Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 249-257

THE CELL SURFACE HYALURONATE BINDING SITES OF INVASIVE HUMAN BLADDER CARCINOMA CELLS

Raymond E. Nemec[1], Bryan P. Toole[2] and Warren Knudson[1,*]

[1] Departments of Biochemistry and Pathology, Rush-Presbyterian St.Luke's Medical Center, Chicago, Il 60612

[2] Department of Anatomy and Cellular Biology, Tufts Medical School, Boston, MA 02111

Received October 2, 1987

SUMMARY: High-affinity, cell surface binding sites for hyaluronate were demonstrated on highly invasive human bladder carcinoma cells. These binding sites were shown to be specific for hyaluronate, saturable and exhibit a Km of 0.94 x 10^{-9}M and a Bmax of 65ng hyaluronate/ 10^6 cells. The binding of [^{3}H]hyaluronate to a fixed cell-affinity column was competed with unlabeled hyaluronate and hyaluronate-hexasaccharide but not with hyaluronate-tetrasaccharide, chondroitin sulfate, heparin or non-sulfated dextran. Pre-treatment of cells with protease destroyed the binding activity whereas pre-treatment with Streptomyces hyaluronidase to reveal occupied binding sites had no effect. No hyaluronate-binding activity was observed on normal human fibroblasts.

Many cell types interact with their extracellular environment via specific cell surface binding proteins or receptors for various extracellular macromolecules. Cell surface receptors have been described for fibronectin (1), laminin (2), collagen (3-5), heparan sulfate (6,7) and hyaluronate (8-12). These receptors have been postulated to anchor the pericellular matrix to the plasma membrane and underlying cytoskeleton (13); bridge cell-cell and cell-matrix associations (14); mediate endocytosis of matrix macromolecules (15) and, promote locomotion of cells through extracellular matrices (16,17).

*Send correspondence to Dr. Warren Knudson.

ABBREVIATIONS: HA: hyaluronate BSA: bovine serum albumin
CMF-PBS: Calcium-magnesium free phosphate buffered saline
EDTA: Ethlylenediaminetetraacetic acid

0006-291X/87 $1.50

Hyaluronate (HA) receptors have been demonstrated on several cell types (9-12), including several virally-transformed rodent tumor cell lines (8,18). Since the nontransformed parental lines failed to show significant HA binding capacity (18),it was suggested that there may be a correlation between the presence of HA receptors and the transformed phenotype. However, there is no report on the expression of HA binding proteins on cells from naturally occurring tumors. In this report we demonstrate the presence of saturable, high-affinity, specific binding sites for HA on highly invasive human urinary bladder transitional cell carcinoma cell lines.

METHODS

Cell culture and harvesting: HCV-29T cells were obtained from Dr. Kieler, Fibiger Institute, Copenhagen, Denmark; SV-3T3 cells were the same as used previously (8); and human fibroblasts were grown in our laboratory from explant cultures of normal adult human skin. All cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and 10% fetal bovine serum, in a humidified 37 ^{o}C incubator supplied with 5% CO_2, 95% air. Cells from nearly confluent monolayer cultures were harvested by treatment with 1 mM EDTA in CMF-PBS for 15 min at room temperature with gentle shaking. The resultant single cell suspension was collected by centrifugation, counted and resuspended in an appropriate volume of assay or fixation buffer.

Preparation of [^{3}H]HA ligand: The [^{3}H]HA used as radioactive ligand was prepared from the conditioned medium of rat fibrosarcoma cells labeled with [^{3}H]acetate as described by Underhill and Toole (8). The molecular weight of the HA synthesized by these rat fibrosarcoma has been previously reported as 1.3 x 10^{6} as determined by viscometric analysis (8).

