Hyaluronan Enters Keratinocytes by a Novel Endocytic Route for Catabolism

Raija Tammi**, Kirsi Rilla*, Juha-Pekka Pienimäki*, Donald K. MacCallum‡, Michael Hogg #, Merja Luukkonen*, Vincent C. Hascall # and Markku Tammi**#

*Department of Anatomy, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland, #Department of Biomedical Engineering/ND20, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195 and ‡Department of Anatomy and Cell Biology, University of Michigan, Ann Arbor, MI 48109

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Correspondence: Raija Tammi, MD, PhD
Department of Anatomy, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland
tel. 358-71-163009
fax 358-71-163032
email: raija.tammi@uku.fi
Hyaluronan synthesized in the epidermis has an exceptionally short half life, indicative of its catabolism by epidermal keratinocytes. An intracellular pool of endogenously synthesized hyaluronan, from 1 to 20 fg/cell, inversely related to cell density, was observed in cultured rat epidermal keratinocytes. More than 80% of the intracellular hyaluronan was small (<90 kDa). Approximately 25% of newly synthesized hyaluronan was endocytosed by the keratinocytes and had a half life of 2-3 h. A biotinylated aggrecan G1 domain/link protein probe demonstrated hyaluronan in small vesicles of ~100 nm diameter close to the plasma membrane, and in large vesicles and multivesicular bodies up to 1300 nm diameter around the nucleus. Hyaluronan did not colocalize with markers of lysosomes. However, inhibition of lysosomal acidification with NH$_4$Cl or chloroquine, or treating the cells with the hyaluronidase inhibitor apigenin increased intracellular hyaluronan staining, suggesting that it resided in prelysosomal endosomes. Competitive displacement of hyaluronan from surface receptors using hyaluronan decasaccharides, resulted in a rapid disappearance of this endosomal hyaluronan ($T_{1/2}$ ~5 min), indicating its transitory nature. The ultrastructure of the hyaluronan-containing vesicles, colocalization with marker proteins for different vesicle types, and application of specific uptake inhibitors demonstrated that the formation of hyaluronan-containing vesicles did not involve clathrin coated pits or caveolae. Treatment of rat epidermal keratinocytes with the OX50 mab against the hyaluronan receptor CD44 increased endosomal hyaluronan. However, no CD44-hyaluronan colocalization was observed intracellularly unless endosomal trafficking was retarded by monensin, or cultivation at 20°C, suggesting CD44 recycling. Rat epidermal keratinocytes thus internalize a large proportion of their newly synthesized hyaluronan into non-clathrin coated endosomes in a receptor mediated way, and rapidly transport it to slower degradation in the endosomal/lysosomal system.
INTRODUCTION

Hyaluronan is produced by the hyaluronan synthase family of enzymes (1), which directly extrude the growing glycosaminoglycan chain through the plasma membrane into the extracellular matrix or onto the cell surface. Most of the hyaluronan synthesized in tissues eventually diffuses into lymph, and is subsequently catabolized by lymph nodes (2), or the liver (3,4). However, partial degradation of radiolabeled hyaluronan injected into joints (5) and skin (6) occurs, indicating that there are also local catabolic systems. Furthermore, cultured chondrocytes (7,8), fibroblasts, smooth muscle cells (9), macrophages (10-12), and some breast cancer cell lines (13) internalize and degrade labeled exogenous hyaluronan.

A protein (HARE) associated with the endocytosis of hyaluronan in liver endothelial cells and lymph nodes has been recently cloned (14), and another (LYVE) may be involved in hyaluronan uptake by lymph vessel endothelium (15). In peripheral tissues, the ubiquitous plasma membrane protein CD44 is known to bind hyaluronan, and is also thought to contribute to hyaluronan internalization (8,12,16). Details of the molecular mechanisms of endocytosis by any of the hyaluronan receptors are largely unknown. However, it is likely that specific uptake processes are required for this macromolecule, that can exceed a molecular mass of $6 \times 10^6$ Da and have a 300 nm radius of gyration in solution (17), a size clearly beyond the ~100 nm diameter of a regular primary endosome.

Footnote 1

Human epidermal keratinocytes actively synthesize hyaluronan (18), and pulse-chase experiments in skin organ cultures demonstrate a short half life (~1 day) for epidermal hyaluronan (19,20). Similar metabolic labeling experiments in rat epidermal keratinocytes (REKs)$^1$ in organotypic cultures also indicate rapid hyaluronan catabolism (21). Partial degradation of epidermal hyaluronan in organ cultures is also indicated by the decrease in the molecular weight of newly synthesized hyaluronan molecules from $>4 \times 10^6$ Da to $1 \times 10^6$ and
0.5x10^6 Da after 24 h and 48 h chases, respectively (20). Keratinocytes express a high level of CD44 (22), a receptor thought to be responsible for hyaluronan uptake in other cells (7,13). Accordingly, mice with blocked epidermal CD44 expression accumulate hyaluronan in skin (16), suggesting impaired hyaluronan uptake by keratinocytes.

We found earlier that REKs contain a pool of hyaluronan resistant to trypsin and *Streptomyces* hyaluronidase treatments (23), suggesting an intracellular location. The present study characterizes the size, location, turnover, molecular weight, and regulation of this hyaluronan pool in more detail, particularly because it has been recently suggested that intracellular hyaluronan may be involved in cell signaling, mitosis and cell cycle regulation (24,25). Our experiments show that even in open monolayer cultures, where hyaluronan can easily escape into the culture medium, REKs actively internalize and rapidly metabolize newly synthesized hyaluronan in a receptor-mediated fashion through an endocytic route which is not dependent on coated pits or caveolae, but which involves CD44.

**EXPERIMENTAL PROCEDURES**

*Cell Culture* - A newborn rat epidermal keratinocyte (REK) cell line was developed by MacCallum and Lillie (26) from neonatal rat epidermal cells originally isolated by Baden and Kubilus (27). REKs were cultured in Dulbecco’s MEM (low glucose, Life Technologies, Paisley, UK) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 4 mM L-glutamine, and penicillin and streptomycin (50 mU/ml and 50 µg/ml, respectively, Sigma, St. Louis, MO) at 37°C in a humidified atmosphere containing 5% CO₂ in air. Cells were passaged when confluent using 0.05% trypsin (w/v)/ 0.02% EDTA (w/v) (Biochrom, Berlin, Germany) in phosphate buffered saline (PBS). For biochemical assays and radiolabeling, the cells were seeded at 100,000/ml and grown to near confluency in 6-well plates (Costar Corp., Cambridge, MA). For microscopic studies, the cells were plated at 20,000 cells/well in 8-well chamber slides (Lab-Tek,
Nalge Nunc Int. Corp., Naperville, IL) precoated with FBS for 30 min at 37°C.

Manipulation of Hyaluronan Uptake and Degradation - To study the receptor mediated uptake of hyaluronan, we treated recently confluent or nearly confluent REK cultures with hyaluronan oligosaccharides consisting of 4, 6, 10, 12 and 14 monosaccharide units in length (HA$_4$, HA$_6$, HA$_{10}$, HA$_{12}$ and HA$_{14}$, respectively) (23,28) at final concentrations of 0.2-0.5 mg/ml (23) or with 0.3 mg/ml of chondroitin, chondroitin sulfate A (mainly chondroitin-4-sulfate), chondroitin sulfate C (mainly chondroitin-6-sulfate) or heparan sulfate (all from Seikagaku Kogyo Co., Tokyo, Japan). Other cultures were incubated with the anti-CD44 mabs Ox 50 (Biosource Int., Camarillo, CA), or Hermes 3 (a gift from Dr. Sirpa Jalkanen, Turku), or with non-immune mouse IgG (Sigma).

To block hyaluronan uptake through clathrin coated pits, we: 1) added 2.5-10.0 µg/ml chlorpromazine (Sigma) for 0.5-4 h in the culture medium, or 2) used serum free, hypertonic medium with 0.4 M sucrose and 0.15% BSA for 60 min as described (29). Control cells were kept in the same medium without sucrose.

To block uptake through caveolae, we added: 1) filipin III (Sigma) to final concentrations of 0.4-23 µM for 0.5 to 6 h, or 2) nystatin (Sigma) (1-10 µM) for 0.5 to 1 h (30).

