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Original Contribution

Targeting the isoprenoid pathway to abrogate progression of pulmonary fibrosis

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ABSTRACT

Fibrotic remodeling in lung injury is a major cause of morbidity. The mechanism that mediates the ongoing fibrosis is unclear, and there is no available treatment to abate the aberrant repair. Reactive oxygen species (ROS) have a critical role in inducing fibrosis by modulating extracellular matrix deposition. Specifically, mitochondrial hydrogen peroxide (H_2O_2) production by alveolar macrophages is directly linked to pulmonary fibrosis as inhibition of mitochondrial H_2O_2 attenuates the fibrotic response in mice. Prior studies indicate that the small GTP-binding protein, Rac1, directly mediates H_2O_2 generation in the mitochondrial intermembrane space. Geranylgeranylation of the C-terminal cysteine residue (Cys^{189}) is required for Rac1 activation and mitochondrial import. We hypothesized that impairment of geranylgeranylation would limit mitochondrial oxidative stress and, thus, abrogate progression of pulmonary fibrosis. By targeting the isoprenoid pathway with a novel agent, digeranyl bisphosphonate (DGBP), which impairs geranylgeranylation, we demonstrate that Rac1 mitochondrial import, mitochondrial oxidative stress, and progression of the fibrotic response to lung injury are significantly attenuated. These observations reveal that targeting the isoprenoid pathway to alter Rac1 geranylgeranylation halts the progression of pulmonary fibrosis after lung injury.

Introduction

Pulmonary fibrosis is a devastating lung disease that is increasing in incidence, and no current therapeutic modalities are available to halt its progression. In particular, idiopathic pulmonary fibrosis (IPF), which is the most common form, has a median survival of 3–5 years after the diagnosis [1–3]. The factors that regulate the process of tissue remodeling in pulmonary fibrosis are poorly understood. Defining the molecular mechanisms that mediate pulmonary fibrosis is urgently needed to prevent the development and/or halt the progression of the disease.

Reactive oxygen species (ROS) have a crucial role in inducing a fibrotic response to lung injury by modulating extracellular matrix deposition. Alveolar macrophages are critical in regulating host responses to lung injury, and H_2O_2 production by macrophages is directly linked to pulmonary fibrosis [4,5]. The primary source of H_2O_2 in alveolar macrophages in the setting of fibrosis is the mitochondria [4–6]. Moreover, inhibition of mitochondrial H_2O_2 or administration of catalase attenuates the fibrotic phenotype in mice [4,5,7].

The Rho GTP-binding proteins, including Rac1, play an important role in host defense. Rac1 regulates several cellular functions in macrophages, such as cell adhesion, actin polymerization and migration, and phagocytosis[8–10]. Rac1 activation also increases

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the generation of H_2O_2 in nearly every cell type [7,11–14]. In macrophages, Rac1 directly mediates H_2O_2 generation in the mitochondrial intermembrane space [6]. Rac1 is biologically relevant in that mice harboring a conditional deletion of Rac1 in macrophages are protected from developing asbestos-induced pulmonary fibrosis [6,7].

The C-terminal cysteine residue in Rho GTPases, such as Cys^{189} in Rac1, can be modified by geranylgeranylation with the requisite geranylgeranyl moiety derived from the isoprenoid pathway. This posttranslational modification is necessary for activation, interaction with other proteins, and mitochondrial import [6,15]. Because mitochondrial Rac1 activity is linked to the development of the fibrotic phenotype in mice, we sought to target the isoprenoid pathway to inhibit Rac1 mitochondrial import as a therapeutic maneuver to prevent the fibrotic response to lung injury. Statins, which block the rate-limiting enzyme HMG-CoA reductase of the isoprenoid pathway, have been associated with interstitial lung abnormalities in smoking individuals likely due to inhibition of several intermediates in the isoprenoid pathway [16]. Thus, we chose to use a more specific inhibitor of geranylgeranylation by inhibiting geranylgeranylpyrophosphate (GGPP) synthase, the enzyme that catalyzes the next to the last step in the posttranslational modification of Rac1. Our novel observations reveal that targeting the isoprenoid pathway to alter Rac1 geranylgeranylation halts progression of pulmonary fibrosis after lung injury.

