The Hyaluronan Receptor (CD44) Participates in the Uptake and Degradation of Hyaluronan

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Abstract. The hyaluronan receptor belongs to the polymorphic family of CD44 glycoproteins, which have been implicated in a variety of cellular functions including adhesion to hyaluronan and collagen, the binding of lymphocytes to high endothelial cells during extravasation, and conferring metastatic potential to carcinoma cells. Here, we demonstrate that the receptor also participates in the uptake and degradation of hyaluronan by both transformed fibroblasts (SV-3T3 cells) and alveolar macrophages. These cells were incubated with isotopically labeled hyaluronan for various periods of time, and the extent of degradation was determined by either molecular-sieve chromatography or centrifugation through Centricon 30 microconcentrators. The macrophages degraded the hyaluronan at a faster rate than the SV-3T3 cells, which may reflect the fact that they contained a greater number of receptors. More importantly, in both cell types, the degradation of hyaluronan was specifically blocked by antibodies directed against the receptor. However, the receptor by itself did not have the ability to degrade hyaluronan, since preparations of SV-3T3 membranes containing the receptor did not break down hyaluronan. Subsequent experiments revealed that macrophages can internalize fluorescein-tagged hyaluronan, and this process was blocked by antibodies against the receptor. Furthermore, the subsequent degradation of hyaluronan was inhibited by agents that block the acidification of lysosomes (chloroquine and NH_4Cl). Thus, the most likely explanation for these results is that the receptor mediates the uptake of hyaluronan into the cell where it can be degraded by acid hydrolases in lysosomes. The ability of cells expressing the receptor to degrade hyaluronan may be important during tissue morphogenesis and cell migration.

THE hyaluronan receptor is a cell-surface glycoprotein of 85–100 kD, which recognizes a six sugar sequence of hyaluronan and mediates the divalent-cation independent aggregation of cells (32, 33, 34, 35, 39). Indeed, mAbs to this receptor specifically block the hyaluronan-induced aggregation of both cultured fibroblasts and macrophages (12). Biochemical studies of this receptor have revealed that it is an elliptical glycoprotein which is phosphorylated and has a high negative charge (4, 33, 39). In addition, its cytoplasmic domain appears to be associated with actin filaments (16), perhaps through an ankyrin-like molecule (15). This receptor is present on a wide variety of cells including most epithelial cells, macrophages and lymphocytes (1, 11, 33). Interestingly, in the case of epithelia, it is preferentially expressed on those cells undergoing active cell division (1).

Recently, a number of studies have shown that the hyaluronan receptor is identical to CD44, which has been implicated in the homing of lymphocytes to mucosal lymphoid tissues (2, 4, 19, 22). CD44 on the surfaces of lymphocytes binds to a protein termed mucosal addressin, which is present on the high-endothelial cells of gut-associated lymphoid tissues (14, 23, 28, 30). However, the ability of CD44 to bind addressin is distinct from its ability to bind hyaluronan. Indeed, sequence analysis of the receptor (CD44) molecule has revealed that its extracellular domain is composed of two distinct regions; an NH_2-terminal region, which is homologous to the link protein of cartilage and presumably mediates the binding of hyaluronan, and a more medial region, which is responsible for the binding to vascular addressin (4, 28). Thus, the hyaluronan receptor (CD44) is an example of a protein which can bind to multiple ligands and carry out different functions.

In this study, we present evidence that this protein also participates in the degradation of hyaluronan. More specifically, cultured fibroblasts and macrophages can degrade hyaluronan, and this process is blocked by antibodies directed against the receptor. This ability to degrade hyaluronan may be an important factor during embryonic development and other processes in which hyaluronan is lost from the matrix in a highly ordered fashion.

Materials and Methods

Assay for [^{3}H]Hyaluronan Degradation

The mouse SV-3T3 cells were grown and cultured in 10% NuSerum (Collaborative Research, Lexington, MA), 90% DME, plus 100 U/ml penicillin

and 100 μg/ml streptomycin as described previously (40), while the alveolar macrophages were collected by lavage of lungs from adult hamsters (Charles River, Wilmington, MA) and maintained in 10% FCS, 90% McCoy's medium plus 50 μg/ml gentamicin. In initial experiments, the cells were cultured in media containing [^{3}H]hyaluronan (2 μg/ml). After 40 h, the medium was removed and the cell layer was digested overnight with 20 mg/ml pronase E (Sigma Chemical Co., St. Louis, MO), which had been predialyzed against distilled water to remove Ca^{++} salts along with NaN_3 as a preservative. The cell layer digest was then recombined with the medium and further incubated for several hours. The digest was lyophilized, reconstituted in 1 ml of calcium- and magnesium-free PBS (CMF-PBS)[1], and applied to a 1.5× 110-cm column of Sephadex G-15 (Pharmacia, Piscataway, NJ). The column was eluted with CMF-PBS and 2-ml fractions were collected. The column was standardized with [^{3}H]glucosamine, [^{3}H]H_2O and various oligosaccharide fragments of hyaluronan which were detected by the uronic acid assay of Bitter and Muir (3).

