Regular Article

Efficient Delivery to Human Lung Fibroblasts (WI-38) of Pirfenidone Incorporated into Liposomes Modified with Truncated Basic Fibroblast Growth Factor and Its Inhibitory Effect on Collagen Synthesis in Idiopathic Pulmonary Fibrosis

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In the present in vitro study, we assessed the delivery of pirfenidone incorporated into liposomes modified with truncated basic fibroblast growth factor (tbFGF) to lung fibroblasts and investigated the anti-fibrotic effect of the drug. The tbFGF peptide, KRTGQYKLC, was used to modify the surface of liposomes (tbFGF-liposomes). We used the thin-layer evaporation method, followed by sonication, to prepare tbFGF-liposomes containing pirfenidone. The cellular accumulation of tbFGF-liposomes was 1.7-fold greater than that of non-modified liposomes in WI-38 cells used as a model of lung fibroblasts. Confocal laser scanning microscopy showed that tbFGF-liposomes were widely localized in WI-38 cells. The inhibitory effects of pirfenidone incorporated into tbFGF-liposomes on transforming growth factor-β1 (TGF-β1)-induced collagen synthesis in WI-38 cells were evaluated by measuring the level of intracellular hydroxyproline, a major component of the protein collagen. Pirfenidone incorporated into tbFGF-liposomes at concentrations of 10, 30, and 100 µM significantly decreased the TGF-β1-induced hydroxyproline content in WI-38 cells. The anti-fibrotic effect of pirfenidone incorporated into tbFGF-liposomes was enhanced compared with that of pirfenidone solution. These results indicate that tbFGF-liposomes are a useful drug delivery system of anti-fibrotic drugs to lung fibroblasts for the treatment of idiopathic pulmonary fibrosis.

Key words liposome; lung fibroblast (WI-38); truncated basic fibroblast growth factor; idiopathic pulmonary fibrosis; pirfenidone

Idiopathic pulmonary fibrosis (IPF) is associated with poor health-related quality of life and is detrimental with an extremely low five-year survival rate (<50%).1,2 This chronic lung disease is characterized by an aberrant accumulation of the extracellular matrix (ECM), including collagen I, and progressive abnormal remodeling and scarring of the lung parenchyma. Lung fibroblasts localize to the sites of active fibrosis and are primary cells responsible for the synthesis and accumulation of ECM induced by the transforming growth factor (TGF)-β1 for structural remodelling from the alveolar epithelial cell injury.3 Therefore, lung fibroblasts are key target cells for the inhibition of collagen synthesis in the treatment of IPF.

We have previously reported that aerosolized formulations using polyethylene glycol (PEG)ylated liposomes enabled sustained drug distribution in the lung by avoiding uptake by alveolar macrophages.4 However, fibroblasts showed poor intracellular accumulation compared with other cells such as macrophages and epithelial cells. Terada et al. reported that liposomes modified with truncated basic fibroblast growth factor (tbFGF) peptide KRTGQYKLC accumulate in cells by binding to the fibroblast growth factor receptors (FGFRs) on cell surface.5,6 Therefore, tbFGF-modified PEGylated liposomes (tbFGF-liposomes) may efficiently accumulate in lung fibroblast and can be a viable drug delivery system for the treatment of IPF.

Pirfenidone is the first and only clinically used anti-fibrotic drug for the treatment of IPF in Japan (Pirespa®), Europe (Esbriet®), and India (Pirfenex®).7 Pirfenidone has anti-fibrotic, anti-inflammatory, and anti-oxidative actions.8–11 In particular, pirfenidone inhibits the collagen synthesis by lung fibroblasts. However, pirfenidone frequently causes systemic adverse effects, such as photosensitivity (51%), anorexia (17%), and nausea (44%), during clinical use.12,13 Therefore, it is an important goal to reduce the incidence of adverse effects using an efficient system for the delivery of pirfenidone to lung fibroblasts.

In the present study, we compared the accumulation of tbFGF-liposomes and non-modified liposomes in an in vitro model of lung fibroblasts (WI-38 cells). In addition, we used WI-38 cells to evaluate the anti-fibrotic effects of pirfenidone incorporated into liposomes.

