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Aryl hydrocarbon receptor–ligand axis mediates pulmonary fibroblast migration and differentiation through increased arachidonic acid metabolism

Hsiang-Han Su[a], Hsin-Ting Lin[b], Jau-Ling Suen[a,c,h], Chau Chyun Sheu[i,j], Kazunari K. Yokoyama[a,c,d,e,f,g], Shau-Ku Huang[c,k,**,1], Chih Mei Cheng[b,c,*,1]

[a] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Taiwan
[c] Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Center for Stem Cell Research, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Center for Infectious Diseases and Cancer, Kaohsiung Medical University, Kaohsiung, Taiwan
[f] Faculty of Science and Engineering, Department of Pharmacological Science, Tokushima Bunri University, Sanuki, Japan
[g] Department of Molecular Prevention Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[h] Center for Research Resources and Development, Kaohsiung Medical University, Taiwan
[i] Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[j] Department of Internal Medicine, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[k] National Institute of Environmental Health Sciences, National Health Research Institutes, Miaoli County, Taiwan

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ABSTRACT

Pulmonary fibroblast migration and differentiation are critical events in fibrogenesis; meanwhile, fibrosis characterizes the pathology of many respiratory diseases. The role of aryl hydrocarbon receptor (AhR), a unique cellular chemical sensor, has been suggested in tissue fibrosis, but the mechanisms through which the AhR-ligand axis influences the fibrotic process remain undefined. In this study, the potential impact of the AhR-ligand axis on pulmonary fibroblast migration and differentiation was analyzed using human primary lung fibroblasts HFL-1 and CCL-202 cells. Boyden chamber-based cell migration assay showed that activated AhR in HFL-1cells significantly enhanced cell migration in response to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), and a known AhR antagonist, CH223191, inhibited its migratory activity. Furthermore, the calcium mobilization and subsequent upregulated expression of arachidonic acid metabolizing enzymes, including cyclooxygenase2 (COX-2) and 5-lipoxygenase (5-LOX), were observed in TCDD-treated HFL-1 cells, concomitant with elevated levels of prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) secretion. Also, significantly increased expression of α-smooth muscle actin α-SMA), a fibroblast differentiation marker, was also noted in TCDD-treated HFL-1 cells ($p < 0.05$), resulting in a dynamic change in cytoskeleton protein levels and an increase in the nuclear translocation of the myocardin-related transcription factor. Moreover, the enhanced levels of α-SMA expression and fibroblast migration induced by TCDD, PGE2 and LTB4 were abrogated by selective inhibitors for COX-2 and 5-LOX. Knockdown of AhR by siRNA completely diminished intracellular calcium uptake and reduced α-SMA protein verified by promoter-reporter assays and chromatin immunoprecipitation. Taken together, our results suggested the importance of the AhR-ligand axis in fibroblast migration and differentiation through its capacity in enhancing arachidonic acid metabolism.

1. Introduction

Common pulmonary diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are the major public-health problem worldwide (Papaiwannou et al., 2014). Both genetic and environmental factors play major roles in their pathogenesis (Wynn, 2011). Respiratory diseases are often

* Corresponding author at: Department of Biomedical Science and Environmental Biology, 100 Shih Chuan 1st Rd, Kaohsiung Medical University, Kaohsiung, Taiwan.
** Corresponding author at: National Institute of Environmental Health Sciences, National Health Research Institutes, Miaoli County, Taiwan.
E-mail addresses: skhuang@nhri.org.tw (S.-K. Huang), chmech@kmu.edu.tw (C.M. Cheng).
[1] These two authors contribute equally to the manuscript.

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associated with pulmonary fibrosis and are characterized by the progressive and irreversible destruction of lung tissues in severe cases. Moreover, these diseases are triggered by multiple factors, including exposure to environmental contaminants (Chen and Stubbe, 2005). Although the major pathways associated with pulmonary fibrosis have been recognized, the sequence of events causing pulmonary fibrosis, particularly in response to environmental stimuli, remains undefined.

Fibroblasts are critical in excessive scar tissue formation or increased extracellular matrix accumulation in response to long-term damage or environmental stimuli (Hinz, 2012). Fibrogenic gene expression (Huang et al., 2012) and wound healing process (Xu and Chisholm, 2011) are closely associated with the cytoskeleton remodeling of actin filament. Dynamic changes in actin filaments initiated by calcium signaling activation (Follonier Castella et al., 2010) or various intracellular and environmental stimuli (Olson and Nordheim, 2010) may contribute to various physiological functions and serve as a platform for signal transduction (Chhabra and Higgs, 2007; Coso et al., 1995, 1996; Fisher et al., 2012).