In subsequent experiments, the extent of [^{3}H]hyaluronan degradation was determined by passage through a size-specific membrane. For this, the cells were suspended in culture medium, and 1-ml aliquots were distributed into a 24 multi-well plate to which was added 2 μg/ml of [^{3}H]hyaluronan along with the various agents to be tested. At the end of the incubation, the cultures were digested with pronase E (200 μl of a 20-mg/ml solution) as described above and applied onto Centricon 30 Microconcentrators (Amicon, Danvers, MA). After centrifugation, the material passing through the membrane was collected and processed for scintillation counting. Control experiments demonstrated that this protocol gave similar results to molecular-sieve chromatography for determining the degradation of [^{3}H]-hyaluronan. In each case, the background level of degradation was determined by incubating [^{3}H]hyaluronan in a cell-free medium for the appropriate length of time. This value varied from 25 to 200 cpm for the SV-3T3 cells and 60 to 1,000 cpm for the macrophages, depending upon the incubation time and the particular medium used.

Assay for [^{3}H]Hyaluronan Binding Activity

Hyaluronan binding activity was determined using a modified version of that previously described (4, 38). The samples were dissolved in 200 μl of 0.1% Na deoxycholate, 0.5 M NaCl, 0.02 M Tris, pH 8.0 (DOC buffer), to which was added 1 μg of [^{3}H]hyaluronan and the volume was adjusted to 250 μl. After shaking for at least 20 min, 250 μl of saturated $(NH_4)_2SO_4$ was added followed by 25 μl of nonfat milk and then the samples were centrifuged at 9,000 *g* for 5 min. The tubes were rinsed twice with 50% saturated $(NH_4)_2SO_4$ and the pellets were dissolved in water and processed for scintillation counting. The background level of binding was determined by including an excess of nonlabeled hyaluronan (60 μg) in the initial assay mixture.

Isolation of SV-3T3 Membranes

To determine if the receptor itself has hyaluronidase activity, membranes were examined for their ability to degrade hyaluronan. Approximately 1.5 × 10^8 SV-3T3 cells were suspended in a solution of 0.02% EDTA in CMF-PBS and centrifuged to pellet the cells. The pellet was resuspended in 5 ml of 10 mM Na_2HPO_4, 1 mM PMSF, pH 7.3, and maintained for 20 min on ice. The cells were then lysed using a Potter-Elvejhem homogenizer, and mixed with 2 vol of 20 mM Tris, 500 mM sucrose, 1 mM PMSF. The homogenate was subjected to low speed centrifugation (2,000 *g*, 15 min) to remove nuclei and unlysed cells, and the resulting supernatant was centrifuged at high speed (100,000 *g*, 30 min) to pellet the membrane-containing fraction. The membrane preparation was analyzed for [^{3}H]-hyaluronan binding activity and protein content. A portion of the preparation was suspended in culture medium along with [^{3}H]hyaluronan and analyzed for degradation as described above.

Fluorescent Microscopy

Alveolar macrophages were suspended in 10% FCS, 90% McCoy's medium, and then 300-μl aliquots were distributed to the wells of chambered slides (eight chambers, Lab-Tek). For the experiments involving the fluorescein-tagged hyaluronan, the cells were pretreated for 15 min with the following agents; K-3 mAb (20 μg/ml); control mouse IgG (20 μg/ml); Fab fragment of the K-3 mAb (20 μg/ml); and hyaluronan (1 mg/ml, Type I, Sigma Chemical Co.). Next, the fluorescein-tagged hyaluronan (100 μg/ml) was added to the wells and the cultures were maintained at 37°C for the indicated period of time. The slides were washed with PBS, fixed for 3 min in methanol at −20°C, and mounted in glycerol/PBS (Citifluor). The cells were photographed on an Olympus Microscope equipped with epifluorescent optics using the fluorescein filter cube. For staining with the b-K-3 mAb, the cells were fixed in formalin, and then incubated with 4 μg/ml b-K-3 mAb in 10% calf serum, 90% CMF-PBS, followed by a 1 to 50 dilution of FITC-Streptavidin (Zymed, South San Francisco, CA). The slides were mounted and photographed as described above. In each case, representative cells were photographed. However, macrophages that contained large autofluorescent granules (easily distinguished by their yellow color) as well as cells which were clearly not macrophages were excluded.

Preparation of Biochemicals

The K-3 mAb is a mouse IgG directed against the hamster hyaluronan receptor (36), which blocks the binding of hyaluronan to receptors from both hamsters and mice (12). It was isolated from ascites fluid by chromatography on a protein A-Sepharose (Sigma Chemical Co.) using the Monopure buffer system (Pierce Chemical Co., Rockford, IL). The control consisted of IgG isolated from pooled mouse serum. The purified K-3 mAb was coupled to biotin (b-K-3) using sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce Chemical Co.) as previously described (1, 41). Fab fragments of the K-3 mAb were prepared by digesting the purified K-3 mAb with immobilized papain according to the method provided by the Pierce Chemical Co. The Fab fragments were purified by chromatography on a protein A-Sepharose column.

The KM-201 mAb is a rat IgG directed against the hyaluronan receptor from mouse, which blocks the binding of hyaluronan to the receptor from both mouse and hamster cells (4, 22). This mAb was purified from ascites fluid using the Econo-Pac serum IgG purification kit from Bio-Rad Laboratories (Richmond, CA). The protocol was modified by applying 3 ml of ascites fluid to the DEAE Affi-gel blue column in 0.028 M NaCl, 0.02 M Tris, pH 8.0, followed by 20 ml of 0.028 M NaCl, 0.02 M Tris, 20 ml of 0.038 M NaCl, 0.02 M Tris and finally, the antibody was eluted with 20 ml of 0.128 M NaCl, 0.02 M Tris, pH 8.0. The antibody was at least 80% pure as judged by SDS-PAGE (17).

