HYALURONIDASE ACTIVITY IN CULTURED CHICK EMBRYO SKIN FIBROBLASTS*

Roslyn W. Orkin, Geraldine Jackson and Bryan P. Toole

Developmental Biology Laboratory
Departments of Medicine and Anatomy,
Massachusetts General Hospital and Harvard Medical School,
Boston, Massachusetts 02114

Received May 16,1977

SUMMARY

Primary cultures of fibroblasts isolated from ten day old embryonic chick skin contain hyaluronidase activity, both associated with the cells and secreted into the medium. The enzyme has a pH optimum at 3.7, and is most active against hyaluronate as a substrate. The oligosaccharide products of fibroblast hyaluronidase action are similar in size to those of testicular hyaluronidase, ruling out significant degradation by exoglycosidases.

INTRODUCTION

Macromolecules of the extracellular matrix (collagen and proteoglycans) appear to be involved in a number of developmental phenomena, including muscle (1,2) and cartilage (3,4) differentiation, and the cell migratory activity of several embryonic cell types (5-7). The presence of hyaluronate has been correlated with cell migration, and its removal by hyaluronidase with the onset of overt differentiation (5,8,9). In addition to its developmental role, hyaluronidase may be an important regulator of glycosaminoglycan turnover in adult cells since hyaluronate accumulates in cultured fibroblasts from patients with Marfan's syndrome (10,11) and Hurler's syndrome (12).

To date, hyaluronidase activity has been extracted from a number of intact tissues, including skin (13-15). However, activity has not been detected in cell cultures of human skin fibroblasts (11,16). This communication describes

* This is publication No. 726 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. Supported by grants from the NIH (AM3564 and DE04220). Dr. Orkin is a fellow of the Arthritis Foundation and Dr. Toole is an Established Investigator of the American Heart Association (#73138).

ISSN 0006-291X

the production of hyaluronidase activity by cultures of fibroblasts derived from embryonic chick skin.

MATERIALS AND METHODS

Fibroblasts were isolated from ten day old embryonic chick skin by incubation of skin segments at 37°C, 1 hour, in 0.1% crude collagenase (Worthington Biochemicals, Freehold, New Jersey), plated at 10^5 cells/100 mm culture dish, and grown to confluency in medium F-12 containing 10% fetal calf serum and 1% antibiotic-antimycotic solution (Gibco, Grand Island, New York) over an 8-10 day culture period. Cultures were refed once during the incubation period, 24-48 hours prior to harvest. The medium from this final period of culture was collected from the confluent cultures and processed separately. The cell layer was rinsed in Hank's Balanced Salt Solution. Cells were then removed from the culture plates with a rubber policeman, hand homogenized in cold formate extraction buffer (0.1M sodium formate-0.15M NaCl-0.1% triton X, pH 3.7) and then sonicated for two 15 second intervals at 0°C to disperse the cell suspension more evenly. Sonication for this period was found not to inhibit enzyme activity. Aliquots of the whole sonicate or of the supernatant obtained after centrifugation at 35,000 RPM for 1 hour at 4°C, were used for enzyme assays. Samples of culture medium were either precipitated with 65% saturated ammonium sulfate, or first allowed to incubate for an additional 24 hours at 37°C in the absence of cells, prior to precipitation with ammonium sulfate. The ammonium sulfate precipitates were redissolved in formate buffer (0.1M sodium formate-0.15M NaCl, pH 3.7), and then assayed for enzyme activity as indicated below.

Hyaluronidase activities of the cell- or medium-derived samples were assayed after incubation at 37°C with exogenous substrate, usually for 16 hours, and the newly formed terminal N-acetylglucosamine measured by the colorimetric assay of Reissig et al. (17). Routinely, hyaluronate (Sigma, grade I or III) (100 µg/250 µl assay mixture) was used as substrate. Hyaluronidase activity was expressed on the basis of total cellular protein (18) as µg of terminal N-acetylglucosamine released per mg protein. Where indicated, saccharolactone (1.5mM, Sigma) an inhibitor of exoglycosidase activity, was included in the incubation mixtures.