To block macropinocytosis, we treated cells either with 2-3 mM amiloride (Sigma,) or 0.05-0.5 mM 5-(N,N-dimethyl)-amiloride (Sigma) (31,32).

The function of lysosomes was perturbed by using either 2-10 mM ammonium chloride, or 0.5-2.5 mM chloroquine (Sigma), for 0.5-6.0 h (33). Receptor recycling was inhibited by 2-32 µM monensin (Sigma) for 0.5 to 4 h (34).

Isolation of Intracellular Hyaluronan - The REK cultures were radiolabeled with 20 and 100 µCi/ml of [${}^3$H]glucosamine and [${}^{35}$S]sulfate (Amersham, Little Chalfont, UK), respectively, for different times in 6-well plates (9.6 cm$^2$/well), and washed with 2 x 250 µl of Hank’s balanced salt solution (HBSS, Euroclone, Milano, Italy). The medium and washes were combined and designated as ”medium”. To remove cell surface associated hyaluronan, the cells were usually
incubated for 10 min at 37°C with the trypsin-EDTA as above, pelleted and washed with HBSS. The trypsin solution and combined washes were designated as “trypsinate” and the pellet as “intracellular” fraction.

In some experiments, incubation was done with 0.2 % trypsin (Sigma type XI, 6000-9000 BAEE units/mg) and 0.1% EDTA in Ca++, Mg++ free HBSS at 4°C for 1 h, the cells were pelleted by low speed centrifugation in the cold, washed with cold HBSS containing 10% FBS, and washed 4 times with HBSS. Thereafter, the cells were digested with Streptomyces hyaluronidase (15 TRU/ ml in HBSS) for 4-12 h at 4 °C, washed 4 times with cold HBSS, and suspended in 200 µl HBSS. The cells were boiled for 15 min to inactivate any exogenous or endogenous hyaluronidase. Alternatively, the cultures were directly digested with Streptomyces hyaluronidase at 4 °C, washed, and incubated with proteinase K as described below.

After adding carrier hyaluronan (Healon®, Pharmacia, Uppsala, Sweden) (4 µg in 40 µl) to each, the medium, trypsinate, and intracellular samples were digested with 50 µg of proteinase K at 60°C for 4 h, then heated at 100°C for 10 min, and centrifuged at 13,000 x g for 15 min. Hyaluronan and other glycosaminoglycans in the supernatants were precipitated with 4 volumes of ethanol for 2 h at -20°C, and collected by centrifugation at 13,000 x g for 15 min. Each precipitate was analyzed for specific disaccharides as described below.

To estimate the proportion of intracellular hyaluronan originating from the cell surface and culture medium, cells were radiolabeled as above for 6 h in medium with 1-5 U/ml of Streptomyces hyaluronidase. The half life of intracellular hyaluronan was determined by overnight labeling of the cultures as described above, then washing the cultures with unlabeled medium and analyzing intracellular hyaluronan after 1-6 h chase in the presence of 1 TRU/ml of Streptomyces hyaluronidase.

Assay of Hyaluronan Disaccharides - Samples were dialyzed in mini-dialysis units (Slide-A-Lyzer, molecular mass cut off 3500, Pierce, Rockford, IL) against distilled water containing 20% ethanol, and then evaporated to dryness under vacuum centrifugation. Each sample was then dissolved in 40 µl of 0.5 M sodium acetate, pH 6.2, and digested for 3 h at 37°C
with 1 mU of \textit{Streptococcus} hyaluronidase, followed by 2 h incubation with 25 mU of chondroitinase ABC (both enzymes from Seikagaku). Aliquots of 10-45 µl were injected onto a 1 x 30 cm Superdex Peptide column eluted at 0.5 ml/min with 0.012 M NH$_4$HCO$_3$. The eluent was monitored at 232 nm, and for $^3$H and $^{35}$S activities to quantitate chondroitin sulfate and hyaluronan disaccharides, as described in detail before (21,23,35).

\textbf{Hyaluronan Size Assays} - The samples were chromatographed on 1x30 cm columns of Sephacryl S-400 and S-1000 (Pharmacia, Uppsala, Sweden), eluted at 24 ml/h with 0.15 M sodium acetate, 0.1% CHAPS, pH 6.8. Fractions of 0.5 ml were collected. Healon® (4 µg) was added as a carrier in each fraction containing radioactivity, followed by precipitation with 4 volumes of ethanol for 2 h at -20°C and pelleting by centrifugation at 13,000 x g for 15 min. The precipitates of two consecutive fractions were redissolved in 20 µl of 0.5 M sodium acetate, pH 6.2, pooled and incubated with 1 mU of \textit{Streptococcus} hyaluronidase (Seikagaku) for 3 h at 37°C. 200 µl of ethanol was added, and the precipitate centrifuged as above. The supernatants were dried in a centrifugal evaporator, dissolved in 40 µl of 0.012 M NH$_4$HCO$_3$ and analyzed for hyaluronan disaccharides as described above.

\textbf{Hyaluronan Oligosaccharide Analysis} - Medium and intracellular samples were digested with proteinase K (250 µg/ml) at 60 °C for 4 h, boiled for 10 min, and evaporated until the volume of the sample was about 100 µl. The specimens were run on a Superdex Peptide column (Pharmacia), eluted at 0.5 ml/min with 0.012 M NH$_4$HCO$_3$, and 100 µl aliquots from 250 µl fractions were counted for radioactivity. The included fractions were combined in three pools (fractions 27-29, 30-32, and 33-35), and 4 µg of Healon was added as a carrier. Each pool was then evaporated to dryness, redissolved in 20 µl of 0.5 M sodium acetate, pH 6.2, digested with \textit{Streptococcus} hyaluronidase, and analyzed for hyaluronan disaccharides as described above.

\textbf{Fluorescent Hyaluronan} - Fluorescein-labeled hyaluronan preparations (FL-HA, gifts from Drs. Ronald Midura, Cleveland Clinic Foundation, Cleveland, OH and Jayne Lesley, Salk Institute, La Jolla, CA) were made as described (36). The molecular mass of the intact FL-HA showed a wide distribution between 200 and 2000 kDa on Sephacryl S-1000 gel filtration and
was completely susceptible to *Streptomyces* hyaluronidase digestion, demonstrated by the shift of all UV-absorbing and fluorescent material from the excluded volume to positions corresponding to tetra- to tetradecasaccharides on Superdex Peptide gel filtration. Hyaluronan oligosaccharides were labeled at their reducing ends by 2-aminoacridone (37).

**Staining for Endogenous Intracellular Hyaluronan** - The basic staining protocol was essentially as described before (23,38). The cells were washed once with HBSS and fixed for 20 min in 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB) at room temperature. After fixation, the cells were washed 5 x 2 min with PB, and digested with *Streptomyces* hyaluronidase, 5-10 TRU/ml of PB for 10 min at 37°C. After washes in PB, the cells were permeabilized for 10 min in 1% BSA containing 0.1% Triton-X100. Thereafter, a biotinylated complex of hyaluronan binding region of bovine articular cartilage aggrecan G1 domain and link protein (bHABC) (38), diluted to 3-5 µg/ml in 1% BSA, was applied to the cells, and incubated overnight at 4°C, followed by 1 h in avidin-biotin peroxidase (ABC-standard kit, Vector Laboratories, Inc., Burlingame, CA) at room temperature. The color was developed with 0.05% 3,3’-diaminobenzidine (DAB, Sigma) and 0.03% H₂O₂. Specimens for photography were counterstained with hematoxylin for 2 min, and embedded in Supermount (Biogenex, San Ramon, CA). Specimens for densitometry were embedded in Supermount without counterstaining. For fluorescence microscopy, the procedure was modified as follows: Either Texas Red streptavidin or FITC-avidin D (Vector, 1:1000 and 1:500 dilutions, respectively, in PB) was used instead of the ABC kit. The cells were coverslipped using Vectashield (Vector) mounting medium.

