Published in final edited form as:

Inflamm Res. 2014 June ; 63(6): 475–484. doi:10.1007/s00011-014-0719-3.

Hyaluronan deposition and co-localization with inflammatory cells and collagen in a murine model of fungal allergic asthma

Sumit Ghosh^{§,*}, Amali E. Samarasinghe[#], Scott A. Hoselton[§], Glenn P. Dorsam[§], and Jane M. Schuh[§]

[§]Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58105, USA

[#]University of Tennessee Health Sciences Center/Le Bonheur Children's Hospital, Memphis, TN 38163, USA

Abstract

Objective—Allergic asthma is a chronic inflammatory disease of the airways characterized by excessive inflammation and remodeling of the extracellular matrix (ECM) and associated cells of the airway wall. Under inflammatory conditions, hyaluronan (HA), a major component of the ECM, undergoes dynamic changes, which may in turn affect the recruitment and activation of inflammatory cells leading to acute and chronic immunopathology of allergic asthma.

Methods—In the present study, we measured the changes in HA levels generated at sites of inflammation and examined its effect on inflammatory responses and collagen deposition in an *Aspergillus fumigatus* murine inhalational model of allergic asthma.

Results—We found that HA levels are elevated in allergic animals and that the increase correlated with the influx of inflammatory cells 5 days after the second allergen challenge. This increase in HA levels appeared largely due to up regulation of hyaluronidase-1 (HYAL1) and hyaluronidase-2 (HYAL2). Furthermore, HA co-localizes with areas of new collagen synthesis and deposition.

Conclusions—Overall our findings contribute to the growing literature that focuses on the components of ECM as inflammatory mediators rather than mere structural support products. The evidence of HA localization in fungal allergic asthma provides the impetus to study HA more closely with allergic leukocytes in murine models. Further studies examining HA's role in mediating cellular responses may help to develop targets for treatment in patients with severe asthma due to fungal sensitization.

*Corresponding author: Sumit Ghosh, Ph.D., Department of Veterinary and Microbiological Sciences, North Dakota State University, P.O. Box 6050, Dept 7690, Fargo, ND 58108-6050, FAX: 701-231-9692, Office: 701-231-7696, Sumit.Ghosh@ndsu.edu.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of North Dakota State University.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

S Ghosh, AE Samarasinghe, SA Hoselton, GP Dorsam, and JM Schuh participated in the design of the study, data analyses, and manuscript preparation. S Ghosh conducted the assays and analyses. All authors read and approved the final manuscript.

Keywords

Hyaluronan; inflammation; *Aspergillus fumigatus*; extracellular matrix

INTRODUCTION

Asthma is a debilitating disease of the airways that affects over 235 million people globally [1]. Asthmatic airways sensitized to a specific allergen respond violently to subsequent exposures, resulting in asthma attacks, which can be fatal. In the context of allergic asthma, sensitization to fungi, with the production of IgE and/or colonization by fungal species, presents a severe clinical scenario that is difficult to treat and accounts for increased morbidity, use of health care support, and health care costs [2].

Characteristic pathological changes associated with fungal asthma include an accumulation of inflammatory cells (eosinophils, neutrophils, lymphocytes, macrophages, mast cells, and basophils) and airway remodeling, which involves an abnormal accumulation of ECM [3–7]. As a consequence of repeated and persistent inflammation and ECM turnover, lung morphology eventually becomes altered, causing chronic dysfunction of the lung [8]. Many of the acute and chronic features of clinical fungal asthma are recapitulated in the experimental inhalation model of allergic asthma with *Aspergillus* sensitization that we have developed in our laboratory [4, 7, 9–12]. In particular, we observe increased eosinophilia, IgE production, and a robust ECM turn over, which is exaggerated upon subsequent challenges with conidia [9, 11]. However, the relationship between ECM accumulation and inflammatory cell recruitment has not been investigated in fungal allergic asthma.

In the lung, the ECM was once considered to be inert scaffolding with a mechanical role in supporting and maintaining tissue structure. However, recent findings indicate that the role of ECM components is much broader than previously thought [13, 14]. We now know that the ECM has roles in cell attachment, movement, activation, tissue growth and repair, proliferation, and differentiation, and thereby can play a role in mediating inflammation [13–17]. Clearly, it functions in processes of both health and disease [18, 19], and understanding the contribution of ECM components to asthma pathogenesis may lead to new therapeutics for patients with asthma.

HA is a major component of the ECM. It is a non-sulfated glycosaminoglycan polymer consisting of repeating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine that is synthesized by a variety of cell types, including stromal cells [20], fibroblasts [21], epithelial cells [22], and smooth muscle cells [23]. In the healthy lung, HA exists as a high molecular mass hyaluronan (HMM HA) polymer in excess of 10^6 D found in the peribronchial and perialveolar spaces where it assists in structural integrity and cushioning, as well as cell movement [13, 15]. During inflammation, however, HA undergoes dynamic regulation [19, 24] and can be broken down into low molecular mass hyaluronan (LMM HA) fragments by the activity of hyaluronidases [13, 25]. Several studies have shown that LMM HA exhibits pronounced biological effects on cells and in tissues [13, 18]. Most importantly, LMM HA has multiple pro-inflammatory effects not observed for HMM HA

[15, 26]. In fact, HMM HA can block the pro-inflammatory effects of LMM HA and help support tissue integrity [13, 26].