Binding assay (19): EDTA-released cells, suspended in PBS were aliquoted into duplicate BSA-coated microcentrifuge tubes containing various concentrations of [^{3}H]HA or [^{3}H]HA plus 250 ug non-labeled HA (added to determine non-specific background binding), in a total volume of 0.5ml. The cell density used in these experiments was 1 x 10^{6} cells/tube. Cells plus [^{3}H]HA were allowed to interact for 60 min at room temperature with gentle shaking. Following this incubation, cells were pelleted by centrifugation at 500 xg, washed two times with 5x PBS, redissolved in 0.5% SDS, mixed with cocktail and counted. Specific binding was determined by subtraction of background binding.

Fixed cell affinity column. Fixation of HCV-29T cells and their use as a cell-affinity column followed protocols outlined by Goldberg et al. (20). Briefly, EDTA-released single cell suspensions of HCV-29T cells were mixed with an equal volume of 2% glutaraldehyde in PBS, and shaken at room temperature for 60 min. The fixed cells were then washed extensively in CMF-PBS containing 1% BSA and a 1.0 ml volume of packed cells loaded into a 0.9mm x 4cm glass column. Once column was packed and equilibrated, approximately 50,000 cpm [^{3}H]HA

(~0.3 ug) diluted in 0.5ml PBS was applied to the column at a flow rate of 3.0 ml/min. Once the sample entered the column, the flow was stopped and the [^{3}H]HA allowed to interact with the cell-column for 60 min at room temperature. Following this incubation, the column was washed with 5-column volumes (5.0ml) of PBS to elute non-bound material. Then, 0.25 mg of various glycosaminoglycans or, HA-oligosaccharides, in 0.5ml PBS were applied to the column followed by 5-column volumes of PBS. Residual [^{3}H]HA remaining attached to the column was removed by elution with low ionic strength buffer, 0.02 M Tris, pH 7.4.

RESULTS AND DISCUSSION

Hyaluronate binding sites (putative receptors) were detected by the incubation of purified [^{3}H]HA ligand with EDTA-released, living cells. As shown in Figure 1, addition of increasing concentrations of [^{3}H]HA to the HCV-29T cells resulted in increased binding until reaching saturation. Scatchard analysis of this binding curve, Figure 2, suggest the presence of a single binding activity, with a Bmax of 65 ng HA/10^6 cells and a Kd of 1.2 ug/ml which is equivalent to 0.94 x 10^{-9}M assuming a MW of 1.3 x 10^{-6} for the [^{3}H]HA. These values are similar to those reported previously for SV3T3 cells (21), namely, a Bmax of 46 ng HA/10^6 cells and a Kd of 1.9 ug/ml. The Bmax values are based on cell number and thus would be influenced by cell size. By morphometric analysis (data not shown) of phase contrast photographs we determined that the HCV-29T cells have approximately 1.5 times larger surface area than the SV3T3 cells. If the Bmax for the HCV-29T cells is normalized to the surface area of a SV3T3 cell, we obtain a relative Bmax of 44 ng/10^6 cells. Thus the density of available HA binding sites on the two cell types is virtually identical. As well, HA binding to HCV-29T was shown to be directly proportional to cell number (data not shown).

It was shown previously that the number of available binding sites on SV-3T3 cells is increased by treatment of the cells with hyaluronidase to remove endogenous HA occupying receptors (19). The HCV-29T cells produce small quantitites of HA (22), some of which is

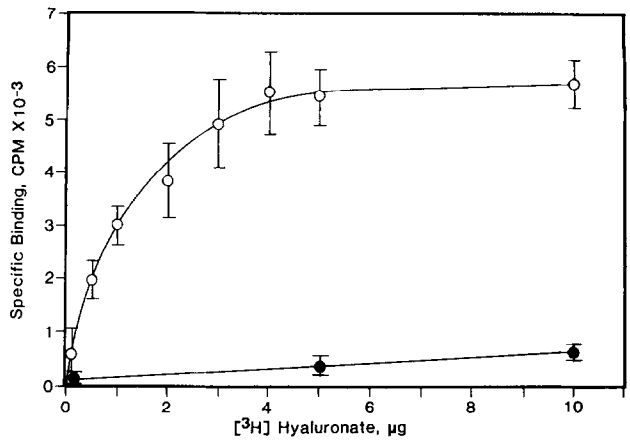

FIGURE 1. Effect of Increasing Concentrations of [^{3}H]HA on the Specific Binding of HA to HCV-29T Cells and Normal Human Fibroblasts.

