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Pulmonary administration of integrin-nanoparticles regenerates collapsed alveoli

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an intractable pulmonary disease, causes widespread and irreversible alveoli collapse. In search of a treatment target molecule, which is able to regenerate collapsed alveoli, we sought to identify a factor that induces differentiation in human alveolar epithelial stem cells using all-*trans* retinoic acid (ATRA), whose alveolar repair capacity has been reported in animal experiments. When human alveolar epithelial stem cells were exposed to ATRA at a concentration of 10 μM for over seven days, approximately 20% of the cells differentiated into each of the type-I and type-II alveolar epithelial cells that constitute the alveoli. In a microarray analysis, *integrin-α1* and *integrin-β3* showed the largest variation in the ATRA-treated group compared with the controls. Furthermore, the effect of the induction of differentiation in human alveolar epithelial stem cells using ATRA was suppressed by approximately one-fourth by siRNA treatments with *integrin α1* and *integrin β3*. These results suggested that integrin *α1* and *β3* are factors responsible for the induction of differentiation in human alveolar epithelial stem cells. We accordingly investigated whether integrin nanoparticles also had a regenerative effect *in vivo*. Elastase-induced COPD model mouse was produced, and the alveolar repair effect of pulmonary administration using nanoparticles of integrin protein was evaluated by X-ray CT scanning. Improvement in the CT value in comparison with an untreated group indicated that there was an alveolar repair effect. In this study, it was shown that the differentiation-inducing effect on human alveolar epithelial stem cells by ATRA was induced by increased expression of integrin, and that the induced integrin enhanced phosphorylation signaling of AKT, resulting in inducing differentiations. Furthermore, the study demonstrated that lung administration of nanoparticles with increased solubility and stability of integrin repaired the alveolus of an Elastase-induced COPD model mouse. Those results show that those integrin nanoparticles are effective as novel COPD treatment target compounds.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disease characterized by cough, primarily dyspnea, sputum production, and other respiratory symptoms, causes widespread and irreversible alveoli collapse [1]. Recent reports suggest that COPD mortality is increasing, and COPD is estimated to emerge as the third leading cause of death worldwide by 2020 [2]. COPD is caused by harmful gasses including smoke from tobacco or biomass fuels. Abnormal chronic inflammation of lungs will be induced. Destruction of alveolar tissue (formation of emphysema), and inhibition of normal repair and defense mechanisms (fibrosis formation in distal airways) will result in airflow obstruction (Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines). Among the symptoms of COPD, there is no effective treatment for the formation of emphysema caused by the destruction of alveolar tissue, and it is one of the biggest challenges in the development of therapeutic agents for COPD. Therefore, identification of effective target molecules for therapy for the destruction of alveolar tissue in COPD was attempted in this study. However, no cure is available for COPD. Regenerative medicine shows potential for addressing this intractable disease, but no regenerative treatments for human alveoli have been described to date.

The targets of human lung alveolar remodeling are resident stem and progenitor cells in the lung that function in tissue repair and homeostasis. The adult lung consists of the following four major biologically distinct components: the trachea, bronchi, bronchioles, and alveoli. Each component is biologically distinct and has its own stem and progenitor population [3–6]. Alveoli are terminal structures of distal airways specialized for gas exchange. The gaseous alveolar surface is lined

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by alveolar type I (ATI) and alveolar type II (ATII) cells [7]. Recent studies have characterized resident alveolar stem cells in human [8]. However, potent regenerative compounds have not been identified for human alveolar stem cells.

Retinoids are necessary for normal lung development and have shown promise in small animal models of lung regeneration [9]. High concentrations of retinoic acid accumulate within the developing lung concurrent with the process of alveolar septation [10]. The essential role that retinoids play in the generation of alveoli has been confirmed by studies involving retinoid receptor knock-out animals [11]. These reports support the continued investigation of retinoids as a potential treatment for human emphysema. However, molecular mechanism of retinoids-induce regenerative on alveoli is not been identified.

The aim of this study was to identify a treatment target molecule which is able to regenerate collapsed alveoli. This study attempted to identify a factor responsible for the induction of differentiation in human alveolar epithelial stem cells using all-*trans* retinoic acid (ATRA). And nanoparticles of those factors attempt to effectively as novel COPD treatment target compound.

2. Materials and methods

2.1. Animals and cells

Male ICR mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). Animals were housed in a temperature-controlled (24 ± 1 °C) facility maintained on a 12-h:12-h light:dark cycle with standard food available *ad libitum*. All animal procedures followed the guidelines established by the Animal Care and Use Committee of the Tokyo University of Science.

Six-week-old male mice were anesthetized with isoflurane, and a solution of porcine pancreatic elastase (Elastin Products Company, Owensville, MO) (4.5 U/100 μl of saline) was administered intratracheally. We administered pulmonary administration of 2.5 mg/kg ATRA or 50 μg/kg of DDM (n-Dodecyl-β-D-maltoside)-nanoparticles include integrin α1β1 to elastase-induced COPD model mouse twice a week and evaluated the results using X-ray CT scanning. Mice were sacrificed after 4 weeks.

