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Adenosine-Dependent Pulmonary Fibrosis in Adenosine Deaminase-Deficient Mice

Janci L. Chunn, Jose G. Molina, Tiejuan Mi, Yang Xia, Rodney E. Kellems, and Michael R. Blackburn

Pulmonary fibrosis is a common feature of numerous lung disorders, including interstitial lung diseases, asthma, and chronic obstructive pulmonary disease. Despite the prevalence of pulmonary fibrosis, the molecular mechanisms governing inflammatory and fibroproliferative aspects of the disorder are not clear. Adenosine is a purine-signaling nucleoside that is generated in excess during cellular stress and damage. This signaling molecule has been implicated in the regulation of features of chronic lung disease; however, the impact of adenosine on pulmonary fibrosis is not well understood. The goal of this study was to explore the impact of endogenous adenosine elevations on pulmonary fibrosis. To accomplish this, adenosine deaminase (ADA)-deficient mice were treated with various levels of ADA enzyme replacement therapy to regulate endogenous adenosine levels in the lung. Maintaining ADA-deficient mice on low dosages of ADA enzyme therapy led to chronic elevations in lung adenosine levels that were associated with pulmonary inflammation, expression of profibrotic molecules, collagen deposition, and extreme alteration in airway structure. These features could be blocked by preventing elevations in lung adenosine. Furthermore, lowering lung adenosine levels after the establishment of pulmonary fibrosis resulted in a resolution of fibrosis. These findings demonstrate that chronic adenosine elevations are associated with pulmonary fibrosis in ADA-deficient mice and suggest that the adenosine functions as a profibrotic signal in the lung. The Journal of Immunology, 2005, 175: 1937–1946.

Interstitial lung disease describes a group of heterogeneous lung disorders with variable degrees of pulmonary inflammation and fibrosis. Pulmonary fibrosis is characterized by inflammation, aberrant fibroblast proliferation, and extracellular matrix deposition that results in pathogenic remodeling that eventually disrupts pulmonary architecture and compromises pulmonary function (1–3). There are many causes of pulmonary fibrosis, including exposure to fibrosis-inducing agents such as silica (4) and coal dust (5). Pulmonary fibrosis is also a feature found in disorders such as scleroderma (6), sarcoidosis (7), and cystic fibrosis (8). Idiopathic pulmonary fibrosis (IPF) is a particularly deadly form of pulmonary fibrosis with unknown causes (2). In addition to these classical forms of pulmonary fibrosis, it is becoming increasingly evident that patients with severe asthma and chronic obstructive pulmonary disease (COPD) also develop features of pulmonary fibrosis (9–11), which greatly broadens the number of patients afflicted with this disorder. Despite its prevalence, the pathogenesis of pulmonary fibrosis is not completely understood due to a lack of knowledge of the molecular mechanisms governing its onset and progression. Moreover, treatment options for the resolution of pulmonary fibrosis are lacking.

Adenosine is a purine-signaling nucleoside that is generated in excess during cellular stress and damage as the result of ATP catabolism. Once produced, adenosine can engage specific G protein-coupled receptors on the surface of cells. Four adenosine receptors (ARs) have been identified (A1AR, A2AAR, A2BAR, and A3AR) (12). Their expression patterns in tissues and cells are diverse, and their activation can elicit a wide array of cellular responses, including the modulation of inflammatory cell function (13, 14), mast cell degranulation (15, 16), bronchoconstriction (17, 18), apoptosis (19), and cell proliferation (20). A recent study demonstrated that AR engagement can promote the differentiation of pulmonary fibroblasts into myofibroblast, suggesting that adenosine may direct profibrotic activities in the lung (21). Adenosine concentrations and AR levels are elevated in the lungs of patients with asthma and COPD (22–24). These patients often develop fibrotic foci within the lung ostensibly due to damage incurred by repeated bouts of pulmonary inflammation (9). Thus, excessive adenosine generation in the lung may access profibrotic pathways and hence contribute to the development and/or maintenance of pulmonary fibrosis. The major goal of the current study was to test this hypothesis in an in vivo model where endogenous adenosine levels are regulated.

