Effect of Lecithinized-Superoxide Dismutase on the Interstitial Pneumonia Model Induced by Bleomycin in Mice

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ABSTRACT—Superoxide anion (O_2^-) acts as an exacerbation factor in interstitial pneumonia. Lecithinized-superoxide dismutase (PC-SOD), which is synthesized with a lecithin derivative bound covalently to recombinant human Cu,Zn-SOD, has a longer half-life in plasma and higher affinity to cell membranes than unmodified SOD. The effect of PC-SOD was evaluated using the bleomycin-induced interstitial pneumonia mouse model. Treatment with PC-SOD at 10 mg/kg significantly reduced the hydroxyproline content and fibrosis score. Namely, PC-SOD suppressed the progression of pulmonary fibrosis on the bleomycin-induced interstitial pneumonia mouse model. PC-SOD may be a potential drug for interstitial pneumonia therapy.

Keywords: Lecithinized-superoxide dismutase, Interstitial pneumonia, Bleomycin

Recently, oxygen radicals such as superoxide anion (O_2^-) have been suggested to be related to the occurrence and exacerbation of interstitial pneumonia (1). Superoxide dismutase (SOD) is normally present in cells at a sufficient level to scavenge intracellular O_2^- . Although extracellular SOD is insufficient, exogenous SOD has been suggested as a possible therapeutic drug for treating this disease. However, the clinical application of SOD has been limited by its low cell membrane or tissue affinity and its rapid metabolic clearance (a few minutes). Therefore, chemically modified preparations of SOD have been investigated to overcome these problems. Mizushima et al. have developed a drug delivery system of lipid microspheres that consists of soybean oil surrounded by lecithin (2). Some investigators, including Mizushima and Igarashi, have found that lecithin is highly cytotropic and safe (3). Igarashi et al. developed lecithinized-superoxide dismutase (PC-SOD), in which 4 molecules of a lecithin (phosphatidylcholine, PC) derivative were covalently bound to each dimer of recombinant human Cu,Zn-SOD (r-hSOD) (4). PC-SOD has a longer half-life in plasma, a higher cell affinity and has more pharmacological potency than unmodified SOD (4–8). Additionally, the lecithin derivatives do not have pharmacological potency (4).

In this study, we examined the effect of PC-SOD as a therapeutic drug for interstitial pneumonia using the bleomycin (BLM)-induced interstitial pneumonia mouse

model.

Six-week-old healthy male ICR mice (SPF) (Charles River Japan, Inc., Yokohama) were used for this experiment. These mice were housed under controlled temperature, humidity and light, with food and water ad libitum. Mice were randomly placed into 5 groups: the control group (20 mice), BLM plus 5% mannitol; 3 PC-SOD treatment groups (20 mice/group), BLM plus PC-SOD/5% mannitol; and the normal group (6 mice), no BLM treatment. Bleomycin (Wako Pure Chemicals Industries, Ltd., Osaka) was dissolved in sterile saline (Otsuka Pharmaceutical Company, Tokyo). Mice were given 100 mg/10 ml/kg of BLM by single intravenous injection. PC-SOD (Lot. No. FU95002; Asahi Glass Co., Ltd., Tokyo) was diluted in 5% mannitol (Wako Pure Chemicals Industries, Ltd.) and prepared for doses of 1, 3 and 10 mg/5 ml/kg. We had already confirmed that 5% mannitol as an isotonic solvent had no effect on an animal model of inflammatory disease. PC-SOD or 5% mannitol as a vehicle was injected intravenously for 7 days starting from the next day after BLM intravenous injection. Fourteen days after they were given BLM, all animals were anesthetized with Nembutal (Abbot Laboratories, North Chicago, IL, USA) and sacrificed by bleeding. The right lung was removed after sacrifice, added with 0.25% protease (actinase; Kaken Pharmaceutical Co., Ltd, Chiba) solution, and then digested at 55°C for over 12 hr.

