Discoidin Domain Receptor 1 Contributes to the Survival of Lung Fibroblast in Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF), characterized by fibroblast proliferation and accumulation of extracellular matrix, including collagen, is a chronic progressive disorder that results in lung remodeling and fibrosis. However, the cellular mechanisms that may make fibroblasts resistant to apoptosis have not been completely elucidated. Discoidin domain receptor 1 (DDR1), a receptor tyrosine kinase whose ligand is collagen, is expressed *in vivo* and contributes *in vitro* to leukocyte differentiation and nuclear factor (NF)- κ B activation, which may play an important role in fibroblast survival. In this study, we examined *in vivo* and *in vitro* DDR1 expression and its role in cell survival using fibroblasts obtained from IPF and non-IPF patients. Immunohistochemically, fibroblasts present in fibroblastic foci expressed endogenous DDR1. The DDR1 expression level was significantly higher in fibroblasts from IPF patients, and the predominant isoform was DDR1b. In IPF patients, DDR1 activation in fibroblasts inhibited Fas ligand-induced apoptosis and resulted in NF- κ B nuclear translocation. Suppression of DDR1 expression in fibroblasts by siRNA abolished these effects, and an NF- κ B inhibitor abrogated the anti-apoptotic effect of DDR1 activation. We propose that DDR1 contributes to fibroblast survival in the tissue microenvironment of IPF and that DDR1 up-regulation may occur in other fibroproliferative lung diseases as well. (*Am J Pathol* 2006, 168:866–877; DOI: 10.2353/ajpath.2006.050801)

Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal pulmonary disorder that is characterized by fibroblast proliferation and abnormal accumulation of ex-

tracellular matrix (ECM) molecules, particularly fibrillar collagens.¹ An important feature of IPF is the presence of fibroblast foci, which are widely distributed throughout the lung parenchyma.¹ The fibroblastic foci represent microscopic zones of acute lung injury (ALI) in which fibroblasts migrate, proliferate, and contribute to the accumulation of ECM molecules in the damaged alveolus. Subsequently, abnormal remodeling of the lung architecture results from interstitial and intraluminal deposition of connective tissue.² In these processes, the release of fibrogenic cytokines may result in fibroblast proliferation and migration to various sites in the lung, followed by differentiation of the fibroblast phenotype.^{3,4} This differentiation of fibroblasts is considered key to the chronic nature of IPF, and several reports suggest that fibroblasts in IPF appear to be more resistant to apoptosis,^{5,6} a process that is important in both the pathogenesis and resolution of pulmonary fibrotic lesions.⁷ However, the cellular mechanisms specifically involved in fibroblast apoptosis have not been completely elucidated. Furthermore, the assumption that fibroblasts in IPF are more resistant to apoptosis remains controversial to date.

Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase that is activated by binding with its ligand, collagen.^{8,9} DDR1 has a unique extracellular domain that is homologous to discoidin 1 of *Dictyostelium discoideum*.¹⁰ DDR1 is constitutively expressed in normal tissues such as lungs, kidneys, colon, and brain; in tumor cells of epithelial origins, such as those from mammary, ovarian, and lung carcinomas¹⁰; and also in dermal fibroblasts.¹¹ Five DDR1 isoforms (a, b, c, d, and e) can be generated by alternative splicing of the *DDR1* gene,^{10,12} and two of these isoforms (1a and 1b) have known func-

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tions.^{13,14} The DDR1a and DDR1b isoforms differ from each other by an in-frame insertion of 111 bp that codes for an additional 37-amino acid peptide in the proline-rich juxtamembrane region. The 37-amino acid insertion in DDR1b contains the LXNPXY motif that corresponds to the consensus-binding motif of the Shc phosphotyrosine-binding domain.¹⁰ Disruption of the *DDR1* gene in mice resulted in viable animals that were significantly smaller in size than their littermates, whereas female DDR1-null mice showed defects in blastocyst implantation and mammary gland development.¹⁵ These previous observations indicate that DDR1 contributes to tissue development. In addition, we recently found that DDR1b activation can induce leukocyte differentiation¹⁶ and activate transcriptional factor nuclear factor (NF)- κ B,¹⁷ which is reported to play an important role in fibroblast survival.¹⁸

In this study, we obtained primary cultures of fibroblasts from IPF patients and non-IPF patients and examined the DDR1 expression. We observed that fibroblasts obtained from IPF patients predominantly expressed DDR1b and DDR1 activation on IPF fibroblasts inhibited Fas ligand (FasL)-induced apoptosis.

