Therapeutic effect of lecithinized superoxide dismutase (PC-SOD) on bleomycin-induced pulmonary fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is thought to involve inflammatory infiltration of leukocytes, lung injury induced by reactive oxygen species (ROS), in particular superoxide anion, and fibrosis (collagen deposition). No treatment has been shown to improve definitively the prognosis for IPF patients. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified by catalase. Lecithinized SOD (PC-SOD) has overcome clinical limitations of SOD, including low tissue affinity and low stability in plasma. In this study, we examined the effect of PC-SOD on bleomycin-induced pulmonary fibrosis. Severity of the bleomycin-induced fibrosis in mice was assessed by various methods, including determination of hydroxyproline levels in lung tissue. Intravenous administration of PC-SOD suppressed the bleomycin-induced increase in the number of leukocytes in bronchoalveolar lavage fluid. Bleomycin-induced collagen deposition and increased hydroxyproline levels in the lung were also suppressed in animals treated with PC-SOD, suggesting that PC-SOD suppresses bleomycin-induced pulmonary fibrosis. The dose-response profile of PC-SOD was bell-shaped, but concurrent administration of
catalase restored the ameliorative effect at high doses of PC-SOD. Intratracheal administration or inhalation of PC-SOD also attenuated the bleomycin-induced inflammatory response and fibrosis. The bell-shaped dose-response profile of PC-SOD was not observed for these routes of administration. We consider that, compared to intravenous administration, inhalation of PC-SOD may be a more therapeutically beneficial route of administration due to the higher safety and quality of life of the patient treated with this drug.

**Keywords:** PC-SOD, bleomycin, idiopathic pulmonary fibrosis, reactive oxygen species, superoxide dismutase
**Abbreviations:** BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; DMBA, 4-(Dimethylamino)-benzaldehyde; EMT, epithelial-mesenchymal transition; H & E, hematoxylin and eosin; IPF, idiopathic pulmonary fibrosis; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; PC, phosphatidylcholine; PC-SOD, lecithinized superoxide dismutase; QOL, quality of life; ROS, reactive oxygen species; SOD, superoxide dismutase; SP-A, surfactant protein-A; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UC, ulcerative colitis.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating chronic lung condition with poor prognosis; the mean length of survival from the time of diagnosis is 2.8-4.2 years. IPF progresses insidiously and slowly, and acute exacerbation of IPF is a highly lethal clinical event (1, 4, 21, 36). Current agents for the treatment of IPF, such as steroids and immunosuppressors have not been found to improve the prognosis (1, 2, 26, 47), thus requiring the development of new types of drugs to treat IPF. To evaluate candidate drugs, the bleomycin-induced pulmonary fibrosis animal model provides a convenient option for the study (33).

Although the etiology of IPF is not yet fully understood, recent studies have suggested that it is triggered by lung injury and inflammation (infiltration of leukocytes (such as alveolar macrophages, lymphocytes and neutrophils) and activation of cytokines). Reactive oxygen species (ROS) that are released from the activated leukocytes cause further lung injury and inflammation. On the other hand, ROS and activated cytokines, especially TGF-β1 stimulate abnormal fibrosis (abnormal wound repair and remodelling), that is characterized by collagen deposition (22, 40). TGF-β1
seems to stimulate the production of interstitial collagen through both activation of fibroblasts and transformation of epithelial cells to fibroblasts (epithelial-mesenchymal transition, EMT) (3, 6, 48). This abnormal process of fibrosis is responsible for the pulmonary dysfunction associated with IPF. Supporting this idea, genetic inhibition of neutrophil elastase, of the TGF-β-dependent signal transduction pathway, or of collagen synthesis was reported to suppress the progress of bleomycin-induced pulmonary fibrosis (5, 9, 14, 52). However, it is not clear whether pharmacological inhibition of these factors can improve the prognosis for IPF in humans.

A number of previous studies have suggested that the cellular redox state, determined by the balance between ROS (such as the superoxide anion) and antioxidant molecules (such as superoxide dismutase (SOD) and glutathione), plays an important role in the pathogenesis of IPF. Pulmonary inflammatory cells prepared from IPF patients generated higher level of ROS than those from controls (25, 45). An increase in the level of ROS was reported in pulmonary tissues, blood and bronchoalveolar lavage fluid (BALF) of IPF patients and bleomycin-administered animals (8, 18, 38, 41). Genetic modulation that increases or decreases the pulmonary level of ROS resulted in
stimulation or suppression, respectively, of bleomycin-induced pulmonary fibrosis (11, 29). Thus, antioxidant molecules have attracted considerable attention as therapeutic candidates for the treatment of IPF. In fact, administration of N-acetylcysteine (NAC), which stimulates the synthesis of glutathione, exhibited therapeutic effects on IPF patients and bleomycin-induced pulmonary fibrosis in animals (10, 30, 31, 39).

SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified to oxygen and water by catalase or glutathione peroxidase (23). A decreased level of SOD was observed both in IPF patients and in animals with bleomycin-induced pulmonary fibrosis (37, 53), thus suggesting that increasing SOD could be of therapeutic benefit in the treatment of IPF. However, the low affinity of SOD to the cell membrane where superoxide anion is produced, and its low stability in plasma, with a half-life of only a few minutes, were obstacles to the application of SOD in a clinical setting (13, 16, 17, 46). As a result of this, various SOD drug delivery systems have been devised to help overcome these limitations (16, 17, 20, 51).

