Endocytosis of hyaluronan in rat Kupffer cells

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The binding, uptake and degradation of hyaluronan (HA) labelled with ^3H in its acetyl group were studied in cultured rat Kupffer cells (KC). At 4°C the binding increased with increasing concentrations of HA in the culture medium up to at least $1\text{ }\mu\text{g/ml}$, when saturation occurred. Binding could be prevented efficiently by the addition of an excess of unlabelled HA, and to a lesser extent by chondroitin sulphate and oligosaccharide fragments of HA, consisting of four sugars or more. The labelled HA bound to the cells could be removed by incubating the cells with *Streptomyces* hyaluronidase, or trypsin, indicating that the HA-binding sites are located on the cell surface. At 37°C HA was internalized in a concentration-dependent manner, and degradation products appeared in the supernatant after 1–5 h, depending on the concentration applied. At 50 ng of free HA/ml, each KC accumulated 60 ag of the polysaccharide/min in the first 1 h, and degraded a total amount of 10 fg of HA during an 8 h period. Addition of the negatively charged polysaccharide dextran sulphate reduced binding, and to an even greater extent internalization, of HA in KC, while no effect was observed with dextran. Depletion of intracellular potassium caused a marked reduction in the rate of endocytosis of cell-membrane-associated HA into KC, without affecting binding. Addition of KCl to the culture medium returned endocytosis of [^3H]HA to normal levels. There was no effect on binding and a partial effect on internalization by depletion of bivalent cations or in the presence of EDTA. The degradation of [^3H]HA by KC cultures was abolished in the presence of weak bases, NH_4Cl and chloroquine, supporting the idea that HA is endocytosed into lysosomes prior to degradation. The fluid-phase marker [^{14}C]sucrose was internalized in the cells at much lower rate than was HA. Rates of binding, internalization and degradation of HA in KC point therefore to a specific endocytosis followed by an intracellular degradation to low- M_r compounds. It was estimated that, under physiological conditions, KC only clear a minor proportion of circulating HA.

INTRODUCTION

Hyaluronan (sodium hyaluronate; HA), a polysaccharide consisting of alternating units of glucuronic acid and *N*-acetylglucosamine, is a major component of the extracellular matrix, particularly in soft connective tissue (for a review, see Laurent, 1989).

The non-parenchymal cells of the hepatic sinusoids play an important role as scavengers of macromolecular substances in blood plasma. In the case of the liver endothelial cells (LEC), it has been shown that they take up major components derived from the intercellular matrix by facilitated endocytosis through specific receptors (Smedsrød *et al.*, 1990). That HA is taken up in the liver sinusoids was first described by Fraser *et al.* (1981), and it was subsequently confirmed that LEC are the main site for the removal of HA by Smedsrød *et al.* (1984) and Fraser *et al.* (1985). LEC carry receptors for HA (Smedsrød *et al.*, 1984; Laurent *et al.*, 1986; McGary *et al.*, 1989), and because of the effective clearance, only a low concentration of the polysaccharide is found in the serum, i.e. $<100\text{ ng/ml}$ in healthy adult humans (Engström-Laurent *et al.*, 1985), with a turnover in the range $0.3\text{--}1.0\text{ }\mu\text{g/min}$ per kg body weight (Fraser & Laurent, 1989). Increased levels of circulating HA have been found in many diseases (Engström-Laurent, 1989) and the rates of clearance and metabolism of the polysaccharide by LEC, and perhaps by other non-parenchymal cells under those conditions, are not yet well understood.

Kupffer cells (KC) are thought to constitute the largest population of fixed macrophages in humans and other vertebrate species (for a review see Jones & Summerfield, 1988). These cells are anchored to the endothelial wall and are responsible for the clearance of endotoxin and other substances. Under physiological conditions there has been no evidence for a major involvement of

KC in the metabolism of HA (Smedsrød *et al.*, 1984; Fraser *et al.*, 1985). However, the surface binding of [^3H]HA to 10^6 KC was estimated to be one-third of the binding to 10^6 LEC (Smedsrød *et al.*, 1984). More recently, Dahl *et al.* (1988), after intravenous administration to rats of HA coupled to ^{125}I -labelled tyramine cellobiose, found part of the ligand in the KC population.

There are several reports in the literature on interactions between HA and macrophages. Love *et al.* (1979) found a macrophage-agglutinating factor that consisted of HA. Underhill (1989) observed that most macrophages react with an antibody directed towards a HA-binding protein, although the receptors were notably absent in KC. HA-binding proteins have also been identified in hamster alveolar macrophages (Green *et al.*, 1988) and on cultured J774 macrophages (Gustafson & Forsberg, 1991). Furthermore, it has been demonstrated that HA increases the uptake of opsonized yeast particles in monocytes (Ahlgren & Jarstrand, 1984), and at relatively high concentrations inhibits phagocytosis in monocytes (Forrester & Balazs, 1980). Altogether, these observations have induced us to reinvestigate the role of KC in HA metabolism and we have found them to be active, although to a lesser extent than LEC.

Several methods have recently been developed to block receptor-mediated endocytosis. One of them involves depletion of potassium (Larkin *et al.*, 1983; Madshus *et al.*, 1987), which causes profound changes in the distribution of cytoplasmic clathrin. We have used this method to study whether or not the uptake of HA in KC is affected by blocking endocytosis. Moreover, the effect of dextran sulphate on the rate of internalization of HA in KC was measured; this polysaccharide inhibits phagocytosis in macrophages (Patel *et al.*, 1983; Das *et al.*, 1987).

