Role of hyaluronan and hyaluronan-binding proteins in human asthma

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Background: The characteristics of human asthma are chronic inflammation and airway remodeling. Hyaluronan, a major extracellular matrix component, accumulates during inflammatory lung diseases, including asthma. Hyaluronan fragments stimulate macrophages to produce inflammatory cytokines. We hypothesized that hyaluronan and its receptors would play a role in human asthma.

Objective: To investigate the role of hyaluronan and hyaluronan-binding proteins in human asthma.

Methods: Twenty-one subjects with asthma and 25 healthy control subjects underwent bronchoscopy with endobronchial biopsy and bronchoalveolar lavage. Fibroblasts were cultured, and hyaluronan and hyaluronan synthase expression was determined at baseline and after exposure to several mediators relevant to asthma pathobiology. The expression of hyaluronan-binding proteins CD44, TLR (Toll-like receptor)–2, and TLR4 on bronchoalveolar lavage macrophages was determined by flow cytometry. IL-8 production by macrophages in response to hyaluronan fragment stimulation was compared.

Results: Airway fibroblasts from patients with asthma produced significantly increased concentrations of lower-molecular-weight hyaluronan compared with those of normal fibroblasts. Hyaluronan synthase 2 mRNA was markedly increased in asthmatic fibroblasts. Asthmatic macrophages showed a decrease in cell surface CD44 expression and an increase in TLR2 and TLR4 expression. Macrophages from subjects with asthma showed an increase in responsiveness to low-molecular-weight hyaluronan stimulation, as demonstrated by increased IL-8 production.

Conclusion: Hyaluronan homeostasis is deranged in asthma, with increased production by fibroblasts and decreased CD44 expression on alveolar macrophages. Upregulation of TLR2 and TLR4 on macrophages with increased sensitivity to hyaluronan fragments suggests a novel proinflammatory mechanism by which persistence of hyaluronan fragments could contribute to chronic inflammation and airway remodeling in asthma. (J Allergy Clin Immunol 2011;128:403-11.)

Key words: *Asthma, hyaluronan, cytokines, fibroblasts, macrophages*

Chronic inflammation and airway remodeling are important characteristics of human asthma. These include the infiltration of inflammatory cells and an abnormal accumulation of extracellular matrix (ECM) in the subepithelial basement membrane region and submucosa.[1,2] Fibroblasts from patients with hyperresponsive airways have been shown to produce more total proteoglycans than cells from subjects with normoresponsive airways.[3] Fibronectin matrix accumulates in asthma and may contribute to the progression of asthma by altering both the airway remodeling and the functional properties of cells of the airway wall.[4] Therefore, increases in ECM degradation products may be associated with airway fibrosis and decline in lung function.[1,2,5] Understanding the contribution of ECM accumulation to asthma pathogenesis may lead to new therapeutics for patients with asthma.

Hyaluronan is a major component of ECM.[6] It exists as a high-molecular-weight polymer under normal physiological conditions and undergoes dynamic regulation resulting in accumulation of lower-molecular-weight species during tissue injury and inflammation.[7-10] Clearance of hyaluronan degradation products is essential for inflammatory resolution and restoration of tissue integrity. Hyaluronan fragment clearance from sites of inflammation requires the major hyaluronan-binding protein, CD44, to be expressed on hematopoietic cells.[7]

Low-molecular-weight hyaluronan induces the expression of a variety of genes by inflammatory cells *in vitro*, including chemokines, cytokines, and growth factors.[11-13] However, low-molecular-weight hyaluronan signaling can be CD44-independent under some circumstances and use Toll-like receptor (TLR)–4 and TLR2.[14-16] Growing evidence shows that TLR signaling plays an important role in innate immunity in asthma[17,18] and is critical for T_H2 response in murine asthma models.[19-21] In addition, TLR2 gene expression was upregulated in human airway epithelial cells isolated from subjects with asthma and infected with *Mycoplasma pneumoniae*.[22]

