Hyaluronan Bound to CD44 on Keratinocytes Is Displaced by Hyaluronan Decasaccharides and Not Hexasaccharides*

(Received for publication, June 29, 1998, and in revised form, August 24, 1998)

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Abundant hyaluronan is present between epidermal keratinocytes. However, virtually nothing is known regarding its organization in the limited extracellular space between these cells. We have used metabolic labeling with [3H]glucosamine and [35S]sulfate and a hyaluronan-specific biotinylated probe to study the metabolism of hyaluronan and its localization in monolayer cultures of a rat epidermal keratinocyte cell line. Hyaluronan (~20 fg/cell) was present on the apical and lateral surfaces of the cells in two nearly equal pools, either in patches (~160/cell) or diffusely spread. The hyaluronan in the patches is bound to CD44 as indicated by co-localization with an antibody to CD44, and by displacement with hyaluronan decasaccharides as well as with an antibody that blocks hyaluronan binding to CD44. The inability of hyaluronan oligomers shorter than 10 monosaccharides to displace hyaluronan suggests that CD44 dimerization or cooperative interactions are required for tight binding. The diffuse hyaluronan pool is likely bound to hyaluronan synthase during its biosynthesis.

Hyaluronan is well known as a constituent of connective tissue extracellular matrices, but more recent studies have also demonstrated its abundance in stratified squamous epithelia including the epidermis (1–3). In contrast with connective tissue extracellular matrices that contain mixtures of collagens, fibronectins, other multiahesive glycoproteins, proteoglycans, and hyaluronan, hyaluronan is the only known extracellular matrix macromolecule present in high concentration, ~2 mg/ml, in the small extracellular space between adjacent epithelial cells (keratinocytes) that form the epidermis (4, 5). Additionally, studies of human skin organ cultures have shown that the hyaluronan within the epidermis is rapidly turned over (6), an observation that suggests that the epidermis possesses efficient mechanisms to catabolize hyaluronan that are closely coordinated with its synthesis.

Although the coating of keratinocytes by hyaluronan is not generally appreciated, it is widely known that cell types of mesodermal origin (7), including fibroblasts (8), chondrocytes (9, 10), and mesothelial cells (11) display surface coats, often several micrometers in thickness, visualized indirectly as a domain excluding particles such as red blood cells. These coats 1) are removed by digestion with highly specific hyaluronidase, 2) can be stabilized by the serum-derived protein inter-alpha-trypsin inhibitor which interacts with hyaluronan (12–14), and 3) can be increased in size and reinforced by proteoglycans that bind specifically to hyaluronan (15, 16).

Extracellular hyaluronan is often anchored to CD44, a ubiquitous, abundant, and structurally variable plasma membrane receptor that has a hyaluronan binding domain (17). Smaller amounts of hyaluronan may bind to RHAMM, a receptor involved in cell motility and cell transformation through hyaluronan-dependent signaling involving tyrosine phosphorylation (18). In addition, some cell-surface hyaluronan appears to remain tethered to hyaluronan synthase, while biosynthesis continues on the cytoplasmic side of the plasma membrane (19–21). Variations in CD44 polypeptide sequence (22, 23), glycosylation (22, 24–27), and oligomerization (28, 29) all influence its avidity for binding hyaluronan. Still, current understanding of the mechanisms of hyaluronan binding to CD44 and other receptors on normal adherent cells, and the role that these interactions have in hyaluronan organization and catabolism, remains far from complete.

We have developed a rat keratinocyte model for epidermal differentiation that is suitable to explore these issues. This epidermal keratinocyte cell line is unique in its ability to reform an epidermis essentially identical to native tissue in the absence of feeder cells when cultured at an air-liquid interface (30) and exhibits a nearly complete set of morphological differentiation markers (31, 32). These cells can also be maintained in a continuous growth phase when regularly trypsinized and cultured as conventional monolayers on plastic dishes. In this report, the essentially monolayer character of recently subcultivated rat keratinocytes enabled us to quantitate and characterize the hyaluronan bound on keratinocyte cell surfaces in ways that are not technically feasible in complete epidermis covered with an impermeable stratum corneum. Our results show that about half of the hyaluronan is bound on the apical and lateral keratinocyte surfaces to CD44 and about half remains bound, most likely to hyaluronan synthase. Furthermore, hyaluronan oligosaccharides with at least five repeat units (decasaccharides) are required to displace hyaluronan from CD44.

EXPERIMENTAL PROCEDURES

Cell Culture—A newborn rat epidermal keratinocyte (REK)³ cell line was developed by MacCallum and Lillie (30) from neonatal rat epidermis.

