Hyaluronidase Expression and Activity Is Regulated by Pro-Inflammatory Cytokines in Human Airway Epithelial Cells

María Elena Monzón[1], Dahis Manzanares[1], Nathalie Schmid[1], S. Marina Casalino-Matsuda[1], and Rosanna M. Forteza[1]

[1]Division of Pulmonary and Critical Care Medicine, University of Miami Miller School of Medicine, Miami, Florida

Hyaluronan (HA) is present at the apical surface of airway epithelium as a high-molecular-weight polymer. Since HA depolymerization initiates a cascade of events that results in kinin generation and growth factor processing, in the present work we used primary cultures of human bronchial epithelial (HBE) cells grown at the air–liquid interface (ALI) to assess hyaluronidase (Hyal) activity by HA zymography, gene expression by quantitative real-time PCR, and localization by confocal microscopy. Because TNF-α and IL-1β induce Hyals in other cells, we tested their effects on Hyals expression and activity. We found that Hyal-like activity is present in the apical and basolateral secretions from HBE cells where Hyals 1, 2, and 3 are expressed, and that IL-1β acts synergistically with TNF-α to increase gene expression and activity. Confocal microscopy showed that Hyals 1, 2, and 3 were localized intracellularly, while Hyal2 was also expressed at the apical pole associated with the plasma membrane, and in a soluble form on the apical secretions. Tissue sections from normal individuals and from individuals with asthma showed a Hyal distribution pattern similar to that observed on nontreated HBE cells or exposed to cytokines, respectively. In addition, increased expression and activity were observed in tracheal sections and in bronchoalveolar lavage (BAL) obtained from subjects with asthma when compared with normal lung donors and healthy volunteers. Our observations indicate that Hyal 1, 2, and 3 are expressed in airway epithelium and may operate in a coordinated fashion to depolymerize HA during inflammation associated with up-regulation of TNF-α and IL-1β, such as allergen-induced asthmatic responses.

Keywords: hyaluronidase; airway; hyaluronan; inflammation; asthma

Hyaluronan (HA) is a glycosaminoglycan widely distributed in tissues and is a normal constituent of airway secretions (1, 2). In the airways, HA is produced by submucosal glands and by superficial airway epithelial cells (3, 4), where it is synthesized by hyaluronan synthases (HAS) at the plasma membrane and released as a high-molecular-weight polymer into the extracellular space (5, 6). Although hyaluronan was discovered in 1934 (7) and its structure defined in 1951 (8), only recently it has become clear that, besides its structural role as a component of the extracellular matrix (ECM) (9), HA is involved in a broad range of biological processes associated with health and disease (for review *see* Refs. 10, 11).

(*Received in original form October 3, 2007 and in final form February 19, 2008*)

This work was supported by National Institutes of Health grant HL073156, James and Esther King Team Science Program and Biomedical Research grants 07KB-02-12292 (to R.M.F.) and 07KN-02-12324, and AHA Scientist Development grant 635093N (to S.M.C.-M.).

Correspondence and requests for reprints should be addressed to: María E. Monzón, Division of Pulmonary and Critical Care Medicine, University of Miami School of Medicine, 1600 NW 10th Ave., RMSB 7072A (R-47), Miami, FL 33136. E-mail: mmonzonmedina@med.miami.edu or Rosanna Forteza, rforteza@miami.edu

Am J Respir Cell Mol Biol Vol 39. pp 289–295, 2008
Originally Published in Press as DOI: 10.1165/rcmb.2007-0361OC on April 3, 2008
Internet address: www.atsjournals.org

CLINICAL RELEVANCE

This article addresses mechanisms of hyaluronan degradation in human airways associated with inflammatory responses.

In the lungs, HA is involved in injury and repair processes through its receptor CD44 (12), in interstitial fluid balance regulation due to its high water-binding capacity (13), and in the induction of inflammatory mediators in alveolar macrophages and lung fibroblasts (14, 15).

Most HA functions have been shown to be size-dependent: the high-molecular-weight molecules have been reported to exert anti-inflammatory and immunosuppressive effects (16), while smaller fragments stimulate gene expression and protein synthesis of pro-inflammatory mediators such as cytokines and chemokines (for review *see* Ref. 17). We have previously reported that high-molecular-weight HA found at the surface of airway epithelium is involved in airway homeostatic mechanisms by binding and regulating the activity of enzymes such as lactoperoxidase and tissue kallikrein (18, 19), while small HA fragments stimulate ciliary beating through a mechanism that involves the receptor for hyaluronic acid–mediated motility (RHAMM) (20, 21).

Hyaluronan fragments can be generated by reactive oxygen species (ROS)-induced depolymerization and/or by the activity of the hyaluronan degrading enzymes, the hyaluronidases (Hyals). ROS induce HA depolymerization in a variety of tissues, including skin (22), cartilage (23), and at the airway surface (21, 24, 25). In contrast, the role of Hyals in HA degradation at the airway surface and subepithelial tissues has not been previously explored.

Six hyaluronidase genes have been reported in humans: Hyal1, Hyal2, and Hyal3, clustered on chromosome 3p21.3, and Hyal4, PH20 and PHyal1, clustered on chromosome 7q31.3 (26). Among them, Hyals 1, 2, and 3 are widely expressed in somatic tissue and in cancer (26, 27). PH20 expression seems to be limited to testes, but its mRNA can be detected in other tissues and in malignancies (28, 29).

It has been shown that the activity of both HA synthases and Hyals can be regulated by cytokines and growth factors (30, 31). Asthma and a variety of airways inflammatory responses are characterized by increased levels of the pro-inflammatory cytokines IL-1β and TNF-α (32, 33). Thus, it is likely that the expression and activity of Hyals can be induced by these cytokines in human airways, and therefore be responsible, at least in part, for the increased levels of soluble HA observed in the bronchoalveolar lavage (BAL) of individuals with asthma (34) with the associated decrease in HA average molecular size observed after allergen challenge (35).

In the present work we report that Hyal 1, 2, and 3 are expressed in the airway epithelium and that TNF-α induces

up-regulation of both gene and protein expression as evidenced by QPCR and increased glycosidase activity in human airway epithelial cells *in vitro*. These effects were potentiated by simultaneous exposure to IL-1β. The biological relevance of these observations is supported by the evidence of augmented Hyal 1, 2, and 3 expressions in tissue sections obtained from lung donors with asthma, and the increased Hyal activity on the BAL of individuals with asthma after segmental allergen challenge when compared with normal lung donors and healthy volunteers, respectively.