**Microscopy of Uptake Markers** - The endocytic compartment was visualized by incubating the cells for 5 min - 4 h with a fluorescent lipid derivative [N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM 1-43, Molecular Probes, Eugene, Oregon; 1 µM], which incorporates into the outer leaflet of the plasma membrane and a part of which is subsequently transported to endocytic vesicles (39). The cells were washed to remove the compound from the plasma membrane, fixed, and stained for endogenous intracellular hyaluronan using Alexa Fluor 633-labeled streptavidin as a reporter
Monoclonal antibodies for transferrin receptor (Chemicon International Inc., Temecula, CA), anti-caveolin 1 (Transduction Laboratories, Lexington, KY), beta-COP (Sigma) and anti CD44 mab (OX50, Biosource, Camarillo, CA) were used at a dilution of 1:100, and the mab against cathepsin D (Transduction Laboratories) was used at a 1:50 dilution. Texas Red-labeled anti-mouse antibody (Vector) at 1:100 dilution was used as the secondary step. In the double staining protocols the primary mab was added together with the bHABC, and the secondary antibody simultaneously with FITC-avidin.

Acidic compartments in keratinocytes were visualized by feeding the cells with 30 µM DAMP (N-(3-(2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine dihydrochloride) (Molecular Probes) for 30 min (40). The cells were then washed with PB, fixed, permeabilized as described above, and allowed to react overnight at 4°C with a polyclonal antibody (FITC-labeled anti-DAMP, Molecular Probes, dilution 1:100). After incubation, the cells were washed and mounted in Vectashield. For hyaluronan double stainings, the anti-DAMP mab was added to the bHABC solution; otherwise the staining was done as described above for bHABC.

Fluid phase uptake and coated pit pathways were visualized by incubating REKs in the presence of lysine fixable, Texas Red-labeled dextran (MW 10,000, Sigma, 10 µg/ml) (41) and Texas Red labeled human transferrin (Molecular Probes, 4 µg/ml) for 10-120 min (42). The cells were then fixed and processed for microscopy as above. For dual staining of dextran or transferrin, and endogenous hyaluronan, cells were first fed with Texas Red-labeled dextran or transferrin for 10-60 min, and then fixed and stained for hyaluronan as described above.

Confocal Microscopy - Laser scanning confocal microscopy was done using an Ultraview confocal scanner, built on a Nikon TE300 microscope with a 100 x NA 1.3 oil immersion objective, and equipped with an UltraPix CCD camera as a detector (PE-Wallac-LSR, Oxford, U.K.). Recordings consisted of 5-10 optical sections from the top to the bottom of the cell layer. Images were processed with Scanware image analysis system for Microsoft Windows. Further
contrast enhancement was done with Photoshop software (Adobe, Mountain View, CA).

**Electron Microscopy** - After fixation *in situ* with 2% paraformaldehyde and 0.5% glutaraldehyde for 20 min and blocking with 3% BSA in 0.1% Triton X-100 for 10 min, the cells were incubated with bHABC (5 µg/ml) overnight at 4°C, washed with PB, and visualized with DAB as described above. The cells were postfixed with reduced osmium tetroxide by adding potassium ferrocyanide (2.5 mg/ml final concentration) into aqueous 1% OsO$_4$ (43). The cells were dehydrated in graded ethanol and embedded in Spurr’s resin. Thin sections were mounted on Formvar coated copper grids, stained with uranyl acetate, and viewed using a JEOL 1200 EX microscope.

For dual staining of hyaluronan and CD44, the Ox50 anti-CD44 antibody was added to the bHABC solution at a 1:50 final dilution. The anti-mouse secondary antibody, conjugated to 5 nm gold particles (Amersham, Little Chalfont, UK) and diluted 1:40, was applied together with the streptavidin-peroxidase (1:500, Vector) solution. The peroxidase activity was visualized as described above.

**Internalization of Exogenous Hyaluronan** - The experiments were done in MEM supplemented with 1% BSA (binding medium). The cells were first washed three times with cold binding medium, then incubated in binding medium containing 10 µg/ml fluorescein-labeled hyaluronan (FL-HA) at 4°C for 1-2 h. The cells were washed 3 x 2 min with cold binding medium, and fixed after incubation at 37°C or at room temperature for 0, 30, 60, 90 or 120 min. Fixation was done in 2% paraformaldehyde for 20 min at room temperature, and the cell layer then washed with PB and mounted in Vectashield.

In preliminary experiments, cells were digested with testicular hyaluronidase in culture prior to the incubation with FL-HA. However, no obvious difference in the binding was observed following hyaluronidase digestion, which was omitted from later experiments.

The specificity of the binding was tested in parallel cultures incubated in: 1) FL-HA digested by *Streptomyces* hyaluronidase, 2) FL-HA together with hyaluronan oligosaccharides
HA_{14}, 0.3-1.0 mg/ml), and 3) FL-HA together with 1 mg/ml of commercially available chondroitin sulfate or heparan sulfate (Seikagaku).

For quantitative measurements of FL-HA binding, cells were seeded onto 6-well plates at 20,000 cells/cm^2, grown to near confluency and incubated with FL-HA with and without the competing substances described above. Each cell layer was washed 2x2 min with PBS and stripped of surface hyaluronan by 0.025% trypsin and 0.02% EDTA for 10 min at 37°C. The cells were then pelleted and washed with PBS before lysis in distilled water. The amount of FL-HA was determined using a fluorescence plate reader (SpectraFluor, Tecan, Salzburg, Austria) calibrated with known amounts of FL-HA.
RESULTS

**Content of Intracellular Hyaluronan** - Our previous biochemical studies demonstrated that REKs contain a pool of hyaluronan which is resistant to trypsin digestion, a treatment that removes virtually all hyaluronan from the cell surfaces, and which comprised about 3% of the total hyaluronan recovered after a 24 h radiolabeling period in confluent cultures (23). The present study shows that the size of this trypsin-resistant hyaluronan pool is larger in low cell density cultures, as demonstrated by analysis of consecutive 24 h synthesis periods on a set of REK cultures, allowed to grow from an initial density of $9 \times 10^4$ to a final density of $156 \times 10^4$ cells/cm$^2$ during 4 days (Fig. 1). In another experiment, parallel cultures were seeded at different densities and metabolically labeled on the next day. This resulted in a tenfold higher intracellular hyaluronan content in cells seeded at $14 \times 10^4$ vs $88 \times 10^4$ cells/cm$^2$ (Fig. 1, inset).

*Insert Fig. 1*

In order to minimize internalization of hyaluronan during enzymatic treatment, we conducted sequential trypsin and *Streptomyces* hyaluronidase digestions at 4°C. This cold, sequential enzymatic treatment reduced the amount of intracellular hyaluronan to 2-4 fg/cell, 30-50% less than the amount determined when digestions were carried out at 37°C, reflecting the high rate of hyaluronan uptake. Despite the relatively small quantity of hyaluronan in the REKs, metabolic studies suggest that ~25% of the total hyaluronan synthesized is ultimately catabolized intracellularly (see Discussion, Hyaluronan Turnover in Keratinocytes).

**Molecular Mass Distribution of Intracellular Hyaluronan** - For analysis of the size distribution of the intracellular hyaluronan, pericellular hyaluronan was first removed by treating the 20 h radiolabeled cultures with *Streptomyces* hyaluronidase in the cold, and then solubilizing intracellular hyaluronan with proteinase K as described in Experimental Procedures. For comparison, pericellular hyaluronan was isolated by regular trypsinization in parallel cultures.
Aliquots of each sample were eluted on Sephacryl S-1000. In the fractions collected, hyaluronan was distinguished from other \(^3\)H-labeled macromolecules as described in Experimental Procedures. Most of the intracellular hyaluronan eluted after \(K_{av} 0.5\), indicating a molecular mass below 400 kDa (Fig. 2a), with the most abundant hyaluronan eluting at \(K_{av} 0.67\), a molecular mass of \(~30\) kDa. In contrast, hyaluronan in the medium and that released from the cell surface by trypsin treatment, both eluted close to the excluded volume, indicating a molecular mass greater than 2000 kDa (Fig. 2b, c). Although hyaluronan of \(<400\) kDa size formed a minor proportion of the total hyaluronan in the extracellular compartments, the total amount in this size range was somewhat higher than that within the cell (Fig. 2b,c).

*Insert Fig. 2*

Superdex Peptide column chromatography demonstrated negligible amounts of hyaluronan oligosaccharides less than 5 kDa in any of the cell compartments (data not shown).

