Hyaluronan serves a novel role in airway mucosal host defense

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ABSTRACT **Enzymes secreted onto epithelial surfaces play a vital role in innate mucosal defense, but are believed to be steadily removed from the surface by mechanical actions. Thus, the amount and availability of enzymes on the surface are thought to be maintained by secretion. In contrast to this paradigm, we show here that enzymes are retained at the apical surface of the airway epithelium by binding to surface-associated hyaluronan, providing an apical enzyme pool 'ready for use' and protected from ciliary clearance. We have studied lactoperoxidase, which prevents bacterial colonization of the airway, and kallikrein, which mediates allergic bronchoconstriction that limits the inhalation of noxious substances. Binding to hyaluronan inhibits kallikrein, which is needed only in certain situations, whereas lactoperoxidase, useful at all times, does not change its activity. Hyaluronan itself interacts with the receptor for hyaluronic acid-mediated motility (RHAMM or CD168) that is expressed at the apex of ciliated airway epithelial cells. Functionally, hyaluronan binding to RHAMM stimulates ciliary beating. Thus, hyaluronan plays a previously unrecognized pivotal role in mucosal host defense by stimulating ciliary clearance of foreign material while simultaneously retaining enzymes important for homeostasis at the apical surface so that they cannot be removed by ciliary action.—Forteza, R., Lieb, T., Aoki, T., Savani, R. C., Conner, G. E., Salathe, M. Hyaluronan serves a novel role in airway mucosal host defense. *FASEB J.* 15, 2179–2186 (2001)**

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Secretions on epithelial surfaces are constantly cleared by mechanical action. For instance, tears are removed by blinking, gastric and bowel secretions by peristalsis, and respiratory secretions (including mucus components) by ciliary beating. Consequently, secretion has been postulated to be the main determinant of enzyme availability and activity on mucosal surfaces. Since many aspects of innate mucosal defense depend on the continued presence of secreted proteins and enzymes on the mucosa, rapid removal requires continued secretion to maintain the surface presence of secreted components important for host defense. In the respiratory system, earlier data suggested that the turnover rate of certain substances inhaled into the airways may be longer than expected for immediate removal by mucociliary clearance (1). These data indicated that rapid clearing of all material from the airway epithelial surface by mucociliary action may not be universal.

We have been studying two key enzymes of airway mucosal defense, both of which are secreted onto the airway surface: tissue kallikrein (TK), which is involved in allergic bronchoconstriction to limit the inhalation of noxious substances (2), and airway lactoperoxidase (LPO), which increases bacterial clearance from ovine airways (3). TK is produced in submucosal gland cells (4–6). LPO is produced in both surface epithelial goblet cells and submucosal gland cells (7–9). We have previously shown that TK from broncho-alveolar lavage is bound to secreted hyaluronan that regulates its activity (4). We have also shown that ciliated cells in culture bind endogenously produced hyaluronan and that exogenously applied hyaluronan can stimulate ciliary beat frequency (CBF) (10).

Hyaluronan is a glycosaminoglycan made of repeating disaccharides formed from glucuronic acid and N-acetylglucosamine, linked through alternating beta-1,4 and beta-1,3 glycosidic bonds (11). In recent years, evidence has accumulated that hyaluronan serves important biological roles beyond its generally accepted function as a structural component of interstitial and connective tissues. For instance, hyaluronan has been shown to promote wound healing (12), tumor transformation, and metastasis (13, 14), as well as cell motility and migration (15–18). Many of these biological activities are mediated by either CD44 or the receptor for hyaluronic acid-mediated motility (RHAMM or CD168) (15). RHAMM is expressed in a variety of mammalian cell types including fibroblasts, smooth muscle cells, endothelial cells, macrophages, sperm, nerve cells, and

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malignant tumors (19), but no data on RHAMM in the airways are available. Since the ultrastructure of sperm tails is similar to the one of cilia in the tracheobronchial tree, data showing that hyaluronan addition to media was critical to preserve sperm motility and able to increase sperm velocity in vitro (20, 21) through its interaction with RHAMM (22) hinted that RHAMM could have functional relevance in the airways. We therefore conducted a series of experiments to examine the role of hyaluronan in airway host defense, specifically as it relates to TK, LPO, and ciliary beating.

MATERIALS AND METHODS

Antibodies

A polyclonal, affinity-purified antibody (R36) raised to a specific peptide sequence (aa 268–288) from the deduced amino acid sequence of the murine RHAMM cDNA was used. This antibody is specific for RHAMM and is able to block hyaluronan-mediated cellular effects (15, 23, 24). Rabbit antiserum to anti-human urinary kallikrein (Calbiochem), previously demonstrated to recognize bronchial TK (4), was used. Antiserum to purified sheep airway LPO (9) was made in rabbits (Covance, Hazleton, PA). Specificity was determined by Western blotting with purified sheep and bovine LPO as well as canine and human MPO. Rabbit anti-chicken IgG, used as a control in CBF experiments, was from Cappel (Cochranville, PA).

