Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice

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IDIOPATHIC PULMONARY FIBROSIS (IPF) is defined as a specific form of chronic fibrosing interstitial pneumonia associated with the histopathological appearance of usual interstitial pneumonia on surgical lung biopsy. The median survival of patients with IPF is reported to be 3–4 yr from the onset of respiratory symptoms (1). Despite such poor prognosis, the etiology of IPF with IPF is reported to be 3–4 yr from the onset of respiratory symptoms (1). Despite such poor prognosis, the etiology of IPF is still not fully understood.

METHODS

Model of bleomycin-induced pneumopathy. The present experiments were approved by the Committee on Ethics regarding Animal Experiments of Kyushu University Faculty of Medicine and were performed according to the guidelines of the American Physiological Society. Six- to eight-wk-old C57BL/6 male mice were purchased from SLC Japan and used in all the experiments. The body weight of the mice was approximately 20–25 g. After measurement of their body weight, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Schering-Plough). The anesthetized mice received 50 μl of bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) solution containing 1.5 units bleomycin/kg body wt in sterile saline intratracheally. At 1, 3, 7, 10, and 14 days after bleomycin instillation, the mice were killed and right lung tissues were fixed in 10% buffered formalin, while left lung tissues were snap-frozen in liquid nitrogen and stored at −80°C until use.

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Analysis of MCP-1 kinetics in bleomycin-induced pulmonary fibrosis.

The frozen left lung was homogenized in a hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml each of leupeptin and aprotinin using a poltron homogenizer (Kinematica, Lucerne, Switzerland). Protein concentrations of lung homogenates were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatants were assayed for MCP-1 concentrations with the mouse MCP-1 ELISA kit (BioSource International, Camarillo, CA). The frozen left lung was homogenized in a hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM fluoride, and 1 mg/ml each of leupeptin and aprotinin using a poltron homogenizer (Kinematica, Lucerne, Switzerland). Protein concentrations of lung homogenates were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatants were assayed for MCP-1 concentrations with the mouse MCP-1 ELISA kit (BioSource International, Camarillo, CA).

RESULTS

Kinetics of endogenous MCP-1 in lung tissue after bleomycin administration. Endogenous MCP-1 concentration in the lung tissue homogenate after bleomycin instillation was significantly elevated between days 3 and 10 after the bleomycin instillation and slightly increased at day 1 and at day 14, but with no statistical significance, compared with the concentration in saline-instilled mice (Fig. 1).

Kinetics of 7ND concentration in serum and lung tissue after gene transfection. 7ND was detectable between days 1 and 10 in serum and markedly increased between days 3 and 7 after the 7ND gene transfer in normal mice. 7ND was not detectable at 14 days after the transfer. On the basis of these findings, we injected the 7ND gene at 3 days before or at 7 days after bleomycin instillation, respectively.

Effect of 7ND gene transfer on bleomycin-induced pulmonary fibrosis. The alveolar wall had begun to thicken with infiltration of neutrophils and lymphocytes at 7 days after the bleomycin instillation (Fig. 3B) compared with the wall of saline-instilled mice (Fig. 3A). After 14 days, a large number of lymphocytes infiltrated into the lung interstitium, and thickening of the alveolar septa, collapse of the alveolar spaces, and proliferation of fibroblasts were all observed (Fig. 3C). 7ND gene transfection reduced the degree of fibrosis involving 30% of the lung; and 3, lesions involving >50% of the lung.

DNA damage and apoptosis in lung tissues. DNA damage and apoptosis were assessed by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method utilizing the DeadEnd Colorimetric Apoptosis Detection system (Promega). After proteinase digestion and removal of endogenous peroxidase, the sections were reacted with fluorescein isothiocyanate antibody. The reaction products were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with methyl green. The number of positive cells for TUNEL was counted in the whole field of each section under a microscope with ×200 magnification.

BALF. A tracheotomy was performed in killed mice. After insertion of the tracheal tube, the trachea was lavaged with five times with volumes of 1 ml of sterile saline at room temperature. The recovered fluids were filtered through a single layer of gauze to remove mucus. Cells in the lavage fluid were counted with a hemocytometer. Differential counts of BAL cells were performed on 200 cells stained with Diff-Quick (Baxter Diagnostics, Düdingen, Switzerland). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories).

Hydroxyproline assay. Samples of the lung tissue were frozen in liquid nitrogen, lyophilized with a freeze-dry system (LABCONCO, Kansas City, MO), weighed, and minced into a fine homogenate mixture. The lung tissue was hydrolyzed with 6 N HCl for 16 h at 12°C. The hydroxyproline content of each sample was determined according to the protocol of Woessner (35).