MATERIALS AND METHODS

Materials: The peptide KRTGQYKLC (tbFGF) was synthesized by Bex Co., Ltd. (Tokyo, Japan). Egg-yolk phosphatidylcholine (EPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-DPPE), 1,2-distearyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), and DSPE-PEG2000-maleimide were purchased from NOF Co. (Tokyo, Japan). Cholesterol (Chol) and L-cysteine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pirfenidone was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Recombinant bFGF and TGF-β1 were purchased from Peprotech Inc. (Rocky Hill, NJ, USA).

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U.S.A.). All other reagents were commercially available and of analytical grade.

**Synthesis of DSPE-PEG<sub>2000</sub>-tbFGF** DSPE-PEG<sub>2000</sub>-tbFGF was synthesized using the method of Terada et al. In brief, 10 mg tbFGF dissolved in 800 µL 100 mM N-(2-hydroxyethyl)pyperazine-N'-(2-ethanesulfonic acid (HEPES) buffer (pH 7.0) and 18 mg DSPE-PEG<sub>2000</sub>-maleimide dissolved in 80 µL methanol were mixed, and the mixture was reacted for 48 h at 4°C. The unreacted tbFGF was removed by dialysis against unionized water using a dialysis cellulose membrane (cut-off size: 3500 Da) and lyophilized. TLC and ninhydrin assay showed the reduction of free tbFGF and the appearance of DSPE-PEG<sub>2000</sub>-tbFGF in the product. The purity of the synthetic DSPE-PEG<sub>2000</sub>-tbFGF was determined using an OPA Protein Quantitation Kit (AnaSpec Inc., Fremont, CA, U.S.A.) and was found to be 92%. Furthermore, to consume any free DSPE-PEG<sub>2000</sub>-maleimide, the product was mixed with 10 mM L-cysteine at room temperature for 1 h, followed by dialysis and lyophilization.

**Preparation of Liposomes** Liposomes were prepared using the lipid thin film hydration method. EPC, Chol, DSPE-PEG<sub>2000</sub>, bFGF, and NBD-DPPE in a lipid molar ratio of 20/10/1/0/10 or 20/10/0.6/0.4/10 were dissolved in chloroform–methanol (9:1) for tbFGF-liposomes and non-modified liposomes, respectively, followed by evaporation to obtain a thin film. To obtain liposomes, the film was completely hydrated using 10 mM HEPES buffer (pH 7.4 in 140 mM NaCl), followed by sonication at 65°C for 2 min with or without pirfenidone (50 mM). The liposomes were then extruded ten times through polycarbonate filters with a pore size of 400, 200, and 100 nm (Nucleopore; Whatman, Piscataway, NJ, U.S.A.). The particle sizes and the zeta-potential of liposomes were determined by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). The particle sizes of tbFGF-liposomes and non-modified liposomes were 109.6 and 104.6 nm, and the polydispersity index was 0.096 and 0.070, respectively. The zeta-potential of tbFGF-liposomes and non-modified liposomes was -0.01 and -0.04 mV, respectively. According to the HPLC analysis after gel filtration, the amount of incorporated pirfenidone into tbFGF-liposomes and non-modified liposomes were 1.86 and 1.66 mol pirfenidone/mol total lipid and the encapsulation efficiency of pirfenidone were 37 and 33%, respectively.

**Cell Culture** WI-38 cells (Riken Gene Bank, Tsukuba, Japan), a human lung fibroblast cell line, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 40 µg/mL gentamicin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells from passage numbers 12–15 were used. HEK293 cells (Riken Gene Bank), a human embryonic kidney cell line, were maintained in minimum essential medium (MEM) containing 10% heat-inactivated FBS, 40 µg/mL gentamicin, and 1% nonessential amino acids in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells from passage numbers 10–11 were used.

**Immunoblotting Analysis** Immunoblotting was performed on WI-38 cells after 2 d incubation on 60 mm culture dishes. HEK293 cells served as a negative control. Samples of cell lysates (5 µg protein/lane) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene difluoride membranes (20 V, 30 min). After blocking with 0.1% Tween 20 and 5% non-fat milk overnight at 4°C, the polyvinylidene difluoride membranes were reacted with rabbit polyclonal anti-FGRF1 antibody (Sigma) at a 1:250 dilution for 1 h at room temperature. The membranes were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 for 5 min and then were reacted with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Pollards Wood, U.K.) at a 1:2000 dilution. Antibody-protein complexes were finally detected using an enhanced chemiluminescence assay (Bio-Rad, Hercules, CA, U.S.A.).

