BINDING OF HYALURONATE TO THE SURFACE OF CULTURED CELLS

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ABSTRACT

The binding of hyaluronate to SV-3T3 cells was measured by incubating a suspension of cells (released from the substratum with EDTA) with ^{3}H-labeled hyaluronate and then applying the suspension to glass fiber filters which retained the cells and the bound hyaluronate. The extent of binding was a function of both the concentration of labeled hyaluronate and the cell number. Most of the binding took place within the first 2 min of the incubation and was not influenced by the presence or absence of divalent cations. The binding of labeled hyaluronate to SV-3T3 cells could be prevented by the addition of an excess of unlabeled hyaluronate. High molecular weight preparations of hyaluronate were more effective in preventing binding than low molecular weight preparations. The binding of [^{3}H]hyaluronate was inhibited by high concentrations of oligosaccharide fragments of hyaluronate consisting of six sugars or more, and by chondroitin. The sulfated glycosaminoglycans (chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin, and heparan sulfate) had little or no effect on the binding. The labeled hyaluronate bound to the cells could be totally removed by incubating the cells with testicular hyaluronidase, streptomyces hyaluronidase, or trypsin, indicating that the hyaluronate-binding sites are located on the cell surface.

KEY WORDS   hyaluronate · cell surface binding · cell aggregation

Hyaluronate has been implicated in a number of phenomena in vivo which involve cell-to-cell interactions. Studies of a variety of systems have revealed a very close correlation between the presence of large amounts of hyaluronate in the extracellular matrix and the migration of cells through the matrix. Examples include: developing embryonic tissues such as the cornea (24), vertebral column (19), neural crest (3, 11), heart (9) and primary mesenchyme (15), as well as amphibian limb regeneration (22) and mammalian tissue remodeling (4, 8, 10). In virtually all of these systems, it has been demonstrated also that subsequent cessation of migration and onset of differentiation are associated with a dramatic decrease in extracellular hyaluronate concentration. Consequently, it has been proposed that hyaluronate acts to weaken cell-to-cell adhesion and thereby facilitate migration (see reference 21 for review).

Studies in vitro have also implicated hyaluronate in cell-to-cell interactions. Toole et al. (23) have shown that when small amounts of hyaluronate are added to cultures of dissociated somite

cells (stage-26 chick embryo), the subsequent appearance of cartilage nodules is inhibited. In addition, Corsin (2) has demonstrated hyaluronate-induced inhibition of chondrogenesis in organ cultures of amphibian cephalic neural crest recombined with endoderm and mesoderm. Possibly, the exogenous hyaluronate is preventing specific adhesion of the prechondrogenic cells.

Recently, evidence was presented which strongly suggests binding of hyaluronate to the surface of cells (26). The aggregation of SV-3T3 cells, and several other types of cultured mouse cells, has been found to consist of two components, one dependent upon and the other independent of the presence of divalent cations (17, 26). The latter component was found to be blocked by several enzymes which degrade hyaluronate, including streptomyces hyaluronidase which is specific for this polysaccharide. The divalent-cation-independent aggregation was also inhibited by high concentrations of exogenously added hyaluronate, chondroitin, and oligosaccharide fragments of hyaluronate consisting of six sugars or more. Other sulfated glycosaminoglycans had little or no effect on aggregation. These results have been interpreted to mean that the divalent-cation-independent aggregation is mediated by the interaction between endogenous hyaluronate on one cell and hyaluronate-binding receptors on adjacent cells. This interaction would then be prevented by either destroying the endogenous hyaluronate with enzymes or by saturating the binding sites with exogenous hyaluronate (26).

In the present study, binding of radioactively labeled hyaluronate to the surface of SV-3T3 cells is directly demonstrated. The results of this study suggest the presence of receptor sites identical to those that mediate the divalent-cation-independent aggregation of cells.

MATERIALS AND METHODS

Culture Procedures

The SV-3T3 cell line was obtained from Dr. J. Keller, and rat fibrosarcoma cells from Dr. A. Dorfman. The cells were grown on 100-mm Falcon plastic dishes (Falkon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in Dulbecco's Modified Eagle's Medium (18) containing 10% fetal calf serum plus 10^3 U/ml penicillin G and 100 μg/ml streptomycin. All cultures were maintained in a 5% CO_2-air atmosphere, and the medium was changed every 2 or 3 d.

Isolation of ^{3}H-Labeled Hyaluronate

For each preparation, 12 dishes of confluent rat fibrosarcoma cells were grown in the presence of 250 μCi/ml of [^{3}H]acetate (New England Nuclear, Boston, Mass., 6 Ci/mmol, or Amersham Corp., Arlington Heights, Ill., 2 Ci/mmol) for 2 d. The medium was collected and digested with 1.0 mg/ml pronase at 37°C in the presence of 0.02% sodium azide. The solution was then exhaustively dialyzed against saline followed by distilled water.