³ The abbreviations used are: REK, rat epidermal keratinocyte; EBSS, Earle’s balanced salt solution; HHABC, biotinylated hyaluronan binding complex; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; D-PBS, Dulbecco’s phosphate-buffered saline.
mal cells originally isolated by Baden and Kubilus (31). REKs were cultured in Dulbecco's minimum essential medium (low glucose, Life Technologies, Inc.) with 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were passaged when confluent using 0.05% trypsin (w/v), 0.02% EDTA (w/v) in PBS (105). When seeded at 10,000 cells/cm², the cells grew to confluence in about 2 days and formed multilayers 2–4 cells thick after 4–6 additional days of culture. For biochemical assays and radiolabeling, the cells were grown close to confluency in 6-well plates and incubated in the presence of 20 and 100 μCi/ml [³H]glucosamine and [³⁵S]sulfate (Amersham Pharmacia Biotech, Aarhus, Little Chalfont, UK), respectively. For microscopic studies, the cells were plated in 8-well chamber slides (Lab-Tek and Nagle, Naperville, IL), 10,000–30,000 cells/well, precoated for 30 min at 37 °C with fetal bovine serum. For ELISA-like assays of cell-associated hyaluronan in situ, the cells were grown on Costar Transwell inserts (polycarbonate, 0.4-μm pore size, 4.5 cm², 6-well plate, Costar Corp., Cambridge, MA) seeded at 10,000 cells/cm².

"Compartments" Defined—Cells were grown in 6-well plates (9.6 cm²/well) in 1–2 ml of medium and subsequently washed with 2 × 250 μl of Earle's balanced salt solution (EBBS, Life Technologies, Inc.). The combined medium and washes were designated as "medium." Each precipitate was trypsinized in 0.5 ml of 0.025% trypsin (w/v) and 0.02% EDTA (w/v) (Sigma). After removing the cells, each plate was washed twice with 200 μl of PBS (106). The fractions corresponding to the peak of each oligosaccharide were dried, redissolved in water, and then with 200 μl of 0.25% (w/v) soybean trypsin inhibitor (Sigma) in 100 mM sodium acetate, pH 5.8, containing 5 mM cysteine HCl and 5 mM EDTA to which 50 μl of NH₄HCO₃. The cell pellet was suspended in 200 μl of EBBS and designated as the "extracellular" hyaluronan fraction. The combined washes, centrifuged, and the supernatant designated as "trypsinate" or "cell-surface hyaluronan." The cell pellet was centrifuged in 200 μl of EBBS and designated as the "intracellular" hyaluronan fraction.

Purification of Radiolabeled Hyaluronan—Carrier (4 μg of hyaluronan in 40 μl, Healon®, Amersham Pharmacia Biotech, Uppsala, Sweden) and 4 volumes of ethanol saturated with NaCl were added to each medium, trypsinate, and intracellular sample. After 1 h at 20 °C, precipitates were centrifuged for 15 min at 2500 × g. Each precipitate was suspended in 200 μl of 50 mM sodium acetate, pH 5.8, containing 5 mM cysteine HCl and 5 mM EDTA to which 50 μg of papain (Sigma) in 50 μl of water was added followed by incubation at 60 °C for 4 h. The samples were heated at 100 °C for 10 min, centrifuged at 13,000 × g for 15 min, and supernatants containing hyaluronan and other glycosaminoglycans recovered. Cetylpyridinium chloride (1% in 20 mM NaCl, 4 volumes) was added to each supernatant followed by incubation for 1 h at room temperature. After centrifugation at 13,000 × g for 15 min, each supernatant was carefully removed by aspiration. Each precipitate was washed with 1 ml of H₂O, centrifuged, and the supernatant discarded. Each cetylpyridinium chloride precipitate was dissolved in 50 μl of 4 M guanidine HCl, and 900 μl of ethanol, saturated with NaCl, was added. After 1 h at 20 °C, each sample was centrifuged, and the supernatant removed as described above.

Superdex Chromatography—Each purified sample was dissolved in 50 μl of 50 mM sodium acetate, pH 6.7, and digested for 3 h at 37 °C with 25 milliunits of chondroitinase ABC and 1 milliunit of Streptococcus hyaluronidase (both from Seikagaku Kogyo Co., Tokyo, Japan), and 10–45 μl were injected onto a 1 × 30 cm Superdex Peptide column (Pharmacia) and eluted at 0.5 ml/min with 0.1 M NH₄HCO₃ (Sigma). After removing the cells, each plate was washed twice with 200 μl of PBS (106). The fractions corresponding to the peak of each oligosaccharide were pooled, lyophilized twice, dissolved in the appropriate medium, and filtered sterile. Each purified sample was dissolved in 50 μl of EBSS and designated as the "intracellular" hyaluronan fraction. The combined washes, centrifuged, and the supernatant designated as "trypsinate" or "cell-surface hyaluronan." The cell pellet was suspended in 200 μl of EBBS and designated as the "intracellular" hyaluronan fraction.