Materials and Methods

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research.

Immunohistochemistry

Biopsied lung tissues obtained from three IPF patients and three non-IPF patients were examined for the presence of DDR1 by immunohistochemical staining using rabbit anti-DDR1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by the diaminobenzidine method, as described previously.¹⁹ Briefly, 4- μ m-thick sections were mounted on poly-L-lysine-coated slides, dewaxed, and washed in Tris-buffered saline (pH 7.4) for 10 minutes. For optimal antigen retrieval, the sections were pressure cooked in 0.01 mol/L citrate buffer (pH 6.0) for 90 seconds. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution in methanol for 10 minutes. After two washes in phosphate-buffered saline (PBS) containing 1% saponin, the blocking reaction was performed as reported previously.²⁰ The sections were incubated with a 1:50 dilution of the primary antibody solution for 2 hours at room temperature. Negative control slides were incubated with rabbit IgG (R&D Systems, Minneapolis, MN). Secondary biotinylated anti-immunoglobulin antibodies (R&D Systems) were added, and the mixture was incubated for 30 minutes at room temperature. After washing, the sections were incubated with streptavidin conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) and then rinsed with deionized water. Diaminobenzidine substrate solution was added, and the mixture was incubated for 10 minutes. A positive result was indicated by a brown color reaction.

Patients with Lung Fibrosis

Fibroblasts were derived from lung tissue samples obtained from seven IPF patients. The lung tissue samples were obtained by video-assisted lung biopsy for diagnosis. IPF was diagnosed in accordance with the American Thoracic Society/European Respiratory Society consensus criteria,^{21,22} including the characteristic morphology of usual interstitial pneumonia. The average age of the seven patients (six men and one woman) was 59.5 years (range, 47 to 68 years). Of the seven patients, six were ex-smokers and one was a nonsmoker. None of the patients were treated with immunosuppressive drugs, including corticosteroids.

Non-IPF Patients

Fibroblasts were derived from lung tissue samples obtained from six patients (four men and two women) undergoing lung surgery for the removal of a primary lung tumor. Normal lung tissue from a noninvolved segment, at a distance from the solitary lesion, was obtained. The average age of the six patients was 62.4 years (range, 41 to 70 years); two patients were nonsmokers and four were ex-smokers.

Acute Lung Injury (ALI) Patients

Fibroblasts were also derived from lung samples obtained from four patients who suffered from adult respiratory distress syndrome (two biopsy samples and two autopsy samples). The average age of the four patients was 52.3 years (range, 31 to 61 years). All of the patients were nonsmokers.

Culture of Fibroblasts

Human lung fibroblasts were cultured from lung explants according to the method described by Akamine and colleagues.²³ The fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with 100 U/ml penicillin and 100 g/ml streptomycin (complete medium). The cells were used at passage 5. All of the cell cultures were immunohistochemically evaluated at passage 5. Essentially, 100% of the cells were fibroblasts as indicated by the strong labeling with anti-prolyl-4-hydroxylase, anti-vimentin, and anti-CD90 monoclonal antibodies (PharMingen, San Diego, CA).²⁴ Staining with anti-smooth muscle myosin heavy chain-1, anti-cytokeratin, and anti-CD31 antibodies (PharMingen) was always negative, indicating that the cultures did not contain smooth muscle cells or epithelial or endothelial cells.

Flow Cytometry Analysis

To detect DDR1 and α -smooth muscle actin (α -SMA) expression on fibroblasts, 5×10^5 cells were collected after five passages. The cells were washed three times with PBS and then incubated with human serum (pooled

Figure 1. Immunohistochemistry of the biopsied lung of an IPF patient. **C:** Fibroblasts in the fibroblastic foci show strong positive staining for DDR1. **C and E:** Inflammatory cells in the IPF lesion are also stained positive for DDR1. **E:** The bronchoepithelial cells are negative for DDR1. In the non-IPF lung, only alveolar macrophages are stained weakly positive for DDR1 (**arrowheads**). **A and B:** H&E staining; **D and G:** nonspecific rabbit IgG; **E:** negative control for second antibody. Original magnifications: $\times 300$ (**A**); $\times 500$ (**B-H**).