Preparation of tracheal epithelial cultures

Primary cultures of tracheal epithelial cells were prepared as described previously (25).

Immunohisto- and cytochemistry

Sheep trachea and cell cultures were fixed with acid formalin (26) and processed according to standard procedures for immunohisto- and immunocytochemistry. Primary antibodies were used at the following dilutions: anti-RHAMM antibody (10 μg/ml); anti-TK (1:500); and anti-LPO (1:500). Preimmune serum was diluted 1:500. Secondary antibodies were alkaline phosphatase or horseradish peroxidase-labeled, affinity-purified goat anti-rabbit IgG used at 5 μg/ml in 50 mM Tris-buffered saline (Kirkegaard and Perry, Gaithersburg, MD). Color was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate or diaminobenzidine, respectively. Hyaluronan was visualized according to Bray et al. (27) using a biotinylated hyaluronan binding protein (Seikagaku, Tokyo, Japan) and avidin coupled with alkaline phosphatase. Hyaluronidase digestion (50 IU/ml from *Streptomyces hyalurolyticus*; Seikagaku) was performed in 10 mM potassium-acetate, pH 5.6, containing pepstatin 0.1 μg/ml, aprotinin and leupeptin 0.1 μM, at 37°C overnight. Chondroitinase ABC (ICN, Costa Mesa, CA) was used as a control at 0.5 IU/ml in 50 mM Tris-buffered saline containing pepstatin (0.1 μg/ml), aprotinin and leupeptin (0.1 μM), at pH 7.5 and 37°C overnight. The chondroitinase ABC units used represent a 10-fold excess over those used for experiments digesting chondroitin sulfate produced by submucosal gland cells (28). Chondroitinase ABC was also used in 10 mM potassium-acetate, pH 5.6, at 0.5 IU/ml.

PCR

PCR was done with our ovine mucosa cDNA library (3) as template and a specific 5′ oligonucleotide (TGTTGAATGAACATGGTGCA) as well as a sixfold degenerate 3′ primer (CCTTDGAAGGRTCAAAGTG), both designed from consensus RHAMM sequences. The FailSafe™ PCR system (Epicentre Technologies, Madison, WI) was used successfully with all buffers provided, annealing at 52°C for a total of 30 cycles.

Measurement of CBF

CBF was measured and analyzed as described previously using continuous perfusion at room temperature (29). To reveal the CBF response to exogenously applied hyaluronan, endogenous hyaluronan was removed from cells with 5 IU/ml hyaluronidase (from bovis testis; Worthington, Freehold, NJ) in buffered Hank's balanced salt solution, 10 mM HEPES, pH 7.4 (referred to as HBSS) for 2 h at room temperature (10). For blocking experiments, cells were incubated for 30 min with anti-RHAMM or control rabbit anti-chicken IgG antibodies (both at 25 μg/ml in HBSS). This concentration of anti-RHAMM antibody inhibited locomotion of smooth muscle cells (24). The cells were then exposed within 60 min to hyaluronan (Seikagaku or Worthington; see Results; both with an average molecular mass of 200 kDa) at 50 μg/ml, previously demonstrated to increase CBF (10). As a control, the cells were exposed to 20 μM ATP to ascertain their response to a known stimulator of CBF. Exchanges of the chamber volume with HBSS alone had no influence on CBF at the perfusion rates used, ruling out that changes in CBF were due to mechanical disturbance of cilia.

Tracheal transport measurements

Recombinant TK (gift kindly provided by Amgen Pharmaceuticals, Thousand Oaks, CA), purified bovine milk LPO, and bovine serum albumin (Sigma, St. Louis, MO) were labeled with fluorescein or rhodamine isothiocyanate (Sigma) according to published methods (30). The products were purified on Sephadex G50 and concentrated to 1 mg/ml in PBS. The coupling efficiency was assessed to be optimal with less than 2 mol fluorochrome per mole of protein by the ratio of absorbance at 495/280 nm for fluorescein (measured to be 0.9) and 575/280 nm for rhodamine (measured to be 0.5). Labeled enzyme (LPO or TK) and albumin were applied in equimolar amounts to the mucosal surface of a trachea in a total volume of 10 μl. Tracheas were obtained from freshly killed sheep, opened by cutting through the membranous portion, and kept in a humidified chamber at 37°C. Movement of the applied fluorescent substances was monitored using a broad-spectrum UV illuminator and a digital camera every 10 min for a total of 30 min.