**Accumulation Experiments in Vitro** WI-38 cells were suspended at a concentration of 2.0×10<sup>5</sup> cells/mL in DMEM containing 0.4% FBS. HEK293 cells were suspended at a concentration of 2.0×10<sup>5</sup> cells/mL in MEM containing 0.4% FBS and 1% nonessential amino acids. Aliquots (500 µL) of the cell suspension were subsequently transferred to 48-well culture plates, and the cells were incubated for 24 h at 37°C with 5% CO<sub>2</sub>. After incubation, non-adherent cells were removed. The tbFGF-liposomes and non-modified liposomes (40 µmol lipid/mL) with or without pirfenidone (pirfenidone concentration: 1 µmol/L) were applied to WI-38 and HEK293 cells, and cells were incubated at 37°C for designated times. The medium was removed by aspiration at designated times and washed three times with ice-cold PBS. Cells were then extracted using 400 µL 2× NaCl. The NBD-DPPE concentration in WI-38 cell extracts was 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 pirfenidone concentration in cell extracts was measured by HPLC as reported previously. The protein concentration in cell extracts was determined using Coomassie protein assay reagent (Pierce Chemical Company, Rockford, IL, U.S.A.) with bovine serum albumin as a standard. In the accumulation inhibition experiments, WI-38 cells were pre-treated with tbFGF (5 µg/mL) at 37°C for 30 min. Subsequently, the cells were treated with tbFGF-liposomes and non-modified liposomes (40 µmol lipid/mL) at 37°C for 2 h.

**Laser Scanning Confocal Microscopy** Laser scanning confocal microscopy was used to evaluate the localization of liposomes in WI-38 cells. WI-38 cells were seeded (2.0×10<sup>4</sup> cells/well) on 2-well glass chamber slides (Matsunami, Osaka, Japan) and were incubated for 24 h at 37°C with 5% CO<sub>2</sub>. After incubation, non-adherent cells were removed. The tbFGF-liposomes and non-modified liposomes (40 µmol lipid/mL) were applied to WI-38 cells, which were then incubated for 4 h at 37°C. The medium was removed by aspiration, and cells were washed twice with PBS, followed by fixation with 4% p-formaldehyde for 10 min at room temperature. Thereafter, green fluorescence of NBD-DPPE as a marker of liposome was analyzed using a laser scanning confocal microscope (LSM 700; Zeiss, Oberkochen, Germany).

**Cell Proliferation Assay** Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). In brief, WI-38 cells were seeded (4.0×10<sup>4</sup> cells/well) on 96-well culture plates. Liposomes (40 µmol lipid/mL) or tbFGF (100 ng/mL) were applied to WI-38 cells, which were then incubated for 24 h at 37°C with 5% CO<sub>2</sub>. After incubation, 100 µL medium was replaced and 10 µL of the CCK-8 solution was added to each well of the plate. After incubation for 3 h at 37°C, the absorbance at 450 nm was measured.
**Antifibrotic Experiments in Vitro**

WI-38 cells were seeded (2.0×10⁵ cells/well) on 24-well culture plates. At 70–80% confluence, medium was replaced with DMEM containing 0.4% FBS and 50 µg/mL of ascorbate. After an incubation of 24 h, TGF-β1 (10 ng/mL) and pirfenidone incorporated into tbFGF-liposomes, pirfenidone incorporated into non-modified liposomes, or pirfenidone solution were applied to the WI-38 cells, and cells were subsequently incubated at 5% CO₂ at 37°C for 24 h. After incubation, medium was removed by aspiration and washed twice with ice-cold PBS. The cells were then extracted with 300 µL 2 M NaCl, and the concentration of hydroxyproline in cell extracts was measured by HPLC as described previously.¹⁶) The DNA concentration in cell extracts was determined using a fluorescent DNA quantitation kit (Bio-Rad).

**Statistical Analysis**

Statistical analysis was performed using Mann–Whitney U-tests and Dunnett’s t-tests. SPSS software version 21 was used for all analyses (IBM Inc., Armonk, NY, U.S.A.). A p value of <0.05 was considered to be statistically significant.

![Figure 1](image1.png)

Fig. 1. Western Blot for FGFR1 Expression in WI-38 Cells

FGFR1 was not detected in HEK293 cells used as a negative control.