sample from healthy volunteer) for 10 minutes. Subsequently, the cells were incubated with biotinylated anti-DDR1 antibodies¹⁴ or fluorescein isothiocyanate-conjugated anti- α -SMA monoclonal antibodies (Sigma Chemical Co., St. Louis, MO) for 20 minutes at 4°C. After washing three times with PBS, the cells were incubated with streptavidin-PE (PharMingen) for 15 minutes at 4°C. Flow cytometry analysis was performed using a FACScan flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Assay for Apoptosis

After five passages, 1×10^6 fibroblasts were seeded and incubated in the presence or absence of FasL (Calbiochem, La Jolla, CA), 50 μ g/ml of type I collagen (Sigma-Aldrich, St. Louis, MO), CAPE (NF- κ B inhibitor; Calbiochem), DDR1 agonistic antibodies, or control IgM.^{14,17,25} To evaluate the effect of β 1-integrin, another collagen receptor, we used monoclonal β 1-integrin neutralizing antibodies (DE9, 10 μ g/ml; Upstate Biotechnology, Lake Placid, NY), as previously described.^{17,25} The apoptosis assay was performed by flow cytometry analysis using monoclonal antibodies for Annexin V-FITC and 7AAD (PharMingen), as described above. In all of the experiments, the apoptotic data were confirmed by terminal dUTP nick-end labeling assay, which was performed using a commercially available kit according to the manufacturer's instructions (TUNEL label mix; Roche Diagnostics Corp., Indianapolis, IN).

Western Blot Analysis

To detect the DDR1 isoforms, 1×10^7 fibroblasts after five passages or 1 mg of total lung tissue was lysed on ice for 20 minutes in 1 ml of lysis buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, and a cocktail of protease inhibitors (Roche). The lysates were centrifuged and 20 μ l of the supernatant was collected. Subsequently, 20 μ l of double-strength sample buffer (20% glycerol, 6% sodium dodecyl sulfate, and 10% 2-mercaptoethanol) was added to the supernatants. The samples were boiled for 10 minutes. The proteins were analyzed on 8% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 hour using a semidry system. The membranes were incubated with rabbit IgGs that specifically recognize DDR1a,¹⁶ DDR1b,²⁵ or both forms of DDR1 (Santa Cruz Biotechnology) or with anti-human actin monoclonal mouse IgG antibodies (Santa Cruz Biotechnology) followed by sheep anti-rabbit or mouse IgGs coupled with horseradish peroxidase (Amersham). The peroxidase activity was visualized by the enhanced chemiluminescence detection system (Amersham). The intensities of the DDR1 isoforms and actin were analyzed using the NIH Image Program (National Institutes of Health, Bethesda, MD), and the relative amount of each DDR1 isoform (DDR1 amount ratio) in each patient was then calculated.

Figure 2. Flow cytometry analysis of cultured fibroblasts. The percentage of α -SMA-positive fibroblasts was significantly higher in the IPF patients than in the non-IPF patients. The percentage of α -SMA-positive fibroblasts was significantly higher in the ALI patients than in the non-IPF patients. The percentage of DDR1-positive fibroblasts was significantly higher in the IPF patients than in the ALI and non-IPF patients. The percentage of DDR1-positive/ α -SMA-positive fibroblasts was significantly higher in the IPF patients than in the ALI and non-IPF patients. **A:** Representative data; **B:** comparison between seven different fibroblasts from seven different IPF patients, four different fibroblasts from four different ALI patients, and six different fibroblasts from six different non-IPF patients. * $P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

To evaluate whether DDR1 activation by type I collagen or DDR1 agonistic antibodies induces autophosphorylation of DDR1 and DDR1 signal transduction, 1×10^7 fibroblasts after five passages were plated on dishes, serum-starved in RPMI 1640 containing 1% fetal calf serum for 10 hours, and subsequently activated with 50 μ g/ml of type I collagen (Sigma) or DDR1 agonistic antibodies (513DDR1 ab).^{17,25} The fibroblasts were then cultured for 1 hour. To evaluate the effect of monomeric collagen, 1×10^7 fibroblasts were seeded to type I collagen-coated dish (Iwaki Glass, Tokyo, Japan) and cultured for 1 hour after serum starvation. Cell lysates were prepared and DDR1 in the cell lysates was immunoprecipitated using anti-DDR1 antibodies (C-20; Santa Cruz Biotechnology) and recombinant protein G-agarose (Invitrogen, Gaithersburg, MD), as previously reported.^{16,17,25} Additionally, tyrosine phosphorylation of DDR1 and Shc recruitment were analyzed by Western blotting by using mouse monoclonal anti-phosphotyrosine IgGs (4G10, Upstate Biotechnology) or mouse monoclonal anti-Shc antibodies (R&D Systems), followed by sheep anti-mouse IgGs coupled with horseradish peroxidase (Amersham). The peroxidase activity was visualized by the enhanced chemiluminescence detection system (Amersham).