Preparation of Hyaluronan Oligosaccharides—Commercial hyaluronan from human umbilical cord (Sigma) was dissolved in 0.1 M sodium acetate, 0.1 M NaCl, pH 6.0. Bovine testicular hyaluronidase (6000 units/mg, Calbiochem) was incubated with the hyaluronan at 37 °C until the size distribution peaked at 10–20 monosaccharide units, as determined by elution on an analytical Superdex 75 gel filtration column (Pharmacia). Preparative fractionation of the oligosaccharides was done on a 5 × 95-cm column of Superdex 30 (Pharmacia), eluted with 0.1 M NH₄HCO₃. The fractions corresponding to the peak of each oligosaccharide were pooled, lyophilized twice, dissolved in the appropriate medium, and filtered sterile. Each purified sample was dissolved in 50 μl of EBBS and designated as the "intracellular" hyaluronan fraction. The combined washes, centrifuged, and the supernatant designated as "trypsinate" or "cell-surface hyaluronan." The cell pellet was suspended in 200 μl of EBBS and designated as the "intracellular" hyaluronan fraction. The combined washes, centrifuged, and the supernatant designated as "trypsinate" or "cell-surface hyaluronan." The cell pellet was suspended in 200 μl of EBBS and designated as the "intracellular" hyaluronan fraction.

Cellular localization of hyaluronan—Each cell layer to be analyzed was washed with Dulbecco's PBS (105), Life Technologies, Inc. at 22 °C and fixed for 20 min either in 2% paraformaldehyde (w/v) and 0.5% glutaraldehyde (w/v) in D-PBS for electron microscopy and light microscopy with peroxidase detection or in paraformaldehyde alone for fluorescence microscopy. After fixation, the cells were washed 3 times for 2 min each with D-PBS and then blocked in 1% BSA (w/v) containing 0.3% Triton X-100 (v/v) and 50 mM glycine in D-PBS, for 30 min at 37 °C.

Keratinocyte CD44 Oligosaccharide Specificity
Keratinocyte CD44 Oligosaccharide Specificity

For light microscopy, the hHABC probe, diluted to 2–10 μg/ml in 3% BSA(w/v), was added to the fixed cells, and incubated overnight at 4 °C. The slides were then washed and treated with avidin-biotin peroxidase (ABC standard kit, Vector) for 1 h. The color was developed using DAB and H2O2 (kit from Vector) according to the manufacturer’s instructions. Counterstaining was done with hematoxylin for 2 min before mounting either in Crystalmount® or DPX® (Biomeda, Foster City, CA).

For electron microscopy, fixation, incubation with hHABC, blocking, and washes were done as for light microscopy. The samples were then incubated with streptavidin/gold (LM grade, Amersham Corp.) 1:40 dilution for 1 h. After washing, the samples were postfixed in 1% glutaraldehyde (v/v) for 20 min and washed 5 times for 1 min with distilled water. Silver enhancement was done according to the manufacturer’s instructions (IntenSE M, Amersham Corp.). The cells were dehydrated in graded ethanol and embedded in Spurr’s resin. Thin sections were cut, placed onto Formvar-coated copper grids, stained with uranyl acetate, and viewed with a JEOL 1200 EX microscope.

The specificity of the staining was controlled by pre-digesting the fixed cultures with Streptomyces hyaluronidase (100 turbidity reducing units/ml, 50 mM sodium acetate buffer, pH 5.0, 3 h at 37 °C) in the presence of protease inhibitors (4) or by preincubating the hHABC probe with HA oligosaccharides (length = 20 monosaccharides, 3 μg/1 μg hHABC) to reveal possible nonspecific binding of the probe.

Image Analysis of Cell Associated Hyaluronan—The cells were grown on 8-well chamber slides and stained for hyaluronan with DAB as a chromogen, as described above, but without hematoxylin counterstaining. A Leitz BK II microscope with 16 ×/0.45 numerical aperture objective (Leitz, Wetzlar, Germany) was connected with a 12-bit digital camera (Photometrics CH 200, Tucson, AZ) equipped with a KAF 1400 CCD detector (Eastman Kodak Co.). The optimum wavelength for DAB was found at 543 nm using an interference filter (Schott, Wiesbaden, Germany). Camera control and image analysis were done with IPLab software (Signal Analytics, Vienna, VA). The settings of the microscope and camera were kept constant during the study. Spatial resolution of the system was 1.43 μm/pixel. During system calibration and measuring, each image of interest was corrected using a flat fielding algorithm. The imaging system was calibrated with neutral density filters (Schott) in the range of 0 to 3 OD to convert the pixel density values into the linear, standardized scale of OD. Multiple fields (10–20) were systematically sampled from each well. Area-integrated mean OD values, including both DAB-positive and background intensities, were calculated for each whole digitized area, excluding possible artifact areas. In addition, DAB-positive staining areas were estimated from binary images with a cut-off at an OD value of 0.13. Based on the positive area data and the sum of the pixels that fulfilled the positivity criteria, the mean area-integrated OD values for the DAB-positive material were calculated accordingly, and those data were used for the presentation of the results.