**Origin and Turnover of Intracellular Hyaluronan** - An experiment was designed to show that the intracellular hyaluronan is derived from the cell surface or the culture medium. REK cultures were incubated with a radiolabeled precursor of hyaluronan while *Streptomyces* hyaluronidase was present in the culture medium. At a concentration (5 U/ml) that removed >90% of the cell surface hyaluronan, there was a concomitant 85% decrease of the intracellular hyaluronan (Fig. 3a), indicating that hyaluronan that is present within the cell had its origin at the cell surface and/or from the culture medium, compartments accessible to hyaluronidase. An almost equal reduction in the amount of intracellular hyaluronan was obtained by using a lower enzyme concentration (1 TRU/ml) (Fig. 3a). Therefore, most of the intracellular hyaluronan in the untreated cultures was probably endocytosed after its biosynthesis and extrusion across the plasma membrane.

*Insert Fig. 3*

The turnover time of the intracellular hyaluronan was studied by steady state
radiolabeling the REK cultures, then chasing in a precursor-free medium containing *Streptomyces* hyaluronidase to prevent the entry of cell surface hyaluronan and that subsequently synthesized from the labeled precursor pool still remaining in the cell. The amount of intracellular radiolabeled hyaluronan was 70% of the starting value after 1 h chase and 60% after 2 h (Fig. 3b). A plateau (~30% of the starting value) in the intracellular hyaluronan was reached in 4 h. The chase experiment thus showed that the half life of most of the intracellular hyaluronan is 2-3 hours (See Fig. 3, lower panel).

**Hyaluronan in Cytoplasmic Vesicles** - The cytochemical hyaluronan probe (bHABC) showed that most of the cell-associated hyaluronan was distributed in patches on plasma membrane (23) (Fig. 4a). However, some was also located in vesicle-like structures, often close to the nucleus, suggesting an intracellular location. This was confirmed by short treatment of live REK cultures with *Streptomyces* hyaluronidase before staining for hyaluronan. The perinuclear signal was retained, while plasma membrane staining was removed by the enzyme (Fig. 4b). To avoid possible translocation of hyaluronan in live cells in response to the enzyme, we treated fixed cells with hyaluronidase prior to permeabilization of the cell membranes. This resulted in the same vesicular staining pattern as that seen with an enzyme digestion prior to the fixation (Fig. 4c). Confocal microscopy of cells pretreated with hyaluronidase after fixation also exhibited vesicle-like hyaluronan signals distributed in a perinuclear position (Fig. 4g). Cultures incubated with the bHABC probe preblocked with hyaluronan oligosaccharides ((44); data not shown), or digested with *Streptomyces* hyaluronidase after permeabilization (Fig. 4d), were completely negative, demonstrating the specificity of the staining.

*Insert Fig. 4.*

Transmission electron microscopy unequivocally confirmed the vesicular residence of the hyaluronan (Figs. 5a-f). Fig. 5a shows the hyaluronan-positive intracellular structures using gold-labeled streptavidin and silver enhancement. Figs. 5b-f show sections from cultures stained with streptavidin peroxidase for hyaluronan and with gold-labeled secondary antibody for CD44.
The hyaluronan-positive vesicles varied from 60 to 600 nm in diameter, with a few even larger (800-1300 nm). The small diameter vesicles (~100 nm) were typically found close to the plasma membrane and were often elongated, forming tube-like structures (Fig. 5b,d). Larger vesicles were distributed around the nucleus with some appearing to be multivesicular bodies (Fig. 5a,e). Cells pretreated with *Streptomyces* hyaluronidase prior to permeabilization lost the fuzzy surface coat visible in Fig. 5b, while both small (Fig. 5c) and large (Fig. 5e) vesicles remained hyaluronan positive. This finding excluded the possibility that the hyaluronan positive vesicles represented deep invaginations of the plasma membrane. Interestingly, hyaluronan found in liver endothelial cells by Fraser et al. (3) was found mainly in large 0.3 to 1.2 µm vesicles resembling the perinuclear hyaluronan-positive structures seen in the REKs.

*Insert Fig. 5.*

**Colocalization of Intracellular Hyaluronan with a Membrane Endocytic Tracer, but not with Markers of Lysosomes and Golgi Vesicles** - The endocytic compartment was visualized using a fluorescent lipid derivative (FM 1-43) which incorporates into the outer leaflet of the plasma membrane, and translocates to endocytic vesicles as a part of the plasma membrane (39). After a few minutes treatment, FM 1-43 positive vesicles appeared in REKs. The post-staining with bHABC reduced the signal of FM 1-43. However, enough was retained to confirm a colocalization with intracellular hyaluronan (Fig. 6a). The data suggest that intracellular hyaluronan is derived from pericellular/extracellular locations, although the intracellular origin is not totally ruled out because of possible intracellular vesicle fusion events.

*Insert Fig. 6.*

Most of the lysosomes, visualized with anti-cathepsin D staining (Fig. 6c), were hyaluronan negative, and most of the hyaluronan positive structures were negative for cathepsin D (Fig. 6c), indicating that the demonstratable intracellular hyaluronan in REKs was not localized in lysosomes. Likewise, REKs fed with DAMP, a tracer that seeks its way into acidic
compartments of the cell (40), showed only rare colocalization with intracellular hyaluronan (Fig. 6b). The data suggest that most of the hyaluronan-containing vesicles had close to neutral pH, and probably represented early endosomes.

Golgi compartments were localized using an anti-COP mab (45) (Fig. 6d, red). While the COP-positive structures were grouped around the nucleus, like most of the vesicles positive for hyaluronan, they did not colocalize (Fig. 6d).

**Intracellular Hyaluronan Accumulation Following Lysosome Inhibition** - Lysosomal functions of the REKs were inhibited by ammonium chloride or chloroquine treatment, followed by staining for intracellular hyaluronan. Optical density (OD) of the intracellular hyaluronan signal was also measured, and the mean OD per area as well as the relative area of intracellular hyaluronan-positive structures were determined. Both reagents increased the OD and the hyaluronan positive area ~ 3-4 times over that of controls (Table I). Ammonium chloride and chloroquine gave their maximum responses at 5 mM and 1 mM concentrations, respectively (data not shown). Accumulation of intracellular hyaluronan was increased by 30 min, and more pronounced after 2 h, reaching a maximum at 4 h after which it declined (data not shown). An image of the intracellular hyaluronan in a REK culture treated with 10 mM ammonium chloride for 4 h is shown in Fig. 4f. Most cells showed both an increased number and size of hyaluronan positive vesicles. Fig. 5g shows a TEM image of a REK culture treated with 5 mM ammonium chloride for 4 h with a very large hyaluronan positive intracellular vesicle. Monensin, an ionophore which has been reported to inhibit lysosomal activity and receptor recycling (34), also caused an increase in the intracellular hyaluronan staining (Figs. 6g and 7e). The hyaluronan-positive structures in monensin-treated cultures often showed a ring-like morphology (Fig. 7e).

*Insert Table I and Fig. 7.*

The molecular mass distribution of metabolically labeled intracellular hyaluronan was also
analyzed in ammonium chloride and chloroquine treated cultures using S-400 gel filtration (Fig. 2d-f). In control cultures about 23% of the intracellular hyaluronan had the apparent molecular mass larger than 90 kDa, while the corresponding figures for chloroquine and ammonium chloride treated cultures were 50% and 53%, respectively, indicating a shift towards higher molecular mass.

Apigenin reportedly inhibits hyaluronidase in vitro (46), in sperm penetration assays (47), and in mammary and cervix tumors (48). When added to the REK culture medium, apigenin caused a rapid and dose-dependent increase in intracellular hyaluronan staining although the accumulation was less pronounced than with ammonium chloride (Table 1).

Taken together, these data in which lysosomal function was perturbed suggest that the intracellular hyaluronan demonstrated by the bHABC probe resides in vesicles targeted to lysosomes.