The [^{3}H]hyaluronan was prepared by growing rat fibrosarcoma cells in the presence of [^{3}H]acetate as previously described (34). The medium was collected, digested with pronase and dialyzed extensively against distilled water. The [^{3}H]hyaluronan was purified by precipitation with cetylpyridinium chloride followed by differential solubilization in salt solutions (34, 39). The amount of hyaluronan was determined by the uronic acid assay of Bitter and Muir (3). The preparation of [^{3}H]hyaluronan used in this study had a specific activity of ~6 × 10^4 cpm per μg of Na hyaluronan.

The fluorescein-tagged hyaluronan was prepared by mixing 200 μg fluorescein amine, isomer 1 (Aldrich, Milwaukee, WI), 200 μg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma Chemical Co.) and 1 mg of hyaluronan (Healon, Pharmacia) in a final volume of 2 ml. The pH was quickly adjusted to 4.5 and the mixture was maintained for 1 h at room temperature. The reaction mixture was extensively dialyzed against 0.15 M NaCl to remove uncoupled fluorescein amine.

The oligosaccharide fragments of hyaluronan were prepared by digestion with testicular hyaluronidase followed by acid hydrolysis and reacetylation (34). The resulting fragments were separated by chromatography on Sephadex G-50 (34).

Protein levels were determined using the bicinchoninic acid reagent (Pierce Chemical Co.).

Results

Degradation of [^{3}H]Hyaluronan by Cells

In initial experiments, we examined the degradation of labeled [^{3}H]hyaluronan by mouse SV-3T3 cells and hamster pulmonary macrophages, both of which are known to express the hyaluronan receptor (CD44) on their surfaces (11, 37). The cells were cultured for 2 d in the presence of [^{3}H]hyaluronan and then digested extensively with pronase. The resulting digests were examined by molecular-sieve chromatography on a column of Sephadex G-15. As shown in Fig. 1 (*a* and *b*), both cell types degraded the labeled

1. *Abbreviations used in this paper*: b-K-3 mAb, biotinylated K-3 mAb; CMF-PBS, calcium- and magnesium-free PBS.

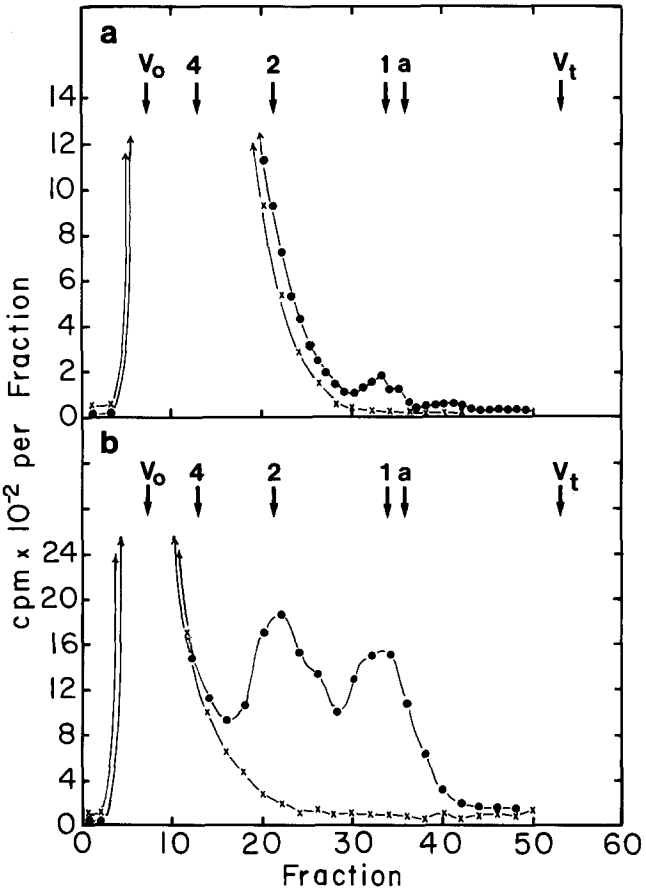

Figure 1. The degradation of [^{3}H]hyaluronan by SV-3T3 cells and alveolar macrophages. (*a*) The SV-3T3 cells were cultured in 10% NuSerum, 90% DME in the presence of 2 μg/ml [^{3}H]hyaluronan (4 ml total volume). After 40 h, the medium was removed, the cell layer was digested with pronase, recombined with the medium and further digested. The sample was lyophilized, dissolved in 1 ml of distilled water and applied to a column of Sephadex G-15 and eluted with CMF-PBS. The background level of degradation was determined by processing a similar amount of [^{3}H]hyaluronan in the absence of cells. (*b*) Hamster alveolar macrophages were cultured in 10% FCS, 90% McCoy's medium as described above, except that the sample size was only 1 ml. The void volume (*Vo*) and total volume (*Vt*) were determined with blue dextran 2,000 and [^{3}H] H_2O, respectively. The elution positions of the various sized fragments of hyaluronan were determined on subsequent chromatographic runs and are indicated on the figure (*4*, tetrasaccharide fragment of hyaluronan; *2*, disaccharide fragment of hyaluronan; *1*, glucuronolactone; *a*, [^{3}H] glucosamine).

hyaluronan as compared to control samples. The resulting fragments ranged in size from monosaccharides to disaccharides. Fragments smaller than this were not detected. These results are consistent with those previously reported by a number of investigators (7, 8, 25).