RESULTS

LPO and TK bind to cilia via hyaluronan

Immunohistochemistry of ovine tracheal sections revealed specific staining not only in submucosal gland cells for both TK and LPO and in goblet cells for LPO, but also along the ciliated border of the airway epithelium (**Fig. 1**). Immunocytochemical localization of LPO and TK in submerged primary cultures of ovine airway epithelial cells (which contained also submucosal gland

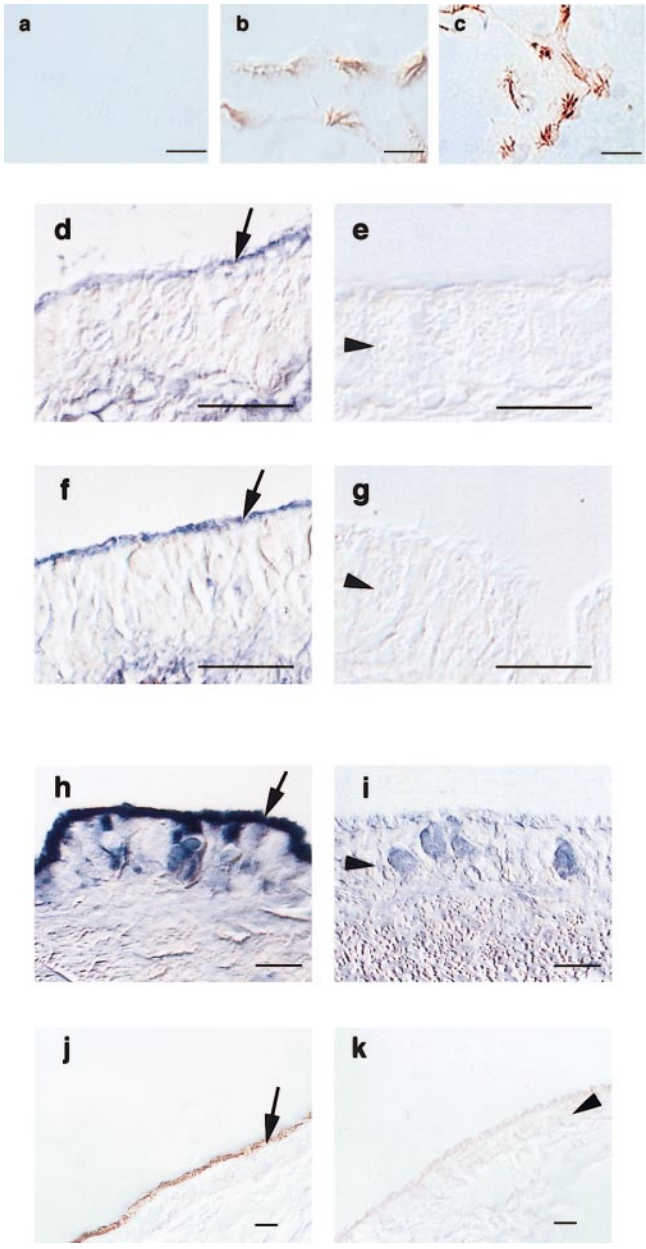

Figure 1. Staining for TK, LPO, and hyaluronan in airway epithelial cells (differential interference contrast images). Cultured airway epithelial cells (*a–c*) were fixed, permeabilized, labeled using preimmune serum (*a*), anti-LPO (*b*), or anti-TK antibodies (*c*), and visualized with horseradish peroxidase-conjugated second antibodies. Specific staining of cilia can be seen. Paraffin sections of trachea were stained with a biotinylated hyaluronan binding protein and avidin-alkaline phosphatase (*d–g*) showing that hyaluronan is localized to the ciliary border of the epithelium in addition to its known localization in the submucosal interstitium. Incubation with hyaluronidase (37°C overnight) removed specific staining for hyaluronan (*e*), whereas incubation with chondroitinase ABC at pH 7.5 did not change the staining pattern for hyaluronan (*f*). When chondroitinase ABC was used at pH 5.6, where it has hyaluronidase activity, hyaluronan staining was also removed from the sections (*g*). Labeling with anti-LPO antibodies and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (*h*) or anti-TK antibodies and diaminobenzidine (*j*) revealed specific staining along the ciliary border of the airway epithelium. Incubation with hyaluronidase removed LPO (*i*) and TK (*k*). All bars are 10 μM. Arrows depict the ciliary border of the epithelium. Arrowheads point to the epithelial cell layer (*i*: to a goblet cell).

cells; 10) also revealed specific staining along cilia with antibodies to both enzymes (Fig. 1). Preimmune serum revealed no nonspecific staining in the ciliary border of tissue sections or cell cultures. Direct visualization of LPO's activity in tissue sections using H_2O_2 and diaminobenzidine (9) confirmed the results obtained by immunostaining, again ruling out nonspecific adherence of antibodies to the ciliary border. Since we have shown that TK obtained from airway lavages was bound to hyaluronan (4), we wondered whether hyaluronan immobilizes enzymes at the apex of epithelial cells. Histochemistry of tracheal sections for hyaluronan using a biotinylated hyaluronan binding protein also labeled the ciliated border of the epithelium (Fig. 1). However, not all tissue sections revealed the ciliary staining for LPO, TK, and hyaluronan, perhaps due to differences in processing, especially the use of fixative (acid formalin seems critical). Digestion with hyaluronidase eliminated the apical staining for hyaluronan, LPO, and TK (Fig. 1). Hyaluronidase did not remove all glycoconjugates from the apical border of the epithelium, as evidenced by the retention of Alcian-blue-PAS positive material at the apical border. In addition, chondroitinase ABC (at pH 7.5) did not eliminate staining for hyaluronan (Fig. 1*f*), LPO, or TK from the apical surface. Although chondroitinase ABC has hyaluronidase activity, the chondroitinase activity is 10-fold higher than the hyaluronidase activity at pH 7.5 (31). When chondroitinase ABC was used at pH 5.6, however, where it has high hyaluronidase activity, apical staining for hyaluronan was also eliminated (Fig. 1*g*). Thus, these data suggest that cell membrane-bound hyaluronan was retaining LPO and TK at the airway epithelial surface.