MATERIALS AND METHODS

All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Cell Cultures

Isolation procedure. Human tracheas and main bronchi from donor lungs were obtained through the University of Miami Life Alliance Organ Recovery Agency with approval from the local Institutional Review Board. The trachea and main bronchi were opened at the membranous portion and the mucosa was dissected off the cartilage. Mucosal strips were digested with 0.05% protease (type 14) incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA) and incubated at 4°C. Human bronchial epithelial (HBE) cells were released by vigorous shaking and harvested by centrifugation as previously described (25, 36).

HBE cells grown at the ALI. Human tracheobronchial epithelial cells were counted and their viability was determined by trypan blue exclusion (viability was always > 80%). Cells (1×10^6; referred to as P0) were plated on collagen-coated plastic dishes (100 mm; Corning Costar Corporation, Cambridge, MA), and grown in bronchial epithelial growth medium (BEGM) (37), yielding undifferentiated airway epithelial cells. After reaching confluence, cells were dissociated with trypsin (referred to as P1 [38]), and 5×10^5 cells from P1 were plated onto 24-mm Transwell-clear culture inserts (Corning), coated with human placental collagen. The culture medium (ALI medium) contained 50% DMEM and 50% Lechner and LaVeck (LHC) basal medium supplemented with insulin (5 μg/ml), hydrocortisone (0.072 ng/ml), epidermal growth factor (0.5 ng/ml), triiodothyronine (T3, 6.5 ng/ml), transferrin (10 μg/ml), epinephrine (0.6 μg/ml), phospholethanolamine (0.5 μM), ethanolamine (0.5 μM), bovine pituitary extract (1% vol/vol), bovine serum albumin (0.5 mg/ml), $CaCl_2$ (0.08 mM), trace elements, penicillin/streptomycin (100 μg/ml), and retinoic acid (1 μM) (36). Cells were grown in an incubator at 37°C in ambient air supplemented with 5% CO_2. Their apical surface was exposed to air as soon as they reached confluence (∼ 7 d). These conditions were maintained until the cells reached full re-differentiation, as evidenced by ciliary beating and the presence of mucus on the apical surface (∼ 4 wk), and used for the experiments described below.

Treatments

Each experimental condition described below was performed in individual cultures obtained from three different lung donors ($n = 3$). Fully differentiated HBE cultures grown at ALI were exposed basolaterally to ALI media containing TNF-α (20 ng/ml), IL-1β (2.5 ng/ml), or their combination (TNF-α 20 ng/ml + IL-1β 2.5 ng/ml) for 18 hours at 37°C. Cultures exposed to ALI media were used as controls. After treatments, basolateral media and apical secretions, obtained by washing with 500 μl of PBS (apical washes), were collected. Cell layers were treated with 1% Triton X-100 in 20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES pH 7.8 (lysis buffer) containing 50 mM NaF, 1 mM sodium orthovanadate, 5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, spun at 14,000 rpm for 5 minutes at 4°C, and stored at −20°C for later analysis.

Quantitative Real-Time PCR

RNA from HBE cultures were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Quantitative real-time PCR (QPCR) was performed using pre-made TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) with TaqMan MGB probes, FAM labeled Hyal1 (ID:Hs00738390_m1), Hyal2 (ID:Hs00186841_m1), Hyal3 (ID:Hs00185910_m1), and Ph20 (Hs00162139_m1). The QPCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's instructions. Thermal cycling was performed using ICycler IQ apparatus (Bio-Rad). The comparative C_T method ($^{\Delta\Delta}C_T$) was used for relative quantification. All results were normalized using the housekeeping gene GAPDH (Hs99999905_m1).

HA-Zymography

Samples were assessed for Hyal activity by zymography as described (39). Briefly, samples were electrophoresed in 10% SDS-polyacrylamide gels containing 0.17 mg/ml of HA (vitreous humor; US Biological, Swampscott, MA). After electrophoresis, gels were washed in 50 mM HEPES buffer pH 7.4 containing 3% Triton X-100 and subsequently incubated in 0.15 M NaCl–0.1 M sodium formate, pH 4.2 at 37°C for 18 hours. Gels were then stained with 0.5% Alcian blue in 3% acetic acid where hyaluronidase activity was visualized as clear bands on the blue background. For the assessment of relative activity, intensities of the bands were recorded using the GelDoc XRS system (Bio-Rad) and analyzed using Quantity One software (Bio-Rad).

Immunofluorescence

Cell cultures. After removing culture media, filter inserts were fixed in 4% paraformaldehyde in PBS, pH 7.4 for 20 minutes at room temperature (RT) and permeabilized with 0.05% Triton X-100 in PBS for 10 minutes at RT. After washing with PBS, each filter was blocked with 5% BSA in PBS for 1 hour at room temperature. Primary antibodies diluted in blocking solution were used as 1:500 dilutions for the following antibodies: rabbit anti-Hyal1 antiserum (a generous gift from Dr. Lokeshwar, University of Miami Miller School Of Medicine [40]), and mouse anti-Hyal2 and Hyal3 antiserum (both from Novus Biologicals, Littleton, CO). Visualization was achieved using Alexa 555– or Alexa 488–labeled anti-rabbit IgG (4 μg/ml) and Alexa 488– or Alexa 555–labeled anti-mouse IgG (4 μg/ml; Molecular Probes, Carlsbad, CA). Nuclei were visualized with 4′6-diamidine-2-phenylindole (DAPI; Molecular Probes). Samples were mounted on slides with gel/mount (Biomeda, Foster City, CA) and images were obtained using a Zeiss LSM-510 confocal laser scanning microscope (Zeiss LSM, Thornwood, NY) at the University of Miami Analytical Imaging Core Facility.

Human tracheas. Tissue sections of human trachea were obtained from normal organ donors and from organ donors with asthma through the University of Miami Life Alliance Organ Recovery Agency with approval from the local Institutional Review Board. Paraffin-embedded sections were hydrated and incubated in EDTA 2 mM pH 8.0 for 15 minutes at 100°C (antigen retrieval) followed by acetone at −20°C for 10 minutes and blocked with Image-iTTM FX (Signal Enhancer; Invitrogen) following manufacturer's instructions. Immunolocalization was preformed as described above in the cell culture section.