To examine the time course for the degradation of hyaluronan in greater detail, the cells were incubated with [^{3}H]-hyaluronan for various periods of time, and the resulting fragments were separated from intact [^{3}H]hyaluronan using Centricon 30 Microconcentrators, which gives similar results to the molecular-sieve chromatography. As shown in Fig. 2 (*a* and *b*), while both cell types degraded the [^{3}H]hyaluronan, the macrophages degraded it at a much faster rate than the

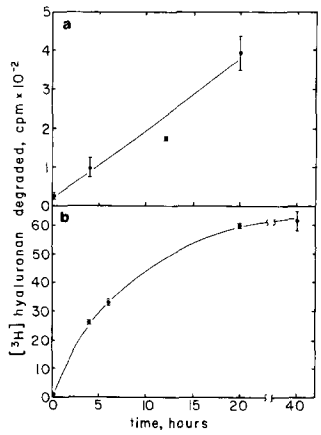

Figure 2. Time course for the degradation of [^{3}H]hyaluronan by SV-3T3 cells and macrophages. (*a*) SV-3T3 cells were suspended in 10% NuSerum, 90% DME and 1 ml aliquots were distributed into a 24 multiwell culture dish (3.3×10^6 cells per well). (*b*) Alveolar macrophages from adult hamsters were suspended in 10% FCS, 90% McCoy's medium (0.5×10^6 cells per well) and processed as above. To each of the wells was added 2 μg of [^{3}H]hyaluronan (1.2×10^5 cpm), and after varying periods of time, the contents of the wells was digested with pronase and assayed for fragments of [^{3}H]hyaluronan using Centricon 30 Microconcentrators. The background (i.e., without cells) was subtracted from each point. The error bars indicate the range of duplicate or triplicate determinations.

SV-3T3 cells. The slopes of the initial portion of the curve suggest that macrophages degraded hyaluronan ~250 times faster than the SV-3T3 cells (1,240 cpm/h/10^6 for macrophages versus 4.8 cpm/h/10^6 for SV-3T3 cells). Part of this difference may be due to the fact that macrophages contain approximately five times the amount of receptor relative to SV-3T3 cells, as determined by the [^{3}H]hyaluronan binding activity of detergent-solubilized cells (7,461 cpm/10^6 for macrophages versus 1,447 cpm/10^6 for SV-3T3 cells). In addition, SV-3T3 cells synthesize hyaluronan which may compete with the exogenously added [^{3}H]hyaluronan, whereas macrophages synthesize little or no hyaluronan (our unpublished observations). Despite the differences in the rate, it is clear that both cell types have the capacity to degrade [^{3}H]hyaluronan.

To determine if the hyaluronan receptor (CD44) plays a role in the degradation of hyaluronan, the effects of various agents on this process were examined. In the case of the SV-3T3 cells (Table I), the degradation was inhibited 50–60% by both intact mAbs and Fab fragments of mAbs directed against the hyaluronan receptor (K-3 and KM-201), whereas control mouse IgG had little or no effect on the extent of degradation. These results indicate that the receptor is indeed involved in the degradation of hyaluronan.

The effect of antibodies was even more striking in the case of the hamster alveolar macrophages, in which the degradation was inhibited by >80% by the K-3 mAb (Table II). Further evidence for the role of the receptor in this process is indicated by the fact that the degradation was also blocked by the addition of an excess of nonlabeled hyaluronan, while the addition of other glycosaminoglycans such as dermatan sulfate, chondroitin-4-sulfate and heparin had only a small inhibitory effect (Table II, experiment 2). This is in keeping with previous studies indicating that the receptor binds with relative specificity to hyaluronan as compared to other glycosaminoglycans (35). Along a similar line, oligosaccharide fragments smaller than a hexasaccharide had only a modest inhibitory effect on the degradation, which is consistent with the size specificity for recognition by the receptor (35).

Taken together, these results strongly suggest that the

Table I. The Effect of Various Agents on the Degradation of [³H]Hyaluronan by SV-3T3 Cells

Experiment No.	[³H]Hyaluronan				
	Time	Treatment	Concentration	Degraded	Inhibition
	h		*μg/ml*	*cpm ± range*	*%*
1	40	Control	0	500 ± 71	0
	40	Mouse IgG	20	600 ± 91	−20
	40	K-3	20	248 ± 63	50
	40	Fab K-3	20	220 ± 76	56
2	20	Control	0	391 ± 44	0
	20	Mouse IgG	20	359 ± 25	8
	20	KM-201	20	154 ± 34	61

Mouse SV-3T3 cells were cultured in 1 ml of medium (10% NuSerum, 90% DME) along with 2 μg of [³H]hyaluronan (1.2×10^5 cpm) for the indicated period of time and then digested with pronase. The extent of [³H]hyaluronan degradation was determined using Centricon 30 Microconcentrators as described in Materials and Methods. The background level of degradation (i.e., without cells) has been subtracted from each value.

receptor (CD44) molecule is critically involved in the process of hyaluronan degradation.

Receptor Lacks Hyaluronidase Activity

One possible explanation for the results described above is that the receptor itself possesses hyaluronidase activity. To investigate this possibility, membranes were isolated from SV-3T3 cells by differential centrifugation, and then incubated with [³H]hyaluronan under conditions identical to that for the intact cells. As shown in Table III, no significant degradation of [³H]hyaluronan took place in this membrane preparation, despite the fact that it contained more receptors than intact cells in which degradation could be readily detected. Thus, the receptor itself lacks the ability to degrade hyaluronan.

