Induction of CDK inhibitor p21 gene as a new therapeutic strategy against pulmonary fibrosis

Ichiro Inoshima, Kazuyoshi Kuwano, Naoki Hamada, Michihiro Yoshimi, Takashi Maeyama, Naoki Hagimoto, Yoichi Nakanishi, and Nobuyuki Hara. Induction of CDK inhibitor p21 gene as a new therapeutic strategy against pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 286: L727–L733, 2004; 10.1152/ajplung.00209.2003.—Alveolar epithelial cells are known to be present at the primary site of lung damage in pulmonary fibrosis. Apoptosis has been implicated as being involved in epithelial cell damage and pulmonary fibrosis. Because the cyclin-dependent kinase inhibitor p21 induces G1 arrest and DNA repair and because it also prevents apoptosis in some cells, we hypothesized that p21 gene transfer may attenuate bleomycin-induced pulmonary fibrosis in mice, the pathogenesis of which likely involves epithelial cell apoptosis. Human p21 protein was expressed in mouse alveolar epithelial cells at 1–7 days in vitro and was detected predominantly in lung epithelial cells at 1–7 days in vivo after adenoviral transfer of the human p21 gene. Inflammatory cell infiltration and fibrosis had already begun at 7 days in this model. Adenoviral transfer of the human p21 gene at 7 days after intratracheal instillation of bleomycin led to a decrease in the number of apoptotic cells, lung inflammation, and fibrosis at 14 days. Therefore, the forced expression of p21 exerted both anti-apoptotic and anti-fibrotic effects, which would facilitate the ultimate goal of treatment for pulmonary fibrosis.

idiopathic pulmonary fibrosis

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 2.5 to 3.5 yr from the onset of respiratory symptoms (2). Despite this poor prognosis, the etiology of IPF is unknown, and no effective therapeutic strategy has been established. The effects of current immunosuppressive therapy with corticosteroids and cytotoxic agents are limited, and the resulting adverse effects are not inconsiderable. Thus the establishment of additional therapeutic strategies is urgently needed.

Alveolar epithelial cells are known to be present at the primary site of lung damage in pulmonary fibrosis. Recently, increasing attention has been paid to the importance of epithelial cell death during the process of pulmonary fibrosis. There is both DNA damage and apoptosis in bronchiolar and alveolar epithelial cells in IPF (3, 16). Damage to and apoptosis of epithelial cells in acute lung injury have also been demonstrated (4, 7). Although the mechanism of how epithelial cell apoptosis leads to pulmonary fibrosis is not clear, it would seem to have an important role to play in the pathogenesis of pulmonary fibrosis.

Severe injury and insufficient repair of lung epithelial cells disturb the normal epithelial-fibroblast interaction. If epithelial cell repair does not proceed smoothly and completely, fibroblasts will proliferate, eventually leading to pulmonary fibrosis. Adamson et al. (1) demonstrated that mouse lung explants with severe epithelial damage induced by prior hyperoxic lung injury exhibited marked fibroblast proliferation and collagen deposition in culture, whereas less severely injured explants did not. Therefore, they speculated that the critical determinants in the pathway to fibrogenesis may not be the infiltrating inflammatory cells themselves but rather the epithelial damage and repair. Uhal et al. (31) demonstrated that abnormal fibroblast phenotypes isolated from the fibrotic human lung produce factors capable of inducing apoptosis and necrosis of alveolar epithelial cells in vitro.

Terzaghi et al. (28) demonstrated that the presence of intact epithelial cells controls fibroblast proliferation. Studies on the repopulation of denuded tracheal explants by epithelial cells show that the denuded tracheal implants are rapidly replaced by fibroblasts, unless enough epithelial cells are introduced in the lumen to control fibroblast proliferation (28). The authors concluded that fibroblast proliferation was a direct result of epithelial damage and not blood-borne factors or the influx of inflammatory cells. These abnormal epithelial-mesenchymal interactions contribute to the pathogenesis and exacerbation of fibrotic lung disease by preventing normal epithelial repair and by allowing abnormal fibroblast proliferation.

