Macrophage Chitinase 1 Stratifies Chronic Obstructive Lung Disease

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Diagnosis and therapy of chronic inflammatory lung disease is limited by the need for individualized biomarkers that provide insight into pathogenesis. Herein we show that mouse models of chronic obstructive lung disease exhibit an increase in lung chitinase production but cannot predict which chitinase family member may be equivalently increased in humans with corresponding lung disease. Moreover, we demonstrate that lung macrophage production of chitinase 1 is selectively increased in a subset of subjects with severe chronic obstructive pulmonary disease, and this increase is reflected in plasma levels. The findings provide a means to noninvasively track alternatively activated macrophages in chronic lung disease and thereby better differentiate molecular phenotypes in heterogeneous patient populations.

Present-day diagnosis of chronic inflammatory lung disease relies almost entirely on physiologic and radiologic approaches. It is therefore critical to develop diagnostic methods that are oriented more specifically to cellular and molecular mechanisms relevant to pathogenesis. In that context, we recently identified an innate immune pathway that depends on invariant NKT cells to activate IL-13 production by macrophages and drive chronic obstructive lung disease in a mouse model and in humans with severe asthma or chronic obstructive pulmonary disease (COPD) (1). Because this immune pathway leads to the generation of alternatively activated macrophages in the lung, we reasoned that products of these macrophages could provide molecular targets to aid in diagnosis and treatment in humans with chronic lung disease. In particular, in a mouse model of chronic obstructive lung disease, we identified IL-13-driven chitinase-like proteins (mouse chitinase 3l3 and 3l4) that were markedly up-regulated in macrophages in concert with disease progression. These proteins belong to a family of secreted chitinase and chitinase-like proteins, at least some of which act as an endoglucosaminidase that cleaves chitin, the linear polymer (N-acetyl-D-glucosamine) found in fungi, nematodes, and insects (2). Whether chitinase or chitinase-like proteins protect or promote inflammatory disease still needs to be

CLINICAL RELEVANCE

The research findings provide a new means to noninvasively track the type of inflammation that occurs in chronic obstructive lung disease and thereby better guide diagnosis and treatment for this disease process.

determined (3, 4), but a critical first step is to define the levels of expression in diseased tissue and the corresponding levels in the circulation. Thus, in the present study, we perform a comprehensive analysis of the family of chitinase and chitinase-like gene expression levels in mouse models of chronic obstructive lung disease due to allergen challenge or viral infection, and we extend this analysis to humans with chronic obstructive lung disease due to COPD. We demonstrate discordance between mouse and human chitinase family members at the level of gene expression, but still uncover useful markers of disease activity in both species. Indeed, in humans, we show that chitinase 1 provides for quantitative stratification of COPD based on plasma levels of the corresponding chitinase 1 protein.

METHODS AND MATERIALS

Mouse mRNA Analysis

Infection with SeV in C57BL/6J mice and challenge with ovalbumin (OVA) in Balb/cJ mice were performed as described previously (1, 5). For the present experiments, mice were sensitized with OVA at 14 and 7 days before challenge, then challenged with OVA twice on Study Day 0 and again once on Days 1 and 2, and then analyzed for gene expression on Day 3. Lung RNA isolation and real-time PCR for mouse *Chi3l3/4* and *Gapdh* mRNA were also performed as described previously (1). Additional primers and probes were designed using PrimerExpress software (Applied Biosystems, Foster City, CA) to overlap intron/exon boundaries and were not recognized by genomic DNA. Sequences of the forward and reverse primers and probes were: 5'-TTGAACAAAGCCCTTGGCA-3', 5'-GGAAGGCACGTCAGGAGCT-3', and 5'-ATCCACTGAAGGTTGCA-3' for *Chi3l3*; 5'-TGGCTCTACCCTCGCTTAAGAG-3', 5'-TCCGCATTTCAGTTCCTTGATC-3', and 5'-CAAGCAGTATTTCTCCACC-3' for *Chi3l4*; and 5'-ACCGGACGGAGGCAGTTC-3', 5'-AAGATGACGTGGGTACACAGGTT-3', and 5'-TCTTTCCAGGGATGTGGA-3' for *Chit1*.

