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Research Article

Targeted inhibition of disheveled PDZ domain via NSC668036 depresses fibrotic process

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ABSTRACT

In this study, we determined the effects of transforming growth factor-beta (TGF-β) and Wnt/β-catenin signaling on myofibroblast differentiation of NIH/3T3 fibroblasts in vitro and evaluated the therapeutic efficacy of NSC668036 in bleomycin-induced pulmonary fibrosis murine model. In vitro study, NSC668036, a small organic inhibitor of the PDZ domain in Dvl, suppressed β-catenin-driven gene transcription and abolished TGF-β1-induced migration, expression of collagen I and α-smooth muscle actin (α-SMA) in fibroblasts. In vivo study, we found that NSC668036 significantly suppressed accumulation of collagen I, α-SMA, and TGF-β1 but increased the expression of CK19, Occludin and E-cadherin that can inhibit pulmonary fibrogenesis. Because fibrotic lung exhibit aberrant activation of Wnt/β-catenin signaling, these data collectively suggest that inhibition of Wnt/β-catenin signaling at the Dvl level may be an effective approach to the treatment of fibrotic lung diseases.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a serious disease characterized by excessive fibroblast proliferation and extracellular matrix (ECM) deposition that disrupts the normal architecture of the pulmonary alveoli [1]. The key pathological feature of IPF is fibroblastic foci diffused distribution in the lung that is highly collagen synthetic, lead to pulmonary ventilation disorder [2]. Residual lifetime is generally not more than 5 years after diagnosis [3]. Unfortunately, current therapies are ineffective for the disease.

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Replacement of injured tissue with a fibrotic scar is a complex and dynamic process involving the interplay of many cell types, matrix proteins and some important signal pathways [1,4,5]. Numerous studies have shown that myofibroblast is an important cell during tissue repair and fibroblast-to-myofibroblast transdifferentiation plays a key role in pulmonary fibrogenesis process [6–8]. Myofibroblasts are specialized fibroblasts that possess the morphologic and biochemical characteristics of both fibroblasts and smooth muscle cells, which have been proposed as the main source of ECM within the impaired lung of patients with IPF [7,9].

Transforming growth factor β (TGF-β) is highly expressed in pulmonary fibrosis and generally acknowledged as the master regulator of fibroblast activation and myofibroblast differentiation [10–12]. Ligand binding to TGF-β receptor causes phosphorylation of cytoplasmic Smad2 or Smad3, promoting Smad hetero complex formation and translocation to nuclear. The Smad complex selectively binds to Smad-binding elements to direct transcriptional regulation of target genes [13]. It has been reported that both in vivo and in vitro TGF-β alters the fibroblast phenotype to myofibroblasts including increased expression of α-smooth muscle actin (α-SMA) and collagen synthesis [14]. TGF-β1, one of the TGF-β isoforms, is the most potent mediator of fibrosis.

Many studies have shown that the Wnt signaling pathway was involved in the development of diseases [15,16]. Evidence suggests the secreted glycoproteins of Wnt family and their associated signaling pathways are overexpressed during wound repair and regeneration events. Researches showed that the Wnt/β-catenin pathway is hyperactivated in lung from a subset of patients with IPF [17,18], and Wnt-3α promoted myofibroblast differentiation via Smad-dependent TGF-β signaling [19]. Our group have found that activated Wnt signaling induces myofibroblast differentiation of mesenchymal stem cells and inhibition of Wnt/β-catenin signaling promotes engraftment and epithelial differentiation of mesenchymal stem cells to repair lung injury [20–22]. Upon activation, β-catenin and Lef1/Tcf, which are downstream components of the Wnt signaling cascade, form a complex with Smad4, an essential mediator of signals initiated by TGF-β, then translocate to the nucleus to participate in transcription [23]. An active glycogen synthase kinase-3β, the important component of Wnt signaling, is requisite in TGF-β1 upregulation of connective tissue growth factor levels [24]. However, the effect of TGF-β on myofibroblast differentiation after inhibition of Wnt signaling remains unclear.

The Wnt family is comprised of 19 secreted glycoproteins that bind the frizzled receptor (Fz) and subsequent recruit the downstream signal mediators disheveled (Dvl/Dsh) and lead to the nuclear accumulation of β-catenin to initiate transcription of target genes [25]. The Dvl proteins are composed of an N-terminal DIX domain, a central PDZ motif, and a C-terminal DEP domain. It has been showed that PDZ domain is a direct interaction between Fz and Dvl, the special role of the Dvl PDZ domain in the Wnt/β-catenin pathway makes it as an ideal pharmaceutical target to effectively block the Wnt signaling pathway at the Dvl level [26]. NSC668036, which binds to the Dvl PDZ domain [27], can inhibit Wnt signaling pathways through block the PDZ-mediated interactions. In addition, NSC668036 is a peptide mimetic in which two peptide bonds are substituted with two ether bonds and NSC668036 is very stable and highly soluble. Therefore, NSC668036 is expected to be more resistant to proteases in vivo [28]. NSC668036, a small organic inhibitor of the PDZ domain in Dvl, might be useful in exploration of the effect of TGF-β on myofibroblast differentiation after inhibition of Wnt signaling.

Considering that the differentiation of myofibroblast is crucial in IPF, in the present study, we examined the effects of TGF-β1 on fibroblast activation after inhibition of Wnt signaling in vitro and evaluated the therapeutic efficacy of NSC668036 in bleomycin-induced pulmonary fibrosis mice.

Materials and methods

Ethical approval

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Experimentation Ethics Review Committee of Nanjing University (ethics approval no. A9089). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

NIH/3T3 fibroblast culture

NIH/3T3 fibroblasts (Chinese Academy of Sciences) were cultured in high DMEM (Hyclon) with 10% FBS (Gibco), 1% L-glutamine, and a 1% solution of penicillin and streptomycin, at 37□ in a humidified atmosphere with 5% (v/v) CO_2. Fibroblasts were plated 24 h before treatment with recombinant mouse TGF-β1 (Cell Signaling Technology) for the indicated times.

CCK-8 assay

Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) which can measure cell viability, is based on the conversion of an orange-colored product from water-soluble tetrazolium salt (WST-8) by dehydrogenases in live cells. When NIH/3T3 fibroblasts reached 80% confluence, they were routinely passaged using 0.25% trypsin and were diluted 1:3 at each passage. NIH/3T3 fibroblasts were cultured in various concentrations of NSC668036 (0, 1 μM, 10 μM, 100 μM) or mitomycin (0, 10^{-3} g/L, 10^{-2} g/L, 5×10^{-2} g/L, 10^{-1} g/L) in 96-well plates followed by the CCK-8 assay at 12 h, 24 h, 36 h or 48 h according to the instruction from the manufacturer.

Migration assay

The modulation of cell migration was evaluated by in vitro wound-healing assays. Briefly, NIH/3T3 fibroblasts were cultured with TGF-β (10 ng/ml) or Wnt-3α (200 ng/ml) in the presence or absence of NSC668036 (10 μM) for 12 h. In the meantime, NIH/3T3 fibroblasts were treated with mitomycin (5×10^{-2} g/L) in every group, and scratch wounds were induced using standard P1000 pipette tips. Wounds were monitored for up to 36 h in serum-free medium by phase-contrast microscopy, and the wound gap length was determined at 5 different sites in each sample at the indicated intervals.