TK and LPO interact with hyaluronan

Using a nondenaturing gel system and affinity chromatography, we previously showed that TK binds to hyaluronan (4). Since TK has an acid pI, nonspecific electrostatic interactions are not expected to account for this interaction. We therefore searched for and found the putative hyaluronan binding motif $B(X_7)B$ (32) in the amino acid sequence of TK, where B is a basic residue and X is any amino acid not negatively charged. Since this binding motif contains a portion of the active TK site (**Fig. 2**), it may provide a basis for specific interactions between hyaluronan and TK. Using nondenaturing agarose gel electrophoresis, bovine milk LPO was also found to bind to hyaluronan (200 kDa average molecular mass, Seikagaku) after incubation times of 60 min (not shown). The binding could be reversed by hyaluronan digestion with hyaluronidase (20 IU/ml for 2 h at 37°C). Analysis of the LPO amino acid sequence, however, did not reveal the presence of the $B(X_7)B$ hyaluronan binding motif. Since LPO has an alkaline pI, nonspecific electrostatic interactions could account for its association with hyaluronan.

Although binding to hyaluronan inhibits TK activity (4), measurements of LPO activity using the 3,3′,5,5′-

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  1  MWFLVLCLAL SLGGTGAAPP IQSRIVGGWE
 31  CEQHSQPWQA ALYHFSTFQC GGILVHRQWV
 61  LTAAHCISDN YQLWLGRHNL FDDENTAQFV
 91  HVSESFPHPG FNMSLLENHT RQADEDYSHD
121  LMLLRLTEPA DTITDAVKVV ELPTQEPEVG
151  STCLASGWGS IEPENFSFPD DLQCVDLKIL
181  PNDECEKAHV QKVTDFMLCV GHLEGGKDTC
211  VGDSGGPLMC DGVLQGVTSW GYVPCGTPNK
241  PSVAVRVLSY VKWIEDTIAE NS
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Figure 2. Amino acid sequence of human glandular tissue kallikrein (GenBank accession #P06870). The putative hyaluronan binding site in form of the $B(X)_7B$ motif is marked by boldface letters. The catalytic sites of TK are underlined.

tetramethylbenzidine assay (9) revealed that LPO was not inhibited by addition of hyaluronan (200 kDa average) in molar ratios of 5:1, 10:1, 50:1, and 100:1 (hyaluronan:LPO). Relative LPO activity after 60 min of incubation with hyaluronan compared to LPO alone (control) was 95 ± 3%, 92 ± 5%, 99 ± 0.5%, and 92 ± 6% (mean±SE) of control, respectively, not reaching statistical significance at any ratio using ANOVA (n=4 at each ratio measured in triplicates). Hyaluronan alone did not affect the 3,3′,5,5′-tetramethylbenzidine assay of LPO's activity.

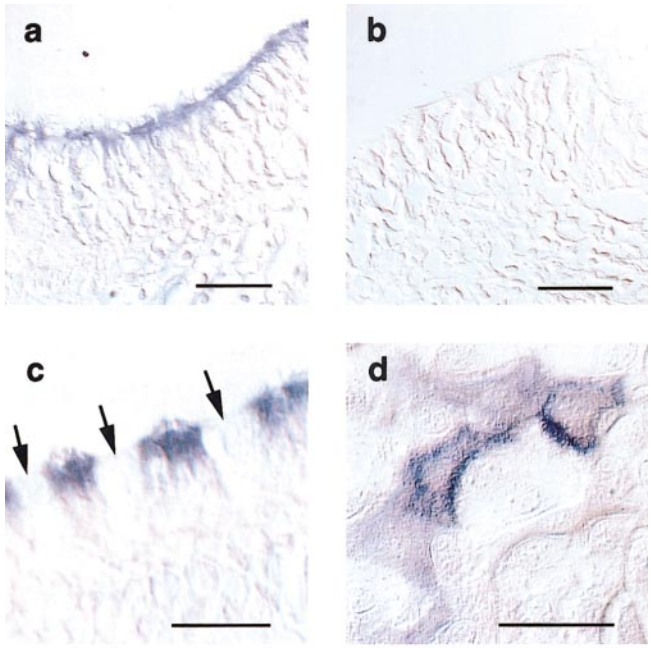
RHAMM is expressed on the apical airway epithelial surface

