Production of Superoxide and Nitric Oxide by Alveolar Macrophages in the Bleomycin-Induced Interstitial Pneumonia Mice Model

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ABSTRACT—To elucidate the potential role of superoxide (O_2^-) and nitric oxide (NO) in the pathogenesis of interstitial pneumonia, the quantity of O_2^- and NO produced by the alveolar macrophages (AM) were determined in the bleomycin (BLM)-induced interstitial pneumonia mouse model. The production of O_2^- and NO increased on days 7, 14 and 21 after BLM injection. Strong expression of peroxynitrite ($ONOO^-$) was seen in AM by using immunostaining for nitrotyrosine. The hydroxyproline contents increased on day 21 after BLM injection. O_2^- and NO are thought to play an important role in the pathology of fibrosis.

Keywords: Superoxide, Nitric oxide, Fibrosis

Several inflammatory mediators have been implicated in the pathogenesis of pulmonary fibrosis. These include inflammatory-derived cytokines and oxygen radicals. The process of cellular injury in lung fibrosis is thought to be mediated by oxygen radicals such as superoxide (O_2^-) produced by infiltrated alveolar macrophages (AM) (1). Thus, we reported previously that lecithinized-superoxide dismutase (PC-SOD, an O_2^- scavenger) suppressed the progression of pulmonary fibrosis in the bleomycin (BLM)-induced interstitial pneumonia mouse model (2). However, O_2^- does not have a strong toxicity to cause tissue injury by itself. O_2^- reacts with nitric oxide (NO) to form the stable peroxynitrite ($ONOO^-$), which is a strong oxidant that causes tissue injury (3). Therefore, we investigated the production of NO, O_2^- and $ONOO^-$ by activated AM in the BLM-induced interstitial pneumonia mouse model.

Six-week-old healthy mice (SPF) (Charles River Japan, Inc., Kanagawa) were used for this experiment. These mice were housed in a controlled temperature, humidity and light, with food and water ad libitum. Bleomycin hydrochloride (Wako Pure Chemicals Industries, Ltd., Osaka) was dissolved in sterile saline (Otsuka Pharmaceutical Company, Tokyo). Mice were given 150 mg/10 ml/kg of BLM by single intravenous injection. All animals were sacrificed by bleeding under anesthesia with Nembutal (Abbot Laboratories, North Chicago, IL, USA) at 3, 7, 14 and 21 days after BLM administration. Animals were killed and the bronchoalveolar lavage fluid

(BALF) was obtained by cannulating the trachea and infusing the lungs with a total of 4.8 ml cold saline. AM were isolated from the BALF, adjusted to 3×10^5 cells/ml and allowed to adhere for 2 hr. The plates were then washed with phenol red free RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) and cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) (Gibco) and 10 μ g/ml lipopolysaccharide (LPS) (*E. coli*, 055:B5; Difco Laboratories, Detroit, MI, USA) for 48 hr. Nitrite concentrations in the cell-free supernatant were determined with the NO_2/NO_3 Assay Kit-F (Fluorometric) (Wako Pure Chemicals industries, Ltd.). Superoxide production by AM was determined by the chemiluminescence (CL) assay. The reaction mixture consisted of 0.25 mM bis-*N*-methylacridinium nitrite (lucigenin) (Sigma Co., St. Louis, MO, USA) in Hank's balanced salt solution (HBSS) (Nissui Pharmaceutical Co., Tokyo) (pH 7.8); cell suspension (6×10^5 cells); and 500 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Co.). The CL was measured for 60 min at 37°C by CCD imaging digital systems (Microtec Nichi-on, Chiba). The production of nitrotyrosine, a byproduct of protein nitration by $ONOO^-$, was investigated using immunohistochemical staining. On day 7 after BLM injection, AM were cultured for 24 hr in RPMI medium with 10% FBS, 10 μ g/ml LPS and 500 ng/ml PMA. AM were fixed in 2% periodate/lysine/paraformaldehyde, frozen and thawed, and then stained with a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY,

Fig. 1. Time course of O_2^- (A) and NO (B) production in the AM with BLM (150 mg/kg) injection (●) and without BLM (○). Each value represents the mean $\pm$ S.E.M. of 3 to 7 mice. * $P < 0.05$, ** $P < 0.01$, significantly different from normal mouse. Direct proof of O_2^- generation in the lung on day 7 after BLM (150 mg/kg) injection (C1) and that in nontreated lung (C2) as determined by chemiluminescence assay.