Localization of CD44—The cells were fixed and blocked with BSA as described above and then incubated overnight at 4 °C with the OX 50 antibody (BioSource Inc., Camarillo, CA) at 1:100 dilution in 3% BSA. After washing, the cells were incubated for 1 h with biotinylated anti-mouse secondary antibody (Vector) diluted to 1:100 in 1% BSA. After washing, they were treated with avidin-biotin peroxidase, developed with DAB, counterstained with hematoxylin, and mounted in Crystalmount® or DPX®, as described above for hHABC. Negative controls included isotype-specific control serum (Sigma) and omission of the primary antibody.

For double staining of hyaluronan and CD44, the cells were fixed in 2% paraformaldehyde, blocked as described above, and then incubated overnight at 4 °C with the OX 50 antibody (1:100 dilution) and hHABC (3 μg/ml) in 3% BSA. After washing, the cells were incubated for 1 h with fluorescein isothiocyanate-labeled anti-mouse secondary antibody (Boehringer GMBH, Mannheim, Germany, 1:50) and Texas Red-labeled streptavidin (Vector) 1:1000 in 1% BSA, washed, and mounted in Vectashield (Vector).

Modification of Hyaluronan Binding with CD44 Antibodies—Recently confluent or near-confluent cultures of REKs were incubated in the presence of monoclonal antibodies against CD44: 1M7 (Zymed Laboratories Inc., San Francisco, CA, and a gift from Dr. W. Knudson, Chicago), Ox 50 (BioSource Int., Camarillo, CA), and Hermes 3 (a gift from Dr. Sirpa Jalkanen, Turku) and with non-immune IgG (Sigma) before staining for hyaluronan as described above.

RESULTS

Steady State Content of [3H]Hyaluronan on Cell Surface—Recently confluent cultures of rat keratinocytes were labeled for times between 6 and 24 h with [3H]glucosamine and [35S]sulfate. The [3H]labeled hyaluronan was determined for

Fig. 2. Hyaluronan displacement from the cell surface by hyaluronan oligosaccharides. a, confluent keratinocyte cultures were incubated for 2 h with purified hyaluronan hexasaccharides (HA6), deacssacharides (HA10), chondroitin 6-sulfate (C6S), chondroitin (C), and the hyaluronan content in cell layers were determined with the in situ ELISA-like assay described under “Experimental Procedures.” b, cultures were labeled for 24 h with [3H]glucosamine in the presence of HA6 or HA14 oligosaccharides. [3H]Hyaluronan content in the trypsinates were determined as described under “Experimental Procedures” and are expressed as a percentage of the control cultures. The bars show the range of duplicate cultures.

FIG. 1. Accumulation of [3H]hyaluronan in different compartments during 24 h labeling with [3H]glucosamine. Confluent cultures of keratinocytes were incubated in medium with [3H]glucosamine and [35S]sulfate for times between 6 and 24 h. The hyaluronan content, calculated from the double label method, was determined in the medium, trypsinate, and intracellular compartments as described under “Experimental Procedures.” The content of newly synthesized hyaluronan at the end of the indicated labeling periods is shown. The bars show the range of duplicate cultures.
medium, cell surface (trypsinate), and intracellular compartments, and the values were used to determine the mass of \([\text{3H}]\)hyaluronan with the double label method. As shown in Fig. 1, the content of newly synthesized hyaluronan in the trypsinate (i.e. on the cell surface) reached a plateau value by \(\pm 18\) h. The \([\text{3H}]\)hyaluronan in the medium continued to increase linearly, after an initial lag, during 24 h indicating continued synthesis of hyaluronan throughout. The intracellular \([\text{3H}]\)hyaluronan (i.e. the cell pellet after trypsin digestion) remained constant and low (\(\sim 4\%\) of the total at 24 h). The kinetics to steady state content of \([\text{3H}]\)hyaluronan in the trypsinates indicates that the half-life of a newly synthesized hyaluronan molecule on the cell surface is only \(\sim 8\) h and the equilibrium content is \(\sim 200\) pg/10\(^4\) cells (or \(\sim 20\) fg/cell). The loss of

