Noninvasive Imaging of Experimental Lung Fibrosis

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Abstract

Small animal models of lung fibrosis are essential for unraveling the molecular mechanisms underlying human fibrotic lung diseases; additionally, they are useful for preclinical testing of candidate antifibrotic agents. The current end-point measures of experimental lung fibrosis involve labor-intensive histological and biochemical analyses. These measures fail to account for dynamic changes in the disease process in individual animals and are limited by the need for large numbers of animals for longitudinal studies. The emergence of noninvasive imaging technologies provides exciting opportunities to image lung fibrosis in live animals as often as needed and to longitudinally track the efficacy of novel antifibrotic compounds. Data obtained by noninvasive imaging provide complementary information to histological and biochemical

measurements. In addition, the use of noninvasive imaging in animal studies reduces animal usage, thus satisfying animal welfare concerns. In this article, we review these new imaging modalities with the potential for evaluation of lung fibrosis in small animal models. Such techniques include micro-computed tomography (micro-CT), magnetic resonance imaging, positron emission tomography (PET), single photon emission computed tomography (SPECT), and multimodal imaging systems including PET/CT and SPECT/CT. It is anticipated that noninvasive imaging will be increasingly used in animal models of fibrosis to gain insights into disease pathogenesis and as preclinical tools to assess drug efficacy.

Keywords: lung fibrosis; small animal models; micro-CT; MRI; nuclear imaging

Idiopathic pulmonary fibrosis (IPF) is a relentless and invariably fatal fibrotic lung disorder that primarily affects aging adults. A U.S. Food and Drug Administration–approved and effective drug therapy for patients with IPF has only recently become available. Recent phase III clinical trials with pirfenidone and nintedanib (also known as BIBF-1120) in the treatment of IPF suggest that we may be entering a new era of developing novel antifibrotic drug therapies. We currently lack insights into which patients may benefit from these drugs and the extent of clinical benefit that might be expected. Clearly, there is a need for continued research efforts in finding better pharmacologic treatments for fibrotic lung diseases.

Small animal models of lung fibrosis are essential for preclinical evaluation of the efficacy of novel antifibrotic compounds. Animal studies are also important in identification of the molecular mechanisms involved in the pathogenesis of fibrotic process, despite their limitation of reproducing the irreversible and progressive nature of human IPF. Current outcome measurements in experimental lung fibrosis involve labor-intensive histological and/or biochemical analyses. These evaluations require killing animals at designated time points, precluding data acquisition of the dynamic nature of the fibrotic process in individual animals. The commonly used measurements of lung fibrosis—hydroxyproline content and morphometric

scores—are known to be highly variable among individual animals. To achieve adequate statistical power, a large number of animals is often required, particularly in experiments in which lung fibrosis is assessed at multiple time points.

In humans, characterization of the extent and longitudinal changes of fibrotic lung diseases by noninvasive imaging techniques, such as high-resolution computed tomography, magnetic resonance imaging (MRI), and positron emission tomography (PET), have been well established. The development of similar, proven imaging techniques optimized for animal imaging provides opportunities to assess lung fibrosis at different time points in living animals and enables each animal to

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serve as its own control. Noninvasive imaging reduces the number of animals used for experimentation, which satisfies the ethical aspects of using fewer animals and allows for multiple, longitudinal measurements in individual animals. Furthermore, noninvasive imaging does not preclude complementary histological and biochemical measurements after the imaging study and allows the temporal tracking of the efficacy of antifibrotic therapies in a single animal. In this article, we review various noninvasive imaging methods that are currently used for evaluation of lung fibrosis in small animal models. These include micro-CT, MRI, nuclear imaging (specifically PET and single photon emission computed tomography [SPECT]), and multimodal PET/CT and SPECT/CT scans. For a detailed description of the principles of these techniques and their broader applications in lung imaging, we recommend a recent review article by Gammon and colleagues (1).