A.

B.

C.

D.

E.

F.

Electrophoretic Mobility Shift Assay

A total of 1×10^8 fibroblasts after five passages were serum-starved as described above; the fibroblasts were then incubated in the presence or absence of type I collagen (50 $\mu\text{g/ml}$, Sigma) or 513DDR1 antibody^{17,25} for 6 hours. Nuclear extracts were prepared as described previously,¹⁷ and aliquots were frozen at -80°C . To evaluate the effect of monomeric collagen, 1×10^8 fibroblasts were seeded onto a collagen-coated dish (Iwaki Glass) and cultured for 6 hours after serum starvation. For electrophoretic mobility shift assay, end-labeled ^{32}P -oligonucleotide probes corresponding to the NF- κB binding site of the *Ig κ -chain* gene (5'-AGTTGAGGGGACTTCCAGGC-3') were incubated with 5 μg of nuclear extracts in a 20- μl binding mixture (50 mmol/L Tris-HCl, pH 7.4, 25 mmol/L MgCl_2 , 0.5 mmol/L dithiothreitol, and 50% glycerol) at 4°C for 15 minutes. The DNA-protein complexes were resolved on a 5% polyacrylamide gel. The gels were dried and then exposed to X-ray films.

RNA Interference

A mixture of four small interfering RNAs (siRNAs) specific for DDR1 and a negative control siRNA were purchased from Santa Cruz Biotechnology. Fibroblasts after five passages were cultured at a density of 70% confluence in the complete medium and transfected with the siRNA at a final concentration of 100 nmol/L by using siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's protocol. After a 48-hour incubation, the cells were rinsed with PBS and used for further analysis as described above.

Statistical Analysis

We used the Bonferroni-Dunn test with one-way factorial analysis of variance. A *P* value below 0.05 was considered statistically significant. The values are presented as mean $\pm$ SD unless stated otherwise.

Results

Expression of DDR1 in the Fibroblastic Foci

As shown in Figure 1, spindle-shaped cells in the fibroblastic foci stained strongly positive for DDR1 (Figure 1C). Infiltrating inflammatory cells and alveolar macrophages were also stained positive for DDR1. However,

the bronchoepithelial cells were negative for DDR1 (Figure 1F). In the non-IPF lung, only alveolar macrophages were stained weakly positive for DDR1 (Figure 1H).

α -SMA-Positive Fibroblasts Expressed DDR1

As shown in Figure 2, the percentage of α -SMA-positive fibroblasts, which are considered myofibroblasts, was significantly higher in IPF patients than in the non-IPF patients after five passages. The percentage of α -SMA-positive fibroblasts was also significantly higher in the ALI patients than in the non-IPF patients. Almost all of the DDR1-positive cells were α -SMA-positive. The percentages of DDR1-positive fibroblasts and DDR1-positive/ α -SMA-positive fibroblasts were significantly higher in IPF patients than in the ALI and non-IPF patients.

DDR1b Was the Predominant Isoform in Fibroblasts and Total Lung Tissue from IPF Patients

As shown in Figure 3, A and B, the total amount of DDR1 was significantly higher in fibroblasts from the IPF patients than in those from the ALI and non-IPF patients. The amount of DDR1b was also significantly higher in fibroblasts from the IPF patients than in those from the ALI and non-IPF patients. No significant difference in DDR1a amount was observed between fibroblasts from the IPF patients and those from the ALI and non-IPF patients. The fibroblasts from IPF patients predominantly expressed DDR1b. The DDR1b expression level did not vary with the number of culture passages (Figure 3, C and D).