Study Samples

Lung tissue was obtained from the explanted lungs of 16 patients with very severe (GOLD Stage IV) COPD at the time of lung transplantation (1, 6). Excess lung tissue from six lung

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transplant donors without COPD was used as a control. Subject characteristics are provided in Table E1 in the online supplement. Plasma samples were obtained from subjects with all severities of COPD based on GOLD Stage (7). Subject characteristics are provided in Table E2. The University Human Studies Committee approved all protocols, and all subjects provided informed consent for participation in the study.

Human mRNA Analysis

Total RNA was isolated from lung tissue using an RNase kit (Qiagen, Valencia, CA). Sample RNA was reverse transcribed by the High Capacity cDNA RT kit (Applied Biosystems) and amplified by the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) using flourogenic primer/probe combinations. Primers and probes were designed with PrimerExpress software (Applied Biosystems) to overlap intron/exon boundaries and to avoid reaction with genomic DNA. Sequences of the forward and reverse primers and probes were: 5'-CCCTAATCTCCACCCTGAAGAA-3', 5'-AGCTGGAGCCGTGCAACTT-3', and 5'-TCGGCCTGCAGAGTG-3' for *CHIA*; 5'-TGTCTGTCGGAGGATGGAAGT-3', 5'-TCTGGGTGTTGGAGGCTATCTT-3', and 5'-TGGGTCTCAAAGA TTT-3' for *CHI3L1*; and 5'-GCATCATGGTGCAGGTCTGT-3',

5'-CCATGGGATCATCAGCAGG-3', and 5'-TGGGCAGGTTCATGG-3' for *CHIT1*. All samples were normalized to the level of *GAPDH* mRNA as described previously (1).

Immunohistochemistry

Immunostaining of human explant lung was performed on formalin-fixed and paraffin-embedded tissue. Tissue sections were deparaffinized, rehydrated in graded alcohol, and incubated in Antigen Unmasking Solution (Vector Labs, Burlingame, CA) at 90°C for 10 minutes for antigen retrieval. Peroxidase activity was quenched with 0.02 N HCl for 20 minutes at 25°C. Nonspecific protein binding was blocked with 1% blocking solution using the Tyramide Signal Amplification (TSA) kit (Invitrogen, Carlsbad, CA) for 60 minutes at 25°C. Lung sections were incubated overnight with goat anti-human chitinase A antibody at 4 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) or chitinase 3L1 or chitinase 1 antibody at 2.5 µg/ml (R&D Systems, Minneapolis, MN) followed by biotinylated anti-goat secondary antibody and avidin-biotin peroxidase complex (VECTASTAIN ABC kit; Vector Laboratories). Staining was visualized with TSA using Alexa Fluor 488 (Invitrogen). Double staining for chitinase A, chitinase 3L1, and chitinase 1 with CD68 was performed as described for

Figure 1. Phylogeny and regulation of mouse chitinase and chitinase-like genes. (a) Phylogenetic analysis of homologies for chitinase and chitinase-like protein sequences from humans and mice. The guide tree shows the relationships that ClustalW (MacVector) generates for pairwise alignments, and is shown as a phylogram with the distances between nodes shown in phylogenetic units. As an approximate guide, a value of 0.1 corresponds to difference of 10% between two sequences. (b) Levels of chitinase and chitinase-like mRNA species in lung tissue from C57BL/6J mice on the indicated days after inoculation with SeV. (c) Corresponding analysis of lung tissue from Balb/c mice after sensitization with ovalbumin (OVA) and then challenge with PBS or OVA.

chitinase single staining followed by a 0.02-N HCl peroxidase quenching step and then incubation with mouse anti-human CD68 (KP1 clone; Dako, Carpinteria, CA) at 2 $\mu\text{g}/\text{ml}$ overnight at 25°C. Sections were then incubated with anti-mouse HRP (Invitrogen) for 1 hour at 25°C followed by TSA using Alexa Fluor 488.