Micro-CT

Micro-CT imaging uses the inherent contrast between air and tissue in the lung to study lung morphometry and function. The CT density of the lung is currently quantified by Hounsfield units (HUs) in micro-CT scan analysis. HUs define air density as $-1,000$ and water density as 0 . Based on this definition, lung tissues that are less dense than water are represented by <0 HUs, whereas areas with higher density exhibit values >0 on micro-CT images.

Evaluation of Lung Injury and Fibrosis

Micro-CT-based outcome measurements have been validated by comparison to the established histologic and physiologic measurements in animal models of lung injury and fibrosis. In bleomycin (BLM)-induced murine lung damage, Cavanaugh and colleagues (2) reported that higher total percent lung damage with micro-CT measurements correlates with severity of lung damage by histologic analysis. De Langhe and colleagues (3) measured the degree of aerated lung with micro-CT and used micro-CT values as a surrogate marker to evaluate lung fibrosis in BLM-treated mice; they demonstrated that lungs of BLM-treated mice had significantly lower CT scan-derived aerated lung volumes than saline-treated control mice. Additionally,

the micro-CT values significantly correlated with the histopathologic scores of fibrosis, total lung collagen content, and measurements of physiologic lung function. A more recent *ex vivo* study by Scotton and colleagues (4) also demonstrated that high-resolution micro-CT provides a highly sensitive and quantitative measure for BLM-induced lung fibrosis in mice. Rodt and colleagues (5) reported a significant correlation between micro-CT-derived aerated lung volumes and histopathologic scores of fibrosis in an adenoviral TGF- β -induced murine model of lung fibrosis. Shofer and colleagues (6) developed a novel micro-CT technique to measure lung compliance; this innovative methodology was validated by quasistatic compliance measurement with a commercial ventilator system. This latter study suggests that micro-CT may also offer a noninvasive means for quantification of pulmonary physiology.

Inflammation and fibrosis are manifested by relative increases in tissue density with micro-CT scanning. Although micro-CT scans are sensitive enough to detect changes in lung tissue density, the current techniques do not distinguish between lung inflammation and fibrosis. Discrimination of lung inflammation from fibrosis will need subsequent histological analysis and related cellular (e.g., flow cytometric) analyses.

Three-Dimensional Assessment of Lung Fibrosis

Neither histomorphometry nor biochemical analyses provides information on the spatial and temporal distribution of lung fibrosis. Serial micro-CT scanning generates quantitative datasets that can be reconstructed to assess the topographical distribution of lung fibrosis and quantitative evaluation of disease severity (7). The fibrotic lesions in current animal models of lung fibrosis are characterized by spatial heterogeneity. Because three-dimensional reconstruction of micro-CT scans provides information of the entire thoracic cavity, micro-CT-based measurements are less likely to be influenced by the nonuniform distribution of lung fibrosis. Several micro-CT analysis protocols have been developed and validated for quantification of aerated lung volume (an inverse surrogate marker for pulmonary fibrosis) in mice and rats (3, 5, 7). A major challenge for quantification of aerated lung volume by

micro-CT scans is correct segmentation of airspaces from intrapulmonary fibrotic tissues and from the surrounding extrapulmonary tissues. Rodt and colleagues (5) applied a modified region-growing segmentation algorithm on each individual micro-CT scan using MevisLab software (MeVis Research, Bremen, Germany). They found a significant correlation between micro-CT-derived aerated lung volumes and the histopathology scores in adenoviral TGF- β -induced mouse lung fibrosis. However, the semiautomated quantification algorithm used in this study involves manual setting of the seeding points for the region growing algorithm. This may potentially result in an observer-induced assessment bias. To eliminate observer interference, De Langhe and colleagues (3) recently developed a fully automated micro-CT image analysis algorithm that used CTAn software (SkyScan, Kontich, Belgium) for segmentation of aerated lung volumes and CTVol software (SkyScan) for three-dimensional image construction. The validity of this technique in longitudinal *in vivo* quantitative assessment of pulmonary fibrosis was demonstrated in BLM-induced mouse lung fibrosis. Micro-CT imaging provides complementary data to more conventional measurements of lung fibrosis and, importantly, provides information on the distinct spatial distribution of fibrosis.