The total amount of DDR1 and the amount of DDR1b in 1-mg samples of total lung biopsy tissue were significantly higher in the IPF patients than in the non-IPF patients (Figure 3, E and F). No significant difference in DDR1a amount was observed between the 1-mg samples of total lung tissue from the IPF patients and those from the ALI and non-IPF patients (Figure 3, E and F).

Stimulation with Collagen or DDR1 Agonistic Antibodies Prevented FasL-Induced Apoptosis of IPF Fibroblasts

In a preliminary study, we cultured fibroblasts in various concentrations of FasL (1, 5, 10, 50, 100, and 500 $\mu\text{g/ml}$) and found that 50 $\mu\text{g/ml}$ is the optimal concentration to

Figure 3. Western blot analysis for DDR1 expression in 1×10^7 fibroblasts. The amount of total DDR1 was significantly higher in fibroblasts from the IPF patients than in those from the ALI and non-IPF patients. In IPF patients, DDR1b was the predominant isoform, whereas no significant difference in DDR1 isoforms was observed between ALI and non-IPF patients. The amount of DDR1b was significantly higher in fibroblasts from the IPF patients than in those from the ALI and non-IPF patients. **A:** Representative data; **B:** comparison of DDR1/actin ratio between seven different fibroblasts from seven different IPF patients, four different fibroblasts from four different ALI patients, and six different fibroblasts from six different non-IPF patients. $*P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance. With regard to DDR1b expression levels, no significant difference was observed among the passage numbers. **C:** Representative data; **D:** comparison between seven different fibroblasts from seven different IPF patients and six different fibroblasts from six different non-IPF patients. The amount of total DDR1 from 1-mg samples of total lung tissue was significantly higher in fibroblasts from the IPF patients than in those from the non-IPF patients. In the IPF patients, DDR1b was the predominant isoform, whereas no significant difference was observed between the DDR1 isoforms in the non-IPF patients. The amount of DDR1b in the total lung tissue was significantly higher in the IPF patients than in the non-IPF patients. **E:** Representative data; **F:** comparison of DDR1/actin amount ratio between five different fibroblasts from five different IPF patients and four different fibroblasts from four different non-IPF patients. $*P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

induce apoptosis. The apoptosis rate was highest at the concentration of 50 $\mu\text{g/ml}$ and then peaked in both IPF and non-IPF patients (data not shown). No significant difference in the dose response of FasL was observed between IPF, ALI patients, and non-IPF patients (data not shown). As shown in Figure 4, collagen or DDR1 agonistic antibodies significantly inhibited the FasL-induced

Figure 5. A: Western blot analysis of DDR1 autophosphorylation in 1×10^7 fibroblasts from IPF patients. The membrane was probed with anti-phosphotyrosine antibodies, anti-DDR1 antibodies (C-20), or anti-Shc antibodies. **Arrows** indicate phosphorylated DDR1 or phosphorylated Shc. DDR1 autophosphorylation (**top arrow**) and the Shc recruitment (**bottom arrow**) were observed after stimulation with monomeric collagen, fibrillar collagen, and DDR1 agonistic antibodies but not with control IgM. Representative data of five individual experiments using samples from five different donors are shown. **B and C:** Electrophoretic mobility shift assay analysis of NF- κB translocation. Monomeric collagen, fibrillar collagen, and DDR1 agonistic antibodies induced NF- κB nuclear translocation in fibroblasts from the IPF patients; however, this was not observed in the case of the non-IPF patients. **B:** Representative data; **C:** data from five different patients in each group; $*P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

apoptosis. The inhibitory effect was higher at a concentration of 1 $\mu\text{g/ml}$ than at a concentration of 0.1 $\mu\text{g/ml}$. Control IgM (1 $\mu\text{g/ml}$) did not show any effect on FasL-

