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Chitinase 1 Is a Biomarker for and Therapeutic Target in Scleroderma-Associated Interstitial Lung Disease That Augments TGF-β1 Signaling

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Interstitial lung disease (ILD) with pulmonary fibrosis is an important manifestation in systemic sclerosis (SSc, scleroderma) where it portends a poor prognosis. However, biomarkers that predict the development and or severity of SSc-ILD have not been validated, and the pathogenetic mechanisms that engender this pulmonary response are poorly understood. In this study, we demonstrate in two different patient cohorts that the levels of chitotriosidase (Chit1) bioactivity and protein are significantly increased in the circulation and lungs of SSc patients compared with demographically matched controls. We also demonstrate that, compared with patients without lung involvement, patients with ILD show high levels of circulating Chit1 activity that correlate with disease severity. Murine modeling shows that in comparison with wild-type mice, bleomycin-induced pulmonary fibrosis was significantly reduced in Chit1−/− mice and significantly enhanced in lungs from Chit1 overexpressing transgenic animals. In vitro studies also demonstrated that Chit1 interacts with TGF-β1 to augment fibroblast TGF-β receptors 1 and 2 expression and TGF-β1-induced Smad and MAPK/ERK activation. These studies indicate that Chit1 is a potential biomarker for ILD in SSc and a therapeutic target in SSc-associated lung fibrosis and demonstrate that Chit1 augments TGF-β1 effects by increasing receptor expression and canonical and noncanonical TGF-β1 signaling. The Journal of Immunology, 2012, 189: 2635–2644.

Scleroderma, or systemic sclerosis (SSc), is a multisystem disease characterized by cutaneous and visceral fibrosis. Nearly 70% of patients with SSc have pulmonary involvement, which has emerged as the greatest cause of mortality for patients with this disorder (1). Many of these patients have SSc-associated interstitial lung disease (SSc-ILD) in which normal lung is replaced with inflamed fibrotic tissue (2). Although 42% of patients with SSc-ILD will die of disease progression within 10 y diagnosis (1), the natural history of the disorder is quite variable, with some patients experiencing accelerated loss of lung function and others manifesting stability over time or progressing very slowly (3, 4). At present, there is no way to predict which patients will progress and are candidates for more intensive therapy and/or referral for lung transplantation and which patients will follow a more indolent course and can be spared the potential side effects inherent in these interventions. The development and validation of a clinically predictive and readily accessible measure of disease progression would therefore be of great benefit. Unfortunately, a biomarker that predicts the occurrence or course of SSc-ILD has not been validated.

TGF-β1 is a well-known regulator of inflammation, injury, remodeling, and repair that has been implicated in the pathogenesis of numerous disorders characterized by pathologic tissue inflammation and fibrosis (5–7). In keeping with the importance of these responses in SSc-ILD, efforts have been directed at defining the roles of TGF-β1 in this disorder. These studies have highlighted a TGF-β1-like molecular signature in fibroblasts from patients with SSc-ILD (8). In contrast, increased fibroblast production of TGF-β1 itself has not been as readily appreciated (9, 10). Scleroderma fibroblasts also demonstrate exaggerated TGF-β1 receptor expression and signaling in the absence of exogenous TGF-β1 (11–13) that appears to be related to enhanced activation of downstream signaling pathways (8, 14) or reduced activity of inhibitory SMADs (15). However, the mechanisms that underlie these exaggerated signaling responses and biomarkers that can be used to assess them have not been appropriately defined.

The GH18 gene family contains true chitinases that bind and cleave chitin. These moieties have been retained during speciation and are found from lower life forms to humans. However, their role in mammalian biology is enigmatic because chitin (the only known substrate of chitinases) is not used as a nutrient by higher life forms and chitin and chitin synthase do not exist in mammals (16). Chitinotriosidase (Chit1, chitinase 1) is the most readily appreciated true chitinase in mammals and man. It can be found in the circulation of normal individuals and has been shown to be increased
in the circulation of patients with Gaucher disease and other disorders characterized by inflammation and remodeling (17–20). Animal experimentation has also demonstrated significant changes in Chit1 expression at sites of inflammation, remodeling, and fibrosis (21, 22). However, the levels of Chit1 in SSc-ILD and the roles of Chit1 in the pathogenesis of tissue fibrosis and TGF-β1 sensitivity have not been fully addressed.

We hypothesized that Chit1 is a biomarker for and a therapeutic target in SSc-ILD. To address the former, we quantified circulating Chit1 activity in two separate cohorts of patients with SSc-ILD and compared these values to parameters of disease severity. To address the role of Chit1 in the fibrotic process we generated mice with null mutations of Chit1 and characterized the effects of this mutation in models of pulmonary inflammation and fibrosis. We also used in vitro studies to define the effects of Chit1 on TGF-β1 receptor expression and signaling. Our studies demonstrate that Chit1 activity is increased in the circulation of patients with SSc-ILD and correlates with objective indices of disease severity. They also demonstrate that Chit1 plays a critical role in the pathogenesis of SSc-like fibrotic pulmonary disorders in mice. Lastly, they provide mechanistic insights by highlighting a previously unrecognized ability of Chit1 to augment TGF-β1 receptor expression and signaling.