Among these applications, lecithinized SOD (PC-SOD) has potentially beneficial effects for the treatment of IPF. PC-SOD is lecithinized human Cu/Zn-SOD in which
four phosphatidylcholine (PC) derivative molecules are covalently bound to each SOD dimer (17). *In vitro* experiments with cultured cells have shown that this modification drastically improves the cell membrane affinity of SOD without decreasing its activity (16, 17), while *in vivo* experiments have demonstrated that it also greatly improves plasma stability (17). In phase I clinical study, intravenously administered PC-SOD (40-160 mg) had a terminal half-life of more than 24 h, with good safety and tolerability (7, 42) and recently published results of a phase II clinical study have shown that intravenously administered PC-SOD (40 or 80 mg) significantly improved the symptoms of patients of ulcerative colitis (UC), which also involves ROS-induced tissue damage (43). Furthermore, intravenously administered PC-SOD ameliorated bleomycin-induced pulmonary fibrosis in mouse (44, 50), suggesting that PC-SOD could be effective in the treatment of IPF patients. However, a bell-shaped dose-response profile of PC-SOD has been reported for its ameliorative effect against bleomycin-induced pulmonary fibrosis (44, 50). Furthermore, when considering the quality of life (QOL) of patients, the present clinical protocol of PC-SOD administration (daily intravenous infusion for 4 weeks) is expected to be improved. In this study, we provide evidence that the ineffectiveness of
higher doses of PC-SOD is due to the accumulation of hydrogen peroxide. Furthermore, based on the results obtained here, we propose that administration of PC-SOD by inhalation is a clinically viable option to improve the QOL of IPF patients treated with this drug.
MATERIALS AND METHODS

Chemicals and animals.

Paraformaldehyde, FBS, catalase from bovine liver (1340 U/mg), an antibody against human Cu/Zn-SOD, 4-(Dimethylamino)-benzaldehyde (DMBA), chloramine T, potassium dichromate, phosphotungstic acid, phosphomolybdic acid, Orange G and acid fuchsin were obtained from Sigma (St. Louis, MO). Bleomycin was from Nippon Kayaku (Tokyo, Japan). Novo-Heparin (5000 units) for injection was from Mochida Pharmaceutical Co. (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex Corporation (Kobe, Japan). Terminal deoxynucleotidyl transferase was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). An ELISA kit for TGF-β1 was from R&D systems, Inc. (Minneapolis, MN). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). Cytospin® 4 was purchased from Thermo Electron Corporation (Massachusetts, USA), while L-hydroxyproline, sodium acetate, TCA, azophloxin, aniline blue were from WAKO.
Pure Chemicals (Tokyo, Japan). Xylidine ponceau was from WALDECK GmbH & Co. KG, DIVISION CHROMA (Muenster, Germany), and Mayer’s hematoxylin, 1% eosin alcohol solution, mounting medium for histological examination (malinol) and Weigert’s iron hematoxylin were from MUTO Pure Chemicals (Tokyo, Japan). PC-SOD (3000 U/mg) was from our laboratory stock (17). DAPI was from Dojindo (Kumamoto, Japan).

Wild-type mice (6-8 weeks old, ICR, male) were used. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

**Administration of bleomycin, PC-SOD and catalase.**

ICR mice maintained under anesthesia with chloral hydrate (500 mg/kg) were given one intratracheal injection of bleomycin (5 mg/kg) in PBS (1 ml/kg) by use of micropipette (p200) to induce an inflammatory response and fibrosis. PC-SOD and catalase were dissolved in 5% xylitol and administered intravenously (tail vein) or intratracheally. For
control mice, 5% xylitol solution was administered. The first administration of PC-SOD was performed just before the bleomycin administration.

For administration of PC-SOD by inhalation, 5 mice were placed in a chamber (volume, 45 L) and maintained under normoxic and normocapnic conditions. PC-SOD was dissolved in 10 ml of 5% xylitol and an ultrasonic nebulizer (NE-U17 from Omron, Tokyo, Japan) that was connected to the chamber, was used to nebulize the entire volume of the PC-SOD solution in 30 min. For control mice, 5% xylitol solution was subjected to nebulizer. Mice were kept in the chamber for a further 10 min after the 30 min of nebulizing.

**Preparation of BALF and cell count.**

BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 units/ml heparin (two times). About 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents and the ratios of alveolar macrophages,
lymphocytes and neutrophils to total cells were determined. More than 100 cells were counted for each sample.

**Histological and immunohistochemical analyses and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.**

Lung tissue samples were fixed in 4% buffered paraformaldehyde, then embedded in paraffin before being cut into 4 µm-thick sections.

For histological examination, sections were stained first with Mayer’s hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For staining of collagen (Masson’s trichrome staining), sections were sequentially treated with solution A (5% (w/v) potassium dichromate and 5% (w/v) trichloroacetic acid), Weigert’s iron hematoxylin, solution B (1.25% (w/v) phosphotungstic acid and 1.25% (w/v) phosphomolybdic acid), 0.75% (w/v) Orange G solution, solution C (0.12% (w/v) xylidine ponceau, 0.04% (w/v) acid fuchsin and 0.02% (w/v) azophloxin), 2.5% (w/v) phosphotungstic acid, and finally Aniline Blue solution.
Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope.

For immunohistochemical analysis, sections were treated with 20 µg/ml Protease K for antigen activation and incubated with 0.3% hydrogen peroxide in methanol for removal of endogenous peroxidase. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with an antibody against human Cu/Zn-SOD (1:200 dilution) in the presence of 2.5% BSA and then incubated for 1 h with peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins. Then, 3, 3’-diaminobenzidine was applied to the sections and the sections were finally incubated with Mayer’s hematoxylin. Samples were mounted with malinol and inspected using a fluorescence microscope (Olympus BX51).

