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

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Submitted 28 May 2003; accepted in final form 2 January 2004

Inoshima, Ichiro, Kazuyoshi Kuwano, Naoki Hamada, Naoki Hagimoto, Michihiro Yoshimi, Takashige Maeyama, Akira Takeshita, Shiro Kitamoto, Kensuke Egashira, and Nobuyuki Hara. Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol 286: L1038–L1044, 2004; 10.1152/ajplung.00167.2003.—Monocyte chemoattractant protein-1 (MCP-1) is a proinflammatory chemokine and may play an important role in the development of pulmonary fibrosis. We examined a new therapeutic strategy that comprises the transfection of the mutant MCP-1 gene into skeletal muscles as a biofactory for anti-MCP-1 therapy against bleomycin-induced pulmonary fibrosis in mice. Overexpression of the mutant MCP-1 gene at 10–14 days after intratracheal instillation of bleomycin resulted in decreased DNA damage, apoptosis, and pulmonary fibrosis at 14 days. However, overexpression of the mutant MCP-1 at 0–4 days after bleomycin instillation did not result in decreased pathological grade, DNA damage, or apoptosis at 7 and 14 days. Because, in this model, inflammatory cell infiltration begins at 3 days and is followed by interstitial fibrosis, it is likely that MCP-1 has an important role to play in the development of fibrogenesis but not in the development of early lung inflammation. This method does not require the use of viral vector or neutralizing antibody, and, as such, it is possible to avoid problems regarding the pathogenicity of the viral vector or immunocomplex. This new strategy may be a beneficial method of treating pulmonary fibrosis from the viewpoint of clinical application.

APPROACHES TO THE TREATMENT OF IPF

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 is as yet unknown, and no effective therapeutic strategy has been established. The effects of current immunosuppressive therapy with corticosteroids and cytotoxic agents are limited, and the adverse effects cannot be ignored. Thus establishment of an alternative therapeutic strategy is urgently needed.

Monocyte chemoattractant protein-1 (MCP-1) is a proinflammatory chemokine that is produced by various types of cells. It has been reported that MCP-1 mRNA and protein are strongly expressed in epithelial cells, macrophages, and endothelial cells from patients with IPF (2, 14). Serum and bronchoalveolar lavage (BAL) fluid (BALF) MCP-1 levels in IPF subjects are significantly higher than those in healthy volunteers (5, 31). In bleomycin-induced pulmonary fibrosis in rats, MCP-1 mRNA expression in lung tissue was significantly elevated between days 3 and 21 (36). Moore et al. (25) reported that CC chemokine receptor 2 (CCR2; receptor of MCP-1, MCP-3, and MCP-5 in mice) deficient mice were protected from FITC- and bleomycin-induced pulmonary fibrosis. These data suggest that MCP-1 may play an important role in the development of pulmonary fibrosis.

MCP-1 forms dimers at physiological concentrations. The NH2-terminal deletion mutant of MCP-1 (deletion of amino acids 2–8), so-called 7ND, specifically forms heterodimers with wild-type MCP-1 and has a greatly reduced capacity to attract monocytes compared with wild-type MCP-1, and, as such, it acts as a specific and dominant negative inhibitor of wild-type MCP-1 (26, 37). This mutant binds to CCR2 and blocks wild-type MCP-1 from binding to CCR2 and from mediating monocyte chemotaxis (26, 37). Skeletal muscle cells infected with an expression plasmid can produce a secreted protein into the circulating blood (33). Human 7ND gene transfer with intramuscular injection of plasmid vector accompanied by in vivo electroporation suppressed the vascular remodeling induced by a chronic blockade of NO synthesis (8). We hypothesized that muscle cells infected with the 7ND gene secrete 7ND protein into the circulating blood, and 7ND protein then binds to CCR2 on the target cells in the lung tissue, thereby blocking its signaling. On this basis, we examined this new strategy whereby 7ND gene transfection into muscle cells prevents the development of pulmonary fibrosis.

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 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 pneumopathy.** The frozen left lung was homogenized in a hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml each of leupeptin and aprotinin using a polytron 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).

2ND gene transfection. Human 2ND cDNA and the expression plasmid vector were constructed as previously described (8). Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (Schering-Plough), and in vivo electroporation was performed as previously described (18). Briefly, the 7ND expression plasmid vector (50 μg/50 μl of saline) was injected into the femoral muscle with a 27-gauge needle. Immediately after the plasmoid injection, a pair of electrode needles (Tokiwa Science) spaced 5 mm apart were inserted into the femoral muscle, one on each side of the injected site. Six 100-V square wave pulses (spaced 1 s apart) were applied with an electric pulse generator CUY201 (BTX, San Diego, CA), and the wound was closed. At 1, 3, 7, 10, and 14 days after 7ND gene transfection, the mice were killed, and whole blood and lung tissues were collected. 7ND concentrations in serum and lung homogenates were assayed with a human MCP-1 ELISA kit (BioSource), which is not reactive to mouse MCP-1. To assess the effect of 7ND gene transfection on the early inflammatory phase and the late fibrotic phase in bleomycin-induced pneumopathy, we administered the 7ND expression plasmid at 3 days before or at 7 days after bleomycin instillation, respectively.

**Histopathological examination.** After thoracotomy, the pulmonary circulation was flushed with saline, and the lungs were explored. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. A 3-μm paraffin section was adhered to slides and stained with hematoxylin and eosin. The pathological grade of inflammation and fibrosis in the whole area of the midsgittal section was evaluated under ×40 magnification and determined according to the following criteria: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2, lesions involving 25–50% 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 incubated in a mixture containing TdT and fluorescein isothiocyanate-labeled dUTP. The sections were then treated with peroxidase labeled with anti-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 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).

