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Role of allograft inflammatory factor-1 in bleomycin-induced lung fibrosis

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ABSTRACT

Allograft inflammatory factor-1 (AIF-1) is a protein expressed by macrophages infiltrating the area around the coronary arteries in a rat ectopic cardiac allograft model. We previously reported that AIF-1 is associated with the pathogenesis of rheumatoid arthritis and skin fibrosis in sclerodermatous graft-versus-host disease mice. Here, we used an animal model of bleomycin-induced lung fibrosis to analyze the expression of AIF-1 and examine its function in lung fibrosis. The results showed that AIF-1 was expressed on lung tissues, specifically macrophages, from mice with bleomycin-induced lung fibrosis. Recombinant AIF-1 increased the production of TGF-β which plays crucial roles in the mechanism of fibrosis by mouse macrophage cell line RAW264.7. Recombinant AIF-1 also increased both the proliferation and migration of lung fibroblasts compared with control group. These results suggest that AIF-1 plays an important role in the mechanism underlying lung fibrosis, and may provide an attractive new therapeutic target.

1. Introduction

Allograft inflammatory factor-1 (AIF-1) is a 17 kDa Ca^{2+}-binding EF-hand intracellular protein that is encoded by the HLA class III genomic region [1,2]. AIF-1 was originally cloned from activated macrophages infiltrating areas around the coronary arteries in a rat ectopic cardiac allograft model [3]. In mice, AIF-1 is expressed at high levels in the testis, at moderate levels in the spleen and lymph nodes, and at low levels in the liver and thymus, although its detailed physiological functions remain unclear [4].

Systemic sclerosis (SSc) is an autoimmune connective tissue disease that affects skin and internal organs such as the lung, heart, kidney, and gastrointestinal tract [5]. Collagen-rich extracellular matrix produced by activated fibroblasts induces functional disorder of affected organs. Pulmonary complications such as pulmonary fibrosis are the main cause of SSc-associated mortality [6]. Clinical evidence from chest radiographs and high-resolution computerized tomography indicates that interstitial lung disease is present in about 40% and 90% of SSc cases, respectively [7]. Del Galdo et al. reported that AIF-1 is expressed in affected vessels, macrophages, and T cells within the lung lesions of patients with SSc [8]. We previously identified the pathogenic mechanism underlying skin fibrosis in sclerodermatous GVHD mice [9], and showed that the levels of both AIF-1 and IL-6 expressed by infiltrating mononuclear cells and fibroblasts in the thickened skin of sclerodermatous GVHD mice are significantly higher than those in control mice. We also showed that recombinant AIF-1 increases the migration of normal human dermal fibroblasts (NHDFs) and stimulated IL-6 secretion by these cells. These results suggest that AIF-1 plays an important molecule in promoting skin fibrosis in a GVHD model. However, the pathophysiologic role of AIF-1 in lung fibrosis is unclear. The histopathology of pulmonary fibrosis resembles that of bleomycin-induced lung fibrosis [10]. Here, we used a mouse model of bleomycin-induced lung fibrosis to examine the expression and

Abbreviations: AIF-1, allograft inflammatory factor-1; GVHD, graft versus host disease; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharide; NHDF, normal human dermal fibroblast; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SSc, systemic sclerosis; rAIF-1, recombinant allograft inflammatory factor-1.

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function of AIF-1 in lung fibrosis.

2. Material and methods

2.1. Mouse model of bleomycin-induced lung fibrosis

C57BL/6 mice (8–10 weeks-of-age) were purchased from Shimizu Laboratory Supplies Co. Ltd. Mice were anesthetized by intraperitoneal administration of pentobarbital. Next, 30 μl of bleomycin hydrochloride (Nippon Kayaku Co, Tokyo, Japan) solution containing 2.15 U/kg of bleomycin dissolved in sterile saline was injected directly into the trachea using a 0.9 mm feeding needle (KN-348 Natsume Seisakusho Co, Tokyo, Japan). Control mice received the same volume of sterile saline alone. Mice were sacrificed by intraperitoneal administration of excess pentobarbital (120–150 mg/kg) on Day7, Day14, Day21 and Day 28 post-intratracheal administration of bleomycin or saline. Mice were euthanized if their body weight fell below 80% of that measured at baseline. The study was approved by the Animal Research Committee, Graduate School of Medical Science, Kyoto Prefectural University of Medicine.

2.2. Histology

The lungs were excised on Day7, Day14, Day21 and Day 28 post-intratracheal administration of bleomycin or saline and immediately fixed with 4% paraformaldehyde. The samples were then embedded in paraffin and stained with H&E and Masson's trichrome method. The degree of lung fibrosis was quantified using Ashcroft scoring method [11] by observers who were blinded as to whether the samples were from bleomycin or saline induced mice. Images were acquired using a DMBA210 microscope (Shimadzu Rika, Tokyo, Japan) equipped with Motic Images Plus2.2s software (Shimadzu Rika).

