UPTAKE OF HYALURONATE BY CULTURED CELLS

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Received August 18, 1977

SUMMARY: [^{14}C]hyaluronate is internalized by adsorptive pinocytosis by cultured rat hepatocytes and human synovial cells, but not by human skin fibroblasts and smooth muscle cells. Hyaluronate oligosaccharides compete for the uptake of hyaluronate by hepatocytes without being internalized themselves at the doses used. It is suggested that for adsorptive pinocytosis a hyaluronate molecule has to bind to at least two receptors on the cell membrane.

Mammalian cells internalize extracellular macromolecules by endocytic processes thereby usually delivering the materials to the degradative apparatus of the cells. Endocytosis can be remarkably selective. Molecules possessing binding sites for receptors on the cell surface can be incorporated in endocytic vesicles at concentrations that are substantially elevated above their concentration in the bulk medium adjacent to the cells.

The enzymatic degradation of glycosaminoglycan chains is considered to take place only within the lysosomes. Secreted glycosaminoglycans must therefore reenter the cells prior to degradation. Previous studies gave evidence that proteoglycans and sulfated glycosaminoglycan chains are internalized by adsorptive pinocytosis by a variety of cultured cells (1-3). Proteoglycans interact more efficiently than glycosaminoglycan chains with the proposed surface receptors since lower concentrations of proteoglycans than of protein-free carbohydrate chains are required for half-maximal uptake (4). Studies on the endocytosis of hyaluronate, however, have not been described in the literature.

ISSN 0006-291X

MATERIALS AND METHODS

Cell culture: Human skin fibroblasts, smooth muscle cells from human thoracic aorta, synovial cells, and rat hepatocytes were maintained in culture as previously described (5).

Preparation of [^{14}C]hyaluronate and hyaluronate oligosaccharides: Skin fibroblast cultures grown to confluency in 75m^2 Falcon plastic flasks were incubated in the presence of 15 ml culture medium and 25 µCi [1-^{14}C]glucosamine (58 mCi/mmol, The Radiochemical Center, Amersham) per flask for 72 h. The medium was dialyzed against 0.4 M Tris/HCl buffer, pH 7.0, in 0.15 M NaCl, and then chromatographed on a Dowex 1 x 2 column according to l.c.3. Material eluting as authentic hyaluronate was dialyzed against 0.15 M NaCl and concentrated by ultrafiltration under reduced pressure.
The purity of the preparation was monitored by cellulose acetate electrophoresis in two different buffer systems (6) and by digestion with trypsin (3) and testes hyaluronidase (7), respectively. The digests were subjected to chromatography on Sepharose 4B in 4M guanidinium chloride in case of proteolytic degradation or on Sephadex G-25 in 1.0 M NaCl after depolymerization by hyaluronidase. Up to 5% of the [^{14}C]radioactivity exhibited a different electrophoretic mobility than hyaluronate and was sensitive to proteolytic digestion.
The specific radioactivity of the preparation was determined by a double-labeling technique using [^{3}H]acetic anhydride (100 mCi/mmol, The Radiochemical Center, Amersham) as described (8) except that the amino sugar derivative was further purified by chromatography on Dowex 1 x 2.
From [^{14}C]hyaluronate and umbilical cord hyaluronic acid even numbered oligosaccharides were obtained and analyzed after degradation with hyaluronidase as described (6,7).

Measurement of [^{14}C]hyaluronate uptake: Confluent monolayer cultures were prepared in 25 cm^2 Falcon plastic flasks and incubated with 3 ml medium containing up to 100 µl of glycosaminoglycan solutions. After 8 h the cultures were worked up according to the published procedure (3) except that the cells were washed only 4 times with 3 ml each of Hanks' Balanced Salt Solution. The fifth washing cycle resulted in an increased liberation of radioactivity from the cell surface. In addition, the intracellular ethanol-soluble radioactivity was determined separately.

RESULTS AND DISCUSSION

Comparing the uptake of [^{14}C]hyaluronate by human skin fibroblasts, smooth muscle cells, synovial cells, and rat hepatocytes it is evident that only synovial cells and hepatocytes are able to incorporate measurable amounts of ^{14}C-radioactivity, whereas all cell types internalized efficiently [^{35}S]proteoglycans (Table 1). Prolonged incubation for up to 72 hours failed to result in measurable endocytosis of hyaluronate by fibroblasts

Table 1

Pinocytosis, degradation and adsorption of [^{14}C]hyaluronate and [^{35}S]proteoglycans by various types of cultured cells

	Cell type	Pinocytosis[a]	Degradation[b]	Adsorption[a]
[^{14}C] Hyaluronate	Skin fibroblasts	< 0.1	none	2.7
	Smooth muscle cells	< 0.1	none	2.6
	Hepatocytes	1.8	41	3.4
	Synovial cells	1.6	76	0.5
[^{35}S] Proteoglycans	Skin fibroblasts	8.7	64	5.6
	Smooth muscle cells	7.8	53	5.1
	Hepatocytes	3.2	40	1.9
	Synovial cells	1.2	45	0.03

[a]Expressed as % of added amount (17 000 cpm x ml^{-1})

[b]Expressed as % of pinocytosed amount

[^{35}S]proteoglycans were prepared from skin fibroblast secretions as described (3). Determination of the radioactivity associated with the trypsin-removable pericellular glycosaminoglycan pool (adsorption) and of the radioactivity present as ethanol-insoluble and -soluble material in the cell pellet and as ethanol-soluble radioactivity in the medium (pinocytosis) were made after incubation for 8 h. The values were corrected for 1 mg cell protein. Degradation is defined as ethanol-soluble radioactivity x 100 x pinocytosed radioactivity^{-1}.