The acidification of the endocytic pathway is a crucial step for

Abbreviations used: HA, hyaluronan; KC, Kupffer cells; LEC, liver endothelial cells; HSA, human serum albumin; PBS, phosphate-buffered saline (KCl 0.2 g/l , KH_2PO_4 0.2 g/l , NaCl 8 g/l , $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 2.16 g/l , pH 7.4); FITC, fluorescein isothiocyanate.

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the process of ligand–receptor degradation. It has been demonstrated that NH_4Cl and chloroquine cause an elevation of the endosomal and lysosomal pH above that required to trigger fusion, and therefore disturb the degradation cascade (for a review, see Marsh, 1984). The effects of weak bases on the degradation of HA by KC were therefore analysed.

Finally, the rates of internalization of HA in KC were compared with those of the fluid-phase marker [^{14}C]sucrose, a compound which is known to be taken up solely by fluid-phase endocytosis, with no affinity for the cell surface and which is not degradable in lysosomes (Munniksmä *et al.*, 1980).

MATERIALS AND METHODS

Chemicals

Type IV collagenase (400 units of collagenase activity, 65 units of caseinase, 0.43 units of clostripain and < 0.04 units of tryptic activity per mg dry weight), BSA fraction V, NH_4Cl , chloroquine and EDTA were bought from Sigma, St. Louis, MO, U.S.A. Trypsin (1–300; porcine pancreas; 8 units trypsin and ≤ 0.002 units chymotrypsin per mg of protein) was purchased from United States Biochemical Corp., Cleveland, OH, U.S.A. Hyaluronidase from *Streptomyces hyalurolyticus* [2000 turbidity-reducing units (TRU)/mg of protein; proteinase activity < 0.05 units] was obtained from Seikagaku Kogyo Co., Tokyo, Japan. Sephadex G-50, Percoll, dextran sulphates (M_r 2×10^6 and 5×10^5) and dextran (M_r 5×10^5) were obtained from AB Pharmacia, Uppsala, Sweden. Chondroitin sulphate (M_r 2×10^4) was a gift from Åke Wasteson (Department of Cell Biology, Linköping University, Sweden) (Wasteson, 1971). Glutaraldehyde and SDS were from E. Merck, Darmstadt, Germany. Human serum albumin (HSA; 200 mg/ml) was purchased from AB Kabi, Stockholm, Sweden. RPMI 1640 medium and phosphate-buffered saline (PBS) without $\text{Ca}(\text{II})$ and $\text{Mg}(\text{II})$ were purchased from Statens Veterinärmedicinska, Anstalt, Sweden. [$\text{U}-^{14}\text{C}$]Sucrose (code CFB.146; batch 73 A; 22.3 GBq/mmol; 604 mCi/mmol) was from Amersham International, Amersham, Bucks, U.K.

Preparation of KC

Animals were provided with standard laboratory chow and water *ad libitum*, and were killed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (no. 46; September 15th, 1988). Livers were obtained from male Sprague–Dawley rats (delivered in Sweden by ALAB). Animals of body wt. 200–250 g were fasted overnight. Using an *in situ* technique (Berry & Friend, 1969), the livers were perfused with 0.05% (w/v) collagenase for 8 min at 37 °C through the portal vein and inferior vena cava. The liver cells were dispersed and non-parenchymal cells were isolated in a yield of about 200×10^6 cells/liver (Pertoft & Smedsrød, 1987). KC were separated and plated on 2.5% (v/v) HSA + 2.5% (v/v) glutaraldehyde-coated plastic Petri dishes (Laakso & Smedsrød, 1987) with surface areas of 9.6, 4 or 2 cm², at a concentration of $(1.5\text{--}2.0) \times 10^6$ cells/cm². The cultures were incubated at 37 °C with 5% CO_2 in air and a nutrient medium of RPMI 1640 containing 2 mM-L-glutamine, in 0.6, 0.4 and 0.3 ml respectively.

Cell counts and morphology

Microscopy was carried out in Leitz microscopes with phase-contrast and fluorescence optics (Leitz, Wetzlar, Germany). Cell counts in suspension were performed in Bürker chambers. Cells in monolayers were quantified in photomicrographs of measured areas on the culture surfaces. The purity of non-parenchymal cell

preparations was determined by observing the size of cells in a phase-contrast microscope. Contaminating parenchymal cells are recognized by their larger size (Pertoft & Smedsrød, 1987). In the KC cultures, contaminating LEC were visualized by intake of fluorescent fluorescein isothiocyanate (FITC)-labelled ovalbumin (Pertoft & Smedsrød, 1987), and Ito cells were identified by fading autofluorescence of vitamin A when irradiated at 328 nm in the fluorescence microscope (Blouin, 1977). KC were identified by uptake of fluorescent FITC-labelled *Staphylococcus aureus* (Friedman & Joseph Roll, 1987).