![Graphs](image2.png)

Fig. 2. Time Profiles of the Cellular Accumulation of tbFGF-Liposomes (●) and Non-modified Liposomes (○) in WI-38 (A) and HEK293 (B) Cells

Liposomes (40 µmol lipid/mL) were applied to cells, followed by incubation at 37°C. At each time point (0.25, 0.5, 1, 2, and 4 h) after incubation, the cellular NBD-DPPE concentration was determined. Each point represents the mean±S.D. (n=5). *p<0.05 and **p<0.01: significantly different from non-modified liposomes.

![Microscopy Images](image3.png)

Fig. 3. Confocal Microscopy Images Showing the Cellular Distribution of tbFGF-Liposomes and Non-modified Liposomes in WI-38 Cells

Liposomes (40 µmol lipid/mL) were applied to WI-38 cells, followed by incubation at 37°C for 4 h. Fluorescence of NBD-DPPE indicates the distribution of liposomes in WI-38 cells. Scale bar=50 µm.
RESULTS AND DISCUSSION

In this study, the efficacy of the delivery of pirfenidone incorporated into tbFGF-liposomes to lung fibroblasts and the anti-fibrotic effect of the drug was evaluated in vitro. The expression profiles of FGFR1 protein in WI-38 cells on immunoblotting are shown in Fig. 1. FGFR1 was expressed in WI-38 cells but not in HEK293 cells used as a negative control. Although FGFRs are overexpressed on the surface of cancer cells,17) the receptors are also expressed in fibroblast-derived cells,18) similar to WI-38 cells used in this study. In addition, FGFRs are more highly expressed in fibrotic lungs compared with normal lungs.19) These findings suggested that FGFRs are becoming potential targets for drug delivery systems for the treatment of IPF.

The time profiles of the accumulation of liposomes in WI-38 cells and HEK293 cells are shown in Fig. 2. The tbFGF-liposomes accumulated and reached saturation after 2 h of incubation. The equilibrium of the accumulation of tbFGF-liposomes in WI-38 cells was 1.7-fold higher than that for non-modified liposomes. On the other hand, the accumulation of tbFGF-liposomes in HEK293 cells without the expression of FGFR1 was not different from that for non-modified liposomes. Confocal microscopy images showing the cellular distribution of liposomes in WI-38 cells are shown in Fig. 3. The green fluorescence of NBD-DPPE in tbFGF-liposomes was stronger and more widely distributed than that in non-modified liposomes. The effects of tbFGF on the accumulation of liposomes in WI-38 cells shown in Fig. 4. tbFGF inhibited the accumulation of tbFGF-liposomes, but didn’t inhibit that of non-modified liposomes. These results agree with a previous report, which showed that bFGF can interact with FGFR1 on tumor cells.20) These findings suggest that tbFGF-liposomes were highly accumulated in WI-38 cells by binding to FGFR1 on cell surface. PEGylated liposomes may be taken up by fibroblasts via non-receptor mediated endocytosis.20) The complex of FGFRs and their ligands is internalized by receptor-mediated endocytosis.17) Therefore, tbFGF-liposomes are thought to be internalized by WI-38 cells via non-receptor and FGFR1-mediated endocytosis.

Because bFGF stimulates the proliferation of fibroblasts, it is believed to be pivotal in the fibrotic process in IPF.21) If tbFGF-liposomes have a proliferation effect on lung fibroblasts, the use of liposomes as a drug delivery system may exacerbate IPF. To evaluate the influence of tbFGF-liposomes on the proliferation of lung fibroblasts, we performed a CCK-8 cell proliferation assay. The effects of liposomes and bFGF on the proliferation of WI-38 cells are shown in Fig. 5. The tbFGF-liposomes had no effect on the proliferation of WI-38

Fig. 4. Effect of tbFGF on the Cellular Accumulation of tbFGF-Liposomes and Non-modified Liposomes in WI-38 Cells

The cells were first pre-treated with tbFGF (5µg/mL) at 37°C for 30 min, then treated with liposomes (40µmol lipid/mL) at 37°C for 2 h. After incubation, the cellular NBD-DPPE concentration was determined. Each point represents the mean±S.D. (n=5). * p<0.05: significantly different from non-modified liposomes.