We have developed a model of adenosine-mediated pulmonary injury that enables the examination of the effect of endogenous adenosine elevations on pulmonary inflammation and injury (25). Adenosine deaminase (ADA) is the purine catabolic enzyme responsible for the deamination of adenosine to inosine. Mice that have a targeted deletion of the gene encoding this enzyme have elevated lung adenosine levels and develop features of chronic pulmonary disease, including progressive airway inflammation, mucus metaplasia, and alveolar airway destruction (25). These mice succumb by 3 wk of age, limiting the ability to study the...
long-term consequences of adenosine elevations on pulmonary injury. In the current study, exogenous ADA enzyme therapy was used to extend the life span of these mice, allowing for investigation of the impact of chronic elevations in lung adenosine on pulmonary disease. In this model, adenosine elevations were associated with pulmonary inflammation and excessive pulmonary fibrosis. This fibrosis could be blocked by preventing the elevations in lung adenosine, suggesting that altered adenosine signaling was causative. Furthermore, lowering lung adenosine levels after the establishment of pulmonary fibrosis resulted in a resolution of fibrosis in the lung, suggesting that adenosine signaling plays an essential role in the regulation of profibrotic pathways.

Materials and Methods

Mice

ADA-deficient mice were generated and genotyped as described previously (25). Mice homozygous for the null Ada allele were designated ADA-deficient (AΔ^−/−), whereas mice heterozygous for the null Ada allele were designated as ADA control mice (AΔ^+). All mice were on a 129sv/ C57BL/6J mixed background, and all phenotype comparisons were performed among littermates. Animal care was in accordance with institutional and National Institutes of Health guidelines. All mice were housed in ventilated cages equipped with microisolator lids and maintained under strict containment protocols. No evidence of bacterial, parasitic, or fungal infection was found, and serology on cage littermates was negative for 12 of the most common murine viruses.

ADA enzyme therapy

Polyethylene glycol-conjugated ADA (PEG-ADA) was prepared as described previously (26). AΔ^−/− mice received i.m. injections of PEG-ADA on postnatal days 1, 5, 9, 13, and 17 (0.625, 1.25, 2.5, 2.5, and 2.5 U, respectively) (1 U is defined as the amount necessary to convert 1 μM of adenosine to inosine/min at 25°C). Mice were then injected i.p. with 5 U of PEG-ADA on a weekly basis until 1 mo of age. At 1 mo of age, a group of mice were tapered down to a low dose of PEG-ADA using the following weekly i.p. injection schedule (0.625 U for 1 wk, 0.25 U for 4 wk, and then 0.125 U for 4–7 wk). This dosing protocol was designated a “low-dose” ADA treatment regimen (LD-ADA). Other mice were maintained on a weekly 5-U i.p. dose of PEG-ADA for the duration of the experimental period (16 wk). This dosing protocol was designated a “high-dose” ADA treatment regimen (HD-ADA). To examine the impact of ADA enzyme therapy on established pulmonary end points, mice were treated according to the LD-ADA regimen described above for 13 wk and were then given weekly i.p. injections of 5 U of PEG-ADA for 5 wk. For all experiments, AΔ^+ mice and AΔ^− mice treated with HD-ADA were used as controls.

FIGURE 1. Modulation of lung adenosine levels using ADA enzyme therapy. Mice were administered on weekly injections of PEG-ADA according to the low/high-dose regimens outlined in the Materials and Methods, where LD-ADA denotes low-dose regimen and HD-ADA denotes high-dose regimen. Mice were sacrificed at 16 wk of age, and lung adenosine levels were measured using reversed-phase HPLC. Values are given as mean nanomoles of adenosine per milligram of protein ± SEM (n = 8 samples for each group). *, significant elevation in adenosine levels over the other groups; #, significant reduction in adenosine levels from AΔ^−/− LD-ADA group; p < 0.05 using a Student’s t test.

FIGURE 2. Lung collagen deposition and α-smα expression following ADA enzyme therapy. Masson’s trichrome staining of paraffin-embedded lung sections from 16-wk-old mice. A, AΔ^+ mice; B, AΔ^−/− on a LD-ADA regimen; C, AΔ^−/− mice on a HD-ADA regimen. D, Immunostaining for α-smα, AΔ^+ mice; E, AΔ^−/− on a LD-ADA regimen; and F, AΔ^−/− mice on a HD-ADA regimen. Findings are representative of at least five mice per group. Scale bars = 100 μm. G, Real-time quantitative RT-PCR for α1-procollagen transcript levels in whole lung RNA extracts taken at 16 wk of age. Data are presented as mean α1-procollagen transcripts per picogram RNA ± SEM, n = 5 for each group. H, Mean total collagen levels ± SEM in 16-wk-old mice (n = 4–8 samples/treatment). I, Mean Ashcroft scores ± SEM at 16 wk of age (n = 4–8 samples/group). *, significant elevation over all groups; #, significant reduction from AΔ^−/− LD-ADA group; p < 0.05 using a Student’s t test.
Quantification of lung adenosine levels

Mice were anesthetized, and the lungs were removed rapidly and frozen in liquid nitrogen. Adenine nucleosides were extracted from frozen lungs using 0.4 N perchloric acid as previously described (27), and adenosine was separated and quantified using reversed-phase HPLC.