Hyaluronate was isolated by cetylpyridinium chloride precipitation. For this, the solution was made 0.03 M in NaCl and a small quantity of celite was added. A 10% solution of cetylpyridinium chloride was then added dropwise until no further precipitate was formed. After centrifugation, the precipitate was extracted twice in a small volume of 0.1% cetylpyridinium chloride in 0.4 M NaCl. The hyaluronate was then precipitated by the addition of 4 vol of ethanol to the combined extracts at 4°C. After centrifugation, the pellet was washed several times with 100% ethanol and dissolved in distilled water.

Some preparations (preps. 2 and 3) were further purified by ion-exchange chromatography. The solution of hyaluronate was applied to a 1.5 × 10 cm column of DEAE-cellulose (Whatman DE-52) and eluted with a linear gradient of 0.02 M Tris, pH 7.5 (250 ml) to 1.0 M NaCl, 0.02 M Tris, pH 7.5 (250 ml), collecting 5-ml fractions. The fractions containing the hyaluronate were pooled, concentrated, and dialyzed against saline. Greater than 98% of the recovered label was associated with the hyaluronate peak, and no detectable amount of label was associated with other glycosaminoglycan fractions. Omission of the DEAE-cellulose chromatography step (prep. 1) did not significantly alter the results. The uronic acid content of the various preparations of hyaluronate was determined by the method of Bitter and Muir (1).

The specific activities of the various preparations of [^{3}H]hyaluronate used in this study are: prep. 1, 6.5 × 10^7 cpm/mg hyaluronate; prep. 2, 1.1 × 10^8 cpm/mg; prep. 3, 1.0 × 10^8 cpm/mg.

Viscosity Measurements

The intrinsic viscosity (η) was determined with a Cannon 75K 511 dilution viscometer (Cannon Instrument Co., State College, Pa.) having a buffer flow time of 112 s at 25°C. All measurements were done in 0.2 M NaCl, 0.02 M Tris, pH 7.5, and at least four dilutions were tested. The concentration of hyaluronate was determined by uronic acid assay (1) using glucuronolactone as a standard. The amount of sodium hyaluronate was obtained by multiplying the mass of glucuronolactone by a factor of 2.27. Approximate estimates of molecular weight can be calculated from these viscosity values using the equation η (100 ml/g) = (2.28 × 10^{-4})mol wt $^{0.816}$ (13).

Binding Assay

The SV-3T3 cells were subcultured at approx. 1×10^6 cells per 100-mm plate and allowed to grow for 24 h before each experiment. The plates were rinsed twice with calcium and magnesium-free phosphate-buffered saline (CMF-PBS), and then 8 ml of 0.02% solution of EDTA in CMF-PBS was added. After 10 min the EDTA solution was removed, the plates were gently rinsed three times with CMF-PBS, and the cells were suspended in a measured amount of CMF-PBS by flushing with a Pasteur pipette. The concentration of suspended cells was determined with a hemocytometer.

1-ml aliquots of the cell suspension (approx. 1×10^6 cells) were then added to siliconized conical flasks (25 ml) containing labeled hyaluronate and the various agents to be tested. Unless indicated otherwise, the flasks were shaken at 70 rpm for 15 min at room temperature. A 1-ml aliquot was then removed from each flask with a plastic pipette (Falcon Labware) and applied to glass fiber filters (Whatman 934 AH) on a Millipore filter manifold (Millipore Corp., Bedford, Mass.), followed by thorough washing with CMF-PBS. The filters were dried under a heat lamp, placed in 10 ml of Aquasol (New England Nuclear), and the radioactivity was determined in a scintillation counter.

All values represent the average of either duplicate or triplicate determinations minus the nonspecific background. Unless specified otherwise, the background was determined as the amount of label retained on the filters in the absence of cells. In some specified cases, background was measured as the amount retained on the filters in the presence of cells plus a large excess of unlabeled hyaluronate (1.0 mg/ml; mol wt, 2.0×10^5). The backgrounds determined by the two methods were similar and varied from 15 to 40% of the total counts retained by $1-2 \times 10^6$ cells, depending upon the particular batch of [^{3}H]hyaluronate. In most cases, the [^{3}H]-hyaluronate was centrifuged (75,000 g, 60 min) before the binding assay, which considerably reduced the background.

Identification of Hyaluronate

Samples of [^{3}H]hyaluronate were digested with chondroitinase ABC (1 U/ml) in 0.1 M Tris buffer, pH 7.5 at 37°C for 5 h, then mixed with disaccharide standards and aliquots applied to thin-layer chromatography sheets (plastic-backed cellulose, 30 × 20 cm; Merck & Co., Inc., Rahway, N. J.). The samples were desalted overnight using n-butanol-ethanol-water (52:32:16), dried, and the chromatogram was developed with n-butanol-acetic acid-IM ammonia (2:3:1) for 8 h (27). The chromatography sheet was dried and the disaccharide spots were visualized under UV light. The sheet was then cut into small sections and the radioactivity in each was counted using a cocktail of Omnifluor (New England Nuclear) dissolved in toluene. Each section was then washed by

dipping in toluene, dried, and the cellulose was scraped into a tube for N-acetylhexosamine assay by the method of Reissig et al. (12). The cellulose was removed by centrifugation before measurement in the spectrophotometer.

