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'-ctccccttttgcacaaagctca-3'; *E-cadherin*: 5'-tgcccagaaaatgaaaaagg-3', 5'-gtgtatgtggcaatgcgttc-3'; *Actin*: 5'-ggacttcgagcaagagatgg-3', 5'- agcactgtgttggcgtacag-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-I cells (human embryonic lung fibroblast) with TGF-β1 induced the expression of *Col1a1* and *Col1a3* 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 *Col1a1* 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.