^3H -labelled HA, unlabelled HA and oligosaccharides

HA labelled with ^3H in the acetyl group (^3H]HA) was a gift from Dr. J. R. E. Fraser, Department of Medicine, University of Melbourne, Melbourne, Australia. It was prepared by biosynthesis from [^3H]acetate in bovine synovial cell cultures and had an M_r of 6×10^6 and a specific radioactivity of 5.1×10^6 d.p.m./ μg (Fraser *et al.*, 1981). More than 99% of the label was found in hyaluronidase-degradable polymers.

Unlabelled HA in sterile solution [M_r $(2\text{--}3) \times 10^6$; 10 mg/ml; Healon] was a gift from AB Pharmacia, Uppsala, Sweden.

Preparation of oligosaccharides of HA has been described (Smedsrød *et al.*, 1984).

Binding experiments at 4 °C

Increasing concentrations of [^3H]HA (and in competition experiments, unlabelled HA) were added to cold RPMI medium containing 0.1% (w/v) BSA. Aliquots of 0.4 ml were added to dishes (4 cm²) containing 5×10^4 KC per cm², and the cells were incubated for 16 h at 4 °C. After termination of the incubations, the medium was removed and the cells were washed four times in RPMI 1640. Each cell layer was then dissolved in 2×0.4 ml of 0.3 M-NaOH/1% (w/v) SDS. After 1 h at room temperature, the extract was neutralized with HCl (2 mol/litre), and added to 10 ml of Beckman Ready-Safe scintillant. Radioactivity was determined in a Pharmacia LKB Wallac Scintillation counter. Background values of 0.05, 0.2 and 1 μg of [^3H]HA/ml added to dishes without cells (0.4 ml) were approx. 35, 50 and 65 d.p.m., respectively. From now on, 'cell-bound radioactivity' refers to experiments at 4 °C. Unless specified otherwise, the background value in all experiments at 4 °C or 37 °C was determined as the amount of label retained on the culture dishes in the absence of cells.

Localization of [^3H]HA bound to KC

Six 4 cm² cultures of KC were incubated at 4 °C for 2 h in RPMI medium containing 0.2 μg of [^3H]HA/ml. The medium was removed and the cell layers were washed four times with RPMI. Two dishes were used for measuring binding of [^3H]HA to the cells (see above). Another two dishes were incubated at 4 °C for 2 h and then exposed to *Streptomyces hyaluronidase* for 30 min at 4 °C, washed and analysed for bound [^3H]HA material. Finally, two dishes were kept at 37 °C for 10 min in the absence of hyaluronidase, followed by treatment with hyaluronidase as above before analysis.

Experiments at 37 °C

KC cultures were incubated with [^3H]HA in RPMI 1640 medium containing 0.1% (w/v) BSA. Aliquots of 0.3 ml were added to 2 cm² dishes, which were kept at 37 °C for up to 8 h. After termination of the incubations, the medium was removed and the cells were washed and analysed for radioactivity as described above. From now on, 'cell-associated radioactivity' refers to experiments at 37 °C. The media from the 1, 2, 3, 5 and 8 h time points were checked for the presence of degradation products of HA by chromatography on Sephadex G-50. A

1 cm × 10 cm column packed in PBS at a flow rate of 20 ml/h was used. The radioactive material that had moved from the V_0 position to an included position was regarded as degraded.

Inhibition of degradation of HA by weak bases

KC were seeded in 4 cm² dishes (0.4 ml) and incubated at 37 °C with 0.25 µg of [³H]HA/ml in the absence or the presence of 10 mM-NH₄Cl or 100 µM-chloroquine (Magnusson & Berg, 1989). After an 8 h period, the supernatant was removed and the cells were washed twice with RPMI. Supernatants and washing media from each experiment were pooled and checked for the presence of degradation products as described above.

Inhibition of binding and internalization of HA in KC

KC were seeded in 4 cm² dishes and incubated for 60 min at 37 °C (internalization studies) or for 120 min at 4 °C (binding studies) with 0.2 µg of [³H]HA/ml in the absence or presence of the following compounds (50 µg/ml): chondroitin sulphate (M_r 2 × 10⁴), dextran (M_r 5 × 10⁵), dextran sulphates (M_r 5 × 10⁵ and 2 × 10⁶) or HA-oligosaccharides of increasing size (HA-4, HA-6, or HA-10 containing 4, 6 or 10 monosaccharide residues respectively). Cell-associated and cell-bound radioactivities were calculated as above.

Exposure of KC to trypsin

KC were cultivated in 4 cm² dishes and exposed to trypsin (0.025 % or 0.050 %) for 15 min at 37 °C. The medium was removed and the cells were washed extensively with RPMI, followed by incubation with 0.2 µg of [³H]HA/ml for 60 min at 37 °C (internalization studies) or for 120 min at 4 °C (binding studies). Cell-associated and cell-bound radioactivities were calculated as above.

Protocols for preparing K⁺-depleted KC

The buffers used were as follows. Buffer A: 50 mM-Hepes, 100 mM-NaCl, 0.1 % (w/v) BSA, pH 7.4. Buffer B: 50 mM-Hepes, 100 mM-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 1 mg of glucose/ml, 0.1 % (w/v) BSA, pH 7.4. Buffer C: 50 mM-Hepes, 100 mM-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 1 mg of glucose/ml, 0.1 % (w/v) BSA, 10 mM-KCl, pH 7.4. Hypo-osmotic medium consisted of RPMI/water (1:1, v/v).