For the TUNEL assay, sections were incubated first with proteinase K (20 µg/ml) for 15 min at 37°C, then with TdTase and biotin 14-ATP for 1 h at 37°C and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 µg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).
Hydroxyproline determination.

Hydroxyproline content was determined as described (49). Briefly, the right lung was removed and homogenized in 0.5 ml of 5% TCA. After centrifugation, pellets were hydrolysed in 0.5 ml of 10 N HCl for 16 h at 110°C. Each sample was incubated for 20 min at room temperature after addition of 0.5 ml of 1.4% (w/v) chloramine T solution and then incubated at 65°C for 10 min after addition of 0.5 ml of Ehrlich’s reagent (1M DMBA, 70% (v/v) isopropanol and 30% (v/v) perchloric acid). Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined.

Determination of the amount of PC-SOD, TGF-β1 and hydrogen peroxide in vivo.

Determination of the amount of PC-SOD in serum and tissue was carried out as previously described (17). After administration of PC-SOD, the blood was collected and serum samples were obtained by centrifugation. Furthermore, lungs were dissected, cut into small pieces, homogenized and centrifuged to obtain the supernatants. The amount of PC-SOD in samples was determined using a human Cu/Zn-SOD ELISA kit (Bender
MedSystem, Burlingame, CA). The amount of TGF-β1 in the lung tissue was also measured by ELISA according to the manufacturer’s protocol.

For determination of hydrogen peroxide levels, lungs were dissected, cut into small pieces, suspended in PBS and incubated for 30 min at 4°C with rotation. After centrifugation, the supernatants were applied to the NWLSS™ NWK-HYP01 assay kit (Northwest Life Science Specialties, Vancouver, WA).

**Real-time RT-PCR analysis.**

Real-time RT-PCR was performed as previously described (32) with some modifications. Total RNA was extracted from pulmonary tissues using an RNeasy kit according to the manufacturer’s protocol. Samples (2.5 µg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad Laboratories, Hercules, CA) experiments using iQ SYBR GREEN Supermix, and analyzed with Opticon Monitor Software. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of
template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, actin cDNA was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were (name: forward primer, reverse primer): collagen type 1 (Col1a1):

5’-ccctgtctgcttcctgtaaact-3’, 5’-catgttcggttggtcaaagata-3’; collagen type 3 (Col1a3):

5’-agggcagggaacaacttgatg-3’, 5’-ctcccctttgcacaaagctca-3’; E-cadherin:

5’-tgcccaaaaaatgaaaaagg-3’, 5’-ctccccctttgcacaaagctca-3’; Actin:

5’-ggacttcgagcaagagatgg-3’, 5’-agcactgtgtggtgcctacag-3’.

**Statistical analysis.**

All values are expressed as the mean ± S.E.M. Two-way analysis of variance (ANOVA) followed by the Tukey test or the Student's t-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P<0.05$. 
RESULTS

Effect of PC-SOD on bleomycin-induced pulmonary fibrosis

Pulmonary fibrosis was induced in mice given a once-only (at day 0) intratracheal administration of bleomycin. The bleomycin-induced inflammatory response can be monitored as a function of the number of inflammatory cells (alveolar macrophages, lymphocytes and neutrophils) in BALF 3 days after the administration of bleomycin. As shown in Fig. 1A, the total number of inflammatory cells, and individual numbers of alveolar macrophages, lymphocytes and neutrophils were all increased by the bleomycin treatment. This effect, however, could be suppressed by the simultaneous intravenous administration of PC-SOD, suggesting that PC-SOD ameliorates the bleomycin-induced pulmonary inflammatory response. PC-SOD produced a maximum beneficial effect at a dosage of 1.5-3 kU/kg, whereas a higher dose (30 kU/kg) did not suppress the bleomycin-induced pulmonary inflammatory response (bell-shaped dose-response profile) (Fig. 1A). Administration of the higher dose (30 kU/kg) PC-SOD alone (without bleomycin administration) did not affect the number of inflammatory cells in BALF (data not shown).
Bleomycin-induced pulmonary fibrosis can be monitored by histopathological analysis and measurement of pulmonary hydroxyproline levels (an indicator of collagen levels), 14 days after the administration of bleomycin. Histopathological analysis of pulmonary tissue using hematoxylin and eosin (H & E) staining revealed that the bleomycin administration induced severe pulmonary damage (thickened and edematous alveolar walls and interstitium) and infiltration of inflammatory cells into these regions (Fig. 2A). These phenomena were suppressed by the intravenous administration of PC-SOD (Fig. 2A). Again, a bell-shaped dose-response profile was observed; PC-SOD produced a maximum beneficial effect at 1.5-3 kU/kg, whereas at a higher dose (30 kU/kg) this ameliorative effect was not evident (Fig. 2A).

Masson’s trichrome staining of collagen showed that bleomycin-induced collagen deposition was clearly suppressed by simultaneous intravenous administration of low doses (1.5-3.0 kU/kg) of PC-SOD, but not so clearly for a high dose (30 kU/kg) (Fig. 2B). As shown in Fig. 2C, a bell-shaped dose-response profile was also observed for the effect of PC-SOD on the bleomycin-induced elevation of pulmonary hydroxyproline content. The results in Fig. 2 thus support the fact that intravenous administration of PC-SOD
ameliorates bleomycin-induced pulmonary fibrosis. We used an ELISA assay to
determine the level of PC-SOD in serum and pulmonary tissue after its intravenous
administration. As shown in Table 1, PC-SOD was detected in serum and pulmonary
tissue 6 h after the final injection.