The purity of the [^{3}H]hyaluronate as measured in this manner is demonstrated in Fig. 1 a. At least 95% of the total radioactivity corresponds to the position of the hyaluronate-derived disaccharide produced by chondroitinase ABC digestion.

Samples of labeled material derived from the cell-binding experiments were first precipitated with 3 vol of 1.3% potassium acetate in 95% ethanol at 4°C overnight, then redissolved in a small volume of 0.1 M Tris buffer, pH 7.5 for chondroitinase digestion and chromatography with internal standards as described above. At least 90% of the labeled material from these experiments was precipitated and redissolved in the above step.

Materials

Two preparations of hyaluronate, both from human umbilical cord, were used. The first, obtained from Dr. Martin Mathews, had a viscosity of 450 ml/g corresponding to a mol wt of approx. 2.0×10^5; the second, from Drs. E. A. Balazs and B. Jacobson, had a viscosity of 3,800 ml/g corresponding to a mol wt of approx. 2.5×10^6. The oligosaccharide fragments of hyaluronate were prepared as described previously (26). Dermatan, dermatan sulfate, heparin, and heparan sulfate were donated by Dr. Martin B. Mathews, chondroitin (desulfated chondroitin-6-sulfate) by Dr. Allen L. Horwitz. Chondroitin-4-sulfate, chondroitin-6-sulfate, chondroitinase ABC, chondro-4-sulfatase, and streptomyces hyaluronidase were purchased from Miles Laboratories, Inc. (Elkhart, Ind.); the testicular hyaluronidase (type VI-S) from Sigma Chemical Co. (St. Louis, Mo.); and Trypsin (TRT PCK) from Worthington Biochemical Corp. (Freehold, N. J.). The unsaturated disaccharides, 2-acetomido-2-deoxy-3-O (β-D-gluco-4-enepyranosyluronic acid)-D-glucose (Di-O-HA), 2-acetomido-2-deoxy-3-O- (β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (DI-4-S), and 2-acetomido-2-deoxy-3-O- (β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (Di-6-S) were prepared by digestion of hyaluronate, chondroitin-4-sulfate and chondroitin-6-sulfate respectively with chondroitinase ABC. The 2-acetomido-2-deoxy-3-O (β-D-gluco-4-enepyranosyluronic acid)-D-galactose (Di-O-Ch) was prepared by digesting chondroitin-4-sulfate concurrently with chondroitinase ABC and chondro-4-sulfatase (14).

RESULTS

Binding of [^{3}H]Hyaluronate to SV-3T3 Cells

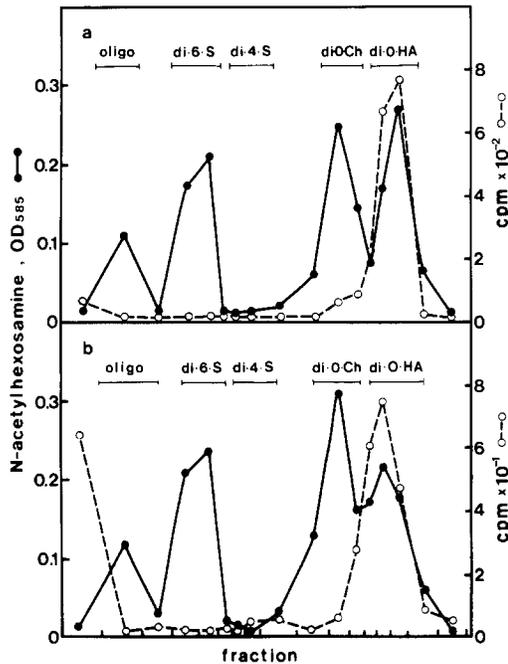Subconfluent SV-3T3 cells were removed from tissue culture dishes with EDTA, incubated with