Various concentrations of [^{3}H]HA were incubated with 1 x 10^6 HCV-29T (-O-) cells or normal human skin fibroblasts (-●-) as described in Methods. Specific cpm bound were determined by subtraction of non-specific background at each concentration. Error bars represent the S.E.M. of 5 experiments, each assayed in duplicate.

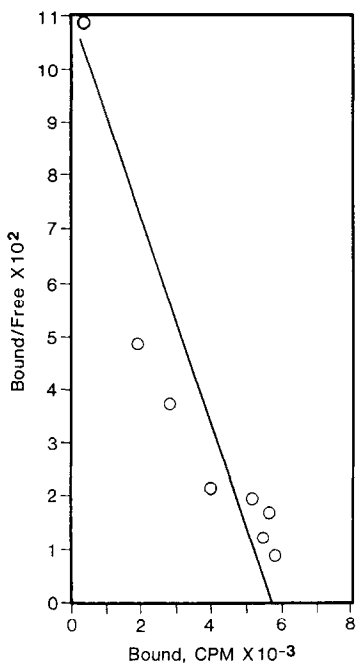

FIGURE 2. Scatchard plot analysis of [^{3}H]HA Binding to HCV-29T Cells.

Data from the specific binding of HA to HCV-29T cells shown in Figure 1 were used to prepare the Scatchard plot analysis. The specific activity of the [^{3}H)HA used in this study was 86,080 cpm/ug HA. Line was drawn from a computer least squares fit of the data with a correlation coefficient of 0.92.

TABLE 1. Effect of Enzymatic Pretreatment of HCV-29T Cells on HA Binding Activity

Sample [a]	cpm [^{3}H]HA bound/10^6 cells [b]
Control (non-treated)	2666 ± 83
Streptomyces hyaluronidase pre-treated	2234 ± 11
Trypsin pre-treated	56 ± 37

[a] EDTA-released single cell suspensions of HCV-29T cells were pretreated with 250 ug/ml trypsin (Gibco) or 1 U/ml Streptomyces hyaluronidase (CalBiochem) in PBS for 15 min at 37 °C. Cells were then centrifuged and washed extensively 2x, resuspended in PBS, counted and assayed for HA binding as described in Methods.

[b] cpm represent the amount of [^{3}H]HA bound specifically to 1 x 10^6 HCV-29T cells following a 1 hr incubation with 1.0 ug HA (equivalent to 86,080 cpm), ± S.E.M. of 4 experiments.

associated with the cell surface. Thus these cells were pretreated with 1 unit/ml of Streptomyces hyaluronidse to remove bound endogenous HA that might occupy binding sites. This treatment however, had no significant effect on HA binding activity (Table 1). Previous reports noted that the normal mouse parental line, 3T3 cells, lacked the high affinity HA binding sites exhibited by the SV-transformed 3T3 cells. In an somewhat analogous fashion, we observed that EDTA-released normal adult human skin fibroblasts also lack HA-binding sites (Figure 1).

As shown in Table 1, pretreatment of HCV-29T cells with 250 ug/ml protease for 15 min completely destroyed the HA-binding activity. The cells treated by this method were still intact and viable, as determined by trypan blue exclusion analysis. This demonstrates that the HA-binding site is a cell surface-associated protein(s).