Histological analysis

Mice were anesthetized, and the lungs were perfused with 5–10 ml of PBS and then infused with 0.5 ml of fixative (4% paraformaldehyde in PBS) and fixed overnight at 4°C. Fixed lungs were rinsed in PBS, dehydrated through graded ethanol washes, and embedded in paraffin. Sections (5 μm) were collected on slides and stained with H&E or Masson’s trichrome, according to the manufacturer’s instructions.

Collagen quantification

The Sircol collagen assay (Biocolor) was performed on snap frozen whole lungs. Lungs were homogenized in 5 ml of 0.5 M acetic acid with 20 mg of pepsin and incubated with shaking for 24 h at 4°C. Homogenate was spun at 4000 rpm, and supernatant was assayed for pepsin soluble collagen, according to the manufacturer’s instructions.

Ashcroft scoring

Assessment of pulmonary fibrosis was performed on Masson’s trichrome stained lung sections using a minor modification of the method outlined by Ashcroft et al. (28). For our purposes, we analyzed 25 fields at ×40/slide using a two-person randomized blind study. At least five mice were used for each group.

Bronchial alveolar lavage and cellular differentials

Mice were anesthetized and trachea intubated with a blunted 21-gauge needle. Lungs were lavaged with 1–2 ml of PBS, and the recovered bronchoalveolar lavage (BAL) fluid was processed for the determination of cellular differentials. Briefly, total cell counts were performed on initial lavaged aliquots, and cellular differentials (300 cells/sample) were conducted on cells cytospun onto slides and stained with Diff-Quick (Dade Behring).

α-Smooth muscle actin (sma) and TGF-β1 immunohistochemistry

Immunohistochemistry was performed on 5-μm sections cut from formalin-fixed, paraﬃn-embedded lungs. Sections were rehydrated through graded ethanol solutions, dehydrated through graded parafﬁns were incubated with 3% hydrogen peroxide, Ag retrieval was performed (DakoCytomation), and endogenous avidin and biotin was blocked with the Biotin Blocking System (DakoCytomation). For α-sma staining, slides were processed with the Mouse on Mouse kit and the ABC Elite Streptavidin Reagents (Vector Laboratories) and incubated with a 1/500 dilution of a α-sma mAb (monoclonal clone1 A-4; Sigma-Aldrich) overnight at 4°C. Sections were developed with diaminobenzidine and counterstained with methyl green. For TGF-β1, slides were processed according to the Elite ABC Rabbit kit (Vector Laboratories) and incubated overnight at 4°C with a 1/200 dilution of a rabbit polyclonal TGF-β1 antiserum (Santa Cruz Biotechnology). Sections were developed with diaminobenzidine and counterstained with hematoxylin. As a control, TGF-β1 peptide was pre-incubated with the primary Ab at a 5-fold greater concentration before incubation.

TGF-β1 immunoassay

Active and total TGF-β1 levels were assayed in BAL fluid using a kit from R&D Systems.

Analysis of mRNA

Mice were anesthetized, and the lungs were rapidly removed and frozen in liquid nitrogen. RNA was isolated from frozen lung tissue using TRIzol Reagent (Invitrogen Life Technologies). RNA samples were then DNase treated and subjected to quantitative real-time RT-PCR. The primers, probes, and procedures for real-time RT-PCR for the ARs and IL-13 were described previously (29, 30). The primers and TaqMan probe for mouse, α1-procollagen were as follows: forward, 5'-GGTGAGACGTGGTGCAACTC-3’; and reverse, 5'-TCTTACCAAGGAGAACCATCA-3’; and probe, 5'-FAM ACAGAGGTGATGCTGGTCCCAAAGG-3’ (Cyber green was used for analysis of IL-1β, plasminogen activator inhibitor-1 (Pai-1), and osteopontin (OPN) using the following primers: IL-1β, forward, 5’-CAGAGCGGCT GACTGAACTC-3’; and reverse, 5’-AGTGCTGTCTCGGGCTGCTT-3’; Pai-1, forward, 5’-AGTGATGGGACCTTACAGCCGAGG-3’, and reverse, 5’- AGGAGGATGGTTTCCTCCTT-3’; and OPN, forward, 5’-GTGTGAT GAGACCTCAGTC-3’; and reverse, 5’-CCTAGACTCTACGGCCTCT C-3’. Reactions were conducted on a Smart Cycler rapid thermal cycler system (Cepheid). Specific transcript levels were determined using Smart Cycler analysis software through comparison to a standard curve generated from the PCR amplification of template dilutions.