Materials and Methods

Study design and subjects

Cohorts of patients that were ≥18 y age and carried a diagnosis of SSc based on present American College of Rheumatology criteria were recruited in New Haven, CT, and Chicago, IL, and characterized as previously described in our laboratories and others (23–26). Patients were excluded based on the following criteria: 1) inability to provide informed consent; 2) significant noncardiovascular or neuromuscular disease; 3) unstable cardiovascular or neurologic disease in past 6 mo; 4) malignancy; 5) pregnancy; 6) chronic infection; 7) known history of smoking. Comprehensive clinical data, including age, sex, race and ethnicity, comorbidities, medications, and physiologic impairment as measured by the percentage predicted forced vital capacity (FVC) and diffusion capacity of carbon monoxide (DLCO), were collected. For the Chicago cohorts additional scleroderma-specific variables such as the presence or absence of diffuse cutaneous disease, Scl-70 status, and the severity of skin involvement by Rodnan skin scores were also assessed. A cohort of demographically matched normal controls was also recruited from the New Haven, CT, community. These human studies were performed with appropriate institutional approval.

Measurement of Chit1 activity

Chit1 enzyme activity was assessed as described by Hollak et al. (27) with minor modification. Briefly, Chit1 activity was determined by incubating 10 μl plasma or serum with 200 μl 22 mmol/l fluorogenic substrate 4-methylumbelliferyl β-D-N,N′,N′-triacetylchitotrioside (Sigma-Aldrich, St Louis, MO) in McIlvain buffer (100 mmol/l citric acid and 200 mmol/l sodium phosphate [pH 5.2]) for 30 min at 37°C. The reaction was stopped by mixing with 2 ml 0.3 mol/l glycine-NaOH buffer (pH 10.6) at room temperature. Substrate hydrolysis by Chit1 produces the fluorescent molecule 4-methylumbelliferone, which was quantitated with a fluorometer (excitation at 366 nm and emission at 446 nm) and compared with a standard calibration curve. Chit1 activity was expressed as nanomoles of substrate hydrolyzed per hour per milliliter of incubated plasma or serum. The pulmonary Chit1 activity was measured using the same assay and 20 μl murine bronchoalveolar larvage (BAL) fluid.

Quantification of IL-13

Plasma IL-13 protein was measured using Luminex technology as we have previously described (24).

Localization of Chit1 expression in SSc

Immunohistochemistry (IHC) was undertaken to localize Chit1 protein using polyclonal rabbit anti-human Chit1 Abs obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and MedImmune (Gaithersburg, MD), as described by our laboratories (25–30). Lung explants from patients with SSc-ILD and nonfibrotic controls were obtained from the University of Pittsburgh. Chit1-expressing cells were counted in six randomly chosen high-power fields. These values were averaged and the means ± SE calculated for SSc-ILD versus normal lung.

Measurement of Chit1 protein expression by ELISA and Western blot

BAL or lung lysate Chit1 were evaluated by ELISA using an anti-Chit1 rabbit polyclonal IgG to capture and biotinylated anti-Chit1 followed by HRP-labeled streptavidin (Amersham Biosciences/GE Healthcare Life Sciences, Piscataway, NJ) for detection. Human and murine anti-Chit1 rabbit polyclonal Abs were obtained from MedImmune. Western blot evaluations were carried out on the BAL, tissue, or cell lysates according to the procedures established in our laboratory (28).

Measurement of Chit1 mRNA expression

Real-time RT-PCR was used to evaluate the levels of mRNA encoding Chit1 in murine lungs using Chit1 specific primers: 5′-TGACTCTGCTGATGTCGCCAG-3′ (sense) and 5′-TAGGCTGTTCACCTCCTGGT-3′ (antisense).

Generation and characterization of Chit1-null (Chit1−/−) mice

A targeting vector was constructed by replacing ~6 kb genomic region including exons 1–5 and part of the Chit1 promoter with the neomycin resistance gene cassette to ensure complete disruption of Chit1 protein expression (Supplemental Fig. 1A–C). The targeting vector was transfected into embryonic stem cells, which were screened for appropriate recombination and injected into C57BL/6 blastocysts. The resulting chimeric animals were bred to generate homozygous null animals. RT-PCR and Western blot analysis confirmed that Chit1 mRNA and protein expression were completely abolished in Chit1−/− mice (data not shown). The Chit1−/− mice were viable and fertile and appeared normal on gross physical and light microscopic examination. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Yale University.

Generation of Chit1 transgenic mice

Lung-specific, inducible Chit1 overexpressing transgenic (Tg) mice were generated using the CC10 promoter, reverse tetracycline transactivator, and the human Chit1 cDNA according to the methods previously established in our laboratory (28). The constructs that were used to generate Chit1 Tg mice were illustrated in Supplemental Fig. 1D. The Chit1 Tg mice were also viable, fertile, and appeared normal on gross physical and light microscopic examination.