To determine the specificity of the cell surface binding, competition experiments with other glycosaminoglycans were performed. Since the HA-binding protein on HCV-29T cells, similar to that on SV3T3 cells (19,20), is still functional following mild

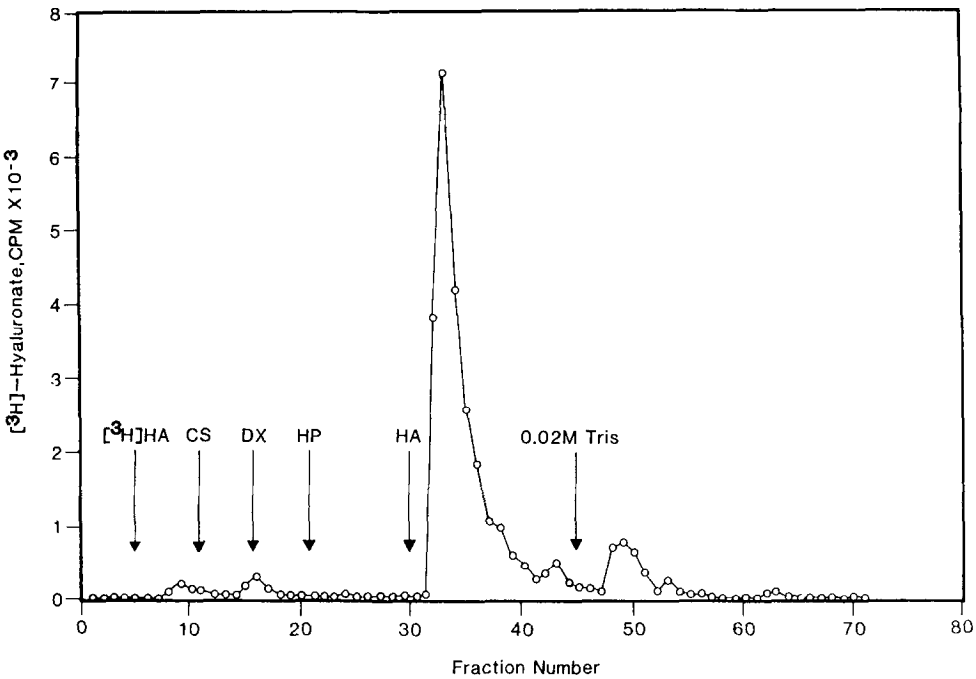

FIGURE 3. The Effect of Glycosaminoglycans on Binding of [^{3}H]HA to a HCV-29T Cell-Affinity Column.

An aliquot of [^{3}H]HA (0.3 ug, ~50,000 cpm) was applied to glutaraldehyde-fixed HCV-29T cell column as described Methods. After a stable baseline was attained, 0.25mg of chondroitin sulfate: mixed isomer (CS), non-sulfated dextran (DX), Heparin (HP) and HA, in 0.5 ml PBS, were applied to the column followed by PBS elution buffer. This was followed by 0.02 M Tris buffer to completely strip the column. Fractions (0.5ml) were collected, mixed with scintillation cocktail and counted.

fixation in glutaraldehyde, a cell affinity column could be set up to test binding specificity. As shown in Figure 3, most of the [^{3}H]HA bound efficiently to the cell column with little flow-through (fractions 4-7). Addition of relatively high concentrations of chondroitin sulfate, heparin, or non-sulfated dextran failed to compete with the binding and elute the bound HA. Addition of non-labeled HA however, caused rapid exchange and elution of the bound ligand. The affinity of HA-binding activity also appeared to decrease with decrease in ionic strength in similar fashion to the SV3T3 cell binding activity (8,20). This was indicated by the removal of additional [^{3}H]HA from the affinity column with low ionic strength buffer (Figure 3) Similar observations were made by Goldberg et al. using the SV3T3 cell-affinity column (20). Although