Chitinase Expression System

The cDNAs for CHIA (Acc# NM_201653), CHI3L1 (Acc# NM_001276), and CHIT1 (Acc# NM_003465) were cloned into pBluescript II (Stratagene, San Diego, CA) and then placed under the 26S Sindbis promoter in the SINrep19 or SINrep5G plasmid (8). Plasmids were linearized by digestion with *Xho*I, and capped RNAs were transcribed using the Cap-Scribe SP6 polymerase kit (Roche, Nutley, NJ). Transcription reaction products were used for electroporation of BHK-21 cells (2 μg of RNA per 1×10^7 cells). Transfected cells were allowed to recover for 12 hours, and stably expressing cells (transfected with SINrep19) were selected using resistance to puromycin (5 $\mu\text{g}/\text{ml}$). These stably expressing cells as well as transiently expressing cells (transfected with SINrep5G) were used to check for any antibody cross-reactivity in chitinase immunostaining. In addition, stable and transient expressing cells were grown in minimum essential medium (MEM) with 2% fetal calf serum, and cell medium containing recombinant chitinase A, chitinase 3L1, and chitinase 1 was used to test for any cross-reactivity in the chitinase 1 enzyme-linked immunosorbent assay (ELISA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at 25°C, washed with phosphate-buffered saline (PBS), permeabilized with 1% Triton X100 for 10 minutes at 25°C, and washed again. Nonspecific protein binding was blocked with 1% bovine serum albumin (BSA) blocking solution. Primary goat anti-human chitinase A, chitinase 3L1, or chitinase 1 antibody were added at 2.5 or 4 $\mu\text{g}/\text{ml}$ at 4°C for 18 hours followed by biotinylated anti-goat secondary antibody and avidin–biotin peroxidase complex. Staining was visualized with TSA using Alexa Fluor 488 or 594. Cross-reactivities of antibodies for chitinase A, chitinase 3L1, and chitinase 1 were checked using transduced BHK-21 cells (Figure E3).

ELISA

Chitinase 3L1 levels were determined using an ELISA kit (Quidel Corp., San Diego, CA) with a lower limit of detection of 20 ng/ml. Chitinase 1 levels were determined using a sandwich ELISA. Microplate wells were coated with 50 μl of goat anti-human chitinase 1 antibody at 1 $\mu\text{g}/\text{ml}$ in PBS at 4°C for 18 hours and then blocked with 4% solution of Carnation milk in PBS for 1 hour at 25°C. Plates were rinsed three times with PBS-T buffer (PBS containing 0.05% Tween-20). Samples of plasma (2.5 μl) were diluted 1:20 (vol/vol) and were added to the well for 1 hour at 25°C. Wells were rinsed with PBS-T, and secondary mouse anti-human chitinase 1 mAb (0.5 $\mu\text{g}/\text{ml}$ in PBS; R&D Systems) was added to the wells for 1 hour at 25°C. After washing with PBS-T, peroxidase-conjugated goat anti-mouse IgG (H + L) (50 μl of 0.32 $\mu\text{g}/\text{ml}$; Jackson Immuno-Research, West Grove, PA) was added to the wells for 30 min at 25°C. After another wash, TMB liquid substrate system (50 μl ; Sigma, St. Louis, MO) was added for 5 minutes at 25°C. The reaction was stopped by the addition of Stop Reagent for TMB (Sigma) and absorbance values were determined at 450 nm using a microplate reader (SpectraMax Plus; Molecular Devices, Sunnyvale, CA). An ELISA standard curve was generated

using serial dilutions of recombinant human chitinase 1 (R&D Systems). The lower limit of detection for chitinase 1 was 30 pg/ml. Cross-reactivity of the chitinase 1 ELISA was checked using recombinant human chitinase A and chitinase 3L1 (Figure E3).

Figure 2. Chitinase and chitinase-like mRNA and protein levels in lung tissue in very severe chronic obstructive pulmonary disease (COPD). (a) Chitinase A (*CHIA*), chitinase 3-like 1 (*CHI3L1*), and chitinase 1 (*CHIT1*) mRNA levels in lung explant tissue from subjects with severe COPD (Gold Stage IV) and from control subjects. (b) Photomicrographs of corresponding lung sections immunostained for chitinase A, chitinase 3L1, or chitinase 1 and co-stained for CD68. Bar = 20 μm . (c) Quantification of results from b. Values represent mean for five subjects.