The tumor suppressor p53 protein is a transcription factor that plays a central role in the cellular response to DNA damage, resulting in either G1 arrest or apoptosis (12). p21Waf1/Cip1/Sdi1 (p21) is induced in cells that contain wild-type p53 after exposure to DNA-damaging agents. p21 inhibits cyclin-CDK complex kinase activity and is a critical downstream effector in the p53-specific pathway of growth control in mammalian cells (6). In normal human cells, p21 exists in a quaternary complex with a cyclin, a Cdk, and the proliferating cell nuclear antigen (PCNA; see Ref. 33). p21 controls Cdk activity and thereby affects cell-cycle control. On the other hand, PCNA functions in both DNA replication and repair. p21 directly inhibits PCNA-dependent DNA replication in the absence of a cyclin/Cdk, but does not inhibit DNA repair (18).

Bleomycin rapidly produces extensive DNA damage in the lung (10). In vitro, bleomycin can induce apoptosis (29).
Electron microscopic findings demonstrate the characteristic features of apoptosis in bronchiolar and alveolar epithelial cells in bleomycin-induced pulmonary fibrosis in mice (9). Mishra et al. (22) also showed that these DNA damage-inducible genes, p53 and p21, were upregulated in this model, and they suggested that DNA damage to the lung appeared to be necessary for the development of pulmonary fibrosis. O’Reilly et al. (24) demonstrated that p21 enhanced survival either by promoting DNA repair or by modifying cell death caused by exposure to hyperoxia using p21-deficient mice. These findings suggest that p21 may be a key regulator of DNA replication and repair after lung injury. Furthermore, forced p21 expression has been shown to have a protective effect against cell death caused by genotoxic stresses, such as radiation or cytotoxic agents (5, 19). Interestingly, activation of caspase-3 is regulated by p21, and procaspase-3-p21 complex formation is necessary for the development of pulmonary fibrosis in mice.

**METHODS**

**Recombinant adenoviruses.** The recombinant replication-defective adenovirus, AxCAp21, containing a human p21 gene, and an AxCALacZ adenovirus containing a bacterial lacZ gene, were purchased from the Riken gene bank (Tsukuba, Japan). The structure of these replication-defective viruses was confirmed by Southern blot analysis. The recombinant adenovirus was propagated in 293 cells, twice purified by cesium chloride density gradient centrifugation, dialyzed in 10% glycerol- PBS solution using dialysis membrane (Wako, Osaka, Japan), and sterilized with a 0.45-mm filter.

**Cell culture.** A mouse lung alveolar epithelial cell line (LA4) was purchased from Riken Cell Bank. LA4 was derived from normal embryonic lung tissue. These cells were grown in 25-cm² tissue culture flasks (Falcon, Franklin Lakes, NJ) containing growth medium that consisted of DMEM (GIBCO, Grand Island, NY) with 10% FBS and 1% penicillin-streptomycin. These cultures were incubated at 37°C in a humidified, 95% air-5% CO₂ atmosphere. When the cells were subconfluent, they were harvested by trypsinization and plated in the same medium within another flask.

**AxCAp21 transfer in LA4 cells.** p21 is an extremely conserved gene, and strongly conserved lesions are critical for functional p21 protein (11). However, we examined whether human p21 induces G₁ arrest in murine lung epithelial cells to verify that human p21 is functional in murine cells. Cells (10⁶) were washed in PBS and resuspended in incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 5 mM CaCl₂) plus Annexin-V-FITC (Roche Diagnostics, Indianapolis, IN) to assess apoptosis and in incubation buffer plus propidium iodide (Calbiochem-Novabiochem, La Jolla, CA) to assess the cell cycle. After 15 min of incubation at 4°C, fluorescence was analyzed by a Coulter EPICS XL flow cytometer (Coulter, Miami, FL). To analyze the expression of human p21 protein after gene transfer, cells were lysed by sample buffer containing 133 mM Tris-HCl (pH 6.8), 0.1% SDS, 5% glycerol, 0.67% 2-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. The total cell lysate of 10⁶ cells was separated by 12% SDS-PAGE, blotted on a nitrocellulose membrane, and blocked with 5% nonfat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 50 mM NaCl)/TWEEN [0.1% Tween 20 in Tris-buffered saline (TBS)]. After being washed by TBS/TWEEN, the blots were incubated for 16 h with rabbit anti-human p21 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which detects human p21 but not mouse p21. Blots were washed again with TBS/TWEEN, incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies (1:500) for 1 h at room temperature, washed again, and then developed with an enhanced chemiluminescence Western blotting detection reagent (Amersham, Buckinghamshire, UK).

