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Pirfenidone plays a biphasic role in inhibition of epithelial-mesenchymal transition in non-small cell lung cancer

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ABSTRACT

Introduction: Epithelial to mesenchymal transition (EMT) relates to both organ fibrosis and malignant behavior of cancer. Pirfenidone (PFD) is an anti-fibrotic agent for idiopathic pulmonary fibrosis and one of its functions may be to inhibit fibrotic EMT. This study aimed to investigate the possibility that PFD might exert an anti-tumor effect through inhibition of EMT in non-small cell lung cancer (NSCLC) cell lines *in vitro* and *in vivo*.
Methods: NSCLC cells (A549, NCI-H358) were used to evaluate PFD effects on TGF-β1 induced phenotypic changes. Possible TGF-β1 signaling pathways modulated by PFD were evaluated. The effects of PFD on EMT induced by an anti-cancer drug was also analyzed. The impact of PFD on tumor growth in nude mice as well as on EMT change *in vivo* was also determined.
Results: PFD significantly inhibited TGF-β1-induced EMT. Smad2 phosphorylation and TGF-β1 receptor I expression were also inhibited as was translocation of Smad2 from the cytoplasm into the nucleus. Carboplatin induced elevation of TGF-β1 production from cancer cells together with induction of EMT, which were suppressed by co-treatment with PFD. In *in vivo* examination, PFD alone did not inhibit tumor progression whereas its combination with carboplatin significantly decreased tumor growth. Immunohistological analysis showed that PFD suppressed EMT change induced by carboplatin.
Conclusions: PFD could attenuate the EMT process induced not only by exogenous TGF-β1 but also by paracrine TGF-β produced from NSCLC cells. PFD may be a promising new therapeutic agent for the treatment of NSCLC through the regulation of EMT.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide, and 80% of affected patients die from the disease [1]. As the overall prognosis for affected patients is still poor due to metastatic disease and lack of curative systemic therapy, the development of new therapeutic strategies is urgently required.

One promising approach to finding new strategies is via investigation of the epithelial to mesenchymal transition (EMT), which is a fundamental biological process during which epithelial cells lose their polarity and change to a mesenchymal phenotype [2]. EMT is classified into three different subtypes: type 1 is associated with development such as implantation or embryo formation, type 2 is associated with wound healing and organ fibrosis, and type 3 is associated with a neoplastic environment [3]. Cancer cells undergoing a type 3 EMT may invade and metastasize in other tissues. Thus, finding an effective way to inhibit EMT should offer an attractive therapeutic strategy for controlling cancer.

Pirfenidone (PFD, 5-methyl-1-2-[1H]-pyridone; Shionogi & Co., Ltd. Osaka, Japan) is a pyridine compound with therapeutic potential for idiopathic pulmonary fibrosis (IPF). The anti-fibrotic effect of this new drug is broad as it suppresses multiple cytokines or growth factors including transforming growth factor β (TGF-β) [4]. It is well known that TGF-β is a strong inducer of EMT [5]. It was recently reported that PFD inhibited TGF-β2 induced EMT, which may thereby prevent the development of a major complication of cataract surgery that is mainly caused by fibrosis in human lens epithelial cells [6]. According to these reports, PFD may have an effect on inhibition of type 2 EMT. As for malignancies, TGF-β is reported to be intimately involved in excessive migratory and inva-

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sive potential in glioma [7], and PFD inhibited TGF-β2 expression in malignant glioma cells, which means that it could serve as a possible adjunct to therapies [8]. Kozono et al. showed that PFD could regulate pancreatic stellate cells (PSC) and that it disrupted the interaction of PSC with pancreatic cancer cells [9]. PSC play a role in desmoplasia, which contributes to the malignant behaviors of cancer cells. They concluded that PFD could be a promising antitumor agent for pancreatic cancer by suppressing desmoplasia through the regulation of PSC. Thus, PFD may have an anti-cancer effect in addition to an anti-fibrotic effect via inhibiting type 3 EMT.

The hypothesis that we developed was that PFD can reduce malignancy in non-small cell lung cancer (NSCLC) via inhibiting EMT. As we have previously shown that EMT is also involved in drug resistance in NSCLC [10], the effect of combining PFD treatment with anti-cancer drug was also examined.

2. Materials and methods

2.1. Cell lines

A549 and NCI-H358 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a humidified incubator of 5% CO_2 at 37 °C.