Statistics. For statistical analysis regarding the comparison of MCP-1 concentration, 7ND concentration, the number of TUNEL-positive cells, and hydroxyproline content, ANOVA followed by Scheffé’s F-test was used. For comparison of pathological grade, Kruskal-Wallis test followed by Mann-Whitney’s U-test was used. P values of <0.05 were considered significant. Statistical analysis was performed with StatView J-4.5 (Abacus Concepts, Berkeley, CA).
tion at 3 days before the bleomycin instillation did not affect the histopathological findings at 7 or at 14 days after the administration. However, 7ND gene transfection at 7 days after the instillation significantly attenuated the histopathological findings at 14 days (Fig. 3D). Empty vector pcDNA3 (Invitrogen, Carlsbad, CA) treatment at 7 days after the administration did not affect the histopathological findings at 14 days. Figure 4 shows that semiquantitative analysis of the pathological grade at 14 days (n = 7) was significantly decreased by 7ND gene transfer at 7 days after, but not at 3 days before, the bleomycin instillation compared with the grade in mice treated with bleomycin alone (n = 7).

7ND transfer at 3 days before the instillation did not affect the pathological grade at 7 days after the instillation (pathological grade: 3, 3, 2, 2, 1; n = 5) compared with the grade in mice treated with bleomycin alone (pathological grade: 3, 2, 2, 2, 1; n = 5). In addition, 7ND gene transfer at 3 days before bleomycin instillation did not affect the protein concentration at 3 days before the bleomycin instillation did not affect the histopathological findings at 7 or at 14 days after the administration. However, 7ND gene transfection at 7 days after the instillation significantly attenuated the histopathological findings at 14 days (Fig. 3D). Empty vector pcDNA3 (Invitrogen, Carlsbad, CA) treatment at 7 days after the administration did not affect the histopathological findings at 14 days. Figure 4 shows that semiquantitative analysis of the pathological grade at 14 days (n = 7) was significantly decreased by 7ND gene transfer at 7 days after, but not at 3 days before, the bleomycin instillation compared with the grade in mice treated with bleomycin alone (n = 7).

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The number of TUNEL-positive cells is correlated with lung injury and fibrosis in this model (17, 21, 32). Although the type of cells was not clearly identified, some of the bronchiolar and alveolar epithelial cells or inflammatory cells in the inflammatory lesions showed evidence of DNA damage and apoptosis at 7 and 14 days after the bleomycin instillation (Fig. 5, B and C, respectively), but this was not so in saline-instilled mice (Fig. 5A). 7ND gene transfer at 7 days after, but not at 3 days before, the instillation significantly decreased the number of positive signals for TUNEL at 14 days (Figs. 5D and 6). 7ND gene transfer at 3 days before the instillation did not decrease the number of TUNEL-positive cells at 7 days after the instillation (mean ± SE = 26.2 ± 4.9/field, n = 5) compared with the number in mice treated with bleomycin alone (31.2 ± 7.1/field, n = 5).

Table 1. The effect of 7ND transfer on BALF analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein, mg/ml</th>
<th>Total Cell Count, × 10^4/ml</th>
<th>Cell Differentiation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05±0.01</td>
<td>0.18±0.05</td>
<td>95.7±0.3</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.05±0.29</td>
<td>2.06±0.39</td>
<td>81.7±9.4</td>
</tr>
<tr>
<td>Bleomycin + 7ND</td>
<td>1.07±0.24</td>
<td>1.83±0.55</td>
<td>82.3±5.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. BALF, bronchoalveolar lavage. *P < 0.05.

DISCUSSION

We hypothesized that 7ND, which is a dominant-negative inhibitor of MCP-1, may prevent the development of bleomycin-induced pneumopathy in mice. We chose this method because it does not require either repeated administrations of 7ND or viral vector transfection. Acute alveolitis develops at 2–3 days followed by interstitial inflammation at 4–12 days after intratracheal instillation of bleomycin in rodents (6, 30). Fibroblast proliferation and collagen synthesis are initiated at 4–14 days after bleomycin instillation (30). We showed that muscle cells infected with the 7ND gene secreted 7ND protein into the circulating blood. 7ND protein was detectable in serum at 1 day after gene transfer. Interestingly, we observed that 7ND gene transfer at 3 days before or 7 days after bleomycin instillation attenuated DNA damage, apoptosis, and pulmonary fibrosis at the late phase, but not at 3 days before the instillation. Accordingly, MCP-1 may play an important role in lung injury and fibrogenesis at the late phase, but not in the initiation of inflammation and lung injury at the early phase in this model.
Brieland et al. (4) reported that MCP-1 mRNA expression in alveolar macrophages is increased at as early as 12 h and peaks at 24 h in bleomycin-treated rats. They also reported that the chemotactic activity of monocytes in BALF and alveolar macrophage-conditioned media obtained at 24 h after bleomycin administration is increased and that this activity was neutralized by anti-MCP-1 antibody. Zhang et al. (36) demonstrated that MCP-1 levels are elevated at 3 days after bleomycin instillation. Moore et al. (25) recently demonstrated that MCP-1/CCR2-mediated signals regulate the production of PGE2 by alveolar epithelial cells. Because PGE2 inhibits fibroblast proliferation, 7ND may prevent pulmonary fibrosis by blocking the suppressive effect of MCP-1 on PGE2 production by alveolar epithelial cells.