Bleomycin administration

Sex-matched, 2-mo-old wild-type (WT), Chit1−/−, and Chit1 Tg mice (≥5 mice/group) were exposed to PBS or bleomycin solution (0.035 U/kg; Teva Parenteral Medicines, Irvine, CA) via i.p. administration every other day for 28 d (31). They were sacrificed 1 d after the final injection and their lungs were evaluated. In selected experiments bleomycin was administered via a single intratracheal injection (2.5 U/kg) and the mice were sacrificed and evaluated 14 d later.

Characterization of the roles of Chit1 in IL-13–induced responses

To define the roles of Chit1 in the pathogenesis of IL-13–induced pulmonary responses, we bred Chit1−/− mice with CC10/IL-13 Tg mice (that had been previously generated in our laboratory) (32) and compared the pulmonary phenotypes of Tg mice with WT and null Chit1 loci.

Assessments of inflammation and fibrosis

BAL was undertaken and the number and differential of the recovered cells were assessed as described by our laboratory (33). The lungs were fixed to prepare and evaluated with H&E in the Research Pathology Laboratory at Yale University School of Medicine. Fibrosis was evaluated histologically using Mallory’s trichrome stains and biochemically using Sircol collagen quantification as described by our laboratory (33).

Evaluation of TGF-β1 receptor expression

MRC-5 human fibroblasts were incubated for the noted period of time in medium with 10% FCS in the presence of rChit1 and rTGF-β1, alone and in combination, or their buffer controls. After incubation for the desired period of time, TGF-β receptor (TGFRI) and TGFRII mRNA and protein
were evaluated by real-time RT-PCR and Western blot analysis, respectively, as previously described by our laboratory (34, 35). The following primers were used for the real-time RT-PCR: 5'-GGTCTTGCCCACCTT-CACAT-3' (sense) and 5'-CCTCTGCTGTTGGAGCACC-3' (antisense) for TGFR1 and 5'-GGGAAAACTAATGGCCTGA-3' (sense) and 5'- GAGCTTTAGGTCCTTGTT-3' (antisense) for TGFR2. Anti-rabbit polyclonal anti-TGFR1 (Millipore, Temecula, CA) and TGFR2 (Cell Signaling Technology, Danvers, MA) were used for the Western blot evaluations.

**Evaluation of TGF-β1 signaling**

Canonical and noncanonical TGF-β1 signaling pathways were assessed using dual reporter assays (SABiosciences, Frederick, MD) and Western blot evaluations. For the canonical Smad-activating pathway, a Smad-using dual reporter assays (SABiosciences, Frederick, MD) and Western luciferase was transfected into NIH3T3 fibroblast cells. After stimulation with TGFβ1 alone or in combination, Smad activation was assessed by measuring the dual luciferase activities. For the noncanonical pathways MAPK/ERK1/2 and Akt activation were assessed using Western blot evaluations as previously described (36).

**Statistical analysis**

Normality of data was assessed using the D’Agostino–Pearson omnibus normality test. Normally distributed data are expressed as the means ± SEM and assessed for significance by a Student t test or ANOVA, as appropriate. Categorical variables were compared using the Fisher exact test. Data that were not normally distributed were assessed for significance using a Kruskal–Wallis test followed by a Dunn post hoc test for multiple comparisons or Mann–Whitney U test for two group comparison. Non-parametric correlation between two variables was evaluated by Spearman’s rank test. Generalized linear modeling was used for multivariate analysis.

**Supplemental material**

Supplemental Tables I and II are demographic characteristics of Yale and Chicago cohorts. Supplemental Table III shows p values for multivariate analyses. Supplemental Fig. 1 is a schematic illustration of targeting constructs used for the generation of Chit1-Δ and Chit1 Tg mice.

**Results**

**Patient characteristics**

To define the regulation of Chit1 in patients with SSc, we have evaluated cohorts from New Haven (hereafter referred to as Yale) and Chicago. Because Chit1 is known to be induced by exposure to cigarette smoke, we performed an initial set of exploratory analyses in control patients with and without a history of smoking. In this study, we found that, as was previously reported (37), circulating Chit1 activity was significantly higher in the plasma of smokers even in the absence of any known lung disease (data not shown). Because we were interested in whether Chit1 reflects pulmonary fibrosis, and not how cigarette smoke confounds these results, patients with a known history of active or prior cigarette smoke exposure were excluded from this study. In the Yale University cohort, we compared healthy individuals and patients with SSc-ILD in whom pulmonary hypertension had been excluded and who were treatment naive. In this cohort, there were no significant differences in age, race, or gender in the patients versus the controls (Supplemental Table I). However, the prevalence of features associated with SSc-ILD such as the presence of esophageal reflux and reductions in the percentage predicted FVC and DLCO were significantly increased in patients with SSc-ILD (Supplemental Table I). In the Chicago cohort, we compared SSc patients who did and did not have evidence for ILD based on radiography and/or pulmonary function testing. In both of these comparisons, there were no significant differences in age, race, gender, age at disease onset, and severity of skin involvement between SSc patients with and without ILD (Supplemental Table II). As expected, in this cohort the ILD patients were more likely to have abnormal lung function (Supplemental Table II).