Figure 4. Effect of DDR1 agonistic antibodies and collagen on the FasL-induced apoptosis of fibroblasts. FasL-induced apoptosis of fibroblasts was observed in both groups. Collagen inhibited the FasL-induced apoptosis of fibroblasts from IPF patients; however, it showed no effect in the case of non-IPF patients. Neutralizing antibodies specific for $\beta 1$ -integrin, another collagen receptor, did not affect the anti-apoptotic effect of collagen. The DDR1 agonistic antibodies inhibited the FasL-induced apoptosis of fibroblasts from the IPF patients in a dose-dependent manner; however, they showed no effect in the case of the non-IPF patients. In IPF patients, the percentage of Annexin V-positive fibroblasts treated with FasL and 50 $\mu\text{g/ml}$ of collagen was significantly lower than those treated with FasL alone. In IPF patients, the percentage of Annexin V-positive fibroblasts treated with FasL and 1 mg/ml DDR1 agonistic antibodies was significantly lower than those treated with FasL alone. Thus, when fibroblasts were treated with collagen or DDR1 agonistic antibodies, the percentage of Annexin V-positive fibroblasts was significantly lower in the IPF patients than in the non-IPF patients. Control IgM did not show any effect on the FasL-induced apoptosis. **A:** Representative data; **B:** comparison of the percentages of Annexin V-positive cells between seven different fibroblasts from seven different IPF patients and six different fibroblasts from six different non-IPF patients. $**P < 0.05$, $*P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

Figure 6. Effect of siRNA specific for DDR1. The siRNA specific for DDR1 purchased from Santa Cruz Biotechnology apparently inhibited the endogenous DDR1 expression in fibroblasts from IPF patients. **A:** Representative data of five individual experiments using samples from five different donors are shown. The siRNA specific for DDR1 abolished the anti-apoptotic effect of collagen and DDR1 agonistic antibodies on fibroblasts from IPF patients. The percentage of Annexin V-positive fibroblasts was significantly higher in fibroblasts treated with FasL and siRNA than those treated with FasL alone. Negative control siRNA did not show any effect on the FasL-induced apoptosis. **B:** Representative data; **C:** comparison of the percentages of Annexin V-positive cells from seven different fibroblasts from seven different IPF patients in each group. * $P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

induced apoptosis. The neutralizing antibodies specific for $\beta 1$ -integrin, another collagen receptor, did not affect the anti-apoptotic effect of collagen. Stimulation with collagen or DDR1 agonistic antibodies did not affect the α -SMA expression on fibroblasts from IPF patients (data not shown).

Stimulation with Collagen or DDR1 Agonistic Antibodies Transduced DDR1 Signaling and Nuclear Translocation of NF- κ B in Fibroblasts from IPF Patients

To evaluate whether collagen or DDR1 agonistic antibodies induce DDR1 activation and signal transduction in fibroblasts from IPF patients, we performed Western blot-

ting against phosphorylated DDR1 and Shc, an adaptor protein for DDR1b signal transduction.¹⁶ As shown in Figure 5A, monomeric collagen, fibrillar collagen, or DDR1 agonistic antibodies induced DDR1 phosphorylation and recruitment of Shc. This experiment was repeated five times by using fibroblasts from five IPF patients, and the same results were obtained for each experiment.

NF- κ B has been reported to play a pivotal role in the prevention of apoptosis.²⁶ Therefore, we examined the nuclear translocation of NF- κ B in fibroblasts. As shown in Figure 5, monomeric collagen, fibrillar collagen, or DDR1 agonistic antibodies induced the nuclear translocation of NF- κ B in fibroblasts from the IPF patients but not in those from the non-IPF patients. Control IgM did not induce the nuclear translocation of NF- κ B.

This experiment was repeated five times by using fibroblasts from five IPF or non-IPF patients, and the same results were obtained from each experiment (Figure 5C).

Inhibition of DDR1 Expression on IPF Fibroblasts Attenuated the Anti-Apoptotic Effect of Collagen or DDR1 Agonistic Antibodies

DDR1 siRNA purchased from Santa Cruz Biotechnology significantly inhibited the endogenous DDR1 expression on fibroblasts obtained from IPF patients (Figure 6A). The inhibition of DDR1 expression by siRNA significantly attenuated the anti-apoptotic effect of the fibrillar type I collagen or DDR1 agonistic antibodies against fibroblasts from IPF patients. Transfection with negative control siRNA did not show any effect on the anti-apoptotic effect of the fibrillar type I collagen or DDR1 agonistic antibodies.

Inhibition of DDR1 Expression on IPF Fibroblasts Reduced the Nuclear Translocation of NF- κ B

As shown in Figure 7, the inhibition of DDR1 expression by siRNA significantly reduced the nuclear translocation of NF- κ B induced by the fibrillar type I collagen or DDR1 agonistic antibodies in fibroblasts from IPF patients. Transfection with negative control siRNA did not show any effect on the nuclear translocation of NF- κ B induced by the DDR1 agonistic antibodies.

