

Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts

Meghna Waghray, Zongbin Cui, Jeffrey C. Horowitz, Indhu M. Subramanian, Fernando J. Martinez, Galen B. Toews, and Victor J. Thannickal¹

Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Michigan Medical Center, Ann Arbor Michigan, USA



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SPECIFIC AIMS

Epithelial-mesenchymal interactions orchestrate complex tissue remodeling events in normal physiological (e.g., organogenesis/development and normal tissue repair) and diverse pathophysiological processes (e.g., carcinogenesis and tissue fibrosis). Reactive oxygen species, such as hydrogen peroxide (H_2O_2), are generated in a compartmentalized manner (intracellular vs. extracellular) in response to specific growth factors in nonphagocytic cells. The aims of this study were to determine whether 1) transforming growth factor- β 1 (TGF- β 1)-induced extracellular H_2O_2 secretion by fibroblasts from patients with idiopathic pulmonary fibrosis (IPF) mediates death of overlying small airway epithelial cells (SAECs) and 2) H_2O_2 secretion by heterogeneous IPF fibroblasts is specific to the myofibroblast phenotype.

PRINCIPAL FINDINGS

1. Fibroblasts, but not airway epithelial cells, are activated by TGF- β 1 to secrete H_2O_2 into the extracellular space

TGF- β 1 is a multifunctional cytokine involved in a number of physiological/pathological processes, including embryogenesis, carcinogenesis and tissue repair/fibrosis. Its effects on target cells are contextual and highly cell specific, often with divergent effects on epithelial cells and mesenchymal cells. We have previously demonstrated that TGF- β 1 stimulates extracellular H_2O_2 production in cultured normal human fetal lung fibroblasts. We first determined whether TGF- β 1 induces extracellular release of H_2O_2 from fibroblasts isolated/cultured from lungs of IPF patients and from normal human SAECs. TGF- β 1 (2 ng/mL \times 16 h) markedly induced the extracellular release of H_2O_2 by IPF fibroblasts; however, similar responses were not observed in SAECs. The TGF- β 1-induced H_2O_2 produc-

tion in IPF fibroblasts was blocked by diphenyliodonium (DPI, 10 μ M), an NAD(P)H oxidase (Nox)/flavoenzyme inhibitor, suggesting that the extracellular H_2O_2 generation by IPF fibroblasts is related to plasma membrane-associated Nox activity.

2. TGF- β 1 signaling of IPF fibroblasts induces death of SAECs by an H_2O_2 -dependent paracrine mechanism

H_2O_2 , and other ROS, may function as *intracellular* signaling molecules; however, *extracellular* (paracrine) signaling roles, particularly in the mediation of cell-cell interactions, are not well defined. In comparison to fibroblasts/mesenchymal cells, epithelial cells are generally more sensitive to death-inducing effects of exogenous H_2O_2 . To determine whether fibroblast-derived H_2O_2 may “signal” the death of overlying epithelial cells, we designed a coculture system of IPF fibroblasts and SAECs separated by a 0.4 μ m porous polyethyleneterephthalate (PET) membrane (Fig. 1A). IPF fibroblasts, which were TGF- β 1-stimulated and washed prior to coculture, induced loss of overlying SAEC viability when assessed by direct counting of cells with a coulter counter at 48 h after coculture (Fig. 1B). This effect was inhibited by introduction of catalase in the coculture system or by pretreating IPF fibroblasts with DPI before coculture (Fig. 1B), suggesting that reduction in the number of SAECs was mediated in part by extracellular H_2O_2 . Assessment of rates of SAEC proliferation by BrdU labeling (ELISA) showed no significant differences under these conditions. Assessment of apoptosis by staining for phosphatidylserine (PS), both qualitatively and quantitatively, demonstrated increased rates of SAEC death when these epithelial cells were cocultured with TGF- β 1-stimulated IPF fibroblasts. Induction

* Correspondence: Division of Pulmonary and Critical Care Medicine, University of Michigan Medical Center, 6301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109, USA. E-mail: vjt@umich.edu

