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Oxidative stress contributes to the induction and persistence of TGF-β1 induced pulmonary fibrosis

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ABSTRACT

Oxidative stress with reactive oxygen species (ROS) can contribute to the pathogenesis of idiopathic pulmonary fibrosis. Antioxidant enzymes, such as extracellular superoxide dismutase (ECSOD), may modulate the injury and repair components of the fibrogenic response. Here we determined whether ECSOD could attenuate experimental TGF-β1-induced persistent lung fibrosis. In this study, primary human lung fibroblasts, MRC-5 fibroblasts and A549 epithelial cells were exposed to recombinant active TGF-β1. An adenovirus vector that expresses human ECSOD (AdECSOD) was constructed and rats were endotracheally intubated with an adenoviral vector encoding active TGF-β1 (AdTGF-β1), AdECSOD or a control vector (AdDL70) alone or in combinations AdTGF-β1/AdDL70 or AdTGF-β1/AdECSOD. TGF-β1 alone induced fibrotic responses and significantly down-regulated endogenous ECSOD gene expression both in vitro and in vivo and caused oxidative stress in rat lung, associated with increased levels of activated TGF-β1 in lung fluid and tissue. ECSOD protein was markedly reduced in the interstitium and fibrotic foci in TGF-β1 induced experimental lung fibrosis. The fibrotic response caused by AdTGF-β1 was markedly attenuated by concomitant gene transfer using AdECSOD, detected by lung function measurements, histologic and morphometric analysis, hydroxyproline content and fibrosis-related gene expression. In addition, the oxidative stress and increased presence of activated TGF-β1 in rat lung induced by AdTGF-β1 was significantly reduced by ECSOD gene transfer. These findings suggest a substantial role for oxidative stress in the pathogenesis of TGF-β1 driven persistent pulmonary fibrosis and enhanced presence of ECSOD can inhibit latent TGF-β1 activation by ROS and diminish subsequent fibrotic responses.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, debilitating and lethal fibrotic disorder with progressive worsening of pulmonary function and characterized by persistent structural alterations of lung parenchyma with an abnormal accumulation of fibroblasts/myofibroblasts and formation of fibroblastic foci in the lung, along with excessive deposition of extracellular matrix (ECM) components (Gross and Hunninghake, 2001; Katzenstein and Myers, 1998; Kuhn and McDonald, 1991; Pardo and Selman, 2002; Selman et al., 2001). The precise etiology of IPF remains elusive. Recent evidence suggests that, amongst other processes, oxidative stress and reactive oxygen species (ROS) may play a role in the pathobiology and progression of this fatal disease (Bocchino et al., 2010; Daniil et al., 2008; Hecker et al., 2009; Kinnula and Myllarniemi, 2008). Possible mechanisms involve ROS damage to elements of ECM, including heparan sulphate (Kliment et al., 2008), hyaluronic acid (Gao et al., 2008; Zelko and Folz, 2010) and syndecan-1(Kliment et al., 2009), or through activation of metalloproteinases (Kinnula et al., 2005). Initiation of repair processes after tissue injury and the onset of scarring or fibrosis involves multiple aspects of the host inflammatory response, including oxidative stress. A recent proteomic analysis of IPF tissue showed down-regulation of anti-oxidant proteins, including catalase (Korfei et al., 2011), and, indeed, enhancing anti-oxidant activity through administration of N-acetyl Cysteine (NAC) has shown some limited therapeutic efficacy in human trials for IPF, thought to mitigate the ROS mediated damage, but possibly involving other anti-oxidant effects (Demedts et al., 2005).

TGF-β1, a potent stimulator of myofibroblast differentiation, proliferation and ECM production, has been widely implicated in the initiation and progression of fibrosis (Verma and Slutsky, 2007).

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Strong evidence for the important role TGF-β1 plays in pulmonary fibrosis has been provided by our previous work demonstrating that transient overexpression (7–10 days) of (exogenous) active TGF-β1 by adenoviral vector gene transfer to rodent lung, induces autocrine expression and activation of endogenous TGF-β1, followed by severe and persistent fibrosis out to 64 days, without persistent overt inflammatory responses in the tissue (Sime et al., 1997). Endogenous TGF-β1 is produced as a latent complex containing a TGF-β1 homodimer noncovalently bound to the latent associated peptide (LAP), which in turn is tightly linked to the matrix through latent TGF-β binding protein (LTBP) (Wipff and Hinz, 2008). TGF-β1 needs to be liberated from this latent complex to be biologically active and functional and this activation is regarded as an essential element in the regulation of the biology of this cytokine (Annes et al., 2003; Khalil, 1999; Rifkin, 2005). Progression of fibrosis is thought to involve continued stimulation of fibroblasts and myofibroblasts by active TGF-β1, acting through signaling pathways involving the Smad3 cytoplasmic cascade (Bonniaud et al., 2005a,b). The activation of TGF-β from its latent complex, targeted to the extracellular matrix, involves a number of seemingly different processes, including, amongst others, proteolysis, integrin interaction, thrombospondin, pH changes and reactive oxygen species (Annes et al., 2003).