The pH optimum of the fibroblast hyaluronidase was determined after dialysis of enzyme samples against formate buffer (0.1M sodium formate - 0.15M NaCl) over a pH range of 2.9 to 4.5, or against 0.2M phosphate buffer at pH 6.0 and 7.0. Saccharolactone was routinely included in these incubations.

The substrate specificity of the fibroblast hyaluronidase was tested in the presence of saccharolactone, using purified chondroitin 6-sulfate, keratan sulfate, and heparin (standard preparations donated by Dr. J. Cifonelli, University of Chicago), as well as hyaluronate as substrates for enzyme activity.

Products of the fibroblast hyaluronidase digestions were examined after incubation of enzyme samples with [^{14}C]-hyaluronate (a kind gift of Dr. Minoru Okayama). After incubation at 37°C for 16 hours, the suspension was centrifuged at 20,000 RPM for 1.5 hours at 4°C, and the clarified digest applied to Sephadex G-50 (2 cm X 90 cm) and 3.8 ml fractions collected. Aliquots of each fraction were counted for radioactivity. [^{14}C]-hyaluronate, products of the digestion of [^{14}C]-hyaluronate with testicular hyaluronidase, [^{3}H]-glucosamine, and $^{3}H_2O$ were also applied to calibrate the column.

TABLE 1. LEVELS OF HYALURONIDASE ACTIVITY IN CULTURED CHICK EMBRYO SKIN FIBROBLASTS

Experiment No.	Without Saccharolactone	With Saccharolactone
I	2.3	-
II	3.5	3.5
III	-	5.5
IV	3.4	2.8
V	3.1	2.4
VI	2.4	1.3
Mean ± Standard Error	2.9 ± 0.3	3.1 ± 0.7

Values represent μg terminal N-acetylglucosamine released/mg protein in 16 hours at 37°C.

RESULTS AND DISCUSSION

Fibroblasts in primary cultures from 10 day embryonic chick skin contain hyaluronidase activity at levels approximately 0.2 μg terminal-N-acetylglucosamine released per mg cellular protein per hour (3 μg/mg/16 hours, Table 1). The reaction is linear over a 16 hour incubation period at 37°C (data not shown). Analysis of the hyaluronidase activity present in medium samples indicate 1-3 times as much enzyme activity as that found in the cell layer. However, these levels of activity may represent only a fraction of the total enzyme released to the medium because of loss of activity; no hyaluronidase activity could be detected in medium which had been allowed to incubate an additional 24 hours at 37°C, in the absence of cells.

Preliminary characterization of the enzyme indicates that, like the tissue hyaluronidases (19-22), the embryonic chick skin fibroblast hyaluronidase has a pH optimum at pH 3.7 (Figure 1). No activity was found when the enzyme was assayed at neutral pH. Activity at pH 3.7 was proportional to protein concentration. Under the conditions of the incubation and assay procedures,

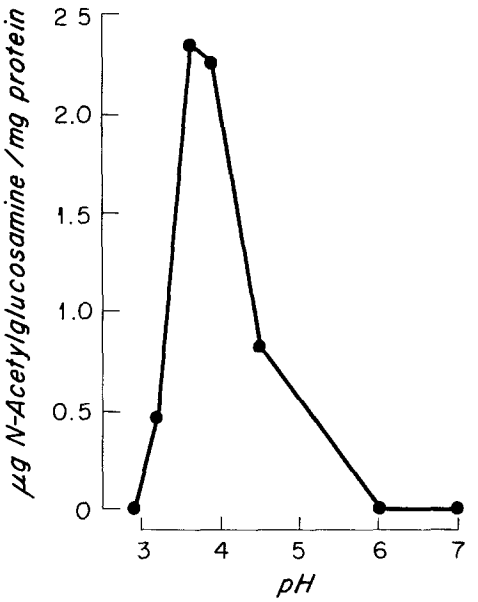

Figure 1.