Materials and methods

Materials

Bleomycin was obtained from the University of Iowa Hospital and Clinics hospital stores. Chrysotile was provided Dr. Peter S. Thorne (College of Public Health, University of Iowa, Iowa City, IA). *p*-Hydroxylphenyl acetic acid (pHPA), horseradish peroxidase (HRP), α-ketoglutarate, and NADPH were purchased from Sigma Chemical Company (St. Louis, MO).

Human subjects

The Human Subjects Review Board of the University of Iowa Carver College of Medicine approved the protocol of obtaining alveolar macrophages from normal volunteers and patients with IPF and asbestosis. Normal volunteers had to meet the following criteria: (1) age between 18 and 55 years; (2) no history of cardiopulmonary disease or other chronic disease; (3) no prescription or nonprescription medication except oral contraceptives; (4) no recent or current evidence of infection; and (5) lifetime nonsmoker. Alveolar macrophages were also obtained from patients with IPF. Patients with IPF had to meet the following criteria: (1) FVC and DLCO at least 50% predicted; (2) current nonsmoker; (3) no recent or current evidence of infection; and (4) evidence of restrictive physiology on pulmonary function tests and interstitial fibrosis on chest computed tomography. Fiberoptic bronchoscopy with bronchoalveolar lavage were performed after subjects received intramuscular atropine (0.6 mg) and local anesthesia. Three subsegments of the lung were lavaged with five 20- ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%.

Mice

Wild-type C57Bl/6 mice were from Jackson Laboratories (Bar Habor, ME). The University of Iowa Institutional Animal Care and Use Committee approved all protocols. After equilibration, osmotic pumps (Alzet, Cupertino, CA) containing either vehicle (water) or DGBP (0.2 mg/kg/day) were implanted subcutaneously, as describe previously [17]. Rac1 null and Rac2 knockout mice have been previously described [5,18]. Briefly, Rac1 null mice are conditional and were generated using $LysM^{cre}$ to selectively delete Rac1 from cells of the granulocyte/monocyte lineage. The Rac2 knockout mice were generated using conventional gene targeting to delete the Rac2 gene as Rac2 is only expressed in cells of the granulocyte/monocyte lineage. Bleomycin (1.3–2.0 U/kg) or chrysotile (100 μg) was administered intratracheally. Mice were euthanized and fibrosis was determined as previously described [6,19].

Cell culture

THP-1 macrophages were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 media supplemented with fetal bovine serum and penicillin/streptomycin. All experiments were performed with media supplemented with 0.5% serum.

Synthesis of digeranyl bisphosphonate (DGBP)

DGBP (U.S. Patent 7,268,164) was synthesized as previously described [20).

Determination of H_2O_2 generation

Extracellular H_2O_2 production was determined fluorometrically, as previously described [4]. Mitochondrial H_2O_2 was measured by suspending mitochondria in phenol red-free Hanks' balanced salt solution supplemented with 6.5 mM glucose, 1 mM Hepes, 6 mM sodium bicarbonate, 1.6 mM pHPA, 0.95 μg/ml HRP, and 5 mM α-ketoglutarate.

Isolation of mitochondria and membrane fractions

Mitochondria and cytoplasm were isolated as previously described [4,7].

Rac1 and Rac2 GTPase activation assays

Rac1 and Rac2 activities were determined using a bead pull-down kit (Cytoskeleton Inc.) or Rac1 activity was determined using the G-LISA kit (Cytoskeleton Inc.), according to the manufacturer's protocols. Negative and positive lysate controls were incubated with GTPγS or GDP, respectively, during PAK-binding domain–GST pull-down for Rac1 and Rac2. Bound protein was eluted and separated by SDS-PAGE. Immunoblots were probed with an antibody specific to Rac1 or Rac2, and GST expression was determined by Coomassie staining, as a loading control. Active Rac1 was also determined by the binding of Rac1 to PAK-PBD beads immobilized in a 96-well plate using G-LISA. The bound active Rac1 was detected with a Rac1-specific antibody. Absorbance was read at 490 nm and normalized to protein concentration in the lysate sample.

Hydroxyproline assay

Lung tissue was dried to stable weight and acid-hydrolyzed with 6 N HCl for 24 h at 120 °C. Hydroxyproline concentration normalized to dry weight of the lung was determined as described previously [5].