to be assayed for lectin activity were centrifuged at 1,000g for 10 min in a clinical centrifuge and used directly in hemagglutination assays.

Hemagglutination assays. Hemagglutination assays were done in microtiter V plates (Fisher Scientific) using serial twofold dilutions in MEPBS of extracts. Each well contained 0.025 ml lectin, 0.025 ml 0.15 M NaCl, 0.025 ml 1% bovine serum albumin in 0.15 M NaCl, and 0.025 ml of either 4% trypsinized glutaraldehyde-fixed rabbit red blood cells (type I cells [1]) or alcohol-washed trypsinized glutaraldehyde-fixed rabbit red blood cells (type II cells [6]) in PBS. Type I cells are specific for lectin-1 activity, while type II cells are specific for lectin-2 activity [6]. Substances to be tested for lectin inhibition were diluted in saline and replaced saline in the assay.

SAM Extraction

The media from 24- to 48-h myoblast cultures were removed and the cells lifted with PBS containing 2 mM EDTA followed by trituration [7]. Care was taken to remove all adherent cells. SAM was extracted in 0.1 N NaOH for 24 h at 37° [7], neutralized

with PBS + 2 mM EDTA, and tested for inhibition of lectin-2 in hemagglutination assays. Each 100-mm culture plate yielded 4 ml of SAM extract containing 18 μg/ml of glucuronic acid, as determined by the orcinol method [8].

Glycosaminoglycan Extraction

Glycosaminoglycans (GAGs) were extracted from pectoral muscle of 15-day embryos by homogenization in three volumes of 0.4% NaCl, followed by centrifugation for 1 h at 100,000g. Subsequent purification was done by the method of Linker and Hovingh [9]. Briefly, the supernatant was precipitated with cetylpyridinium chloride and the precipitate, after washing with ethanol, was resuspended in buffer and digested with pronase. The

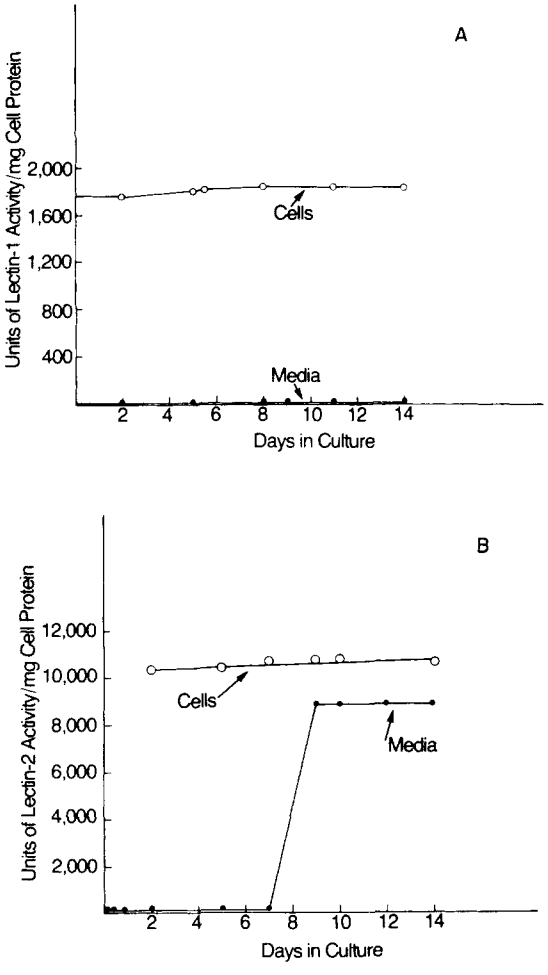

Fig. 1. Lectin-1 (A) and lectin-2 (B) activity in cell extracts and growth media of muscle cultures. Cultures were prepared and extracted as described in Methods. Lectin-1 activity was determined with type I cells and lectin-2 activity with type II cells as described in Methods. The units of lectin activity are the reciprocal of the titers (eg, a titer of 1:800 is referred to as 800 units). Each point is the average of duplicate values obtained from a single experiment. The overall results were confirmed in a replicate experiment.

TABLE I. Comparison of Characteristics of Lectin in Media From Muscle Cultures and Lectin-2

	Lectin-1	Lectin-2	Media
Agglutinates type I cells	+	0	0
Agglutinates type II cells	0	+	+
Inhibited by 10 mM lactose	+	0	0
Inhibited by 5 μg/ml heparin	0	+	+
Inhibited by extracts of SAM	0	+	+
Binds to p-aminophenyl lactoside column	+	0	0
Sedimented at 100,000g for 12 h	0	+	+

TABLE II. Comparative Potency of Extracts of SAM From Muscle Cultures, Muscle Tissue Extract, and Other Inhibitors of Lectin-2

	Concentration (μg/ml) that inhibits lectin-2 activity by 50%
Heparin	2.5
Dermatan sulfate	5.0
Heparan sulfate	12.5
Chondroitin-4-sulfate	>50
Chondroitin-6-sulfate	>50
Hyaluronic acid	>50
Muscle tissue extract	0.1[a]
NaOH extract of SAM	0.1[a]

[a]Specific activity determined by assuming that inhibitor is a glycosaminoglycan and that all uronic acid in these extracts is in active material.