FIGURE 1 Thin-layer chromatography of chondroitinase ABC digests of (a) purified [^{3}H]hyaluronate and (b) [^{3}H]hyaluronate bound to SV-3T3 cells. (a) Purified [^{3}H]hyaluronate (prep. 3) was processed directly as described in Materials and Methods. (b) [^{3}H]hyaluronate bound to SV-3T3 cells was recovered by the following procedure: SV-3T3 cells were incubated with [^{3}H]hyaluronate (2 μg/ml, prep. 3) for 15 min and then applied to glass fiber filters which were thoroughly washed with CMF-PBS. The filters were then transferred to different ports on the manifold filter box and each was treated with a total of 4 ml of 0.5 mg/ml trypsin (TPCK) in CMF-PBS which was added at 15-min intervals over a period of 60 min. The filters were then washed with CMF-PBS. The filtrates from these steps (trypsin plus wash) were pooled, dialyzed against distilled water, and lyophilyzed. The trypsin treatment removed essentially all of the label from the filters and at least 90% of this was recovered after the dialysis and lyophilization steps. The sample was then processed as described in Materials and Methods. The brackets indicate the positions of the various disaccharide standards as they were visualized by UV light. Using these spots as a guide, the chromatogram was cut into sections which were then analyzed for label and N-acetylhexosamine content. The di-4-S disaccharide yields no color in the N-acetylhexosamine assay.

varying concentrations of [^{3}H]labeled hyaluronate, and then filtered. As shown in Fig. 2, the amount of hyaluronate bound increased with increasing concentration of input hyaluronate up to 2–3 μg/
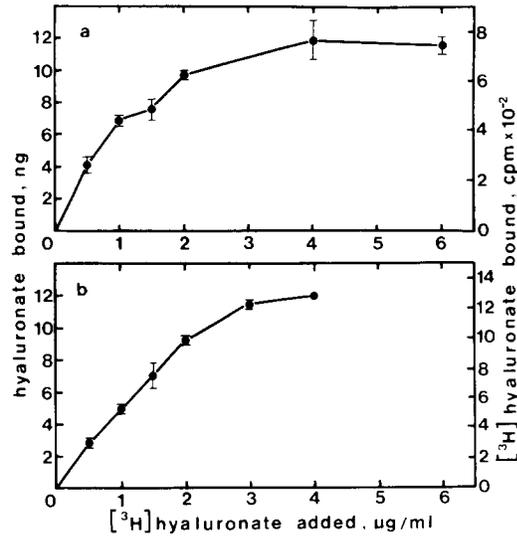
FIGURE 2 Effect of increasing concentrations of [^{3}H]hyaluronate on the binding of hyaluronate to SV-3T3 cells. (a) Prep. 1. [^{3}H]hyaluronate was incubated with 0.93 × 10^6 cells/ml. (b) Prep. 2. [^{3}H]hyaluronate was incubated at 1.2 × 10^6 cells/ml. The background was subtracted from each value. The absolute amount of hyaluronate bound was calculated from the respective specific activities (prep. 1, 6.5 × 10^7 cpm/mg; prep. 2, 1.1 × 10^8 cpm/mg).

ml. At this concentration, the binding sites on the cells appeared to be saturated with hyaluronate.

The three preparations of [^{3}H]hyaluronate used in this study saturated the binding sites at approximately the same concentration (1–3 μg/ml). However, the fraction of hyaluronate which bound to the cells varied widely for other preparations. For preparations of [^{3}H]hyaluronate used in this study (preps. 1, 2, 3), from 0.4 to 0.6% of the input counts were bound by 1 × 10^6 SV-3T3 cells at 1 μg/ml of input hyaluronate. However, for other preparations which were not used in this study, this proportion was as small as 0.01%. Also, for a given preparation of [^{3}H]hyaluronate, there was a gradual decrease in its binding activity with time of storage.

Since only a small fraction of the input hyaluronate was usually bound to the cells under the conditions used, it was important to establish that the bound label was, in fact, hyaluronate rather than a contaminant in the preparation. This was accomplished by releasing the cell-bound material into solution with trypsin (0.5 mg/ml in CMF-PBS for 30 min) and then analyzing it by chon-

droitinase ABC digestion and thin-layer chromatography as described in Materials and Methods. Fig. 1 b shows that ~80% of the bound label was associated with the digestion products of hyaluronate, the rest remaining at the origin. Similar results were obtained for each preparation of [^{3}H]hyaluronate used in this study.

It is possible that only a small unique subfraction of the labeled hyaluronate bound to the cells. To test this possibility, cells were incubated with labeled hyaluronate and filtered. Then the filtrate was used to test the ability of the remaining unbound hyaluronate to bind to a second set of SV-3T3 cells. As shown in Table I, the cells bound similar amounts of labeled hyaluronate from the filtrates and from the original solution. Thus, there does not appear to be a small subpopulation of hyaluronate that preferentially binds to the cells.

Other characteristics of hyaluronate binding to SV-3T3 cells were examined. First, the amount of hyaluronate bound increased linearly with increasing cell number (Fig. 3). Secondly, the time-course of binding (Fig. 4) showed that most of the binding took place within the first 2 min of the incubation and that the amount of hyaluronate bound remained relatively constant over a period of 1 h. Finally, the presence or absence of divalent cations had no effect on the binding of labeled hyaluronate (Table II).