Human lungs are exposed to higher concentrations of oxygen compared to other organs, and, thus are particularly susceptible to oxidative injury (Bargagli et al., 2009; Kliment and Oury, 2010). Under physiologic conditions, a delicate balance is maintained between the generation of ROS and antioxidant defense systems, and disruption of this balance inevitably leads to oxidative stress with subsequent tissue injury and remodeling (Valko et al., 2007).

Oxidative stress induced by generation of superoxide, O_2^-, a type of ROS generated from one-electron reduction of molecular oxygen, contributes to the pathogenesis of a wide range of medical conditions (Pervaiz and Clement, 2007; Dikalova et al., 2010; Guzik et al., 2002; McCann et al., 2008), including pulmonary diseases (Gongora et al., 2008). Superoxide dismutases (SODs), including the Cu,Zn isoform (CuZnSOD or SOD1), the Mn isoform of the mitochondria (MnSOD or SOD2) and the major extracellular isoform (ECSOD or SOD3) are the only family of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (Nozik-Grayck et al., 2005) and play a crucial role in regulating oxidative stress (Kinnula and Crapo, 2003).

The ECSOD isoenzyme is located predominantly in the extracellular space and is the major extracellular antioxidant enzyme present in the lung (Ganguly et al., 2009). Accumulating evidence has implicated disordered regulation of oxidative stress in both IPF and experimental lung fibrosis, with a lack of ECSOD detected in fibrotic lesions in IPF lungs (Kinnula et al., 2006; Kliment et al., 2009). Also, it was demonstrated that lung specific transgenic overexpression of ECSOD could protect against bleomycin- or radiation-induced lung injury and thus interfered in the subsequent development of fibrosis (Bowler et al., 2002; Kang et al., 2003; Rabbani et al., 2005; Van Rheen et al., 2010). Furthermore, ECSOD knockout mice were more susceptible to pulmonary damage after bleomycin or asbestos exposure (Fattman et al., 2003a, 2006). Whether ECSOD protects against ROS mediated damage and reduced requirement for repair and/or is involved in mediating the initiation and/or progression of fibrosis cannot be determined from these studies.

Previously we described an experimental model of pulmonary fibrosis with persistent matrix deposition and alteration of lung compliance (stiffness) that depends solely on adenovirus vector mediated transient expression (7–10 days) of activated TGF-β1 (AdTGF-β1) in the adult lung, without overt evidence of tissue damage while accompanied by a mild and transient innate inflammatory response (Sime et al., 1997). In this model, persistence of fibrosis depends on endogenous gene expression patterns that ensue after stimulation by exogenous active TGF-β1 (Ask et al., 2008a,b; Sime et al., 1997). Here we used this model to show that enhanced expression of active TGF-β1 can directly induce oxidative stress in the lung and that transient co-expression of ECSOD in the lung alleviates the oxidative stress caused by TGF-β1, reduces the innate inflammatory response, as well as levels of activated TGF-β1 present in the lung and attenuates the persistent pulmonary fibrosis.

2. Materials and methods

2.1. Recombinant adenovirus

A replication-deficient adenovirus that expresses biologically active human ECSOD (AdECSOD) was constructed using procedures as previously described (Palmer and Ng, 2008). AdTGF-$\beta1^{223/225}$ expresses biologically active porcine TGF-β1 and control vector (AdDL70) has no insert as described previously (Sime et al., 1997).

2.2. Cell culture

Primary normal (control) adult lung fibroblasts (NLF) were isolated by outgrowth culture from non-involved lung tissue from a patient with lung cancer. Human fetal lung fibroblasts (MRC-5) and human type II alveolar epithelial cells (A549) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were incubated at 37 °C and were serum starved for 24 h before stimulation with 5 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) for the indicated times.

2.3. RNA interference for ECSOD knockdown

For RNA interference (RNAi), MRC-5 cells were transfected with either human SOD3 Pre-design Chimera RNAi (10 mM) for ECSOD RNAi knockdown or Pre-design Chimera RNAi (Naito 1) (10 mM) as negative control (Abnova). Transfections were conducted using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations. The efficacy of RNAi-mediated gene knockdown was assessed 48 h after transfection. Cells were serum starved for 24 h before they were stimulated with 5 ng/ml recombinant human TGF-β1 (R&D Systems) for 48 h.

2.4. Sircol assay

Collagen concentration in MRC-5 cell culture medium was determined by Sircol assay (Biocolor, County Antrim, UK) according to the manufacturer's recommendations.

2.5. Animal transfection

All animal experiments were approved by the Animal Research Ethics Board at McMaster University. Female Sprague-Dawley rats (225–250 g, Charles River Laboratories, Montreal, QC, Canada) received 2×10^8 plaque forming units (PFU) AdTGF-β1 alone or AdDL70 alone or in combinations AdTGF-β1/AdDL70 or AdTGF-β1/AdECSOD (2×10^8 PFU each) via endotracheal intubation under isoflurane anesthesia (MTC Pharmaceuticals, Cambridge, ON, Canada). Rats were sacrificed at day 7, 14, 21 and 28 post adenovirus exposure. BAL was performed as previously described (Sime et al., 1997). Following BAL, the left lung was inflated with and fixed in 10% formalin for 48 h. The right lung was frozen in liquid nitrogen and stored at −80 °C. Lung elastance was assessed by pressure–volume loops at day 21 and 28 with Flexivent (v5.1, Scireq, Montreal, QC, Canada), as described previously (Ask et al., 2008b).