Results

Modulation of lung adenosine levels using ADA enzyme therapy

A primary function of ADA is to control the levels of adenosine in tissues and cells. ADA−/− mice exhibit increases in lung adenosine levels in conjunction with extensive pulmonary inflammation, injury, and death by 3 wk of age (25). The rapid onset of pulmonary inflammation and death prevented the examination of the effects of chronic elevations in adenosine. To establish a model system to investigate the consequences of chronic adenosine elevations, ADA−/− mice were treated with varying doses of exogenous PEG-ADA over a period of 16 wk, and lungs were collected for adenosine quantification (Fig. 1). ADA−/− mice on LD-ADA showed marked increases in lung adenosine levels as compared with ADA−/− mice, whereas ADA−/− mice kept on HD-ADA did not develop significant elevations in lung adenosine. These findings substantiate that continuous HD-ADA enzyme therapy can effectively prevent elevations in lung adenosine in ADA−/− mice, while LD-ADA allows elevations in lung adenosine.

ADA−/− mice with chronic elevations in lung adenosine exhibit extensive lung collagen deposition and α-sma expression

ADA−/− mice treated with LD-ADA showed external features of respiratory distress, including rapid, labored breathing, rib cage

FIGURE 3. Airway cellularity following ADA enzyme therapy. Cellularity was determined in the lungs of 16-wk-old mice treated with LD-ADA or HD-ADA. A, Total cell numbers obtained from BAL fluid. B, BAL cells were collected, cytospun onto slides, and stained with Diff-Quick for determination of cellular differentials. Data are presented as mean cell counts ± SEM (n = 5); *, significant elevation over the other groups; #, significant reduction in total cells from ADA−/− mice treated with LD-ADA (p < 0.05).
deformities, and pulmonary crakles. Histological analysis showed that mice treated with LD-ADA had extensive pulmonary remodeling, including alveolar epithelial cell hyperplasia and hypertrophy, smooth muscle cell thickening and alveolar invasion, severe pulmonary inflammation, and apparent fibrosis and matrix deposition (data not shown). To more accurately characterize the fibrotic phenotype seen, various end points associated with fibrosis were examined. Collagen deposition was found to be increased in the lungs of ADA−/− mice treated with LD-ADA as detected by intense blue staining with Masson’s trichrome (Fig. 2B). These findings were confirmed by demonstrating that the production of α1-procollagen transcripts (Fig. 2G) and total lung collagen content (Fig. 2H) were also markedly increased in the lungs of ADA−/− mice treated with LD-ADA. In addition to collagen deposition, extensive α-sm smooth muscle actin staining was detected in the lungs of ADA−/− mice treated with LD-ADA (Fig. 2E), suggesting increased numbers of myofibroblasts. Collagen production and deposition as well as increases in myofibroblasts were prevented in ADA−/− mice given HD-ADA therapy (Fig. 2, C, F, G, and H). These alterations in fibrosis were also associated with overall changes in Ashcroft fibrotic scores (Fig. 2I). Taken together, these data suggest a correlation between elevations in lung adenosine and the elaboration of a fibrotic phenotype within the lung.

**ADA−/− mice develop severe pulmonary inflammation**

Pulmonary fibrosis is often associated with an inflammatory cell component that is predominantly comprised of alveolar macrophages (2). Histological analysis of the lungs of ADA−/− mice following the LD-ADA regimen revealed extensive pulmonary inflammation within remodeled alveoli as well as the pulmonary interstitium (data not shown). To characterize this inflammation, total cell numbers and cellular differentials were assessed in recovered BAL fluid. There was a 9-fold increase in total cell numbers obtained from the BAL fluid of ADA−/− mice following LD-ADA treatment, as compared with untreated ADA+ mice and ADA+ mice following HD-ADA treatment (Fig. 3A). Furthermore, ADA−/− mice treated with HD-ADA exhibited normal levels of total cells recovered from the BAL fluid (Fig. 3A). Cellular differentials from ADA−/− mice treated with LD-ADA showed a 6-fold increase in alveolar macrophages and a significant elevation in lymphocytes, eosinophils, and neutrophils (Fig. 3B). These increases were not seen in ADA−/− mice treated with HD-ADA. In addition, the macrophages obtained from ADA−/− mice treated with LD-ADA were large and foamy, indicative of an activated state. This was not apparent in the other groups where the macrophages appeared quiescent. Taken together, these results suggest that chronic elevations in lung adenosine promote inflammatory cell infiltration into the lung.