**Reduction of Vesicular Hyaluronan by Hyaluronan-Oligosaccharides** - Subconfluent REK cultures were incubated with hyaluronan oligosaccharides of different sizes at 0.2 to 0.5 mg/ml concentrations. The OD measurement of the hyaluronan staining showed that oligosaccharides with 8 monosaccharide units (HA$_{8}$) or smaller had no effect, while HA$_{10}$ or greater decreased intracellular hyaluronan staining (Fig. 8a). The area of DAB positivity was reduced with HA$_{14}$ to 20% of the control cultures. The structurally related glycosaminoglycans chondroitin sulfate and heparan sulfate had no effect (Fig. 8a). Fig. 4e shows histological staining for intracellular hyaluronan of a culture treated with HA$_{10}$ for 4 h, exhibiting virtually no hyaluronan signal. These findings suggest that there is an uptake receptor in REKs specific for hyaluronan which requires a minimum length of HA$_{10}$ for effective binding.

*Insert Fig. 8.*

The kinetics of clearance of the intracellular hyaluronan signal following addition of HA$_{12}$
into growth medium, visualized with bHABC, is shown in Fig. 8b. The OD of the DAB color was reduced to 60% of the starting value in 5 min and reached a stable basal level, ~20% of control, in 10 min. The half life of the intracellular hyaluronan detected with bHABC was thus considerably shorter than the total hyaluronan assayed by radiolabeling (Fig. 3). This observation is consistent with the hypothesis that the bHABC probe specifically demonstrates recently endocytosed hyaluronan molecules that are rapidly shifted to another pool that cannot be detected with the bHABC probe.

**Intracellular Hyaluronan and CD44** - REKs reacted with the mab OX50 against the hyaluronan receptor CD44 showed a strong plasma membrane signal, especially concentrated at areas of cell-cell contact and in patches on the apical surface, a distribution similar to that of hyaluronan (Fig. 7a, and ref. (23)). Very little CD44 was detected intracellularly by confocal microscopy, and dual staining of CD44 and intracellular hyaluronan exhibited only rare colocalizations close to the plasma membrane (Fig. 7a-c). Immuno-electron microscopy demonstrated CD44 enrichment on plasma membrane projections (Fig. 5b). CD44-positive, small diameter vesicles or tubules close to plasma membrane were occasionally ultrastructurally seen (Fig. 5b, h). These structures were also hyaluronan positive (Fig. 5b,h). CD44 was not found in coated pits or coated vesicles (Fig. 5e,f).

Perturbation of lysosomal activity with ammonium chloride or chloroquine, which markedly increased the amount of vesicular hyaluronan, caused a minor increase in the intracellular CD44 staining (data not shown). However, treatments which have been shown to slow down receptor recycling, *e.g.* monensin (34) (Fig. 7d-f), or incubation at room temperature (49) (Fig. 7g-l), caused accumulation of intracellular CD44 in addition to hyaluronan. Monensin-treated cultures frequently exhibited large circular structures with CD44 on the perimeter and hyaluronan inside (Fig. 7d-f). Confocal analyses confirmed that these structures with a ring-like distribution of CD44 were within the cells. REKs incubated at room temperature also showed a colocalized signal for CD44 and hyaluronan, typically close to the plasma.
membrane (Fig. 7g-l), in addition to vesicles farther removed from the surface (Fig. 7i and j, 4 and 2 μm from the bottom, respectively).

It was previously shown that OX50, a monoclonal anti-CD44 antibody increases pericellular hyaluronan staining on REKs (23). The consequences of this pericellular accumulation were studied by an OD assay of intracellular hyaluronan after a 4 h incubation with OX50. This antibody caused a consistent, dose-dependent increase in the intracellular hyaluronan-positive area (Fig. 9). A normal, isotypically matched mouse IgG, (data not shown) and Hermes 3, an anti CD44 antibody against human CD44, which does not recognize rat CD44, had little or no effect on the level of intracellular hyaluronan (Fig. 9).

Endogenous Hyaluronan Uptake and Coated Pits, Caveolae and Pinocytosis - Two established mechanisms of receptor mediated endocytosis, utilizing clathrin-coated pits and caveolae, were studied. The avid internalization of transferrin, a widely used marker of the coated pit pathway (42), confirmed that this endocytosis pathway operated in REKs (Fig. 6e-g). The formation of coated pits is selectively inhibited by chlorpromazine and hyperosmotic medium (50). Both of these inhibitors significantly reduced the uptake of Texas Red- or FITC-labeled transferrin (data not shown), indicating that the treatments perturb the coated pit pathway in REKs. However, neither of these treatments decreased the amount of intracellular endogenous hyaluronan in REK cultures (Table 2). In fact, chlorpromazine caused an increase in the intracellular hyaluronan staining in all experiments (Table 2). Higher chlorpromazine concentrations and shorter and longer treatment times (30 min - 4 h) also caused a consistent increase in the intracellular hyaluronan staining (data not shown).

Cells with very active transferrin uptake usually exhibited low intracellular hyaluronan content. The converse was also true with cells exhibiting abundant intracellular hyaluronan
demonstrating reduced transferrin uptake (Fig. 6e). Incubation of cells at room temperature permits endocytosis, but slows receptor recycling and transport to lysosomes (49). REKs incubated at room temperature for 1 h (Fig. 6f) contained more intracellular hyaluronan-positive vesicles than cells taken directly from 37°C (Fig. 6e), and also accumulated more Texas Red-transferrin. Most of the intracellular hyaluronan and transferrin resided in separate compartments (Fig. 6f). REKs treated with monensin to reduce receptor recycling (34), showed accumulation of both transferrin and intracellular hyaluronan, again mainly in distinct compartments (Fig. 6g). Staining for the receptor of transferrin also confirmed separate uptake vesicles for hyaluronan and transferrin (data not shown). Furthermore, electron microscopy did not reveal coats on hyaluronan vesicles (Fig. 5e,f). These data indicate that hyaluronan did not enter REKs through coated pits.

REKs showed active pinocytosis of Texas red-labeled dextran, a commonly used fluid phase endocytosis marker (41). Cells with the most active dextran uptake usually had more endogenous intracellular hyaluronan. An example of such a cell is shown in Fig. 6h. Despite this association of intracellular hyaluronan with the fluid phase endocytosis, most of the endogenous hyaluronan vesicles were dextran negative (Fig. 6h).

The large size of the endogenous hyaluronan-positive vesicles and their responsiveness to growth factor stimulation (51) and the fact that large polysaccharides like dextrans, are preferentially taken into macropinosomes rather than micropinosomes (41), suggested that hyaluronan-positive vesicles might be formed via macropinocytosis. The possibility that hyaluronan bound to cell surface receptors is recruited into these macropinocytotic vesicles, was probed with amiloride, an inhibitor of macropinocytosis (31,32). However, amiloride was unable to influence the content of intracellular hyaluronan in REKs (Table 2). A more potent derivative of amiloride, N,N-dimethylamiloride, also had a negligible influence on the amount of intracellular hyaluronan positivity (data not shown).

Basal cells in the epidermis have caveolae on their basolateral surfaces (52), and immunostaining with anti-caveolin 1 antibody demonstrated a positive reaction in the same area
REKs stained with a monoclonal antibody against caveolin 1 showed a diffuse plasma membrane and cytoplasmic signal (Fig. 6l). Occasional cells with more intense punctate caveolin 1 staining were seen (Fig. 6l). The distribution patterns of caveolin 1 and hyaluronan were clearly different (Fig. 6l). Filipin and nystatin, reported to inhibit the formation of caveolae in endothelial cells (30), failed to cause any reduction in the intracellular hyaluronan staining (Table 2). In contrast, both increased intracellular hyaluronan positivity (Table 2). Lower and higher concentrations of inhibitors, and longer and shorter treatment times, were also tested, and they gave similar results (data not shown).

Receptor-Mediated and Bulk Phase Uptake of Exogenous Hyaluronan - Exogenous FITC-labeled macromolecular hyaluronan (size > 100 kDa) given to the REKs, was internalized into vesicular structures (Fig. 4h and 6i). Cells showing efficient uptake of exogenous hyaluronan often had high endogenous intracellular hyaluronan content, and the two signals showed partial colocalization (Fig. 4h). To distinguish between receptor mediated versus bulk phase endocytosis of exogenous hyaluronan, hyaluronan oligosaccharides were added to inhibit the binding and uptake of FITC-labeled hyaluronan. The amount of FITC-hyaluronan bound to the cell surface at 4°C, and that associated with the cells after 30 min chase at 37°C, are shown in Fig. 10. The oligosaccharides partially inhibited FITC-hyaluronan binding on the cell surface, while sulfated glycosaminoglycans at the same concentration were less effective as competitors (Fig. 10). The HA₁₄ oligosaccharide also reduced the internalization of high molecular weight hyaluronan by ~70%, while the other glycosaminoglycans were less effective (Fig. 10). The uptake of exogenous hyaluronan in the presence of competing oligosaccharides and the fact that exogenous hyaluronan showed partial colocalization with dextran (Fig. 6i) suggest that exogenous hyaluronan can be endocytosed both via receptor-mediated (HA₁₄ affected) and non-specific mechanisms.