**RESULTS**

**Kinetics of endogenous MCP-1 in lung tissue after bleomycin administration.** Endogenous MCP-1 concentration in the lung tissue homogenate 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. (Fig. 2A). In lung homogenates, 7ND was detectable between days 1 and 14 and markedly increased between days 7 and 10 days after the 7ND gene transfer in normal mice (Fig. 2B). In serum and lung homogenates obtained from mice at 14 days after bleomycin instillation, 7ND was markedly increased at 7 days after the 7ND gene transfer (Fig. 2, A and B). On the basis of these findings, we injected the 7ND gene at 3 days before or at 7 days after the bleomycin instillation to examine the significance of MCP-1 on the early inflammatory phase (day 0–day 4) or late fibrotic phase (day 10–day 14) in this model, respectively.

**Effect of 7ND gene transfer on bleomycin-induced pneumopathy.** The alveolar wall had began 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 transfec-
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.

Fig. 2. Time course of 7ND concentration in lung homogenate (A) and serum (B) after intramuscular gene transfection. Data are shown as means ± SE obtained from 5 mice. Significance was compared with mice of day 0 (*$P < 0.05$). Experiments were repeated twice, and data were reproducible.

Fig. 3. The effect of 7ND gene transfer on microscopic findings. Normal lung parenchyma from saline-instilled mice (A), thickened alveolar septa infiltrate with neutrophils and lymphocytes at day 7 after the BLM instillation (B), marked thickening of the alveolar septa, collapse of alveolar spaces, a large number of lymphocytes accumulated in the alveolar walls, and proliferation of fibroblasts at day 14 after the BLM instillation (C). 7ND gene transfer at 7 days after BLM instillation significantly attenuated the histopathological findings at 14 days after the administration (D). Original magnification $\times 62.5$.

Fig. 4. The effect of 7ND gene transfer on pathological grade at 14 days after BLM instillation. Each circle corresponds to the data of 1 mouse (*$P < 0.05$).
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, A 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, × 10^6/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>Macrophage: 95.7±0.3, Lymphocytes: 1.2±0.3, Neutrophils: 3.2±0.3, Eosinophils: 0.0±0.0</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.05±0.29</td>
<td>2.06±0.39</td>
<td></td>
</tr>
<tr>
<td>Bleomycin + 7ND</td>
<td>1.07±0.24</td>
<td>1.83±0.55</td>
<td></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 transfection. On the basis of these findings, we injected the 7ND gene at 3 days before or 7 days after bleomycin instillation to examine the significance of MCP-1 on the early inflammatory phase (days 0–4) or the late fibrotic phase (days 10–14) in this model, respectively. We demonstrate that 7ND gene transfer at 7 days after, but not at 3 days before, the instillation, attenuated MCP-1 production, reduced fibrosis at 14 days. 7ND gene transfer at 3 days before the bleomycin instillation did not affect the protein concentration or the number of cell in BALF at 7 days after 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.

Fig. 5. The effect of 7ND gene transfer on terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining in lung tissues after BLM instillation. No positive signals for TUNEL in normal lung parenchyma (A). TUNEL-positive cells in lung tissues at 7 days (arrows in B) and at 14 days (arrows in C). These positive signals for TUNEL at 14 days were abrogated by 7ND gene transfer at 7 days after BLM instillation (D). Original magnification ×250.
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 3–21 days after bleomycin instillation. Moore et al. (25) mentioned that the MCP-1 level in lung homogenate is elevated from day 1 to day 14 after bleomycin instillation, with the peak being day 3 according to ELISA. We also detected increased MCP-1 concentration in lung homogenate at early as 3 days after bleomycin instillation. These results suggest that MCP-1 may play an important role in the development of lung inflammation at the early phase after bleomycin instillation. Therefore, we expected that 7ND gene transfer at 3 days before bleomycin administration, which means that 7ND concentration in serum peaks at day 0–day 4 after the instillation, may suppress the acute lung inflammation and subsequently the development of pulmonary fibrosis. However, 7ND overexpression during the acute phase did not affect DNA damage, apoptosis, and or the histopathological findings of this model.

Moore et al. (25) also demonstrated that both lung fibrosis and interstitial inflammation at 21 days after intratracheal administration of bleomycin in CCR2−/− mice are significantly attenuated, whereas acute lung inflammation at 1–7 days is not attenuated compared with that of wild-type mice. It is possible that MCP-1 is not essential in the development of acute lung inflammation and lung injury immediately after the bleomycin instillation, since various proinflammatory cytokines and chemokines other than MCP-1 are involved in acute lung inflammation. Bless et al. (3) demonstrated that the blockade of MCP-1 has no effect on pulmonary neutrophil recruitment or the extent of lung injury in IgG immune complex-induced lung injury, although MCP-1 is upregulated in the lung shortly after the deposition of IgG immunocomplexes. 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.

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 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.

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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 pneumopathy 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 beneficial method of treating pulmonary fibrosis from the viewpoint of clinical application.

ACKNOWLEDGMENTS

The English used in this manuscript was revised by K. Miller (Royal English Language Centre, Fukuoka, Japan).

GRANTS

This work was supported by Grants-in-Aid for Scientific Research 13470127 and 13670604 from the Ministry of Education, Science and Culture, Japan.

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