BAL

BAL, kindly provided by Dr. Ndukwu (University of Chicago), were obtained from healthy volunteers ($n = 3$) and from volunteers with asthma ($n = 3$) 24 hours after segmental allergen challenge (or saline instillation on the contra lateral lung) as described (41). Hyal activity was assessed by HA-zymography as described above, where equal amounts of protein were loaded per well on the HA-acrylamide gels.

Statistical Analysis

All n refer to the number of different HBE cultures used for each study where cells from each culture were obtained from only one lung donor. Data were expressed as means ± SEM. Differences between multiple groups were compared using a one-way ANOVA followed by the Tuckey Kramer honestly significant difference test. Levene test was used to analyze the homogeneity of variances. Significance was accepted at $P < 0.05$.

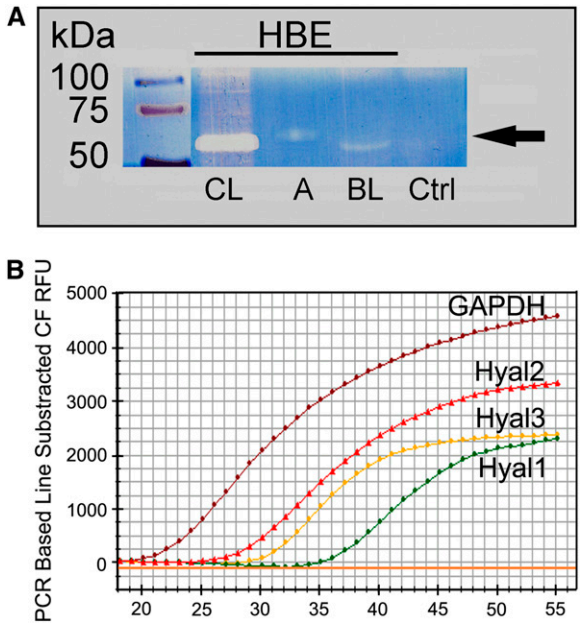

Figure 1. Hyaluronidases 1, 2, and 3 are expressed in human bronchial epithelial (HBE) cells grown at the air–liquid interface. (*A*) Hyals on HBE cells were assessed by HA zymography for Hyal-like activity in: cell lysates (CL), apical washes (A), and basolateral media (BL). Control is culture media not exposed to cell (Ctrl). (*B*) QPCR of Hyal1, Hyal2, and Hyal3 by Taq-Man as described in Materials and Methods. Figure depicts results obtained from one lung, representative of the results observed in cultures obtained from three lung donors.

RESULTS

Hyaluronidase Activity Is Present in HBE Cells Grown at the ALI where Hyals 1, 2, and 3 Are Expressed

To test if primary cultures of HBE cells grown at the ALI had the ability to degrade HA, we determined glycosidase activity by HA-zymography. Cell lysates, apical washes, and basolateral media were collected ($n = 3$) and activity was determined as described in Materials and Methods. As shown in Figure 1A, clear bands were detected in apical washes, cell lysates, and basolateral media, indicating that Hyal-like activity is present in all three compartments. The apparent migration pattern (50–65 kD) shows that the band corresponding to apical washes appear to migrate slower than the one observed in cell lysates, indicating that more than one molecular species are expressed by these cells.

To further investigate Hyals gene expression in these cells, QPCR (Figure 1B) studies were performed using mRNA obtained from cell cultures from three different lung donors. We found that Hyal1, Hyal2, and Hyal3 are normally expressed by these cells, while we did not detect PH20 mRNA expression (data not shown). The fact that Hyal activity was present at the apical surface (evidenced by zymography on apical washes) and in basolateral media, suggests that Hyals likely participate in HA depolymerization at the luminal pole and subepithelial areas of human airways.

Hyals Are Distinctly Localized in HBE Cells Grown at the ALI

To determine protein expression and cellular localization of the individual Hyals in airway epithelial cells, fully differentiated ALI cultures ($n = 3$) were labeled using primary antibodies generated against Hyal1, Hyal2, and Hyal3. Consistent with QPCR data, positive staining for the three Hyals were observed in HBE cells (Figure 2). Z-stack reconstruction images provided a more detailed view of the cellular localization of these enzymes (Figure 2, *upper panels*). Hyal1 (Figures 2A and 2B, *red*), Hyal2 (Figure 2A, *green*), and Hyal3 (Figure 2B, *green*) immunoreactivities were visualized intracellularly, inside vesicle-like structures as reported (42, 43). In addition, frequent colocalizations were observed between Hyal1 and 2 (Figure 2A) and Hyal1 and Hyal3 (Figure 2B).

Hyal2 was also observed at the apical surface, consistent with the glycosyl-phosphatidylinositol (GPI) anchored form reported for this enzyme in airway epithelial cell lines (44). Overall, these findings suggest the participation and a possible cooperation between different Hyals in HA degradation in the airway epithelium.

Tissue Distribution of Hyals in Airways Tracheal Sections from Normal Donors Is Similar to the One Observed in HBE Cells

To determine if the observed Hyals distributions in cell cultures were consistent with localization in human airway epithelium, we used human trachea sections for immunohistochemical analysis. As observed in Figure 3, the three Hyals tested were expressed in the airways. Hyal1 (Figures 3C and 3D) and Hyal3 (Figures 3G and 3H) appeared to be distributed intracellularly as observed in HBE cultures.

Hyal2 labeling (Figures 3E and 3F) was visible inside the cells and at the epithelial surface, where it appears to be associated with the cell surface and also in a "free form" at the mucus layer (Figure 3, *arrows*). These data are consistent with the distribution pattern found in cell cultures and confirm that HBE cells are an appropriate model to evaluate Hyals expression and regulation.