We also examined the effect of intravenous administration of PC-SOD on
pre-existing fibrosis; intravenous administration of PC-SOD was started at day 7 after the
administration of bleomycin. As shown in Fig. 2D-F, bleomycin-induced fibrosis was
suppressed by higher dose of PC-SOD (6 kU/kg) but not its low dose (1.5 kU/kg) under
the conditions.

Mechanism for ameliorative effect of PC-SOD on bleomycin-induced pulmonary
fibrosis

As described in the Introduction, ROS-induced pulmonary cell death and
TGF-β1-dependent stimulation of collagen synthesis and EMT play an important role in
IPF and bleomycin-induced pulmonary fibrosis (24, 48). We examined effect of
intravenous administration of PC-SOD on the extent of pulmonary cell death by
employing the TUNEL assay. TUNEL-positive cells (indicative of cell death) increased in response to administration of bleomycin and this increase was suppressed by simultaneous intravenous administration of PC-SOD (Fig. 1B), showing that PC-SOD protects pulmonary cells from cell death in vivo. We also examined the effect of PC-SOD on ROS-induced cell death in vitro, using A549 cells (human alveolar epithelial cell line). As shown in Fig. 3A, cell death induced by menadione, a superoxide anion-releasing drug, was inhibited by treatment of cells with PC-SOD.

A bleomycin-induced elevation of TGF-β1 levels in lung tissue was also suppressed by the intravenous administration of PC-SOD (Fig. 1C). We then examined effect of PC-SOD on the TGF-β1-dependent induction of collagen expression and EMT in vitro by using real-time RT-PCR analysis. Treatment of HFL-1 cells (human embryonic lung fibroblast) with TGF-β1 induced the expression of Coll1a1 and Colla3 mRNA; the simultaneous treatment of cells with PC-SOD did not affect this induction (Fig. 3B). As shown in Fig. 3C, treatment of A549 cells with TGF-β1 induced or suppressed expression of Coll1a1 or E-cadherin mRNA, respectively, suggesting that EMT was induced. PC-SOD did not affect these TGF-β1-dependent alterations of
mRNA expression (Fig. 3C). These results suggest that PC-SOD does not affect the TGF-β1-induced collagen synthesis and EMT.

**Effect of simultaneous administration of catalase on the ameliorative effect of PC-SOD against bleomycin-induced pulmonary fibrosis**

As described in the introduction, a bell-shaped dose-response profile of PC-SOD against bleomycin-induced pulmonary fibrosis has been also observed in other studies (44, 50). One possible explanation for the ineffectiveness of high doses of PC-SOD to combat the effects of bleomycin is the accumulation of hydrogen peroxide due to the relatively higher activity of SOD compared with catalase. We recently found evidence to support this notion in another animal model; simultaneous administration of catalase restored the ineffectiveness of higher doses of PC-SOD to combat dextran sulfate sodium (DSS)-induced colitis, an animal model of UC (19). On this basis, we tested here the effect of concurrent administration of catalase on the activity of a high dose of PC-SOD in bleomycin-treated animals. Administration of 30 kU/kg PC-SOD improved the bleomycin-induced inflammatory response (increase in inflammatory cells in BALF) in
the presence of the concurrent intravenous administration of catalase (1.5-6 kU/kg), but not in its absence (Fig. 4A). Administration of catalase alone did not significantly affect the bleomycin-induced inflammatory response (Fig. 4A).

We next examined the effect of simultaneous administration of catalase and high doses of PC-SOD on other aspects of bleomycin-induced pulmonary fibrosis. Bleomycin-induced pulmonary damage and infiltration of inflammatory cells into these regions, were clearly suppressed by the simultaneous administration of catalase and a high dose of PC-SOD; however, treatment with either catalase or PC-SOD alone did not bring about such ameliorative effects (Fig. 4B). Collagen deposition and an increase in hydroxyproline levels were also clearly suppressed by the simultaneous administration of catalase and a high dose of PC-SOD (Fig. 4C and D). Again, treatment with either catalase or high dose of PC-SOD alone did not exert these beneficial effects (Fig. 4C and D).

We further tested this idea by direct measurement of the pulmonary level of hydrogen peroxide. As shown in Table 2, administration of a high dose (30 kU/kg) but not low dose (1.5 kU/kg) of PC-SOD increased the pulmonary level of hydrogen peroxide.
The results shown in Fig. 4 and Table 2 suggest that the catalase-dependent restoration of
efficacy of a high dose of PC-SOD on bleomycin-induced pulmonary fibrosis is due to
the detoxification of hydrogen peroxide effects produced by a relatively higher activity of
SOD.

Effect of modified methods of administration on PC-SOD’s capacity to combat
bleomycin-induced pulmonary fibrosis

To obtain some useful clues for refining the clinical guidelines for administration of
PC-SOD, we tested the outcome of other routes of administration in the treatment of
bleomycin-induced pulmonary fibrosis. As illustrated in Fig. 5A, the intratracheal
administration of PC-SOD gave ameliorative effects against the bleomycin-induced
inflammatory response. Interestingly, a bell-shaped dose-response profile was not
observed with this route of administration; the intratracheal administration of higher
doses of PC-SOD (30 or 60 kU/kg) showed similar ameliorative effects to those seen for
lower doses (Fig. 5A). As shown in Fig. 5B-D, the intratracheal administration of
PC-SOD also suppressed bleomycin-induced pulmonary tissue damage and fibrosis.