Competition of Other Carbohydrate Preparations

For competition experiments, the amount of [^{3}H]hyaluronate added was approximately the minimum needed for saturation. For preps. 1 and
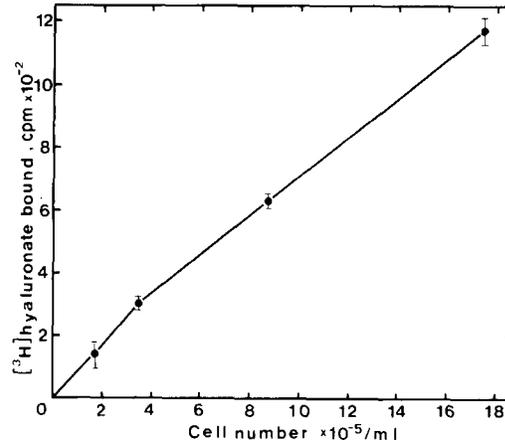
FIGURE 3   Effect of increasing SV-3T3 cell number on the binding of labeled hyaluronate. Cells were incubated for 15 min with 3 μg/ml of prep. 2, [^{3}H]hyaluronate. The background was 164 cpm.
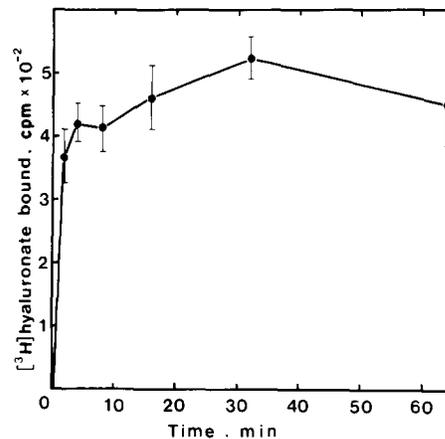
FIGURE 4   Time-course of [^{3}H]hyaluronate binding to SV-3T3 cells. Labeled hyaluronate (3 μg/ml, prep. 2) was incubated with 7 $\times$ 10^5 cells/ml for varying lengths of time. The background was 160 cpm.

TABLE I

Effect of Pretreatment with Cells on the Binding of [^{3}H]Hyaluronate to SV-3T3 Cells

Hyaluronate	cpm Bound ± SD
Control	568 ± 51
Pretreated	561 ± 34

[^{3}H]hyaluronate (6 μg/ml; prep. 2) was mixed with 3.0 $\times$ 10^6 SV-3T3 cells/ml. After 15 min, the cell suspension was filtered and the filtrate collected. The washed filter retained 2835 cpm of bound hyaluronate. Aliquots of a suspension of untreated SV-3T3 cells were then mixed with aliquots of the filtrate containing the residual [^{3}H]hyaluronate or of the original [^{3}H]hyaluronate solution. The final mixtures contained 0.85 $\times$ 10^6 cells/ml and 3 μg/ml hyaluronate. After 15 min the cell suspensions were again filtered. The background was 163 cpm.

TABLE II

Effect of Divalent Cations on the Binding of [^{3}H]-Hyaluronate to SV-3T3 Cells

Component added	cpm Bound ± SD
—	636 ± 69
Ca^{++}, Mg^{++}	623 ± 26

Mixtures contained 3 μg/ml of [^{3}H]hyaluronate (prep. 2) and 1.2 $\times$ 10^6 cells/ml. One set of mixtures was made 0.9 mM in CaCl$_2$ and 0.5 mM in MgCl$_2$. Background was 160 cpm.

2 this was 3 µg/ml, while for prep. 3, which bound to a greater extent, this was 1 µg/ml.

In initial experiments with unlabeled hyaluronate as the competitor, it was found that the order of addition of the components, i.e., labeled hyaluronate added to the cells first, then the unlabeled hyaluronate (or vice versa) had no significant effect on the final extent of binding (data not shown). For this reason, in all subsequent experiments the [³H]hyaluronate and the competitor were added to the cells simultaneously and then incubated for 15 min.

Competition of [³H]hyaluronate binding with unlabeled hyaluronate was dependent upon molecular weight. The concentration of high molecular weight hyaluronate (2.5×10^6) necessary for 50% inhibition of [³H]hyaluronate binding was ~5 µg/ml (Fig. 5a) which was 10- to 20-fold less than that of the lower molecular weight (2.0×10^5) preparation of hyaluronate (Fig. 5b and c). It should be noted that the absolute amount of hyaluronate required for 50% inhibition of binding varied slightly for different preparations of [³H]-hyaluronate. However, the relative competitive efficiencies of the high and low molecular weight preparations of hyaluronate remained constant.
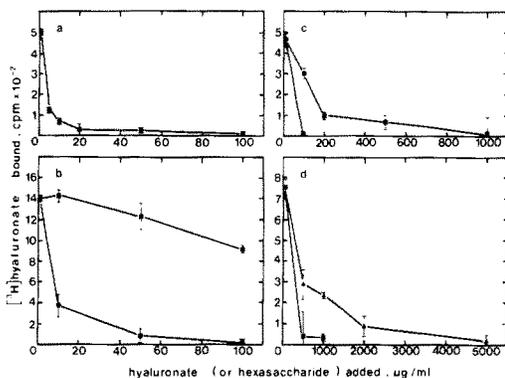

