Journal of Pharmaceutical Sciences 105 (2016) 1327–1334

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Alteration in Intrapulmonary Pharmacokinetics of Aerosolized Model Compounds Due to Disruption of the Alveolar Epithelial Barriers Following Bleomycin-Induced Pulmonary Fibrosis in Rats

Kohei Togami, Sumio Chono, Hitoshi Tada*

Division of Pharmaceutics, Hokkaido Pharmaceutical University School of Pharmacy, Sapporo, Hokkaido 006-8590, Japan

ARTICLE INFO

Article history:
Received 31 August 2015
Revised 6 December 2015
Accepted 9 December 2015
Available online 30 January 2016

Keywords:
idiopathic pulmonary fibrosis
aerosol-based pulmonary drug delivery system
anti-fibrotic agent
tight junction
claudin
alveolar epithelial cells
epithelial-mesenchymal transition

ABSTRACT

Idiopathic pulmonary fibrosis is a lethal lung disease that is characterized by the accumulation of extracellular matrix and a change in lung structure. In this study, intrapulmonary pharmacokinetics of aerosolized model compounds were evaluated using rats with bleomycin-induced pulmonary fibrosis. Aerosol formulations of indocyanine green, 6-carboxyfluorescein (6-CF), and fluorescein isothiocyanate dextrans (FD; 4.4, 10, 70, and 250 kDa) were administered to rat lungs using a MicroSprayer. Indocyanine green fluorescence signals were significantly weaker in fibrotic lungs than in control lungs and 6-CF and FD concentrations in the plasma of pulmonary fibrotic animals were markedly higher than in the plasma of control animals. Moreover, disrupted epithelial tight junctions, including claudins-1, -3, and -5, were observed in pulmonary fibrotic lesions using immunofluorescence microscopy. In addition, destruction of tight junctions on model alveolar epithelial cells (NCI-H441) by transforming growth factor-β_1 treatment enhanced the permeability of 6-CF and FDs through NCI-H441 cell monolayers. These results indicate that aerosolized drugs are easily distributed into the plasma after leakage through damaged tight junctions of alveolar epithelium. Therefore, the development of delivery systems for anti-fibrotic agents to improve intrapulmonary pharmacokinetics may be necessary for effective idiopathic pulmonary fibrosis therapy.

Introduction

Idiopathic pulmonary fibrosis (IPF) occurs during poor health-related quality of life correlated with high morbidity with 90% dyspnea and leads to an extremely low 5-year survival rate of <50%.[1,2] Therefore, current efforts to develop anti-fibrotic agents will improve survival rates and the health-related quality of life for patients with IPF. Clinically, pirfenidone is the first and only anti-fibrotic agent that is used for IPF therapy. However, because orally active pirfenidone is widely distributed via the blood, systemic side effects such as photosensitivity (51%), anorexia (17%), and nausea (44%) are frequently observed.[3,4] Several other drugs, including arsenic trioxide,[5] the intracellular tyrosine kinase inhibitor nintedanib,[6] and the thymic hormone facteur thymique serique,[7] also have anti-fibrotic effects in experimental animal models with bleomycin-induced pulmonary fibrosis. They have not yet been used in the treatment of IPF. Thus, development of IPF therapy requires enhancement of anti-fibrotic effects and avoidance of systemic side effects, warranting investigation of drug delivery to the lungs.

Intrapulmonary administration of aerosolized drugs is an efficient method for direct drug delivery to the lungs. Therefore, enhancements of anti-fibrotic effects and reductions in effective doses to avoid systemic side effects may warrant intrapulmonary administration of aerosolized anti-fibrotic agents for IPF. However, intrapulmonary pharmacokinetics and absorption into blood after intrapulmonary administration of aerosolized drugs in IPF patients remain largely unknown.