Motion Artifacts

During acquisition of CT scans, animals are subject to respiratory motion while under anesthesia. The dynamic respiratory movements can blur micro-CT images, particularly near the diaphragm. Various techniques have been developed to reduce the degree of motion artifacts to produce high-resolution micro-CT images. Prospective respiratory gating limits respiratory motion by synchronizing the scan to the breathing cycle through an external ventilator (8), and retrospective gating improves motion artifacts by postacquisition sorting of images and subjecting each image group to a single respiratory phase (9). Motion artifacts can also be reduced by shortening the scanning time. Flat-panel volumetric CT scans murine lungs at a resolution of $50\text{ }\mu\text{m}$ within 8 seconds, whereas most micro-CT instruments require minutes to scan to achieve the same resolution (10). The rapid acquisition of imaging data by flat-panel

volumetric CT dramatically reduces the susceptibility to motion artifacts. Although higher-energy photons (e.g., 80 keV) may speed up imaging data acquisition, the use of higher X-ray photon energies also reduces soft tissue contrast.

Radiation Toxicity

An inherent concern with the use of micro-CT is the exposure to X-radiation. X-radiation may potentially alter the phenotype and/or genotype of animals. This is a particular concern for longitudinal micro-CT scan studies requiring multiple scans. Several previous studies have examined unscanned and serially scanned mice for potential radiotoxic effects. One study showed that normal C57BL/6 mice scanned (80 kVp/50 mA) three times weekly for 6 weeks (resulting in a weekly dose of 0.84 Gy and a total study dose of 5.04 Gy) had no significant pathologic cardiopulmonary consequences compared with unirradiated mice; this was based on evaluations of the volume and density of the lung, the volume and ejection fraction of the left ventricle, and histological examinations of lung and myocardial tissues for inflammation and fibrosis (11). In a separate study, mice receiving a higher dose of X-radiation (7–9 Gy) did not develop significant lung fibrosis (12). It has been reported that phase shift X-ray and refraction X-ray CT scanners reduce the potential toxicity of radiation exposures (13).

MRI

MRI uses strong magnetic field and radio waves to form detailed images of organs and tissues in the body. It enables detection of anatomical and functional changes in the lung and has the potential to discriminate between inflammatory and fibrotic processes *in vivo*. MRI is free of ionizing radiation and therefore is less likely to influence the injury-repair process. MRI provides a useful tool for assessing the efficacy of antifibrotic drugs in preclinical studies.

Proton MRI

Although the conventional proton MRI has been used to detect BLM-induced lung injury in rats (14), the inherent air–tissue interface generates large magnetic susceptibility gradients, which cause significant loss of signals in standard MR pulse sequences (15). Ultrashort echo time (UTE)-MRI overcomes this limitation and

is capable of detecting small foci of inflammatory and/or fibrotic lesions, which remain undetectable in conventional MRI (16). In addition, the acquisition time in UTE-MRI is considerably reduced compared with conventional proton MRI (16). Thus, UTE-MRI allows for high-throughput measurements of a large number of animals per day.

Respiratory-Gated and Self-Gated MRI

Similar to micro-CT imaging, MRI suffers from breathing-related motion artifacts. Respiratory-gated MRI with synchronous ventilation minimizes respiratory motion artifacts by controlling breathing to the recovery period after data acquisition (17). However, mechanical ventilation in respiratory-gated MRI, particularly if applied repeatedly, may contribute to (or exacerbate) lung injury. AcidoCEST MRI is a technique that was originally developed for measurements of extracellular pH in stationary kidney, bladder, and solid tumors. Recently, respiratory-gated acidoCEST MRI has been developed to repetitively measure extracellular pH in BLM-injured mice (18).