FIGURE 5   Effect of varying concentrations of unlabeled hyaluronate and hexasaccharide on the binding of labeled hyaluronate to SV-3T3 cells. In each case 3 µg/ml of prep. 1, [³H]hyaluronate was used. (a) 9×10^5 cells/ml with hyaluronate, mol wt: 2.5×10^6. (b) 2.2×10^6 cells/ml with hyaluronate, mol wt: 2.5×10^6 (●) and hyaluronate, mol wt: 2.0×10^5 (■). (c) 1.6×10^6 cells/ml with hyaluronate, mol wt: 2.5×10^6 (●) and hyaluronate, mol wt: 2.0×10^5 (■). (d) 2.2×10^6 cells/ml with hyaluronate, mol wt: 2.0×10^5 (■) and hexasaccharide fragment of hyaluronate (▲). The backgrounds for the various experiments were: (a) 259 cpm, (b) 689 cpm, (c) 550 cpm, (d) 757 cpm.

Because of this large effect of molecular weight, we measured the viscosity of [³H]hyaluronate prepared from cultures of rat fibrosarcoma cells in the same fashion as the preparations used here for the binding studies. The viscosity obtained was 3,300 ml/g, which corresponds to a mol wt of approx. 2.0×10^6, almost identical to the high molecular weight preparation used for the preceding competition experiments.

The hexasaccharide fragment of hyaluronate also competed with labeled hyaluronate for binding but was even less efficient than either preparation of intact hyaluronate (Fig. 5d). The effect of various hyaluronate oligosaccharides at a concentration of 5 mg/ml on the binding of [³H]hyaluronate is given in Table III. The disaccharide had no effect and the tetrasaccharide had only a slight inhibitory effect, whereas the hexasaccharide and larger oligosaccharides significantly inhibited binding of labeled hyaluronate.

Chondroitin is a glycosaminoglycan similar in structure to hyaluronate except that it contains N-acetylgalactosamine instead of N-acetylglucosamine. Chondroitin also inhibited the binding of labeled hyaluronate to SV-3T3 cells (Fig. 6), but its activity (500 µg/ml for 50% inhibition) was less than that of the low molecular weight preparation of hyaluronate (Fig. 5c) and similar to that of the hexasaccharide (Fig. 5d).

Dermatan has a structure similar to that of chondroitin except that it contains iduronic acid instead of glucuronic acid. The particular preparation of dermatan used in this study contained ~80% iduronic acid and 20% glucuronic acid. When tested in the competition assay (Table IV), it was found to be distinctly less active than a

TABLE III

Inhibition of [³H]Hyaluronate Binding to SV-3T3 Cells by Oligosaccharide Fragments of Hyaluronate

Component added	cpm Bound ± SD	% Inhibition
—	1,005 ± 38	0
Disaccharide	973 ± 54	3
Tetrasaccharide	644 ± 80	36
Hexasaccharide	322 ± 14	68
Octasaccharide	291 ± 72	71
Decasaccharide	259 ± 65	74

Mixtures contained 1 µg/ml of [³H]hyaluronate (prep. 3) plus 5 mg/ml of oligosaccharide, incubated with 1.8×10^6 SV-3T3 cells/ml for 15 min. The cells were then filtered, washed, and counted. The background was 160 cpm.
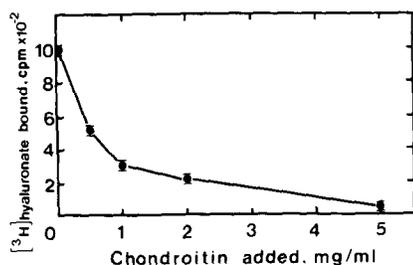
FIGURE 6 Effect of varying concentrations of chondroitin on the binding of [³H]hyaluronate to SV-3T3 cells. Increasing amounts of chondroitin were incubated with 3 μg/ml prep. 2, [³H]hyaluronate and 1.3×10^6 cells/ml. The background was 160 cpm.