**Chit1 activity in the circulation of patients with SSc-ILD**

To determine whether Chit1 activity is increased in the circulation of patients with SSc we applied fluorometric approaches to the plasma samples from the Yale cohort and the normal controls. Compared with control samples, which showed only modest levels of Chit1 activity, the levels of plasma Chit1 activity were higher in patients with SSc-ILD (8.1 ± 3.34 versus 15.5 ± 2.4, p = 0.0009; Fig. 1A). This suggests that Chit1 may be a useful biomarker of disease in this patient population.

**IHC analysis of Chit1 expression in the lung**

IHC was next used to define the sites of Chit1 production in patients with SSc-ILD. These studies revealed very few Chit1-expressing cells in control lungs, as well as significantly increased numbers in lungs from patients with end-stage SSc-ILD (2.0 ± 0.37 versus 18.5 ± 2.35, p < 0.0001; Fig 1B). Consistent with a previous report (37), in the normal lung Chit1 expression was restricted to a low-level signal in macrophages with essentially no staining in epithelial cells (Fig. 1C). In contrast, SSc-ILD lung explants displayed Chit1 expression in macrophages as well as airway and alveolar epithelial cells (Fig. 1D–F). Representative lower power images are shown in Fig 1G and 1H for control and SSc-ILD lungs, respectively. These studies demonstrate that the exaggerated plasma Chit1 activity in patients with SSc-ILD is associated with exaggerated Chit1 protein expression in pulmonary macrophages and epithelial cells.

**Chit1 levels are highest in patients with SSc-ILD**

Having found that Chit1 activity was elevated in SSc patients with ILD, we next examined whether this finding was specific to those SSc patients with parenchymal lung involvement or whether it was related to the SSc disease state in general. Because it has been previously shown that Chit1 activity is equivalent between plasma and serum (38), we were able to explore this question in samples obtained from a well-characterized scleroderma serum bank in Chicago. This cohort was used to determine whether Chit1 levels were higher in those patients with SSc who also had ILD. Patient characteristics are shown in Supplemental Table II. This analysis demonstrated that SSc patients with interstitial lung disease show elevated levels of serum Chit1 activity when compared with SSc patients without ILD (25.22 ± 3.87 versus 13.86 ± 2.54 nM/ml/h, p = 0.04; Fig. 2A). In both cohorts, those subjects with an impaired FVC demonstrated substantially increased levels of Chit1 activity compared with those with preserved pulmonary function (Fig. 2B, 2C, p < 0.05 all comparisons).

**Characteristics of patients with high Chit1 activity**

To further understand the significance of high levels of circulating Chit1 activity, we performed a post hoc analysis using plasma Chit1 activity and the recorded characteristics of the patients in our cohorts (Supplemental Table III). In these evaluations, Chit1 activity was not associated with the age at the time of blood draw or disease onset, duration of disease, or DLCO. The levels of Chit1 activity also did not correlate with the levels of circulating IL-13 (data not shown). A significant relationship between Chit1 activity and severity of skin involvement or Scl-70 positivity was also not appreciated (Supplemental Table III). This multivariate analysis also demonstrated that the correlation between Chit1 activity and the severity of lung disease persisted after adjustments for potential confounders, including age, sex, race or ethnic group, and esophageal reflux (Supplemental Table III). Because the Yale cohort did not include patients with pulmonary hypertension or patients that had been treated with immunosuppression, we were unable to evaluate a relationship between Chit1 activity and immunosuppression or pulmonary hypertension in these subjects.
However, in the Chicago cohort there was no relationship between the use of immunosuppression and the level of Chit1 activity, and there was a mild trend toward increased Chit1 activity in SSc-ILD. \( p = 0.05 \). In both cohorts there was a significant negative correlation between Chit1 activity and the percentage predicted FVC (Fig. 2D, 2E). When viewed in combination, these evaluations demonstrate that in both cohorts the levels of circulating Chit1 activity are independently associated with ventilatory impairment and disease severity.

**Bleomycin and IL-13 induction of Chit1**

The data illustrated above highlight a strong relationship between the levels of circulating Chit1 activity and disease severity in patients with SSc-ILD. These findings do not, however, indicate whether Chit1 activity is simply a biomarker or also contributes to disease pathogenesis. To begin to address this issue studies were undertaken to determine whether fibrogenic stimuli altered Chit1 expression in vivo. After 28 d of every other day i.p. bleomycin administration, pulmonary inflammation and fibrosis were readily appreciated. These alterations were associated with increased lung levels of Chit1 protein, mRNA, and activity (Fig. 3A–C). This induction was not bleomycin-specific because IL-13 also stimulated Chit1 protein and mRNA (Fig. 3D, 3E). As in the SSc human lung samples, immunofluorescence evaluations demonstrated that this Chit1 localized to alveolar epithelial cells and macrophages (Fig. 3F, 3G). These studies demonstrate that bleomycin and IL-13 are potent stimulators of pulmonary Chit1.