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**Fig. 3. Localization of hyaluronan on keratinocytes.** Confluent REK cultures were fixed and stained for hyaluronan using the bHABC probe (a, b, c, and e) or bHABC preincubated with hyaluronan oligosaccharides HA\(_{50}\) (d and f). The bHABC was visualized using the ABC-peroxidase technique with DAB chromogen (a, b, c, and d) or with gold-labeled streptavidin, intensified with silver (e and f). The untreated cultures (a, d, e, and f) were fixed after a brief wash with the buffer, whereas b was incubated for 45 min with Streptomyces hyaluronidase and c for 5 min with trypsin at room temperature, prior to the fixation. Magnification bar in d represents 10 \(\mu\)m for a, b, c, and d and 1 \(\mu\)m for e and f. See text for descriptions.
[3H]hyaluronan from the cell surface reflects loss to the medium and some internalization for catabolism.

**Cell-surface Hyaluronan Is Partially Displaced by Hyaluronan Decasaccharides**—Confluent cultures of keratinocytes were incubated at 37 °C for 2 h with hyaluronan oligosaccharides of different sizes, and the displacement of cell-surface hyaluronan was determined using the *in situ* hyaluronan assay described under “Experimental Procedures.” Oligosaccharides HA10 (Fig. 2a) or larger (not shown), when used at 0.3–1.0 mg/ml, reduced cell layer-associated hyaluronan in this ELISA assay to 45–56% of controls. In contrast, HA6 had no effect (Fig. 2a). Related glycosaminoglycans, such as chondroitin and chondroitin 4-sulfate (d), heparan sulfate (e), and HA10 (f, g, and h). Magnification bar in f represents 10 μm for a–f and 1 μm for h. The bar in g represents 10 μm. See text for descriptions.

A nearly identical displacement of cell-surface [3H]hyaluronan (to 53–55% of controls) was found in cultures labeled for 24 h with [3H]glucosamine in the presence of HA14 oligosaccharides (Fig. 2b). Again, HA6 oligosaccharides had no effect. This confirmed that HA6 was unable to displace endogenous hyaluronan and that [3H]-labeled cell-surface hyaluronan had equilibrated with the total content of hyaluronan on cell surfaces. Increasing oligosaccharide size from 10 to 18 monosaccharides did not significantly increase the release of cell-surface hyaluronan (data not shown). Hyaluronan molecules on the surfaces of the keratinocytes, therefore, are present in two different pools, one with the characteristics of a specific receptor (displaced by HA14-oligosaccharides, but not with other glycosaminoglycans), and one not displaced and likely still attached to hyaluronan synthase.

**Hyaluronan Is Present in the Form of Patches on the Keratinocyte**—Localization of hyaluronan with the biotinylated probe

Fig. 4. Effects of hyaluronan oligosaccharides and other glycosaminoglycans on displacement of hyaluronan from keratinocyte cell surfaces. Confluent REK cultures were fixed and stained for hyaluronan using the bHABC and ABC-peroxidase method (a–g) or the gold-labeled streptavidin and silver enhancement method (h). A control culture fixed after a brief wash with the buffer is presented in a, whereas those in b–h were incubated for 2 h in the presence of 1 mg/ml of the following substances: chondroitin (b), HA6 (c), chondroitin 4-sulfate (d), heparan sulfate (e), and HA10 (f, g, and h). Magnification bar in f represents 10 μm for a–f and 1 μm for h. The bar in g represents 10 μm. See text for descriptions.
(bHABC) in confluent cultures revealed dense patches decorating the cell-cell contact areas, as well as spots and linear deposits on the apical surfaces (Fig. 3a). In contrast, the underside of the keratinocytes, facing the plastic or glass surface (position determined by the focal plane), was almost always devoid of hyaluronan. Digestion with either Streptomyces hyaluronidase (Fig. 3b) or trypsin (Fig. 3c) completely removed the cell-surface signal. An intracellular, granular hyaluronan signal was present in some of the cells (Fig. 3b). The ability of trypsin to release all cell-surface hyaluronan was also confirmed by the absence of any hyaluronan degradation products in the hyaluronidase containing incubation medium of cultures that had been first treated with trypsin (data not shown).

The cell-surface patches were rather uniform in size ranging from 0.8 to 2.4 μm (1.1 ± 0.03 μm, mean ± S.E.). The estimated number of the patches per keratinocyte was 160 ± 20 (mean ± S.E., 6 cultures).

All experiments included control cultures probed with bHABC preincubated with hyaluronan oligosaccharides (HA_20), which combine with the specific binding sites in the probe and prevent its binding to hyaluronan. No signal was detected in these controls, indicating the specificity of the reaction (Fig. 3d).