**Immunohistochemistry for human p21 protein expressed in vivo.** Male C57BL/6 mice (6–8 wk old; SLC Japan, Fukuoka, Japan) were anesthetized with an intraperitoneal injection of pentobarbital sodium (Schering-Plough). The anesthetized mice received AxCAp21 or AxCALacZ (3 × 10⁸ plaque-forming units (PFU)) in 50 μl of saline intratracheally. The mice were killed at day 1, 3, or 7 after instillation, and the lungs were fixed with formalin for 24 h and embedded on paraffin. A 3-μm paraffin section was adhered to slides pretreated with poly-i-lysine. For the purpose of immunohistochemistry for human p21, the tissue sections were autoclaved after dehydration and deparaffinization at 121°C for 5 min in a glass pot filled with enough distilled water to completely immerse the sections and were then washed three times in 0.1 M PBS. Sections were incubated with anti-human p21 antibody (N-20; Santa Cruz Biotechnology), which detects human p21, but not mouse p21. Immunohistochemistry was performed using a streptavidin-biotinylated peroxidase technique using a Histofine SAB-PO kit from Nichirei (Tokyo, Japan). The sections were subsequently counterstained with mithylene green and mounted.

**AxCAp21 gene therapy.** Male C57BL/6 mice (6–8 wk old; SLC Japan) were used in all the experiments. After measurement of their body weight, 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 U bleomycin/kg body wt in sterile saline intratracheally on day 0. Either AxCAp21 or AxCALacZ (3 × 10⁸ PFU) in 50 μl of saline or 50 μl of saline alone was administered intratracheally at day 7, on which interstitial inflammation and fibrosis had begun to be evident. Animals were killed on day 14 for evaluation.

**Western blots analysis for human and mouse p21.** After thoracotomy, the pulmonary circulation was flushed with saline, and the lungs were excised. To analyze p21 protein expression by Western blot analysis in vivo, the left lung was homogenized in buffer A (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) using a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 15,000 g for 30 min at 4°C, and then the supernatant was dissolved in sample buffer and boiled. Protein concentrations were determined with the use of Bio-Rad protein assay (Bio-Rad Laboratories). Thirty micrograms of protein were loaded in each lane of a Western blot. Anti-human p21 antibody (F-5; Santa Cruz Biotechnology) was used as a primary antibody, which detects both human and mouse p21 protein.

**Histopathology of lung tissue.** The right lung was fixed with 10% formalin overnight before being 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 mid-sagittal section was evaluated under ×40 magnification. The pathological grade was 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.

**Apoptosis analysis of lung tissue.** Apoptosis was detected by the terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) method with a commercially available kit (Deadend Colormetric TUNEL System; Promega). After proteinase digestion and removal of endogenous peroxidase, the sections were incubated in a mixture containing terminal deoxynucleotidyltransferase and FITC antibody. The reaction products were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with mithylene green. The number of cells positive for TUNEL in the whole area of the section was counted under a microscope at ×200 magnification.
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 homogeneous mixture. The lung tissue was hydrolyzed with 6 N HCl for 16 h at 120°C. The hydroxyproline content of each sample was determined according to the protocol of Woessner (35).

Statistics. For statistical analysis regarding the comparison of the number of TUNEL-positive cells and hydroxyproline content, ANOVA followed by Scheffe’s F-test was used. For comparison of the pathological grade, Kruskal-Wallis test, followed by Mann-Whitney’s U-test was used. P values <0.05 were considered significant. Statistical analysis was performed with StatView J-4.5 (Abacus Concepts, Berkeley, CA).

RESULTS

Exogenous p21 expression on LA4 cells. Figure 1 shows the time course of human p21 expression at 24 h after gene transfer in LA4 cells. The effects of AxCAp21 infection on cell cycle (B) and apoptosis (C) in LA4 cells. Results shown are from 1 representative experiment from a total of 3 performed. PI, propidium iodide.

Fig. 1. A: Western blot showing time course of human p21 expression at 24 h after gene transfer in LA4 cells. The effects of AxCAp21 infection on cell cycle (B) and apoptosis (C) in LA4 cells. Results shown are from 1 representative experiment from a total of 3 performed. PI, propidium iodide.