The accumulation of HA has been noted in tissue injury following a variety of insults, such as those that occur in Acute Respiratory Distress Syndrome [13], Idiopathic Pulmonary Fibrosis [13], Chronic Obstructive Pulmonary Disease [13, 27], and chronic persistent asthma [13, 14, 17, 28], suggesting a role of HA in pulmonary disease mediation. Although HA has been linked to asthma, little is known about HA in fungal allergy and asthma with regards to HA breakdown, deposition, interaction with inflammatory cells, or interaction with other matrix components and receptors.

The trafficking of inflammatory cells is dependent on integrins, selectins, and cytokine/chemokine gradients. However, hyaluronan-based ECM defines the location of the problem and subsequently promotes adhesion and likely subsequent activation of inflammatory cells. In the current study, we examined the spatial co-localization of HA in areas of the allergic lung which undergo airway inflammation and subepithelial fibrosis after conidia inhalation, hypothesizing that HA would localize to the peribronchial space in order to influence inflammatory cell recruitment and collagen deposition. We found that HA levels were increased and co-localized to areas of inflammation around the airways and blood vessels where collagen deposition was also observed after fungal challenge. Increased HA levels appeared largely due to up-regulation of HYAL1 and HYAL2, which play a role in breakdown of HMM HA to its pro-inflammatory form (LMM HA). Future studies will determine the extent to which structural cells or other immune cells are directly impacted by the dramatic changes in HA over the course of the fungal allergy model.

MATERIALS AND METHODS

Experimental animals

Immunocompetent, 5 to 9 week old C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed on Alpha-dri™ paper bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter-topped cages (Ancare, Bellmore, NY, USA) in a specific pathogen-free facility with *ad libitum* access to food and water. The study described was performed in accordance with the Office of Laboratory Animal Welfare guidelines and was approved by the North Dakota State University Institutional Animal Care and Use Committee.

Model of allergic airways disease

Animals were sensitized as per published protocol [9, 12]. Briefly, mice were sensitized with 10 µg of *A. fumigatus* antigen (Greer Laboratories, Lenoir, NC, USA) in 0.1 ml normal saline (NS) mixed with 0.1 ml of Imject Alum (Pierce, Rockford, IL, USA), which was injected subcutaneously (0.1 ml) and intraperitoneally (0.1 ml). After two weeks, mice were given a series of three weekly, intranasal 20-µg doses of *A. fumigatus* antigen in 20 µl of NS. Animals were challenged with a 10-min nose-only aerosol exposure to live *A. fumigatus* conidia [9]. Each anesthetized mouse was placed supine with its nose in an inoculation port inhaling the live fungal conidia for 10 min. Two weeks after the first allergen challenge,

mice were subjected to a second 10-min aerosol fungal challenge as previously described [9, 12]. Naïve controls were age-matched mice that were neither sensitized nor challenged. After the second allergen exposure, the mice were separated into groups of five for analysis at days 5 and 28 after the second aerosol challenge. Day 5 after the second conidia challenge had been previously demonstrated by us to be the peak of lymphocyte recruitment into the allergic lungs [4], and leukocyte inflammation and HA levels were assessed at this time point. Airway wall remodeling marked by excessive collagen deposition can be seen as early as 5 days after the second aerosol challenge in this model, and the changes to the lung architecture continue to accrue through at least day 28 after the second inhalation of fungal conidia. The day-5 and day-28 time points were chosen to assess peribronchial fibrosis. The experimental protocol is depicted in Figure 1.

AHR measurement

Mice were anesthetized using sodium pentobarbital (Butler, Columbus, OH; 100 mg/kg of mouse body weight), intubated, and ventilated with a Harvard pump ventilator (Harvard Apparatus, Reno, NV, USA) to assess allergic airway responses. Restrained plethysmography (Buxco, Troy, NY, USA) was used to assess AHR. Before performing readings, the system was calibrated and the stroke volume set at 225 with the strokes per minute set at 150. The value for baseline airway resistance was measured for each animal before an i.v. injection of acetyl- β -methacholine (420 μ g/kg) was administered to determine AHR at each time point.