**Profibrotic mediators are elevated in the lungs of ADA−/− mice treated with LD-ADA**

IL-1β (31), IL-13 (32), Pai-1 (33), OPN (34), and matrix metalloproteinase (MMP)-2 (35) are profibrotic mediators that have been correlated with pulmonary fibrosis. Transcript levels of these mediators were assessed in the lungs of ADA−/− mice treated with LD- or HD-ADA to determine whether profibrotic mediators were elevated. IL-1β, IL-13, Pai-1, OPN, and MMP-2 were all found to be elevated in the lungs of ADA−/− mice treated with LD-ADA (Fig. 4, A–E). These increases were prevented in ADA−/− mice following HD-ADA therapy. RNA was isolated from the lungs of 16-wk-old mice treated with LD-ADA or HD-ADA. A, Quantitative RT-PCR analysis for transcripts of IL-1β in whole lung RNA extracts. B, Quantitative RT-PCR analysis for transcripts of IL-13 in whole lung RNA extracts. C, Quantitative RT-PCR analysis for transcripts of Pai-1 in whole lung RNA extracts. D, Quantitative RT-PCR analysis for OPN in whole lung RNA extracts. E, Quantitative RT-PCR analysis for MMP-2 in whole lung RNA extracts. Data are presented as mean transcripts per picogram RNA ± SEM (n = 4); *, significant elevations in transcripts over controls; #, significant reduction from ADA−/− mice treated with LD-ADA (p < 0.05).
FIGURE 5. TGF-β1 levels in ADA−/− mice treated with ADA enzyme therapy. Immunostaining for TGF-β1 was determined in the lungs of 16-wk-old mice treated with LD-ADA or HD-ADA. A, ADA+/− mice; B, ADA−/− mice; C, ADA−/− mice on a LD-ADA regimen; and D, ADA−/− mice on a HD-ADA regimen. E, Lung section incubated with peptide control for Ab specificity. Findings are representative of at least five mice per group. Scale bars = 100 μm. E, Quantitative RT-QPCR analysis for transcripts of TGF-β1 in whole lung RNA extracts of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean transcripts per picogram RNA ± SEM (n = 4). F, Total TGF-β1 protein levels were determined in the BAL fluid of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean pg/ml ± SEM (n = 4–6 in each group). G, Active TGF-β1 protein levels were determined in the BAL fluid of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean pg/ml ± SEM (n = 4–6 in each group). *, Significant elevations in transcripts over controls; #, significant reduction from ADA−/− mice treated with LD-ADA (p < 0.05).

Reversal of lung adenosine levels in ADA−/− mice receiving “late-stage” (LS)-HD-ADA enzyme therapy

The findings described above demonstrate that chronic elevations in lung adenosine correlate with pulmonary fibrosis in the lungs of ADA−/− mice. To determine whether ADA enzyme therapy could lower lung adenosine levels after damage had occurred, ADA−/− mice were treated with the LD-ADA regimen for 13 wk and then brought up to a HD-ADA treatment regimen for 5 wk. Analysis of lung adenosine levels revealed that this LS-HD-ADA treatment was able to return lung adenosine levels to control values (Fig. 6). These data demonstrate that ADA therapy can reverse lung adenosine elevations in ADA−/− mice with established lung disease. ADA therapy can reverse pulmonary fibrosis in the lungs of ADA−/− mice

Collagen production and the presence of myofibroblasts were examined to determine the consequences of lowering adenosine levels in the lungs of ADA−/− mice with established pulmonary fibrosis. ADA−/− mice were treated with the LD-ADA treatment regimen for 13 wk and were then placed on HD-ADA treatment for 5 wk. Lungs from ADA−/− mice on LD-ADA exhibited increased collagen deposition as indicated by increased Masson’s