Fig. 2. Immunohistochemistry for human p21 protein at 1, 3, and 7 days after AxCAp21 infection (B, C, and D, respectively) and 3 days after AxCALacZ transfection (A). Positive signals were predominantly detected in lung epithelial cells (arrows). Original magnification, ×250.
fection in LA4 cells. Human p21 protein was expressed at 1 to 3 days, and decreased at 7 days after AxCAp21 transfection, but not at 7 days after AxCALacZ transfection in LA4 cells according to Western blot analysis (Fig. 1A). Overexpression of human p21 induced G1 arrest in LA4 cells (Fig. 1B). The frequencies of the apoptotic cells in the untreated, the AxCAp21-transfected, and the AxCALacZ-transfected LA4 cells were not significantly different at 24 h after transfection (Fig. 1C).

Exogenous p21 expression in lung tissues. After $3 \times 10^8$ PFU AxCAp21 were instilled intratracheally, human p21 expression was detected by immunohistochemistry in both the nuclei and cytoplasm predominantly in the lung epithelial cells, as well as in some macrophages for a period of at least 7 days after instillation (Fig. 2). Although transient inflammation was detected, there were no TUNEL-positive cells in lung tissues after AxCAp21 or AxCALacZ transfection (data not shown). The primary antibody used for immunohistochemistry specifically detects human p21 protein, whereas that used for Western blot analysis detects both human and mouse p21 protein. Consistent with the results of immunohistochemistry, human p21 expression was detected in lung homogenates at 14 days after bleomycin instillation by Western blot analysis (Fig. 3). Mouse p21 protein was also detected simultaneously as a lower molecular band than human p21 protein. Mouse p21 protein was increased after bleomycin instillation, and the increase of human p21 expression was also remarkable.

p21 gene therapy suppresses histopathology of the lung. Hematoxylin and eosin staining of the lung of mice at 14 days after bleomycin instillation revealed marked inflammatory cell infiltration within the lung parenchyma, thickening of the alveolar septa, collapse of the alveolar spaces, focal intra-alveolar hemorrhage, and lung fibrosis. The inflammatory cell infiltration and fibrosis were attenuated in AxCAp21-infected mice compared with AxCALacZ-infected or saline-treated mice after bleomycin instillation (Fig. 4). The pathological grades of AxCAp21-transfected mice were significantly lower than those of mice treated with AxCALacZ or saline after bleomycin instillation (Fig. 5).
p21 gene therapy decreases the number of TUNEL positive cells. Although the type of apoptotic cells was not clearly identified, some bronchiolar and alveolar epithelial cells or inflammatory cells in the lesions of pneumonitis at 14 days after bleomycin instillation showed evidence of apoptosis, as estimated by the TUNEL method, but not in control mice (Fig. 6). The number of cells positive for TUNEL in the lung tissue of AxCAp21-transfected mice was significantly decreased compared with that of mice treated with bleomycin alone. AxCALacZ transfection did not affect the number of TUNEL-positive cells (Fig. 7).

p21 gene therapy decreases the hydroxyproline content in lung tissue. To estimate the effect of AxCAp21 transfection on the development of bleomycin-induced lung fibrosis, we measured the hydroxyproline content in lung tissues at 14 days after bleomycin administration. At 14 days after bleomycin instillation, the hydroxyproline content in lung tissues was significantly increased compared with that seen in control mice. However, the hydroxyproline content in lung tissues from AxCAp21-transfected mice was significantly decreased compared with that in mice treated with bleomycin alone or that in AxCALacZ-transfected mice (Fig. 8).