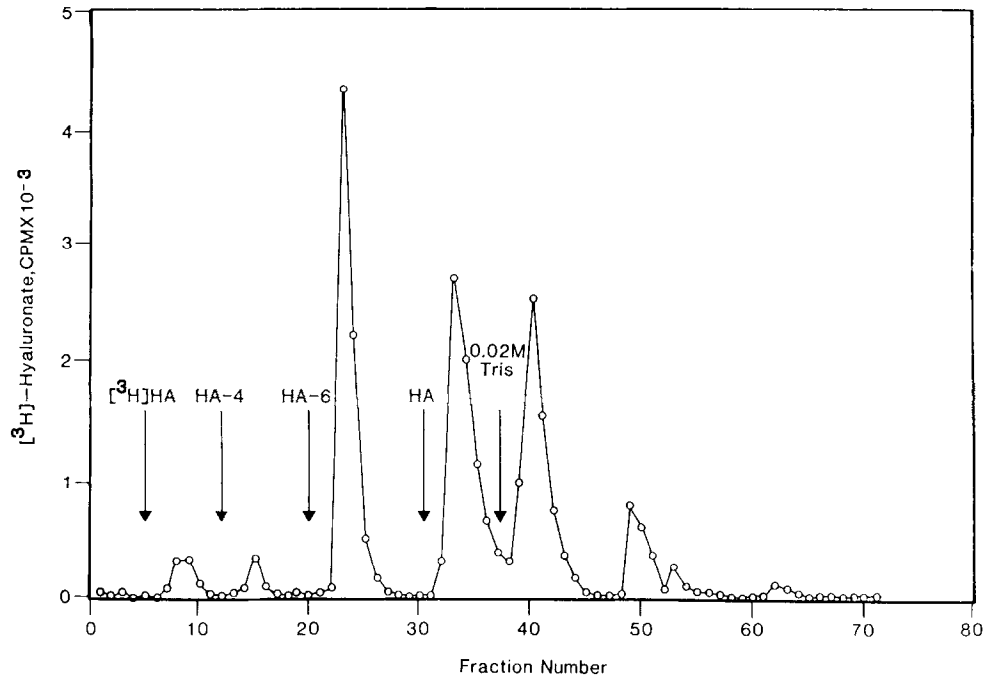

FIGURE 4. The Effect of HA-oligosaccharides on the Binding of [^{3}H]HA to a HCV-29T Cell-Affinity Column.

An aliquot of [^{3}H]HA (0.3 ug) was applied to the HCV-29T cell-affinity column as described in Figure 3. After the establishment of a stable baseline, 0.25 ug of HA-tetrasaccharide (HA-4) or HA-hexasaccharide (HA-6), in 0.5ml PBS were applied to the column followed by PBS. This was followed by addition of 0.25 ug HA in 0.5ml PBS to remove bound ligand remaining. Additional [^{3}H]HA was further removed by addition of 0.02M Tris buffer.

not shown, total bound [^{3}H]HA can also be completely removed by treatment with low ionic strength buffer. To further investigate the characteristics of binding of HA to the HCV-29T cells, elution of [^{3}H]HA from the above columns was attemped with HA-oligosaccharides. As shown in Figure 4, purified hexasaccharide (obtained from Dr. J. Kimura) effectively competed off more than 50% of the [^{3}H]HA as compared with competition by high molecular weight HA. Smaller oligosaccharides such as HA-tetrasaccharide had little ability to displace bound HA (Figure 4). The remaining [^{3}H]HA bound to the column was eluted with non-labeled HA and low ionic strength buffer. This result further confirms the similar characteristics of HA binding to HCV-29T and SV3T3 cells, since the latter is also

competed by HA-hexasaccharide (8), and distinguishes the binding from HA-proteoglycan or HA-link protein interactions which require a minimum size of decasaccharide for competition (23).

In summary, a specific high-affinity binding protein(s) for hyaluronate is present on human bladder carcinoma cells which may allow unique interactions with the HA-enriched extracellular environment characteristic of many tumors (24-29).

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Institutes of Health, CA42614 (to W.K.) and DE05838 (to B.P.T.).

The authors also wish to thank Pankaj Bidani and Kurian Thomas for their expert technical assistance.

REFERENCES

1. Yamada, K.M., Akiyama, S.K., Hasegawa, T., Hasegawa, E., Humphries, M.J., Kennedy, D.W., Nagata, K., Urushihara, J., Olden, K., and Chen, W.T. (1985) J. Cell. Biochem. 28, 79-97.