Self-gated MRI corrects motion artifacts based on MRI signal intensity variations (19). Despite being less sensitive to the earlier stages of lung inflammation, this technique has been proven to provide more accurate information in visualizing and quantifying the progression of lung fibrosis in BLM-treated mice (20). It has been reported that MRI with a gradient-echo sequence produces sharper lung images even in spontaneously breathing rats (21). This technique uses image averaging to suppress motion artifacts due to respiratory movements.

Hyperpolarized Gas-Enhanced MRI

In contrast to the conventional MRI, which detects changes in lung structure with little or no capability to evaluate lung function, MRI with hyperpolarized gases provides both structural and functional information of the lung. Hyperpolarized ^{129}Xe MRI detects inhaled gaseous ^{129}Xe in the airspace, lung interstitium, and red blood cells. MRI of ^{129}Xe in these three different compartments provides a means to measure alveolar–capillary gas transfer. It has been shown that BLM treatment reduces ^{129}Xe signal intensity in all three of these compartments in rat lungs (22).

Impaired ^{129}Xe transfer to red blood cells was observed in regions of severe lung injury. Apparent diffusion coefficient (ADC) and fractional ventilation are commonly used metrics to evaluate lung function and structure. Hyperpolarized ^3He MRI analysis has shown that BLM- or radiation-exposed rats had significantly decreased fractional ventilation and ADC of ^3He compared with control animals (23, 24). Furthermore, areas of decreased ADC in radiation-treated rats correlated with lung fibrosis identified by histological analysis (24). Because ^3He gas has a larger gyromagnetic ratio and a higher polarization level than ^{129}Xe gas, ^3He MRI is more sensitive (by approximately one order of magnitude) than ^{129}Xe MRI.

Distinguishing Lung Fibrosis from Inflammation by MRI

MRI has the potential to differentiate lung inflammation and fibrosis by means of signal intensity and contrast enhancement. Polylysine–gadopentetate dimeglumine and gadolinium–diethylene triamine penta-acetic acid, macromolecular MRI contrast agents, have been used to distinguish alveolitis and pulmonary fibrosis in cadmium chloride–treated rats and in BLM-treated rabbits (25, 26). It has been reported that lung fibrosis in BLM-treated mice is manifested by an increased T_2 , whereas lung inflammation is associated with decreased T_2 , suggesting that spin-spin relaxation time may differentiate inflammation and fibrosis in the lung (27). However, an apparently opposite result has been observed in BLM-treated rats (28). To date, most of the studies suggest that signal intensity (S_0) measurements help differentiate lung inflammation and fibrosis (25, 26, 29, 30). A more recent study failed to confirm altered S_0 and T_2 relaxation in distinguishing pulmonary inflammation and fibrosis in BLM-treated rats (31). Although differentiation of inflammation and fibrosis is a particular interest in pulmonary research, MRI characteristics that reliably distinguish inflammation from fibrosis appear to be lacking.

Nuclear Imaging and Multimodality Imaging

Nuclear imaging uses radioisotope-labeled, small-molecule compounds, known as

tracers, to detect *in vivo* biological processes at the molecular level. PET and SPECT systems are common instruments that record gamma rays emitted by radiotracers. Most current nuclear imaging devices integrate PET and SPECT with high-resolution CT. These bimodal systems coregister molecular imaging data with fine structural data obtained by CT, enabling the precise anatomical localization of nuclear imaging signals.

PET and PET/CT Imaging of Glucose Metabolism

[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F -FDG), a ^{18}F -labeled glucose analog, is the most widely used PET tracer. It competes with glucose for uptake into metabolically active cells through glucose transporter-1. In the cytoplasm, ^{18}F -FDG is phosphorylated by hexokinase to deoxyglucose-6-phosphate. Radiolabeled deoxyglucose-6-phosphate does not undergo further metabolism and accumulates in cells. Metabolically (specifically, glycolysis) active cells have a higher rate of ^{18}F -FDG uptake and therefore have a higher accumulation of ^{18}F -FDG, which distinguishes them from quiescent normal cells on PET scans.

