Introduction of the interleukin-10 gene into mice inhibited bleomycin-induced lung injury in vivo

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Idiopathic pulmonary fibrosis (IPF) is a disease characterized by progressive pulmonary insufficiency due to a fibrotic process of unknown etiology. The pathophysiology of pulmonary fibrosis has not been fully elucidated. However, a number of investigations have indicated sustained and augmented expression of some cytokines in the cells from lung fibrosis, and abnormal expression was suggested to be related to the progression of the disease. Tumor necrosis factor (TNF)-α (48), interleukin (IL)-1β (29), IL-8 (25), monocyte chemoattractant protein-1 (20), platelet-derived growth factor (PDGF) (30), and transforming growth factor (TGF)-β (6) are supposed to play important roles in pulmonary inflammation and fibrosis of IPF. A previous clinical investigation suggested that corticosteroid sometimes ameliorates lung inflammation (3), but in most cases of IPF, it hardly prevents the progression of fibrosis (2) and the prognosis is poor, with a mean survival of 5–6 yr (8, 21). Therefore, new treatment strategies are urgently required.

Intratracheal instillation of bleomycin (Bleo) in mice has been shown to cause lung inflammation and fibrosis (24). This lung injury model in mice caused by Bleo has been used to study the mechanism of lung fibrosis and the antifibrotic effects of many drugs. Anti-TNF-α antibody (33), soluble TNF-α receptor (34), IL-1 receptor antagonist (35), and anti-TGF-β antibody (17) were demonstrated to be useful in suppressing Bleo-induced lung injury. These investigations suggested that inhibiting the function of cytokines related to lung inflammation and fibrosis may be an effective means of treating IPF.

IL-10, which is produced by Th2 cells, B cells, monocytes, macrophages, and keratinocytes, is known to suppress many inflammatory reactions (28). IL-10 reduces the synthesis of proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α by monocytes/macrophages (10, 13) and polymorphonuclear leukocytes (44), and it also reduces free radical release (15) and nitric oxide synthesis (5) by macrophages and synthesis of interferon-γ by T cells (14). IL-10 also exerts anti-inflammatory effects by inducing apoptosis of activated neutrophils (9). Anti-inflammatory effects of IL-10 have been identified in vivo in a collagen-induced arthritis model (43). Therefore, it is of interest to determine whether IL-10 can ameliorate pulmonary inflammation induced by Bleo. In this study, we investigated the inhibitory effects of IL-10 on lung injury induced by Bleo in mice. We also examined whether IL-10 can suppress collagen production and proliferation by human lung fibroblasts.

Materials and Methods

Animals. Seven-week-old male C57BL/6 mice were purchased from SLC Japan (Hamamatsu, Japan) and were kept under specific pathogen-free conditions in our animal facility.
Plasmid vector. Human IL-10 cDNA (American Type Culture Collection) (42) was cloned into the expression vector pCAGGS (31) that contains the chicken β-actin promoter. Plasmids were generated in Escherichia coli JM109 and prepared with a Qiagen (Chatsworth, CA) Endofree Plasmid Mega Kit. To confirm the capacity of human IL-10 expression, pCA-hL10 was transfected into the human lung adenocarcinoma cell line A549 with FuGENETM 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The concentration of human IL-10 in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) with a human IL-10 ELISA kit (Endogen, Cambridge, MA) with a detection limit of 12 pg/ml.

cDNA synthesis. Total RNA was isolated with Isogen (Nippongene, Tokyo, Japan) from BALF cells or human lung fibroblasts. RNA (800 ng) was reverse transcribed in 20 µl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 1 mM each deoxynucleotide triphosphate, 20 U of RNase inhibitor, 2.5 µM random primers, and 5 U of reverse transcriptase with an RNA PCR kit version 2.1 (Takara, Kyoto, Japan) for 10 min at 30°C, for 30 min at 42°C, for 5 min at 99°C, and then for 5 min at 5°C.

PCR. Reaction mixtures contained 1× PCR buffer (Takara), 0.2 mM each deoxynucleotide triphosphate, 400 nM sense and antisense primers, 1.25 U of Taq polymerase (Takara), and cDNA in 50 µl. Amplification was performed in a thermal cycler MP (Takara) for the appropriate number of cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min. The optimal number of PCR cycles and annealing temperature for each primer set were as follows: 32 cycles and 57°C for mouse TNF-α, 27 cycles and 57°C for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 30 cycles and 56°C for human type I collagen (α1 chain), and 30 cycles and 60°C for human GAPDH. PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. The density of the product was calculated with FMBIO II multiview system (Takara).