The Hyaluronan Patches Are Displaced by Hyaluronan Decasaccharides—Since hyaluronan in the trypsinate was present in two different pools, one displaced by HA_10 oligosaccharides, and the other not (Fig. 2), we tested whether the patches belonged to one or both of these pools. Cultures incubated for 2–4 h at 37 °C with 1 mg/ml HA_4 or HA_6 (not shown) appeared identical to controls (Fig. 4a). The culture containing HA_8 showed only a slight reduction in the hyaluronan signal (Fig. 4c), while that with HA_10 resulted in a complete loss of the patches (Fig. 4f and g). Cultures incubated with chondroitin 4-sulfate and heparan sulfate did not differ from controls (Fig.

FIG. 5. Reappearance of hyaluronan after hyaluronidase digestion. Confluent REK cultures were treated with Streptomyces hyaluronidase for 45 min and chased for 2 h (a), and 8 h (b, c, d, and e). Fixed cultures were then stained for hyaluronan using bHABC and the ABC-peroxidase technique. Whereas the other cultures contained 10% serum during the chase, c was chased in serum-free medium. d 1 mg/ml of HA_6 and in e 1 mg/ml HA_10 was added when starting the chase. Magnification bar in d represents 10 μm. See text for descriptions.
4, d and e), and only a slight reduction was observed with chondroitin (Fig. 4b). These findings are consistent with those in the biochemical assays (Fig. 2) and indicate that the patches represent only the pool displaced by HA$_{10}$ oligosaccharides. The hyaluronan not displaced appeared evenly distributed on the plasma membrane and gave a signal of very low contrast (Fig. 4, f and g), unlike the patchy pattern in controls (Figs. 3a and 4a). Interestingly, a stronger signal was observed in plasma membrane areas with slender cytoplasmic extensions (Fig. 4g), possible loci of hyaluronan synthase.

### Ultrastructural Localization of Hyaluronan in Keratinocytes

Gold-labeled streptavidin (1-nm gold particles) was applied on fixed and permeabilized cultures previously incubated with bHABC, and the signal was subsequently enhanced with silver (Fig. 3c). Controls using the bHABC probe presaturated with hyaluronan oligosaccharides (HA$_{20}$), showed very sparse labeling and no specific localization (Fig. 3f), similar to cultures digested with Streptomyces hyaluronidase prior to the staining (data not shown). The silver grains formed clusters in the extracellular pouches between adjacent keratinocytes, corresponding to the patches observed in light microscopy. The label was often associated with the microvilli (Fig. 3e) and most conspicuous around the cytoplasmic extensions between neighboring cells in cells treated with HA$_{10}$ oligosaccharides (Fig. 4, g and h). The oligosaccharide-resistant signal was very close to plasma membranes, as if hyaluronan was tightly associated with the cell surface (Fig. 4h).

### Reappearance of the Hyaluronan Patches after Hyaluronidase Treatment

Confluent keratinocyte cultures were treated with Streptomyces hyaluronidase to remove cell-surface hyaluronan specifically. The cultures were then washed and chased for 2–8 h. After a 2-h chase, new hyaluronan was present on cell surfaces, with a distribution resembling that in untreated cultures, but with smaller and less intensely stained patches (Fig. 5a). With increasing chase time, the patches increased in size and signal intensity (shown at 8 h chase, Fig. 5b). Hyalu-
Ronan distribution was not affected by 8 h chase in the absence of serum (Fig. 5c), suggesting that inter-alpha-trypsin inhibitor is not required (12–14). The presence of HA6 did not prevent the formation of new patches during the chase (Fig. 5d), whereas HA10 completely inhibited their reappearance (Fig. 5e).

Co-localization of CD44 and Hyaluronan—An antibody specific for CD44 (OX50) was used with a biotinylated secondary antibody to localize CD44 in a confluent culture (Fig. 6a). The CD44 signal resembled the staining pattern of hyaluronan, being more intense at cell-cell contact areas and forming small patches on the apical cell surfaces. Cultures probed with control antiserum or without the primary antibody were negative (data not shown). Double staining with bHABC and anti-CD44 using red and green fluorochromes, respectively, showed colocalization on the apical cell surfaces (Fig. 6, b and c). However, images focused at a lower level, corresponding to lateral plasma membrane domains (Fig. 6, d–f) showed areas where CD44 was present, but hyaluronan was absent. This occurred particularly in subconfluent cultures at the leading edges of the growing keratinocyte colonies (Figs. 6, d–f).