GAGs were then precipitated with ethanol, dissolved in buffer, and tested for inhibitory activity in hemagglutination assays.

RESULTS

Extracellular Lectin-2 Activity

Extracts of embryonic chick skeletal muscle cultures had high levels of both lectin-1 and lectin-2 (Fig. 1A, B). Cells had a constant level of both lectin activities through 14 days in culture. During this period myoblast fusion and myotube formation took place.

The culture media showed no detectable lectin activity for seven days, followed by a dramatic rise of lectin-2 activity (Fig. 1B). Total extracellular lectin-2 activity in a given plate reached levels comparable to the lectin-2 activity that could be extracted from the cells on that plate. No lectin-1 activity was ever detected in the media (Fig. 1A). Mixing experiments in which purified lectin-1 was reacted with native or boiled media showed this was not due to the presence of an inhibitor in the culture media (data not shown).

The agglutinin found in the media was shown to meet functional criteria defining lectin-2 activity (Table I). It agglutinated type II but not type I erythrocytes; this agglutination was specifically blocked by heparin and extracts of SAM (see below) and not blocked by lactose. The activity failed to bind to a p-aminophenyl lactoside column, and sedimented in 12 h at 100,000g, as does lectin-2.

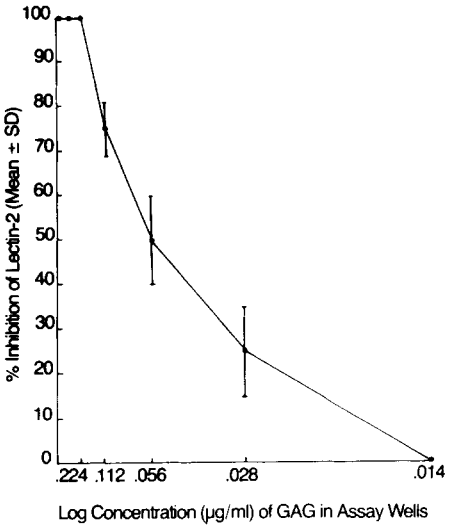

Fig. 2. Inhibition of lectin-2 activity by SAM from primary chick muscle cultures as a function of inhibitor concentration. SAM was extracted in 0.1 NaOH and neutralized with PBS, and serial dilutions were assayed for inhibition of lectin-2 activity. Glycosaminoglycans (GAG) content of the extracts was estimated by the orcionol method [8] assuming that 2.5 mg of GAG contains 1 mg of uronic acid.

SAM Inhibition of Lectin-2 Activity

Extracts prepared from SAM of myoblast cultures in 0.1 N NaOH and crude GAGs prepared from embryonic muscle tissue were extremely potent inhibitors of lectin-2 (Table II, Fig. 2). These substances had no detectable effect on lectin-1 activity. The potency of the extract of SAM as an inhibitor of lectin-2 is expressed in terms of GAG content of the extract, since we had previously found [6] that lectin-2 activity is sensitive to a group of iduronic acid-containg GAGs, including heparin, dermatan sulfate, and heparan sulfate, but not to other GAGs such as chondroitin sulfate or hyaluronic acid. As indicated in Table II, the extracted material from SAM or muscle tissue appears to be more potent than any of the standard GAGs. The specific activity of the GAG in these extracts may actually be much greater than would be estimated from uronic acid determination, since it is likely that, as with fibroblasts [10], there are several different GAGs in the SAM of muscle cultures and not all are active inhibitors.

The assumption that the inhibitor in the extracts is indeed a GAG is supported by a number of observations. First, the inhibitory activity of the extracts is resistant to prolonged digestion with trypsin or with pronase (Table III). It is also resistant to prolonged boiling, and can be precipitated by cetylpyridinium chloride (data not shown). Furthermore, the inhibitor is obviously highly anionic, since it binds tenaciously to a DEAE-cellulose column. In addition, the inhibitory activity in extracts of SAM can be completely destroyed by digestion with chondroitinase ABC (Table III). The same result was found with GAG preparations from embryonic muscle tissue. These results, coupled with studies of the effects of other enzymes, suggest that the inhibitor is similar to dermatan sulfate. This is suggested by the finding that the inhibitor is insensitive to testicular hyaluronidase, which digests hyaluronic acid and chondroitin sulfates, and is also insensitive to chondroitinase AC, which digests hyaluronic acid and chondroitin

TABLE III. Destruction of Inhibitory Activity of SAM by Chondroitinase ABC

	% Inhibition of lectin-2 agglutination activity
SAM	100
SAM + chondroitinase ABC	0
SAM + chondroitinase AC	100
SAM + hyaluronidase (testicular)	100
SAM + trypsin	100
SAM + pronase	100

SAM was incubated alone or with 2.5 units of chondroitinase ABC or AC (Sigma), or with 1 mg/ml of testicular hyaluronidase, trypsin, or pronase for 36 h at 37°C in PBS, pH 7.2 (pH 8 in the case of pronase). After incubation the samples treated with proteolytic enzymes were boiled for 5 min. Serial dilutions of all samples were then assayed for activity as lectin-2 inhibitors. SAM boiled or incubated alone for 36 h maintained full activity. Enzymes incubated alone had no effect on lectin-2 agglutination.