and smooth muscle cells. As suggested earlier binding for integration of glycosaminoglycans into the membrane-associated pericellular pool and binding for pinocytotic uptake appear as separate processes (3,9). Extracellular hyaluronate associates

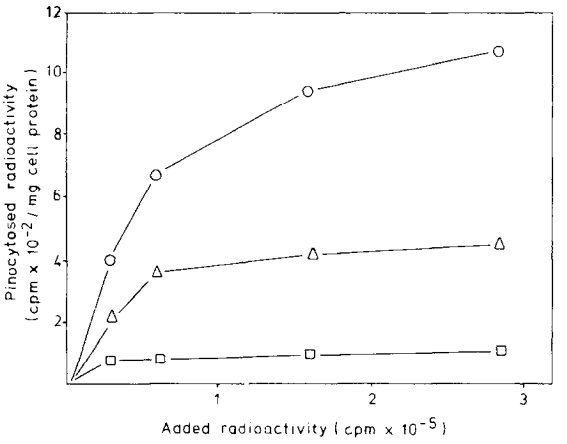

Fig.1. Uptake (O) and degradation of [^{14}C]hyaluronate by rat hepatocytes as a function of hyaluronate concentration.
Measurements were performed after 8 h of incubation. Degraded hyaluronate is represented by the sum of intracellular (Δ) and extracellular (□) ethanol-soluble radioactivity.

with membranes of cells unable for specific endocytosis of that particular glycosaminoglycan (Table 1) in accordance with the fact, that hyaluronate is a normal constituent of the pericellular pool of at least some of those cells (5).

The amount of [^{14}C]hyaluronate which can be internalized by hepatocytes depends on the extracellular concentration. As shown in Fig.1 the uptake of [^{14}C]hyaluronate shows saturation kinetics as expected for adsorptive pinocytosis. By converting the curves according to Lineweaver-Burk the graphic analysis reveals a maximal rate of pinocytosis of 1540 cpm x (mg cell protein x 8 h)$^{-1}$. On the basis of the specific radioactivity of the hyaluronate (3.7×10^5 cpm x mg^{-1}) and the cell number (1.9×10^6 x mg protein^{-1}) it was calculated that on an average a single hepatocyte internalizes up to 0.2 pg hyaluronate per hour.

Pinocytosed hyaluronate is rapidly degraded. In contrast to the

liberated radiosulfate from internalized [^{35}S]proteoglycans, which is mainly found extracellularly, most of the ethanol - soluble hyaluronate degradation products are found within the cells (84 to 86 %).

The adsorption of hyaluronate onto the cell surface is also saturable. From the double-reciprocal plot, a maximum of 4350 cpm x mg cell protein^{-1} corresponding to 4.5 pg/cell might be bound to the cell membrane.

Competition experiments were performed by incubating hepatocytes in the presence of [^{14}C]hyaluronate and unlabelled hyaluronate oligosaccharides. As it is shown in Tab.2 all oligosaccharides tested compete at a concentration of 0.3 mg x ml^{-1} medium for uptake and adsorption of [^{14}C]hyaluronate, but they are less effective than unlabeled hyaluronate at the same concentration. On a molar basis hexa- and octasaccharides are the most efficacious competitors for uptake among the saccharide fragments.

[^{14}C]hyaluronate oligosaccharides, however, are not internalized by hepatocytes when they are added at a concentration of 46 µg x ml^{-1} (17000 cpm x ml^{-1}) to the culture medium. They can be bound to the cell membrane, but we found only between 0.3 and 1.0% of the added material within the pericellular pool. Shortage of material prevented the application of higher doses. Though we cannot exclude the possibility that the addition of a sevenfold higher dose of oligosaccharides would lead to their internalization by adsorptive pinocytosis it seems more likely that vesicle formation will be induced only after interaction of hyaluronate with at least two receptor molecules on the cell membrane. The failure of uptake of hyaluronate oligosaccharides would then be explained by the binding of the saccharide fragments to only one receptor molecule.

Table 2

Pinocytosis, degradation and adsorption of [^{14}C]hyaluronate by rat hepatocytes in the presence of unlabeled hyaluronate oligosaccharides.

Unlabeled compound[a]	Pinocytosis[b]	Degradation[c]	Adsorption[b]
None	1.62	59	5.46
Hyaluronate	0.11	74	0.31
Decasaccharide	0.92	77	1.71
Octasaccharide	0.62	65	1.73
Hexasaccharide	0.52	84	0.88
Tetrasaccharide	0.95	71	3.72

[a]The concentration of unlabeled compounds was 0.3 mg x ml^{-1}

[b]Expressed as % of added amount (20 000 cpm x ml^{-1})

[c]Expressed as % of pinocytosed amount

All cultures were processed after 8 h of incubation. Values for pinocytosis and adsorption were corrected for a cell protein content of 1.0 mg.

In summary, our results give evidence that only some types of hyaluronate producing cells can reincorporate that macromolecule by adsorptive pinocytosis in vitro. Whether the inability of skin fibroblasts and arterial smooth muscle cells to internalize hyaluronate is the consequence of tissue culture conditions or whether it reflects the in vivo situation cannot yet be decided. The occurence of adsorptive pinocytosis of hyaluronate by one type of mesenchymal cells, however, supports the latter hypothesis.

ACKNOWLEDGEMENTS

This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich and by the Deutsche Forschungsgemeinschaft (SFB 104). We are indebted to Dr.L.E. Gershenson, Los Angeles, for kindly providing us with hepatocyte cultures and to Dr.H. Greiling, Aachen, for sending us synovial cells.

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