Since hyaluronan appeared to retain LPO and TK at the surface, we examined whether hyaluronan binding receptors are expressed at the apical surface of the epithelium. Previous reports indicated that CD44, a common extracellular hyaluronan receptor, is found on basal cells but not on the apex of normal, ciliated airway epithelial cells (33). Although this does not rule out its expression on the apical surface of ovine airway epithelial cells, it makes it less likely, and we concentrated on the expression of RHAMM in ovine trachea. Immunohistochemistry revealed specific staining for RHAMM in the apical portion of ciliated cells, but no staining in goblet cells (**Fig. 3**). To confirm expression of RHAMM in tracheal epithelial cells, we used an ovine tracheal mucosa cDNA library (3) and primers for RHAMM (Fig. 3) designed according to consensus regions of the published sequences. PCR reactions yielded a band of expected size (249 bp) and the fragment was sequenced (Fig. 3; GenBank accession no. BankIt354819 AF310973). The deduced amino acid sequence was 89% and 81% identical to the published human and mouse sequences, respectively. Together, these data show that RHAMM is expressed in and localized to the apical portion of polarized ciliated airway epithelial cells.

CBF is regulated by hyaluronan-mediated signaling through RHAMM

To examine whether our previously reported hyaluronan-mediated increase in CBF (10) was mediated through RHAMM, we used primary cultures of ovine airway epithelial cells (25).

Using anti-RHAMM antibody and fixed, nonpermeabilized cells, expression of RHAMM could be shown to occur on the ciliated surface of cultured cells (Fig. 3). These results were confirmed by adding anti-RHAMM antibody to live cells before fixation. The expression of RHAMM increased during the time in culture (18% of all ciliated cells stained positive on day 3 after plating, 57% on day 5, 65% on day 8, and 76% on day 11). This increase in expression correlated with our reported increase in the percentage of ciliated cells in culture staining positive for surface hyaluronan (stemming from submucosal gland cells usually found in primary airway epithelial cell cultures; 10) as well as the increase in the percentage of ciliated cells in culture responding



TGTTGAATGAACATGGTGCAGCTCAGGAACAACTAAATAATCTAAGA
GATTCATATGCTAAATTATTGGGTCATCAGAATCTAAAGCAAAAAATC
AAGCATGTTGTGAAACTGAAAGATGAAAACAGCAATCTCAAATCGGAG
GTGTTAAAACTCCGTGCTCAGCTTACTAAAAGAAAACAAAGTGAAGCA
AAACTTCAGGAGGAATTGAATAAAGTTCTGGGTATCAAA**CACTTTGAC**
CCTTCNAAGG

Figure 3. Immunohistochemistry and immunocytochemistry for RHAMM in airway epithelial cells. Cultured cells or tracheal sections were fixed and stained with a specific, affinity-purified RHAMM antibody (*a, c, d*) or preimmune serum (*b*), followed by alkaline phosphatase-conjugated second antibody. Sections were stained after permeabilization necessary for embedding (*a–c*), whereas cell cultures were not permeabilized (*d*). The apical portion of ciliated cells (including the cilia themselves) were specifically labeled (*a, c*), whereas goblet cells were not (*c*). Arrows indicate goblet cells (no apical cilia). All bars are 10 μm. The text panel below depicts a 249 bp RHAMM cDNA sequence that was amplified from an ovine airway epithelial cDNA library using specific primers. The boldface sections represent primer regions. The deduced amino acid sequence was 89% identical to the human and 81% to the mouse sequence (GenBank accession no. BankIt354819 AF310973).

to exogenous hyaluronan with an increase in CBF (**Fig. 4**; ref 10). This correlation is significant, since other responses of ovine ciliated airway epithelial cells, such as CBF increases in response to ATP or acetylcholine, can be elicited immediately after plating, are maintained over a 14–20 day period, and do not depend on cell growth or adaptation to culture (25, 29, 34).

We next made sure that the CBF stimulating effect of hyaluronan did not depend on the commercial source used. Hyaluronan from two suppliers (Seikagaku and Worthington) yielded the same CBF stimulation when measured in six cells each over 10 days in culture (ΔCBF of 1.8±0.6 Hz for Seikagaku and ΔCBF of 1.2±0.4 Hz for Worthington, $P>0.5$). Hyaluronan digested with hyaluronidase, however, had no effect on CBF.

Then we tested whether the hyaluronan-induced CBF increase could be inhibited with our functionally blocking anti-RHAMM antibody. At room temperature, none of 10 cells (>10 days in culture) preincubated with anti-RHAMM antibody (24) responded with a CBF change (baseline 7.4±0.6 Hz; **Fig. 5**). However, six of eight cells exposed to a nonspecific, control rabbit anti-chicken IgG responded to 50 μg/ml hyaluronan with an increase in CBF from 7.2 ± 0.6 to 9.1 ± 0.4 Hz ($P<0.05$; Fig. 5). This CBF increase in the presence of control antibody was similar to responses measured without any antibody (10). The percentage of responding cells also corresponded to the percentage of RHAMM-expressing ciliated cells. Finally, control responses to 20 μM ATP, a well-known stimulator of CBF (35–37), were statistically indistinguishable between both groups (ΔCBF in the anti-RHAMM group was 2.5±0.5 Hz and in the anti-IgG control group 2.7±0.5 Hz, $P=0.45$) and indistinguishable from cells not exposed to antibodies.