Table II. The Effect of Various Agents on the Degradation of [³H]Hyaluronan by Macrophages

Experiment No.	[³H]Hyaluronan			
	Treatment	Concentration	Degraded	Inhibition
		μg/ml	*cpm ± range*	*%*
1	Control	0	4,976 ± 174	0
	Mouse IgG	20	5,224 ± 196	−5
	K-3	20	468 ± 100	91
	Hyaluronan	200	242 ± 122	95
2	Control	0	3,706 ± 400	0
	Mouse IgG	20	3,142 ± 1,004	15
	K-3	20	644 ± 218	83
	Hyaluronan	100	206 ± 280	94
	Dermatan Sulfate	100	2,520 ± 164	32
	Chondroitin-4-Sulfate	100	2,498 ± 374	33
	Heparin	100	2,416 ± 240	35
	Disaccharide	100	2,998 ± 74	19
	Tetrasaccharide	100	2,252 ± 122	39
	Hexasaccharide	100	1,736 ± 114	53

Hamster alveolar macrophages in 1 ml of 10% FCS, 90% McCoy's medium were incubated for 40 h with 2 μg of [³H]hyaluronan in the presence of the various test substances. The extent of degradation was then determined as described in Materials and Methods.

Table III. Binding and Degradation of [³H]Hyaluronan by Intact SV-3T3 Cells and Membranes Prepared from SV-3T3

Sample	Time	Protein	[³H]Hyaluronan binding	Specific activity	[³H]Hyaluronan degraded
	h	*μg*	*cpm ± range*	*cpm/μg*	*cpm ± range*
Intact Cells	0	810	4,776 ± 512	5.9	26 ± 7
	20	895	5,931 ± 321	6.6	391 ± 44
Membranes	0	61	10,706 ± 1,326	175.5	30 ± 21
	40	42	6,038 ± 518	143.8	37 ± 22

Both intact SV-3T3 cells and membranes prepared from these cells were incubated with [³H]hyaluronan in 10% NuSerum, 90% DME for the indicated periods of time. The protein content and [³H]hyaluronan binding activity were determined both at the beginning and end of the incubation. The background levels of both the [³H]hyaluronan binding and degradation were subtracted from the values shown above.

Localization of Fluorescein-tagged Hyaluronan

Another possible explanation for the above results is that the receptor mediates the uptake of hyaluronan into the cell so that it can be degraded internally by lysosomal enzymes. To explore this possibility, we examined the interaction of macrophages with fluorescently tagged hyaluronan. As shown in Fig. 3 *a*, after incubating for only 1 min, the fluorescein-tagged hyaluronan was bound to the surfaces of the macrophages. After 1 h (Fig. 3 *b*), the bulk of the fluorescein-tagged hyaluronan was still present only at the cell surface. However, after 3 h (Fig. 3 *c*), a small fraction of the fluorescence could be detected in granules within the macrophage. At 24 h, most of the fluorescence was present in cytoplasmic granules of varying sizes.

Both the binding and internalization of the fluorescently tagged hyaluronan appear to involve the receptor. The K-3 mAb blocked the binding of fluorescently tagged hyaluronan to the cell surface while control antibodies had no effect (compare Fig. 4, *b* and *c*). The K-3 mAb also blocked the uptake of the fluorescein-tagged hyaluronan inside macrophages after a 24-h incubation (compare Fig. 4, *d* and *e*). Likewise, Fab fragments of the K-3 mAb inhibited both the binding and uptake of fluorescein tagged hyaluronan by macrophages (data not shown). And finally, the uptake was also inhibited by a large excess of unlabelled hyaluronan (Fig. 4 *f*). These results suggest that the uptake of hyaluronan is a receptor mediated process as opposed to nonspecific phagocytosis.

It should be noted that the distribution of the fluorescein-tagged hyaluronan was similar to that of the receptor. As shown in Fig. 5 *b*, the receptor was present as small patches on the cell surface as well as in granules within the cytoplasm. This is consistent with a functional interaction between these two components.

Degradation Blocked by Lysosomal Inhibitors

To further test the possibility that the hyaluronan was being degraded internally by lysosomal enzymes, the effects of chloroquine and NH_4Cl were examined. These agents prevent the acidification of endocytotic vesicles and thereby inhibit the enzymatic activity of acid hydrolases (5, 6, 21). As shown in Table IV, both of these agents significantly inhibited the degradation of [³H]hyaluronan by alveolar macrophages. These results suggest that the degradation of