Cells were depleted of K⁺ at 37 °C using the hypo-osmotic shock procedure (Larkin *et al.*, 1983). Briefly, KC monolayers were incubated for 5 min in 0.4 ml of hypo-osmotic medium, followed by a 10 min incubation in buffer A. The medium was discarded and the cells were incubated at 37 °C for 15 min in 0.4 ml of buffer B, followed by a 60 min incubation with 0.2 µg of [³H]HA/ml or 0.2 µg of [¹⁴C]sucrose/ml dissolved in buffer B. In one experiment, K⁺-depleted cells were reincubated in the presence of 10 mM-KCl (buffer C) for 30 min before adding [³H]HA in buffer C for 60 min. Cell-associated radioactivity was measured as described above.

To deplete cells of K⁺ at 4 °C, each KC monolayer was subjected to the following sequential incubations: (a) 5 min in hypo-osmotic medium at 37 °C; (b) 10 min buffer A at 37 °C; and (c) 120 min in buffer B containing labelled HA (0.2 µg/ml) at 4 °C. Cell-bound radioactivity was measured as described above.

The content of intracellular K⁺ was determined by flame photometry (Larking *et al.*, 1983).

Experiments with Ca²⁺- and/or Mg²⁺-depleted buffers

The buffers used were as follows. Control buffer: 50 mM-

Hepes, 100 mM-NaCl, 10 mM-KCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 1 mg of glucose/ml, 0.1 % (w/v) BSA, pH 7.4. Ca²⁺-depleted buffer: 50 mM-Hepes, 100 mM-NaCl, 10 mM-KCl, 1 mM-MgCl₂, 1 mg of glucose/ml, 0.1 % (w/v) BSA, pH 7.4. Mg²⁺-depleted buffer: 50 mM-Hepes, 100 mM-NaCl, 10 mM-KCl, 1 mM-CaCl₂, 1 mg of glucose/ml, 0.1 % (w/v) BSA, pH 7.4. Ca²⁺- and Mg²⁺-depleted buffer: 50 mM-Hepes, 100 mM-NaCl, 10 mM-KCl, 1 mg of glucose/ml, 0.1 % (w/v) BSA, pH 7.4.

In experiments performed at 37 °C, KC monolayers seeded in 4 cm² dishes were incubated for 15 min in 0.4 ml of medium in the presence or absence of Ca²⁺ and/or Mg²⁺, followed by a 60 min incubation with labelled HA (0.2 µg/ml) diluted in the respective buffers. At 4 °C, cells were incubated as described above followed by a 120 min incubation with labelled HA (0.2 µg/ml) diluted in the respective buffers at 4 °C for analysis of cell-bound radioactivity. In parallel experiments, KC were incubated with 0.2 µg of labelled HA/ml in the absence or presence of 5 mM-EDTA at 4 °C (2 h) or 37 °C (1 h). In all experiments, cell-bound radioactivity (4 °C experiments) and cell-associated radioactivity (37 °C experiments) were measured as above.

Measurements of fluid-phase pinocytosis

Culture medium (0.6 ml) containing [¹⁴C]sucrose at 0.2 µg/ml was added to KC for different periods of incubation. The medium was then removed and the cells were washed four times with RPMI. Each cell layer was then analysed as described above. The radioactivity measurements (¹⁴C) were performed in a Pharmacia LKB Wallac Scintillation counter. The rates of accumulation of [¹⁴C]sucrose by the cells were calculated after subtracting background values in the solubilizer solution (of the order of 30 c.p.m.).

RESULTS

Liver perfusion and KC preparation

Rat livers were perfused *in situ* and cells were completely dispersed by the collagenase technique. The non-parenchymal cells were then purified from contaminating parenchymal cells and red blood cells by density gradient centrifugation in Percoll. Pure KC were obtained after 30 min of cell attachment to HSA/glutaraldehyde-coated culture dishes. The yield of cultured KC was about 4 × 10⁶ cells/liver, whereas that of KC cultured on to uncoated dishes was about 2.5 × 10⁶ cells/liver. Cells were cultivated in the absence of serum and antibiotics as described in the Materials and methods section.

Binding of HA to KC

KC cultivated in the absence of serum were able to bind [³H]HA when incubated for 16 h at 4 °C. As demonstrated in Fig. 1 (see also Table 3), the amount of HA bound increased with increasing concentration of added HA up to 1–2 µg/ml. At this concentration, the binding sites on KC appeared to be saturated with HA. In competition experiments with 50 µg of unlabelled HA/ml mixed with increasing concentrations of [³H]HA, there was an efficient inhibition of the binding to KC (Fig. 1; see also Table 5), suggesting that the uptake was receptor-mediated (Smedsrød *et al.*, 1990). There were approx. 500 molecules bound/cell at saturation, and half-maximal binding was calculated to be 0.19 µg/ml.

Location of bound HA

To determine whether the bound HA was intracellular or located on the cell surface, cells were first incubated with labelled HA. Table 1 demonstrates that up to 80 % of HA bound to the

Fig. 1. Binding of [^{3} H]HA to KC

KC were incubated with increasing concentrations of [^{3} H]HA in the absence (●) or presence (○) of 50 μ g of unlabelled HA/ml overnight at 4 °C. Cell-bound radioactivity was measured as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of triplicate assays.