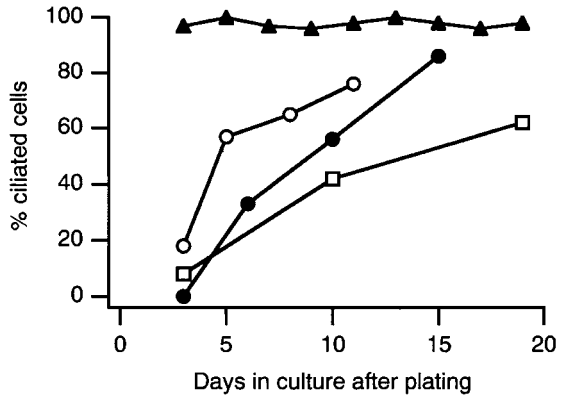


Figure 4. Expression of hyaluronan, RHAMM and hyaluronan-regulated CBF in cultured cells. After plating, ciliated cells were analyzed over time in culture for the percentage that stained positive for endogenous hyaluronan (□), the percentage that stained positive for RHAMM (o), and the percentage that responded to exogenously applied hyaluronan with an increase in CBF (●). For each cytochemical study point, 50 randomly selected high-power fields (400×) from each culture were analyzed assessing only ciliated cells (>100 cells total). As a comparison, CBF responses of ciliated cells to exogenously applied 10 μM ATP are shown (▲); these responses are seen early after plating and show no significant change over time. Each data point of the CBF responses represents analysis from at least 10 cells.

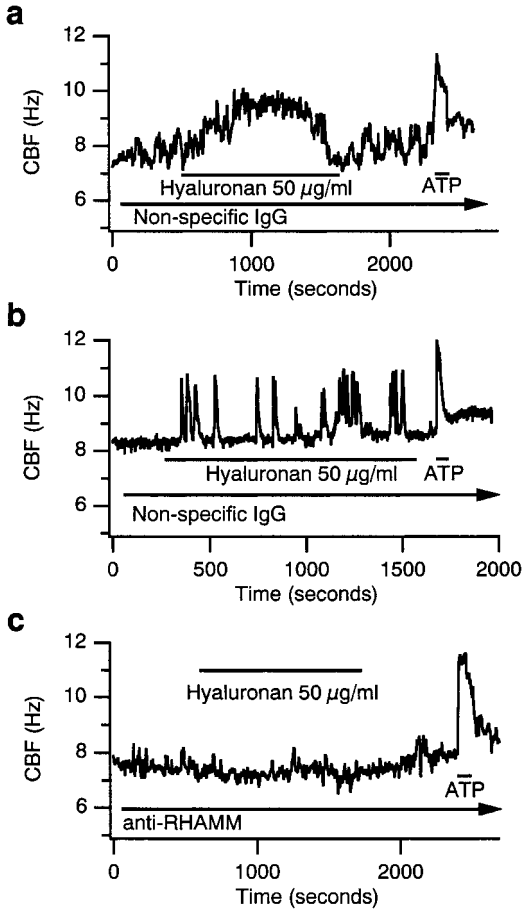


Figure 5. Hyaluronan-induced CBF increase is blocked by anti-RHAMM antibodies. CBF of ovine tracheal epithelial cells in primary cultures was measured as described previously (29) in the presence of IgG control antibodies (*a, b*) or anti-RHAMM antibodies (*c*). Continuous recordings of CBF are shown in response to exogenous 50 μg/ml hyaluronan (each n≥8). There were two types of responses: *a)* a transient but continuous increase in CBF and *b)* an oscillatory response. Both types of CBF responses to hyaluronan were blocked using anti-RHAMM antibodies (*c*). All cells responded to 20 μM ATP with a statistically indistinguishable, transient increase in CBF (*a–c*). The duration of drug application is indicated by labeled horizontal bars (arrows indicate continued presence of drug).

Tracheal transport of TK, LPO, and albumin

To examine whether hyaluronan actually protected these enzymes from removal by mucociliary clearance, we labeled recombinant TK with fluorescein and both LPO and albumin with rhodamine. Labeled TK and albumin were applied together (as a mixture) onto the same region of the surface tracheal epithelium and the migration of the fluorescence was measured over a 30 min period. TK was not transported after application whereas albumin moved forward over the entire 30 min period. Thus, the two substances moved apart, as indicated by a separation of the original orange fluo-

rescence (mixture) into clearly defined green (TK) and red (albumin) bands (**Fig. 6**). Movement of albumin was measured to range between 1 and 5 cm (dye front) at the end of 30 min, whereas TK did not move (n=3). To show that LPO behaved similarly to TK and that fluorescein modification was not responsible for TK's immobilization, we used rhodamine-labeled LPO with the same result (not shown). The immobilization of the enzymes was in fact due to hyaluronan, since TK and albumin did not separate on tracheas pretreated with hyaluronidase, moving at the same rate over the 30 min observation period (Fig. 6). These data show that airway LPO and TK are both bound to the airway epithelial surface by hyaluronan and are not transported away by mucociliary clearance as labeled albumin is.