TABLE IV

Inhibition of [³H]Hyaluronate Binding to SV-3T3 Cells by Various Glycosaminoglycans

Exp No.	Component added	cpm Bound ± SD	% Inhibition
1	—	495 ± 13	0
	Chondroitin	54 ± 24	89
	Dermatan	245 ± 26	50
2	—	425 ± 35	0
	Chondroitin-4-sulfate	367 ± 39	19
	Chondroitin-6-sulfate	385 ± 4	15
	Dermatan sulfate	358 ± 15	21
3	—	653 ± 17	0
	Heparan sulfate	392 ± 10	39
	Heparin	370 ± 31	43

Mixtures contained 3 μg/ml of [³H]hyaluronate (prep. 2) and 1.0 mg/ml of the various glycosaminoglycans. The cell numbers were: exp 1, 1.12×10^6 cells/ml; exp 2, 1.3 $\times$ 10^6 cells/ml; and exp 3, 1.04×10^6 cells/ml. The backgrounds were determined from the amount of label retained on the filters in the presence of 1 mg/ml hyaluronate. They were: exp 1, 204 cpm; exp 2, 208 cpm; and exp 3, 217 cpm.

similar amount of chondroitin. The sulfated glycosaminoglycans chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate had a negligible effect on the binding of hyaluronate to SV-3T3 cells, while heparin and heparan sulfate had a small but consistent inhibitory effect (Table IV).

Location of Bound Hyaluronate

To determine whether the bound hyaluronate was intracellular or located on the cell surface, cells were incubated with labeled hyaluronate for 15 min and then hyaluronidase (testicular or strep-

tomyces) was added to the suspension. After an additional 15 min, the cells were filtered and washed. As shown in Table V, both hyaluronidase preparations eliminated essentially all of the bound label.

Small amounts of trypsin also inhibited the binding of labeled hyaluronate to cells (Table VI). These results are consistent with the cell surface location of the hyaluronate-binding site and suggest that this site may be a protein.

DISCUSSION

The major conclusion which can be drawn from

TABLE V

Effect of Testicular and Streptomyces Hyaluronidases on the Binding of [³H]Hyaluronate to SV-3T3 Cells

Exp No.	Component added	cpm Bound ± SD	% Decrease
1	—	1,578 ± 289	0
	1 μg/ml Testicular hyaluronidase	11 ± 43	99
	10 μg/ml Testicular hyaluronidase	−32 ± 42	102
2	—	916 ± 30	0
	1 U streptomyces hyaluronidase	1 ± 12	100
	10 U streptomyces hyaluronidase	19 ± 25	98

[³H]hyaluronate (3 μg/ml, prep. 2) was incubated with SV-3T3 cells (1.5×10^6 cells/ml and 1.1×10^6 cells/ml for exps 1 and 2, respectively). After 15 min, the indicated amount of either testicular or streptomyces hyaluronidase was added to the suspensions. After 15 additional min, the cells were filtered and the amount of bound label was determined. The backgrounds were 160 and 58 cpm for exps 1 and 2, respectively.

TABLE VI

Effect of Trypsin on the Binding of [³H]Hyaluronate to SV-3T3 Cells

Trypsin added	cpm Bound ± SD	% Decrease
μg		
0	665 ± 58	0
0.1	597 ± 110	10
1.0	57 ± 14	91
10	−39 ± 12	106

SV-3T3 cells (0.82×10^6 cells/ml) were added to flasks containing both [³H]hyaluronate (1 μg/ml, prep. 3) and varying amounts of TPCK trypsin. After incubating for 15 min, the cells were filtered, washed, dried, and counted. The background was 152 cpm.

these experiments is that hyaluronate binds to the surface of SV-3T3 cells. Much of the evidence suggests that this binding is due to specific receptors on the cell surface. First, the binding of labeled hyaluronate to the cells is saturable and is inhibited by addition of unlabeled hyaluronate but not by several other closely related carbohydrates. Secondly, the binding can be prevented by incubating the cells with low concentrations of trypsin, which presumably digests the receptors and thereby stops binding. Thirdly, the binding of labeled hyaluronate can be blocked by fragments of hyaluronate consisting of six or more monosaccharides, but not by smaller oligosaccharides, suggesting that there is a minimum size requirement for recognition by the putative receptor site. Finally, calculation of the dissociation constant for binding of hyaluronate to the SV-3T3 cells gives values in the range $1-2 \times 10^{-9}$ M indicating a relatively high-affinity interaction. We have also calculated from our data that approx. 3×10^3 molecules of hyaluronate (mol wt, 2×10^6) bind to each SV-3T3 cell at saturation. It must be emphasized, however, that, since binding seems to be influenced greatly by the molecular weight of the hyaluronate, absolute figures for dissociation constant and the number of molecules bound will vary accordingly.