PCR primer sequences were designed as follows: mouse TNF-α sense primer, 5'-GCGGTTACATTTAGATGCTTGCGT-3'; mouse TNF-α antisense primer, 5'-TCCCTTTTGCAGACTCACGAAATGG-3'; mouse GAPDH sense primer, 5'-GGTTGAGGTTTCTGTGGTTGACCGATT-3'; mouse GAPDH antisense primer, 5'-ATGCTAAGATTGTCATGATGACCC-3'; human type I collagen (α1 chain) sense primer, 5'-CTGTGTTCAAGGGTAAAAAGCAG-3'; human type I collagen (α1 chain) antisense primer, 5'-GCCAGGAAACCCAGGTCCC-3'; human GAPDH sense primer, 5'-GCGTCTCCGACCACCTAAGCTG-3'; and human GAPDH antisense primer, 5'-AATGGCCAAGCCCAGG-3'.

Semi-quantitative analysis of mouse TNF-α mRNA in BALF cells by RT-PCR. cDNA of mouse BALF cells was synthesized, and half of the cDNA was diluted 10-fold with water. PCR was performed with specific sets of sense and antisense primers, including 4 µl of cDNA for mouse TNF-α or 2 µl of diluted cDNA for mouse GAPDH. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Titration of cytokines in the serum and lavage fluid. Human IL-10 in the serum and abdominal lavage fluid was measured with an UltraSensitive human IL-10 immunosay kit (Bio-source International, Camarillo, CA) with a sensitivity of 208 fg/ml. Mouse TNF-α in the serum was measured with a mouse TNF-α ELISA kit (R&D Systems, Minneapolis, MN) with a detection limit of 5.1 pg/ml. Mouse IL-10 in the serum was measured with a mouse IL-10 ELISA kit (Endogen, Cambridge, MA) with a detection limit of 12 pg/ml.

density of 2.25 x 10^6 cells/well of a 96-well microtiter plate or at a density of 1.5 x 10^5 cells/well of a 12-well plate. At confluence, the medium of the 12-well plates was replaced with DMEM containing 0.4% FBS and 50 µg/ml of ascorbate. After 24 h, the cells were cultured in the presence of recombinant TGF-β (R&D Systems) and/or recombinant IL-10 (PharMingen) for 24 h and used for quantification of type I collagen mRNA. Another 12-well plate was cultured under the same conditions. The cells were recovered by trypsinization, and cell viability was determined with trypsin blue.
staining. Ninety-six-well microtiter plates were used for cell proliferation assay. Cells were seeded at a density of $2.25 \times 10^4$ cells/well. After 24 h, before confluence was reached, the medium was replaced with DMEM containing 0.4% FBS (sparse condition). The cells were cultured in the presence of IL-10 and/or recombinant PDGF (R&D Systems) for 24 h.

Quantification of human type I collagen mRNA in human lung fibroblasts by competitive PCR. Competitor DNA, which could be amplified by specific sets of primers for human type I collagen (α1-chain) or GAPDH were constructed with a competitive DNA construction kit (Takara). For competitive PCR, 1 μl of cDNA from WI-38 cells and the competitor DNA (10^9 to 10^5 copies) were added to the reaction mixture, and PCR was performed. The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Densities of the target DNA (type I collagen or GAPDH) and the respective competitor DNA were quantified with the FMBIO II multiview system, and the ratio of target DNA to competitor DNA was calculated. Statistical analysis was performed. The significance of differences between groups was assessed by the ratio of the copy number of type I collagen to that of GAPDH.

Cell proliferation assay. Cell proliferation was estimated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (7). Cells in 96-well microtiter plates were stimulated with IL-10 and/or PDGF. After 24 h, 50 μg of MTT (Sigma) were added to each well. After 6 h of incubation, the culture medium was replaced with 150 μl of DMSO to solubilize formazan crystals. Proliferation is expressed as absorbance at 490 nm recorded with a microtiter plate reader.

