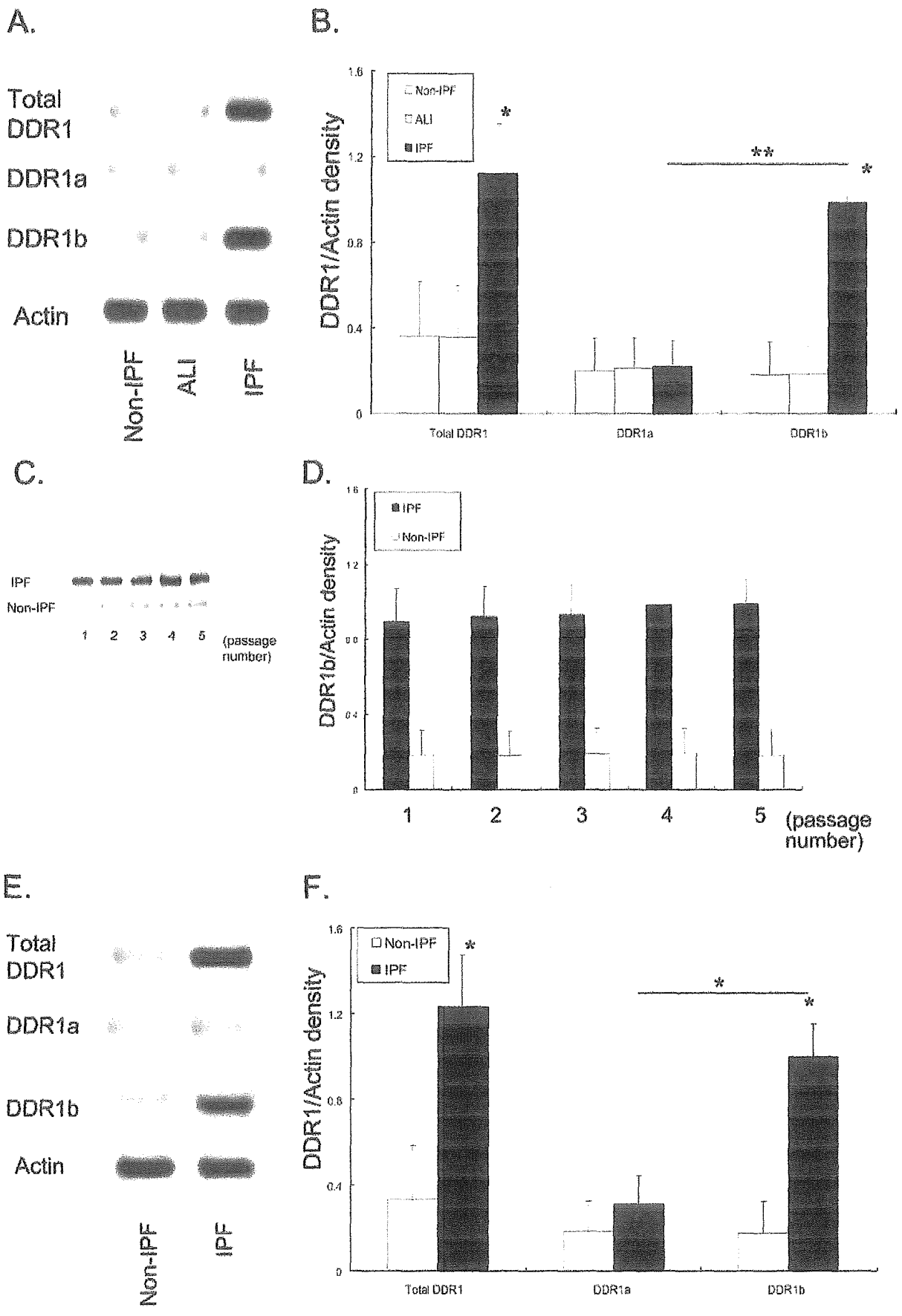
A.
Total DDR1
DDR1a
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Actin
Non-IPF
ALI
IPF
B.
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Total DDR1
DDR1a
DDR1b
C.
IPF
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1 2 3 4 5
(passage number)
D.
IPF
Non-IPF
DDR1b/Actin density
1 2 3 4 5
(passage number)
E.
Total DDR1
DDR1a
DDR1b
Actin
Non-IPF
IPF
F.
Non-IPF
IPF
DDR1/Actin density
Total DDR1
DDR1a
DDR1b

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 μg/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′-AGTTGAGGGGACTTTCCCAGGC-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 ± 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).

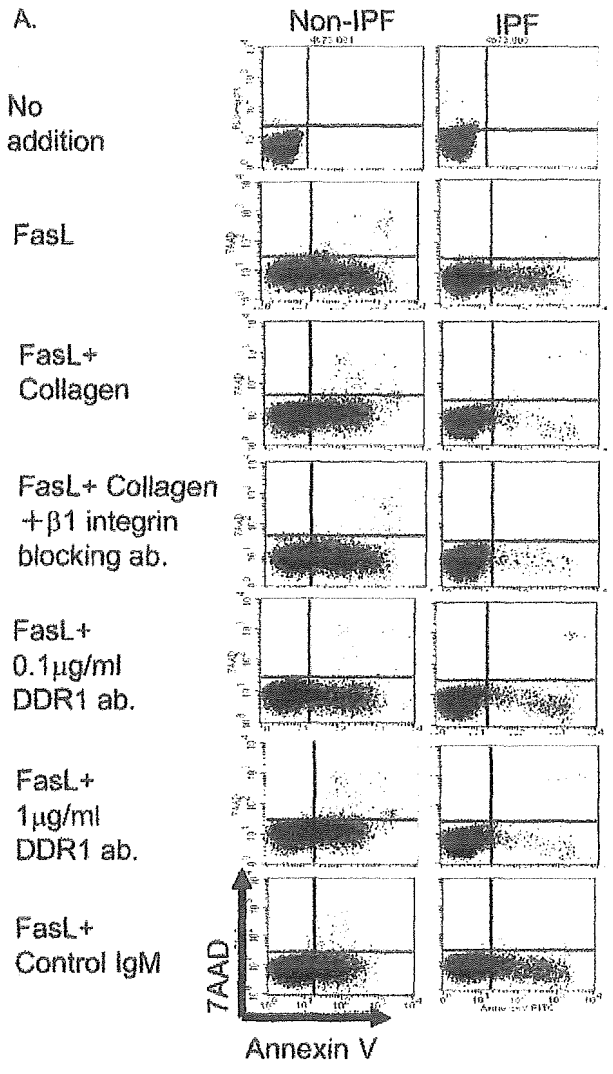
The total amount of DDR1 and the amount of DDR1b in 1-mg samples of total lung biopsy tissue were significantly higher in the IPF patients than in the non-IPF patients (Figure 3, E and F). No significant difference in DDR1a amount was observed between the 1-mg samples of total lung tissue from the IPF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