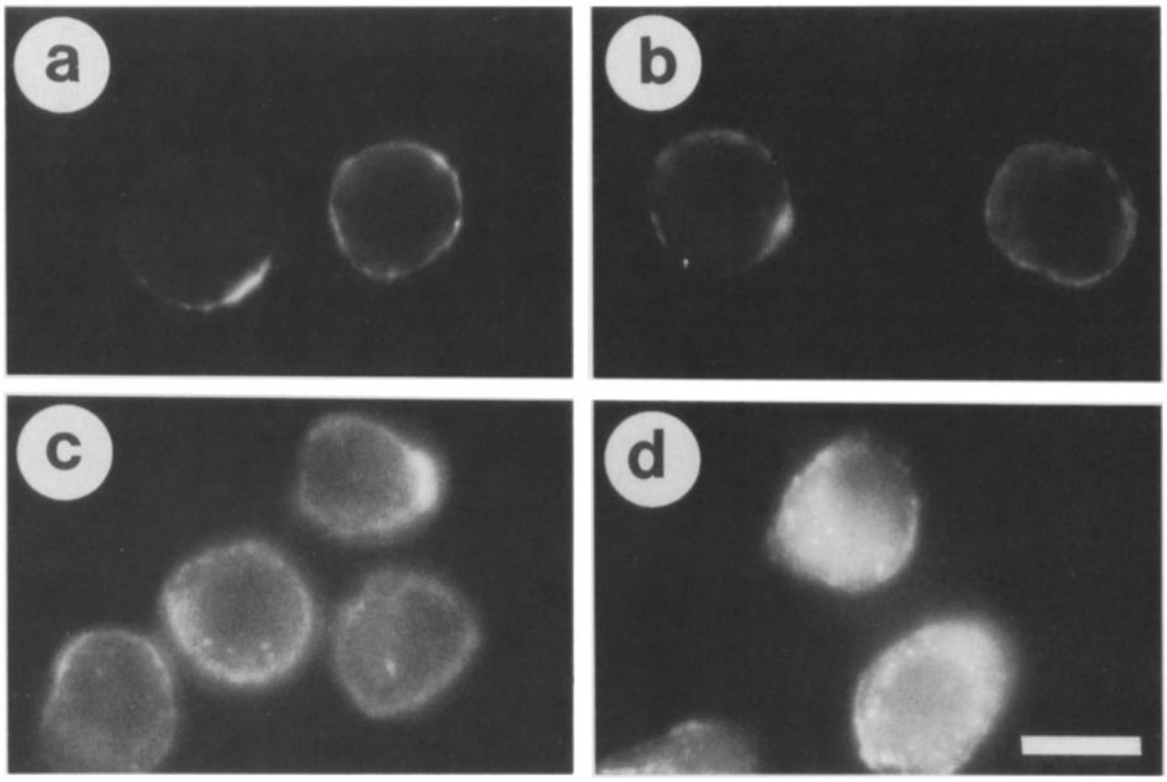

Figure 3. The binding and uptake of fluorescein-tagged hyaluronan by hamster alveolar macrophages. The macrophages were incubated with tagged hyaluronan for various periods of time and then examined under the fluorescent microscope as described in Materials and Methods. (*a*) At 1 min, the fluorescein-tagged hyaluronan was present only on the surface of the macrophage. (*b*) At 1 h, the tagged hyaluronan was still restricted to the cell surface. (*c*) After 3 h, a fraction of the tagged hyaluronan could be observed in intracellular granules. (*d*) After 24 h, the bulk of the hyaluronan was present inside the macrophage in both large granules and granules which are at the limit of resolution of the microscope. Bar, 10 μm.

[^{3}H]hyaluronan takes place in the lysosomal compartments of the cell.

Discussion

The results of this study indicate that the hyaluronan receptor (CD44) plays a key role in the degradation of hyaluronan. More specifically, the receptor is responsible for the initial binding of hyaluronan to the cell surface so that it can be internalized and degraded by acid hydrolases. Thus, the degradation of hyaluronan takes place in a fashion similar to that of other receptor-mediated degradatory processes such as LDL and transferrin (10).

The degradation of hyaluronan appears to involve three distinct steps: first, it binds the cell surface; second, it is endocytosed; and third, it is degraded by acid hydrolases. In the first step, the receptor mediates the attachment of hyaluronan to the cell surface, as previously demonstrated by the fact that mAbs to the receptor block the binding of [^{3}H]hyaluronan to the cell surface (12), and further confirmed in the present study with fluorescein tagged hyaluronan. Clearly, this step is critical in the degradatory process, since blockage of this step also inhibits the subsequent steps leading to the breakdown of hyaluronan.

The second step involves the uptake of hyaluronan into the cells. This process could be directly visualized with the fluorescein tagged hyaluronan, which revealed that it was taken up into granules of varying sizes. More importantly, this uptake was blocked by the mAbs to the receptor as well as by an excess of nonlabeled hyaluronan, suggesting that it is a receptor-mediated process as opposed to nonspecific phagocytosis. This receptor-mediated uptake is consistent with previous studies suggesting that the receptor is associated with the cytoskeleton (15, 16).

In the third step, the hyaluronan is degraded by lysosomal enzymes. This was indirectly suggested by the fact that the receptor itself lacked hyaluronidase activity, since isolated membranes of SV-3T3 cells enriched in the receptor did not have the capacity to degrade [^{3}H]hyaluronan. More direct evidence for the role of lysosomal enzymes in this process came from the fact that the degradation of [^{3}H]hyaluronan was inhibited by chloroquine and NH_4Cl, both of which prevent the acidification of the lysosomal compartment required for the action of lysosomal enzymes (5, 6, 21). These results are consistent with previous studies which have reported that a number of different cell types contain acid hydrolases which are capable of degrading hyaluronan (8, 24, 27).