Biochemical estimates of collagen were based on the total amount of hydroxyproline in lungs of the mice. The hydroxyproline content was determined by the method of Woessner (9). The absorbency of the resulting solutions was measured spectrophotometrically at 557 nm. The left lung was fixed in 10% neutralized formalin. Embedded samples were then sectioned and stained with Azan-Mallory's stain. Histopathologically, fibrosis was assessed by a scoring system similar to that of Ekimoto et al. (10): 0, indicating the absence of fibrosis; 1, the presence of areas with questionable fibrosis in alveolar septa; 2, a few foci of fibrosis, often in the subpleural area (fibrosis areas of 1/8 or less in a lobe); 4, scattered foci of fibrosis (fibrosis areas of 1/8 to 1/4 in a lobe); and 6, diffuse fibrosis (fibrosis areas of 1/4 or more in a lobe). All assessments were done in a blind fashion. All results were presented as values of the mean $\pm$ S.E.M. The significance of the difference between the groups was analyzed by Student's *t*-test or Dunnett's multiple comparison test. Dunnett's nonparametric multiple comparison test was used for the analysis of fibrosis scores. The level of significance was set at 5%.

The control group (BLM plus 5% mannitol) had significantly increased hydroxyproline content compared with the normal group (BLM none). Treatment with PC-SOD at 10 mg/kg (BLM plus PC-SOD, 10 mg/kg/5% mannitol) significantly suppressed the increase in hydroxyproline content (Fig. 1). Histologically, treatment with PC-SOD at 10 mg/kg significantly reduced the fibrosis

score (Fig. 2). In the control group, foci of fibrosis located in the subpleural areas were observed (arrow, Fig. 3a). In the PC-SOD (10 mg/kg) treatment group, questionable fibrosis was present in subpleural areas (arrow, Fig. 3b). In the normal group, no fibrosis was observed (Fig. 3c).

BLM has generally been utilized for preparation of the interstitial pneumonia animal model (11). In this model, it has been reported that BLM increased the number of total cells in bronchoalveolar lavage fluid (BALF) (11) and production of O_2^- by alveolar macrophages in vivo (12) and in vitro (13). These findings suggest that oxygen radicals such as O_2^- produced by activated alveolar macrophages and other inflammatory cells may injure epithelial cells and the basement membrane in the lungs. Repeated tissue injury causes overdeposition of extracellular matrix (ECM) proteins and finally induce fibrosis. Namely, the pulmonary fibrosis may be caused by the excessive repair of the tissue injury caused by oxygen radicals. However, no reports have shown that Cu,Zn-SOD suppresses fibrosis in BLM-induced interstitial pneumonia models because of its rapid metabolic clearance and low affinity to cell membranes. PC-SOD, which is a lecithinized Cu,Zn-SOD, has a longer half-life in plasma and higher cellular affinity than r-hSOD (4, 5). It has been reported that PC-SOD improved some inflammatory diseases in animal models; the dextran sulfate sodium induced rat colitis model is an example of this (4–8). Now, there is no effective drug for interstitial pneumonia. We

Fig. 1. Effect of PC-SOD on the hydroxyproline content of the right lung in mice treated with 100 mg/kg of BLM intravenously. Mice were given BLM plus 5% mannitol in the control group, BLM plus PC-SOD (1, 3 or 10 mg/kg)/5% mannitol in the PC-SOD treatment groups and no BLM in the normal group. Each value represents the mean $\pm$ S.E.M. of 19–20 animals (6 normal animals). * $P < 0.05$.

Fig. 2. Effect of PC-SOD on the pulmonary fibrosis score of the left lung in mice treated with 100 mg/kg of BLM intravenously. Mice were given BLM plus 5% mannitol in the control group, BLM plus PC-SOD (1, 3 or 10 mg/kg)/5% mannitol in the PC-SOD treatment groups and no BLM in the normal group. Each value represents the mean $\pm$ S.E.M. of 19–20 animals. * $P < 0.05$.

Fig. 3. Histological examination of the lung in mice treated with 100 mg/kg of BLM plus 5% mannitol (a), BLM plus PC-SOD, 10 mg/kg/5% mannitol (b) and no BLM (c). Azan-Mallory's stain ($\times 50$).

studied the effect of PC-SOD on the BLM-induced interstitial pneumonia mouse model. PC-SOD significantly suppressed the progression of pulmonary fibrosis. PC-SOD has been reported to suppress the injury of vascular endothelial cells by neutrophils at a lower concentration than r-hSOD *in vitro* because it accumulated in the membrane of neutrophils and the vascular endothelium to scavenge O_2^- efficiently (5). In a recent report, $OH\cdot$ scavengers did not suppress the increase of lung hydroxyproline content by BLM treatment (14). O_2^- does not have such a strong toxicity to cause tissue injury by itself. However, O_2^- reacts with nitric oxide ($NO\cdot$) to form the stable peroxynitrite anion ($ONOO^-$), which is a strong oxidant that causes tissue injury (15). Now, we are obtaining evidence that productions of O_2^- , NO and $ONOO^-$ by alveolar macrophages increase in this model (data not shown). We speculate that PC-SOD may reduce the progression of pulmonary fibrosis as a result of preventing tissue injury by efficiently scavenging O_2^- and then suppression of $ONOO^-$ production.