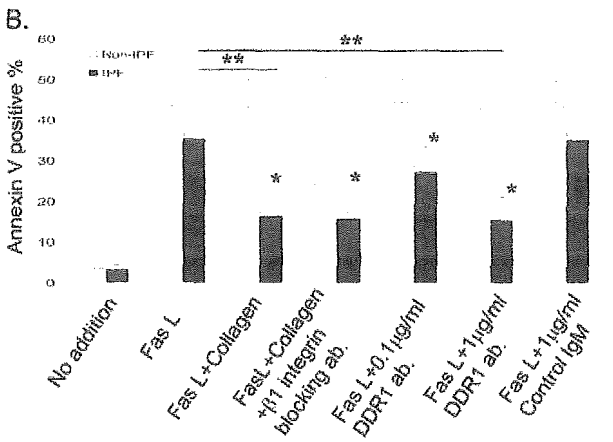
Stimulation with Collagen or DDR1 Agonistic Antibodies Prevented FasL-Induced Apoptosis of IPF Fibroblasts

In a preliminary study, we cultured fibroblasts in various concentrations of FasL (1, 5, 10, 50, 100, and 500 μg/ml) and found that 50 μg/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 μg/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





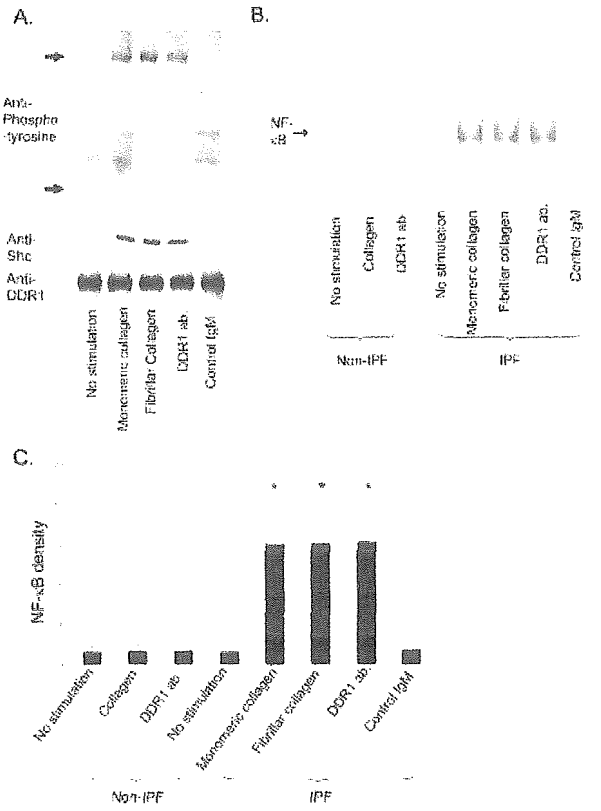


Figure 3. A: Western blot analysis of DDR1 autophosphorylation in 1×10^7 fibroblasts from IPF patients. The membrane was probed with anti-phosphotyrosine antibodies, anti-DDR1 antibodies (C-20), or anti-Shc antibodies. **Arrows** indicate phosphorylated DDR1 or phosphorylated Shc. DDR1 autophosphorylation (**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 μg/ml than at a concentration of 0.1 μg/ml. Control IgM (1 μg/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 β1-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 μg/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.