Sample collection

Approximately 500 μ l of blood was collected from each mouse via ocular bleed and centrifuged at $13,000 \times g$ for 10 min to yield serum which was stored at -20°C until use. Bronchoalveolar lavage (BAL) was performed on five mice per group with 1.0 ml sterile saline. The BAL contents from each animal was centrifuged at $2000 \times g$ for 5 min to separate cells from fluid. The BAL fluid was stored at -20°C until use. Cells were resuspended in phosphate buffered saline (PBS) and macrophages, neutrophils, eosinophils, and lymphocytes were counted from 200 μ l of the cell suspension cytopspin (Shandon Scientific, Runcorn, UK) onto glass microscope slides after staining with Quik-Dip differential stain (Mercedes Medical; Sarasota, FL, USA). The mean number of each cell type was determined by morphometric analysis. Results were reported as mean of each cell type per high-powered field (hpf, $1000\times$) and total cells per hpf.

Quantification of IgE in serum and BAL

The total IgE (BD OptEIA, San Diego, CA, USA) in serum and BAL fluid were quantified via specific ELISA according to manufacturer's guidelines. For IgE serum samples were diluted in PBS 1:100 and BAL samples were diluted 1:5. The detection limit of the kit was 1.6 ng/ml.

Quantification of HA in serum and BAL fluid

The total HA levels in undiluted serum and BAL fluid were quantified via specific competitive ELISA according to the manufacturer's guidelines (Echelon Biosciences, Salt Lake City, Utah, USA). The detection limit of the kit was 50 ng/ml.

Real-Time Quantitative PCR (qRT-PCR) Technique

Total RNA was extracted from lung tissues of naïve and allergic animals using a standardized TRIzol method of phenol extraction (Invitrogen, Carlsbad, CA, USA). Total RNA was measured by a NanoDrop (Wilmington, DE, USA). The A260/280 ratios were between 1.90 and 2.1 for all samples. Total RNA samples were pooled together for each time point for real-time quantitative PCR (qRT-PCR) analysis. A total of 1 µg of RNA was subjected to DNase I (Promega, Madison, WI, USA) treatment and was used to generate cDNA using reverse transcriptase (Promega, Madison, WI, USA) and random primers (Promega, Madison, WI, USA) as described by the manufacturer. Real time reactions contained 5 µl of cDNA template (1/30 dilution) with 12.5 µl of a 2X QuantiTect SYBR Green master mix ([Cat # 204143], Qiagen, Valencia, CA, USA), 2.5 µl of 10X primers (Qiagen, Valencia, CA, USA) and 5 µl nuclease-free H₂O (Qiagen, Valencia, CA, USA). The final volume of the reactions was 25 µl. Transcripts were studied by qRT-PCR in duplicates. HYAL1 and HYAL-2 mRNA levels were determined by qRT-PCR. Transcript-specific primers HYAL1 (Cat # QT00171094) and HYAL2 (Cat # QT00112105) were purchased from Qiagen (Valencia, CA, USA). Mouse Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal control and relative transcript abundance was normalized to the amount of HPRT for the qRT-PCR data. All samples were analyzed for dissociation curves and melting temperatures to confirm single amplification species. Mean fold changes were calculated by averaging the duplicate measurement for each gene. The qRT-PCR reaction was conducted using a 7500 ABI instrument with the following parameters: 2 min at 48°C, 10 min at 94°C to denature the cDNA and activate the Taq polymerase, followed by 40 cycles of 15 s at 94°C and 60 s at 60°C. Reactions for all amplicons were conducted with nuclease-free water alone, and in the absence of reverse transcriptase to ensure 6 cycle thresholds compared to reactions in the presence of reverse transcriptase. This would verify 1.6% genomic DNA contamination in reactions as described by the manufacturer. The relative fold difference calculation uses the 2-Delta Delta CT method.

Tissue harvest and staining

Left lobes of lungs were inflated *ex vivo* and fixed in 10% neutral buffered formalin and paraffin-embedded. Hematoxylin and Eosin (Thermo Scientific, Rockford, IL, USA) and Gomori's trichrome (Richard-Allan Scientific, Kalamazoo, MI, USA) stains were performed on 5-µm sections cut across the coronal planes of the lungs to determine inflammation and subepithelial collagen deposition, respectively.

Immunostaining for HA was performed as previously published [29]. Briefly, sections were incubated overnight at 4°C in 3 µg/ml of HA-binding protein (Seikagaku Corporation, Japan) diluted in PBS with 1% bovine serum albumin (Sigma-Aldrich Corp, St. Louis, MO, USA). Washed sections were then incubated for 1 h in avidin-conjugated horseradish

peroxidase (Vector Labs, Irvine, CA, USA) and developed in 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) for 10 min and counterstained in hematoxylin. Serial sections that were not incubated in HA-binding protein, which, therefore, did not yield brown precipitate served as negative controls.

Statistical analysis

Allergic C57BL/6 wild type animals were compared to their respective naïve controls at each time point. Results were expressed as the mean $\pm$ standard error of the mean (SEM). Data were evaluated with GraphPad Prism software (San Diego, CA, USA) using an unpaired, student's two tailed *t* test with Welch's correction to determine statistical significance. A *p* value of < 0.05 was considered statistically significant and noted by an asterisk.