2.3. Double immunofluorescence staining

Paraffin-embedded sections were deparaffinized in xylol and rehydrated through a graded series of ethanol solutions. Sections were washed 2 times with PBS, blocked in Blocking One (Nacalai tesque) for 10 min, then incubated with a mixture of two primary antibodies: anti-AIF-1 (1:500; Proteintech) and anti-S100A4 (1:500; Acris Antibodies) overnight at 4 °C. They were washed 2 times with PBS before incubation with a mixture of two secondary antibody; donkey anti-rabbit IgG conjugated to Alexa 594(1:1000; abcam) and chicken anti-goat IgG conjugated to Alexa 488(1:1000; molecular probes) for 1 h at 37 °C. Next, sections were washed 2 times with PBS, then incubated with DAPI solution (DOJINDO) for 5 min at 37 °C. Sections were finally washed 2 times with PBS and coverslips were sealed by use of a drop of Fluoro-KEEPER Antifade Reagent (Nacalai tesque). Images were acquired by fluorescence microscope (Keyence BZX-710).

2.4. Triple immunofluorescence staining

Paraffin-embedded sections were deparaffinized in xylol and rehydrated through a graded series of ethanol solutions. Sections were washed 2 times with PBS, blocked in Blocking One for 10 min, then incubated a mixture of two primary antibodies: anti-AIF-1 (1:500; Proteintech) and anti-CD68 (1:500; SantaCruz) overnight at 4 °C. They were washed 2 times with PBS before incubation with a mixture of two secondary antibody; donkey anti-rabbit IgG conjugated to Alexa 594(1:1000; abcam) and chicken anti-goat IgG conjugated to Alexa 488(1:1000; molecular probes) for 1 h at 37 °C. Next, sections were washed 2 times with PBS, blocked in Blocking One for 10 min, then incubated with the primary antibodies anti-TGF-β (1:500; Abcam) overnight at 4 °C. They were washed 2 times with PBS before incubation with secondary antibody; donkey anti-rabbit IgG conjugated to Alexa 350(1:1000; invitrogen) for 1 h at 37 °C. Sections were finally washed 2 times with PBS and coverslips were sealed by use of a drop of Fluoro-KEEPER Antifade Reagent. Images were acquired by fluorescence microscope.

2.5. Western blot analysis

Western blot analysis was performed as previously described [9]. Briefly, lung samples on Day28 were homogenized in liquid nitrogen and solubilized in RIPA buffer (150 mM NaCl, 1% NP-40, 25 mM Tris-HCl (pH 7.6), 1% sodium deoxycholate, and 0.1% SDS) (Thermo Scientific) containing protease inhibitors (GE Healthcare Life Sciences, CT, USA). Total protein extracts from each sample (20 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were then incubated with 2% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (GE Healthcare) for 1 h, followed by anti-AIF-1 Ab (1:3000; Proteintech) overnight at 4 °C. The next day, the membranes were washed three times and incubated with HRP-conjugated anti-goat IgG (1:5000; Santa Cruz) for 1 h at room temperature. After washing again, the antibody-antigen complexes were detected with ECL Prime Western blotting detection reagents (GE Healthcare). The ECL signal was obtained by exposure to the ECL Select LAS500 (GE Healthcare) for 20 sec.

2.6. ELISA

A mouse macrophage cell line, RAW 264.7, was obtained from the RIKEN cell bank (Tsukuba, Japan). RAW264.7 cells were seeded in 12-well plates (5×10^5 cells/well) in RPMI-1640 supplemented with 10% FBS for 24 h. Media were replaced with serum-free RPMI-1640 and the cells were serum-starved overnight. Next, the cells were stimulated for 24 h by addition of RPMI-1640 supplemented with various concentrations of recombinant AIF-1 (CUSABIO, endotoxin removed) or a lipopolysaccharide (LPS) (Sigma). The cell culture supernatant was collected and stored at −80 °C until required. The levels of TGF-β in the cell culture supernatant were measured using commercial ELISA kits (a TGF-β ELISA kit; eBioscience), according to the manufacturer's instructions. The optical density was measured at 450 nm using a SoftMaxPro40 plate reader. Each measurement was determined in three separate experiments.

2.7. Quantitative real-time RT-PCR analyses

RAW264.7 cells were seeded in 12-well plates (5×10^5 cells/well) in RPMI-1640 supplemented with 10% FBS for 24 h. Media were replaced with serum-free RPMI-1640 and the cells were serum-starved overnight. Next, the cells were stimulated for 2 h by addition of RPMI-1640 supplemented with saline or recombinant AIF-1 (100 ng/ml). The medium was washed with PBS three times and total RNA was obtained with an RNeasy Plus Mini Kit (Qiagen). Quantitative real time RT-PCR was performed on StepOne™ & StepOnePlus™ Real-Time PCR Systems (Life Technologies) using the Thunderbird Probe qPCR Mix (TOYOBO). The following TaqMan expression assays were used: Mm00479862_g1 (AIF-1), Mm03928990_g1 (18 S). Results in duplicate were normalized to 18 S expression.

2.8. Isolation of primary lung fibroblasts

Primary lung fibroblasts were isolated from C57BL/6 mice and

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