Again, the bell-shape dose-response profile was not so obvious.

As shown in Table 1, after daily intratracheal administration of PC-SOD, the pulmonary level of PC-SOD was very high compared to that seen following intravenous administration. We therefore compared the distribution of PC-SOD in lung tissue in response to intravenous and intratracheal administration, using immunohistochemical analysis with antibody against human Cu/Zn-SOD. As shown in Fig. 6, SOD was detected depending on the administration of PC-SOD, showing that this antibody specifically recognises administered PC-SOD (not endogenous mouse SOD) under the conditions used. PC-SOD was detected in tissues containing a major airway (region A, Fig. 6) but was not as evident in regions distant from trachea (region B, Fig. 6) after the intratracheal administration of a low dose (1.5 kU/kg) (Fig. 6). On the other hand, PC-SOD was widely detected in both regions after the intravenous administration of a high dose (30 kU/kg) (Fig. 6). No SOD staining was observed in any regions after the intravenous administration of a low dose of PC-SOD (1.5 kU/kg) (data not shown).
PC-SOD was also detected in the serum after intratracheal administration, however, the level was much lower than that measured after its intravenous administration at an equivalent dose (Table 1).

The results shown in Fig. 5 suggest that inhalation of PC-SOD may increase the QOL of patients in the clinical practice. To test this idea, bleomycin-administered mice were placed in a chamber connected to an ultrasonic nebulizer, thus exposing them to PC-SOD-containing vapor. We confirmed by HPLC analysis and measurement of SOD activity that this treatment did not affect the structure and activity of the PC-SOD (data not shown). This treatment was repeated once daily for 3 days or 14 days and bleomycin-induced pulmonary disorders were examined. As shown in Fig. 7A, inhaled PC-SOD (both low dose (60 kU/chamber) and high dose (300 kU/chamber)) ameliorated the bleomycin-induced inflammatory response and suppressed the pulmonary tissue damage and fibrosis (Fig. 7B-D). We also found that inhalation of an even higher dose of PC-SOD (900 kU/chamber) decreased the bleomycin-induced inflammatory response as much as its low dose (60 kU/chamber) (data not shown), suggesting that bell-shaped dose-response profile did not occur with inhalation. As shown in Table 2, administration
of not only low dose (60 kU/chamber) but also a high dose (300 kU/chamber) of PC-SOD did not increase the pulmonary level of hydrogen peroxide, being different from the case of intravenous administration. We also found that inhalation of unmodified SOD (U-SOD) did not affect the bleomycin-induced inflammatory response (Table 3). As shown in Table 1, PC-SOD was detected in the pulmonary tissue after daily sessions of inhalation. Immunohistochemical analysis revealed that inhaled PC-SOD was distributed broadly in the lung tissue (Fig. 6). Furthermore, very little PC-SOD was detected in serum following its delivery in this manner (Table 1).
DISCUSSION

Previous studies showed that intravenous administration of PC-SOD ameliorates bleomycin-induced pulmonary fibrosis, however, its molecular mechanism was not fully understood (44, 50). In these studies, a bell-shaped dose-response profile for PC-SOD was observed, but the mechanism underlying this effect was unclear. In the present study, we reproduced the results of the previous studies and examined underlying mechanisms. Furthermore, as the current clinical protocol for the administration of PC-SOD (once daily intravenous infusion for 4 weeks) does not provide patients with good QOL, we attempted to find other dosing regimes in our animal model with a view to provide better clinical outcomes.

Pulmonary cell death could be a trigger of IPF and bleomycin-induced pulmonary fibrosis because it stimulates the inflammatory response and fibrosis (abnormal wound repair and remodelling) as described in the Introduction section. We showed that pulmonary cell death in bleomycin-treated mice was suppressed by administration of PC-SOD. We also showed that PC-SOD protected cultured lung epithelial cells from menadione-induced cell death. Furthermore, we found that PC-SOD suppresses the
bleomycin-dependent increase in TGF-β1 levels in pulmonary tissue in vivo and
menadione-induced production of TGF-β1 in vitro. On the other hand, PC-SOD did not
affect the TGF-β1-dependent stimulation of collagen synthesis and induction of EMT.
Based on these findings, we consider that PC-SOD ameliorates bleomycin-induced
pulmonary fibrosis through its inhibitory effect on ROS-induced cell death and
expression of TGF-β1 rather than by modulating TGF-β1-dependent cellular responses.