2.2. Drug treatments

A549 cells were plated at a density of 5×10^4 cells/ml in 10-cm dishes. After 24 h of incubation, 5 μM of carboplatin was added to the culture with or without 200 μg/ml of PFD. When the culture reached 70–90% confluence, the cells were passaged and the concentration of carboplatin was sequentially increased by 5 μM up to 25 μM. For NCI-H358 cells, 1×10^5 cells/ml were placed in 10-cm dishes and carboplatin with or without PFD was initially added in the same manner as for A549 cells. These cells were also passaged when grown to 70–90% confluence, but the concentration of carboplatin was maintained at 5 μM. For all *in vitro* studies, each cell line treated with carboplatin was used within 25–40 days from its creation.

2.3. Antibodies and reagents

Antibodies used for Western blotting and immunofluorescence were as follows: anti-N-cadherin mAb (sc-59987; Santa Cruz Biotechnology, Inc, Dallas, TX), anti-*E*-cadherin mAb (M106; Takara, Shiga, Japan), anti-vimentin mAb (V6633; Sigma, St. Louis, MO), anti-fibronectin pAb (ab2413; Abcam, Cambridge, MA), anti-Smad2 mAb (#3103S; Cell Signaling, Beverly, MA), anti-phosphorylated Smad2 mAb (#3101S; Cell Signaling), and anti-TGF-β receptor I pAb (sc-9048; Santa Cruz Biotechnology). Antibodies for the immunohistochemistry experiments were as follows: anti-*E*-cadherin mAb (M3612; Dako, Carpinteria, CA), anti-*N*-cadherin mAb (M3613; Dako), and anti-Ki67 mAb (M7240; Dako). Carboplatin was purchased from Sigma-Aldrich (#C2538, St. Louis, MO). The powder was dissolved in sterile water to a concentration of 25 mM. Transforming growth factor (TGF)-β1 was purchased from R&D Systems (240-B; Minneapolis, MN). For *in vitro* studies, PFD was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and was dissolved in DMSO to a concentration of 50 μg/ml. For *in vivo* studies, Pirespa™ tablets were purchased from Shionogi & Co. and were dissolved in DMSO, using one-fifth of the final volume of the total solvent, after crushing using a micro smash (TOMY SEIKO CO., LTD, Tokyo, Japan). Sterile normal saline at four-fifths of the final total volume was then added to bring PFD to a final concentration of 40 mg/ml.

2.4. RNA extraction and real-time RT-PCR

Cells were treated under the indicated conditions, and then total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Real-time RT-PCR (*E*-cadherin, Hs00170423_m1; *N*-cadherin, Hs00169953_m1 (Applied Biosystems, Tokyo, Japan)) was performed using a CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA), and relative expression levels were calculated by the comparative Ct method. All experiments were performed in triplicate.

2.5. SDS-PAGE and Western blot analysis

Monolayers of cultured cells were treated under the indicated conditions, and proteins were extracted with RIPA buffer (#9806, Cell Signaling). Cell extracts were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as described [11]. Western blotting after extraction of membrane proteins was performed using a Cell Surface Protein Isolation Kit (P74008, Takara) according to the manufacturer's protocol. Quantification of immunoblots was performed by Image Lab software as per the manufacturer's instructions (Bio-Rad Laboratories, Inc.).

2.6. Trans-well motility assays (migration assays)

A549 cells (1×10^6 cells), which were suspended in RPMI containing 1% FBS, were plated in the upper chamber of a transwell with polyethylene terephthalate membranes (pore size 8 μm; Becton Dickinson, Franklin Lakes, NJ). For NCI-H358 cells, 2×10^6 cells were plated and the filters were coated with collagen (50 μg/filter) (Thermo Fisher Scientific Inc. Waltham, MA). In the lower chamber, RPMI containing 10% FBS with or without TGF-β or PFD was added. The number of cells on the lower side of the filter was counted under a microscope after incubation for 24 h. Data were collected from three independently performed experiments.

2.7. Invasion assays

Transwell chambers with 8 μm pores (Becton Dickinson) were coated with 100 μg/ml Matrigel (Becton Dickinson). A549 and NCI-H358 cells (4×10^5 cells/well) suspended in serum-free RPMI were plated in the Matrigel-coated upper chamber. In the lower chamber, RPMI containing 10% FBS with or without TGF-β or PFD was added. The number of cells on the lower side of the filter was counted under a microscope after incubation for 24 (A549) or 48 (NCI-H358) h. Data were collected from three independently performed experiments.

2.8. Colony formation assays

To examine the anchorage-independent tumor growth, the colony-forming capacity was assessed using a Cytoselect 96-Well In Vitro Tumor Sensitivity Assay Kit (Cell Biolabs, Inc, San Diego, CA), according to the manufacture's protocol. Briefly, cell suspension in an agar matrix layer was placed on a base agar matrix layer and treated with various concentration of Pirfenidone (PFD). After incubation for 7 days, the colony formation was detected by the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT) solution.