**Characterization of Chit1−/− mice**

Chit1−/− mice on a C57BL/6 background were successfully generated and maintained. At baseline the Chit1−/− mice were viable and fertile and were not able to be differentiated from the WT littermate controls based on size, physical appearance, rate of growth, or level of physical activity (data not shown). Additionally, at autopsy, light microscopic examinations of skin and visceral organs and H&E, Mallory’s trichrome, and elastin evaluations failed to reveal differences between WT and Chit1−/− animals (data not shown).
Role of Chit1 in bleomycin- and IL-13–induced pulmonary fibrosis

To define the roles of Chit1 in the pathogenesis of SSc-ILD–like pulmonary fibrotic responses we compared the responses that bleomycin and Tg overexpression of IL-13 induce in mice that are deficient in Chit1 and WT controls. Intraperitoneal bleomycin caused significant pulmonary fibrosis and inflammation (Fig. 4A–C). This fibrotic response was at least partially Chit1-dependent because histologically and biochemically detectable collagen accumulation was significantly decreased in lungs from bleomycin-treated Chit1−/− mice (Fig. 4A, 4B). This result was not related to the route of bleomycin administration because similar results were seen with intratracheal bleomycin (data not shown). It was also not specific for bleomycin because similar decreases in collagen accumulation were seen in lungs from IL-13 Tg mice with null Chit1 loci (Fig. 4D, 4E). Interestingly, although BAL cell counts were not significantly reduced in Chit1−/− mice after i.p. or intratracheal bleomycin administration (Fig. 4C and data not shown), IL-13–stimulated inflammation was significantly reduced in the absence of Chit1 (Fig. 4F). These findings demonstrate that Chit1 plays a critical role in the pathogenesis of bleomycin- and IL-13–induced pulmonary fibrosis and IL-13–stimulated tissue inflammation.

Effects of Tg Chit1

The CC10/reverse tetracycline transactivator/Chit1 Tg mice had an appropriately targeted and inducible transgene. At baseline human Chit1 was not detectable in BAL from WT mice and Chit1 Tg mice that did not receive doxycycline. In contrast, Chit1 was readily detected (50–70 ng/ml) in BAL from Chit1 Tg mice treated with doxycycline for ≥48 h. Interestingly, Tg Chit1 by itself did not cause pulmonary inflammation or structural alterations because Chit1 Tg mice had normal lungs on gross and light microscopic examination after 4 wk doxycycline administration (data not shown). In contrast, Tg Chit1 did alter bleomycin-induced responses. This was appreciated in studies in which WT and Tg mice were randomized to normal or doxycycline water and then challenged with intratracheal bleomycin or vehicle. As noted above, bleomycin caused fibrosis and inflammation in the lungs of WT mice (Fig. 5). Importantly, exaggerated fibrotic reactions were noted in lungs from Chit1 Tg mice (Fig. 5A, 5B). Chit1 excess did not quantitatively or qualitatively alter bleomycin-induced BAL and tissue inflammation in a similar manner (Fig. 5C and data not shown). When the WT, Chit1−/−, and Chit1 Tg mice are viewed in combination, they demonstrate that Chit1 is necessary for optimal bleomycin-induced fibrosis and sufficient to directly augment bleomycin-induced tissue fibrotic responses.

Effects of Chit1 on TGF-β1 receptor expression and signaling

Our human studies demonstrate that Chit1 is a biomarker of disease and disease severity in SSc-ILD, and our mouse data demonstrate that Chit1 is a therapeutic target in multiple forms of TGF-β1–driven lung fibrosis (39–41). Because enhanced TGF-β1 signaling has been postulated to contribute to the pathogenesis of SSc-ILD (9, 34, 42), we hypothesized that Chit1 might act by altering TGF-β1–induced receptor expression and signaling. To test this hypothesis, we evaluated the effects of Chit1 on TGF expression by incubating human fibroblast MRC5 cells with rChit1 and rTGF-β1, alone and in combination. In accord with previous studies (43), TGF-β1 stimulated the expression of TGFRI1 while suppressing TGFRI2 expression (Fig. 6). Chit1 by itself did not stimulate and inconsistently inhibited the expression of both receptors (Fig. 6). In contrast, Chit1 and TGF-β1 interacted to augment the expression of both receptors. Specifically, costimulation with Chit1 and TGF-β1 enhanced the expression of TGFRI1 while abrogating the suppression of TGFRI2 (Fig. 6).