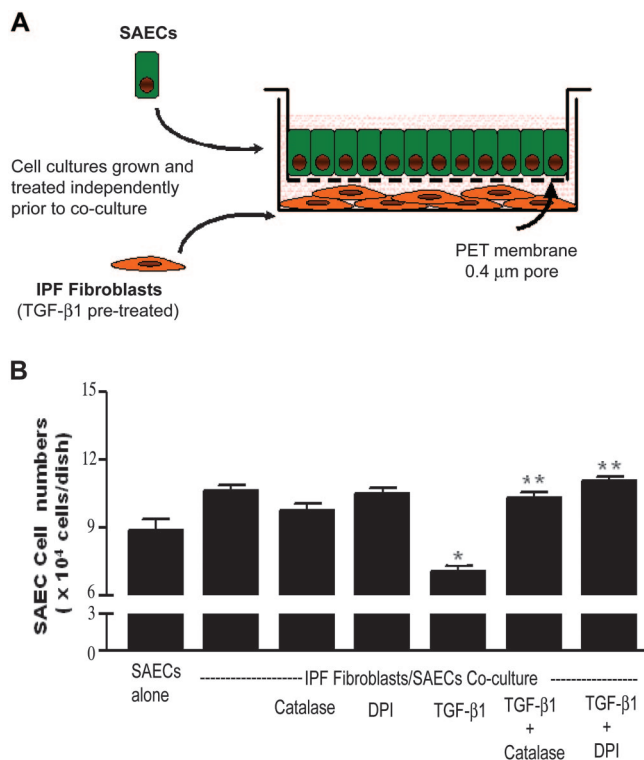


Figure 1. TGF- β 1 signaling of IPF fibroblasts induces H_2O_2 -mediated loss of SAEC viability. **A)** Coculture system of epithelial cells and fibroblasts. IPF fibroblasts and SAECs were grown separately on the lower wells and upper chambers, respectively, of a Transwell coculture system. IPF fibroblasts were growth-arrested at 80–90% confluence, stimulated with/without TGF- β 1, and washed twice before coculture. SAECs were grown to near-confluence before coculture. **B)** Effects of TGF- β 1-stimulated IPF fibroblasts on viability of SAECs. IPF fibroblasts pretreated with/without TGF- β 1 (2 ng/mL \times 16 h) in the absence/presence of diphenyliodonium (DPI, 10 μ M added 30 min prior to coculture) were washed before introduction of SAECs. Catalase (3000 U/mL) was added to coculture media for the duration of coculture. Cell numbers were assessed after 48 h of coculture by Coulter counting. Values represent mean \pm SE ($n=3$, * $P<0.05$ vs. coculture without TGF- β 1, ** $P<0.05$ vs. coculture with TGF- β 1). Three replicate experiments demonstrated similar results.

of SAEC death was inhibited when extracellular H_2O_2 was reduced by addition of catalase to the coculture system or by the selective blockade of H_2O_2 production by IPF fibroblasts. These results suggest that the observed reduction in SAEC numbers when cocultured with TGF- β 1-stimulated IPF fibroblasts is accounted for by death of SAECs and not by inhibition of cell proliferation. Furthermore, SAEC death by apoptosis, and possibly early necrosis, is mediated by the secretion of H_2O_2 selectively induced by TGF- β 1 signaling of IPF fibroblasts.

3. Secretion of H_2O_2 by IPF fibroblasts is specific to the myofibroblast phenotype

Fibroblasts isolated from IPF lungs are phenotypically heterogeneous with regard to myofibroblast

differentiation. Myofibroblasts are contractile cells with high synthetic capacities and are key effector cells in fibrotic diseases. We examined the possibility that secretion of H_2O_2 in response to TGF- β 1 stimulation may be specific to this cellular phenotype. H_2O_2 -secreting cells were identified by inducing tyrosine cross-linking of the overlying matrix with a FITC-labeled phenolic compound, as described previously. Myofibroblasts were identified by immunofluorescence staining for α -smooth muscle actin (SMA). TGF- β 1 stimulation of IPF fibroblasts induced a heterogeneous pattern of H_2O_2 secretion (FITC-green) that appears to closely associate with α -SMA-positive myofibroblasts (TRITC-red) (Fig. 2). These results suggest that myofibroblasts are primarily responsible for the generation of extracellular H_2O_2 in response to TGF- β 1 stimulation.