FIGURE 6. Lowering of lung adenosine levels using ADA enzyme therapy. ADA−/− mice were maintained on the LD-ADA regimen until 13 wk of age. At this stage, half of the group was sacrificed for lung analysis (LD-ADA), and half was placed on a HD-ADA regimen for 5 wk and then sacrificed (LS-HD-ADA). Lung adenosine levels were measured using HPLC analysis. Values are given as mean nanomoles adenosine per microliter protein ± SEM (n = 6); *, significant elevation over ADA+/−; #, significant reduction from ADA−/− LD-ADA (p < 0.05).
trichrome staining (Fig. 7B). In addition, RNA extracts from the lungs of ADA−/− mice receiving LD-ADA had increased levels of α1-procollagen transcripts (Fig. 7D) and increased collagen protein levels in the BAL fluid (Fig. 7E). Consistent with the fibrotic phenotype, there was also an increase in α-sma-positive cells in the airways of these mice (Fig. 7B, inset). Treatment of ADA−/− mice on the LD-ADA regimen with HD-ADA for 5 wk (LS-HD-ADA) resulted in a significant decrease in lung collagen production (Fig. 7D and E) and deposition (Fig. 7C), as well as α-sma staining (Fig. 7C, inset). Alterations in fibrosis were also associated with overall changes in Ashcroft fibrotic scores (Fig. 7F). These results demonstrate that ADA treatment can halt and reverse pulmonary fibrosis and implies a correlation between the modulation of lung adenosine levels and the elaboration of collagen deposition in the lungs of ADA−/− mice.

**Lung inflammation is reduced in ADA−/− mice following ADA treatment**

To determine whether lowering lung adenosine levels could alter the inflammatory status of ADA−/− mice, BAL cellularity was examined. There was a 5-fold increase in total cells recovered from BAL fluid of ADA−/− mice on LD-ADA treatment for 13 wk (Fig. 8A). This increase was reduced 2-fold following HD-ADA treatment for 5 wk (LS-HD-ADA). Cellular differentials of ADA−/− mice following LS-HD-ADA treatment revealed a reduction in macrophages, lymphocytes, eosinophils, and neutrophils (Fig. 8B). These results demonstrate that ADA treatment can improve pulmonary inflammation in ADA−/− mice.

**Profibrotic mediators are reduced in ADA−/− mice following ADA treatment**

To investigate whether lowering lung adenosine levels would alter transcript levels of profibrotic mediators, lung transcripts for IL-1β, Pai-1, OPN, and MMP-2 were assessed in ADA−/− mice following LS-HD-ADA treatment. Results showed that levels of profibrotic mediators were reduced in ADA−/− mice following LS-HD-ADA treatment (Fig. 9, A–D). Similarly, the enhanced levels

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**FIGURE 7.** Normalization of lung collagen and α-sma expression following ADA enzyme therapy. Lung sections were stained with Masson’s trichrome. A, Lung from untreated ADA+ mouse; B, lung from ADA−/− mouse treated with LD-ADA until 13 wk of age; C, ADA−/− mouse treated with LD-ADA until 13 wk of age and then treated with HD-ADA for 5 wk (LS-HD-ADA). Insets represent α-sma immunohistochemistry from the respective samples. Data represent findings from at least six mice per group. Scale bar = 100 μm in figures and 10 μm in insets. D, Quantitative RT-PCR for α1-procollagen in whole lung RNA extracts from mice treated as described in A–C. Data are presented as mean α1-procollagen transcripts per picogram RNA ± SEM (n = 6). E, Soluble collagen protein levels in BAL fluid (n = 4–8 samples/group). F, Mean Ashcroft scores ± SEM (n = 4–8 samples/group). *, Significant elevation over all groups; #, significant reduction from ADA+.}

**FIGURE 8.** Normalization of airway inflammation following ADA enzyme therapy. A, Total cell numbers obtained from BAL fluid of ADA−/− mice treated with LD-ADA for 13 wk or ADA−/− mice treated with LD-ADA for 13 wk and then HD-ADA for 5 wk (LS-HD-ADA). B, Cellular differentials of BAL cells collected from mice described in A. Values are presented as mean total cells ± SEM (n = 5); *, significant elevation over ADA+; #, significant reduction from ADA−/− LD-ADA (p < 0.05).
of TGF-β seen in the lungs of ADA−/− mice on LD-ADA treatment for 13 wk (Fig. 10, A and C–E) were lowered following HD-ADA treatment for 5 wk (Fig. 10, B and C–E). These results demonstrate that exogenous ADA can lower profibrotic mediator production in the lungs of ADA−/− mice.

AR transcripts are altered in the lungs of ADA−/− mice following ADA enzyme therapy

AR levels are known to be altered in chronic lung diseases where adenosine levels are elevated (24). To investigate the levels of various ARs in the lungs of ADA−/− mice exhibiting pulmonary fibrosis, AR transcript levels were quantified in whole lung extracts from ADA−/− mice treated with various dosages of PEG-ADA (Fig. 11). There was a significant elevation in transcript levels for the A1AR, A2AAR, and A3AR in the lungs of ADA−/− mice on a LD-ADA treatment regimen. These elevations were prevented with HD-ADA therapy. There were no significant changes in the transcript levels of the A2BAR in any of the treatment groups. LS-HD-ADA treatment lowered A2AAR transcript levels to control values but did not significantly alter the transcript levels of the A1AR, A2BAR, or A3AR in the lungs of ADA−/− mice. These findings demonstrate that AR levels are altered in the lungs of ADA−/− mice exhibiting pulmonary fibrosis.