Previous work indicated that the divalent-cation-independent aggregation of SV-3T3 cells, as well as other cultured cell lines, is dependent on the presence of intact endogenous hyaluronate associated with their cell surfaces (26). It is very likely that the mechanism which is responsible for binding hyaluronate is identical to that which mediates the cation-independent aggregation of cells. This is suggested by the very close correlation between those substances which inhibited aggregation and those which inhibited hyaluronate binding. For example, macromolecular hyaluronate and fragments of hyaluronate consisting of six sugars or more were active in inhibiting both aggregation and binding of [³H]hyaluronate to SV-3T3 cells, while the tetrasaccharide fragment was only slightly active and the disaccharide was not active in either case. Likewise, chondroitin and, to a lesser extent, dermatan were active in blocking both aggregation and the binding of labeled hyaluronate. On the other hand, the sulfated glycosaminoglycans (chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin, and heparan sulfate) had little or no effect on either aggregation or binding. In addition to the above

characteristics, it was found that the presence of calcium and magnesium did not affect the binding of labeled hyaluronate, nor were they involved in the hyaluronate-mediated aggregation of cells.

Several structural requirements for binding of hyaluronate can be inferred from the competition experiments. First, the fact that chondroitin is a competitor for hyaluronate binding suggests that polymers of N-acetylgalactosamine-glucuronate or of N-acetylglucosamine-glucuronate can bind. The lower efficiency of chondroitin for competition as compared to hyaluronate could be due to either the low molecular weight of the chondroitin ($<5 \times 10^4$ daltons) or the higher affinity for N-acetylglucosamine-containing polymers. Secondly, the very low competitive activity of dermatan suggests that binding is less active for iduronate than for glucuronate-containing polymers. Indeed, the activity of the dermatan could be due entirely to the 20% glucuronate found in the preparation. Thirdly, the lack of competitive activity of chondroitin-4- and 6-sulfate suggests that the presence of a sulfate group on the polymer prevents binding. Finally, the inhibitory effect of the hexasaccharide fragment of hyaluronate but not small oligomers suggests that the binding site requires at least six sugars. This last result agrees with the work of Truppe et al. (25) who found that the binding of labeled hyaluronate to hepatocytes could be prevented by the addition of oligosaccharides of hyaluronate consisting of six or more sugars.

In other studies (5, 16, 20, 29), it has been shown that the addition of exogenous hyaluronate depresses synthesis of chondroitin sulfate-proteoglycan by chondrocytes derived from a variety of sources. This reaction is presumably mediated by the interaction of hyaluronate with the chondrocyte surface, but in this case the effect is not obtained with oligosaccharides smaller than a decasaccharide (28). In addition, the formation of aggregates of chondroitin sulfate-proteoglycan by the interaction of monomer with hyaluronate requires oligosaccharides no smaller than the decasaccharide (6, 7). Thus, we can conclude that cell surface proteoglycan is probably not the receptor site for hyaluronate binding in the present study.

An important characteristic of the binding of hyaluronate to the surface of SV-3T3 cells is the influence of molecular weight of the polysaccharide. Although the hexasaccharide fragment of hyaluronate was found to inhibit binding of labeled macromolecular hyaluronate significantly,

the efficiency of competition was very much less than that of high molecular weight hyaluronate. Also, the large difference in efficiency of competition between hyaluronate preparations of mol wt, 2.0×10^5 and 2.5×10^6, emphasizes this point. One possible explanation for this phenomenon is a cooperative effect caused by the binding of hyaluronate to more than one receptor at a time because of its repetitive structure. Perhaps, when one molecule of hyaluronate binds to one receptor, this enhances the probability that the same molecule will bind to a second receptor. Thus, the larger the hyaluronate molecule, the more sites it can attach to and the more tightly it is bound. An alternative explanation is that some structural feature of the high molecular weight hyaluronate caused it to be bound to the cell surface more strongly than low molecular weight hyaluronate. Clearly, further research is needed to resolve this issue.

The importance of molecular weight in determining the efficiency of binding might explain some of the variations between different preparations of [³H]hyaluronate with respect to their extent of binding. In general, those preparations which were isolated by the gentlest procedures and which presumably have the highest molecular weight were those that bound most efficiently to the cells. It was also noted in this study that, for a particular batch of labeled hyaluronate, the extent of binding gradually decreased with time. Perhaps this effect is caused by slow breakdown of hyaluronate occurring with repeated freezing and thawing of the preparation.

SV-3T3 cells were used throughout this study because the results of the study on aggregation indicated the possibility of hyaluronate-binding sites on the surface of these cells (26). Preliminary studies indicate that many other cell types are also capable of binding labeled hyaluronate to varying degrees. This includes both established cell lines (3T3, 3T6, BHK) as well as cells isolated directly from embryonic chick brain and limb bud. The significance of the differences between the various cell lines with respect to the extent of hyaluronate binding is not as yet clear, since the effect of endogenous hyaluronate on binding is unknown. We are currently investigating this question.

As described in the Introduction, hyaluronate is a major component of the embryonic extracellular matrix in which mesenchymal cells migrate and proliferate. It is also present in areas of tissue remodeling and regeneration. Determination of

the nature of binding of hyaluronate to cells involved in these processes as well as to cells exhibiting malignant properties should shed light on the role of hyaluronate in control of cell behavior.

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