Human alveolar epithelial stem cells were provided by Dr. Hiroshi Kubo (Tohoku University, Japan) [8] and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 20% ReproFF2 Medium (ReproCELL, Yokohama, Japan) in a humidified 5% CO_2 atmosphere maintained at 37 °C. Experiments using human cells were conducted in accordance with the guidelines of the Research Ethics Committee of the Tokyo University of Science and Tohoku University.

2.2. Preparation of integrin nanoparticles

We have purchased a recombinant protein integrin α1β1 and αvβ3 (Millipore, Billerica, MA). We use the Detergent selection kit (GE Healthcare UK Ltd, Buckinghamshire, England), include OG (Octylglucoside), DDM (n-Dodecyl-β-D-maltoside), LDAO (N, N-dimethyldodecylamine-oxide), DM (Decylmaltoside), C12E8 (Octaethyleneglycol mono-n-dodecyl Ether). The recombinant protein integrin solubilized with those surfactants 2% concentration of over the critical micelle concentration (cmc). Those separated the insoluble fraction of the precipitate and the soluble fraction of the supernatant after 60 min ultracentrifugation (105,000 ×*g*), SDS-PAGE check the dimeric integrin amount. We chose DDM(n-Dodecyl-β-D-maltoside) to study the highest solubilization capacity.

2.3. Induction of differentiation to ATI or ATII cells

To induce the differentiation of human alveolar epithelial cells to ATI or ATII cells, a culture system described previously was adopted with some modifications [7]. In brief, cells (5×10^5) at passages 3–6 were plated on cell culture inserts (BD Biosciences,Franklin Lakes, NJ) that had been coated with a mixture of 60% Matrigel (BD Biosciences, Franklin Lakes, NJ) and 40% rat tail collagen I (BD Biosciences,Franklin Lakes, NJ) in 5% FBS/DMEM. After 4 h, media were changed to DMEM containing 5% FBS with or without all-*trans* retinoic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or recombinant proteins of integrin α1β1 and integrin αvβ3. The media were changed every other day until analysis on day 7.

The cultured cells were fixed with 4% paraformaldehyde in phosphate buffer for 15 min at room temperature. Samples were blocked using Blocker BSA (Thermo Fisher, Waltham, MA) for 0 min at room temperature. Cells were then incubated with the following primary antibodies overnight at 4 °C: goat anti-human CD90 (Thy-1) polyclonal antibody (1:200, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-human pro-surfactant protein (SP)-C polyclonal antibody (1:500, Millipore, Billerica, MA), goat anti-human aquaporin-5 (AQP-5) polyclonal antibody (1:200, Santa Cruz Biotechnology, Billerica, MA), and goat anti-human SP-A monoclonal antibody (1:200, Abcam, Cambridge, United Kingdom). Alexa Fluor 546-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-goat IgG (each at 1:100, Molecular Probes, Carlsbad, CA) were used as secondary antibodies. After mounting the samples and staining nuclei using ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes, Carlsbad, CA), samples were observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and an A1Rsi confocal laser scanning microscope system (Nikon, Tokyo, Japan). By using image analysis software, ImageJ (Wayne Rasband (NIH), Bethesda, MD), the number of positive cells in immunostaining images at 20× magnification was counted. Then the ratios of CD90, AQP-5, and SP-A positive cells were determined against the number of DAPI positive cells, *i.e.*, the nuclear marker. Ten views were taken of each sample, and CD90, AQP-5, and SP-A positive cell numbers were measured for each sample, for a total of 500 DAPI positive cells.

2.4. Microarray gene expression analysis

To evaluate gene expression profiles, total RNA was extracted and purified from human alveolar epithelial stem cells treated with all-*trans* retinoic acid or vehicle (saline containing 50% ethanol). RNA quality was analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara CA). Input RNA then was amplified and labeled to generate cRNA using a Low RNA Input Quick Amp Labeling Kit. Labeled cRNA was hybridized to a 44 K Agilent 60-mer oligo microarray (Whole Human Genome Oligo Microarray Kit v.2.0, Agilent Technologies). To identify up- and down-regulated genes, we calculated ratios using the normalized signal intensities of each probe. We set the following criteria: for up-regulated genes, ratio ≧5-fold and for down-regulated genes, ratio ≦0.2. All microarray data were submitted to the Gene Expression Omnibus at the National Center for Biotechnology Information. Differentially expressed genes were functionally annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on the Functional Annotation Clustering Tool in the Database for Annotation, Visualization, and Integrated Discovery v.6.7 (DAVID, http://david.abcc.ncifcrf.gov).

2.5. Quantitative reverse transcription–polymerase chain reaction

The RT-PCR method described previously was adopted with some modifications [12]. Total RNA was extracted using RNAiso reagent (Takara, Shiga, Japan), and 0.4 μg aliquots of RNA were reverse-transcribed (RT) using a ReverTra Ace quantitative polymerase chain reaction (qPCR) RT kit (Toyobo, Osaka, Japan) The cDNA equivalent of 12 ng of input RNA was amplified by real-time PCR (Applied Biosystems, Life Technologies, Carlsbad, CA). The following primers were used for PCR: *integrin α1* (F)

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