The bell-shaped dose-response profile of PC-SOD is of clinical concern, as this
may reflect side effects of the drug. Here, however, we found that the efficacy of higher
doses of PC-SOD is restored by simultaneous administration of catalase, which converts
hydrogen peroxide to water and oxygen. As such, the ineffectiveness of high doses of
PC-SOD on bleomycin-induced pulmonary fibrosis is likely to be caused by
accumulation of hydrogen peroxide. The simultaneous administration of catalase with
PC-SOD to IPF patients may therefore provide a greater therapeutic effect and lower the
risk of side effects. Furthermore, the examination of catalase activity in individuals prior
to PC-SOD administration may result in the establishment of safer treatment protocols for
IPF patients.
We also found that intratracheal administration of PC-SOD significantly suppressed bleomycin-induced pulmonary fibrosis. PC-SOD was detected in the serum following this mode of administration, however, the serum level with intratracheal administration of PC-SOD (1.5 kU/kg, effective dose for bleomycin-induced pulmonary fibrosis) was much lower than that measured following the intravenous administration of PC-SOD (0.75 kU/kg, ineffective dose). Therefore, it seems that the delivery of PC-SOD directly to the lung (but not via the blood) is primarily responsible for the improved effects seen in response to its intratracheal administration. On the other hand, the pulmonary level of PC-SOD administered intravenously (1.5 kU/kg, effective dose) was much lower than that obtained with intratracheal administration (0.15 kU/kg, ineffective dose). This may due to the localization of intratracheally administered PC-SOD close to the trachea rather than regions distant from there. Therefore, it seems that PC-SOD should be delivered in a broad manner to the lung to suppress bleomycin-induced pulmonary fibrosis. It should also be noted that the bell-shaped dose-response profile of PC-SOD was not observed (up to 60 kU/kg) with the intratracheal mode of administration.
We also found that inhalation of PC-SOD ameliorated bleomycin-induced pulmonary fibrosis. This finding is very important because if this mode of administration of PC-SOD can be applied clinically, it should greatly improve the QOL of patients treated with the drug. The lack of a bell-shaped dose-response profile with this route of administration is also therapeutically beneficial. The pulmonary level of PC-SOD after inhalation of PC-SOD (900 kU/chamber, effective dose) was higher than that after the intravenous administration (30 kU/kg, ineffective dose due to the bell-shaped profile). This discrepancy may be due to the difference in the local distribution of PC-SOD (for example, in the alveolar epithelia or in vessel walls). It was recently reported that inhalation of NAC attenuates bleomycin-induced pulmonary fibrosis (15). Since NAC stimulates the conversion of hydrogen peroxide to water and oxygen (12, 27), simultaneous administration of PC-SOD and NAC by inhalation may have a synergistically therapeutic effect on bleomycin-induced pulmonary fibrosis and IPF.

A phase II clinical study has shown that intravenously administered PC-SOD (40 or 80 mg) showed therapeutic effects against IPF as judged by the serum level of markers (lactate dehydrogenase (LDH) and surfactant protein-A (SP-A)) (Azuma, A. et al.,
unpublished results). Based on results in this study, we propose that the inhalation mode for administering PC-SOD could prove beneficial for the treatment of IPF patients. This is because comparing to intravenous administration, this mode of administration would cause improvement of the QOL of patients treated with the drug, equivalent efficacy (judged by immunohistochemical analysis in this study) and superior safety (due to lack of a bell-shaped dose-response profile). This mode of administration may be effective for other pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) and asthma in which ROS-induced pulmonary damage also plays an important role (28, 34, 35).
FOOTNOTES

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, as well as the Japan Science and Technology Agency and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
REFERENCES


8. **Cantin AM, North SL, Fells GA, Hubbard RC, and Crystal RG.**


Table and Figure Legends

Table 1. Serum and pulmonary levels of PC-SOD. Mice treated with or without bleomycin (5 mg/kg) once-only at day 0 were administered indicated doses of PC-SOD (kU/kg or kU/chamber) intravenously, intratracheally or by inhalation once daily for 3 days. Blood and pulmonary tissue were taken 6 h after the final administration of PC-SOD. Levels of PC-SOD in samples were determined by ELISA. Values are mean ± S.E.M. (n.d., not detected).

Table 2. Effect of PC-SOD on pulmonary level of hydrogen peroxide. Mice were administered indicated doses of PC-SOD (kU/kg or kU/chamber) intravenously or by inhalation once daily for 3 days. Lungs were removed and the amount of hydrogen peroxide was determined. Values are mean ± S.E.M. ** P<0.01.

Table 3. Effect of inhalation of U-SOD on bleomycin-induced inflammatory response. Mice were treated with bleomycin and the inflammatory response was
assessed as described in the legends of Fig. 1. Indicated doses of U-SOD (kU/chamber) were inhaled once per day for 3 days. Values are mean ± S.E.M.

**Figure 1.** Effect of intravenous administration of PC-SOD on bleomycin-induced inflammatory response. Mice treated with or without (vehicle) bleomycin (BLM) (5 mg/kg) once-only at day 0 were intravenously administered indicated doses of PC-SOD (kU/kg) once per day for 3 days (A-C). Total cell number, and numbers of alveolar macrophages, lymphocytes and neutrophils were determined after 3 days as described in Materials and Methods (A). Sections of pulmonary tissue were prepared after 3 days and subjected to TUNEL assay and DAPI staining. Similar results were obtained for at least three sections (B). The level of TGF-β1 in pulmonary tissue after 3 days was determined by ELISA (C). Values are mean ± S.E.M. * or # P<0.05; ** or ## P<0.01 (A, C).

**Figure 2.** Effect of intravenous administration of PC-SOD on bleomycin-induced pulmonary fibrosis. Mice treated once-only with or without (Control) bleomycin (BLM) (5 mg/kg) at day 0 were intravenously administered indicated doses of PC-SOD (kU/kg)
once per day for 14 days (A-C). Mice treated once-only with bleomycin (BLM) (5 mg/kg) at day 0 were intravenously administered indicated doses of PC-SOD (kU/kg) once per day from day 7 to day 13 (D-F). Sections of pulmonary tissue were prepared after 14 days and subjected to histopathological examination (H & E staining (A, D) or Masson’s trichrome staining (B, E)) as described in Materials and Methods. Similar results were obtained for at least three sections (A, B, D, E). The pulmonary hydroxyproline level was determined after 14 days as described in Materials and Methods. Values are mean ± S.E.M. * or # P<0.05; ** or ## P<0.01 (C, F).

Figure 3. Effect of PC-SOD on cell death, expression of collagen and EMT in vitro.