An Antibody to CD44 That Blocks the Hyaluronan Binding Reduces Cell-surface Hyaluronan and Inhibits Patch Formation—Nearly confluent cultures were incubated for 8 h with IM7 antibody, which partially inhibits binding of hyaluronan to

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**Fig. 7.** Effect of the anti-CD44 antibodies IM7 and OX50 on hyaluronan distribution on keratinocytes. Nearly confluent REK cultures were incubated without additions (a), with IM7 (60 μg/ml) for 8 h (b), with OX50 for 6 h (c–e) or with Hermes 3 for 6 h (f), prior to fixation and bHABC staining. d and e, hyaluronan oligosaccharides, HA10 and HA6, respectively, were added (at 1 mg/ml) for the last 2 h of the OX50 treatment. Magnification bar in f represents 10 μm. See text for descriptions.
CD44 in rat chondrocytes (10, 35), as well as inducing the shedding of CD44 in mice (36). A reduction in cell-surface hyaluronan staining was observed at 8 h (Fig. 7b) compared with untreated control (Fig. 7a). Control cultures incubated in the presence of non-immune IgG (not shown), or the antibody Hermes 3 specific for human, but not rat CD44 (Fig. 7f), showed an intense signal for hyaluronan, indistinguishable from untreated cultures (Fig. 7a). In cultures treated for 8 h with increasing concentrations of IM7, image analyses of the hyaluronan staining showed a dose-dependent decrease in hyaluronan signal, with a maximum reduction of optical density ∼30% at 120 μg/ml (Fig. 8).

The CD44 Antibody OX50 Increases Binding with HA₆ Specificity—Nearly confluent REK cultures were incubated with OX50 anti-CD44 antibody for different times and then stained with bHABC (6 h cultures are shown in Fig. 7, c−e). The amount of endogenous hyaluronan on keratinocyte surfaces was considerably increased by incubation with this monoclonal antibody, as compared with non-treated (7a) or Hermes 3-treated cultures (Fig. 7f). Other CD44 antibodies that enhance hyaluronan binding have been described previously (28, 37). Image analyses indicated that the enhanced binding was detectable 1 h after adding the antibody and reached its full effect by 4 h (Fig. 9a).

We tested the specificity of this effect by treating cultures with OX50 in the presence of HA₉ and HA₁₀ for 6 h. As shown in Figs. 7d and 9b, HA₁₀ reduced keratinocyte-surface hyaluronan to approximately half of that in control cultures. In contrast, OX50 treated cultures showed hyaluronan levels higher than controls in the presence of HA₉ (Figs. 7e and 9b). These results indicate that enhanced hyaluronan binding to CD44 induced by OX50 exhibits specificity for HA₁₀.

**DISCUSSION**

**Two Pools of Hyaluronan on Keratinocyte Surface**—The steady state content of hyaluronan, bound primarily on the apical and lateral cell surfaces of the rat keratinocytes in nearly confluent monolayer cultures, was determined to be ∼20 fg per cell by independent chemical and double radiolabeling assays. Of this, ∼50% is bound to the cell-surface receptor, CD44, as indicated by the following: (a) colocalization of hyaluronan and the anti-CD44 OX50 antibody, and (b) the ability of anti-CD44 IM7 antibody, which inhibits hyaluronan binding to CD44, to displace hyaluronan from the “patches.” The amount, ∼10 fg per cell, bound to CD44 corresponds to 900−3000 hyaluronan molecules if they average 2−7 million Da in molecular mass (19, 38). Thus, each of the ∼160 patches observed per cell would contain 6−19 molecules of bound hyaluronan. The remaining molecules, ∼50% of the total, are not bound to CD44 as indicated by the inability of HA₁₀ to displace them and by a distinctly different distribution (diffuse pericellular location) from the clusters or patches of hyaluronan that can be displaced by these same oligosaccharides.

This residual hyaluronan could be bound by one or more of several established alternative mechanisms. Hyaluronan may remain bound on hyaluronan synthase (20, 39, 40), bound to matrix components like type VI collagen (41, 42) and aggregating proteoglycans (43−45). However, type VI collagen and aggrecan are not expressed in epidermis, and no proteoglycans with the properties of versican were detected in our rat epidermal keratinocytes when labeled with [³H]glucosamine and [³⁵S]SO₄.² Therefore, association with hyaluronan synthase remains the most likely tether of the hyaluronan that is not bound to CD44. The fact that the endogenous hyaluronan in this compartment is not accessible to extracellular HA₁₀ oligosaccharides fits well with the current model for the hyaluronan synthase activity which includes an intracellular catalytic (binding) domain and a narrow pore for the extrusion of the growing chain into the extracellular space (21). However, if this model is true, the release of all hyaluronan with trypsin suggests that a protein exposed on the cell surface, perhaps a part of synthase itself, is required to hold the growing polysaccharide chain.