RESULTS

Inhalational fungal challenge with *A. fumigatus* results in airway hyperresponsiveness and increased IgE production in allergen challenged mice

To measure disease severity after fungal challenge, the airway physiology, IgE Ab levels, and pulmonary inflammation of C57BL/6 mice were monitored before allergen challenge in naïve animals and at days 5 and 28 after the second conidia inhalation. At day 5 after two conidia challenges, airway hyperresponsiveness was significantly increased in C57BL/6 mice as compared to naïve controls (Fig. 2A). By day 28, after the second conidia challenge, AHR values returned to the baseline level (Fig. 2A). Furthermore, IgE, which is the hallmark of allergic asthma, was elevated in serum and BAL fluid at days 5 and 28 after the second conidia challenge (Fig. 2B, 2C).

Inhalational fungal challenge with *A. fumigatus* results in increased pulmonary inflammation in allergen challenged mice

Morphometric identification of monocytes/macrophages, neutrophils, eosinophils, and lymphocytes was performed to estimate the relative makeup of the cellular inflammation and to monitor leukocyte egress into the airway lumen (Fig. 3A–D). Lymphocytes (Fig. 3D), macrophages (Fig. 3A), and particularly eosinophils (Fig. 3C), were the prominent cell types identified in the BAL 5 days after the second conidia challenge with very few neutrophils (Fig. 3B), emphasizing that multiple inhalations of conidia polarize the immune response in favor of allergy, and that lymphocytes and eosinophils form a major percentage of cells in the allergic lung. The total cell counts from the BAL at day 5 further highlight that eosinophils form the major percentage of cells (~73%) in the BAL compartment, indicating an allergy phenotype (Fig. 3E). By day 28, after the second conidia challenge, macrophages and lymphocytes were the only major cellular component of the BAL compartment with very few eosinophils (Fig. 3A–D).

HA levels are elevated in the serum and BAL fluid of allergen challenged mice

Given the recognized role of inflammatory cells and HA in disease processes including COPD and asthma pathogenesis, we investigated the influx of inflammatory cells with HA levels in a model of fungus-induced pulmonary inflammation. HA associated with asthma

and COPD has a lower molecular mass because, under diseased conditions, HMM HA is broken down into LMM HA [15, 18, 14, 13], which can be detected as increased concentrations of HA in the BAL fluid [30, 31, 13, 32]. In the present study, inhalation of *A. fumigatus* conidia resulted in an increase in the concentration of HA in both serum and BAL fluid of C57BL/6 mice at day 5 after the second conidia challenge (Fig. 4A, 4B). This coincided with increased inflammatory cell recruitment in the murine fungal allergy model system. By day 28 after the second aerosol challenge, HA levels returned to basal levels in both serum and BAL fluid (Fig. 4A, 4B). This time point also coincides with the resolution of inflammation.

HYAL1 and HYAL2 gene expression levels are elevated in the lungs of allergen challenged mice

Our next step was to determine the role of HYAL1 and HYAL2 (genes that encode for hyaluronidases) in increasing HA deposition in allergic animals as they play an important role in hydrolyzing HA of high molecular mass to yield intermediate-sized HA fragments. Given the increase in HA content in the BAL fluid of allergic animals, we determined mRNA changes of HYAL1 and HYAL2 by qRT-PCR in the lung of allergen challenged mice. We found that HYAL1 mRNA was approximately 5 fold higher 5 days after the second conidia challenge when compared to naïve controls (Fig. 5A). Similarly, HYAL2 mRNA was approximately 6 fold higher 5 days after the second conidia challenge (Fig. 5B). By day 28 after the second aerosol challenge, HYAL1 (Fig. 5A) and HYAL2 (Fig. 5B) mRNA levels were not significantly different from naïve levels. This time point also coincides with low HA levels in the BAL fluid of allergic animals (Fig. 4) and the resolution of inflammation (Fig. 3). We also determined mRNA changes of HAS1 (Hyaluronan synthase 1) and HAS2 (Hyaluronan synthase 2) by qRT-PCR in the lung of allergen challenged mice. We found no difference in HAS1 and HAS2 expression at day 5 after the second conidia challenge when compared to naïve controls (data not shown). Taken together, these results suggest that the induction of HYAL1 and HYAL2 may contribute in full or in part to the early phase HA breakdown in the lung and may be responsible for increased HA accumulation around the airways in this fungal model of allergic asthma.

Increased HA deposition in the lung co-localizes with pulmonary inflammation and increased collagen in allergen-challenged mice

We next evaluated whether HA deposition correlates with pulmonary inflammation and collagen deposition. Allergen sensitization and challenge resulted in an influx of inflammatory cells and an increase in HA deposition in the lung tissue (Fig. 6). Naïve lungs showed no signs of inflammation (Fig. 6A&J) and minimal HA staining around the blood vessels, airways (Fig. 6D), and the basement membrane (Fig. 6M). HA and inflammatory cell recruitment increased after allergen challenge and was localized to the perivascular and peribronchial areas. Five days after the second conidia inhalation, intense HA deposition was observed around the distal airways and blood vessels of conidia-challenged allergic lungs where the inflammatory cells were present (Fig. 6E). The basement membrane (Fig. 6K) and surrounding inflammatory cells (Fig. 6N) also showed intense HA staining at day 5. By day 28, intense HA staining was still clearly evident surrounding the blood vessels that lay adjacent to the airways (Fig. 6F) and the basement membrane (Fig. 6O).