Statistical Analysis

Values for mRNA and protein levels were analyzed by Kruskal Wallis (nonparametric ANOVA) for untransformed data and by one-way ANOVA for log transformed data (which exhibited a Gaussian distribution). Both analyses gave the same results for statistical significance (P value < 0.05).

RESULTS

In moving from mouse to human studies, we noted that phylogenetic analysis of the chitinase and chitinase-like protein family provides for sequence homologies (Figure 1a), but does not necessarily predict similarities in gene expression or function (9, 10). In fact, when we analyzed the expression of chitinase and chitinase-like genes in mouse models of chronic obstructive lung disease driven by viral infection or allergen challenge (1, 5), we observed marked induction of lung chitinase 3l3/4 (*Chi3l3/4*) gene expression, but relatively little or no change in the expression of other chitinase and chitinase-like genes (*Chia*, *Chi3l1*, or *Chit1*) (Figures 1b and 1c). Since mouse chitinase 3l3/4 proteins have no human homologs (based on protein sequences), the mouse studies could not predict the optimal chitinase or chitinase-like candidate for induction during chronic lung disease in humans.

Thus, in contrast to previous studies of chitinase and chitinase-like proteins in chronic obstructive lung disease (11–15), we assessed the level of expression for each of the possible chitinase and chitinase-like family members that may be found in human lung: chitinase A (also known as CHIA or AMCase), chitinase 3 like 1 (also known as CHI3L1, HC gp-39, or YKL-40), and chitinase 1 (also known as CHIT1 or chitotriosidase). In addition, we designed our study to continue successful approaches in mice (where whole lung samples for gene expression better avoided sampling errors) and humans (where subjects with more severe lung disease manifest activation of the iNKT cell-macrophage immune axis). We therefore took special advantage of the availability of whole lung explants

obtained from lung transplant recipients with very severe COPD (GOLD Stage IV) as well as control tissue from lung donors who did not have COPD. This approach also avoided the acute effects of cigarette smoke, since transplant recipients and lung donors were not current cigarette smokers. Under these conditions, we found that the lung tissue obtained from subjects with COPD contained a significantly increased level of *CHIT1* mRNA without a significant change in the level of *CHIA* or *CHI3L1* mRNA compared with control subjects (Figure 2a). Chitinase and chitinase-like gene expression at the mRNA level was associated with the presence of cells that immunostained positive for the corresponding proteins in COPD-affected lung tissue (Figure 2b). These chitinase and chitinase-like staining cells were identified mainly as lung macrophages on the basis of typical morphology and positive immunostaining for CD68, although chitinase-positive neutrophils could also be found in some sections. Even though chitinase A⁺ and chitinase 3L1⁺ cells were found at relatively high levels in control and COPD-affected lungs, only chitinase 1⁺ cells were increased in number in COPD-affected compared with non-COPD control lungs (Figure 2c). Chitinase 1⁺ cells were detected adjacent to IL-13⁺ cells (Figure E1), suggesting the presence of distinct subsets of lung macrophages and consistent with IL-13-driven expression of macrophage chitinase and chitinase-like genes noted previously (1).

Because chitinase and chitinase-like proteins are secreted out of the cell after production, it is possible that these proteins may be detectable in the airspaces of the lung and in the circulation. In fact, chitinase 1 (but not chitinase 3L1) may be found at increased levels in bronchoalveolar lavage (BAL) samples from subjects who are current cigarette smokers (14, 15). To determine whether chitinase and chitinase-like proteins might also be detectable in the circulation as a noninvasive biomarker of lung disease, we analyzed plasma levels of chitinase 3L1 and chitinase 1 in smokers without COPD and in current and former smokers with COPD. We found that the plasma levels of chitinase 3L1 were no different among these groups (Figure

Figure 3. Chitinase and chitinase-like protein levels in plasma samples in smokers and in subjects with COPD. (a) Plasma levels of chitinase 3L1 protein in normal control subjects, smokers without COPD, and subjects with COPD (GOLD Stage I–II and III–IV). (b) Corresponding levels of chitinase 1 in the same groups of subjects. (c) Correlation of plasma chitinase 1 levels with FEV₁ (% predicted) in the same subjects. (d) Corresponding correlation for FEV₁/FVC.