Fig. 2. Immunohistochemistry of AM with (A) and without (B) BLM (150 mg/kg) treatment on day 7 by using a specific antibody against nitrotyrosine ($\times 400$). A section of lung on day 7 after BLM (150 mg/kg) treatment (C) and a section of normal lung (D) were stained with anti-nitrotyrosine antibody ($\times 500$).

USA; in a dilution of 1:200). The antibody was detected by peroxidase-conjugated anti-rabbit immunoglobulin and 3-amino-9-ethyl carbazole as a substrate (Histostain SP kit; Zymed Laboratories Inc., San Francisco, CA, USA). Similarly, a section of mouse lung on day 7 after BLM injection was stained with a specific antibody against nitrotyrosine. Direct proof of O_2^- generation in the lung was obtained by using the CL assay. On day 7 after BLM injection, the lung was removed and the surface of it was stripped off. The lung was immersed into lucigenin solution as previously described and exposed to CCD digital imaging systems for 20 min at 37°C. The hydroxyproline contents was determined by the method of Woessner (4). All results were presented as values of the mean $\pm$ S.E.M. The statistical analyses were performed by Wilcoxon's rank sum test. The level of significance was set at 5%.

Superoxide generation by AM significantly increased on day 7 in the BLM treatment group (Fig. 1A), and this was confirmed by CL intensity in the lung 7 days after BLM injection (Fig. 1C). Nitric oxide generation by AM significantly increased on days 7 and 14 in the BLM-treated group (Fig. 1B). Strong immunohistochemical staining for nitrotyrosine residues was observed in the AM and tissue of lung 7 days after BLM injection (Fig. 2: A and C). All of the positive staining was nullified by free nitrotyrosine (Aldrich Chemical Co., Milwaukee, WI, USA) (10 mM) added into the incubation mixture containing the anti-nitrotyrosine antibody (data not shown). Very little staining was observed in the normal AM and mouse lung (Fig. 2: B and D). Hydroxyproline contents significantly increased 21 days after BLM injection (Fig.

3).

Oxidants are thought to play an important role in the pathology of pulmonary fibrosis (5). In the BLM-induced interstitial pneumonia mouse model, it has been reported that the treatment with BLM increased the number of total cells in BALF (6) and production of O_2^- by AM in vivo (7) and in vitro (8). Furthermore, treatment with PC-SOD, a scavenger of O_2^- , suppressed the progression of lung fibrosis and the increase in of hydroxyproline contents in a BLM-induced interstitial pneumonia mouse model (2). These findings suggest that oxygen radicals such as O_2^- produced by activated AM and other inflammatory cells may injure epithelial cells and cause lung fibrosis. However, O_2^- does not have such a strong toxicity to cause tissue injury by itself, and in a recent report, OH $\cdot$ -scavengers did not suppress the increase of lung hydroxyproline content by BLM treatment (9). As reported earlier, NO reacts very rapidly with O_2^- , resulting in the formation of $ONOO^-$, which is a more reactive and toxic oxidant than NO or O_2^- (3). However, there is no report about NO or $ONOO^-$ production in the BLM-induced interstitial pneumonia mouse model. Our present study indicated that production of O_2^- and NO increased in the initial stage of inflammation (i.e., on 7 and 14 days after BLM administration), and then expression of nitrotyrosine, which is a byproduct of protein nitration by $ONOO^-$, was seen in AM and lung tissue. In the late stage of the process (i.e., on 21 days after BLM administration), hydroxyproline contents increased in the lung. These results show that O_2^- , NO and $ONOO^-$ play an important role in the progression of pulmonary fibrosis in this model. It is known that $ONOO^-$ may contribute to

Fig. 3. Time course of hydroxyproline contents in the lung with BLM (150 mg/kg) injection (●) and without BLM (○). Each value represents the mean $\pm$ S.E.M. of 6 to 7 mice. ** $P < 0.01$, significantly different from normal mouse.

tissue injury. In fact, it was reported that in the lungs of patients with idiopathic pulmonary fibrosis, strong expression of nitrotyrosine and NOS was seen in inflammatory cells (10). Recently, ONOO⁻ has been reported to induce apoptosis in vitro (11), and excessive apoptosis leads to persistent inflammation and pulmonary fibrosis in the BLM-induced pulmonary fibrosis mouse model (12, 13). In view of these findings, we hypothesize that O₂⁻ and NO, which may account for the ONOO⁻, are associated with lung injury of this model in the initial stage. In the late stage of the process, repeated tissue injury causes overdeposition of extracellular matrix (ECM) protein and finally induces fibrosis. Namely, the pulmonary fibrosis may be caused by the excessive repair of the tissue injury by oxygen radicals.

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