We next evaluated the expression of a TGF-β1–responsive plasminogen activator inhibitor promoter-driven luciferase construct in Mv1Lu cells treated with rChit1 and rTGF-β1, alone and in combination. In agreement with the receptor alterations noted above, rChit1 enhanced the ability of TGF-β1 to activate this construct (Fig. 7A). To evaluate canonical signaling, NIH 3T3
cells were stimulated with rTGF-β1 in the presence or absence of rChit1, and SMAD2 phosphorylation was assessed by Western blot (Fig. 7B). We also transfected HEK 293 cells with a SMAD-luciferase reporter and evaluated the activation of this construct after treatment with rTGF-β1 and rChit1, alone and in combination. These studies demonstrated that Chit1 augments TGF-β1-induced SMAD2 phosphorylation and Smad2 reporter activation (Fig. 7B, 7C). To evaluate noncanonical TGF-β1 signaling, we

FIGURE 3. Bleomycin and IL-13 stimulate Chit1 in the lung. (A–C) WT mice were exposed to i.p. bleomycin (Bleo) for 28 d and the levels of Chit1 protein, mRNA, and activity were evaluated (n = 4–5 mice/group, *p < 0.05). (D and E) BAL Chit1 protein and whole-lung mRNA expression in CC10/IL-13 Tg mice. (F and G) IHC and confocal analysis demonstrating the colocalization of Chit1 and the cell-specific markers pro-SPC (alveolar type 2 cells) and F4/80 (macrophages) after bleomycin administration. The values in the panels (A)–(E) and are the means ± SEM of evaluations in a minimum of four animals. Panels in (F) and (G) are representative of a minimum of five similar experiments. Arrows highlight Chit1. Scale bars, 10 μm.

FIGURE 4. Role of Chit1 in bleomycin- and IL-13–induced pulmonary fibrosis and inflammation. (A–C) Two-month-old WT (Chit1+/+) and Chit1−/− were exposed to i.p. bleomycin (Bleo) and pulmonary fibrosis (A), lung collagen content (B), and BAL inflammation (C) were evaluated by Mallory’s trichrome stains, Sircol collagen assays, and BAL cell counts, respectively. (D–F) Evaluation of pulmonary fibrosis (D), collagen content (E), and BAL inflammation (F) in lungs from IL-13 Tg mice with and without Chit1-null mutations. The values in (B), (C), (E), and (F) are the means ± SEM of evaluations in a minimum of four animals. Panels in (A) and (C) are representative of a minimum of five mice per group. Scale bars, 200 μm. *p < 0.05.
evaluated the effects of rChit1 and rTGF-β1 on the phosphorylation of the MAPK ERK1/2. These studies demonstrated that Chit1 augments TGF-β1–induced ERK phosphorylation (Fig. 7D). Thus Chit1 interacts with TGF-β1 to augment TGF-β1 receptor expression and canonical and noncanonical TGF-β1 signaling.

Discussion
To determine whether there is a relationship between SSc and chitinases, we compared the levels of Chit1 activity in the circulation and the accumulation of Chit1 in tissues from patients with SSc and controls. We also generated Chit1−/− mice and Chit1 Tg mice and used them to define the roles of Chit1 in the pathogenesis of pulmonary fibrosis and define novel mechanisms that contribute to these responses. These studies demonstrate that Chit1 is a potential biomarker of SSc-ILD that is produced by lung macrophages and epithelial cells and correlates with disease severity. The murine studies also demonstrate that Chit1 is induced by bleomycin and IL-13 and is necessary for optimal pulmonary fibrosis and sufficient to augment responses to fibrogenic stimuli. Lastly, they demonstrate that Chit1 mediates these effects, at least in part, by interacting with TGF-β1 to augment TGFRI and TGFR2 expression and canonical and noncanonical TGF-β1 signaling.

Our studies demonstrate that the levels of circulating Chit1 activity are increased in a subpopulation of patients with SSc. On superficial analysis this might appear to differ from SSc studies by Bargagli et al. (44). However, differences in the populations that were studied and the methods that were employed likely explain these contrasting results. Specifically, the studies by Bargagli et al. (44) did not include significant numbers of patients with severe disease, but only assessed a single patient cohort, used historical controls with high levels of circulating Chit1 activity, and did not exclude smokers from their populations. In contrast, using two cohorts from different institutions we noted that the levels of Chit1 correlated with disease severity. Interestingly, in agreement with the studies by Bargagli et al. (44), patients with less severe disease did not show increased Chit1 activity. Additionally, our simultaneously processed controls had lower levels of Chit1 activity than did those in the Bargagli et al. (44) study. Lastly, smokers were excluded from our studies but not from their studies. It is well known that cigarette smoke increases Chit1 expression in otherwise normal humans (37, 45), and our data support this, as the levels of Chit1 activity were very high in current or former smokers and did not show increased Chit1 activity. Additionally, our simultaneously processed controls had lower levels of Chit1 activity than did those in the Bargagli et al. (44) study. Lastly, smokers were excluded from our studies but not from their studies. It is well known that cigarette smoke increases Chit1 expression in otherwise normal humans (37, 45), and our data support this, as the levels of Chit1 activity were very high in current or former smokers in both the control and SSc-ILD subjects (data not shown).