Insert Fig. 10.
Short hyaluronan oligosaccharides (HA$_{\geq10}$) can displace hyaluronan from its receptors on REKs (23). To study whether they were also internalized, oligosaccharides were labeled with the fluorescent AMAC group at their reducing end to avoid possible interference of receptor binding along the relatively short chain. The biological properties of the tagged oligosaccharides were similar to those of unlabeled oligosaccharides in that they were equally effective in displacing intact hyaluronan from the cell surface (data not shown). Throughout the HA$_4$-HA$_{40}$ size range examined, the labeled oligosaccharides were avidly internalized by REKs when incubated at 37°C (Fig. 6j). However, this internalization was not inhibited by unlabeled oligosaccharides (Fig. 6k). Unlike macromolecular hyaluronan, the labeled oligosaccharides were not bound to the cells when incubated at 4°C, or internalized upon subsequent chase at 37°C (data not shown). This suggests that these small hyaluronan oligosaccharides have a low affinity for the hyaluronan receptor, and that their uptake is not receptor mediated. The labeled oligosaccharides were taken up into the same vesicles as the Texas Red labeled-dextran (Fig. 6j and k), supporting the idea of bulk phase endocytosis as their primary route for internalization.

DISCUSSION

**Origin of Intracellular Hyaluronan** - The present data demonstrate that intracellular vesicles in rat epidermal keratinocytes contain hyaluronan derived from either endogenous or exogenous sources. Most of the endogenous intracellular hyaluronan originates from the cell surface via receptor-mediated endocytosis and is destined for lysosomal degradation since: 1) intracellular hyaluronan colocalized with a plasma membrane endocytic tracer, 2) the content of intracellular hyaluronan decreased by competition with HA$_{\geq10}$ oligosaccharides which block binding of hyaluronan to cell-surface receptors, 3) continuous hyaluronidase digestion of extracellular hyaluronan reduced intracellular hyaluronan, and 4) the inhibition of endogenous hyaluronidase and lysosomal functions lead to accumulation of intracellular hyaluronan and a
shift of the size towards higher molecular mass (see diagram in Fig. 11). Exogenous hyaluronan is partly taken up via receptor mediated uptake, and partly via bulk phase endocytosis, because: 1) its uptake was only partially reduced with hyaluronan-oligosaccharides, and 2) it showed a marked colocalization with bulk fluid phase endocytosis markers.

**Properties of the Hyaluronan Uptake Receptor** - The hyaluronan uptake receptor in keratinocytes is distinct in that it is not sensitive to sulphated glycosaminoglycans, unlike those in chondrocytes and liver endothelial cells (17,54,55). Likewise, HA$_6$ or HA$_8$ oligosaccharides compete for hyaluronan uptake in chondrocytes (8) and liver endothelial cells, but not in REKs, which require HA$_{10}$.

While an excess of end-labeled fluorescent oligosaccharides in the size range HA$_{10}$ to $\approx$HA$_{40}$ competed for the binding and internalization of hyaluronan, they were not bound to the receptors tightly enough to be demonstrated on the cell surface by fluorescence microscopy. Furthermore, they were mainly endocytosed through bulk phase pinocytosis as indicated by the lack of competition with unlabelled oligosaccharides. This suggests that the uptake of intact hyaluronan involves multivalent binding to several receptor molecules (28), probably clustered at defined sites on the plasma membrane (23,56). Coalescence of such cholesterol-enriched (56) and detergent-resistant (57) receptor clusters is compatible with the patchy distribution of hyaluronan and CD44 on keratinocytes (23). The patching of CD44 may enhance its affinity for hyaluronan, and may also be required for receptor-mediated uptake. It has been shown by several studies that the proximity of CD44 molecules, spontaneous or induced by crosslinking in the membrane spanning domain or by antibodies, enhance hyaluronan binding (57-59).

**Role of CD44 in Hyaluronan Uptake of Keratinocytes** - While the increase of endogenous intracellular hyaluronan by the anti CD44 antibody OX50 suggested that CD44 is involved in hyaluronan endocytosis in REKs, the mechanism remains incompletely understood. Immunostaining of REKs with a mab against CD44 showed only occasional intracellular signal, similar to that observed in human keratinocytes *in vivo* (22). The absence of intracellular CD44
staining may indicate that CD44 does not act alone in the uptake of hyaluronan. The fact that decasaccharides are required to compete for the binding and uptake in keratinocytes, while hexasaccharide size is large enough to compete for CD44 binding in many other cells and tissues, can be taken to support this alternative. On the other hand, incubation of REKs with monensin or at room temperature to interfere with receptor recycling, increased the number and size of CD44 positive intracellular vesicles, the number of hyaluronan positive vesicles, and colocalization of intracellular CD44 and hyaluronan. These experiments suggest that the low number of CD44 positive vesicles and the lack of colocalization with hyaluronan reflect recycling of CD44 back to the cell surface from early endosomes (Fig. 11).

Routes of Hyaluronan Endocytosis - It is puzzling that clathrin-coated pits, a common vehicle for wrapping and detaching receptor bound cargo into endosomal vesicles, and also shown to be active in REKs, seems not to operate in hyaluronan uptake. This is suggested by: 1) the lack of transferrin and transferrin receptor colocalizations with hyaluronan, 2) the inability of the inhibitors of coated pit formation to reduce intracellular hyaluronan although they effectively reduced the uptake of transferrin, and 3) the lack of electron microscopically detectable coats around hyaluronan-containing vesicles. There is previous evidence for the involvement of coated pits with the hyaluronan uptake mediated by the receptor HARE on liver endothelial cells (60) although non-saturable, fluid-phase endocytosis has also been reported in these cells (4). Coated pits were not associated with the CD44-associated hyaluronan endocytosis in chondrocytes (7,61), nor was CD44 found in the coated pits of the present keratinocytes. Keratinocytes are known to use unusual mechanisms not corresponding to the classical endocytic routes to internalize subdomains of their plasma membrane. Thus, hemidesmosomal and desmosomal plaques are internalized by a pathway not involving clathrin coated pits (62,63). A similar mechanism might operate in the uptake of membrane bound hyaluronan.

Caveolae, the other common mechanism of receptor mediated endocytosis, were also excluded as the hyaluronan-uptake route because: 1) inhibitors of caveola formation did not reduce intracellular hyaluronan content, and 2) immunostaining for caveolae showed no
colocalization with hyaluronan. The fact that inhibitors of endocytosis through coated pits and caveolae actually lead to accumulation of intracellular hyaluronan may reflect the pleiotropic effects of the inhibitors, or activation of alternate uptake systems when one route is blocked (64).

While the hyaluronan endocytosed by specific receptors, and labeled dextran taken up by bulk phase endocytosis, resided in separate vesicles, both were often abundant in the same cells, suggesting that the two processes are somehow linked. Both uptake systems may correlate with the rate of membrane turnover. High molecular mass hyaluronan when present in the forming endosome may sterically exclude other macromolecules like dextran, thereby explaining the lack of colocalization of hyaluronan and dextran in most vesicles.