Airway remodeling characterized by increased collagen deposition around the airways is a key feature of allergic asthma. Therefore, we stained lung sections from naïve and allergic animals for collagen. A small amount of collagen was observed in naïve lungs (Fig. 6G, 6P). There was an increase in the amount of collagen deposition between the cells in the zone of inflammation around the blood vessels and bronchioles at day 5 after the second conidia challenge, which presented a similar pattern as that of HA deposition (Fig. 6E, 6H, 6N, 6Q). A similar phenomenon was observed at day 28 after the second conidia challenge (Fig. 6F, 6I, 6O, 6R).

DISCUSSION

In the normal lung, the inhalation of fungus is met by resident alveolar macrophages and an influx of recruited monocytes and neutrophils, which phagocytose inhaled conidia and prevent its growth. The ECM has an important role in the lung, both in normal homeostasis and disease. HA, a component of the ECM, has been implicated in regulating host responses to lung injury [24, 28, 33]. While accumulation of HA during pathological states has been reported, the dynamic regulation of HA after conidia inhalation in the allergic lung has not been investigated. In the current fungal model of allergic asthma, we report increased HA concentrations in the serum and BAL fluid of allergic animals, indicating a diseased state. There was a significant correlation between the severity of asthma as measured by airway hyperresponsiveness, IgE levels, and inflammation and the HA levels in BAL fluid, serum, and lung tissue sections indicating significant pulmonary damage and dynamic regulation of HA in response to fungal challenge.

Asthma is an inflammatory disease of the lungs that is characterized by increased inflammatory cell infiltration into the airways and poor respiratory function mediated by excessive airway remodeling. This fungal asthma model in the present study, which mimics both the acute and chronic features associated with asthma, was utilized to better understand HA deposition in relation to pulmonary inflammatory cell recruitment and collagen deposition in the allergic lung. We show that HA was increased and co-localized to areas of inflammation around the airways and blood vessels where collagen deposition was also noted in the allergic lung after the inhalation of fungal spores. Eosinophil's were a major component of the pulmonary inflammation associated with allergic asthma when the HA levels are elevated in the allergic animals. Furthermore, increased mRNA levels of both HYAL1 and HYAL2 in allergic animals indicate that these hyaluronidases may be responsible for the rapid increase in HA in our fungal model system. These observations suggest that HA may play a particularly prominent role in supporting immunological changes associated with the inhalation of fungus in the sensitized lungs. This is in agreement with previous reports, which show an increase in HA accumulation under pathological conditions. [13–15, 17]. However, future studies will be needed to determine if immune cells recruited to the allergic lung are impacted by the dramatic changes in HA over the course of the model.

HA exists as a HMM HA polymer usually in excess of 10^6 D in the native form found in the lung [13, 15, 34]. Under conditions of inflammation, HMM HA is broken down into LMM HA, which exhibits multiple pro-inflammatory effects on cells and tissues [13, 18, 26].

HMM HA is broken down into LMM HA by the combined actions of hyaluronidases and reactive oxygen species (ROS) that are produced by inflammatory cells [35]. LMM HA has inflammatory properties and regulates local effects on cell activation at sites of chronic inflammation [16, 32, 36, 37]. Elevated HYAL1 and HYAL2 expression at day 5 after the second conidia challenge suggest that these hyaluronidases may be responsible for the rapid hydrolysis of HMM HA to LMM HA in our fungal model system. The source of these hyaluronidases is still unclear, although the inflammatory cells present in the lungs are a likely reservoir for these hyaluronidases.

Eosinophils are major producers of ROS in the pulmonary compartment, suggesting a source and role for ROS in mediating HMM HA breakdown and maintaining an inflammatory milieu. Recent studies have shown that the production of ROS by eosinophils can be triggered by the binding of antigen-antibody conjugates on the surface of eosinophils [38]. The inhalation model of fungal asthma reiterates the eosinophilia associated with the clinical allergic asthma which precedes lymphocyte recruitment [7, 9, 10, 12, 17]. Generation of ROS by these eosinophils at sites of inflammation may have a role in the breakdown of HMM HA to LMM HA to generate an inflammatory reaction.