Pro-Inflammatory Cytokines Induce Hyal Gene Expression in HBE Cells

Since HA depolymerization is associated with airway inflammatory responses, to test if pro-inflammatory cytokines that have been reported to regulate Hyals in other tissues (31, 45)

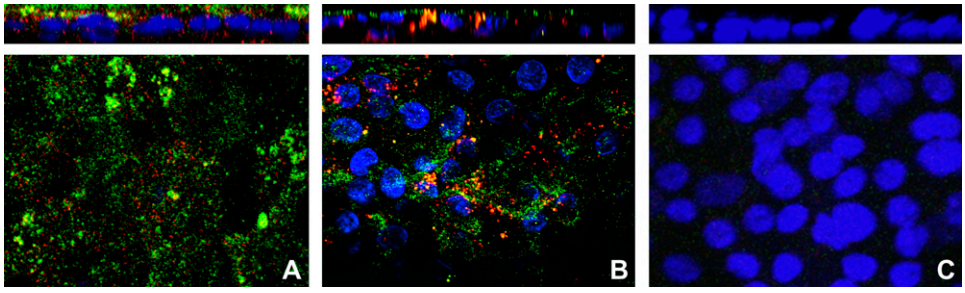

Figure 2. Hyaluronidases 1, 2, and 3 are localized intracellularly. Hyal 2 it is also present at the apical membrane and in a soluble form at the mucus layer in HBE cells. Cultures were labeled with (*A*) Hyal1- (*red*) and Hyal2- (*green*), or (*B*) Hyal1- (*red*) and Hyal3- (*green*) specific antibodies as described in Materials and Methods. (*C*) Control cultures were incubated with mouse and rabbit non-immune serum. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained by confocal microscopy (*magnification*: ×63). Above each panel the corresponding *Z*-axis reconstruction images are included.

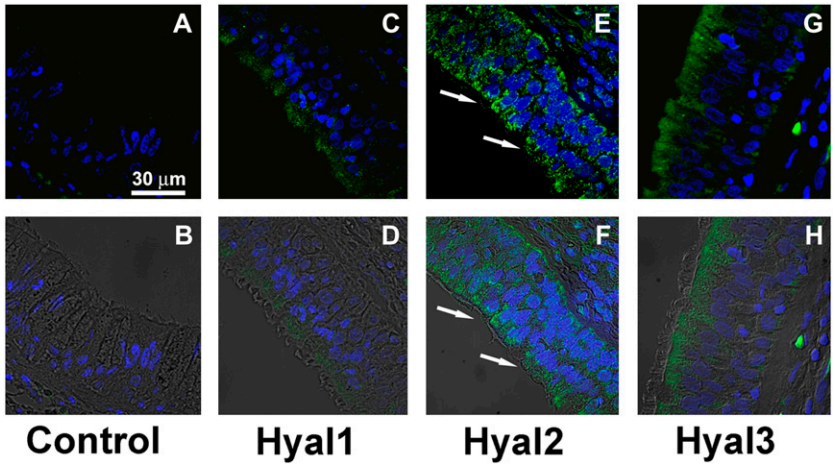

Figure 3. Tissue distribution of Hyals in human airways is similar to the one observed in HBE cultures. Confocal microscopy (*magnification*: ×63) of human tracheal sections immuno-labeled with rabbit anti-Hyal1 (*C* and *D*), mouse anti-Hyal2 (*E* and *F*) or mouse anti-Hyal3 (*G* and *H*), control nonimmune (*A* and *B*). Specific staining was visualized using Alexa 488–conjugated secondary antibodies. *Bottom*: same as *top images* merged with differential interference contrast for better visualization of the ciliary border. *White arrows* show the epithelial surface localization of Hyal2. Nuclei were visualized with DAPI.

have similar effect in airway epithelium, HBE cells were incubated with media alone (controls) or media containing TNF-α and/or IL-1β. As depicted in Figure 4, exposure to TNF-α alone resulted in increased Hyals1, 2, and 3 mRNA expressions, but only increases in Hyal1 expression achieved statistical significance (1.6 ± 0.21) from control cells ($P < 0.05$) on the three cultures tested. Exposure to IL-1β did not result in changes in gene expression in any of the three Hyals tested. In contrast, when cells were treated with TNF-α + IL-1β, all three (Hyal1 [2.3 ± 0.22], Hyal2 [2.2 ± 0.21], and Hyal3 [1.9 ± 0 0.25]) expressions were significantly increased when compared with controls ($P < 0.05$). These results suggest that IL-1β and TNF-α act in a synergistic fashion to induce Hyals gene expression in airway epithelial cells.

Pro-Inflammatory Cytokines Induce Hyal Activity in HBE Cells

To further investigate if the stimulatory effect on Hyals gene expression observed after cytokine exposure resulted in increased in glycosidase activity, we evaluated Hyal activity by HA-zymography in cell lysates obtained from cells exposed to TNF-α and/or IL-1β. While TNF-α induced an increase of 1.5 ± 0.2 ($P < 0.05$) in Hyal activity, no effect was observed with IL-1β in accordance with gene expression results. Likewise, the exposure to both TNF-α + IL-1β induced a higher increase (2.2 ± 0.3, $P < 0.05$) in enzymatic activity with respect to controls (Figure 5). These data further confirmed the synergistic/potentiating effect between IL-1β and TNF-α on Hyal activity in airway epithelial cells.

Hyaluronidase Activity Increases after Segmental Allergen Challenge in Individuals with Asthma

To explore if Hyals are up-regulated in the airways in conditions that are associated with increases of TNF-α and IL-1β *in vivo* such as allergen-induced airway hyperreactivity (46), we assessed Hyal activity on BAL obtained from healthy volunteers and from volunteers with asthma 24 hours after segmental allergen challenge (41, 47). Equal amounts of protein were loaded in each well where Hyal activity was analyzed by HA-zymography as described on Materials and Methods. As seen in Figure 6, basal Hyal activity (saline) is increased in individ-

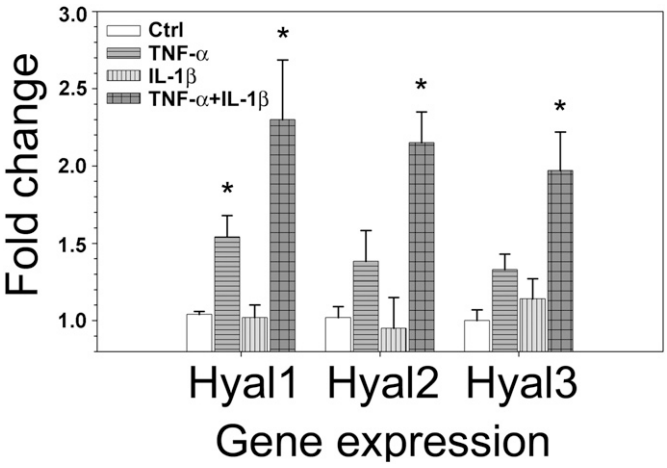

Figure 4. Pro-inflammatory cytokines induce Hyals gene expression in HBE cells. Hyaluronidases gene expression was assessed by QPCR after basolateral exposure to media alone (controls), TNF-α, IL-1β, or both (TNF-α + IL-1β). Results were normalized to GAPDH and expressed as fold changes with respect to media control. *Bars* represent mean ± SEM obtained from three different lung donors. *$P < 0.05$ compared with controls.