The MCP-1 effect on T cell differentiation may also be associated with the attenuation of fibrosis. It has recently been reported that MCP-1 can act as a T helper (h) 2 cytokine (15). CCR2 is expressed on activated CD4+ T cells. In the presence of MCP-1/CD4+ T cells. In the presence of MCP-1, CD4+ T cells produce increased amount of IL-4 but not IFN-γ (20). MCP-1 overexpression enhances Th2 response (16), whereas MCP-1 deficiency prevents Th2 response in vivo (23). Although the roles of Th1 and Th2 cytokines in pulmonary fibrosis have not been confirmed, it has been suggested that Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 may promote pulmonary fibrosis (10–13, 19, 28). 7ND may alter T helper cell polarization and attenuate the development of lung fibrosis in this model.

If one takes these findings into consideration, preventing MCP-1 function may be insufficient to suppress lung inflammation or lung injury during the early phase of this model.

Pulmonary fibrosis develops gradually from 7 to 14 days after bleomycin instillation. Macrophages and lymphocytes are the predominant infiltrating cells in this phase (29). 7ND gene transfection at day 7, which means 7ND concentration in serum peaked at day 10–day 14 after bleomycin administration, attenuated lung injury and pulmonary fibrosis at day 14. Macrophages produce reactive oxygen species, transforming growth factor-β1 (TGF-β1), and TNF-α. Activated lymphocytes express Fas ligand, perforin, and other cytotoxic molecules. MCP-1 may play a central role in the accumulation of these proapoptotic and fibrogenic molecules in lung tissue at late fibrotic phase in this model. Accordingly, 7ND gene transfer may attenuate apoptosis and fibrosis in this model.

7ND may suppress macrophage migration to the site of inflammation and reduce the amount of fibrogenic cytokines released from macrophages, such as TGF-β1, and platelet-derived growth factor (7, 22, 27, 34). Additionally, 7ND may prevent the direct effect of MCP-1 on fibroblasts such as the induction of collagen and TGF-β1 expression (9). Moore et al. (25) recently demonstrated that MCP-1/CCR2-mediated signals regulate the production of PGE2 by alveolar epithelial cells. Because PGE2 inhibits fibroblast proliferation, 7ND may prevent pulmonary fibrosis by blocking the suppressive effect of MCP-1 on PGE2 production by alveolar epithelial cells.
There are several advantages in this method compared with that using viral vector. Plasmid costs less and can be prepared easily in large amounts, compared with viral vector, and in vivo transfection of plasmid is safe and can be done without substantial inflammation or activation of immune reactions. However, the efficiency of gene transfer using plasmid is one of the limiting steps in gene therapy. Mir et al. (24) summarizes the advantages of in vivo electroporation in muscle tissues as follows. In vivo electroporation increases the efficiency of gene transfer not only by cell permeabilization but also by promoting DNA migration and cellular uptake, resulting in a 2-to 4-log enhancement of gene expression. There is no fast extracellular degradation of plasmid DNA in muscle tissue, and there is less interindividual variability. The expression level can be regulated by modulating the amount of DNA injected, electric-pulse parameters, or the volume of tissue exposed to the electric pulses. Therefore, this strategy may have broad applications in the therapeutic field.

In conclusion, 7ND gene transfer at the later phase, but not at the acute phase, ameliorated the development of bleomycin-induced pulmonary fibrosis in mice. These results suggest that MCP-1 may play an important role in the development of pulmonary fibrosis, but not in the initiation of acute lung inflammation. The inhibition of MCP-1 activity may be a possible therapeutic strategy for treating chronic interstitial inflammation and fibrosis in IPF. This is the first report about the protective effect of 7ND gene transfer into muscles against pulmonary fibrosis. Because this method does not require the use of viral vector or MCP-1-neutralizing antibody, it is possible to avoid problems regarding the pathogenicity of viral vector and immunocomplex. 7ND gene transfer into muscles may be a beneficial method of treating pulmonary fibrosis from the viewpoint of clinical application.

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