Statistical analysis. All data are expressed as means ± SE. The significance of differences between groups was assessed with analysis of variance in conjunction with Fisher’s least squares difference test. Only the difference in serum TNF-α levels induced by Bleo treatment with and without IL-10 was analyzed by nonparametric Mann-Whitney U-test. Probability values of $<0.05$ were considered significant.

RESULTS

Time course of lung inflammation and fibrosis in Bleo-induced lung injury. To investigate the time course of lung injury induced by intratracheal instillation of Bleo, MPO activity of BALF and hydroxyproline content of pairs of lungs were quantified. MPO activity, which was taken as an indicator of the extent of inflammation, increased significantly on day 7 (0.123 ± 0.017) compared with the baseline level on day 0 (0.007 ± 0.001; $P < 0.01$) and decreased significantly on day 21 (0.029 ± 0.017; $P < 0.05$; Fig. 1A). In addition, serum TNF-α level, which is another parameter of inflammation, was significantly elevated on day 7 ($P < 0.05$), and its peak concentration was 15.20 ± 5.38 pg/ml (Fig. 1B). TNF-α concentration then decreased gradually. After the peak concentration of TNF-α, serum IL-10 began to increase and reached a plateau 7 days after the peak concentration of TNF-α. Serum IL-10 was elevated significantly on day 21 (67.29 ± 25.79 pg/ml; $P < 0.05$; Fig. 1B). Hydroxyproline content, which was used to monitor the extent of fibrosis, was increased significantly on days 7, 14, and 21 ($P < 0.05$).
0.05), with a maximum of 417.0 ± 76.0 μg/pair of lungs on day 21 (Fig. 1C).

In the following experiments, the effects of IL-10 on Bleo-induced lung injury were evaluated by determining MPO activity on day 7 and hydroxyproline on day 21 after Bleo treatment.

Human IL-10 expression in the hIL10-HVJ-treated mice. To confirm successful IL-10 gene transfer and expression, hIL10-HVJ or Cont-HVJ was intraperitoneally injected into 7-wk-old male C57BL/6 mice. Values are means ± SE. Human IL-10 concentration in abdominal lavage fluid of hIL10-HVJ-treated mice (2.47 ± 0.42 pg/ml) were detected. However, significant elevation of IL-10 was not detected in Cont-HVJ-treated mice (Fig. 2B).

Inhibitory effects of IL-10 on Bleo-induced lung injury. To determine whether IL-10 can suppress pulmonary inflammation induced by Bleo treatment, MPO activity (Fig. 3) and TNF-α mRNA in the lungs (Fig. 4) were quantified. Mice received an intraperitoneal injection of HVJ-liposomes on day −3, and Bleo was instilled intratracheally on day 0. Then the mice were killed on day 7, and BAL was performed. MPO activity was increased in mice treated with Bleo (Bleo+), without HVJ (HVJ−) group (0.202 ± 0.036) compared with the without Bleo (Bleo−), HVJ− group (0.006 ± 0.002; P < 0.01). However, the increase in MPO activity was significantly reduced by pretreatment with hIL10-HVJ (0.110 ± 0.013; P < 0.05). The HVJ-liposomes without the IL-10 expression vector (Cont-HVJ) showed no suppression (0.232 ± 0.040; Fig. 3). Treatment with Cont-HVJ or hIL10-HVJ without Bleo instillation did not affect the MPO activity level in BALF (0.005 ± 0.001 and 0.006 ± 0.001, respectively).

According to the same schedule, the TNF-α mRNA level in BALF cells was quantified by RT-PCR (Fig. 4A). hIL10-HVJ treatment resulted in a significant decrease in TNF-α mRNA in BALF cells (0.120 ± 0.023) compared with Cont-HVJ treatment (0.229 ± 0.034; P < 0.05). Serum levels of TNF-α were lower in hIL10-HVJ-treated mice (1.78 ± 1.95 pg/ml) than in Cont-HVJ-treated mice (28.54 ± 17.62 pg/ml), but this difference was not significant (P < 0.10; Fig. 4B).