DISCUSSION

The data presented here contradict the commonly held notion that enzymes secreted onto epithelial surfaces are rapidly cleared by mechanical action. The data provide evidence that some enzymes are specifically retained at the surface, and therefore availability and activity of mucosal enzymes do not depend solely on exocytosis from secretory cells. In the respiratory system, we showed that TK and LPO are bound to the airway epithelial surface by hyaluronan and not cleared by ciliary action. Since other mucosal secretions such as tears and conjunctival cell culture supernatants contain hyaluronan (38) and possibly other glycosaminoglycans, these observations may be relevant to all epithelia bathed in secretions and cleared by mechanical processes.

Proteins can bind to hyaluronan by several mechanisms (32, 39, 40). We have shown here that the deduced amino acid sequence of TK contains the putative hyaluronan binding motif $B(X_7)B$ (32) in the active site of TK (Fig. 2). We have also shown that LPO binds to hyaluronan, but that the LPO amino acid sequence does not contain the $B(X_7)B$ binding motif. LPO has an alkaline pI (~10) that by itself allows nonspecific electrostatic interactions with hyaluronan. In fact, hyaluronan may act in general as a cation exchanger (39) and therefore may be able to bind several other cationic proteins present in the airway to the epithelial surface. Among those could be a variety of cationic antimicrobial substances—for example, those studied by Cole et al. (41).

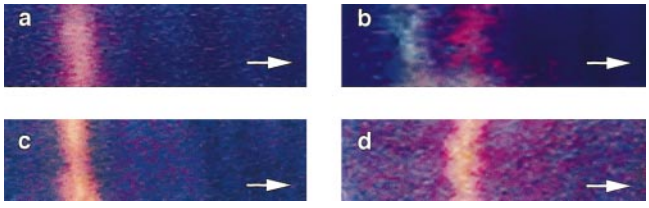


Figure 6. Binding to hyaluronan modulates TK but not albumin movement by the mucociliary transport system. Tracheas from freshly killed sheep were opened at their membranous portion. White arrows point to the proximal end of the trachea and represent a surface length of 2 cm. The same trachea at time 0 (*a, c*) and after 30 min (*b, d*). Fluorescein-labeled TK and rhodamine-labeled albumin were mixed and applied to the tracheal surface, revealing an orange fluorescence mixture at time 0 (*a*). After 30 min of incubation at 37°C (humidified), fluorescein-labeled TK did not move as indicated by the stripe of green fluorescence at the location of application, whereas albumin (represented by red fluorescence) separated from TK toward the proximal end of the trachea (movement ~ 2.5 cm in this experiment) (*b*). *c, d)* Trachea was pretreated with hyaluronidase as described in Materials and Methods. A TK/albumin mixture was applied, represented by an orange fluorescence (*c*). After digestion of hyaluronan, both TK and albumin were transported without separation for ~ 2.5 cm during the 30 min observation period (*d*).

In addition to binding TK to the epithelial surface, hyaluronan also inhibits its activity (4). This is important because TK can lead to bronchoconstriction, which is useful only during exposure to certain stimuli. Airway LPO, on the other hand, should be active at all times, because it contributes to host defense against inhaled bacteria (3). In fact, measurements of airway LPO activity in vitro presented here revealed that the enzyme did not change its activity when bound to hyaluronan over a wide range of molar ratios. Thus, hyaluronan perhaps serves as a broad anchor for many enzymes at the airway surface, either through electrostatic interactions or through specific binding. Note that the specific interaction occurs with TK, an enzyme that needs to be inhibited while present at the airway surface. On the other hand, electrostatic interactions seem to be important for enzymes immobilized in their active form. In this sense, it is remarkable that many antimicrobial substances in nasal secretions and in airway secretions are cationic in nature (41).

The data showing RHAMM expression at the apical surface suggest a mechanism for hyaluronan binding to the airway epithelial surface. Whereas the presence of RHAMM is certainly important, we cannot rule out that other hyaluronan anchoring proteins are present at the apical surface including CD44, although it has been reported not to be expressed there in the normal, undamaged epithelium (33). Whether or not RHAMM is the sole anchor for hyaluronan, the experiments using anti-RHAMM antibody reveal a clear functional role of RHAMM in the airway epithelium: upon hyaluronan binding, RHAMM signals to increase CBF. Although it would be interesting to know how RHAMM increases CBF without having a transmembrane signaling component, such experiments go beyond the scope of this paper, especially since the few signaling pathways known to be activated by RHAMM (e.g., ERK stimulation) have not been reported to regulate CBF and are usually slower than the observed reactions of CBF to hyaluronan.