Table 1. Internalization of [^{3} H]HA bound to the cell surface

KC were seeded in 4 cm² dishes and incubated with 0.2 μ g of [^{3} H]HA/ml at 4 °C. The radioactivity associated with the cell layer was determined after the treatment with 1 unit of *Streptomyces* hyaluronidase/ml before or after incubation at 37 °C. Results are expressed as percentages of the values in control cells incubated without enzyme. Each value represents the mean $\pm$ S.E.M. of duplicate assays.

Treatment	Cell-associated [^{3} H]HA (% of control)
Incubation with [^{3} H]HA at 4 °C	100.0 $\pm$ 11.0
Incubation with [^{3} H]HA at 4 °C followed by hyaluronidase treatment	20.3 $\pm$ 8.8
Incubation with [^{3} H]HA at 4 °C followed by incubation at 37 °C for 10 min (without hyaluronidase) and hyaluronidase treatment	45.6 $\pm$ 6.2

Table 2. Effect of trypsin on the binding and internalization of [^{3} H]HA to KC

KC seeded in 4 cm² dishes were exposed to 0.025 % or 0.050 % trypsin for 15 min at 37 °C in a volume of 0.4 ml. Cells were then washed extensively and incubated with 0.2 μ g of [^{3} H]HA/ml for 2 h at 4 °C or for 1 h at 37 °C. Cell-bound radioactivity (4 °C experiments) and cell-associated radioactivity (37 °C experiments) were measured as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of quadruplicate assays.

Treatment	Cell-associated radioactivity (% of control)	
	Binding (4 °C)	Internalization (37 °C)
None	100.0 $\pm$ 13.5	100.0 $\pm$ 9.2
Trypsin (0.025 %)	44.8 $\pm$ 5.1	28.0 $\pm$ 3.4
Trypsin (0.050 %)	16.2 $\pm$ 1.9	12.1 $\pm$ 2.2

cells in the cold can be removed by hyaluronidase treatment. After 10 min at 37 °C (in the absence of hyaluronidase), about half of the material becomes insensitive to extracellular hyaluronidase treatment, indicative of an internalization of HA.

Table 3. Cell-associated and degraded [^{3} H]HA in KC cultures at 37 °C

KC were cultivated (2 cm² dishes) in a volume of 0.3 ml and incubated with increasing concentrations of [^{3} H]HA, i.e. 0.05 μ g/ml (A), 0.2 μ g/ml (B), 1 μ g/ml (C) or 5 μ g/ml (D). The cells were harvested after various times of incubation at 37 °C and washed. Cell-associated HA and degradation products in the medium were measured as described in the Materials and methods section. Each value represents the mean of triplicate assays. The variation between the determinations was about 10 %.

Incubation time (h)	Cell-associated HA (ng/mg of cell protein)				Degraded HA (ng/mg of cell protein)			
	A	B	C	D	A	B	C	D
1	33	112	188	380	0	0	54	224
2	30	84	103	336	0	54	147	540
3	28	81	102	328	0	146	317	1263
5	23	52	92	272	17	152	559	1917
8	23	34	87	259	84	261	1074	3148

Table 4. Effect of NH₄Cl and chloroquine on the degradation of [^{3} H]HA by Kupffer cell cultures

KC cultivated in 4 cm² dishes (0.4 ml) were incubated with 0.25 μ g of [^{3} H]HA/ml in the presence or absence of 10 mM-NH₄Cl or 100 μ M-chloroquine for 8 h at 37 °C. Degradation products in the medium were measured as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of duplicate assays.

Agent	Degraded [^{3} H]HA (% of control)
None	100.0 $\pm$ 4.9
NH ₄ Cl	4.8 $\pm$ 3.7
Chloroquine	6.5 $\pm$ 2.8

Trypsin also inhibited the binding and internalization of labelled HA to cells (Table 2). These results are consistent with the cell surface location of the HA-binding site, and suggest that this site may be a protein.

Internalization and degradation of HA by cultured KC

To evaluate the capacity of KC to endocytose HA, 0.05–5.0 μ g of [^{3} H]HA/ml was applied, and the cells were incubated for up to 8 h. Cell-associated [^{3} H]HA and degraded products in the culture medium were analysed (Table 3). Cell-associated HA decreased and degraded HA increased with time and with increasing concentrations of [^{3} H]HA in the culture medium. Comparative experiments demonstrated that the amount of degraded HA present in the supernatant of KC cultures was 4–5 times lower than in LEC cultures (calculated per mg of cell protein) (Smedsrød, *et al.*, 1984).

Inhibition of degradation of HA by weak bases

To eliminate the possibility that any surface-associated hyaluronidase could be involved in the release of HA-associated oligosaccharides, the effects of NH₄Cl and chloroquine on the degradation of HA by KC were examined. These compounds are known to prevent the acidification of intracellular vesicles and thereby inhibit the enzymic activity of acid hydrolases (Mellman *et al.*, 1986). As shown in Table 4, both of these agents almost completely abolished the degradation of [^{3} H]HA by Kupffer cells.

Table 5. Effects of uncharged and anionic molecules on the binding and internalization of [³H]HA by Kupffer cells

KC were cultured in 4 cm² dishes with [³H]HA (0.2 µg/ml) in a volume of 0.4 ml as described in the Materials and methods section. Cells were exposed to the labelled HA in the presence or absence of 50 µg of inhibitor/ml for 2 h at 4 °C or for 1 h at 37 °C. Cell-bound radioactivity (4 °C experiments) and cell-associated radioactivity (37 °C experiments) were measured as described in the Materials and methods section. Each value represents the mean ± S.E.M. of duplicate or triplicate assays.