Fig. 5. The Effect of tbFGF-Liposomes and Non-modified Liposomes on the Proliferation of WI-38 Cells

Liposomes (40µmol lipid/mL) and bFGF (100ng/mL) were applied to WI-38 cells, followed by incubation at 37°C for 24h. The number of cells was determined by the CCK-8 assay. * p<0.01: significantly different from control.
cells. In a previous report, tbFGF had no influence on cell proliferation by stimulation of the bFGF pathway, although the peptide was capable of binding to FGFR1. These findings indicate that tbFGF-liposomes do not have a proliferation effect and are safe as a drug delivery system for the treatment of IPF.

The time profiles of pirfenidone accumulation in WI-38 cells are shown in Fig. 6. The accumulation of pirfenidone in WI-38 cells after the application of liposomes containing pirfenidone was higher than the accumulation of pirfenidone solution at each time point. These results indicate that liposomes are useful for enhancing the accumulation of pirfenidone in lung fibroblasts. Since pirfenidone has small molecular size (MW: 185.22) and moderate hydrophobicity (log $P=1.1$), the permeability by passive diffusion is thought to be high. However, if pirfenidone accumulated in WI-38 cells by passive diffusion only, intracellular concentration of pirfenidone in equilibrium become equal to extracellular concentration. In general, the high concentration of drugs in cells is based on the active transport systems (e.g. drug transporter and endocytosis) and intracellular binding. Pirfenidone permeation through cellular membrane is not mediated by drug transporters. These finding indicate that pirfenidone incorporated into liposomes is highly concentrated in cells by endocytosis of lung fibroblast. In addition, similar to the cellular accumulation of liposomes, the equilibrium of the cellular accumulation of pirfenidone incorporated into tbFGF-liposomes was 1.8-fold higher than that for pirfenidone incorporated into non-modified liposomes. These results indicate that the surface tbFGF modification of liposomes is an efficient pharmaceutical technique to enhance the efficiency of pirfenidone accumulation.

The effects of the different concentrations of pirfenidone incorporated into tbFGF-liposomes on the content of hydroxyproline, a major component of the protein collagen, in WI-38 cells are shown in Fig. 7. Pirfenidone incorporated into tbFGF-liposomes at 10, 30, and 100 µM, pirfenidone incorporated into non-modified liposomes at 30 and 100 µM, and pirfenidone solution at 100 µM significantly decreased the TGF-β1-induced

![Fig. 6. Time Profiles of the Cellular Accumulation of Pirfenidone in WI-38 Cells](image)

![Fig. 7. Effects of Pirfenidone Incorporated into Liposomes on the TGF-β1-Stimulated Increase in Hydroxyproline in WI-38 Cells](image)
hydroxyproline content in WI-38 cells. As above, pirfenidone has small molecular size, moderate hydrophobicity, and high water solubility (>15 mg/mL). In addition, since pirfenidone has low pK_a values (~0.34) and, pirfenidone is not ionized at physiological pH. This information suggests that pirfenidone located in inner water phase of liposomes as non-ionized physiological pH. This information suggests that pirfenidone has an inhibitory effect on the collagen I synthesis in lung fibroblasts mediated by the inhibition of intracellular heat-shock protein 47 and the inhibition of Smad3, p38, and Akt factors in the TGF-β1 pathway involved in lung fibrosis.1,2,3 These findings strongly suggest that pirfenidone incorporated into tbFGF-liposomes is efficiently accumulated in WI-38 cells, and then pirfenidone is released from liposomes into the cytoplasm where it inhibits the synthesis of collagen in cell.

Researchers have reported that several drugs, including macrolide compounds,24 thymic hormone,25 and herbal compounds,26 have anti-fibrotic actions in the experimental animal models of pulmonary fibrosis. The mechanisms underlying the anti-fibrotic actions of these drugs on lung fibroblasts are yet to be determined. If these drugs do have anti-fibrotic effects on lung fibroblasts, tbFGF-liposomes may be useful for the delivery of these drugs for the treatment of IPF.

CONCLUSION

This study examined the effects of liposome surface tbFGF modification on drug delivery to lung fibroblasts. We have shown that pirfenidone incorporated into tbFGF-liposomes was efficiently delivered into WI-38 cells used as a model for lung fibroblasts. Furthermore, anti-fibrotic effects could be achieved in WI-38 cells at a lower dose than that used in clinical situations. This study indicates that tbFGF-liposomes is an efficient drug delivery system for the treatment of IPF.

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Conflict of Interest The authors declare no conflict of interest.

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