IPF is characterized by aberrant accumulation of fibroblasts and myofibroblasts and progressively abnormal remodeling and reconstruction of lung tissue, followed by irreversible scarring of alveolar epithelium.[8] Alveolar epithelial cells are connected by tight junction proteins such as claudins and occludin, which together present a significant alveolar barrier.[9] Hence, disruptions of alveolar tight junctions have been observed in other lung diseases such

* *Correspondence to:* Hitoshi Tada (Telephone: +81-11-676-8731; Fax: +81-11-676-8666).
E-mail address: h-tada@hokuyakudai.ac.jp (H. Tada).

http://dx.doi.org/10.1016/j.xphs.2015.12.010
0022-3549/

as lung cancer and acute lung inflammation.[10,11] In addition, lung inflammation reportedly increases the permeability of the alveolar barrier.[12] Because IPF lesions compromised alveolar epithelial tight junctions, the intrapulmonary pharmacokinetics of aerosolized drugs may be altered under these conditions.

In this study, we demonstrate altered pharmacokinetics of aerosolized model compounds in rats with bleomycin-induced pulmonary fibrosis. Subsequently, altered expression of tight junctions in alveolar epithelial cells was observed and *in vivo* and *in vitro* pharmacokinetic effects were investigated.

Materials and Methods

Materials and Animals

Indocyanine green was purchased from Wako Pure Chemicals Company, Ltd. (Osaka, Japan). 6-Carboxyfluorescein (6-CF) and fluorescein isothiocyanate dextrans of 4.4, 10, 70, and 250 kDa (FD-4, -10, -70, and -250) were purchased from Sigma Aldrich Company (St. Louis, MO). Bleomycin chlorate was purchased from Nippon Kayaku Company (Tokyo, Japan). Recombinant transforming growth factor-β_1 (TGF-β_1) was purchased from PeproTech, Inc. (Rocky Hill, NJ). All other reagents were commercially available and were of analytical grade. Male 6-week-old Wistar rats weighing 130-150 g were purchased from Japan SLC (Shizuoka, Japan). The animal experimental protocol was approved by the Committee of the Laboratory Animal Center (No. 14-002) and conformed to the Guiding Principles for the Care and Use of Experimental Animals at Hokkaido Pharmaceutical University.

Model of Bleomycin-Induced Pulmonary Fibrosis

Bleomycin chlorate was dissolved in phosphate buffered saline solution (PBS, pH 7.4) and intrapulmonary administration of 10 mg/0.25 mL/kg was performed using a Liquid MicroSprayer® (Model IA-1C; Penn Century, Inc., Philadelphia, PA) in rats under pentobarbital anesthesia. Intrapulmonary treatments with PBS were administered to negative control rats following the same procedures. After administration of bleomycin for 14 days, rats were used in experiments as a model of pulmonary fibrosis.[13]

Ex Vivo *Imaging of Lung Tissues*

Indocyanine green was dissolved in PBS and intrapulmonary administrations of 75 μg/0.25 mL/kg in rats were performed using a Liquid MicroSprayer after pentobarbital anesthesia. At each designated time point (15 min, 1 h, and 4 h), the rats were exsanguinated by severing the abdominal aorta, and the lungs were quickly excised for indocyanine green imaging using a Clairvivo OPT instrument (Shimadzu Company, Kyoto, Japan) with an excitation filter of 785 nm and an emission filter of 845 nm. Imaging analyses were performed using Clairvivo OPT measurement and display software version 3.0.0.0. (Shimadzu Company).

Pharmacokinetic Analysis After Intrapulmonary Administration

6-CF and FDs were dissolved in PBS for intrapulmonary administration into rats at doses of 250 μg/0.25 mL/kg and 10 mg/0.25 mL/kg, respectively, using a Liquid MicroSprayer after pentobarbital anesthesia. At each designated time point (15 min, 30 min, 1 h, 2 h, and 4 h), rats were anesthetized using pentobarbital and blood was collected from the jugular veins. After 4-h treatments, tracheas were immediately cannulated and lungs were lavaged 3 times with 5 mL of ice-cold PBS (pH 7.4). Bronchoalveolar lavage fluid was immediately centrifuged at 1600 × g for 5 min at 4°C and alveolar cells such as alveolar macrophages were removed. Concentrations of 6-CF and FDs in plasma and supernatants of bronchoalveolar lavage fluid were measured using a microplate reader (Powerscan HT; DS Pharma Biomedical, Osaka, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The area under the compound concentration time curve from time 0-4 h (AUC) was calculated using the trapezoidal rule for pharmacokinetic analyses.