A549 (A, C) or HFL-I (B) cells were preincubated with the indicated concentration of PC-SOD for 1 h and further incubated with the indicated concentrations of menadione (A) or TGF-β1 (B, C) for 24 h in the presence of the same concentrations of PC-SOD as in the preincubation step. Cell viability was determined by MTT assay (A). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for each
gene. Values were normalized to the actin gene, expressed relative to the control sample (B, C). Values shown are mean ± S.E.M. (n=3). **P<0.01 (A-C).

**Figure 4.** Effect of concurrent administration of catalase on the ameliorative effect of PC-SOD on the bleomycin-induced inflammatory response and fibrosis. Mice were treated with bleomycin (BLM) and PC-SOD, and the inflammatory response (A) and pulmonary fibrosis (B-D) were assessed as described in the legends of Figs. 1 and 2. Indicated dose of catalase (Cat) (kU/kg) was intravenously administered once per day for 3 days (A) or 14 days (B-D). Similar results were obtained for at least three sections (B, C). Values are mean ± S.E.M. * or #P<0.05; ** or ##P<0.01.

**Figure 5.** Effect of intratracheal administration of PC-SOD on bleomycin-induced inflammatory response and pulmonary fibrosis. Mice were treated with bleomycin (BLM) and the inflammatory response (A) and pulmonary fibrosis (B-D) were assessed as described in the legends of Figs. 1 and 2. Indicated doses of PC-SOD (kU/kg) were administered intratracheally once per day for 3 days (A) or 14 days (B-D). Similar results
were obtained for at least three sections (B, C). Values are mean ± S.E.M. * or # P<0.05; ** or ## P<0.01.

**Figure 6.** Distribution of PC-SOD in the lung. Mice were treated with bleomycin (BLM) and indicated doses of PC-SOD (kU/kg or kU/chamber) were administered intravenously, intratracheally, or by inhalation once per day for 3 days. Sections of pulmonary tissue (from the two regions shown in the figure) were prepared 6 h after the final administration of PC-SOD (after 3 days) and subjected to immunohistochemical analysis with an antibody against human Cu/Zn-SOD. Similar results were obtained for at least three sections.

**Figure 7.** Effect of inhalation of PC-SOD on bleomycin-induced inflammatory response and pulmonary fibrosis. Mice were treated with bleomycin (BLM) and the inflammatory response (A) and pulmonary fibrosis (B-D) were assessed as described in the legends of Figs. 1 and 2. Indicated doses of PC-SOD (kU/kg) were inhaled once per
day for 3 days (A) or 14 days (B-D). Similar results were obtained for at least three sections (B, C). Values are mean ± S.E.M. * or \( P < 0.05 \), ** or \( P < 0.01 \).
<table>
<thead>
<tr>
<th></th>
<th>PC-SOD, intravenous (kU/kg)</th>
<th>plasma (U/ml)</th>
<th>lung (mU/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-SOD, intravenous (kU/kg)</td>
<td>0.75</td>
<td>7.80 ± 1.38</td>
<td>3.09 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>16.9 ± 0.93</td>
<td>9.06 ± 1.29</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>128 ± 9.5</td>
<td>63.9 ± 1.86</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>245 ± 7.6</td>
<td>109 ± 4.3</td>
</tr>
<tr>
<td>PC-SOD, intratracheal (kU/kg)</td>
<td>0.15</td>
<td>n.d.</td>
<td>20.6 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.30 ± 0.03</td>
<td>72.9 ± 5.31</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.75 ± 0.18</td>
<td>131 ± 28.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.34 ± 2.58</td>
<td>1050 ± 381</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>12.5 ± 6.60</td>
<td>2052 ± 702</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>26.6 ± 7.17</td>
<td>5412 ± 183</td>
</tr>
<tr>
<td>PC-SOD, inhalation (kU/chamber)</td>
<td>60</td>
<td>0.12 ± 0.06</td>
<td>22.7 ± 2.97</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.36 ± 0.12</td>
<td>51.9 ± 3.66</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>0.57 ± 0.12</td>
<td>198 ± 49.8</td>
</tr>
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### Tanaka et al. Table 2

<table>
<thead>
<tr>
<th>PC-SOD, intravenous (kU/kg)</th>
<th>Hydrogen Peroxide (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>15.7 ± 0.81</td>
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<tr>
<td>1.5</td>
<td>17.4 ± 0.75</td>
</tr>
<tr>
<td>30</td>
<td>22.8 ± 1.33 **</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PC-SOD, inhalation (kU/chamber)</th>
<th>Hydrogen Peroxide (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>15.1 ± 1.79</td>
</tr>
<tr>
<td>60</td>
<td>13.1 ± 1.93</td>
</tr>
<tr>
<td>300</td>
<td>11.5 ± 0.95</td>
</tr>
</tbody>
</table>

* (v.s. control)
<table>
<thead>
<tr>
<th>U-SOD, inhalation (kU/chamber)</th>
<th>Total cells (x $10^5$ cells)</th>
<th>Alveolar macrophages (x $10^5$ cells)</th>
<th>Lymphocytes (x $10^4$ cells)</th>
<th>Neutrophils (x $10^4$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>5.1 ± 0.28</td>
<td>4.8 ± 0.26</td>
<td>0.5 ± 0.01</td>
<td>1.7 ± 0.15</td>
</tr>
<tr>
<td>60</td>
<td>5.1 ± 0.14</td>
<td>4.8 ± 0.14</td>
<td>0.5 ± 0.07</td>
<td>1.6 ± 0.08</td>
</tr>
<tr>
<td>300</td>
<td>4.6 ± 0.23</td>
<td>4.4 ± 0.25</td>
<td>0.5 ± 0.08</td>
<td>1.5 ± 0.22</td>
</tr>
</tbody>
</table>
Tanaka et al. Fig. 1