3a). By contrast, chitinase 1 plasma levels were significantly increased in severe and very severe COPD (Gold Stage III–IV) but not in mild or moderate disease (Gold Stage I–II) compared with subjects without COPD (Figure 3b). Similarly, the level of plasma chitinase 1 correlated significantly with the degree of airway obstruction (as marked by the decline in FEV₁ or FEV₁/FVC) in the overall group of subjects with COPD (Figures 3c and 3d). In each of these analyses, there was no relationship between plasma chitinase 1 level and smoking status. Therefore, it appears that the increased lung levels of chitinase 1 found in smokers without lung disease are not necessarily detected in the circulation unless there is the concomitant development of inflammatory lung disease. This observation is further supported by our evidence that at least one determinant of increased plasma chitinase 1 level is the severity of chronic obstructive lung disease, which likely causes increased chitinase 1 production and greater movement of the protein into the circulation.

Together, our results suggest that chitinase 1 represents the human counterpart of what we observe in mouse models of chronic obstructive lung disease, where chitinase 3l3/4 expression is an informative marker for the alternative pathway for macrophage activation. Further definition of the factors that regulate chitinase gene expression is needed to better define the structural and functional relationships among chitinase and chitinase-like family members, but present evidence suggests that the overlap in mouse *Chi3l3/4* and human *CHIT1* gene expression may be due to shared regulatory elements responsive to IL-13 signaling. In addition to our report of IL-13-dependent expression of chitinase 3l3/4 after viral infection in mice, others have observed increases in IL-13-dependent chitinase A gene expression in the allergen-challenge mouse model and in lung tissue from humans with asthma (11). Follow-up reports showed increased chitinase 3L1 levels in bronchial biopsy specimens and serum of subjects with severe asthma and in BAL fluid and serum from subjects with COPD (12, 14). Here we demonstrate relatively low induction of chitinase A and chitinase 3L1 gene expression in mouse models of chronic obstructive lung disease and no significant up-regulation of the expression of these genes at mRNA or protein levels in humans with COPD. The difference in COPD study results does not appear to be based on technical approaches. For example, the present methods were the same as those used previously to detect blood levels of chitinase 3L1 in studies of asthma and other inflammatory conditions (16–18). In addition, we used supplementary methods that also showed no increase in *CHI3L1* mRNA or chitinase 3L1-immunostaining cells in COPD-affected lungs. Thus, differences between studies (and among subjects in our study) are more likely attributed to the heterogeneity of a complex disease. This heterogeneity underscores the need for more precise molecular and physiologic phenotyping of chronic obstructive lung disease. We now recognize that expression of chitinase 1 is restricted to a narrow range of inflammatory and inherited disorders (17, 19, 20) and is significantly increased relative to other chitinase and chitinase-like proteins found in the lung. Therefore, chitinase 1 appears to be an appropriate marker to stratify patients into a subset that may benefit from personalized therapy directed to a specific type of immunopathology in chronic lung disease. In comparison to previous studies restricted to human samples (21–23), our study offers how an experimental model can guide the identification of biomarkers in humans, and how these biomarkers can be used to develop noninvasive methods for stratification of chronic obstructive lung disease.

Conflict of Interest Statement: M.J.H. has received consultancy fees from Roche (\$1,001–\$5,000) and Sepracor (\$1,001–\$5,000), lecture fees from Merck (\$5,001–\$10,000) for speaking at academic institutions, received industry-

sponsored grants from GlaxoSmithKline (\$10,000–\$50,000) and Forest Labs (\$10,000–\$50,000) for collaborative research projects, and received sponsored grants from the NIH (\$100,000+) for NIH-funded research at Washington University. R.H. has received lecture fees from Astellas (\$1,000) and an industry-sponsored educational grant from Cyles (\$10,000). None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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