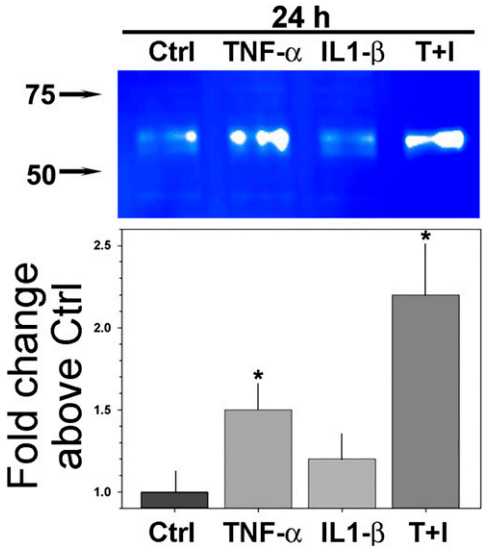

Figure 5. Hyaluronidase activity is induced by pro-inflammatory cytokines in HBE cells. *Top*: Hyal activity was determined by HA zymography in cell lysates (25 μg of proteins were loaded per well) after exposure to PBS (controls), TNF-α, IL-1β, or both (T+I). *Bottom*: Relative activity expressed as fold changes above media control.

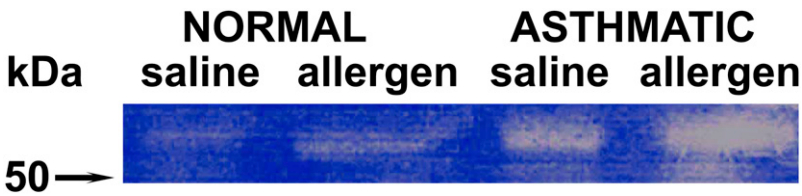

Figure 6. Hyaluronidase activity is elevated in the BAL of individuals with asthma and is further increased after segmental allergen challenge: HA-zymogram was used to assess Hyal activity on BAL samples obtained from healthy subjects and from subjects with asthma 24 hours after segmental allergen challenge (allergen). Saline instillation (Saline) on the contralateral lung was used as a control. A total of 50 μg (total proteins) from each sample were loaded per well.

uals with asthma when compared with normal subjects. In addition, Hyal activity in response to allergen challenge was visibly higher in the asthma group (allergen). These data provide *in vivo* evidence that Hyal activity is increased in airway inflammatory responses associated with TNF-α and IL-1β.

Expression of Hyas Are Increased in the Airways of Individuals with Asthma

To test if the increases in Hyal activity observed on the BAL of individuals with asthma were due to infiltrated leukocytes or/and activated macrophages alone, or were associated with increases in epithelial Hyals expression, double immunolabeling for Hyal1 (*green*) and Hyals 2 or 3 (*red*) was performed in human tracheal sections obtained from subjects without asthma, as depicted in Figures 7A, 7B, 7F, and 7G, and from subjects with asthma (Figures 7C–7E and 7H–7J) donors, as described in Materials and Methods. We intentionally used three lung donors, each showing different characteristics of pathological findings in asthmatic airways: (*1*) denuded epithelium with abundant leukocyte infiltration, (*2*) intact epithelium, and (*3*) with increased numbers of goblet cells (associated with hypersecretion in these patients). In all of the asthmatic tissues, Hyal1 and 2 (Figures 7C–7E) and Hyal1 and 3 (Figures 7H–7J) expression were clearly increased when compared with those from individuals without asthma (Hyal1 and 2, Figures 7A and 7B) and Hyal1 and 3 (Figures 7F and 7G). Hyal1 staining resulted particularly strong in infiltrated leukocytes (Figures 7C and 7H, *green arrows*) and in the interstitial space in subepithelial tissues. Hyal2 and Hyal3 immunoreactivity were prominently visible in ciliated cell in both shedded and intact epithelium (Figures 7C and 7H, *yellow arrows*) of subjects with asthma. In addition, Hyals 1 and 2 were found increased at the apical pole of cells and at the ciliary border of epithelium from subjects with asthma (Figures 7D, 7E, and 7J). No staining for Hyals 1, 2 or 3 was observed in goblet cell (Figures 7D, 7E, 7I, and 7J, *white arrows*), suggesting that Hyals expression is limited to the ciliated and basal cells of airway epithelium. These data suggests that the airway epithelial cells can contribute to the increased Hyal activity observed on the BAL of individuals with asthma.

DISCUSSION

We have previously shown that HA is present at the apical pole of airway epithelial cells (3, 18), where it plays important roles in both health and disease (18, 21, 24, 25). In the present study we show that Hyal1, Hyal2, and Hyal3 are expressed in primary cultures of HBE cells grown at the ALI and are up-regulated by TNF-α and IL-1β. These *in vitro* findings correlated *in vivo* with observations on tissue sections and BAL obtained from individuals with asthma, suggesting that Hyals participate in HA fragmentation at the airway lumen during inflammatory responses.

HA zymography from samples obtained from HBE cells confirmed that Hyal activity is present in these cultures not only in the cell layers but also in the basolateral media and in apical washes. The presence of Hyal activity in the basolateral media suggests that Hyals produced by airway epithelial cells can be involved in the HA degradation in the subepithelial tissues as well. These results, together with the data indicating that Hyal1, 2, and 3 are expressed by HBE cells, suggest that more than one Hyal isoenzyme participates in HA degradation in airway epithelium and at the mucus layer.