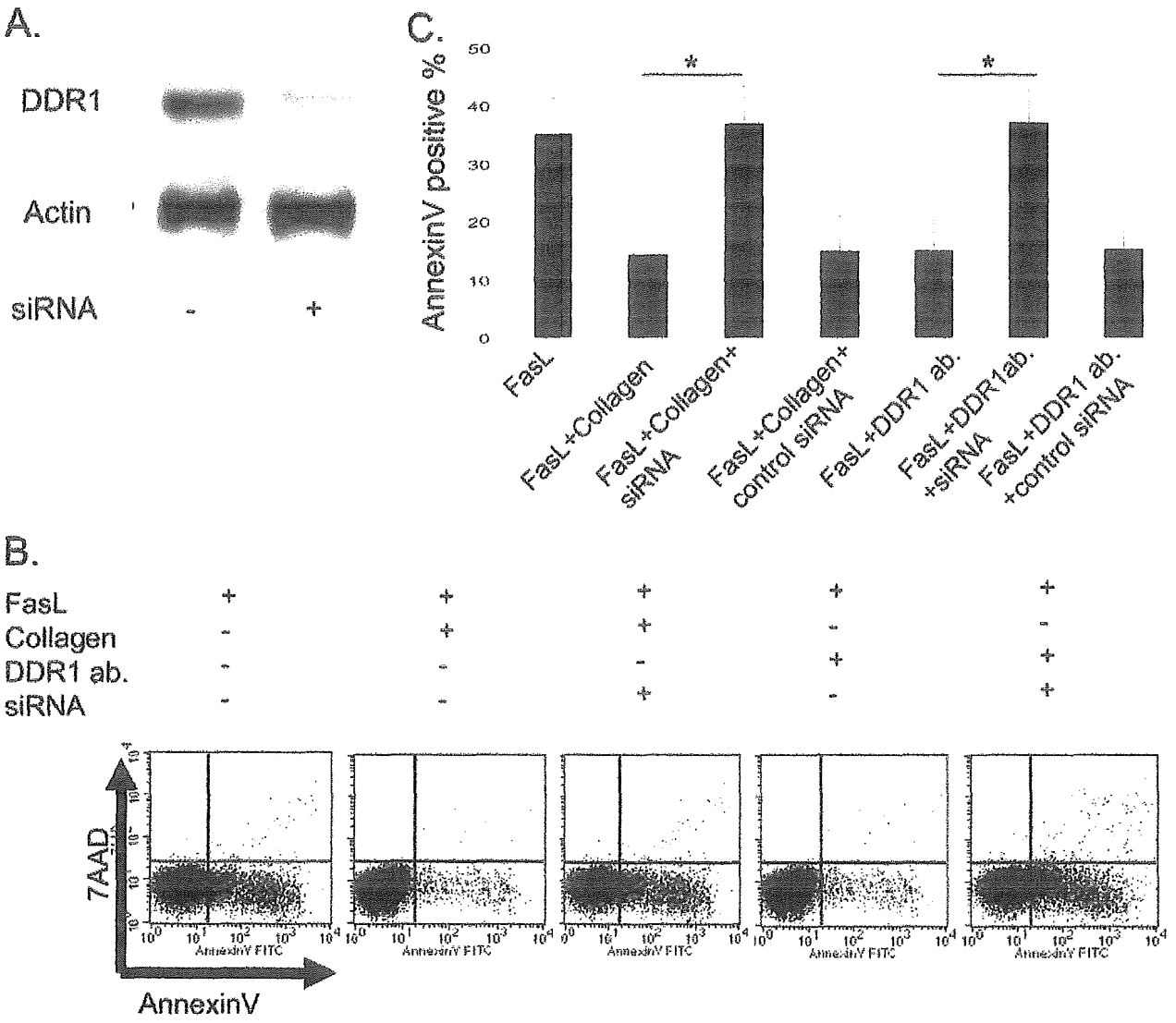


Figure 6. Effect of siRNA specific for DDR1. The siRNA specific for DDR1 purchased from Santa Cruz Biotechnology apparently inhibited the endogenous DDR1 expression in fibroblasts from IPF patients. **A:** Representative data of five individual experiments using samples from five different donors are 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 β1-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-κB 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 blotting 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-κB has been reported to play a pivotal role in the prevention of apoptosis.[26] Therefore, we examined the nuclear translocation of NF-κB in fibroblasts. As shown in Figure 5, monomeric collagen, fibrillar collagen, or DDR1 agonistic antibodies induced the nuclear translocation of NF-κB 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-κB.

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-κB

As shown in Figure 7, the inhibition of DDR1 expression by siRNA significantly reduced the nuclear translocation of NF-κB 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-κB induced by the DDR1 agonistic antibodies.

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

To evaluate whether NF-κB is involved in the anti-apoptotic effect of collagen or DDR1 agonistic antibodies, we cultured fibroblasts with CAPE, a NF-κB inhibitor. In a preliminary study, we cultured fibroblasts in various concentrations of CAPE (0.1, 1, 5, 10, 50, and 100 μg/ml) and found that 10 μg/ml is the optimal concentration to suppress NF-κB 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.

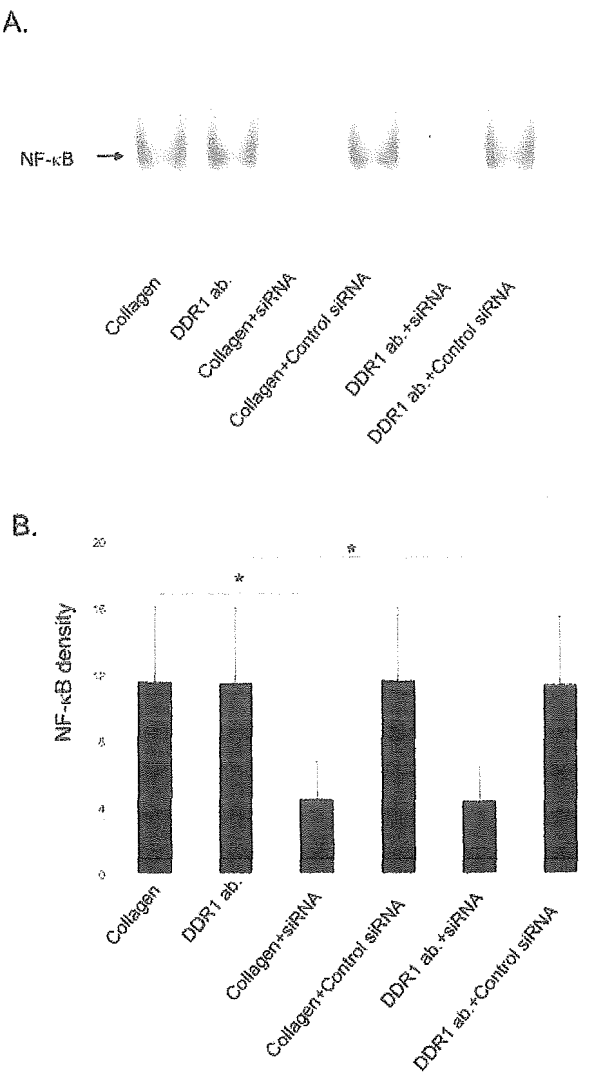


Figure 7. Effect of DDR1 siRNA on the NF-κB nuclear translocation. DDR1 siRNA significantly attenuated the NF-κB nuclear translocation that was induced by collagen or DDR1 agonistic antibodies in fibroblasts from IPF patients. Negative control siRNA did not show any affect on the NF-κB nuclear translocation. **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.

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 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