In summary, PC-SOD suppressed the progression of pulmonary fibrosis in the BLM-induced interstitial pneumonia mouse model. This result suggests that O_2^- relates to fibrosis in the BLM-induced interstitial pneumonia mouse model, and PC-SOD may be a potential drug for interstitial pneumonia therapy.

REFERENCES

- 1 Strausz J, Müller-Quernheim J, Stepling H and Ferlinz R: Oxygen radical production by alveolar inflammatory cells in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* **141**, 124–128 (1990)
- 2 Mizushima Y, Igarashi R, Hoshi K, Sim AK, Cleland ME, Hayashi H and Goto J: Marked enhancement in anti-thrombotic activity of isocarbacyclin following its incorporation into lipid microspheres. *Prostaglandins* **33**, 161–168 (1987)
- 3 Mizushima Y and Igarashi R: Studies on polypeptide drug delivery system: tissue distribution of immunoglobulin G conjugated with lecithin. *J Controlled Release* **17**, 99–104 (1991)
- 4 Igarashi R, Hoshino J, Takenaga M, Kawai S, Morizawa Y, Yasuda A, Otani M and Mizushima Y: Lecithinization of superoxide dismutase potentiates its protective effect against Forssman antiserum-induced elevation in guinea pig airway resistance. *J Pharmacol Exp Ther* **262**, 1214–1219 (1992)
- 5 Igarashi R, Hoshino J, Ochiai A, Morizawa Y and Mizushima Y: Lecithinized superoxide dismutase enhances its pharmacologic potency by increasing its cell membrane affinity. *J Pharmacol Exp Ther* **271**, 1672–1677 (1994)
- 6 Ikeda K, Kinoshita M, Iwasaki N, Tagaya N and Shiojima T: Lecithinized superoxide dismutase retards wobblers mouse motoneuron disease. *Neuromuscul Disord* **5**, 383–390 (1995)
- 7 Hori Y, Hoshino J, Yamazaki C, Sekiguchi T, Miyauchi S, Mizuno S and Horie K: Effect of lecithinized-superoxide dismutase on the rat colitis model induced by dextran sulfate sodium. *Jpn J Pharmacol* **74**, 99–103 (1997)
- 8 Nakauchi K, Ikata T, Katoh S, Hamada Y, Tsuchiya K and Fukuzawa K: Effect of lecithinized superoxide dismutase on rat spinal cord injury. *J Neurotrauma* **13**, 573–582 (1996)
- 9 Woessner LF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* **93**, 440–447 (1961)
- 10 Ekimoto H, Takahashi K, Matsuda A and Umezawa H: Animal model on BLM-induced pulmonary fibrosis – comparison of the systemic (intraperitoneal) administration with the local (intratracheal) instillation – *Jpn J Cancer Chemother* **10**, 2550–2557 (1983)
- 11 Yasui M: Effect of the administration of interleukin-1 *in vivo* on bleomycin-induced pneumonitis in mice. *Juzen Med Soc* **103**, 425–440 (1994)
- 12 Slosman DO, Costabella PM, Roth M, Werlen G and Polla BS: Bleomycin primes monocytes-macrophages for superoxide production. *Eur Respir J* **3**, 772–778 (1990)
- 13 Conley NS, Yarbrow JW, Ferrari HA and Zeidler RB: Bleomycin increases superoxide anion generation by pig peripheral alveolar macrophages. *Mol Pharmacol* **30**, 48–52 (1986)
- 14 Mori H, Tanaka H, Kawada K, Nagai H and Koda A: Suppressive effects of tranilast on pulmonary fibrosis and activation of alveolar macrophages in mice treated with bleomycin: role of alveolar macrophages in the fibrosis. *Jpn J Pharmacol* **67**, 279–289 (1995)
- 15 Beckman JS, Beckman TW, Chen J, Marshall PA and Bruce AF: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* **87**, 1620–1624 (1990)