HA has also been demonstrated to promote the survival of eosinophils [37, 39] thus its distribution around the allergic airways may promote eosinophilia. Inflammatory cells express HA-binding proteins: CD44, receptor for hyaluronan mediated motility, lymphatic vessel endothelial hyaluronan receptor-1, and toll-like receptors. The asthmatic phenotype may be promoted by HA bound receptors on eosinophils. Based on these observations, one may hypothesize that the decrease in HA at day 28 in the BAL fluid may function to reduce the number of eosinophils as noted in our model, thereby protecting the tissue from the damaging effects of accumulated activated eosinophils. As the results presented in this study do not mechanistically mimic the effect of eosinophils or other leukocytes on HA breakdown and cellular recruitment, further studies will be needed to address this issue.

Increased collagen synthesis is a key feature of human asthma and is also noted in murine models of antigen-induced pulmonary inflammation. The hallmark of chronic asthma, as characterized by robust collagen deposition was produced in allergic animals after conidia inhalation. A parallel increase in HA and collagen deposition in the allergic lungs suggests that the increase in HA levels is closely linked to collagen deposition. The co-localization of HA in areas of collagen deposition further highlights HA's role in regulating the matrix turnover. Future research will determine if HA appears to provide the scaffolding for new collagen synthesis and deposition.

CONCLUSIONS

In summary, our study contributes to the growing literature that focuses on the components of ECM as inflammatory mediators rather than mere structural support products. The evidence of HA localization in fungal allergic asthma provides the impetus to study HA more closely with allergic leukocytes in murine models. Future studies will focus on the various molecular mass products of HA and the different CD44 isoforms present on leukocytes and their role in regulating the allergic phenotype in response to fungal antigens.

This will include selective interference with HA production or interaction with receptors, which may present a therapeutic target that can be exploited to minimize long-term damage associated with excessive leukocyte recruitment/collagen deposition. The *A. fumigatus* inhalation model, which produces a massive accumulation of HA, may further help to elucidate the role of HA in patients with severe asthma with fungal sensitization and other pulmonary diseases.

Acknowledgments

This work was funded by NIH grants (1R15HL117254-01 to JMS and 1R15AI101968-01A1 to GPD). S.G. was supported through a predoctoral fellowship through North Dakota Experimental Program to Stimulate Competitive Research (NSF, EPS-0814442).

The authors wish to thank Jennifer Carlson and James McCarthy from Department of Laboratory Medicine and Pathology, University of Minnesota for HA staining. The authors also wish to thank Dr. Pawel Borowicz, co-Director of the Advanced Imaging and Microscopy Core Laboratory at NDSU, for his help with imaging using the Zeiss Z1 AxioObserver inverted microscope. The Advanced Imaging and Microscopy Core Laboratory at NDSU was supported through NSF (MRI-0959512 to A. Grazul-Bilska and JMS). And the Core Biology Facility at NDSU was supported by the NIH (2P20RR015566 and P30GM103332-01 to M Sibi).

ABBREVIATIONS

ECM	Extracellular Matrix
HA	Hyaluronan
LMM HA	low molecular mass HA
HMM HA	high molecular mass HA
IL	Interleukin
BAL	Bronchoalveolar lavage
ROS	Reactive oxygen species

References

1. Manion AB. Asthma and obesity: the dose effect. *Nurs Clin North Am.* 2013; 48(1):151–8. [PubMed: 23465448]
2. Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol.* 2012; 129(2):280–91. quiz 92–3. [PubMed: 22284927]
3. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med.* 2012; 18(5):693–704. [PubMed: 22561833]
4. Ghosh S, Hoselton SA, Schuh JM. Characterization of CD19(+)CD23(+)B2 lymphocytes in the allergic airways of BALB/c mice in response to the inhalation of *Aspergillus fumigatus* conidia. *Open Immunol J.* 2012; 5:46–54. [PubMed: 23316260]
5. Schuh JM, Blease K, Hogaboam CM. The role of CC chemokine receptor 5 (CCR5) and RANTES/CCL5 during chronic fungal asthma in mice. *FASEB J.* 2002; 16(2):228–30. [PubMed: 11744622]
6. Sharma M, Hegde P, Aimaganianda V, Beau R, Senechal H, Poncet P, et al. Circulating human basophils lack the features of professional antigen presenting cells. *Sci Rep.* 2013; 3:1188. [PubMed: 23378919]
7. Ghosh S, Hoselton SA, Dorsam GP, Schuh JM. Eosinophils in fungus-associated allergic pulmonary disease. *Front Pharmacol.* 2013; 4:8. [PubMed: 23378838]

