ROS Mediates Radiation-Induced Differentiation in Human Lung Fibroblast

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1. Introduction

One of the most common tumors worldwide is lung cancer and the number of patients with lung cancer received radiotherapy is increasing rapidly. Although radiotherapy may have lots of advantages, it can also induce serious adverse effects such as acute radiation pneumonitis and pulmonary fibrosis (1, 2). Pulmonary fibrosis is characterized by excessive production of smooth muscle actin-alpha (α-SMA) and accumulation of extracellular matrix (ECM) such as collagen and fibronectin.

There has been a great amount of research about fibrosis but the exact mechanism causing the reaction is not elucidated especially in radiation-induced fibrosis. Until now it has been known that several factors such as transforming growth factor (TGF-β), tumor necrosis factor (TNF), IL-6, platelet-derived growth factor (PDGF) and reactive oxygen species are related to fibrosis.

It is also reported that reactive oxygen species (ROS) can be induced by radiation and can act as a second messenger in various signaling pathways (3, 4). Therefore we focused on the role of ROS in radiation induced fibrosis. Here, we suggest that irradiation generate ROS mainly through NOX4, result in differentiation of lung fibroblast into myofibroblast.

2. Materials and Methods

2.1. Cell culture

IMR90 (ATCC CCL186) derived from human fetal lung fibroblast and WI38 (ATCC CCL75) derived from human lung fibroblast were obtained from the American Type Culture Collection (Rockville, MD, USA). IMR90 was grown in MEM medium and WI38 was cultured in RPMI medium supplemented with 10% fetal bovine serum and non-essential amino acid at 37°C in a humidified incubator with 5% CO_2.

2.2. Irradiation

Cells were irradiated with 4 Gy using a gamma-cell irradiator with ^{137}Cs source (Gamma cell 3000 Elan, MDS Nordion, Canada).

2.3. Small interference RNA transfection

Human lung fibroblast cells were transfected with siNOX4 (sense; 5'CAACUCAUAUGGGACAAGAtt-3', antisense: 5'-UCUUGUCCCAUAUGAGUUGtt-3') and scrambled siRNA (siScr) which were purchased from Ambion. Each siNOX4 and scrambled siRNA were mixed with siPORT NeoFX transfection agent (Ambion) and were incubated for 10 minutes before adding to trypsinized cells. After 24 hours of transfection, cells were irradiated and harvested at an appropriated time.

2.4. Confocal microscopy

ROS detection was measured by 2′,7′-Dichloro dihydrofluorescein diacetate (H_2DCFH-DA) staining using confocal microscope. In brief, cells were incubated for 24 hrs after irradiation and then treated with 10μM of H_2DCFH-DA for 30min.

Prepared samples were mounted with DAPI included mounting solution and were examined under the confocal microscope (Zeiss LSM , Germany).

2.5. Western blot analysis

Cell lysates were prepared by extracting proteins with RIPA supplemented with protease. Equal amounts of the 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 incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) reagents according to the manufacturer's recommendation(Amersham Biosciences England). The experiments were repeated at least three times.

2.6. Flow cytometric analysis

Intracellular ROS measurement using Dichlorofluoresc ein (DCF) was followed as previously described [1]. In brief, radiation exposed and non exposed cells were treated with 10uM Dichlorofluorescein (DCF) for 30min and were washed with PBS before trypsinization. After detaching cells with trypsin, cells were collected, washed and resuspended in 800 ul of PBS. Fluorescence was detected with excitation at 488nm and emission at 525nm by FACSort flow cytometer (Becton Dickinson, CA) 24hrs after irradiation.

3. Results

3.1. Effects of Radiation on α-SMA, fibronectin and collagen expression in human lung fibroblast.

Previous research has shown that radiation induces fibrotic phenomena on skin, liver, lung, intestine and kidney, etc. Therefore, to investigate whether radiation induces fibrosis in human lung fibroblast, we examined the expression level of a-SMA, fibronectin and collagen in IMR-90 and WI-38 cells as it is a well known key marker for fibrosis. As a result, a-SMA and collagen expression was increased significantly high at day 3 after 4Gy irradiation and fibronectin expression was increased time dependently.

3.2. Effects of radiation on ROS production in human lung fibroblast.

It is known that UV, γ-radiation, TGF-b and other factors induce ROS production in various types of cells. Therefore to confirm whether radiation induces ROS in IMR-90 cells, ROS production was measured by flow cytometry and confocal microscope. As a result, 24hrs after 4Gy irradiation, ROS level was increased ~1.6 fold higher than that of non-irradiated control cells. DPI, a NADPH oxidase inhibitor and NAC, a general ROS scavenger, decreased the radiation induced ROS level.

3.3. Effects of radiation induced ROS on trans-differentiation of human lung fibroblast.

To determine the radiation induced ROS could be involved in the process of fibrosis, α-SMA and fibronectin expression was examined in the presence of antioxidants such as NAC and DPI which decreased radiation induced ROS production. DPI significantly decreased both α-SMA and fibronectin expression compared to NAC, suggesting that NADPH oxidase may be involved in this process.

3.4. Radiation induces ROS production via NOX4.

To identify the sources of ROS production, various NOX homologues were screened by RT-PCR and western blotting. As a result, only Nox4 protein and mRNA level were increased after irradiation. Nox4 protein expression was increased after 72 hours whereas mRNA levels were increased as early as 1 hour, peaked at 3 hour, and sustained until 24hour. The treatment with siRNA of Nox4 significantly reduced α-SMA and fibronectin protein expression, whereas no reduction was observed in scrambled siRNA as a negative control.

4. Conclusion

In this study, we aim to elucidate the mechanisms of radiation induced lung fibrosis. Exposure to radiation at 4 Gy on human lung fibroblast cells significantly increased fibronectin, collagen and α-SMA protein expression especially at day 3. Moreover, ROS induction by irradiation was also shown 1.6 fold higher than that of non-irradiated cells. The treatment of DPI and NAC decreased not only the ROS levels but also the α-SMA and fibronectin expression, showing that radiation induced ROS may be an important role in lung fibrosis. Among several kinds of ROS generators, Nox4 expression was only increased after irradiation, and knock-down of Nox4 using siRNA exhibited marked reduction of α-SMA and fibronectin expression.

In conclusion, these results show that ROS mainly generated through Nox4 mediates radiation induced transdifferentiation in human lung fibroblast and Nox4 may be a good molecular candidate for developing antifibrotic agents.

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