Next, the effects of IL-10 on pulmonary fibrosis 21 days after Bleo instillation were assessed. Hydroxypro-
was cultured in the presence of IL-10 (0.2, 2.0, and 20 ng/ml) and/or TGF-β (0.1, 1.0, and 10 ng/ml), and expression of type I collagen mRNA was quantified by competitive PCR. Constitutive type I collagen mRNA expression in WI-38 cells was suppressed by IL-10 in a dose-dependent manner (Fig. 6A). Twenty nanograms per milliliter of IL-10 reduced type I collagen mRNA expression to 41%. In contrast, TGF-β augmented type I collagen mRNA expression dose dependently (Fig. 6B). To assess the interaction of these factors, WI-38 cells were cultured with 10 ng/ml of TGF-β and 20 ng/ml of IL-10. Only the constitutive level of type I collagen gene expression was detected in cells cultured with TGF-β and IL-10 (Fig. 6C). Cell viability as determined by trypan blue staining was not affected by stimulation of IL-10 and/or TGF-β (data not shown).

Human fibroblast proliferation. To verify the direct effect of IL-10 on fibroblast proliferation, cells were cultured under sparse conditions in the presence of IL-10 and/or PDGF. PDGF induced fibroblast proliferation at concentrations of 5 and 20 ng/ml (Fig. 7B), but IL-10 did not influence fibroblast proliferation (Fig. 7A). IL-10 also did not affect fibroblast proliferation induced by PDGF (20 ng/ml; Fig. 7C).

DISCUSSION

A number of investigations (5, 9, 10, 13–15, 28, 44) have shown that IL-10 is involved in regulation of various aspects of inflammation. Production of TNF-α, which is one of the most important factors in the pathogenesis of interstitial pneumonia, is inhibited by IL-10. TNF-α induces the expression of adhesion molecules by vascular endothelial cells (22) and intensifies...
the recruitment of inflammatory cells into the lungs (38). Thus suppressing the effects of TNF-α is supposed to weaken pulmonary inflammation. For example, it has been reported that the intratracheal instillation of recombinant IL-10 can suppress TNF-α content in BALF and the expression of intercellular adhesion molecule-1 on pulmonary endothelial cells in immune complex-induced lung injury (40). It has also been shown that IL-10 treatment attenuates silica-induced pulmonary inflammation by reducing the production of chemokines, superoxide anions, and nitric oxide by BALF cells (12). However, the effects of IL-10 on pulmonary inflammation induced by Bleo have not been reported previously.

In our experiments, the endogenous TNF-α level in the serum of mice treated with intratracheal instillation of Bleo was elevated transiently on day 7 compared with the baseline level on day 0 and decreased gradually thereafter. However, serum IL-10 began to increase after the peak concentration of TNF-α, and the maximum concentration of IL-10 was detected from days 14 to 21. The endogenous IL-10 expression may be interpreted as a negative feedback reaction in response to inflammation. Hence it is reasonable to suppose that endogenous IL-10 before the elevation in TNF-α can attenuate lung inflammation induced by Bleo. To test the anti-inflammatory effect, we introduced the human IL-10 gene into the peritoneum of mice before Bleo treatment. As anticipated, MPO activity in BALF and TNF-α mRNA expression in BALF cells on day 7 after Bleo instillation were significantly inhibited by in vivo human IL-10 gene transfer with HVJ-liposomes. In addition, hydroxyproline content, which is an indicator of the extent of pulmonary fibrosis, was also reduced in our experiments on day 21 after Bleo treatment. The results of the present investigation suggested that IL-10 inhibited not only pulmonary inflammation but also the pulmonary fibrosis induced by Bleo.

Previous in vitro experiments indicated that TNF-α is relevant to the induction of fibrosis by augmenting synthesis of fibronectin (4), prostaglandin (18), and TGF-β (32). Fibrogenic effects of TNF-α have also been demonstrated by in vivo experiments. Sime et al. (41) reported that the transfer of TNF-α into rat lungs by an adenovirus vector induced severe pulmonary inflammation and patchy interstitial fibrogenesis with the induction of TGF-β. Moreover, it has been shown that lung-specific persistent expression of TNF-α in transgenic mice results in the development of chronic inflammation and severe fibrosis of the lungs (27). These observations are compatible with our findings, and it is reasonable to assume that blocking the effects of TNF-α...
would lead to inhibition of pulmonary fibrosis. Thus our observations have shown that IL-10 exerts its antifibrotic activity partially through reducing TNF-α expression.