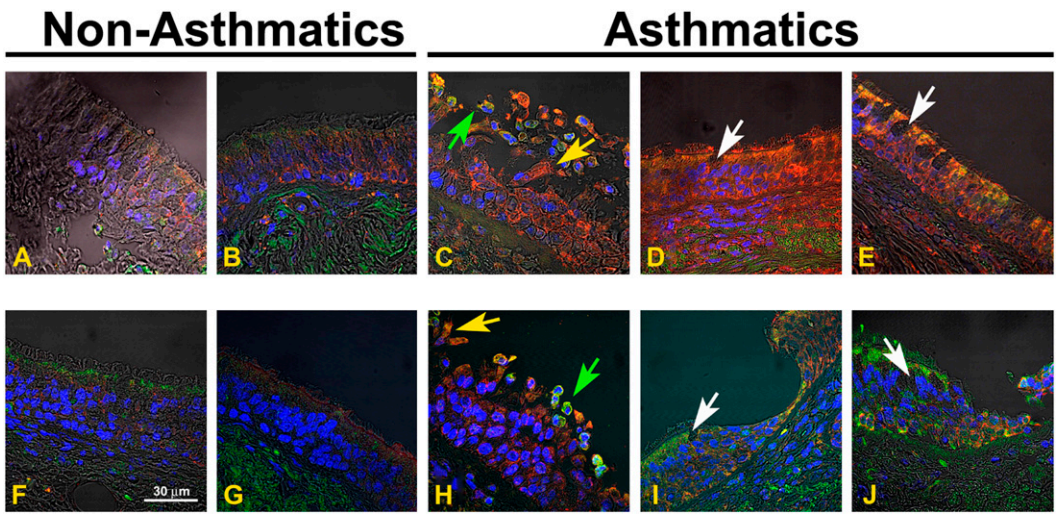

Figure 7. Hyaluronidases 1, 2, and 3 immunoreactivity is increased in airway tissue sections obtained from asthmatic lung donors: Human tracheal sections from subjects without (*A*, *B*, *F*, and *G*) and from subjects with asthma (*C–E* and *H–J*) were double-labeled with (*A–E*) anti-Hyal1 (*green*) and anti-Hyal2 (*red*) or (*F–J*) anti-Hyal1 (*green*) and anti-Hyal3 (*red*). Specific staining was visualized using Alexa 488–conjugated (Hyal1, *green*) and Alexa 555–conjugated (Hyals 2 and 3, *red*) secondary antibodies. Tissue samples obtained from three different individuals with asthma depicting denuded (*C*, *H*), intact (*D*, *I*), goblet cells (*E*, *J*) tissues were chosen. Images were merged with differential interference contrast to better visualize the epithelial morphology. *Green arrows* indicate infiltrated leukocyte expressing Hyals, *yellow arrows* indicate ciliated epithelial shedding, and *white arrows* show goblet cells. Nuclei were visualized with DAPI.

Differences in the Hyal isoenzymes degradation properties have been described (*see* Ref. 26). For instance, Hyal1 is active only at acidic pH (optimum pH 3.8–4.3 [48, 49]) and degrades HA of any molecular size generating mainly tetrasaccharides/hexasaccharides (42, 50), while Hyal2 cleaves high-molecular-weight HA into fragments of approximately 20 kD (42). The optimum pH for Hyal2 activity is still controversial and appears to depend on the Hyal2 form expressed: a free form of Hyal2 has been shown to be active under acidic conditions (50, 51), while membrane-associated Hyal2 is active under acid-neutral conditions (pH 6.0–7.0 [52]).

Hyaluronidase 2 localization seems to be tissue specific: while it is exclusively intralysosomal in chondrocytes (43), there is also evidence that can be expressed bound to the plasma membrane through a GPI anchor in airway epithelial cell lines (53).

We found that in our primary cultures as well as in human tracheal tissue sections, Hyal2-like immunoreactivity was present intracellularly, associated with the apical epithelial surface and as a free form at the mucus layer, suggesting that in human airways, both forms are likely expressed.

The distribution pattern of Hyals 1 and 2 that we found in primary cultures and in tracheal tissue sections agrees with the differential contribution of these enzymes in intracellular and extracellular HA degradation, respectively, as has been proposed in keratinocytes (54).

A seminal work from Harada and Takahashi (52) on HEK 293 cells showed that intracellular and extracellular Hyals 1 and 2 activities are dependent on the association with CD44 (52). If the mechanism mentioned above operates in airway epithelial cells, other considerations need to be in place: CD44 is found at the basolateral compartment in normal airway epithelium (55) and therefore could not participate in hyaluronidase activity at the apical surface. The crystal structure of Hyal1, recently determined (56), evidenced EGF-like domains, close to the catalytic site. These domains, characteristic of protein–protein interaction, could be involved in Hyals interaction with other surface receptors, as has been shown for the tyrosine receptor RON (44). We speculate that RON (21), other apically expressed HA receptors such as RHAMM (19, 21), or other undetermined protein may regulate Hyal activity on the epithelial surface.

In the present work we report that TNF-α and IL-1β (known to regulate HA metabolism [30, 31] and to be associated with inflammation [57, 58], chronic obstructive pulmonary disease [59], and asthma [32]) stimulate Hyals gene expression and activity. The induction of Hyals 1, 2, and 3 by TNF-α and the potentiation by IL-1β is a phenomenon that has been previously reported. For instance, TNF-α and IL-1β have synergistic effects to stimulate matrix metalloprotease-3 (60) in the trabecular meshwork; prostaglandin E (PGE) and collagenase production in fibroblasts (61), and PGE2 and cyclooxygenase activity in chondrocytes (62). Although we did not determine the nature of the synergistic effect induced by TNF-α + IL-1β in our cells, it has been suggested that these cytokines can be involved in transcriptional versus post-transcriptional events (62) in lymphocytes, where IL-1β induces TNF receptor expression and up-regulates mRNA transcription and membrane expression (63). Asthma, and particularly allergen exposure in these patients, is characterized by synthesis and release of IL-1β and TNF-α (46). Consistent with *in vitro* data, we found that Hyal activity was augmented in the BAL of individuals with asthma and further increased after segmental allergen challenge.

We have previously shown that exogenous sources of ROS are involved in HA depolymerization in the airway lumen (19, 21, 25). In the present study we provide additional evidence that airway epithelial cells themselves can participate in HA degradation at the lumen and in subepithelial tissues, particularly during inflammatory responses in which TNF-α and IL-1β cytokines are up-regulated.

The specific roles of individual Hyals on HA metabolism in the airway lumen as well as in epithelial and subepithelial tissues under normal and pathological conditions remains to be elucidated.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Vinata Lokeshwar from the Departments of Urology, Cell Biology and Anatomy at the University of Miami Miller School Of Medicine for providing the Hyal1 antibodies and for critical reading of the manuscript. The authors are particularly thankful to Dr. Ndukwu from the University of Chicago for providing the BAL samples. They also thank Drs. Gregory Conner, Matthias Salathe, and Nevis Fregien for helpful comments and support. This work was possible with the support of University of Miami Life Alliance Organ Recovery Agency, and the Analytical Imaging Core at the University of Miami and to the Histology Laboratory at the University of Miami Hospital and Clinics, Sylvester Comprehensive Cancer Center.