NF- κ B Inhibitor Attenuated the Anti-Apoptotic Effect of Collagen or DDR1 Agonistic Antibodies

To evaluate whether NF- κ B is involved in the anti-apoptotic effect of collagen or DDR1 agonistic antibodies, we cultured fibroblasts with CAPE, a NF- κ B inhibitor. In a preliminary study, we cultured fibroblasts in various concentrations of CAPE (0.1, 1, 5, 10, 50, and 100 μ g/ml) and found that 10 μ g/ml is the optimal concentration to suppress NF- κ B translocation (data not shown). As shown in Figure 8, CAPE significantly attenuated the anti-apoptotic effect of the fibrillar type I collagen or DDR1 agonistic antibodies. Dimethyl sulfoxide, the buffer for CAPE, did not affect the anti-apoptotic effect of the fibrillar type I collagen or DDR1 agonistic antibodies.

Discussion

The present study is the first to report the anti-apoptotic effect of DDR1 on fibroblasts from IPF patients. Fibroblasts are not a homogeneous population. There are phenotypically distinct populations of lung fibroblasts that differ in surface markers, receptor expression, cytoskeletal arrangement, and cytokine profiles.^{4,23} The major fibroblasts observed in fibroblastic foci are myofibroblasts, which are characterized by the expression of

Figure 7. Effect of DDR1 siRNA on the NF- κ B nuclear translocation. DDR1 siRNA significantly attenuated the NF- κ B nuclear translocation that was induced by collagen or DDR1 agonistic antibodies in fibroblasts from IPF patients. Negative control siRNA did not show any effect on the NF- κ B nuclear translocation. **A:** Representative data; **B:** data from five different patients in each group; * $P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

markers of smooth muscle differentiation, such as α -SMA. These myofibroblasts are also believed to be the major source of collagen and profibrogenic growth factors within the lung.^{2,27} Under normal conditions, myofibroblasts are transient but essential cells that function in the resolution of inflammation and scar formation; these cells are cleared from the wound site by apoptosis.^{28,29} However, in IPF, because of the release of fibrogenic cytokines such as tumor necrosis factor (TNF)- α ,^{30,31} interleukin (IL)-1,³¹ and basic fibroblast growth factor,³² these myofibroblasts are differentiated and activated.³ An *in vitro* study showed that the primary cultures of fibroblasts from IPF patients contain significantly greater numbers of myofibroblasts than the cultures from patients without pulmonary fibrosis.^{4,28} In addition, fibroblasts from IPF patients have been reported to be more resistant to apoptosis than those from patients without pulmonary

Figure 8. Effect of the NF- κ B inhibitor CAPE on the anti-apoptotic effect of collagen or DDR1 agonistic antibodies. CAPE abolished the NF- κ B nuclear translocation in fibroblasts from IPF patients. **A:** Representative data of five individual experiments using samples from five different donors are shown. CAPE significantly attenuated the NF- κ B nuclear translocation that was induced by collagen or DDR1 agonistic antibodies in fibroblasts from IPF patients. Dimethyl sulfoxide, the buffer of CAPE, did not show any effect on the NF- κ B nuclear translocation. **B:** Representative data; **C:** data from seven different patients in each group. * $P < 0.01$, Bonferroni-Dunn test with one-way factorial analysis of variance.

fibrosis.^{5,6,33} Moodley and co-workers⁵ have suggested that the altered IL-6 signaling in fibroblasts from IPF patients may enhance the resistance of these cells to apoptosis and thus contribute to pulmonary fibrosis. Tanaka and co-workers⁶ showed that resistance to FasL-induced apoptosis in human primary lung fibroblasts is associated with the expression of anti-apoptotic proteins such as X chromosome-linked inhibitor of apoptosis and FLICE-like inhibitor protein, which are up-regulated in the lungs of IPF patients. Thus, our findings with regard to apoptosis are in agreement with the recent reports. In our study, DDR1 could inhibit the FasL-induced apoptosis of fibroblasts from IPF patients. The Fas-Fas ligand pathway is up-regulated in IPF³⁴ and is associated with the deterioration of IPF.³⁵ This up-regulated Fas-Fas ligand pathway contributes to the apoptosis of the bronchoepithelial

cells leading to fibroblast replacement in IPF.³⁶ DDR1 activation inhibited the FasL-induced apoptosis of fibroblasts. Taken together, we believe that DDR1 might be associated with fibroblast replacement in IPF through the inhibition of the FasL-induced apoptosis.