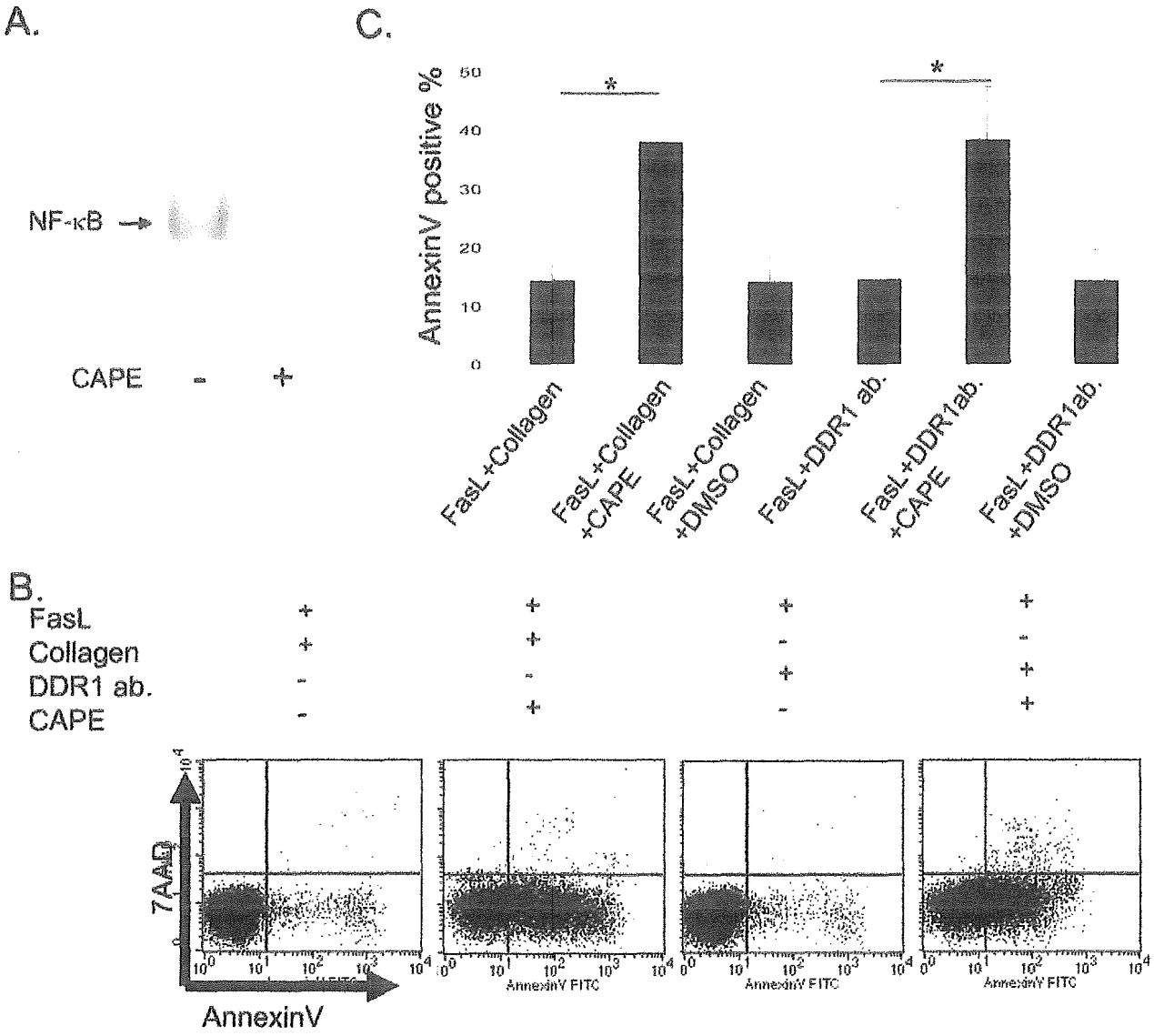


Figure 8. Effect of the NF-κB inhibitor CAPE on the anti-apoptotic effect of collagen or DDR1 agonistic antibodies. CAPE abolished the NF-κB nuclear translocation in fibroblasts from IPF patients. **A:** Representative data of five individual experiments using samples from five different donors are shown. CAPE significantly attenuated the NF-κB 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-κB 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-workers[5] 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-workers[6] 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 IPF[34] 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-κB in fibroblasts from IPF patients. NF-κB plays a central role in the prevention of apoptosis and activation of inflammatory responses.[26] Classical NF-κB is a heterodimer consisting of the DNA binding subunit p50 and the transactivation subunit RelA/p65. In nonstimulated cells, the NF-κB dimer exists as a cytoplasmic latent complex through binding with IκB. Various stimuli such as proinflammatory cytokines induce the degradation of IκB and activate NF-κB by phosphorylation. This allows NF-κB to translocate into the nucleus and transcriptionally activate the NF-κB target genes that include anti-apoptotic genes and growth-promoting genes.[44] The prosurvival function of NF-κB was also established by the finding that mice lacking the p65 subunit of NF-κB 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-κB activation to IPF is lacking,[47] we believe that NF-κB activation might be associated with the FasL-induced fibroblast apoptosis observed in our study. Because the activation of NF-κB could prevent hypoxia or TNF-induced apoptosis of fibroblasts *in vitro*, proinflammatory cytokines that are up-regulated in IPF can activate NF-κB,[48,49] and inhibition of NF-κB can attenuate lung fibrosis in the bleomycin mouse model.[50] DDR1b activation can induce NF-κB nuclear translocation via a unique signaling pathway.[17] Taken together, we propose that DDR1 might contribute to the survival of fibroblasts via NF-κB 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 proliferation[51] 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.

Acknowledgments

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References

1. Katzenstein AL, Myers JL: Idiopathic pulmonary fibrosis: clinical relevance of pathological classification. Am J Respir Crit Care Med 1998, 157:1301–1315
2. Kuhn C, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am J Pathol 1991, 138:1257–1265
3. Khalil N, O'Connor R: Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. CMAJ 2004, 171:153–160
4. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A: Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol 2001, 24:591–598
5. Moodley YP, Misso NL, Scaffidi AK, Fogel-Petrovic M, McAnulty RJ, Laurent GJ, Thompson PJ, Knight DA: Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. Am J Respir Cell Mol Biol 2003, 29:490–498
6. Tanaka T, Yoshimi M, Maeyama T, Hagimoto N, Kuwano K, Hara N: Resistance to Fas-mediated apoptosis in human lung fibroblast. Eur Respir J 2002, 20:359–368
7. Uhal BD: Apoptosis in lung fibrosis and repair. Chest 2002, 122:293S–298S
8. Shrivastava A, Radziejewski C, Campbell E, Kovac L, McGlynn M, Ryan TE, Davis S, Goldfarb MP, Glass DJ, Lemke G, Yancopoulos GD: An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. Mol Cell 1997, 1:25–34
9. Vogel W, Gish GD, Alves F, Pawson T: The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell 1997, 1:13–23
10. Vogel W: Discoidin domain receptors: structural relations and functional implications. FASEB J 1999, 13:S77–S82
11. Chin GS, Kim WJ, Lee TY, Liu W, Saadeh PB, Lee S, Levinson H, Gittes GK, Longaker MT: Differential expression of receptor tyrosine kinases and Shc in fetal and adult rat fibroblasts: toward defining scarless versus scarring fibroblast phenotypes. Plast Reconstr Surg 2000, 105:972–979
12. Alves F, Saupe S, Ledwon M, Schaub F, Hiddemann W, Vogel WF: Identification of two novel, kinase-deficient variants of discoidin domain receptor 1: differential expression in human colon cancer cell lines. FASEB J 2001, 15:1321–1323
13. Kamohara H, Yamashiro S, Galligan C, Yoshimura T: Discoidin domain receptor 1 isoform-a (DDR1alpha) promotes migration of leukocytes in three-dimensional collagen lattices. FASEB J 2001, 15:2724–2726
14. Matsuyama W, Watanabe M, Shirahama Y, Oonakahara K, Higashimoto I, Yoshimura T, Osame M, Arimura K: Activation of discoidin domain receptor 1 on CD14-positive bronchoalveolar lavage fluid cells induces chemokine production in idiopathic pulmonary fibrosis. J Immunol 2005, 174:6490–6498
15. Vogel WF, Aszodi A, Alves F, Pawson T: Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. Mol Cell Biol 2001, 21:2906–2917
16. Matsuyama W, Kamohara H, Galligan C, Faure M, Yoshimura T: Interaction of discoidin domain receptor 1 isoform b (DDR1b) with