References

1. Schmekel B, Hornblad Y, Hvatum M, Norlund AL, Venge P. Kinetic retrieval of eosinophil cationic protein, hyaluronan, secretory IgA, albumin, and urea during BAL in healthy subjects. *Chest* 1995;108:62–67.
2. Midulla F, Villani A, Merolla R, Bjermer L, Sandstrom T, Ronchetti R. Bronchoalveolar lavage studies in children without parenchymal lung disease: cellular constituents and protein levels. *Pediatr Pulmonol* 1995;20:112–118.
3. Monzon ME, Casalino-Matsuda SM, Forteza RM. Identification of glycosaminoglycans in human airway secretions. *Am J Respir Cell Mol Biol* 2006;34:135–141.
4. Basbaum CB, Finkbeiner WE. Airway secretion: a cell-specific analysis. *Horm Metab Res* 1988;20:661–667.
5. Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life* 2002;54:195–199.
6. Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. *J Biol Chem* 1997;272:13997–14000.
7. Meyer KP, John W. The polysaccharide of the vitreous humor. *J Biol Chem* 1934;107:629–634.
8. Rapport MM, Weissmann B, Linker A, Meyer K. Isolation of a crystalline disaccharide, hyalobiuronic acid, from hyaluronic acid. *Nature* 1951;168:996–997.
9. Fraser JR, Laurent TC, Laurent UB. Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* 1997;242:27–33.
10. Hascall VC, Majors AK, De La Motte CA, Evanko SP, Wang A, Drazba JA, Strong SA, Wight TN. 2004. Intracellular hyaluronan: a new frontier for inflammation? *Biochim Biophys Acta* 2004;1673:3–12.
11. Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. *J Biol Chem* 2002;277:4589–4592.
12. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E, Henson PM, Noble PW. Resolution of lung inflammation by CD44. *Science* 2002; 296:155–158.
13. Allen SJ, Gunnar Sedin E, Jonzon A, Wells AF, Laurent TC. Lung hyaluronan during development: a quantitative and morphological study. *Am J Physiol* 1991;260:H1449–H1454.
14. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages: the role of HA size and CD44. *J Clin Invest* 1996;98:2403–2413.
15. Mascarenhas MM, Day RM, Ochoa CD, Choi WI, Yu L, Ouyang B, Garg HG, Hales CA, Quinn DA. Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression. *Am J Respir Cell Mol Biol* 2004;30:51–60.
16. Delmage JM, Powars DR, Jaynes PK, Allerton SE. The selective suppression of immunogenicity by hyaluronic acid. *Ann Clin Lab Sci* 1986;16:303–310.
17. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol* 2002;21:25–29.
18. Forteza R, Lauredo I, Abraham WM, Conner GE. Bronchial tissue kallikrein activity is regulated by hyaluronic acid binding. *Am J Respir Cell Mol Biol* 1999;21:666–674.
19. Forteza R, Lieb T, Aoki T, Savani RC, Conner GE, Salathe M. Hyaluronan serves a novel role in airway mucosal host defense. *FASEB J* 2001;15:2179–2186.
20. Lieb T, Forteza R, Salathe M. Hyaluronic acid in cultured ovine tracheal cells and its effect on ciliary beat frequency in vitro. *J Aerosol Med* 2000;13:231–237.