TABLE IV. Comparison of Lectin-2 and Fibronectin

Lectin-2	Fibronectin
Does not agglutinate formalinized sheep red blood cells	Agglutinates formalinized sheep red blood cells
EDTA-insensitive	EDTA blocks agglutination activity
β-mercaptoethanol preserves agglutination activity	β-mercaptoethanol dissociates to monomers with impaired binding
Does not bind to collagen[a]	Binds to collagen
Hemagglutination inhibited by N-acetyl-D-galactosamine but not N-acetyl-D-glucosamine	Hemagglutination not inhibited by N-acetyl-D-galactosamine; inhibited somewhat by D-galactosamine and D-glucosamine

Properties of fibronectin are documented in Yamada and Olden [11]. Properties of lectin-2 are based on Mir-Lechaire and Barondes [5] and Kobiler and Barondes [6], and on original observations made in the course of the present work.

[a]Lectin-2 was reacted with a collagen coated culture dish and the supernatant was assayed after incubation for 30 min. No activity was lost even when very dilute solutions of crude lectin were used.

sulfates (Table III). The only known glycosaminogylcan degraded by chondroitinase ABC but not by chondroitinase AC is dermatan sulfate.

Comparison of Lectin-2 and Fibronectin

Because fibronectin is also a known hemagglutinin that is present both on cell surfaces and in culture media of several cell types [11] and because it also interacts with heparin [12], it was necessary to discriminate between lectin-2 and fibronectin. The differences between lectin-2 and fibronectin, summarized in Table IV, indicate that they are distinct.

DISCUSSION

These results show that lectin-2 activity can be found in the medium of embryonic muscle culture, presumably owing to secretion by the muscle cells. This inference is based on our finding (data not shown) that there is no lectin-2 activity detectable in the medium of confluent or growing fibroblast cultures, although extracts of these cultures do contain lectin-2 activity. Presumably, then, the source of the lectin-2 in the medium of the muscle cultures is the muscle cells themselves, rather than fibroblasts in the cultures. Appearance of lectin-2 in the medium is not due to cell breakage, since the cultures show no visible deterioration and since another intracellular protein, lectin-1, was never found in the medium.Why lectin-2 appears in the medium, and only after substantial differentiation of the muscle cells, is presently unclear.

A possible extracellular function of lectin-2 is suggested by the finding that SAM from muscle cultures is rich in an inhibitor of this lectin. The inhibitor appears to be a glycosaminoglycan and the results suggest that the GAG binds to an active site on the lectin. It is notable that among the group of GAGs that act as inhibitors of lectin-2 some heparan sulfate is found associated with cell surfaces [13] and heparin binds to a cell surface protein [14]. One possible interpretation of these observations is that lectin-2 appears on the cell surface and plays a role in interactions of cells with GAGs either associated with other cells or bound within the extracellular matrix. An interaction of this complex with fibronectin might also occur. Appearance of free lectin-2 in the medium might result from an artifactual sloughing of this material but could also play some role in cellular attachment or detachment. Whatever the details, the observation that both lectin-2 and GAG that interacts with it are present outside muscle cells is suggestive of a functional interaction between these substances.

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REFERENCES

1. Nowak T, Kobiler D, Roel LE, Barondes SH: J Biol Chem 252:6026, 1977.
2. Den H, Malinzak D, Rosenberg A: Biochem Biophys Res Commun 69:621, 1976.
3. Nowak T, Harwood PL, Barondes SH: Biochem Biophys Res Commun 68:650, 1976.
4. Kobiler D, Barondes SH: Develop Biol 60:326, 1977.
5. Mir-Lechaire FJ, Barondes SH: Nature 272:256, 1978.
6. Kobiler D, Barondes SH: FEBS Lett 101:257, 1979.
7. Culp LA, Rollins BJ, Baniel J, Hitri S: J Cell Biol 79:788, 1978.
8. Mejbaum R: J Physiol Chem 258:117, 1939.
9. Linker A, Hovingh P: Carbohydrate Res 29:41, 1973.
10. Rollins BJ, Culp LA: Biochemistry 18:141, 1979.
11. Yamada K, Olden K: Nature 275:179, 1978.
12. Ruoslahti E, Hayman EG: FEBS Lett 97:221, 1979.
13. Kraemer PM: Biochem Biophys Res Commun 78:1334, 1977.
14. Alimedia B, Busch C, Höök M: Thrombosis Res 12:773, 1978.