^{18}F -FDG PET is a sensitive imaging modality for the detection of cellular glucose uptake in rabbit models of lung inflammation and fibrosis as well as in human IPF (32, 33). PET scans have shown that lung uptake of ^{18}F -FDG after intravenous injection was increased in microcrystalline silica- or BLM-treated rabbits compared with control rabbits, and the ^{18}F -FDG signals persisted for several weeks in the lung (34).

Microautoradiographic analysis suggested that neutrophils were the primary cell type responsible for increased ^{18}F -FDG uptake in microcrystalline silica-challenged rabbits (34). (Myo)fibroblasts may contribute to prolonged ^{18}F -FDG uptake during the fibrotic phase in rabbit lungs; previous studies demonstrated that activated fibroblasts had increased glucose uptake and glycolysis (35). It has been found that pulmonary ^{18}F -FDG uptake in humans was related to disease severity of IPF, as assessed by quality of life measurements, lung volumes, and gas transfer (36). In approximately three fourths of patients with IPF, the most intense pulmonary ^{18}F -FDG uptake was localized to honeycomb cystic regions (34). Furthermore, pulmonary ^{18}F -FDG uptake significantly correlated

with lung function and global health score of patients with IPF (34). Despite these recent advances, current ^{18}F -FDG PET and PET/CT techniques are unable to distinguish IPF and nonspecific interstitial pneumonia (36).

PET and PET/CT Imaging of Proline Uptake

Pulmonary fibrosis is characterized by excessive production of extracellular matrix proteins, primarily collagens. Because collagens are rich in proline residues, specific PET scans have been developed to track the uptake of isotope-labeled proline analogs for the evaluation of lung fibrosis (37). In microcrystalline silica-induced rabbit model of silicosis, PET imaging detected heightened pulmonary uptake of Cis-4- ^{18}F -fluoro-L-proline (^{18}F -proline) in microcrystalline silica-challenged regions. The ^{18}F -proline signals were found to correlate with histopathologic scores of lung fibrosis in rabbits (38). A separate study with PET imaging confirmed increased ^{18}F -proline signals in the lungs of microcrystalline silica-treated rabbits (34). It appears that radiolabeled proline localized to fibroblasts in the silica-challenged regions, whereas fibroblasts at the nonfibrotic regions and the acellular fibrotic extracellular matrix were largely unlabeled (34). These results suggest that uptake of ^{18}F -proline by lung fibroblasts rather than incorporation of ^{18}F -proline into newly synthesized collagen contributes to increased PET signals in fibrotic rabbit lungs. ^{11}C -proline has also been used as a radiotracer in PET imaging of pulmonary fibrosis (34). The shorter half-life of ^{11}C (20 min) restricts PET imaging in a limited time, rendering ^{11}C -proline PET less practical than ^{18}F PET.

PET/CT and SPECT/CT Imaging of Receptors on the Cell Surface

Fibroblasts isolated from BLM-treated mice and patients with IPF express increased levels of somatostatin receptor (SSTR) (39). SSTR blockers reduce collagen deposition, improve pathologic scores, and increase the survival rate in BLM-treated mice (39). A ^{68}Ga -labeled peptide tracer (^{68}Ga -DOTANOC) that specifically binds SSTR with high affinity has been developed to track SSTR expression in patients with IPF (40). PET/CT studies found that the uptake of ^{68}Ga -DOTANOC was primarily located at the peripheral and subpleural regions,

typically affected in IPF lungs. More intense ^{68}Ga -DOTANOC signals were observed at the regions between honeycomb cysts and ground-glass opacity, where fibroblastic foci are most represented. Patients with NSIP had no significant pulmonary uptake of ^{68}Ga -DOTANOC (41). A previous study has shown that normal human subjects did not exhibit uptake of ^{68}Ga -DOTANOC in the lungs (42). The PET/CT imaging studies confirm increased SSTR expression in IPF; further studies are required to determine if ^{68}Ga -DOTANOC PET/CT imaging is a valid approach for testing the antifibrotic efficacy of candidate drugs.