Human lung fibroblasts are an important source of hyaluronan production.[23-25] There are 3 hyaluronan synthase (HAS) enzyme isoforms: HAS1, HAS2, and HAS3.[26,27] HAS2 is the major isoform expressed in human lung fibroblasts.[28] Multiple studies have shown that human fibroblasts produce hyaluronan *in vitro* in response to stimulation by cytokines and growth factors

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Supported by NIH grants AI052201, HL06539, P50-HL084917, HL77291 (P.W.N.), and P50-HL084917 (M.K.).

Disclosure of potential conflict of interest: J. Ingram receives research support from the NIH-NHLBI and the Parker B. Francis Family Foundation. M. Kraft receives research support from GlaxoSmithKline, Merck, Genentech, Novartis, and GE Healthcare and is vice president of the American Thoracic Society. P. W. Noble receives research support from the NIH. The rest of the authors have declared that they have no conflict of interest.

Received for publication December 2, 2010; revised March 29, 2011; accepted for publication April 1, 2011.

Available online May 13, 2011.

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0091-6749/$36.00

doi:10.1016/j.jaci.2011.04.006

Abbreviations used
BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
bHABP: Biotinylated hyaluronan-binding protein
ECM: Extracellular matrix
HAS: Hyaluronan synthase
HYAL: Hyaluronidase
NOX: Nicotinamide adenine dinucleotide phosphate oxidase
PMA: Phorbol 12-myristate 13-acetate
ROS: Reactive oxygen species
TLR: Toll-like receptor

including IL-1β, TNF-α, and TGF-β.[25,28-31] Hyaluronan appears in low concentrations in bronchoalveolar lavage (BAL) fluid (BALF) from healthy individuals and is elevated in BALF of patients with asthma.[32-34] The concentration of hyaluronan in BALF was found to correlate significantly with the severity of asthma.[34] However, the role of hyaluronan homeostasis in human asthma has not been thoroughly explored.

We hypothesized that increased hyaluronan accumulation in the lungs of patients with asthma contributes to chronic inflammation and airway remodeling through processes mediated by both fibroblasts and macrophages. In the current study, we isolated airway fibroblasts and alveolar macrophages from patients with asthma and healthy subjects. We have examined hyaluronan production by airway fibroblasts, cell surface expression of hyaluronan-binding proteins on alveolar macrophages, and inflammatory mediator production by alveolar macrophages in response to low-molecular-weight hyaluronan and LPS stimulation. Samples were obtained from a total of 21 patients with asthma and 25 healthy control subjects. Our results demonstrate an increase in hyaluronan production by airway fibroblasts and an imbalance in hyaluronan-binding protein expression on alveolar macrophages in asthma that favors reduced clearance of hyaluronan fragments and increased production of cytokines in response to hyaluronan fragments and LPS.

METHODS

Study population

Subjects age 18 to 60 years were recruited by advertisement. Samples used for this study are from a total of 21 patients with asthma and 25 healthy control subjects. Not all studies were performed on all subjects; numbers studied in each experiment are denoted in the Results section. Subjects were of mild severity per National Asthma Education and Prevention Program criteria[35] and used no controller medications. Healthy subjects demonstrated normal lung function, had no history of asthma, and used no medications. All the subjects were never-smokers. The characteristics of subjects are shown in Table I. The study was approved by the Duke University Institutional Review Board. All individuals gave informed consent.

Bronchoscopy

Subjects with asthma and healthy controls underwent bronchoscopy with BAL as previously described.[36] Three hundred milliliters of warm, sterile saline in 60-mL aliquots was used for the BAL. Cells derived from the BAL fluid were pelleted by centrifugation at 1000 rpm for 10 minutes. BAL cells were cultured for cytokine production analysis or fixed in 3.7% formaldehyde for flow cytometry.