The findings of this study are also in keeping with the earlier studies of Orkin and her coworkers (20, 24, 25), who showed that the rate limiting step for the degradation of hyaluronan was its internalization. Furthermore, several characteristics of the degradation process were consistent with the role of the hyaluronan receptor in this process. First,

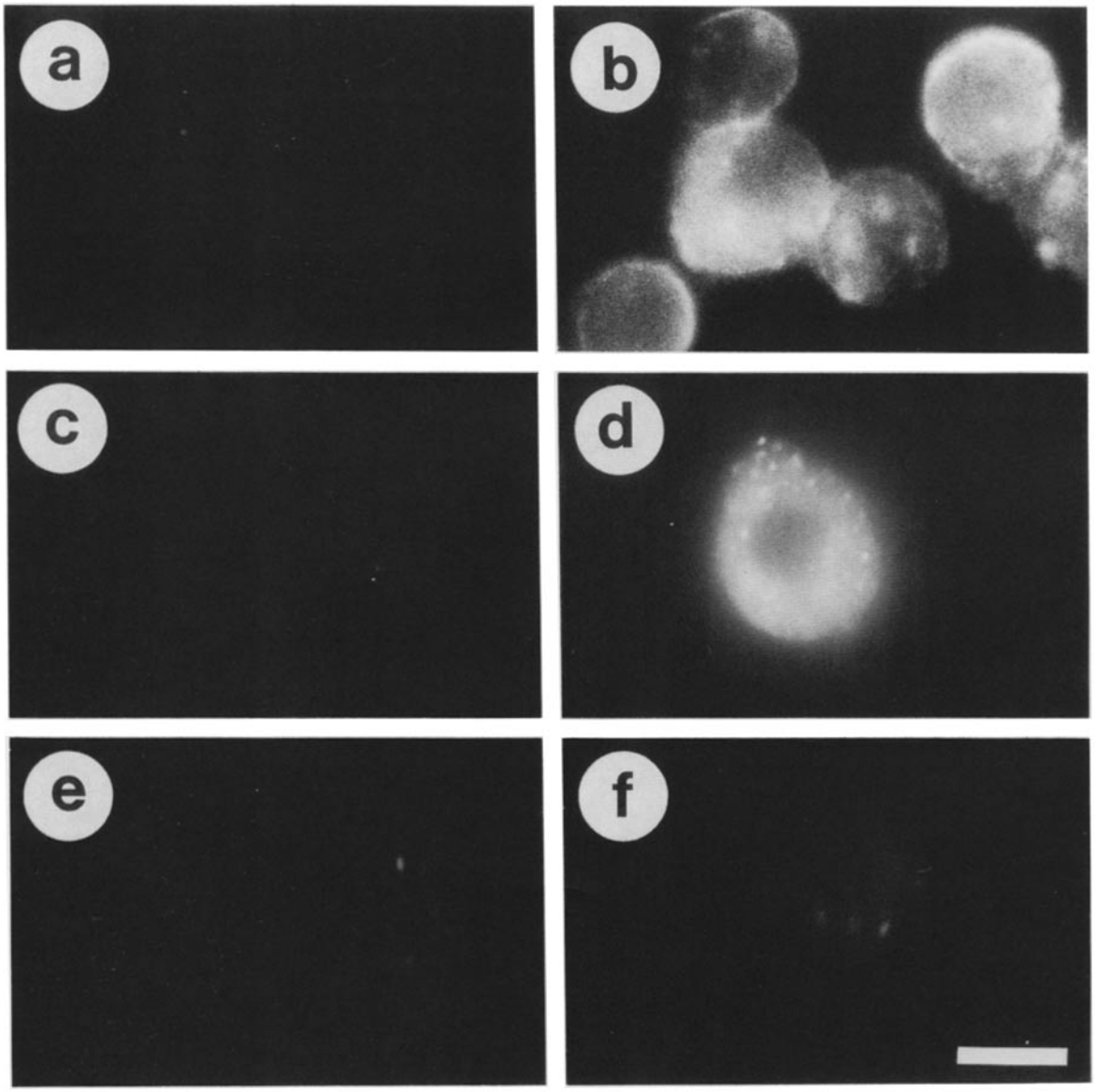

Figure 4. The effect of various agents on the binding of fluorescein-tagged hyaluronan to macrophages after 3 and 24 h. (*a*) Macrophages incubated in the absence of fluorescein tagged hyaluronan show the background level of fluorescence. (*b*) Macrophages pretreated with control mouse IgG (20 μg/ml) and then incubated with fluorescein-tagged hyaluronan (100 μg/ml) for 3 h show fluorescence both on the cell surface and intracellularly. (*c*) Macrophages pretreated with the K-3 mAb (20 μg/ml) and then incubated with fluorescein-tagged hyaluronan show a lower level of fluorescence. (*d*) Macrophages incubated with tagged hyaluronan for 24 h demonstrate the presence of fluorescence predominantly in cytoplasmic granules. (*e*) Macrophages pretreated with the K-3 mAb followed by a 24-h incubation with tagged hyaluronan show a greatly diminished level of fluorescence. (*f*) Macrophages premixed with nonlabeled hyaluronan (1 mg/ml) and then incubated with the tagged hyaluronan for 24 h, show that most of the uptake is blocked. The photography and processing within sets *a–c* and *d–f* were carried out under identical conditions. Bar, 10 μm.

SV-3T3 cells degraded [^{3}H]hyaluronan at a much faster rate than 3T3 cells (25), which correlates with the fact that they have a greater number of receptors available on their surfaces (40). And secondly, larger molecules of hyaluronan were degraded more readily than smaller molecules (20). This size-dependence was also one of the characteristic features of hyaluronan binding to the cell surface receptor (36).