The effect of pH on the activity of embryonic chick skin fibroblast hyaluronidase. Cell extracts (35,000 RPM supernatant) were dialyzed into formate (pH 2.9-4.5) or phosphate (pH 6.0-7.0) buffers and then assayed for hyaluronidase activity as described in the text.

hyaluronate was a significantly better substrate for the enzyme than were the other glycosaminoglycans which were tested. No activity was detected against keratan sulfate or heparin. As with testicular and lysosomal hyaluronidases, chondroitin 6-sulfate was degraded by the fibroblast enzyme (approximately 65% of that obtained with hyaluronate as substrate, under the conditions of our assay procedure).

Since the exoglycosidases, β-glucuronidase and β-N-acetylglucosaminidase are both present in the fibroblast cell extracts, we used precautions to eliminate the possibility of their contributing significantly to the total reducing N-acetylglucosamine measured in the colorimetric assay. First, the buffer system used for enzyme assay is known to be inhibitory to exoglycosidase activity (21,22). Second, the addition of saccharolactone (1.5mM), an inhibitor of β-glucuronidase, did not significantly reduce the hyaluronidase activity

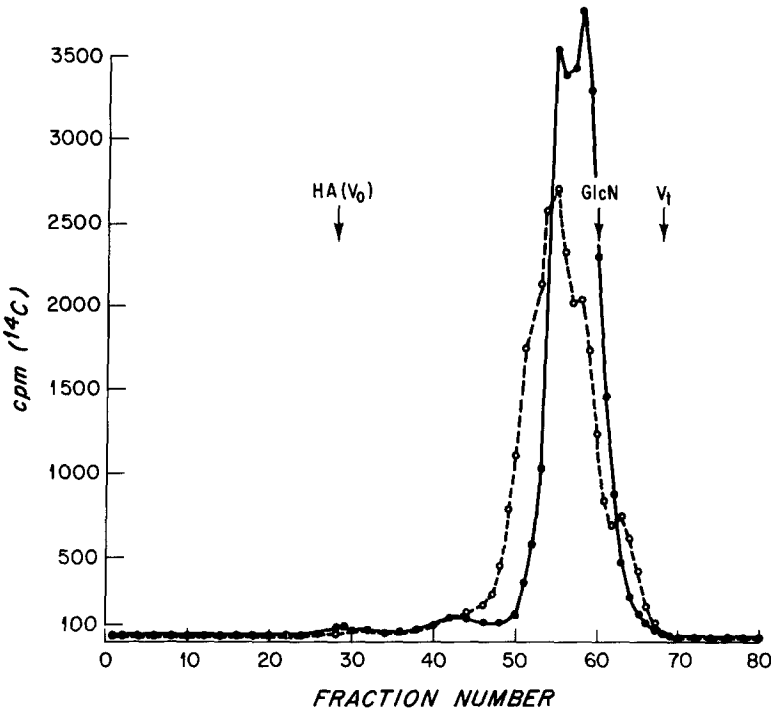

Figure 2.

The elution profiles, on Sephadex G-50, of the digestion products of embryonic chick skin fibroblast (o—o--o) and testicular (●—●—●) hyaluronidase after incubation with [^{14}C]-hyaluronate (HA). The elution position of hyaluronate, which eluted in the void volume (V_o), of [^{3}H]-glucosamine (GlcN), and of $^{3}H_2O$ (total volume V_t), are also indicated.

(Table 1). Third, the elution profile obtained from Sephadex G-50 chromatography of the products of fibroblast hyaluronidase digestion demonstrated that the products are of a similar size range to the oligosaccharides obtained after digestion of hyaluronic acid with testicular hyaluronidase (Figure 2). Only a small percentage of the digestion products overlapped the elution position of monomeric glucosamine. The elution pattern clearly indicates that the majority of digestion products are typical of those produced by the endoglycosidase, hyaluronidase, rather than the monomeric products of exoglycosidase activity.