SSc is a clinically heterogeneous condition characterized by fibroproliferative vasculopathy and tissue fibrosis affecting the skin and multiple internal organs. The extent of organ involvement and rate of disease progression are the main determinants of morbidity and mortality (25, 46). Pulmonary involvement is the main cause of mortality in SSc, with the most common form of lung involvement being ILD (47). However, organ involvement and disease phenotype are not easy to predict in patient populations or individuals (25). To address this issue, investigators have focused on biomarkers in this disorder and the criteria that should be used to evaluate them. These efforts have led to a number of candidate molecules and scoring systems (reviewed in Refs. 25, 48). They also highlighted the importance of biomarker validity and reproducibility, the ability of the biomarker to discriminate between the situations of interest, and the feasibility of biomarker evaluation (25). Despite these efforts, validated biomarkers that can serve as surrogate outcome measures in SSc are lacking (25). Our demonstration that Chit1 activity associates with SSc-ILD and correlates with disease severity in two different patient cohorts supports the potential utility and reproducibility of this parameter in patient evaluations. The ease with which this assay is performed on readily available clinical samples also speaks to its feasibility. Because we did not find a relationship between Chit1 activity and

FIGURE 5. Transgenic Chit1 enhances bleomycin-induced pulmonary fibrosis. (A–C) Two-month-old WT and Chit1 Tg mice were treated with doxycycline and exposed to intratracheal bleomycin (Bleo; 1.25 U/kg). Pulmonary fibrosis and BAL inflammation were quantitated using Mallory’s trichrome stains (A), Sircol collagen assays (B), and BAL cell counts (C). The values in (B) and (C) are the means ± SEM of evaluations in a minimum of four animals. Panels in (A) are representative of a minimum of five mice per group. Scale bars, 200 μm. *p < 0.05.

FIGURE 6. Chit1 increases TGF-β receptor expression. (A) mRNA expression of TGFRI and TGFR2 assessed by real-time RT-PCR. (B) The expression of TGFRI and TGFR2 evaluated by Western blot in MRC5 human lung fibroblast cells after stimulation with rChit1 (250 ng/ml) alone or in combination with rTGF-β1 (10 ng/ml) for 24 h. The values in the (A) are the means ± SEM of triplicate evaluations; (B) is representative of a minimum of three separate experiments. **p < 0.01.
FIGURE 7. Chit1 increases TGF-β signaling. (A) Mv1Lu cells were stimulated with rChit1 and rtGF-β1, alone and in combination, and luciferase activity was assessed. (B) Smad 2 activation (phosphorylation) was assessed by Western blot in the MRC5 cells stimulated by rChit1 and rtGF-β1, alone and in combination. (C) Smad activation was evaluated by dual luciferase reporter assay in HEK293 cells after stimulation with rChit1 and rtGF-β1, alone and in combination. (D) MAPK/ERK activation in MRC5 cells detected by Western blot after stimulation with rChit1 and rtGF-β1, alone and in combination. The values in (A) and (C) are the means \( \pm \) SEM of triplicate evaluations. (B) and (D) are representative of a minimum of three separate experiments. **\( p < 0.01 \).

The presence of many SSc-relevant comorbidities, it is likely that the presence of parenchymal lung disease accounts for the documented increase in Chit1. We did find a possible association between Chit1 activity and pulmonary hypertension in the Chicago cohort. However, because this association only reached borderline statistical significance and because Chit1 was also elevated in the Yale samples in which patients with pulmonary hypertension were specifically excluded, it is likely that the relationship between Chit1 activity and SSc-ILD is independent of pulmonary vascular disease. As with all bench to bedside undertakings, the limitations of these assessments must also be kept in mind. Specifically, our cohorts were relatively small and did not assess the relationship between Chit1 and disease progression. Additionally, they did not address the utility of Chit1 assessments in the diagnosis of SSc-ILD or the degree to which Chit1 activity levels are increased in other fibrotic lung diseases. Even with these limitations, importantly note that our human data are accompanied by animal data demonstrating that Chit1 plays an important role in the pathogenesis of pulmonary fibrosis. This allows for the exciting speculation that interventions that abrogate Chit1 may be therapeutically useful in SSc-ILD and that assessments of circulating Chit1 activity may predict who will respond and who will not respond to these interventions.

TGF-β1 has long been considered to be a central mediator of wound healing and tissue fibrosis (49, 50). In keeping with this conceptualization, the “scleroderma phenotype” has been attributed, in part, to exaggerated autocrine stimulation by TGF-β1 (8, 49, 50). This assumption is based on animal modeling systems and studies of SSc tissues. The former includes studies from our laboratory and others highlighting the importance of TGF-β1 and or Smad signaling in bleomycin-, IL-13–, and graft versus host-induced tissue fibrosis (49, 50). The latter includes studies highlighting the exaggerated accumulation of TGF-β1 at the periphery of SSc scars (51) and the presence of a TGF-β1 molecular signature in SSc tissues (8). However, the exact role that TGF-β1 plays in SSc is not clear because it is now known that exaggerated TGF-β1 accumulation and its genetic signature are only seen in a subpopulation of SSc patients (8, 52–54). Additionally, the overexpression of type I collagen by SSc fibroblasts is known to be TGF-β1–dependent even though the cells do not produce elevated levels of active or latent TGF-β1 (55). Furthermore, it is now appreciated that SSc tissues manifest an exaggerated response to endogenous TGF-β1 (55) and that optimal TGF-β1–induced fibrotic responses often require interactions with a variety of cofactors such as connective tissue growth factor (52). Our studies add to our understanding of the complexities of these issues by demonstrating that the levels of circulating Chit1 are increased in patients with SSc-ILD and that Chit1 plays a critical role in the pathogenesis of bleomycin- and IL-13–induced fibrosis. They also demonstrate that Chit1 is an important TGF-β1 cofactor that enhances TGFRI and TGFRII expression, as well as canonical and noncanonical TGF-β1 signaling. These findings are in agreement with prior studies that demonstrate that TGF-β1 signatures are more common in SSc patients with ILD (8). In combination, they demonstrate that Chit1 is an important therapeutic target in SSc and suggest that elevated Chit1 levels contribute to the enhanced TGF-β1 responsiveness that is characteristic of the disorder.