A number of studies have shown that liver endothelial cells also degrade hyaluronan (7, 18, 26). However, this does not appear to involve the hyaluronan receptor (CD44), since immunohistochemical studies have indicated that the receptor is absent from these cells of adult hamsters or mice (unpublished observations). Furthermore, the characteristics of hyaluronan binding to endothelial cells are different from that of the CD44 with respect to both oligosaccharide recognition (18) and size dependency of the interaction (8). It is possible that a different type of hyaluronan binding protein is present on liver endothelial cells, which carries out a similar type of function. Indeed, a number of other hyaluronan binding proteins have been described.

The ability of cells expressing the receptor to degrade hyaluronan may have a number of important biological consequences. For example, macrophages probably function to degrade hyaluronan from connective tissues of the adult.

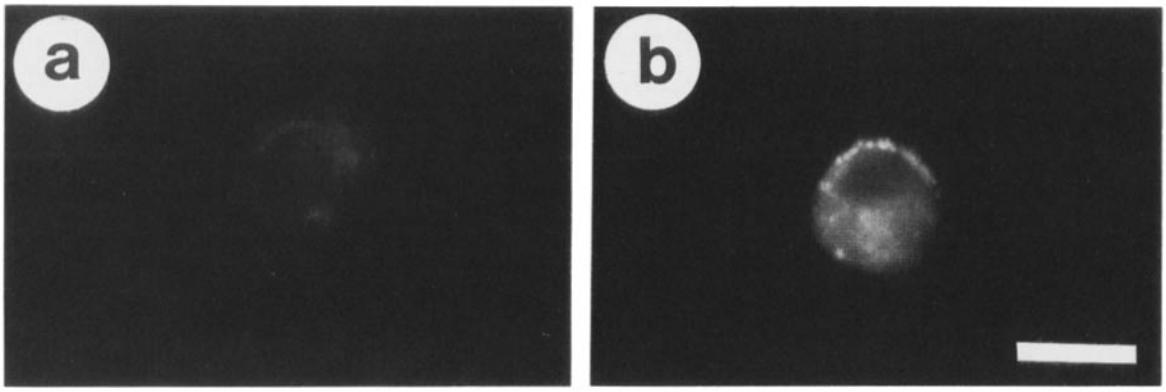

Figure 5. The distribution of the hyaluronan receptor on macrophages. (*a*) The background level of fluorescence in shown (i.e., b-K-3 mAb omitted). (*b*) The pattern of fluorescence observed with b-K-3 mAb followed by FITC-Streptavidin. The receptor is present on the cell surface as well as in cytoplasmic granules. Bar, 10 μm.

This may be particularly important in the case of the lung, where hyaluronan on the surfaces of alveoli could interfere with air-blood exchange.

Dendritic cells represent another family of cells (29) that is involved in the turn-over of hyaluronan. Fraser and coworkers (8, 9) have shown that when [^{3}H]hyaluronan was injected either intravenously or into afferent lymphatic vessels, a large fraction was removed by dendritic cells of the spleen and lymph nodes. Histochemical studies have indicated that dendritic cells express large amounts of the receptor (CD44) (our unpublished observations). Furthermore, the characteristics of the removal of [^{3}H]hyaluronan by lymphatic tissue were consistent with the involvement of the hyaluronan receptor (CD44) with regard to specificity of the binding and the size dependency.

The receptor-mediated degradation of hyaluronan may be an important factor during cell migration. For example, the ability of macrophages to degrade hyaluronan may be important during the process of migration through the extracellular matrix. Along similar lines, many tumor cells degrade the matrix through which they migrate as part of the invasive process. A number of proteases have been described in tumor cells which hydrolyze collagen, fibronectin and laminin. It is possible that these cells may also degrade hyaluronan. Recently, Gunthert et al. (13) have shown that the expression of a high molecular weight form of CD44 is directly correlated with the metastatic properties of cells. Indeed, nonmetastatic cells were converted to metastatic cells by transfecting them with cDNA of this form of CD44 (13). It would be interesting to determine if this form of CD44 has the capacity to bind hyaluronan.

The turnover of hyaluronan is also important during embryonic development. In a series of studies, Toole and his associates (31) have shown that hyaluronan changes in a dynamic fashion during the development of a number of tissues (cornea, brain, limb bud, etc.). During early developmental stages, these tissues contain relatively large amount of hyaluronan, which functions to keep cells separated, so that cell migration and other morphological events can take place. Then, at a defined stage, the concentration of hyaluronan is abruptly reduced, leading to the condensation of the tissue. It is possible that the hyaluronan receptor participates in this developmentally programmed loss of hyaluronan. Indeed, recently we have found that the expression of the receptor coincides with the stages during which the hyaluronan is removed (our unpublished observations).

In conclusion, our results suggest that CD44 is responsible for the receptor-mediated uptake of hyaluronan leading to its degradation. In view of hyaluronan's widespread distribution and importance in connective tissue, it is reasonable that this mechanism has evolved to facilitate its removal.

Table IV. Effect of Acidotropic Agents of [^{3}H]Hyaluronan Degradation by Macrophages

Agent	Concentration	[^{3}H]Hyaluronan	
		Degraded	Inhibition
	M	*cpm ± range*	%
Control	0	1,394 ± 46	0
Chloroquine	1×10^{-5}	978 ± 20	30
	5×10^{-5}	80 ± 34	94
	1×10^{-4}	8 ± 20	99
NH_4Cl	1×10^{-3}	726 ± 86	48
	1×10^{-2}	44 ± 2	97

Hamster alveolar macrophages were preincubated with the test substances for 20 min and then further incubated for 6 h in the presence of [^{3}H]hyaluronan. The background level of [^{3}H]hyaluronan degradation was subtracted from each value.

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