IM-1662 Attenuates Radiation-Induced Fibroblast Differentiation through Restoration of TGF-β type III Receptor

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1. Introduction

Although pulmonary fibrosis occurs 5-20% of lung cancer patients who underwent radiotherapy, clinically standard treatment for fibrotic disease has not been developed yet. Among fibrosis mediating factors such as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin-13 (IL-13), IL-4, interferon-γ (IFN-γ), and tumor necrosis factor (TNF-α), TGF-β is considered as a critical mediator in normal wound healing as well as pathological fibrogenic processes [1-2]. The TGF-β transmits signals either directly or indirectly through types I, II and III (TβRI, II, and III) receptor complexes and activates downstream Smad signaling [3]. The type III TGF-β receptor (TβRIII or betaglycan) is a transmembrane proteoglycan without a functional kinase domain, and is regarded as a co-receptor to increase the affinity of ligand binding to TβRII [4]. In addition, TβRIII act as a regulator in cell migration, invasion and cell growth in cancer models [5-6]. However, in contrast to a great number of studies about TGF-β ligand and TβRII signaling, the relationship between TGF-β and TβRIII (or betaglycan) remains largely unknown.

In this study, we searched for a new compound which inhibited TGF-β responses using cell-based chemical screening and investigated the effects of the novel compound on radiation induced myofibroblast differentiation. We suggest that a novel small molecule, pyrazolopyrimidine compound IM-1662, can act as an anti-fibrotic agent through inhibiting expression of TGF-β receptor type I and type II whereas, preserving the levels of TβRIII which seems to act as a negative regulator in TGF-β signaling

2. Methods and Results

2.1 Cell culture

Human lung fibroblast CCD-18Lu cells and human embryonic kidney HEK293 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in MEM and DMEM respectively which were supplemented with 10% FBS. After 24 h of starvation with serum-free medium, cells were treated with IM-1662 at the indicated doses for 2 h before irradiation and then, harvested and analyzed by Western blotting.

2.2 Irradiation

Cells were irradiated using a gamma-cell irradiator with 4 Gy [137Cs] γ-ray radiation (Gammacell 3000 Elan, MDS Nordion, Canada).

2.3 Chemical library

A library consists of 1,040 natural single products, 1,040 US drug collection compounds, and 5,040 single synthetic compounds, was purchased from ChemBridge Co. (San Diego, USA).

2.4 Luciferase assay

HEK293 cells were stably transfected with 3TP-Lux reporter gene, which contains 3 copies of TGF-β responsive elements and PAI promoter region, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

The cells were inoculated into 96-well plates at 15,000 cells per well in duplicate and grown for 24 h. Each chemical was adjusted to several doses and was added with TGF-β (5ng/ml) to the wells. After 24 h, the plates were assayed for cell viability and luciferase activity using the luciferase assay kit (Promega, Madison, USA). For transient transfection on CCD-18Lu cells with p3TP-lux vector, 0.2μg of DNA and Lipofectamine 2000 (Invitrogen) was mixed and incubated with cells for 24 h.

2.5 Western blot analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitors. Equal amounts of proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked with 5% skim milk in Tris-buffered saline and then were incubated overnight with primary antibodies at 4℃. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by Enhanced Chemiluminescence (ECL) reagents according to the manufacturer's instructions (Amersham Biosciences, England).

3. Results

3.1. Radiation accelerates human lung fibroblast differentiation into myofibroblast

Previous studies have shown that radiation induces fibrotic phenomena on skin, liver, lung, and kidney. Therefore, to investigate whether radiation induces fibroblast differentiation into myofibroblast in human lung fibroblast, we examined the expression level of a-SMA, fibronectin and collagen in CCD-18Lu cells as it is a well known key marker for fibrosis. After 4Gy of irradiation, the expression levels of α-SMA, fibronectin and collagen were significantly increased, indicating that radiation induced fibroblast differentiation.

3.2. Radiation-induced fibrosis is attributed to the TGF-β related signaling pathways.

As TGF-β is a key mediator in fibrosis, we investigated whether TGF-β was generated by irradiation on CCD-18Lu human lung fibroblasts. As a result, radiation increased the generation of TGF- β as well as the phosphorylation levels of Smad 2 and 3. The expression level of TβRI and TβRII was also increased by radiation, suggesting that radiation-induced fibroblast differentiation seems to be due to the activation of TGF- β dependent signaling.

3.3. Chemical screening to inhibit TGF-β responses with cell-based luciferase assay.

To search for novel small molecules that inhibit TGF-β responses, HEK293 cell-based chemical screening was performed. Among 8,000 small molecules, IM-1662, a pyrazolopyrimidine compound, was one of the active compounds to exhibit significant inhibitory effect against TGF-β induced firefly activity. The 5 ng/ml of TGF-β treatment increased luciferase activity about 12 fold compared with pGL2-luc control vector transfected HEK293 cells, whereas over 50% of luciferase activity was attenuated after IM-1662 treatment in a dose-dependent manner without affecting cell viability (>90% of survival). Thus, we selected IM-1662 for further investigation.

3.4. Novel compound IM-1662 differentially modulates TGF-β receptors.

We next investigated the effects of IM-1662 on radiation induced fibroblast differentiation. IM-1662 significantly blocked radiation induced α-SMA, fibronectin, and collagen expression, as well as decreased phosphorylation levels of Smad 2 and 3. The radiation induced TGF-β receptors type I and type II expression was also down regulated by IM-1662 treatment. Interestingly, IM-1662 restored expression levels of TβRIII to the basal level which was downregulated by radiation or TGF-β treatment. These results indicate that IM-1662 attenuates fibroblast differentiation through modulating TGF-β receptors.

4. Conclusion

The aim of this study was to search a novel compound which could be applied in the attenuation of radiation induced fibrotic process. Exposure to 4 Gy radiation on human lung fibroblast cells significantly increased the protein levels of fibronectin, collagen, and α-SMA. Generation of TGF-β and phosphorylation levels of Smad 2 and 3 were increased after irradiation. Expression levels of TGF-β receptors type I and type II were also increased after irradiation, showing that TGF-β signaling is involved in radiation-induced fibroblast differentiation. A pyrazolopyrimidine compound IM-1662 significantly inhibited radiation-induced fibroblast differentiation through restoring TβRIII expression level and down regulating the levels of TβRI and TβRII.

In conclusion, these results suggest that IM-1662 can be a promising novel agent for the treatment of radiation -induced fibrosis through differential modulation of TGF-β receptors.

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