CONCLUSIONS AND SIGNIFICANCE

Our studies demonstrate for the first time a paracrine signaling role for H_2O_2 in selective induction of epithe-

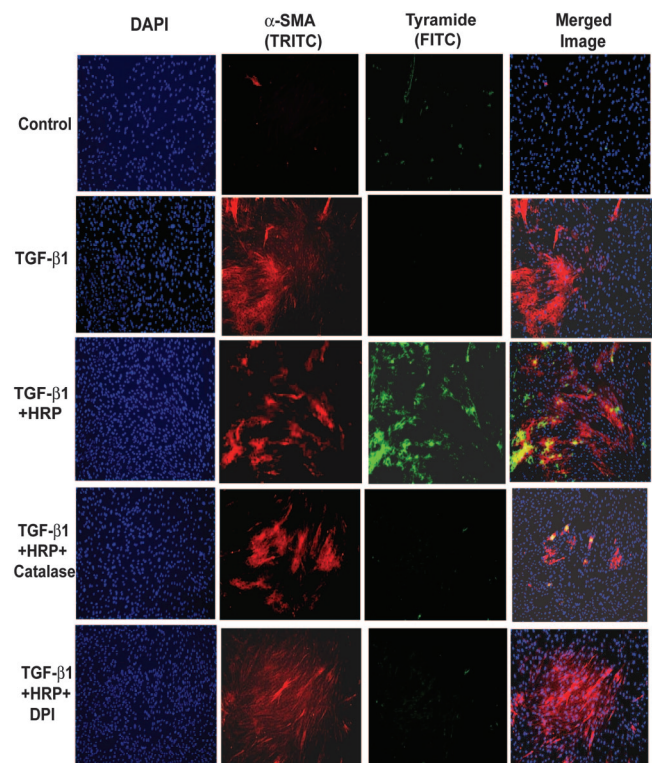
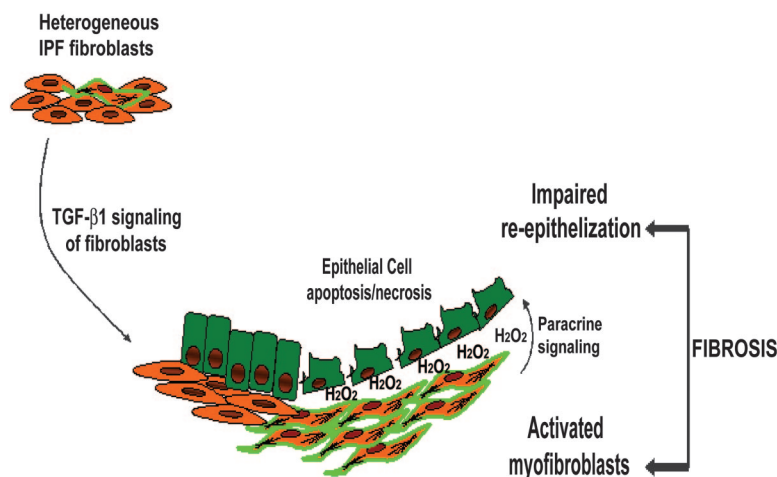


Figure 2. Localization of cellular H_2O_2 production. IPF fibroblasts were grown to 80–90% confluence, serum-starved for 24 h and treated with/without TGF- β 1 (2 ng/mL) for 16 h. Cells were washed and incubated in assay medium containing tyramide-FITC (green) in the absence/presence of horseradish peroxidase (HRP, 5 U/mL, incubated during the assay period) and DPI (10 μ M, added 30 min before assay) for 1 h. Cells were then washed, fixed, and stained for α -smooth muscle actin (SMA) with secondary antibody conjugated to TRITC (red). Counterstaining for nuclei was with DAPI (blue).

Figure 3. H_2O_2 secreted by myofibroblasts is a diffusible, paracrine mediator of epithelial cell death. IPF tissue-derived fibroblasts represent a heterogeneous population of cells, including myofibroblasts. TGF- β 1 signaling of IPF fibroblasts induces death of adjacent epithelial cells. This effect is mediated, at least in part, by diffusible paracrine signaling by H_2O_2 . Myofibroblasts are the principal cells responsible for extracellular H_2O_2 production. This novel mechanism for paracrine signaling by H_2O_2 may be important in the dysregulated epithelial-mesenchymal cross-talk that characterizes chronic fibrotic diseases, including IPF.



lial cell death by a specific population of mesenchymal cells (**Fig. 3**). Epithelial-mesenchymal interactions are critical events in tissue remodeling and fibrosis. Increased rates of epithelial cell injury/death characterize many progressive fibrotic diseases, including IPF. Human fibrotic diseases are also characterized by over-expression/activation of TGF- β 1 and the persistence of

myofibroblasts in injured tissues. Our study supports the concept that a dysregulated tissue repair process characterized by activated myofibroblasts may induce epithelial cell death and impair re-epithelialization; this may result in a repetitive cycle of epithelial injury and aberrant repair responses that culminates in progressive organ fibrosis. **FJ**