Discussion

Adenosine has been implicated in the regulation of chronic lung disease; however, there are few studies that have examined the contributions of endogenous adenosine to specific cellular processes within the inflamed lung. In the current study, ADA enzyme replacement therapy was used to regulate the levels of endogenous adenosine in ADA−/− mice. This approach demonstrated that chronic elevations in lung adenosine levels led to the development of severe pulmonary fibrosis. Preventing or reversing elevations in adenosine levels in this model was associated with a prevention or...
reversal of pulmonary fibrosis, suggesting that elevations in lung adenosine were causative in the resulting condition.

IPF is a particularly severe form of pulmonary fibrosis in humans (2). It is progressive in nature, does not respond well to corticosteroid treatments, and usually leads to death within 5 years. The poor response to corticosteroid treatment has lead to the hypothesis that it is not remitting pulmonary inflammation that leads to the progression of fibrosis in IPF but rather structural changes in the lung architecture (36). Structural abnormalities of the lung appear to result from pathways that mediate airway epithelial cell integrity (37), extracellular matrix turnover (38), and myofibroblast recruitment and maintenance (39). Understanding the complex regulation of these processes in IPF has been difficult due to the lack of model systems that exhibit features of progressive pulmonary fibrosis. The progressive nature of the pulmonary fibrosis seen in ADA−/− mice suggests that adenosine may access pathways that maintain or promote the progression of pulmonary fibrosis. This is supported by the observation that lowering adenosine levels during the progression of pulmonary fibrosis can halt and even reverse aspects of pulmonary inflammation and fibrosis in ADA−/− mice. This model will be useful in examining mechanisms by which adenosine mediates fibrosis, as well as investigating the cellular signaling pathways involved in the maintenance, progression, and resolution of pulmonary fibrosis.

A key finding of this study was the adenosine-dependent elevation of key regulators of pulmonary inflammation and fibrosis in the lungs of ADA−/− mice. Numerous cellular mediators have been implicated in the ontogeny and maintenance of pulmonary fibrosis (2, 36, 40). Among these, IL-1β and IL-13 are important proinflammatory signals (31, 32), whereas TGF-β1, Pai-1, OPN, and MMP-2 are involved in the regulation of the extracellular matrix environment and structural integrity of the airways (41–44). The levels of all these mediators are elevated in patients and animal models exhibiting pulmonary fibrosis (35, 45–48), and studies in transgenic and knockout mice demonstrate their importance in bleomycin-induced pulmonary fibrosis (34, 42, 49). The current study demonstrates that these mediators are all substantially elevated in the lungs of ADA−/− mice that exhibit chronic elevations of adenosine and fibrosis. Moreover, lowering levels of endogenous adenosine in ADA−/− mice with established pulmonary fibrosis was able to reverse elevations of these mediators. Although these findings suggest that adenosine elevations lead to the activation of pathways classically associated with fibrosis, they do not distinguish which of these mediators are directly regulated by adenosine in this model. Along these lines, the regulation of TGF-β1 and IL-13 are of particular interest because they have been shown to be sufficient for the induction of fibrotic pathways such as is seen in the lungs of ADA−/− mice (32, 50). Furthermore, IL-13 has been implicated in an adenosine amplification pathway in the lungs of ADA−/− mice (51), and TGF-β1 and IL-13 are themselves in parallel pathways (50). Current efforts are underway to determine the ability of adenosine to directly regulate these and other profibrotic mediators, as well as to determine the relative contribution of these mediators to the pulmonary fibrosis seen in ADA−/− mice. These studies will provide important information into the mechanisms by which adenosine regulates fibrosis in the lung.

Many of the cellular processes that have been implicated in pulmonary inflammation and airway damage have been directly or indirectly associated with adenosine signaling (52, 53). Adenosine elicits many of its actions by engaging cell surface ARs. Four ARs have been characterized, each with a unique affinity for adenosine and unique connections to cellular signaling components via various G proteins (12). Engagement of ARs can promote aspects of inflammation and remodeling, including the induction of apoptosis (54), the promotion of cell survival or differentiation (55), cytokine and chemokine production (29, 56–61), extracellular matrix deposition (62), and protease activation (30, 63). Thus, it is reasonable to suggest that adenosine generated in the inflamed and damaged lung may contribute to various cellular processes in the fibrotic program. Perhaps the most compelling evidence that adenosine can directly activate profibrotic pathways is a recent study in human pulmonary fibroblasts cultured ex vivo, where adenosine was shown to promote the differentiation of pulmonary fibroblasts into collagen-producing myofibroblasts (21). This response involved the engagement of the A3AR and the release of IL-6, and these responses were enhanced in the presence of hypoxia. The transformation of pulmonary fibroblasts into myofibroblasts is considered a major mechanism underlying pulmonary fibrosis (36, 39).