A

Bar charts show the following:

- **Total cells (x 10^5 cells)**
- **Alveolar macrophages (x 10^5 cells)**
- **Lymphocytes (x 10^4 cells)**
- **Neutrophils (x 10^4 cells)**

Different conditions are compared:

- Control
- BLM (5) alone
- + PC (0.75)
- + PC (1.5)
- + PC (3)
- + PC (15)

Significance levels:

* (v.s. control)
# (v.s. BLM (5) alone)
** (v.s. control and BLM (5) alone)

B

Images show TUNEL and DAPI staining:

- **control**
- **BLM (5) alone**
- **+ PC (1.5)**

C

Bar chart shows TGF-β1 (pg/mg tissue):

- **control**
- **+ PC (1.5)**
- **BLM (5) alone**

Significance levels:

* (v.s. control)
# (v.s. BLM (5) alone)
Tanaka et al. Fig. 2

A

control

BLM (5) alone

+ PC (0.75)

+ PC (1.5)

+ PC (3)

+ PC (0.75) + PC (1.5)

+ PC (15)

+ PC (30)

+ PC (30)

B

control

BLM (5) alone

+ PC (0.75)

+ PC (1.5)

+ PC (3)

+ PC (15)

+ PC (30)

+ PC (30)

C

Hydroxyproline (µg/right lung)

control

BLM (5) alone

+ PC (0.75)

+ PC (1.5)

+ PC (3)

+ PC (15)

+ PC (30)

* (v.s. control)

# (v.s. BLM (5) alone)
Tanaka et al. Fig.3

A

Menadione (12.5 μM)

Cell viability (%)

Menadione (15 μM)

PC-SOD (U/ml)

B

Col1a1

(Relative expression)

Col1a3

(Relative expression)

Cont  TGF-β1  200  400

(1 ng/ml)  + PC-SOD

(U/ml)

C

Col1a1

(Relative expression)

E-cadherin

(Relative expression)

Cont  TGF-β1  200  400

(5 ng/ml)  + PC-SOD

(U/ml)
A

**Tanaka et al. Fig. 4**

**A**

- **Total cells** (x 10^5 cells):
  - BLM (5) alone
  - BLM (5) alone + PC (30)
  - BLM (5) alone + PC (30) + Cat (1.5)
  - BLM (5) alone + PC (30) + Cat (3)
  - BLM (5) alone + PC (30) + Cat (6)

- **Lymphocytes** (x 10^4 cells):
  - BLM (5) alone
  - BLM (5) alone + PC (30)
  - BLM (5) alone + PC (30) + Cat (1.5)
  - BLM (5) alone + PC (30) + Cat (3)
  - BLM (5) alone + PC (30) + Cat (6)

- **Neutrophils** (x 10^4 cells):
  - BLM (5) alone
  - BLM (5) alone + PC (30)
  - BLM (5) alone + PC (30) + Cat (1.5)
  - BLM (5) alone + PC (30) + Cat (3)
  - BLM (5) alone + PC (30) + Cat (6)

B

- BLM (5) alone
- BLM (5) alone + PC (30)
- BLM (5) alone + PC (30) + Cat (6)

C

- BLM (5) alone
- BLM (5) alone + PC (30)
- BLM (5) alone + PC (30) + Cat (6)

D

- **Hydroxyproline** (µg/right lung):
  - control
  - BLM (5) alone
  - BLM (5) alone + PC (30)
  - BLM (5) alone + PC (30) + Cat (6)

* (v.s. control)
# (v.s. BLM (5) alone)
Tanaka et al. Fig. 5

A

Bar charts showing the number of total cells, alveolar macrophages, lymphocytes, and neutrophils with different treatments.

B

Representative images of tissue sections with different treatments.
Hydroxyproline

Tanaka et al. Fig. 5

C

BLM (5) alone + PC (0.15) + PC (0.75) + PC (1.5)

+ PC (15) + PC (30) + PC (60)

D

Hydroxyproline (μg/right lung)

control
BLM (5) alone
+ PC (0.15)
+ PC (0.75)
+ PC (1.5)
+ PC (15)
+ PC (30)
+ PC (60)

* (v.s. control)
# (v.s. BLM (5) alone)
Tanaka et al. Fig. 6

BLM (5) alone

+ PC (30, intravenous)

+ PC (1.5, intratracheal)

+ PC (300, inhalation)
Tanaka et al. Fig.7

A

**Total cells** (x 10^5 cells)  
BLM (5) alone  
+ PC (60)  
+ PC (300)  
* (v.s. BLM (5) alone)

**Alveolar macrophages** (x 10^5 cells)  
BLM (5) alone  
+ PC (60)  
+ PC (300)  
* (v.s. BLM (5) alone)

**Lymphocytes** (x 10^4 cells)  
BLM (5) alone  
+ PC (60)  
+ PC (300)  
* (v.s. BLM (5) alone)

**Neutrophils** (x 10^4 cells)  
BLM (5) alone  
+ PC (60)  
+ PC (300)  
* (v.s. BLM (5) alone)

B

BLM (5) alone  
+ PC (60)  
+ PC (300)

C

BLM (5) alone  
+ PC (60)  
+ PC (300)

D

**Hydroxyproline** (µg/right lung)  
BLM (5) alone  
+ PC (60)  
+ PC (300)  
* (v.s. control)  
# (v.s. BLM (5) alone)