The detectability of hyaluronidase activity in cultures of embryonic chick skin fibroblasts may be a species or age-related phenomenon, or it may be due to our use of the formate buffer system which appears to be particularly favor-

able for activity of the fibroblast enzyme. We have found that the acetate buffer system routinely used for measuring testicular hyaluronidase activity, and also used to search for activity in human skin fibroblast cultures (10,16) is strongly inhibitory to the hyaluronidase activity of the cultured chick embryo skin fibroblasts.

In work to be reported elsewhere, we have also detected hyaluronidase activity, having similar properties to those reported here, in cultures of fibroblasts isolated from embryonic chick skeletal muscle. In both skin- and muscle-derived fibroblast cultures, levels of hyaluronidase activity are maintained after repeated subculture. While this work was in progress, similar studies on cultures of the rat pituitary cell line GH_1 were being conducted by Polansky et al. (23). These studies indicate that the GH_1 cell line produces hyaluronidase activity at similar levels, and with similar characteristics, to those of the embryonic chick skin fibroblast cultures reported here. These two studies constitute the first reports of hyaluronidase activity produced by cultured cells.

ACKNOWLEDGMENT

We wish to thank Drs. Chitra Biswas and Jerome Gross for their useful discussions.

REFERENCES

1. Hauschka, S.D. and Konigsberg, I.R. (1966) Proc. Natl. Acad. Sci. U.S.A. 55,119-126.
2. Ketley, J.N., Orkin, R.W. and Martin, G.R. (1976) Exptl. Cell Res. 99, 261-268.
3. Levitt, D., Ho, P.-L. and Dorfman, A. (1974) In: The Cell Surface in Development (A.A. Moscona, ed.) pp.101-128, John Wiley and Sons, Inc., New York.
4. Kosher, R.A., Lash, J.W. and Minor, R.R. (1973) Devel. Biol. 35,210-220.
5. Toole, B.P. (1973) Amer. Zool. 13,1061-1065.
6. Solursh, M. (1976) Devel. Biol. 50,525-530.
7. Pratt, R.M., Larsen, M.A. and Johnston, M.C. (1975) Devel. Biol. 44,298-305.
8. Toole, B.P. and Trelstad, R.L. (1971) Devel. Biol. 26,28-35.
9. Smith, G.N., Jr., Toole, B.P. and Gross, J. (1975) Devel. Biol. 43,221-232.
10. Matalon, R. and Dorfman, A. (1973) Biochem. Biophys. Res. Commun. 32,150-154.
11. Lamberg, S.I. and Dorfman, A. (1973) J. Clin. Invest. 52,2428-2433.
12. Germinario, R.J., Kahlenberg, A. and Pinsky, L. (1973) Biochem. J. 132, 403-408.

13. Cashman, D.C., Laryea, J.V. and Weissmann, B. (1969) Arch. Biochem. Biophys 135,387-395.
14. Silbert, J.E., Nagai, Y. and Gross, J. (1965) J. Biol. Chem. 240,1509-1511.
15. Polansky, J.R. and Toole, B.P. (1976) Devel. Biol. 53,30-35.
16. Arbogast, B., Hopwood, J.J. and Dorfman, A. (1975) Biochem. Biophys. Res. Commun. 67,376-382.
17. Reissig, J.L., Strominger, J.L. and Leloir, L.R. (1955) 217,959-966.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193,265-275.
19. Aronson, N.N. and Davidson, E.A. (1967) J. Biol. Chem. 242,441-444.
20. Vaes, G. (1967) Biochem. J. 103,802-804.
21. Margolis, R.U., Margolis, R.K., Santilla, R. and Atherton, D.M. (1972) J. Neurochem. 19,2325-2332.
22. Polansky, J.R., Toole, B.P. and Gross, J. (1974) Science 183,862-864.
23. Polansky, J.R., Corbett, J.M. and Baxter, J.D. (1977) Personal Communication.