Furthermore, recent evidence has suggested that the exposure to environmental contaminants is closely associated with activation of arachidonic acid cascade (Dong and Matsumura, 2008; Lupo et al., 2007), and that bioactive eicosanoids not only mediate inflammatory responses but also regulate fibrogenesis (Charbeneau and Peters-Golden, 2005). Arachidonic acid metabolites, such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), are essential mediators in both acute and chronic inflammation. Increased PGE2 synthesis as well as cyclooxygenase-2 (COX-2) and α-smooth muscle actin (α-SMA) protein colocalization have been observed in lesions of the stromal area of the cornea, suggesting that PGE2 is involved in accelerating the corneal wound-healing process (Kawamura et al., 2008). The assembly of actin filaments after the activation of α-SMA promoter activity by the cooperative regulation of FAK and myocardin-related transcription factor (MRTF)-A was observed in NIH 3T3 cells (Chan et al., 2009). Collectively, these results, suggest the possible crosstalk between arachidonic acid metabolism and MRTF axis activation. However, in the context of exposure to environmental stimuli, the association between the generation of arachidonic acid metabolites and fibrogenesis remains unclear.

Aryl hydrocarbon receptor (AhR) is a ligand-activated receptor for various environmental contaminants and endogenous metabolites. Environmental contaminant-induced AhR activation influences inflammatory, fibrotic responses and lung functions in various disease models (Chiba et al., 2011; Ramirez et al., 2010), although the detailed mechanisms remain to be elucidated. Particularly, the potential role of the AhR–ligand axis in regulating pulmonary fibroblast migration and differentiation and its underlying mechanisms has not been completely defined. We hypothesize that AhR–ligand axis activation is involved in the imbalance of arachidonic acid metabolism, pulmonary fibroblast migration and subsequent fibrotic progression. Herein, we provide evidence supporting the involvement of the AhR–ligand axis in regulating pulmonary fibroblast migration and differentiation through, at least in part, its ability to increase the generation of arachidonic acid metabolites.

2. Materials and methods

2.1. Reagents and antibodies

Ham's F12K medium, minimal essential medium, L-glutamine, fetal bovine serum (FBS), sodium pyruvate, and a nonessential amino acid solution were purchased from Gibco (Gaithersburg, MD, USA). Cell Titer 96 Aqueous One Solution Reagent was purchased from Promega (San Luis Obispo, CA, USA). The Abs used for Western blotting analysis were as follows: anti-α-SMA (Millipore), anti-cPLA2 (Santa Cruz Biotechnology), anti-COX-2 (Santa Cruz Biotechnology), anti-5-LOX (BD), anti-MRTF-A (Santa Cruz), anti- β-actin (Santa Cruz Biotechnology), anti-GAPDH (GeneTex), anti-PARP (Santa Cruz), anti-mouse IgG–HRP (Cell Signaling Technology), anti-rabbit IgG–HRP (Cell Signaling Technology), anti-goat IgG–HRP Abs; for immunofluorescence staining, anti α-SMA (Sigma–Aldrich Co.), anti-AhR (Santa Cruz Biotechnology), anti-mouse IgG2a and IgG2b isotype control (e-Bioscience), goat anti-mouse IgG-conjugated Alexa Fluor 568 (Cell Signaling Technology), anti-rabbit IgG-conjugated Alexa Fluor 568 (Cell Signaling Technology), and Alexa Fluor 488-conjugated phalloidin (Invitrogen) Abs were used. *si*RNAs for AhR and scrambled control *si*RNAs were purchased from Invitrogen. Anti-AhR Abs, used for the chromatin immunoprecipitation assay, was purchased from Genetex.

2.2. Cell culture

Human normal fetal lung fibroblast, HFL-1 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and normal male CCL-202 human lung fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA). For sub culturing, HFL-1 cells were maintained in Ham's F12K medium with 2 mM L-glutamine and 10% fetal bovine serum (FBS). The CCL-202 cells were maintained in Eagle's minimal essential medium with Earle's balanced salts solution (BSS), supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, and 1% penicillium/streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Proliferation assay

Proliferation of HFL-1 cells was assessed by MTT assay. HFL-1 cells (6×10^3 cells/well) were seeded in a 96 well microplate for 24 h. Cells were incubated in the serum free medium before TCDD treatment. After treatment with TCDD, cells were then incubated with MTT (5 mg/ml) at 37 °C for 4 h. Proliferation of HFL-1 cell was monitored by measuring the formation of formazan at the O.D. 570 nm by spectrophotometer.

2.4. Migration assay

Boyden chamber-based cell migration assay was performed using a 24-well micro-chemotaxis chamber and polycarbonate filters (BD) (8-μm diameter pores). HFL-1 cells (80% confluence) were seeded on the upper chamber of a trans well chamber containing Ham's F12 serum-free medium, medium containing TCDD or FICZ was replaced in the lower chamber. The chambers were incubated at 37 °C under 5% CO_2 for 24 h. Cell migration was examined following the manufacture's protocol. The top of filters were mechanically scraped, and the fibroblasts, which migrated to the undersurface of the filter, were fixed in 100% methanol and stained with 1% crystal violet. The number of migrated cells was examined under a microscope.

2.5. Western blotting

Cells (1×10^5 cells/mL) were seeded in 100-mm^2 dishes. At 90% confluence, the cells were starved with serum-free medium for 24 h and treated with various experimental conditions. Subsequently, the cells were harvested and homogenized in RIPA cell