collagen activates p38 mitogen-activated protein kinase and promotes differentiation of macrophages. FASEB J 2003, 17:1286–1288
17. Matsuyama W, Wang L, Farrar WL, Faure M, Yoshimura T: Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-κB. J Immunol 2004, 172:2332–2340
18. Qanungo S, Wang M, Nieminen AL: N-Acetyl-L-cysteine enhances apoptosis through inhibition of nuclear factor-κB in hypoxic murine embryonic fibroblasts. J Biol Chem 2004, 279:50455–50464
19. Matsuyama W, Hashiguchi T, Matsumuro K, Iwami F, Hirotsu Y, Kawabata M, Arimura K, Osame M: Increased serum level of vascular endothelial growth factor in pulmonary tuberculosis. Am J Respir Crit Care Med 2000, 162:1120–1122
20. Matsuyama W, Hashiguchi T, Umehara F, Matsuura E, Kawabata M, Arimura K, Maruyama I, Osame M: Expression of vascular endothelial growth factor in tuberculous meningitis. J Neurol Sci 2001, 186:75–79
21. American Thoracic Society: Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med 2000, 161:646–664
22. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias: This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. Am J Respir Crit Care Med 2002, 165:277–304
23. Akamine A, Raghu G, Narayanan AS: Human lung fibroblast subpopulations with different C1q binding and functional properties. Am J Respir Cell Mol Biol 1992, 6:382–389
24. Strutz F, Renziehausen A, Dietrich M, Amin J, Becker V, Heeg M, Rastaldi MP, Muller GA: Cortical fibroblast culture from human biopsies. J Nephrol 2001, 14:190–197
25. Matsuyama W, Faure M, Yoshimura T: Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-β-activated protein kinase 1 binding protein 1 β/p38 α mitogen-activated protein kinase signaling cascade. J Immunol 2003, 171:3520–3532
26. Karin M, Lin A: NF-κB at the crossroads of life and death. Nat Immunol 2002, 3:221–227
27. Zhang K, Rekhter MD, Gordon D, Phan SH: Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. Am J Pathol 1994, 145:114–125
28. Moodley Y, Rigby P, Bundell C, Bunt S, Hayashi H, Misso N, McAnulty R, Laurent G, Scaffidi A, Thompson P, Knight D: Macrophage recognition and phagocytosis of apoptotic fibroblasts is critically dependent on fibroblast-derived thrombospondin 1 and CD36. Am J Pathol 2003, 162:771–779
29. Zhang HY, Phan SH: Inhibition of myofibroblast apoptosis by transforming growth factor β(1). Am J Respir Cell Mol Biol 1999, 21:658–665
30. Nash JR, McLaughlin PJ, Butcher D, Corrin B: Expression of tumor necrosis factor-α in cryptogenic fibrosing alveolitis. Histopathology 1993, 22:343–347
31. Pan LH, Ohtani H, Yamauchi K, Nagura H: Co-expression of TNF α and IL-1 β in human acute pulmonary fibrotic diseases: an immunohistochemical analysis. Pathol Int 1996, 46:91–99
32. Inoue Y, King Jr TE, Barker E, Daniloff E, Newman LS: Basic fibroblast growth factor and its receptors in idiopathic pulmonary fibrosis and lymphangioleiomyomatosis. Am J Respir Crit Care Med 2002, 166:765–773
33. Moodley YP, Scaffidi AK, Misso NL, Keerthisingam C, McAnulty RJ, Laurent GJ, Mutsaers SE, Thompson PJ, Knight DA: Fibroblasts isolated from normal lungs and those with idiopathic pulmonary fibrosis differ in interleukin-6/gp130-mediated cell signaling and proliferation. Am J Pathol 2003, 163:345–354
34. Kuwano K, Miyazaki H, Hagimoto N, Kawasaki M, Fujita M, Kunitake R, Kaneko Y, Hara N: The involvement of Fas-Fas ligand pathway in fibrosing lung diseases. Am J Respir Cell Mol Biol 1999, 20:53–60
35. Kuwano K, Kawasaki M, Maeyama T, Hagimoto N, Nakamura N, Shirakawa K, Hara N: Soluble form of fas and fas ligand in BAL fluid from patients with pulmonary fibrosis and bronchiolitis obliterans organizing pneumonia. Chest 2000, 118:451–458
36. Maeyama T, Kuwano K, Kawasaki M, Kunitake R, Hagimoto N, Matsuba T, Yoshimi M, Inoshima I, Yoshida K, Hara N: Upregulation of Fas-signaling molecules in lung epithelial cells from patients with idiopathic pulmonary fibrosis. Eur Respir J 2001, 17:180–189
37. McDonald JA, Broekelmann TJ, Matheke ML, Crouch E, Koo M, Kuhn III C: A monoclonal antibody to the carboxyterminal domain of procollagen type I visualizes collagen-synthesizing fibroblasts. Detection of an altered fibroblast phenotype in lungs of patients with pulmonary fibrosis. J Clin Invest 1986, 78:1237–1244
38. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG: Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proc Natl Acad Sci USA 1981, 78:7147–7151
39. White ES, Thannickal VJ, Carskadon SL, Dickie EG, Livant DL, Markwart S, Toews GB, Arenberg DA: Integrin $\alpha_4\beta_1$ regulates migration across basement membranes by lung fibroblasts: a role for phosphatase and tensin homologue deleted on chromosome 10. Am J Respir Crit Care Med 2003, 168:436–442
40. Vogel W, Brakebusch C, Fassler R, Alves F, Ruggiero F, Pawson T: Discoidin domain receptor 1 is activated independently of β_1 integrin. J Biol Chem 2000, 275:5779–5784
41. Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, Crystal RG: Intraluminal fibrosis in interstitial lung disorders. Am J Pathol 1986, 122:443–461
42. Suga M, Iyonaga K, Okamoto T, Gushima Y, Miyakawa H, Akaike T, Ando M: Characteristic elevation of matrix metalloproteinase activity in idiopathic interstitial pneumonias. Am J Respir Crit Care Med 2000, 162:1949–1956
43. Raghu G, Striker LJ, Hudson LD, Striker GE: Extracellular matrix in normal and fibrotic human lungs. Am Rev Respir Dis 1985, 131:281–289
44. Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S, Mak TW: Regulation of PTEN transcription by p53. Mol Cell 2001, 8:317–325
45 Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, Doerschuk CM, Hynes RO, Baltimore D: Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-κB in leukocyte recruitment. J Immunol 2001, 167:1592–1600
46. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 1995, 376:167–170
47. Rahman I, MacNee W: Role of transcription factors in inflammatory lung diseases. Thorax 1998, 53:601–612
48. Jany B, Betz R, Schreck R: Activation of the transcription factor NF-κB in human tracheobronchial epithelial cells by inflammatory stimuli. Eur Respir J 1995, 8:387–391
49. Schwartz MD, Moore EE, Moore FA, Shenkar R, Moine P, Haenel JB, Abraham E: Nuclear factor-κB is activated in alveolar macrophages from patients with acute respiratory distress syndrome. Crit Care Med 1996, 24:1285–1292
50. Zhang XY, Shimura S, Masuda T, Saitoh H, Shirato K: Antisense oligonucleotides to NF-κB improve survival in bleomycin-induced pneumopathy of the mouse. Am J Respir Crit Care Med 2000, 162:1561–1568
51. Olaso E, Labrador JP, Wang L, Ikeda K, Eng FJ, Klein R, Lovett DH, Lin HC, Friedman SL: Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2. J Biol Chem 2002, 277:3606–3613
52. Wang J, Lu H, Liu X, Deng Y, Sun T, Li F, Ji S, Nie X, Yao L: Functional analysis of discoidin domain receptor 2 in synovial fibroblasts in rheumatoid arthritis. J Autoimmun 2002, 19:161–168
53. Limb GA, Matter K, Murphy G, Cambrey AD, Bishop PN, Morris GE, Khaw PT: Matrix metalloproteinase-1 associates with intracellular organelles and confers resistance to lamin A/C degradation during apoptosis. Am J Pathol 2005, 166:1555–1563