Treatment of cells	Cell-associated radioactivity (% of control)	
	Binding (4 °C)	Internalization (37 °C)
None	100.0 ± 18.5	100.0 ± 12.3
Uncharged molecule		
Dextran (<i>M_r</i> 2 × 10 ⁵)	90.3 ± 6.5	98.4 ± 3.9
Anionic molecules		
Hyaluronan [<i>M_r</i> (2–3) × 10 ⁶]	< 5	20.3 ± 3.8
Oligosaccharides*		
HA-4	66.3 ± 5.9	98.0 ± 0.9
HA-6	49.5 ± 6.1	82.4 ± 3.3
HA-10	57.5 ± 4.7	80.9 ± 8.6
Chondroitin sulphate (<i>M_r</i> 2 × 10 ⁴)	53.2 ± 6.8	41.4 ± 2.9
Dextran sulphate (<i>M_r</i> 2 × 10 ⁶)	51.0 ± 2.9	31.3 ± 4.0
Dextran sulphate (<i>M_r</i> 5 × 10 ⁵)	52.0 ± 5.8	27.2 ± 3.9

* The oligosaccharides are denoted by the number of monosaccharide units/molecule.

Table 6. Internalization of [³H]HA and [¹⁴C]sucrose in Kupffer cells after intracellular K⁺ depletion

KC seeded in 4 cm² dishes were used to study uptake of 0.2 µg of [³H]HA/ml or of 0.2 µg of [¹⁴C]sucrose/ml in a volume of 0.4 ml without K⁺. After a 5 min exposure to hypo-osmotic shock, followed by 10 min in the presence of buffer A and 15 min exposure to K⁺-depleted buffer (buffer B), [³H]HA or [¹⁴C]sucrose, both in buffer B, was added for further 60 min at 37 °C. Cell-associated radioactivity (37 °C) was measured as described in the Materials and methods section. Control cells were exposed to buffer C during the experimental procedure. Each value represents the mean ± S.E.M. of triplicate assays.

Buffer used	Cell-associated radioactivity (% of control)	
	[³ H]HA	[¹⁴ C]Sucrose
Control	100.0 ± 7.9	100.0 ± 9.4
K ⁺ -depleted	43.1 ± 6.1	103.2 ± 2.3

Inhibition of binding and internalization by uncharged and anionic molecules

Table 5 demonstrates that negatively charged polymers inhibited both binding to and internalization in KC of labelled HA. Unlabelled HA was by far the most effective inhibitor of both binding and internalization of [³H]HA. The dextran sulphates and the chondroitin sulphate, which are highly negatively charged polymers, decreased binding to 50 %, and internalization to an even greater extent. The neutral dextran had no effect. A partial decrease in cell-bound HA was found with HA-

Table 7. Binding and internalization of [³H]HA to Kupffer cells after Ca²⁺ and/or Mg²⁺ depletion and after EDTA exposure

KC were seeded in 4 cm² dishes in 0.4 ml of medium to analyse the effect of bivalent cations and EDTA on the binding and uptake of [³H]HA (0.2 µg/ml), as described in the Materials and methods section. Control cells were exposed to control buffer during the experimental procedure. Cell-bound radioactivity (4 °C experiments) and cell-associated radioactivity (37 °C experiments) were measured as described. Each value represents the mean ± S.E.M. of triplicate assays.

Treatment	Cell-associated radioactivity (% of control)	
	Binding (4 °C)	Internalization (37 °C)
Control buffer	100.0 ± 6.9	100.0 ± 12.6
Ca ²⁺ -depleted	121.4 ± 4.8	105.3 ± 6.6
Mg ²⁺ -depleted	92.9 ± 7.4	79.0 ± 8.3
Ca ²⁺ - and Mg ²⁺ -depleted	100.4 ± 5.2	63.1 ± 7.7
EDTA (5 mM)	98.9 ± 11.3	68.7 ± 4.1

oligosaccharides of different sizes, but there was only a small effect on intracellular HA uptake.

Effect of K⁺ depletion on the association of HA with KC

To determine whether the uptake was mediated by specific surface structures, the cells were depleted of intracellular K⁺ by a method involving hypo-osmotic shock and subsequent incubation in the absence of K⁺. After a 5 min hypo-osmotic shock treatment, followed by a 15 min incubation in K⁺-free buffer, there was a 60 % drop in intracellular K⁺. Therefore we used these conditions to study the initial stages of binding and internalization of HA. Table 6 shows that the uptake of HA at 37 °C was markedly decreased in the absence of K⁺, whereas no effect was found on sucrose internalization. Moreover, addition of KCl to the culture medium, as well as restoring intracellular K⁺ levels, also restored endocytosis of HA to normal levels (results not shown). There was a slight increase in the surface binding of both HA (15 %) and sucrose (10 %) to KC during K⁺ depletion at 4 °C.

Effect of Ca²⁺ and/or Mg²⁺ depletion and of EDTA on the association of HA with KC

To investigate the role of bivalent cations on the binding and internalization of [³H]HA, KC were incubated in the presence or absence of Ca²⁺ and/or Mg²⁺ or with 5 mM-EDTA at 4 °C and 37 °C respectively. Results shown in Table 7 demonstrate no effect on binding but a partial effect on the internalization of HA, especially after Mg²⁺ depletion.