DISCUSSION

This is the first study to demonstrate that adenovirus-mediated p21 gene transfer suppresses the development of bleomycin-induced pneumopathy in mice. The forced expression of p21 gene transfer on terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in lung tissues after bleomycin instillation. A: no positive signals for TUNEL in normal lung parenchyma. B: TUNEL-positive cells in lung tissues at 14 days after bleomycin instillation (arrows). These positive signals for TUNEL at 14 days were abrogated by AxCAp21 infection (D), but not AxCALacZ infection (C), after bleomycin instillation. Original magnification, ×125.
p21 in alveolar epithelial cells suppressed apoptosis and pulmonary fibrosis. Although the TUNEL assay is not specific for epithelial cells, we have previously determined that most apoptotic cells are in fact epithelial cells by electron microscopy (9). We transfected adenovirus p21 at 7 days after apoptotic cells are in fact epithelial cells by electron microscopy. Therefore, the anti-fibrotic effects are not the result of the direct suppression of bleomycin-induced cell damage but are postulated to result from a p21-mediated reduction during the process of progression from inflammation to fibrosis, especially in the case of epithelial cell damage and apoptosis.

O’Reilly et al. (23) demonstrated that bronchiolar and alveolar epithelial cells damaged by hyperoxia express molecules such as p21, which may participate in the regulation of cell proliferation, DNA repair, and cell death (25). They also demonstrated that p21 protects the lung from oxidative stress, in part by inhibiting DNA replication and thereby allowing additional time to repair damaged DNA using p21-deficient mice (24). The absence of p21 results in rapid necrotic alveolar cell death and mortality and also results in premature and extended proliferation of parenchymal cells, thereby creating hyperplastic regions enriched in proliferating fibroblasts after oxidant injury (25). p21 is also present in epithelial cells during bleomycin-induced lung injury, which seems to suggest that p21 is a key molecule responding to DNA damage (15, 22). We consider that alveolar epithelial cells transfected with AxCap21 were induced to growth arrest and become resistant to apoptosis. Because this overexpression of p21 is transient, transfected cells could be alive at least for 7 days, as shown by the results of TUNEL staining. Therefore, adenoviral p21 gene transfer is a novel strategy for the treatment of lung injury and fibrosis through the regulation of apoptosis and the repair of epithelial cells.

We previously demonstrated that epithelial cell apoptosis is involved in the pathophysiology of bleomycin-induced pulmonary fibrosis in mice (9). The Fas-Fas ligand pathway is important for inducing epithelial cell apoptosis and pulmonary fibrosis (14). The angiotensin-converting enzyme inhibitor captopril or the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone is able to block epithelial cell apoptosis and lung fibrosis (17, 34). A specific inhibitor of p38 mitogen-activated protein kinase or anti-interleukin-12 antibody inhibits the augmented expression of tumor necrosis factor-α and the apoptosis of lung epithelial cells (20, 21). It has been reported that 14-membered ring macrolides inhibit the development of this model, a finding that is associated with a decreased number of apoptotic cells in the lung (13). Heme oxygenase 1 overexpression using adenovirus prevents bleomycin-induced pulmonary fibrosis by attenuating apoptotic cell death (30). These studies support the probability that epithelial cell apoptosis and various types of apoptosis-related signaling are involved in the pathophysiology of this model.

The significance of transforming growth factor (TGF)-β1 in pulmonary inflammation, fibrosis, and remodeling is well known. We previously demonstrated that TGF-β1 is a potent inducer of apoptosis through caspase-3 activation and the downregulation of p21 and also that it is an enhancer of Fas-mediated apoptosis in lung epithelial cells (8). AxCap21 inhibited apoptosis of human primary small airway epithelial cells induced by TGF-β1 (8). The activation of caspase-3 is regulated by p21, and Procaspase-3-p21 complex formation is an essential system for cell survival (26, 27). Alveolar epithelial cell apoptosis and caspase-3 activation were detected in the animal model of pulmonary fibrosis induced by amiodarone or bleomycin (32, 34). In these models, inhibition of caspase-3 activation resulted in the attenuation of epithelial cell apoptosis and pulmonary fibrosis. In this study, we detected exogenous expression of the human p21 gene predominantly in lung epithelial cells, as well as in some macrophages. Because various molecules and signaling pathways participate in epithelial cell apoptosis in this model, the molecular mechanisms whereby overexpressed p21 attenuates the development of this model are now under investigation. However, it is likely that p21 is one of the key molecules that helps to regulate apoptosis of lung epithelial cells. We have shown here that the forced expression of p21 blocked the apoptosis of epithelial cells and may therefore prevent the development of pulmonary fibrosis. Accordingly, administration of the adenovirus p21 gene may lead to a reduction in epithelial cell damage and apoptosis and the prevention of pulmonary fibrosis in human diseases.

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