It is also important to determine whether IL-10 modulates the functions of lung fibroblasts because IL-4, which is a Th2 cytokine and has anti-inflammatory effects similar to IL-10, is known to directly upregulate collagen synthesis by human fibroblasts (36). In addition, it may be possible that endogenous IL-10 elevation in the serum induced by Bleo treatment, which we have shown, intensifies collagen synthesis. The direct effects of IL-10 on collagen production and proliferation of lung fibroblasts have not been extensively investigated. In skin fibroblasts (37, 46), it has been reported that IL-10 can suppress collagen production in the static state. Hepatic stellate cells, the cells that produce collagen in the liver, produce more collagen when cultured with anti-IL-10 antibody (45), and IL-10 is supposed to modulate collagen synthesis by activated hepatic stellate cells in the fibrotic liver. In addition, proliferation of smooth muscle cells is inhibited by IL-10 (39). Thus IL-10 may directly influence the functions of lung fibroblasts. We have shown in this investigation that IL-10 inhibited collagen production by WI-38 cells, a human lung fibroblast cell line, in the steady state and also in the presence of TGF-β. Proliferation of WI-38 cells was not influenced by IL-10, and proliferation induced by PDGF was not suppressed. The precise inhibitory mechanisms of IL-10 on collagen production have not yet been fully clarified. In addition to prevention of TNF-α expression, the present results strongly suggested that IL-10 may exert its in vivo antifibrotic effects through the direct inhibition of collagen gene expression. Hence it may be possible that the elevation in endogenous IL-10 in the serum was too late to prevent pulmonary fibrosis induced by Bleo treatment.

In this investigation, we clarified the biological effects of IL-10 by in vivo gene transfer. The in vivo effects of IL-10 on lung disease have also been studied in IL-10-deficient mice. Huaux et al. (19) reported that in a silica-induced lung fibrosis model, the amount of total protein, lactate dehydrogenase activity, and number of total cells in BALF were increased in IL-10 knockout mice compared with those in normal littermates. However, the hydroxyproline content of lungs on day 30 after silica exposure was decreased in IL-10 knockout mice. They concluded that IL-10 suppresses pulmonary inflammation but may promote the process of pulmonary fibrosis. The apparent contradiction between our results and their observations may be explained by the differences between the Bleo and silica models. In the silica exposure model, intensity of pulmonary fibrosis is inversely correlated with strength of initial inflammation. Adamson et al. (1) explained this by suggesting that intratracheal instillation of leukocyte chemotactic factor can enhance clearance of silica from the lung interstitium by intensifying pulmonary inflammation and thus reduce pulmonary fibrosis. Therefore, the results of this experiment with IL-10 knockout mice do not necessarily indicate that IL-10 exerts fibrogenic effects, and it remains to be determined whether Bleo
causes more severe fibrosis in IL-10 knockout mice than in normal mice. Martinez et al. (26) studied the clinical significance of IL-10 expression in IPF. They reported that macrophages from patients with IPF expressed increased levels of IL-10 mRNA compared with those from healthy control subjects, but less IL-10 was detected in the BALF from IPF patients compared with that from healthy control subjects. The clinical significance of this discrepancy between IL-10 mRNA and protein levels is not clear, but it is possible that the low concentration of IL-10 in the lungs enhances pulmonary inflammation and fibrosis in IPF. The inflammatory and fibrotic foci are present in the same area and at the same time in the lungs of IPF patients. Histopathological observations strongly suggested that IL-10, which exerts both anti-inflammatory and antifibrotic effects, may be useful in the treatment of IPF.

We used in vivo gene transfer utilizing HVJ-liposomes for the administration of IL-10. Because the half-life of IL-10 in mouse serum is no more than 20 min (16), we supposed that in vivo persistent expression of human IL-10 by gene transfer would be an effective strategy to achieve high serum levels of this cytokine. Although the actual duration of IL-10 expression was <2 days, the gene expression was sufficient to suppress lung injury induced by Bleo. There are some problems to be resolved in administering IL-10 by in vivo gene transfer with the use of HVJ-liposomes. The major problem is that the efficiency and duration of IL-10 expression may not be sufficient to ameliorate the lung disease completely. By improving these drawbacks, gene transfer may become a feasible method for the treatment of lung fibrosis.

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