Overexpression of $\alpha_v\beta_6$ integrin by alveolar epithelial cells plays a crucial role in the pathogenesis of pulmonary fibrosis. Measurements of $\alpha_v\beta_6$ levels in lung tissues have been restricted to *ex vivo* immunohistochemical analysis. In a recent study, ^{111}In -labeled peptides specific to $\alpha_v\beta_6$ (A20FMDV2) were developed and used to measure $\alpha_v\beta_6$ expression in BLM-treated mouse lungs by SPECT/CT (43). The authors found that lung ^{111}In -A20FMDV2 signals were increased in BLM-treated mice compared with saline-treated control mice. The highest level of radioactive signals was detected 1 hour after intravenous administration of ^{111}In -A20FMDV2. Pretreatment of the mice with $\alpha_v\beta_6$ -blocking antibodies attenuated the binding of ^{111}In -A20FMDV2 in mouse lungs. In contrast, ^{111}In -labeled control peptides (^{111}In -A20FMDVran) did not show significant binding to mouse lungs. Quantitative analysis demonstrated that SPECT signals correlate with the levels of hydroxyproline, $\alpha_v\beta_6$ protein, and messenger RNA of the β_6 subunit. Together, this study suggests that SPECT/CT provides a useful means for noninvasive measures of endogenous $\alpha_v\beta_6$ and may potentially be used for stratifying therapies for patients with IPF.

Macrophages express a high level of benzodiazepine-like receptors. ^{11}C -R-PK11195 tracers have been developed to track macrophage accumulation in the lung. PET scan studies have shown that an increase in lung ^{11}C -R-PK11195 signals is associated with decreased macrophage clearance in microcrystalline silica-treated rabbits (34). This finding supports the hypothesis that a deficient macrophage clearance is associated with pulmonary fibrosis.

Table 1. Comparison of Current Imaging Modalities

Feature	Modality			
	Micro-CT	MRI	Nuclear Medicine Imaging (PET/SPECT)	Bimodality Imaging (PET/CT, SPECT/CT)
Spatial resolution	Excellent (up to 1 μm)	Good (up to 25–100 μm)	Poor (~ 1 mm)	Excellent
3D lung assessment	Yes	Yes	Yes	Yes
Assessment of lung structure, function, and/or molecular mechanisms	Structure	Structure and function	Molecular mechanisms	Molecular mechanisms and structure
Potential for distinguishing lung inflammation versus fibrosis	No	Yes	Yes (with specific molecular tracers)	Yes (with specific molecular tracers)
Acquisition time	Fast (minutes)	Slow (minutes to hours)	Fast (minutes)	Fast
Ionizing radiation	Yes	No	Yes	Yes
Cost	Less expensive (\$200,000–\$400,000)	Most expensive ($\sim$ \$2,000,000)	Expensive ($<$ \$1,000,000)	Expensive

Definition of abbreviations: CT, computed tomography; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single photon emission computed tomography.

Conclusions

Noninvasive imaging offers exciting new opportunities for preclinical research in lung fibrosis. Each of these imaging modalities has advantages and disadvantages (Table 1). Micro-CT enables dynamic assessment of the progression and topographic distribution of lung fibrosis in small animal models. MRI provides valuable information in the context of profiling antifibrotic compounds. Of particular interest is the potential of

hyperpolarized gaseous MRI for the evaluation of compounds designed to improve lung function. Lung fibrosis in most animal models results from inflammation, whereas IPF is primarily a fibrotic disease with relatively less inflammation in its late stages. The effectiveness of preclinical testing of antifibrotic compounds in animal models requires distinguishing the effects of compounds on the inflammatory process versus fibrosis progression. MRI has shown the potential to fulfill this requirement.

Finally, nuclear imaging provides a versatile tool to study specific molecular events in lung fibrosis, validate biomarkers for the diagnosis of lung fibrosis, and test the efficacy of potential antifibrotic drugs in live animals. It is clear that noninvasive imaging techniques will play an increasingly important role in preclinical lung fibrosis research. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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