Quantitative Real-Time PCR of Lung Tissue

After bleomycin treatments for 14 days, lung tissues were homogenized with Trizol® (Life Technologies Company, Carlsbad, CA) and RNA was isolated using phenol chloroform purification. RNA samples were stored at −80°C before performing real-time PCR and cDNA reverse transcription from RNA was performed using High-Capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) with a Power SYBR® Green Master Mix (Applied Biosystems). Primer sequences for rat claudins-1, -3, -4, -5, and -18, occludin, zonula occuldens-1 (ZO-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: claudin-1 (sense 5′-CTGGGAGGTGCCCTACTTT-3′ and antisense 5′-CCGCTGTCACACGTAGTCTT-3′), claudin-3 (sense 5′-AGATGTACGACTCGCTGCTG-3′ and antisense 5′-CTTGGCCGTCTCATCTTGT-3′), claudin-4 (sense 5′-CTCTCGCCTCCACGTTACTC-3′ and antisense 5′-TCAGTCATCCTCGACACCAA-3′), claudin-5 (sense 5′-CAGGCTCTTGTGAGGACTTG-3′ and antisense 5′-TGCCCTTTCAGGTTAGCAG-3′), claudin-18 (sense 5′-AAGGGCTCTGGAGGAGTTG-3′ and antisense 5′-GCCCAGGATGGTGAAGTATG-3′), occludin (sense 5′-GCTCAGGGAATATCCACCTATC-3′ and antisense 5′-TTCTCCAGCAACCAGCATC-3′), ZO-1 (sense 5′-CCACAGACATCCAACCAGC-3′ and antisense 5′-AGCCCAAAGAACAGAAGACC-3′), and GAPDH (sense 5′-GATTTGGCCGTATCGGAC-3′ and antisense 5′-GAAGACGCCAGTAGACTC-3′). Subsequently, mRNA expression in tight junctions was calculated from cycle thresholds of the PCR products.

Immunofluorescence Microscopy of Lung Tissue Section

Cryobiopsies were performed as described by Saitoh et al.[14] during phenobarbital anesthesia. Briefly, tracheas were cannulated to an animal respirator (SN-480-7; Shinano Manufacturing Company Ltd., Tokyo, Japan) at 1.5 respirations/s. The thorax was then opened to expose the lungs and the right lungs were snap frozen by directly adding liquid isopentane cryogen (−160°C) that had been precooled in liquid nitrogen. Frozen lung samples were stored in acetone at −80°C for 2 days. The frozen samples were transferred at −30°C for 2 h, and then transferred at 4°C. The lung samples were transferred into PBS containing 30% sucrose and were stored for 2 days at 4°C. Frozen lung samples were cut into 4-μm thick frozen sections using a cryostat and were directly mounted onto aminosilane-coated glass slides (Matsunami Trading Company Ltd., Osaka, Japan). After blocking with Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) at room temperature for 15 min, lung tissue sections were exposed to the following antibodies (1:250 dilution) at 4°C overnight: rabbit polyclonal anti-claudin-1 antibody (Sigma, Catalog number: SAB4503546), rabbit polyclonal anti-claudin-3 antibody (Novus Biologicals, Inc., Littleton, CO, Catalog number: NBP1-67517), or rabbit polyclonal anti-claudin-5 antibody (Sigma, Catalog number: SAB4502981). Later, the specimens were washed 3 times in PBS for 5 min and then reacted with Alexa Fluor 555-conjugated anti-rabbit IgG secondary antibodies (Life Technologies, Catalog number: A-21428) at a 1:200 dilution for 1 h at room temperature.