Suppression of Discoidin Domain Receptor 1 by RNA Interference Attenuates Lung Inflammation[1]

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Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase whose ligand is collagen. Recently, we have reported the association of DDR1 in the cytokine production of human leukocytes in in vitro and in vivo expression in idiopathic pulmonary fibrosis. However, its role in in vivo inflammation has not been fully elucidated. Small interference RNA (siRNA) can induce specific suppression of in vitro and in vivo gene expression. In this study, using a bleomycin-induced pulmonary fibrosis mouse model, we administered siRNA against DDR1 transnasally and evaluated histological changes, cytokine expression, and signaling molecule activation in the lungs. Histologically, siRNA against DDR1 successfully reduced in vivo DDR1 expression and attenuated bleomycin-induced infiltration of inflammatory cells. Furthermore, it significantly reduced inflammatory cell counts and concentrations of cytokines such as MCP-1, MIP-1α, and MIP-2 in bronchoalveolar lavage fluid. Subsequently, bleomycin-induced up-regulation of TGF-β in bronchoalveolar lavage fluid was significantly inhibited, and collagen deposition in the lungs was reduced. Furthermore, siRNA against DDR1 significantly inhibited bleomycin-induced P38 MAPK activation in the lungs. Considered together, we propose that DDR1 contributes to the development of bleomycin-induced pulmonary inflammation and fibrosis. *The Journal of Immunology, 2006, 176: 1928–1936.*

Discoidin domain receptor 1 (DDR1)[3] is a receptor tyrosine kinase that is activated by binding to its ligand-collagen, including collagen type IV (1, 2), a component of the extracellular matrix (ECM) of the lungs. DDR1 possesses a unique extracellular domain that is homologous to discoidin 1 of *Dictyostelium discoideum* (3). DDR1 is constitutively expressed in the normal tissues of organs, such as the lungs, kidneys, colon, and brain, as well as in the tumor cells of epithelial origin, such as those of mammary, ovarian, and lung carcinomas (3). We have previously reported that in vitro DDR1 expression could be induced in human leukocytes, including neutrophils, monocytes, lymphocytes, and macrophages (4). The tissue-infiltrating mononuclear cells in vivo, macrophages in particular, were positive for DDR1 (5). We have recently discovered that DDR1 activation up-regulates the production of chemokines, such as MCP-1, in macrophages. These chemokines play an important role in the pathogenesis of idiopathic pulmonary fibrosis (IPF) in a P38 MAPK-dependent manner and are likely to contribute to the development of inflammatory responses in the tissue microenvironment (6). Furthermore, we found that increased DDR1 expression in alveolar macrophages of IPF patients and its activation induce the production of cytokines, including MCP-1 and matrix metalloproteinase-9, which are key molecules in the pathogenesis of IPF (7). These findings led us to hypothesize that DDR1 suppression might affect or attenuate inflammation in the lungs.

To suppress a specific gene, antisense or ribozyme-based techniques were used in the past decades. In addition, gene silencing using synthetic small interfering RNA (siRNA) has emerged as a powerful tool in the suppression of target gene expression (8). It has been reported recently that transnasal administration of siRNA is able to down-regulate protein expression (9) as well as the respiratory virus replication (10) specifically in the lungs. In this study, by using a bleomycin-induced pulmonary fibrosis mouse model, we investigated whether DDR1 suppression by siRNA is capable of attenuating bleomycin-induced lung inflammation.

Materials and Methods

This study was conducted using female C57BL/6 mice (weight, 17–20 g; age, 8 wk) in specific pathogen-free conditions and was approved by the Kagoshima University Ethics Committee for Animal Experiments.

Treatment of animals

The mice were anesthetized by i.p. administration of 1.5 mg of ketamine hydrochloride (Sankyo) and 0.3 mg of xylazine hydrochloride (Bayer), and the trachea was exposed via a cervical incision. Bleomycin (90 μg; Nippon Kayaku) was dissolved in 50 μl of saline and then instilled intratracheally with a 27-gauge needle. For additional experiments, the mice were ether anesthetized and then sacrificed by axillary artery exsanguination. Table I lists the oligonucleotides used in this study. Each siRNA was diluted in PBS and then administered intranasally after anesthetizing the animals, as described above.

Bronchoalveolar lavage fluid (BALF)

BALF was obtained by cannulating the trachea with a 20-gauge needle and infusing the lungs four times with 1 ml of saline. The recovery of BALF ranged between 2.0 and 3.5 ml, with no significant difference in the volume recovered from each mouse. The BALF cells were collected after centrifugation (1000 × *g*, 10 min, 4°C). The supernatants were stored immediately at −80°C until use for further analysis.

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[3] Abbreviations used in this paper: DDR1, discoidin domain receptor 1; BALF, bronchoalveolar lavage fluid; ECM, extracellular matrix; IPF, idiopathic pulmonary fibrosis; siRNA, small interference RNA.

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Table I. *Oligonucleotides used in this study*

Name	Target	Sequence
siRNA1	DDR1	5′-AAGTGCCGCTATGCCCTGGdTdT-3′
		5′-dTdTCCAGGGCATAGCGGCACTT-3′
siRNA2	DDR1	5′-AAGCTATCGGTTGCGTTACdTdT-3′
		5′-dTdTGTAACGCAACCGATAGCTT-3′
siRNA-Luc	Luciferase	5′-CGUACGCGGAAUACUUCGAdTdT-3′
		3′-dTdTGCAUGCGCCUUAUGAAGCU-5′
Negative	None	5′-UUCUCCGAACGUGUCACGUdTdT-3′
		3′-dTdTTAAGAGGCUUGCACAGUGCA-5′

Transfection into mammalian cells

BALF cells were obtained from the bleomycin-treated mice (day 14). The cells (2×10^6) were maintained under aerobic conditions in 5% CO_2 at 37°C in 6-well culture dishes containing 6 ml of DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (JRH Biosciences). To evaluate the suppressive effects of siRNA, we transfected each siRNA construct into the mice BALF cells by using Transfectene Reagents (Qiagen), according to the manufacturer's protocol.