The “type 2 cytokine hypothesis of fibrosis” posits that Th2 inflammation is a major driving force in the development of end-organ fibrosis (56). In keeping with this hypothesis, IL-13, the major fibrogenic effector at sites of Th2 responses, is elevated in the lung and circulation of patients with SSc and patients with linear scleroderma or SSc-like reactions to prosthetics (24, 57, 58). Additionally, genetic inquiries and murine modeling have provided lines of evidence that suggest that IL-13 plays a critical role in the pathogenesis of the fibrosis in SSc (59–61). We thus hypothesized that Chit1 exerts its effects by contributing to the pathogenesis of IL-13–induced fibrosis. To test this hypothesis, Tg mice constitutively expressing IL-13 were crossed with Chit1-null mice and the fibrotic effects of IL-13 were assessed in mice with normal and null Chit1 loci. These studies demonstrate that Chit1...
plays a critical role in the pathogenesis of IL-13–induced fibrosis because the levels of fibrosis and collagen accumulation were decreased in lungs from IL-13 Tg mice that were deficient versus sufficient in Chit1. In contrast to our findings with bleomycin, a deficiency of Chit1 also reduced the ability of IL-13 to induce tissue inflammation. When viewed in combination, these studies demonstrate that Chit1 plays a critical role in the pathogenesis of the fibrosis and inflammation induced by IL-13. They also suggest that Chit1 might also play an important role in the pathogenesis of other diseases characterized by IL-13–driven remodeling such as asthma (32).

Chit1, the human homolog of chitinases from nonvertebrate species, is one of the most abundant proteins produced by activated macrophages (20). Its production is increased in patients with Gaucher disease where the level of Chit1 is a validated biomarker of disease activity and response to therapy (20). It is also increased in other disorders characterized by lysosomal dysfunction and or macrophage activation such as lysosomal storage disorders, atherosclerosis, and nonalcoholic steatohepatitis (20). However, our very limited understanding of the biology of Chit1 makes it difficult to appreciate the true meaning of these disease/Chit1 associations and the ways that Chit1 contributes to disease-relevant pathologic responses. This is due, in part, to the fact that mice with null mutations of Chit1 and mice that overexpress Chit1 had not been generated, thereby limiting murine modeling of these molecules. To address this deficiency and further define the biology of Chit1, we generated and characterized mice with null mutations of Chit1 and mice that overexpress Chit1 in a lung-specific fashion. These studies demonstrate that Chit1 plays an important role in the pathogenesis of pulmonary fibrosis and that elevated levels of Chit1 further augment tissue fibrotic responses. They also provide novel insights into mechanisms that can contribute to these responses by demonstrating that Chit1 augments TGF expression and canonical and noncanonical TGF-β1 signaling. Interestingly, previous studies from our laboratory demonstrated that acidic mammalian chitinase and chitinase 3-like 1 (called BRP-39 in the mouse and YKL40 in man) also contribute to the pathogenesis of pulmonary fibrosis and the induction of TGF-β1 elaboration (28, 30). These findings allow for the speculation that Chit1 contributes to the pathogenesis of the tissue remodeling in diseases characterized by Chit1 excess. They also demonstrate that the ability to contribute to wound healing, TGF-β1 elaboration and signaling, and tissue fibrosis is likely a general property of GH18 family moieties.

In summary, these studies demonstrate in two separate patient cohorts that the levels of circulating Chit1 activity are elevated in patients with SSC-ILD where they correlate with objective measures of disease severity. They also demonstrate that Chit1 plays a critical role in the pathogenesis of SSC-relevant tissue fibrotic responses because bleomycin- and IL-13–induced pulmonary fibrosis are ameliorated in Chit1-null mice and bleomycin-induced fibrosis is increased in lung-targeted Chit1 Tg mice. Lastly, they provide insights into the mechanisms that are involved in these responses by demonstrating that Chit1 interacts with TGF-β1 to augment TGF expression, as well as canonical and noncanonical TGF-β1 signaling. These studies validate Chit1 as a biomarker for SSC-ILD and a therapeutic target against which interventions can be directed to control SSC-ILD and other fibrotic disorders.

Disclosures

The authors have no financial conflicts of interest.

References