**Hyaluronan Receptors on Keratinocytes**—The contribution of RHAMM to the immobilization of endogenous hyaluronan has not been established in quantitative terms but is probably minor and transient (46). Our unpublished findings indicate that a RHAMM antibody known to block hyaluronan binding does not influence the signal intensity of the keratinocyte hyaluronan patches. The inability of hexasaccharides to displace the hyaluronan patches should rule out the involvement of the novel hyaluronan receptor Cdc37, identified by the antibody IVd4, from which hyaluronan can be displaced by HA₆ (47, 48).

In addition to localizing CD44 on rat keratinocytes and blocking HA binding with anti-CD44 antibody IM7 in this study, further evidence for CD44 as the main hyaluronan receptor on keratinocytes comes from the fact that epicain, the major CD44 variant on epidermal keratinocytes (with variant exons v3−v10), binds hyaluronan and that this binding is blocked by an antibody to CD44/epican (49). Moreover, a transgenic mouse with selective suppression of CD44 gene expression in epidermis, shows a 75% reduction in endogenous hyaluronan on the surface of cultured keratinocytes derived from this mouse (50). Collectively, these data indicate that CD44 is the major receptor for hyaluronan in epidermal keratinocytes.

**Molecular Basis of the HA₁₀ Specificity of Epidermal CD44**—Our observation that HA₁₀ oligosaccharides are required to displace hyaluronan from CD44 on keratinocytes is particularly striking. CD44 belongs to the class of hyaladherins with a domain called the “link module,” recently modeled in three dimensions from the TSG-6 member of the family (51) and adapted to CD44 (52). The proposed hyaluronan site on the link module of TSG-6 is unlikely to require a length greater than HA₆ (51). In fact, many cells in which CD44 functions to bind a pericellular coat of hyaluronan exhibit specificity for HA₆ since they are displaced from the cell surface by hexasaccharides (16, 53), whereas proteins such as link protein and the G₁ domain of aggrecan with two link modules in tandem (54, 55).
show specificity for \( \text{HA}_{10} \) (45).

However, high affinity binding of hyaluronan to CD44 appears to require an amino acid (Lys-68) on the outer edge (52) and an established hyaluronan-binding peptide (amino acids 150–162) completely outside of the link module (56–58). Thus, a larger binding site with greater affinity for hyaluronan than the current one requiring \( \text{HA}_{6} \) may exist. One possibility is that CD44 molecules on keratinocytes can achieve close enough proximity such that the HA binding domains of two adjacent molecules form a cooperative site effectively converting them to a functional site with two link modules equivalent to that in the link protein, which requires \( \text{HA}_{10} \) for binding. Dimerization has, in fact, been implicated in enhanced binding of hyaluronan to CD44, which can occur naturally with rat CD44v4-v7 (29), or can be induced experimentally with certain divalent antibodies to CD44 (28), or by constructing CD44 with cysteine in the mobility of CD44, free to dimerize or change conformation has, in fact, been implicated in enhanced binding of hyaluronan to CD44, which can occur naturally with rat CD44v4-v7 (29), or can be induced experimentally with certain divalent antibodies to CD44 (28), or by constructing CD44 with cysteine in the intramembranous domain that forms disulfide cross-links (59). However, coordinate action of CD44 with another cell-surface molecule cannot be ruled out (60).

**Regulation of CD44 Affinity for Hyaluronan**—The increased hyaluronan binding induced by OX50 antibody observed in our study suggests that CD44 molecules that weakly bind hyaluronan can be made more efficient. Our results make it unlikely that highly mobile CD44, free to dimerize or change conformation, binds additional hyaluronan from the medium compartment. Such binding would be inconsistent with the gradual increase of CD44-bound hyaluronan observed following the addition of the OX50 antibody, an observation more consistent with the possibility that newly synthesized hyaluronan, or CD44, or both are required for the increased hyaluronan present on the cell surface.

Hyaluronan is thus present on the surface of rat epidermal keratinocytes in two different forms as follows: one that can be displaced by hyaluronan oligomers \( \text{HA}_{10} \) that is bound to CD44 and the other that can be removed by subsequent hyaluronidase or trypsin treatment and probably is tethered to hyaluronan synthase. The hyaluronan bound to CD44 can be increased in amount by the use of a monoclonal antibody that probably cross-links or clusters the CD44, as has been shown by other CD44 antibodies and other cells. Having gained some insight into the amount and organization of hyaluronan in these epidermal keratinocytes, we now are studying the biosynthesis and organization of hyaluronan in the completely reformed epidermis that these cells make when cultured at an air-liquid interphase.
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