Degradation of Hyaluronan Following Endocytosis - The molecular mass of the intracellular hyaluronan was strongly skewed towards the lower end, with most of the material below 400 kDa and a peak at ~20 kDa, in contrast to that on the cell surface and in the medium, mostly exceeding 2000 kDa. This suggests that either: 1) the intracellular material has already undergone degradation after endocytosis, or 2) there is selective uptake of low molecular mass hyaluronan from the cell surface. The fact that perturbation of lysosomal activity caused a shift towards higher molecular mass species of intracellular hyaluronan supports the idea that the intracellular hyaluronan detected in control samples was partly degraded inside the cells; quite likely by hyaluronidases in the early endosomal compartment. Interestingly, one of the recently described hyaluronidases (Hyal 2) was reported to produce ~20 kDa hyaluronan fragments as its end product (65). The rapid clearance of the bHABC positive vesicles by the receptor competing oligosaccharides also fits with the hypothesis that these endosomes represent dynamic, transitory vehicles in which hyaluronan is moved towards lysosomes and in which its initial fragmentation takes place. The 20-90 kDa hyaluronan probably does not give a signal in contrast to the high molecular mass hyaluronan, since it may not fix well enough to be retained throughout the staining procedure. The relatively long 2-3 h half life of the total intracellular hyaluronan is best explained by gradual endoglycosidase action in the prelysosomal endosomes (Fig. 11), a process similar to that in heparan sulfate catabolism (66). The final degradation in
lysosomes may be rapid, since no oligosaccharide size intermediates were detected.

_Hyaluronan Size and Endocytosis_ - The alternative that there was preferential uptake of low molecular mass hyaluronan cannot be completely excluded, however, since there was sufficient low molecular mass material in the medium, despite its low proportion of the total, to supply the intracellular pool. Selective endocytosis of relatively low molecular mass material is also in agreement with a study on cultured endothelial cells, fibroblasts, and vascular smooth muscle cells, showing the highest internalization rate in the molecular mass range ≤30 kDa (9). Interestingly, the present study showed efficient non-receptor mediated internalization of 0.8 - 8 kDa hyaluronan oligosaccharides (HA₄-HA₄₀).

The size of pericellular hyaluronan may also influence the probability of its engulfment into a vesicle, since a long chain of hyaluronan simultaneously bound to distant cell surface sites would be expected to resist vesicle budding and detachment. Extracellular and intracellular hyaluronan showed a molecular mass up to 6000 kDa and less than 400 kDa, respectively, corresponding to 300 nm and 65 nm radius of gyration (17). It has been shown that high molecular mass dextran (150 kDa) is taken up by macropinocytosis, i.e. vesicles larger than 200 nm in diameter, while small dextran molecules (4-10 kDa) can enter both in macropinosomes and micropinosomes. Therefore, accommodation of the large hyaluronan species in the micropinosomes of ~120 nm diameter vesicles (41) seems difficult, and fragmentation of cell surface attached hyaluronan could facilitate its uptake. In chondrocytes, the increased content of hyaluronan, and its liberation from other matrix molecules enhances CD44 turnover and internalization (7). Our previous organ culture experiments suggest that epidermal hyaluronan is fragmented into intermediate sizes before final clearance (20), perhaps mediated by reactive oxygen species (19).

_Hyaluronan Turnover in Keratinocytes_ - The present and earlier data (23) suggest that a considerable proportion of newly synthesized hyaluronan is internalized and degraded. In continuous labeling of REK cultures, a steady state in cell surface and intracellular hyaluronan prevails between 12 and 24 h after label introduction (23). It can be calculated that hyaluronan in
the medium increases by ~32 fg/cell during that time (23) and that ~11 fg/cell is degraded in a similar time period in the present experiments, suggesting that 25% of all hyaluronan is endocytosed shortly after synthesis. It is highly likely that this fast hyaluronan synthesis/endocytosis traffic on the cell surface has a biological importance of which little is currently known.

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Footnotes

1The abbreviations used are: REK, rat epidermal keratinocyte; HBSS, Hank’s balanced salt solution; bHABC, biotinylated hyaluronan binding complex, BSA, bovine serum albumin; TRU, turbidity reducing unit; PBS, phosphate buffered saline; PB, phosphate buffer; DAB, 3,3-diaminobenzidine; FL-HA fluorescein labeled hyaluronan; mab, monoclonal antibody; DAMP, [N-(3-(2,4-dinitrophenyl)amino)propyl]-N-(3-aminopropyl)methylamine dihydrochloride; FM1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide; AMAC, 2-aminoacridone.
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**TABLE I**

*Influence of perturbation of lysosomal activity on intracellular hyaluronan*

REK cultures were incubated with ammonium chloride, chloroquine or apigenin for 4 h prior to staining for intracellular hyaluronan. The optical densities (OD) were measured and the area of DAB-positive (above the threshold value) intracellular structures was compared to that in control cultures. Medians and ranges of three experiments are shown.

| Effector                  | % of OD in controls |
|---------------------------|---------------------|
| Ammonium chloride (10 mM) | 494 (216-2045)      |
| Chloroquine (2 mM)        | 345 (340-345)       |
| Apigenin (25 µM)          | 203 (131-264)       |
TABLE II

Influence of perturbation of coated pits, caveolae and macropinosomes on endogenous intracellular hyaluronan

REK cultures were incubated with chlorpromazine, sucrose, nystatin, filipin, or amiloride for 1 h prior to the fixation and staining for intracellular hyaluronan. The optical densities (OD) were measured and the area of DAB-positive (above the threshold value) intracellular structures was compared to that in control cultures. Medians and ranges of three experiments for chlorpromazine, filipin, and nystatin, and two experiments for amiloride and sucrose are shown.

| Effector                | % of OD in controls |
|------------------------|---------------------|
| Chlorpromazine (2 mM)  | 339 (199-2088)      |
| Sucrose (0.4 M)        | 151 (87-214)        |
| Filipin (4 µM)         | 165 (88-442)        |
| Nystatin (2 mM)        | 224 (121-296)       |
| Amiloride (2 mM)       | 91 (70-111)         |
LEGENDS TO THE FIGURES

**Fig. 1.** The influence of cell density on intracellular hyaluronan content. REKs were plated at 10,000 cells per cm$^2$, metabolically labeled with $[^3]$Hglucosamine and $[^{35}]$Ssulfate for 20 h (bars) after 0, 1, 2 or 3 days of culture. The intracellular hyaluronan was recovered after removal of the pericellular hyaluronan with trypsin and quantitated as described in Experimental Procedures. The density of the cultures (cells per cm$^2$ x 10$^{-3}$) at the end of labeling is shown in parenthesis for each time point. The inset shows the data from another experiment where cells were seeded at low and high densities, and labeled 24 h later. The number of cells per cm$^2$ (x10$^{-3}$) at the end of labeling is given on the x-axis.

**Fig. 2.** Molecular mass distributions of hyaluronan. (a-c) Hyaluronan was metabolically labeled with $[^3]$Hglucosamine, recovered from the medium, pericellular and intracellular compartments, and fractionated with Sephacryl S-1000 gel filtration as described in Experimental Procedures. (a) Intracellular compartment, (b) trypsinate, (c) medium. (d-f) REK cultures were treated with 5 mM NH$_4$Cl and 1 mM chloroquine while metabolically labeled with $[^3]$Hglucosamine for 6 h. Hyaluronan was recovered from the intracellular compartment at 4°C as described in Experimental Procedures and fractionated with Sephacryl S-400 gel filtration. The arrows indicate the elution positions of 400 kDa and 90 kDa hyaluronan determined with the calibration curves supplied by the manufacturer of the chromatographic resin, and the position of hyaluronan oligosaccharides of about 5 kDa, prepared in our laboratory.

**Fig. 3.** Effect of extracellular hyaluronidase treatment on intracellular hyaluronan. (upper panels) Nearly confluent cultures were metabolically labeled with $[^3]$Hglucosamine and $[^{35}]$Ssulfate for 6 h in the presence of 1 and 5 units/ml of *Streptomyces* hyaluronidase in order to demonstrate the cell surface or extracellular source of intracellular hyaluronan. Intracellular and
cell surface (trypsinate) hyaluronan were quantitated after separation of the compartments with trypsin at 37°C as described in Experimental Procedures. (lower panel) Nearly confluent cultures were metabolically labeled with $[^3H]$glucosamine and $[^35S]$sulfate for 18 h, washed with unlabeled medium, and chased for 0-6 h in the presence of 1 unit/ml of Streptomyces hyaluronidase before the assay of intracellular hyaluronan to estimate the half life of the intracellular hyaluronan. The bar shows the range of duplicate cultures.