Histological examination

The excised lungs were immediately fixed with 10% formaldehyde neutral buffer solution for 48 h and then embedded in paraffin. Sagittal sections were cut at 2-μm thickness and stained with H&E and Masson-trichrome stains. The total lung area of the sections was used for fibrotic scale microscope evaluation (Olympus; BX50F4). The criteria for grading lung fibrosis were in accordance with the method reported by Ashcroft et al. (11): grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to the lung architecture; grade 5, increased fibrosis with definite damage to the lung architecture and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of architecture and large fibrous area; and grade 8, total fibrous obliteration of the field. The severity of fibrotic changes in each lung section was assessed as the mean score of severity from the observed microscopic fields. The grade of lung fibrosis was scored on a scale from 0 to 8 by examining 10 randomly chosen regions per sample at a magnification of ×200 by four pathologists who were blinded to the treatment. After examining the entire section, the mean of the scores from all the fields was considered as the fibrotic score.

Immunohistochemistry

Lung tissues were examined by immunohistochemical staining for DDR1 using a rabbit anti-DDR1 Ab (Santa Cruz Biotechnology) by using the diaminobenzidine method, as described previously (12). In brief, 4-μm-thick sections were mounted on poly-L-lysine-coated slides, dewaxed, and washed in TBS (pH 7.4) for 10 min. For optimal Ag retrieval, the sections were pressure cooked in 0.01 M citrate buffer (pH 6.0) for 90 s. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution in methanol for 10 min. The blocking reaction was performed after two washes in PBS with 1% saponin. The sections were incubated with the primary Ab solution for 2 h at room temperature by using a 1/50 dilution of the Ab. Negative control slides were incubated with rabbit IgG (R&D Systems). Secondary biotinylated anti-Ig Ab (R&D Systems) was added, and the mixture was incubated for 30 min at room temperature. After washing, the sections were incubated with streptavidin conjugated to HRP (Amersham) and then rinsed with deionized water. Diaminobenzidine substrate solution was added, and the mixture was incubated for 10 min. A brown-colored reaction represented a positive result.

Western blot analysis

To detect DDR1 and actin, we lysed 1 mg of lung tissue from each mouse or 2×10^6 mice BALF cells in 1 ml of lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors (Roche) by using a hypersonic homogenizer (UD-200; Tomy). We used rabbit IgGs that recognize the DDR1 of humans and mice (Santa Cruz Biotechnology) or anti-actin mouse IgG mAb (Santa Cruz Biotechnology). To detect P38 MAPK phosphorylation, we lysed 1 mg of lung tissue from each mouse and used rabbit polyclonal anti-phosphorylated mouse P38α Ab or rabbit polyclonal anti-mouse P38α Ab (Cell Signaling Technology). To detect β_1 integrin, we lysed 1 mg of lung

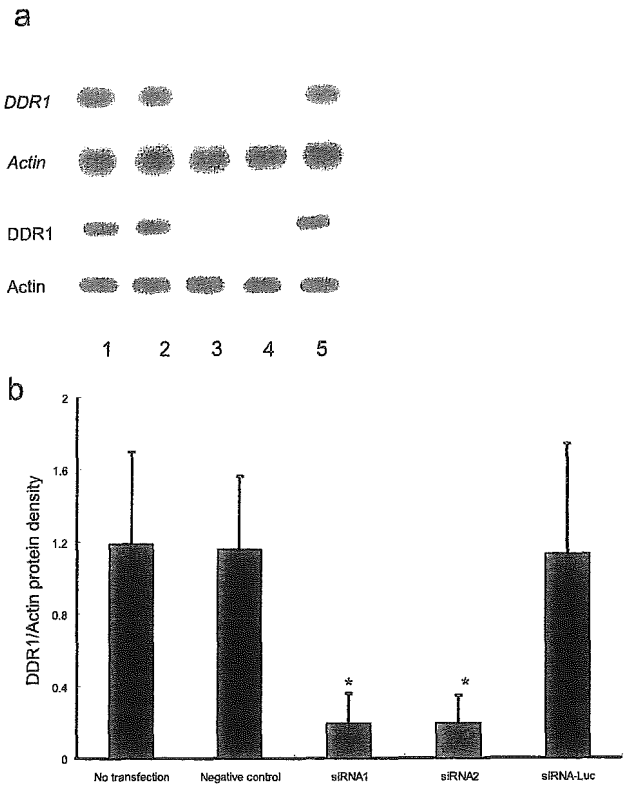

FIGURE 1. The effect of siRNA on in vitro DDR1 expression. The BALF cells obtained from bleomycin-treated mice on day 7 expressed endogenous DDR1 (*a, lane 1*). *a*, The *two upper panels* show Northern blot analysis, and the *two lower panels* show Western blot analysis. Three days after the transfection of 5 nM of each construct, the DDR1 *mRNA* and protein expression levels were significantly lower than in those with siRNA1 (*a, lane 3*) and siRNA2 (*a, lane 4*) when compared with the negative control (*a, lane 2*) and siRNA-Luc (*a, lane 5*). *b*, Shows the statistical result of DDR1/actin protein density ratio. The inhibitory effect on protein expression was statistically significant (*, $p < 0.01$; Bonferroni-Dunn test with one-way factorial ANOVA, $n = 5$ in each group; *b*).

tissue from each mouse and used anti-mouse β_1 integrin rat mAb (R&D Systems). We performed the Western blot analysis, as described previously (4, 6, 13). In brief, the lysates were centrifuged, and 20 μl of the supernatant was collected. Subsequently, 20 μl of double-strength sample buffer (20% glycerol, 6% SDS, and 10% 2-ME) was added to each of supernatants. The samples were boiled for 10 min. Proteins were analyzed on 8 or 12% polyacrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h by using a semidry system. The membranes were incubated with each primary Ab, followed by sheep anti-rabbit or mouse IgG coupled with HRP (Amersham Biosciences). Peroxidase activity was visualized using the Enhanced Chemiluminescence Detection System (Amersham). The density of the bands was measured using a freeware image analysis software (NIH Image, version 1.62; National Institutes of Health (http://rsb.info.nih.gov/nih-image/)).

Northern blotting

Total RNA was extracted from 1 mg of lung tissue or 2×10^6 BALF cells by using TRIzol reagent, and Northern blotting was performed, as described previously (14). The clones of mouse DDR1 cDNA of TGF-β, MCP-1, MIP-2, and MIP-1α were donated by T. Yoshimura (National Cancer Institute, Frederick, MD). Each cDNA was labeled with [α-^{32}P]dCTP using the Rediprime II Random Prime Labeling System (Amersham).

ELISA

The concentrations of TGF-β, MCP-1, MIP-2, and MIP-1α in 1 mg of homogenized lung tissue were measured by using ELISA kits (R&D Systems) according to the manufacturer's protocols.