Fluid-phase pinocytosis by KC

The amount of [¹⁴C]sucrose taken up by KC from a solution containing 0.2 µg of the sugar/ml is compared in Fig. 2 with the amount of HA accumulated from solutions 0.05, 0.2 and 1 µg of HA/ml. Sucrose is known to be taken up by fluid-phase pinocytosis, and the intracellular concentration appeared to have reached a steady state after 15 min, when the cells contained in the order of 500–600 pg of the sugar/mg of cell protein (Fig. 2a). The comparable uptake of HA from a 0.2 µg/ml solution after 15 min was about 30 ng/mg of cell protein (Fig. 2b), i.e. about 50 times greater than the uptake of sucrose. The accumulation of HA had not reached a steady state after 15 min.

Fig. 2. Internalization of $[^{14}\text{C}]$ sucrose (a) and $[^3\text{H}]$ HA (b) by KC during the first hour of incubation at 37 °C

The uptake of $[^{14}\text{C}]$ sucrose was followed in a medium containing 0.2 μg of the sugar/ml. The uptake of $[^3\text{H}]$ HA was measured in media containing 0.05 μg (●), 0.2 μg (□) or 1 μg (▲) of the polysaccharide/ml. Cell-associated radioactivity was measured as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of quadruplicate assays.

DISCUSSION

The following conclusions could be drawn from these experiments: (a) HA binds to the surface of KC; (b) the binding sites seem to be extracellular; (c) the polysaccharide is internalized and degraded by the cells at lower rates than in LEC; (d) the mechanism of internalization, at least when high- M_r HA is under study, points to a receptor-mediated phagocytic event; and (e) KC may play an important role in the clearance of HA in various diseases associated with increased plasma levels.

In our experiments cell-receptor damage was presumably low, since only collagenase was used to disperse the cells and monolayers of KC were cultured in the absence of serum and antibiotics. The experiments were performed 1 h after seeding the KC because previous studies have demonstrated a decline in HA metabolism in LEC after overnight culture (Eriksson *et al.*, 1983; Raja *et al.*, 1988). Markers used to test cell function and to identify KC showed $\leq 5\%$ contamination by other cell types.

The binding curve in Fig. 1 shows 50% saturation at approx. 200 ng of free HA/ml. This value exceeds by 3–10-fold the half-saturation point of the LEC receptor (Smedsrød *et al.*, 1984; Laurent *et al.*, 1986) and the range of the physiological plasma concentrations of HA found in humans and rats (Engström-Laurent *et al.*, 1985). When an excess of unlabelled HA was included in incubations with KC, the binding effectively ceased (Fig. 1, Table 5), showing that a specific membrane structure (receptor) recognizes the HA. The labelled HA bound to the cells at 4 °C could to a large extent be removed by *Streptomyces* hyaluronidase or by trypsin, indicating that the HA-binding sites

are located on the cell surface and are protein in nature (Tables 1 and 2). The different affinities for HA displayed by LEC and KC make it probable that the two cell types carry different types of receptors and that LEC are of greater importance for the physiological HA metabolism, while the importance of KC increases at pathologically high plasma concentrations of HA.

At 37 °C, $[^3\text{H}]$ HA was captured in a concentration-dependent manner and the binding was followed by internalization and degradation (Table 3). At the physiological concentration found *in vivo*, i.e. 50 ng of HA/ml, the rate of clearance of the polysaccharide was approx. 2 pl/min per cell during the first hour of incubation, which is within the same range of clearance rates found in LEC cultures by Smedsrød *et al.* (1984). KC apparently have a higher rate of phagocytosis than LEC, which compensates for the lower surface binding. However, the similarity in clearance *in vitro* does not imply that the two cell populations are equally important *in vivo*. By assuming that the KC population from an adult rat contains 114 μg of protein/ 10^6 cells (Knook & Sleyster, 1980), one can estimate a clearance of approx. 20 μl of culture medium/min per mg of cell protein. The liver of a 250 g rat weighs ~ 8.4 g (Weibel *et al.*, 1969) and contains 15×10^6 KC/g of liver (Knook & Sleyster, 1980), which means that a total volume of 17 ml of blood should be cleared of HA per h by the resident KC in the rat liver, if these cells are equally active *in vivo* and *in vitro*. By assuming the same cell content per g of human liver (1500 g), we can calculate the number of KC to be approx. 2.25×10^{10} . According to our experiments *in vitro*, KC could then turn over 1.4 μg of HA/min, i.e. 6-fold less than the LEC population (Smedsrød *et al.*, 1984). It is, however, conceivable that uptake of HA in KC is even less *in vivo* than *in vitro*, as the KC in culture are spread out on the substrate and expose a larger surface area than *in vivo*.

The KC can effectively degrade HA to low- M_r products (Table 3). It has previously been shown by Gustafson & Forsberg (1991) that a murine macrophage cell line also degrades the polysaccharide. The ability of KC to degrade HA is consistent with the presence of hyaluronidase in KC as well as in LEC (L. Rodén, personal communication) and also of the enzyme *N*-acetylglucosamine 6-phosphate deacetylase, which is required for the formation of free acetate from the acetyl groups of HA (Campbell *et al.*, 1990).