**FIG. 4.** Localization of intracellular hyaluronan. Subconfluent REK cultures were stained with bHABC for intracellular hyaluronan using the avidin-biotin-peroxidase technique and DAB (a, b, c, d, e, f) or with FITC-avidin (g), or with Texas Red streptavidin (h). (a) An untreated REK culture permeabilized with Triton-X100 and stained. (b) A culture treated with Streptomyces hyaluronidase prior to fixation, permeabilization, and staining. (c, e-h) Cultures fixed, first digested with hyaluronidase, then permeabilized and stained. (d) A culture fixed, first permeabilized, then digested with hyaluronidase and stained. Staining with the appearance of intracellular vesicles is present whether hyaluronidase digestion is performed on live (b) or fixed (c) cultures, but not in cultures treated with the enzyme after permeabilization (d) which allows access to intracellular compartments, and abolishes the vesicular hyaluronan. (e) A REK culture treated with HA$_{10}$-oligosaccharides for 4 h prior to fixation, permeabilization, and staining. (f) A culture treated with 10 µmol ammonium chloride for 4 h to block lysosomal activity. (g) A series of optical sections through the cell layer confirming the intracellular localization of the endogenous hyaluronan vesicles around the nucleus. The left image in the top row, and the right image in the lower row represent the upper and lower surfaces of the cell layer, respectively. (h) Localization of endogenous hyaluronan with bHABC (red), and exogenous FITC-labeled hyaluronan (green). Magnification bars 10 µm.

**FIG. 5.** Electron microscopic localization of hyaluronan and CD44. (a) Subconfluent REKs
stained for hyaluronan with bHABC using gold-labeled streptavidin and silver enhancement prior to embedding in plastic.  
(b-h) Double staining for hyaluronan with bHABC-avidin-biotin-peroxidase-DAB technique, and CD44 with a gold-labeled secondary antibody.  
(c,e,g) Cells treated with *Streptomyces* hyaluronidase prior to permeabilization to specifically remove pericellular hyaluronan. Note the strong, fuzzy, pericellular hyaluronan signal (b,d,f,h, large arrowheads in b,f), and the CD44 staining (small arrows), especially abundant on cell processes (e). Intracellular vesicles containing hyaluronan (large arrows) are seen both with the silver enhancement technique (a) and DAB visualization (b,c,d,e,g,h). The hyaluronan vesicles close to the plasma membrane are often elongated (b,d,h). CD44 is also found in some of the vesicles close to the plasma membrane (b,h). Coated vesicles appear to be hyaluronan-negative (e,f, small arrowheads). A culture treated with 5 mM ammonium chloride for 4 h (g) contains a large (diameter 600 nm) hyaluronan-positive vesicle. Magnification bars: 500 nm in (a), 200 nm in (b-g), and 100 nm in (h).

**Fig. 6. Characterization of the hyaluronan vesicles.** Confocal optical sections are shown, each with two fluorescent markers, that when colocalized yield a yellow color.  
(a) Cells incubated with a fluorescent lipid to specifically demonstrate the vesicles derived from plasma membrane (green) and those containing endogenous hyaluronan (red).  
(b) Cells incubated with DAMP to visualize acidic compartments (green) and endogenous hyaluronan (red).  
(c) Lysosomes indicated by staining for cathepsin D (red) and endogenous hyaluronan (green).  
(d) Golgi vesicles localized by staining for COP (red) and endogenous hyaluronan (green).  
(e) Coated pit derived endosomes after 30 min incubation at 37°C with Texas Red labeled transferrin (red) and endogenous hyaluronan (green).  
(f) Coated pit derived endosomes and hyaluronan as in (e) but incubated at 20°C.  
(g) Coated pit derived endosomes and hyaluronan as in (e) but incubated with monensin to inhibit receptor recycling.  
(h) Texas red labeled 10 kDa dextran (10 µg/ml) incubated for 30 min at 37°C to demonstrate bulk phase endocytosis vesicles (red), and endogenous hyaluronan (green).  
(i) Texas red labeled dextran (red) and 10 µg/ml FITC-labeled exogenous hyaluronan (green)
incubated together for 30 min at 37°C. (j) Texas red labeled dextran (red) and ~6 kDa AMAC-labeled hyaluronan oligosaccharides (green) incubated for 30 min at 37°C. (k) Texas red labeled dextran (red) and AMAC-oligosaccharides (green) incubated as in (j) with an excess of unlabeled oligosaccharides. (l) Caveolin containing vesicles (red), and endogenous hyaluronan (green).

**Fig. 7. Localization of CD44 in REKs.** Dual staining for CD44 (red) and endogenous intracellular hyaluronan (green), using Texas-Red labeled secondary antibody and FITC-labeled avidin as reporters, respectively. Confocal optical sections through the cell layer are shown. (a-c) Untreated cells, with the signal of CD44 (a), hyaluronan (b), and both (c). (d-f) Cells treated with 2 µM monensin for 2 h at 37°C, showing CD44 (d), hyaluronan (e) and both (f). (g-l) A series of consecutive optical sections through a cell incubated for 1 h at room temperature, with the topmost section in (g) and lowermost in (l). Note the strong CD44 signal from the plasma membrane, but little intracellular staining in untreated cells (a). Incubation with monensin induced large intracellular structures positive for hyaluronan (e) and CD44 (d) that were often colocalized (f). Intracellular CD44 and its colocalization with hyaluronan was also markedly increased by incubation at room temperature (g-l). Magnification bars 10 µm.

**Fig. 8. Influence of hyaluronan-oligosaccharides and related GAGs on intracellular hyaluronan.** (a) REK cultures were incubated with 0.3 mg/ml of sized hyaluronan-oligosaccharides (HA4-HA14), heparan sulfate (HS) and chondroitin sulfate (CSA) for 4 h prior to fixation and staining for endogenous intracellular hyaluronan. (b) REKs were incubated with 0.2 mg/ml of HA14 for 0-30 min before fixation and staining. The optical densities of the cultures were measured, and the areas of the DAB-positive intracellular structures were compared to that in control cultures. The data represent means ± SE from two independent experiments for hyaluronan oligosaccharides and three for heparan sulfate and chondroitin sulfate.
FIG. 9. **Influence of anti-CD44 monoclonal antibodies on intracellular hyaluronan.** REK cultures were incubated with either the OX50 antibody or the Hermes 3 antibody for 4 h prior to the staining for endogenous intracellular hyaluronan. The optical densities were measured, and the area of DAB-positive intracellular structures were compared to that in control cultures. The data represent means and ± SE from 3 different experiments.

FIG. 10. **Uptake of exogenous hyaluronan.** (a) REK cultures were incubated with FITC-labeled hyaluronan for 120 min at 37°C alone (Co) or with 1 mg/ml of chondroitin sulfate (CS-C), heparan sulfate (HS) or hyaluronan-oligosaccharides (HA_{14}), and the fluorescence released from the cells with trypsin (left panel) and that recovered from lysed cells (right panel) were measured. One of two experiments with similar results is shown.

FIG. 11. **Suggested hyaluronan endocytosis and degradation process in keratinocytes.** 1) Hyaluronan binds to clustered CD44 and possibly other unknown cell surface receptors. 2) Hyaluronan and the receptors are internalized and detected in elongated vesicles close to plasma membrane, a step possibly involving limited cleavage by a hyaluronidase. 3) CD44 is released and recycled back to cell surface. 4) Hyaluronan is found in larger, perinuclear, non-acidic vesicles. The half life of steps 2-4 combined is ~5 min. 5) Fragmentation, perhaps by a hyaluronidase, into a weakly bHABC positive <90 kDa size pool with 2-3 h half life. 6) Degradation in lysosomes. 7) Hyaluronan and particularly hyaluronan oligosaccharides can also enter cells via non-receptor mediated pathway. The dashed lines indicate steps inhibited by the lysosomal inhibitors ammonium chloride and chloroquine, and the endosome recycling inhibitor monensin.
Fig. 3

[Bar graphs showing hyaluronan levels (g/cell) after different doses of hyaluronidase (TRU/ml) for Intracellular and Trypsinate samples.]

[Graph showing the percentage decrease in hyaluronan over time (h).]
Fig. 7.
Fig. 9

![Graph showing HA positive area (% of control) for different volumes and strains.](image-url)
CD44
Hyaluronan
Hyaluronidase?
Monensin
Chloroquine
Ammonium chloride

Fig. 11