21. Manzanares D, Monzon ME, Savani RC, Salathe M. Apical oxidative hyaluronan degradation stimulates airway ciliary beating via RHAMM and RON. *Am J Respir Cell Mol Biol* 2007;37:160–168.
22. Agren UM, Tammi RH, Tammi MI. Reactive oxygen species contribute to epidermal hyaluronan catabolism in human skin organ culture. *Free Radic Biol Med* 1997;23:996–1001.
23. Yamazaki K, Fukuda K, Matsukawa M, Hara F, Matsushita T, Yamamoto N, Yoshida K, Munakata H, Hamanishi C. Cyclic tensile stretch loaded on bovine chondrocytes causes depolymerization of hyaluronan: involvement of reactive oxygen species. *Arthritis Rheum* 2003; 48:3151–3158.
24. Casalino-Matsuda SM, Monzon ME, Conner GE, Salathe M, Forteza RM. Role of hyaluronan and reactive oxygen species in tissue kallikrein-mediated epidermal growth factor receptor activation in human airways. *J Biol Chem* 2004;279:21606–21616.
25. Casalino-Matsuda SM, Monzon ME, Forteza RM. Epidermal growth factor receptor activation by epidermal growth factor mediates oxidant-induced goblet cell metaplasia in human airway epithelium. *Am J Respir Cell Mol Biol* 2006;34:581–591.
26. Stern R. Devising a pathway for hyaluronan catabolism: are we there yet? *Glycobiology* 2003;13:105R–115R.
27. Lokeshwar VB, Cerwinka WH, Isoyama T, Lokeshwar BL. HYAL1 hyaluronidase in prostate cancer: a tumor promoter and suppressor. *Cancer Res* 2005;65:7782–7789.
28. Beech DJ, Madan AK, Deng N. Expression of PH-20 in normal and neoplastic breast tissue. *J Surg Res* 2002;103:203–207.
29. Godin DA, Fitzpatrick PC, Scandurro AB, Belafsky PC, Woodworth BA, Amedee RG, Beech DJ, Beckman BS. PH-20: a novel tumor marker for laryngeal cancer *Arch Otolaryngol Head Neck Surg* 2000; 126:402–404.
30. Pasonen-Seppanen S, Karvinen S, Torronen K, Hyttinen JM, Jokela T, Lammi MJ, Tammi MI, Tammi R. EGF upregulates, whereas TGF-beta downregulates, the hyaluronan synthases Has2 and Has3 in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. *J Invest Dermatol* 2003;120:1038–1044.
31. Flannery CR, Little CB, Hughes CE, Caterson B. Expression and activity of articular cartilage hyaluronidases. *Biochem Biophys Res Commun* 1998;251:824–829.
32. Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC Jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992;146:109–115.
33. Churg A, Dai J, Tai H, Xie C, Wright JL. Tumor necrosis factor-alpha is central to acute cigarette smoke-induced inflammation and connective tissue breakdown. *Am J Respir Crit Care Med* 2002;166:849–854.
34. Soderberg M, Bjermer L, Hallgren R, Lundgren R. Increased hyaluronan (hyaluronic acid) levels in bronchoalveolar lavage fluid after histamine inhalation. *Int Arch Allergy Appl Immunol* 1989;88:373–376.
35. Forteza R, Conner GE, Salathe M. Hyaluronan in the airways. In: Garg HG, Hales CA, editors. Chemistry and biology of hyaluronan. Elsevier Ltd.; 2004. p. 323–337.
36. Nlend MC, Bookman RJ, Conner GE, Salathe M. Regulator of G-protein signaling protein 2 modulates purinergic calcium and ciliary beat frequency responses in airway epithelia. *Am J Respir Cell Mol Biol* 2002;27:436–445.
37. Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* 2005;107:183–206.
38. Bernacki SH, Nelson AL, Abdullah L, Sheehan JK, Harris A, Davis CW, Randell SH. Mucin gene expression during differentiation of human airway epithelia *in vitro*: muc4 and muc5b are strongly induced. *Am J Respir Cell Mol Biol* 1999;20:595–604.
39. Guntenhoner MW, Pogrel MA, Stern R. A substrate-gel assay for hyaluronidase activity. *Matrix* 1992;12:388–396.
40. Lokeshwar V, Rubinowicz D. Hyaluronic acid and hyaluronidase: molecular markers associated with prostate cancer biology and detection. *Prostate Cancer Prostatic Dis* 1999;2:S21.
41. Naureckas ET, Ndukwu IM, Halayko AJ, Maxwell C, Hershenson MB, Solway J. Bronchoalveolar lavage fluid from asthmatic subjects is mitogenic for human airway smooth muscle. *Am J Respir Crit Care Med* 1999;160:2062–2066.
42. Lepperdinger G, Strobl B, Kreil G. HYAL2, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. *J Biol Chem* 1998;273:22466–22470.
43. Chow G, Knudson CB, Knudson W. Expression and cellular localization of human hyaluronidase-2 in articular chondrocytes and cultured cell lines. *Osteoarthritis Cartilage* 2006;14:849–858.
44. Danilkovitch-Miagkova A, Duh FM, Kuzmin I, Angeloni D, Liu SL, Miller AD, Lerman MI. Hyaluronidase 2 negatively regulates RON receptor tyrosine kinase and mediates transformation of epithelial cells by jaagsiekte sheep retrovirus. *Proc Natl Acad Sci USA* 2003;100: 4580–4585.
45. Ohno S, Ijuin C, Doi T, Yoneno K, Tanne K. Expression and activity of hyaluronidase in human periodontal ligament fibroblasts. *J Periodontol* 2002;73:1331–1337.
46. Townley RG, Horiba M. Airway hyperresponsiveness: a story of mice and men and cytokines. *Clin Rev Allergy Immunol* 2003;24:85–110.
47. Ndukwu IM, Naureckas ET, Maxwell C, Waldman M, Leff AR. Relationship of cellular transmigration and airway response after allergen challenge. *Am J Respir Crit Care Med* 1999;160:1516–1524.
48. Frost GI, Stern R. A microtiter-based assay for hyaluronidase activity not requiring specialized reagents. *Anal Biochem* 1997;251:263–269.
49. Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, Nadji M, Lokeshwar BL. Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. *J Biol Chem* 2001;276:11922–11932.
50. Lepperdinger G, Mullegger J, Kreil G. Hyal2–less active, but more versatile? *Matrix Biol* 2001;20:509–514.
51. Vigdorovich V, Miller AD, Strong RK. Ability of hyaluronidase 2 to degrade extracellular hyaluronan is not required for its function as a receptor for jaagsiekte sheep retrovirus. *J Virol* 2007;81:3124–3129.
52. Harada H, Takahashi M. CD44-dependent intracellular and extracellular catabolism of hyaluronic acid by hyaluronidase-1 and -2. *J Biol Chem* 2007;282:5597–5607.
53. Rai SK, Duh FM, Vigdorovich V, Danilkovitch-Miagkova A, Lerman MI, Miller AD. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. *Proc Natl Acad Sci USA* 2001;98:4443–4448.
54. Tammi R, Rilla K, Pienimaki JP, MacCallum DK, Hogg M, Luukkonen M, Hascall VC, Tammi M. Hyaluronan enters keratinocytes by a novel endocytic route for catabolism. *J Biol Chem* 2001;276: 35111–35122.
55. Lackie PM, Baker JE, Gunthert U, Holgate ST. Expression of CD44 isoforms is increased in the airway epithelium of asthmatic subjects. *Am J Respir Cell Mol Biol* 1997;16:14–22.
56. Chao KL, Muthukumar L, Herzberg O. Structure of human hyaluronidase-1, a hyaluronan hydrolyzing enzyme involved in tumor growth and angiogenesis. *Biochemistry* 2007;46:6911–6920.
57. Isomaki P, Punnonen J. Pro- and anti-inflammatory cytokines in rheumatoid arthritis. *Ann Med* 1997;29:499–507.
58. Aringer M, Smolen JS. Tumour necrosis factor and other proinflammatory cytokines in systemic lupus erythematosus: a rationale for therapeutic intervention. *Lupus* 2004;13:344–347.
59. Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001;34:50s–59s.
60. Kelley MJ, Rose AY, Song K, Chen Y, Bradley JM, Rookhuizen D, Acott TS. Synergism of TNF and IL-1 in the induction of matrix metalloproteinase-3 in trabecular meshwork. *Invest Ophthalmol Vis Sci* 2007;48:2634–2643.
61. Meyer FA, Yaron I, Yaron M. Synergistic, additive, and antagonistic effects of interleukin-1 beta, tumor necrosis factor alpha, and gamma-interferon on prostaglandin E, hyaluronic acid, and collagenase production by cultured synovial fibroblasts. *Arthritis Rheum* 1990; 33:1518–1525.
62. Berenbaum F, Jacques C, Thomas G, Corvol MT, Bereziat G, Masliah J. Synergistic effect of interleukin-1 beta and tumor necrosis factor alpha on PGE2 production by articular chondrocytes does not involve PLA2 stimulation. *Exp Cell Res* 1996;222:379–384.
63. Dett CA, Gatanaga M, Ininns EK, Cappuccini F, Yamamoto RS, Granger GA, Gatanaga T. Enhancement of lymphokine-activated T killer cell tumor necrosis factor receptor mRNA transcription, tumor necrosis factor receptor membrane expression, and tumor necrosis factor/lymphotoxin release by IL-1 beta, IL-4, and IL-6 in vitro. *J Immunol* 1991;146:1522–1526.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.