2. Von der Mark, K., and Kuhl, U. (1985) Biochim. Biophys. Acta 823, 147-160.

3. Chiang, T.M., and Kang, A.H. (1982) J. Biol. Chem. 257, 7581-7586.

4. Mollenhauer, J., and K. von der Mark (1983) Eur. Mol. Biol. Organ. 2, 45-50.

5. Dedhar, S., Ruoslahti, E., and Pierschbacher, M.D. (1987) J. Cell Biol. 104, 585-593.

6. Kjellen, L., Oldberg, A., Rubin, K., and Hook, M. (1977) Biochem. Biophys. Res. Commun. 74, 126-132.

7. Kraemer, P.M. (1977) Biochem. Biophys. Res. Commun. 78, 1334-1340.

8. Underhill, C.B., and Toole, B.P. (1979) J. Cell Biol. 82, 475-484.

9. Turley, E.A., Moore, D., and Hayden, L.J. (1987) Biochem. 26, 2997-3005.

10. Smedsrod, B., Pertoft, H., Eriksson, S., Fraser, J.R.E., and Laurent, T.C. (1984) Biochem. J. 223, 617-626.

11. Truppe, W., Basner R., von Figura, K., and Kresse, H. (1977) Biochem. Biophys. Res. Commun. 78, 713-719.

12. Knudson, C.B., and Toole, B.P. Dev. Biol., in Press.

13. Tamkun, J.W., DeSimone, D.W., Fonda, D., Patel, R.S., Buck, C., Horwitz, A.F., and Hynes, R.O. (1986) Cell 46, 271-282.

14. Woods, A., Couchman, J.R., and Hook, M. (1986) J. Biol. Chem. 260, 10872-10879.

15. Orkin, R.W., Underhill, C.B., and Toole, B.P. (1982) J. Biol. Chem. 257, 5821-5826.

16. Terranova, V.P., Liotta, L.A., Russo, R.G., and Martin, G.R. (1982) Cancer. Res. 42, 2265-2269.

17. Terranova, V.P., Rao, C.N., Kalebic, T., Margulies, I.M. and Liotta, L.A. (1983) Proc. Natl. Acad. Sci. USA 80, 444-448.

18. Underhill, C.B. and Toole, B.P. (1981) Exp. Cell. Res. 131, 419-423.

19. Goldberg, R.L., Seidman J.D., Chi-Rosso, G., and Toole, B.P. (1984) J. Biol. Chem. 259, 9440-9446.

20. Goldberg, R.L., Underhill, C.B. and Toole, B.P. (1982) Anal. Biochem. 125, 59-65.

21. Toole, B.P., and Underhill, C.B. (1983) In Cell Interactions and Development (Yamada, K.M., Ed), pp.203-230. Wiley & Sons, New York.

22. Knudson, W., Pauli, B.U. and Toole, B.P. (1986) Organ Systems Newsletter, 2, 21.

23. Kimura, J.H. and Kuettner, K.E. (1986) In Articular Cartilage Biochemistry, (Kuettner, K.E., Schleyerbach, R. and Hascall, V.C. Eds), pp. 113-124. Raven Press, New York.

24. Knudson, W, Biswas, C., Toole, B.P. (1984) J. Cell. Biochem. 25, 183-196.

25. Toole, B.P., Biswas, C., and Gross, J. (1979) Proc. Natl. Acad. Sci. USA 76, 6299-6303.

26. Takeuchi. J., Sobue, M., Sato, E., Shamato, M., Miura, K., and Nakagaki, S. (1976) Can. Res. 36, 2133-2139.

27. Horai, T., Nakamura, N., Tateishi, R., and Hattori, S. (1981) Cancer 48, 2016-2021.

28. Takeuchi, J., Sobue, M., Sato, E., Yoshida, M., Uchibori, N., and Miura, K. (1981) Cancer 47, 2030-2035

29. Kojima, J., Nakamura, N., Kanatani, M., and Ohmori, K. (1975) Can Res 35, 542-547.