Hyaluronan staining and quantitation of human lung sections

Lung sections from biopsy tissues of patients with asthma and healthy subjects were stained with biotinylated hyaluronan-binding protein (bHABP; 4 μg/mL; Associates of Cape Cod Inc, East Falmouth, Mass) for 1 hour, then developed by using a Vectastain-Elite-ABC kit (Vector Laboratories, Burlingame, Calif). The specificity of the staining was determined by preincubating tissue samples with 10 U/mL *Streptomyces* hyaluronidase (HYAL) at 37°C for 2 hours in a humidification chamber and then staining with bHABP.

For each subject, photomicrographs were randomly taken at ×40 magnification, avoiding the regions in proximity of the edges of the biopsies. Quantitative assessment of hyaluronan staining in lung tissue was quantified by using ImageJ (version 140 1.44f; National Institutes of Health). A color deconvolution module was created to threshold automatically only the tissue expressing hyaluronan. The vectors for the color deconvolution module were calculated to omit any marginal staining that was not directly related to hyaluronan. Epithelial cells were excluded because there was no significant staining of hyaluronan in all subjects (healthy and asthma). For such calculation, multiple sampling of the stained area was performed to allow consistency across photomicrographs and sections. Similarly, the total tissue area of the biopsy was calculated but by using a different color deconvolution module to detect the total tissue area. Finally, the thresholded images generated by these modules were visually compared with the photomicrographs for accuracy. Separate Java macro scripts were developed for hyaluronan staining and for total tissue area and applied to all images for automatic data generation. The volume percentage of positive tissue was calculated by the ratio of the value of the reactive tissue to hyaluronan and the total volume of the tissue.

Human airway fibroblast cell culture

Fibroblasts were cultured from endobronchial biopsy tissues as previously described.[37] Briefly, biopsy specimens were rinsed and cut into small pieces and cultured in Dulbecco modified Eagle medium supplemented with FBS (10%), streptomycin (100 μg/mL), penicillin (10,000 U/mL), and gentamicin (100 μg/mL). The purity of fibroblasts was confirmed as shown previously.[37] Cells from passage 2 to passage 4 were used for experiments. Airway fibroblasts were seeded in 12-well plates with 50,000 cells per well. Once the cells reached confluence, they were cultured for 1 additional week. The cells were then incubated in fresh serum-free medium with and without 10 ng/mL IL-13 (10 ng/mL), TNF-α (50 ng/mL), or TGF-β (10 ng/mL) for 48 hours. Conditioned media were collected for hyaluronan measurement, and cells were harvested in Trizol reagent (Invitrogen, Carlsbad, Calif) for RNA isolation.

Hyaluronan content determination

Hyaluronan concentrations in culture medium of airway fibroblasts were measured with a competitive ELISA-like assay by using bHABP (Associates of Cape Cod Inc) as described previously.[7] Briefly, samples and bHABP were incubated in a microtube for 1 hour. The sample-bHABP mixtures were added onto a hyaluronan-coated microtiter CovaLink NH modules (Nunc, Rochester, NY). Bound bHABPs were measured with a colorimetric reaction. Sample concentrations were calculated from a standard curve that was generated by using hyaluronan standards of known concentration (ranging 0-2000 ng/mL).

mRNA analysis

RNA was extracted from airway fibroblasts by using Trizol Reagent (Invitrogen) following the manufacturer's instructions. For real-time PCR analysis, 0.5 μg total RNA was used for reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, Calif). One microliter cDNA was subjected to real-time PCR by using Power SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7500 Detection system (Applied Biosystems). The specific primers were designed on the basis of on cDNA sequences deposited in the GenBank database: HAS1 (NM_001532) sense GAGGCCTGGTACAACCAGAA, antisense TGTACAGCCACTCACGGAAG; HAS2 (NM_005328) sense GCCTCAT CTGTGGAGATGGT, antisense TCCCAGAGGTCCACTAATGC; HAS3