![Figure 1](http://www.jimmunol.org/)

**FIGURE 11.** AR transcript levels in the lungs following ADA enzyme therapy. RNA was isolated from the lungs of ADA−/− mice treated with LD-ADA and HD-ADA for 16 wk or LD-ADA for 13 wk followed by HD-ADA for 5 wk (LS-HD-ADA). Quantitative real-time RT-PCR for the ARs was performed. Values are presented as mean transcripts per nanogram RNA ± SEM (n = 6 for each group). *, Significant elevations in transcripts over ADA−/−; #, significant reduction in lung transcripts as compared with ADA−/− LD-ADA (p < 0.05).
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The demonstration that adenosine can directly promote this process further strengthens the likelihood that this nucleoside is in itself profibrotic. It appears that engagement of the A2B AR may be an important mechanism by which adenosine promotes pulmonary fibrosis (21). This would imply that treatment with selective A2B AR antagonist may have some use in regulating pulmonary fibrosis. In the current study, we demonstrate that there are increased numbers of myofibroblasts and collagen deposition in the lungs of ADA−/− mice and that all of the ARs are expressed in this environment. Interestingly, A2B AR was the most abundant of the four ARs in the lung. Also of interest was the observation that AR levels changed with the degree of inflammation and fibrosis seen. The significance of these changes is not yet clear, but they provide evidence for the existence of adenosine signaling pathways in these mice. Examination of the AR functions in the lungs of ADA−/− mice using AR antagonists and knockout mice will help to define the functions of individual ARs in adenosine-dependent pulmonary fibrosis.

Collagen deposition by pulmonary myofibroblasts is a major feature of pulmonary fibrosis (2, 36, 39). Much of the myofibroblast proliferation and collagen deposition seen in the lungs of ADA−/− mice maintained on LD-ADA was located in the distal alveolar airways. However, collagen deposition was also noted around bronchial airways (data not shown); a feature of airway remodeling that is common in severe asthma and in some instances of COPD (9). This suggests that adenosine may regulate aspects of collagen deposition in these diseases. Consistent with this, relatively high levels of adenosine are found in the lungs of asthmatic and COPD patients in association with severe inflammation (22, 23). In addition, adenosine levels are elevated in a mouse model of chronic pulmonary inflammation and remodeling (51). Mice overexpressing the pleuripotent cytokine IL-13 in the lungs develop features of chronic lung disease, including excessive collagen deposition around the airways (32). Interestingly, treatment of IL-13-overexpressing mice with ADA enzyme therapy was able to lower the levels of endogenous adenosine in the lungs and attenuate the degree of collagen deposition seen (51). These findings demonstrate that, as in the current study, elevations in lung adenosine levels can activate pathways that contribute to collagen deposition. Collectively, these studies suggest that controlling adenosine levels with exogenous ADA treatments may provide a meaningful approach to halt the progression or reverse features of pulmonary fibrosis not only in IPF but also in severe asthma and COPD. More research is needed to understand the regulation of adenosine metabolism in these chronic lung disorders, as well as to uncover the specific mechanisms by which adenosine exerts its proinflammatory and profibrotic activities. Doing so will not only build confidence in the potential use of ADA enzyme therapy in these diseases but identify specific targets for modulating adenosine’s actions.

Several studies of acute injury in the lung and in other tissues have shown that adenosine has potent anti-inflammatory and tissue protective effects (64–67). In contrast, the current study suggests that adenosine has proinflammatory, profibrotic, and tissue destructive effects on chronic lung injury. It is likely that differential responses of adenosine will be governed by the extent and duration of adenosine production, the specific expression of ARs on various effector cells, and the differential regulation of downstream signal transduction pathways. Deciphering these parameters in the context of the whole animal will be essential and will be aided by the emerging availability of mice with genetic modifications in components of adenosine metabolism and signaling and the increasing availability of selective AR ligands. Continued efforts to understand the contribution of adenosine signaling in both acute and chronic aspects of tissue inflammation and injury will be essential for the development of adenosine-based therapeutic approaches for the management of numerous disorders.

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Disclosures

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