Intra-alveolar fibroblasts are found in proximity to both collagen- and fibronectin-rich areas in IPF,³⁷ suggesting the possible role of ECM signaling in the pathophysiology of IPF.³⁸ In fact, integrins, which are receptors of ECM proteins, regulate lung fibroblast migration across the basement membrane in IPF.³⁹ DDR1 activation by collagen is independent of integrin, another collagen receptor.⁴⁰ In the development of IPF, the disruption of the epithelial basement membrane—a specialized form of ECM—is associated with cell activation and migration, including that of fibroblasts, and this interaction is con-

sidered to be the key event leading to intraluminal fibrosis.^{41,42} It is also known that the amount of collagen in ECM increases during this process.⁴³ Thus, the collagen-DDR1 ligand can continuously interact with fibroblasts rather than cytokines such as IL-6, which has an anti-apoptotic effect on fibroblasts in IPF.^{5,33} Therefore, we believe that DDR1 contributes to the survival of fibroblasts in the IPF lesion such as fibroblastic foci.

Our study showed that DDR1 stimulation could induce the nuclear translocation of NF- κ B in fibroblasts from IPF patients. NF- κ B plays a central role in the prevention of apoptosis and activation of inflammatory responses.²⁶ Classical NF- κ B is a heterodimer consisting of the DNA binding subunit p50 and the transactivation subunit RelA/p65. In nonstimulated cells, the NF- κ B dimer exists as a cytoplasmic latent complex through binding with I κ B. Various stimuli such as proinflammatory cytokines induce the degradation of I κ B and activate NF- κ B by phosphorylation. This allows NF- κ B to translocate into the nucleus and transcriptionally activate the NF- κ B target genes that include anti-apoptotic genes and growth-promoting genes.⁴⁴ The prosurvival function of NF- κ B was also established by the finding that mice lacking the p65 subunit of NF- κ B die at embryonic day 12 as a result of massive liver apoptosis triggered by endogenous TNF.^{45,46} Although experimental evidence linking *in vivo* NF- κ B activation to IPF is lacking,⁴⁷ we believe that NF- κ B activation might be associated with the FasL-induced fibroblast apoptosis observed in our study. Because the activation of NF- κ B could prevent hypoxia or TNF-induced apoptosis of fibroblasts *in vitro*, proinflammatory cytokines that are up-regulated in IPF can activate NF- κ B,^{48,49} and inhibition of NF- κ B can attenuate lung fibrosis in the bleomycin mouse model.⁵⁰ DDR1b activation can induce NF- κ B nuclear translocation via a unique signaling pathway.¹⁷ Taken together, we propose that DDR1 might contribute to the survival of fibroblasts via NF- κ B activation in the tissue microenvironments of IPF.

In conclusion, we show the anti-apoptotic effect of DDR1 on lung fibroblasts in IPF patients and propose a possible association of DDR1 with chronic progressive fibrosis in IPF. Of course we cannot conclude that DDR1 up-regulation is specific in IPF from the present data. To date, there have been many reports on the fibrogenic cytokines or on the molecular interactions between the fibrogenic cytokines and fibroblasts in IPF; however, limited information is available regarding the molecular interactions between the ECM components, such as collagen and fibroblasts. DDR2, another DDR, is also able to regulate fibroblast proliferation⁵¹ and can induce the production of matrix metalloproteinase-1,⁵² which is associated with cell survival.⁵³ Thus, DDRs have potential functions that contribute to cell survival. In addition, there are several non-FasL apoptotic factors of fibroblasts, such as hypoxia,¹⁸ which is a common symptom of IPF.¹ In IPF, CD14-positive bronchoalveolar lavage fluid cells also express DDR1b, and activation of DDR1 induces chemokine production from these cells.¹⁴ DDR1 up-regulation noted in IPF fibroblasts may not be specific for IPF but may be up-regulated during the fibroproliferative stage after lung tissue damage and may have other functions.

Therefore, we believe that further studies addressing apoptosis or other functions of DDRs might provide a new insight to clarify the pathogenesis of fibroproliferative lung diseases including IPF.

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