8. Dagenais TR, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin Microbiol Rev.* 2009; 22(3):447–65. [PubMed: 19597008]
9. Hoselton SA, Samarasinghe AE, Seydel JM, Schuh JM. An inhalation model of airway allergic response to inhalation of environmental *Aspergillus fumigatus* conidia in sensitized BALB/c mice. *Med Mycol.* 2010; 48(8):1056–65. [PubMed: 20482452]
10. Ghosh S, Hoselton SA, Schuh JM. mu-chain-deficient mice possess B-1 cells and produce IgG and IgE, but not IgA, following systemic sensitization and inhalational challenge in a fungal asthma model. *J Immunol.* 2012; 189(3):1322–9. [PubMed: 22732592]
11. Samarasinghe AE, Hoselton SA, Schuh JM. A comparison between intratracheal and inhalation delivery of *Aspergillus fumigatus* conidia in the development of fungal allergic asthma in C57BL/6 mice. *Fungal Biol.* 2011; 115(1):21–9. [PubMed: 21215951]
12. Samarasinghe AE, Hoselton SA, Schuh JM. Spatio-temporal localization of vasoactive intestinal peptide and neutral endopeptidase in allergic murine lungs. *Regul Pept.* 2010; 164(2–3):151–7. [PubMed: 20566347]
13. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiol Rev.* 2011; 91(1):221–64. [PubMed: 21248167]
14. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol.* 2007; 23:435–61. [PubMed: 17506690]
15. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol.* 2002; 21(1):25–9. [PubMed: 11827789]
16. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, et al. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest.* 1996; 98(10):2403–13. [PubMed: 8941660]
17. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Correlation of hyaluronan deposition with infiltration of eosinophils and lymphocytes in a cockroach-induced murine model of asthma. *Glycobiology.* 2013; 23(1):43–58. [PubMed: 22917573]
18. Noble PW, Jiang D. Matrix regulation of lung injury, inflammation, and repair: the role of innate immunity. *Proc Am Thorac Soc.* 2006; 3(5):401–4. [PubMed: 16799081]
19. Lennon FE, Singleton PA. Role of hyaluronan and hyaluronan-binding proteins in lung pathobiology. *Am J Physiol Lung Cell Mol Physiol.* 2011; 301(2):L137–47. [PubMed: 21571904]
20. Yue BY, Baum JL, Silbert JE. Synthesis of glycosaminoglycans by cultures of normal human corneal endothelial and stromal cells. *Invest Ophthalmol Vis Sci.* 1978; 17(6):523–7. [PubMed: 659073]
21. Kitchen JR, Cysyk RL. Synthesis and release of hyaluronic acid by Swiss 3T3 fibroblasts. *Biochem J.* 1995; 309 (Pt 2):649–56. [PubMed: 7626032]
22. Toshida H, Tabuchi N, Koike D, Koide M, Sugiyama K, Nakayasu K, et al. The effects of vitamin A compounds on hyaluronic acid released from cultured rabbit corneal epithelial cells and keratocytes. *J Nutr Sci Vitaminol (Tokyo).* 2012; 58(4):223–9. [PubMed: 23132305]
23. Deudon E, Berrou E, Breton M, Picard J. Growth-related production of proteoglycans and hyaluronic acid in synchronous arterial smooth muscle cells. *Int J Biochem.* 1992; 24(3):465–70. [PubMed: 1551458]
24. Jiang D, Liang J, Noble PW. Regulation of non-infectious lung injury, inflammation, and repair by the extracellular matrix glycosaminoglycan hyaluronan. *Anat Rec (Hoboken).* 2010; 293(6):982–5. [PubMed: 20186964]
25. Maharjan AS, Pilling D, Gomer RH. High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. *PLoS One.* 2011; 6(10):e26078. [PubMed: 22022512]
26. Yang C, Cao M, Liu H, He Y, Xu J, Du Y, et al. The high and low molecular weight forms of hyaluronan have distinct effects on CD44 clustering. *J Biol Chem.* 2012; 287(51):43094–107. [PubMed: 23118219]
27. Bracke KR, Dentener MA, Papakonstantinou E, Vernooij JH, Demoor T, Pauwels NS, et al. Enhanced deposition of low-molecular-weight hyaluronan in lungs of cigarette smoke-exposed mice. *Am J Respir Cell Mol Biol.* 2010; 42(6):753–61. [PubMed: 19675307]

28. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Hyaluronan deposition and correlation with inflammation in a murine ovalbumin model of asthma. *Matrix Biol.* 2011; 30(2):126–34. [PubMed: 21251977]
29. Simpson MA, Wilson CM, McCarthy JB. Inhibition of prostate tumor cell hyaluronan synthesis impairs subcutaneous growth and vascularization in immunocompromised mice. *Am J Pathol.* 2002; 161(3):849–57. [PubMed: 12213713]
30. Sahu S, Lynn WS. Hyaluronic acid in the pulmonary secretions of patients with asthma. *Biochem J.* 1978; 173(2):565–8. [PubMed: 697736]
31. Sahu SC. Hyaluronic acid. An indicator of pathological conditions of human lungs? *Inflammation.* 1980; 4(1):107–12. [PubMed: 7390611]
32. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. *Veterinari Medicina.* 2008; 8(53):397–411.
33. 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(12):2179–86. [PubMed: 11641244]
34. 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(5 Pt 2):H1449–54. [PubMed: 2035667]
35. Bates EJ, Harper GS, Lowther DA, Preston BN. Effect of oxygen-derived reactive species on cartilage proteoglycan-hyaluronate aggregates. *Biochem Int.* 1984; 8(5):629–37. [PubMed: 6548142]
36. McKee CM, Lowenstein CJ, Horton MR, Wu J, Bao C, Chin BY, et al. Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. *J Biol Chem.* 1997; 272(12):8013–8. [PubMed: 9065473]
37. Ohkawara Y, Tamura G, Iwasaki T, Tanaka A, Kikuchi T, Shirato K. Activation and transforming growth factor-beta production in eosinophils by hyaluronan. *Am J Respir Cell Mol Biol.* 2000; 23(4):444–51. [PubMed: 11017908]
38. Muraki M, Gleich GJ, Kita H. Antigen-specific IgG and IgA, but not IgE, activate the effector functions of eosinophils in the presence of antigen. *Int Arch Allergy Immunol.* 2011; 154(2):119–27. [PubMed: 20733320]
39. Esnault S, Malter JS. Hyaluronic acid or TNF-alpha plus fibronectin triggers granulocyte macrophage-colony-stimulating factor mRNA stabilization in eosinophils yet engages differential intracellular pathways and mRNA binding proteins. *J Immunol.* 2003; 171(12):6780–7. [PubMed: 14662883]

Figure 1. Sensitization, challenge, and analysis schedule for the *A. fumigatus* murine model of allergic asthma

Mice were sensitized to fungal antigens by subcutaneous and intraperitoneal (IP) injection of *A. fumigatus* antigen mixed with alum in PBS. This was followed by three weekly intranasal inoculations of *A. fumigatus* antigen in PBS. A week later, mice were exposed to live *A. fumigatus* conidia. This was the first inhalational exposure with live conidia. Two weeks after the first inhalational exposure mice were subjected to a second and final inhalational exposure with live *A. fumigatus* conidia. After the second inhalational exposure, the mice were separated into groups of five for analysis at days 5 and 28 after the second conidia challenge. Naïve animals were maintained as negative controls.

Figure 2. Effect of inhalation of *A. fumigatus* conidia on AHR, and IgE Ab levels in allergic C57BL/6 mice

Airway hyperresponsiveness (A) was recorded before (baseline indicated by the dotted line, 1.89 ± 0.09 cm H₂O/ml/s) and after i.v. methacholine injection (420 µg/kg). Total IgE was measured in the serum (B) and BAL fluid (C) of naïve and allergic animals. n = 4–5 mice/group, *p < 0.05.

Figure 3. Effect of inhalation of *A. fumigatus* conidia on pulmonary inflammation in allergic C57BL/6 mice

Airway inflammation marked by the presence of macrophages (A), neutrophils (B), eosinophils (C), and lymphocytes (D) in naïve and allergic animals. (E) Represents the mean of the total inflammatory cells in the BAL compartment of naïve or allergic C57BL/6 mice. n = 4–5 mice/group, *p < 0.05.

Figure 4. Inhalation of *A. fumigatus* conidia increases HA in serum and bronchoalveolar lavage (BAL) fluid

ELISAs were carried out to measure HA levels in C57BL/6 mice after fungal challenge. HA was measured by ELISA in the serum (A) and BAL fluid (B) of naïve and allergic animals. n = 4–5 mice/group, *p < 0.05.

Figure 5. qRT-PCR analysis of mRNA expression for HYAL1 and HYAL2 in lung tissues isolated at different timepoints after *A. fumigatus* exposure
(A) The changes in HYAL1 mRNA expression relative to values from the naïve lung are shown. **(B)** The changes in HYAL2 mRNA expression relative to values from naïve lung are shown. Data represent mean values of fold changes over naïve for HYAL1 and HYAL2 respectively. $n = 5$ mice/group and all the lung sections at each time point were pooled together and run as a single sample for qRT-PCR analysis. This result is an outcome of two independent experiments. * $p < 0.05$.

Figure 6. Histopathologic co-localization of HA, inflammatory cells, and collagen in lung sections during acute and chronic stages of *A. fumigatus* exposure

Distribution of HA, inflammatory cells and collagen are shown in lung sections of the 12 week time course of *A. fumigatus* exposure. (A, B, C, J, K, & L) Hematoxylin and eosin (H&E) stained paraffin sections represent the inflammation infiltrate trend around the distal (A–C) and large airways (J–L) for the acute stage (day 5) and chronic stage (day 28) after the second conidia exposure. A section from a control naïve lung (A and J) is also shown. (D, E, F, M, N, & O) HA deposition around the small (D–F) and large airways (M–O) is shown for lungs from naïve (unchallenged) mouse and *A. fumigatus* sensitized and challenge mice. (G, H, I, P, Q, and R) Distributions of collagen (stained with Gomori's trichrome stain, green color) around the distal (G–I) and large airways (P–R) are shown in lung sections representative of the 12 week time course of *A. fumigatus* exposure. A–R, scale bars = 100 μ m.