RHAMM expression on the surface was initially lost and slowly reappeared on the cell surface in culture. This phenomenon could be due to initial removal of RHAMM from these cells by proteolytic action during cell isolation. The time course of RHAMM reappearance correlated with hyaluronan binding to these cells as well as CBF

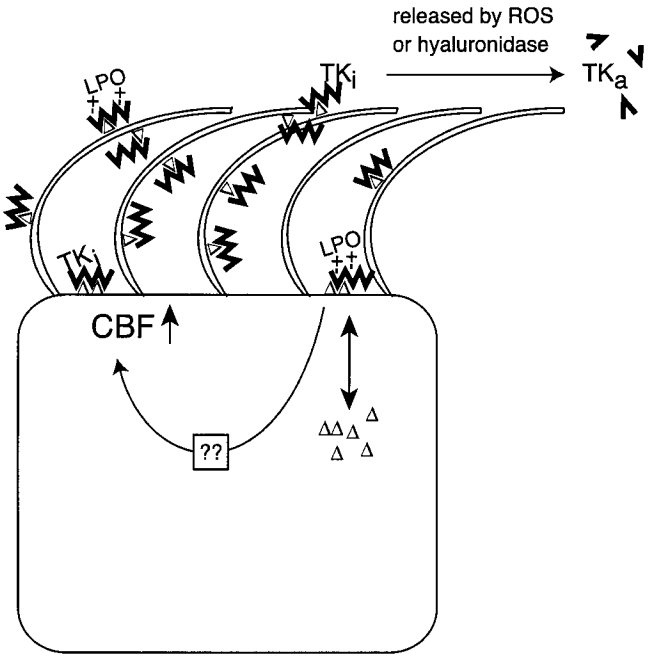


Figure 7. Model of hyaluronan function in the airway. Secretions from submucosal gland cells contain hyaluronan (ww), LPO, and TK. Hyaluronan binds to RHAMM (Δ) and perhaps other surface receptors (not shown). RHAMM is depicted on ciliary membranes, although no formal evidence of its expression at this location was obtained. Interaction of hyaluronan with RHAMM stimulates CBF and thereby mucociliary clearance to enhance removal of foreign particles. Simultaneously, LPO and TK are retained at the apical surface and protected from mucociliary clearance. LPO binds to hyaluronan via electrostatic interactions, whereas TK binding to hyaluronan is specific and inactivates TK (TK_i). The normal turnover rate of hyaluronan at the airway surface remains to be determined. However, if insults induce the release reactive oxygen species (ROS; e.g., in asthma) or the release of hyaluronidases, hyaluronan is broken down, TK is released and thereby activated (TK_a) to produce kinins that cause bronchoconstriction. Several questions remain. Is there another apical surface hyaladherin besides RHAMM; how is RHAMM signaling to increase CBF; and how is the turnover or exchange of apical intracellular and extracellular RHAMM regulated?

responses to exogenously applied hyaluronan. RHAMM expression in fact predicted CBF responses to hyaluronan. The correlation between RHAMM expression, hyaluronan binding, and CBF increases over time in culture is significant since CBF responses to other stimuli such as ATP and acetylcholine do not show such a time course. These responses can be elicited right after plating and are not lost during a 14–20 day culture period. These data therefore show that hyaluronan signals through RHAMM to increase cilia beating on airway epithelial cells similar to the effect of hyaluronan on sperm flagellar bending (22).

Hyaluronan is most often thought to be a component of the extracellular matrix and until now, the airways have not been an exception. Therefore, an increase in the amount of soluble hyaluronan in bronchial lavage fluid has been interpreted as a marker of interstitial damage to the lung (42–44). In the context of chronic airway diseases, where increased amounts of soluble hyaluronan could be measured in bronchial lavage fluid (e.g., ref 45), hyaluronan has been interpreted as a sign of tissue 'remodeling'. These interpretations are surprising since hyaluronan has been known to be secreted from submucosal gland cells, specifically from serous cells (46). Therefore, and as shown here, hyaluronan is secreted into the airways under normal conditions and must have specific functions in the normal airway. At least part of this function is to immobilize TK and LPO on the epithelial surface. In diseased airways, altered amounts of secreted or surface-released hyaluronan could be responsible for changes seen in the airway in asthma and other inflammatory conditions.

In summary, we propose a model in which hyaluronan serves a previously unrecognized pivotal role in mucosal host defense (**Fig. 7**). Hyaluronan stimulates ciliary beating (through its interaction with RHAMM) and hence the clearance of foreign material from mucosal surfaces, but simultaneously retains and regulates enzymes important for homeostasis at the apical mucosal surface. Therefore, the common belief that constitutive and stimulated secretion onto the mucosal surface is the major determinant of enzyme availability has to be revisited. The data shown here suggest a new paradigm that involves an apical enzyme pool 'ready for use' and protected from ciliary clearance. This pool will have to be considered in enzymatic reactions at the mucosal surface, be it in health or disease. FJ

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