The results in Table 4 show that NH_4Cl and chloroquine are capable of blocking the degradation of labelled HA, which supports the notion that HA is endocytosed prior to degradation. This is in agreement with a recent study performed by Culty *et al.* (1992), who clearly demonstrated that the degradation of $[^3\text{H}]$ HA by hamster pulmonary macrophages was blocked in the presence of the two acidotropic agents.

In order to characterize the uptake mechanism of HA in KC, we have compared it with the fluid-phase pinocytosis of $[^{14}\text{C}]$ sucrose. During the first hour of uptake (Fig. 2a), one can calculate that the rate of clearance of sucrose from the medium was 5 fl/min per cell. Pratten *et al.* (1977) found only 0.4 fl/min per cell in rat peritoneal macrophages, and Kaplan & Nielsen (1978) obtained a value of 1.4 fl/min per cell in rabbit alveolar macrophages. In spite of the relatively high rate of fluid-phase pinocytosis in KC, it is still one or two orders of magnitude lower than the clearance of HA, which can be calculated from Fig. 2(b) to be between 0.5 and 2 pl/min per cell for the different concentrations of HA. The experiment confirms that HA is taken up in the KC by a specific mechanism.

Dextran sulphate has been shown to be an effective blocking agent of hepatic phagocytosis *in vivo*. Souhami & Bradfield (1974) studied the effects of dextran sulphate administered intravenously to mice on the hepatic uptake of ^{51}Cr -labelled sheep red blood cells. They observed a profound decrease in

hepatic phagocytosis, also observed by others when dextran sulphate was given intraperitoneally to mice (Patel *et al.*, 1983) and rats (Das *et al.*, 1987), and rates of liver uptake of liposomes were calculated. Dextran sulphate was also effective in protecting perfused rat livers from phagocytosing malarial sporozites (Smith & Sinden, 1982). Thus the blockade observed with dextran sulphate in our experiments (Table 5) points to a general inhibition of the phagocytic mechanism involved in the internalization of HA by KC. The phagocytosis was inhibited to a greater extent than the binding. The uncharged dextran molecule showed no inhibition of either binding or internalization (Table 5), indicating that the charges on dextran sulphate are responsible for the inhibition of HA uptake. Chondroitin sulphate behaved similarly to dextran sulphate, and it is conceivable that its effect is due to an unspecific charge effect than a specific similarity to HA. The HA-oligosaccharides, however, inhibited binding more than internalization, in accordance with a mechanism of competition with a specific receptor.

Most receptor-mediated endocytosis occurs through invaginations or depressions in the plasma membrane, named coated pits, in which the cytoplasmic surface is enriched with a protein termed clathrin. In macrophages the percentage of the surface area capable of being coated is high, of the order of 15% (Aggeler & Werb, 1982). Several authors have reported that K⁺ depletion reduces the endocytosis of various ligands via coated pits. Larkin *et al.* (1983) demonstrated that depletion of intracellular K⁺ caused a profound reduction not only in the number of coated pits, but also in the rate of endocytosis of low-density lipoprotein and epidermal growth factor in human fibroblasts, despite normal or increased receptor binding. They also observed a marked decrease in fluid-phase pinocytosis in those cells as measured by uptake of horseradish peroxidase. More recently, similar results by Madhus *et al.* (1987) also demonstrated a reduction in the number of coated pits at the cell surface and an inhibition of endocytosis of transferrin in Hep 2 cells. In the present investigation, intracellular K⁺ was depleted very quickly when KC were subjected to hypo-osmotic shock prior to incubation in K⁺-free buffer. Rates of internalization of HA were markedly decreased, but this was reversed by the addition of KCl to the culture medium (Table 6). Furthermore, Table 6 demonstrates that the K⁺ depletion procedure did not affect the internalization of the fluid-phase marker [¹⁴C]sucrose by KC. The results reinforce the concept of a receptor-mediated mechanism for the uptake of HA by KC.

As shown in Table 7, bivalent cations were not required for the binding of HA to KC. This finding is in agreement with earlier reports on HA binding to SV-3T3 cells (Underhill & Toole, 1979). Mg²⁺ is the major intracellular bivalent cation, and HA internalization at 37 °C in KC seems to be more sensitive to the absence of this cation in the culture medium than to the absence of Ca²⁺. In another study, Stossel (1973) demonstrated that bivalent cations were not required for cultured macrophages to bind particles, and Mg²⁺ especially played an important role in subsequent ingestion. EDTA treatment or removal of Ca²⁺ and/or Mg²⁺ did not affect the internalization of [¹⁴C]sucrose by KC (results not shown). Previously, Besterman *et al.* (1981) showed an unaffected accumulation of [¹⁴C]sucrose by guinea pig alveolar macrophages in the absence of extracellular Ca²⁺ and Mg²⁺.

Recently it has been shown that HA is internalized at very low rates in hepatocytes, indicating a non-coated pit pathway, probably via a general membrane pinocytosis (Frost *et al.*, 1990). The high affinity of HA towards the LEC receptor *in vivo* could to some extent obscure the interaction of HA with KC and even other liver cell populations. An important question is whether the uptake *in vivo* compares with that *in vitro*. Of special interest

will be the role of KC in the uptake of HA under pathological conditions.

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