Hydroxyproline assay

The lungs were harvested on day 21 after bleomycin administration. Each lung homogenate (0.5 ml) was digested in 1 ml of 6 N HCl for 8 h at 120°C. Five microliters of citrate/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid (pH 6.0)) along with 100 μl of chloramines T solution (282 mg of chloramines T, 2 ml of *n*-propanol, 2 ml of H_2O, and 16 ml of citrate/acetate buffer (pH 6.0)) were added to 5 μl of the sample and incubated for 20 min. Next, 100 μl of Ehrlich's solution (2.5 g of 4-(dimethylamino)benzaldehyde, 9.3 ml of *n*-propanol, and 3.9 ml of 70% perchloric acid) was added to each sample and incubated for 15 min at 65°C. The OD was determined at 550 nm on a DU 640 spectrophotometer (Beckman Instruments). Commercially available hydroxyproline (Sigma-Aldrich) was used to construct a standard curve.

Statistical analysis

The Mann-Whitney *U* test and Bonferroni-Dunn test with one-way factorial ANOVA were used. Kaplan-Meier analysis was used for survival analysis. A p value below 0.05 was considered to be significant. Values have been presented as the mean ± SD, unless otherwise stated. SD was calculated using Microsoft Excel software.

Results

Development of siRNA against DDR1

We first transfected various concentrations of siRNA (0.1, 1, 2, 5, and 10 nM) into mice BALF cells and found that transfection with 2 nM siRNA1 and siRNA2 significantly inhibited in vitro DDR1 mRNA and protein expression in mouse BALF cells (Fig. 1, *a* and *b*). The DDR1 expression was not inhibited by siRNA-Luc. Based on these results, we used siRNA1 for additional experiments.

Transnasal administration of siRNA for DDR1

To evaluate whether transnasal administration is an adequate method for the suppression of in vivo DDR1 expression, we examined pulmonary DDR1 expression by Western blot analysis. We administered each type of siRNA on day 3 and collected the lungs on day 5. As shown in Fig. 2, siRNA1 significantly inhibited DDR1 expression in the lung tissue, while siRNA-Luc did not exert any inhibition. The minimum dose required to obtain adequate suppression of DDR1 expression was 5 nM. Therefore, we used 5 nM siRNA1 for additional experiments. The suppressive effect of DDR1 expression in the lung continued until day 9, and the maximum effect was observed on day 5 (Fig. 2, *c* and *d*). Based on these findings, we administered siRNA every 3 days to obtain continuous DDR1 suppression (Fig. 3, *a* and *b*). The DDR1-suppressive effect was specific to the lungs (Fig. 3*c*).

DDR1 siRNA1 attenuates bleomycin-induced pulmonary inflammation

We started the administration of each type of siRNA on day 3; following this, administration was performed every 3 days. Histological examinations revealed that DDR1 siRNA apparently attenuated the inflammation and pulmonary fibrosis (Fig. 4*a*). Although

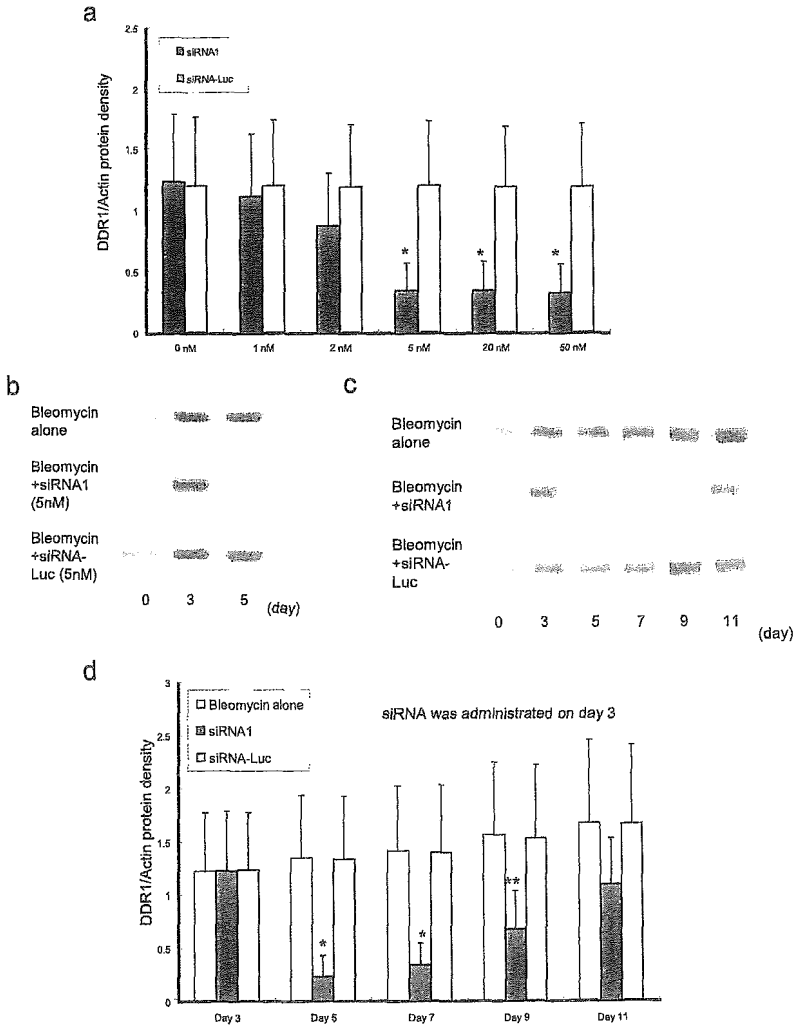

FIGURE 2. The effect of transnasal administration of siRNA1 on day 3 against lung DDR1 expression. *a*, Shows the DDR1/actin protein density ratio at each concentration of siRNA1. DDR1 siRNA1 was administered on day 3, and the lungs were obtained on day 5. The minimum dose of siRNA1 that was required to obtain significant inhibition of DDR1 protein expression in the lung on day 5 was 5 nM (*, $p < 0.01$, Bonferroni-Dunn test with one-way factorial ANOVA, $n = 5$ in each group; *a*). *b*, Shows the representative data obtained from Western blot analysis for DDR1. DDR1 siRNA1 significantly reduced lung DDR1 expression, whereas siRNA-Luc did not affect lung DDR1 expression (*b*). *c* and *d*, Show the suppressive effect of the transnasal administration of DDR1 siRNA1 (5 nM) on day 3 only (*c* shows the representative data obtained from Western blot analysis for lung DDR1 expression, and *d* shows the statistical result of DDR1/actin protein density). The suppressive effect of lung DDR1 expression continued until day 9, and the maximum effect was observed on day 5 (*, $p < 0.01$; **, $p < 0.05$, compared with bleomycin alone, Bonferroni-Dunn test with one-way factorial ANOVA, $n = 5$ in each group; *d*).

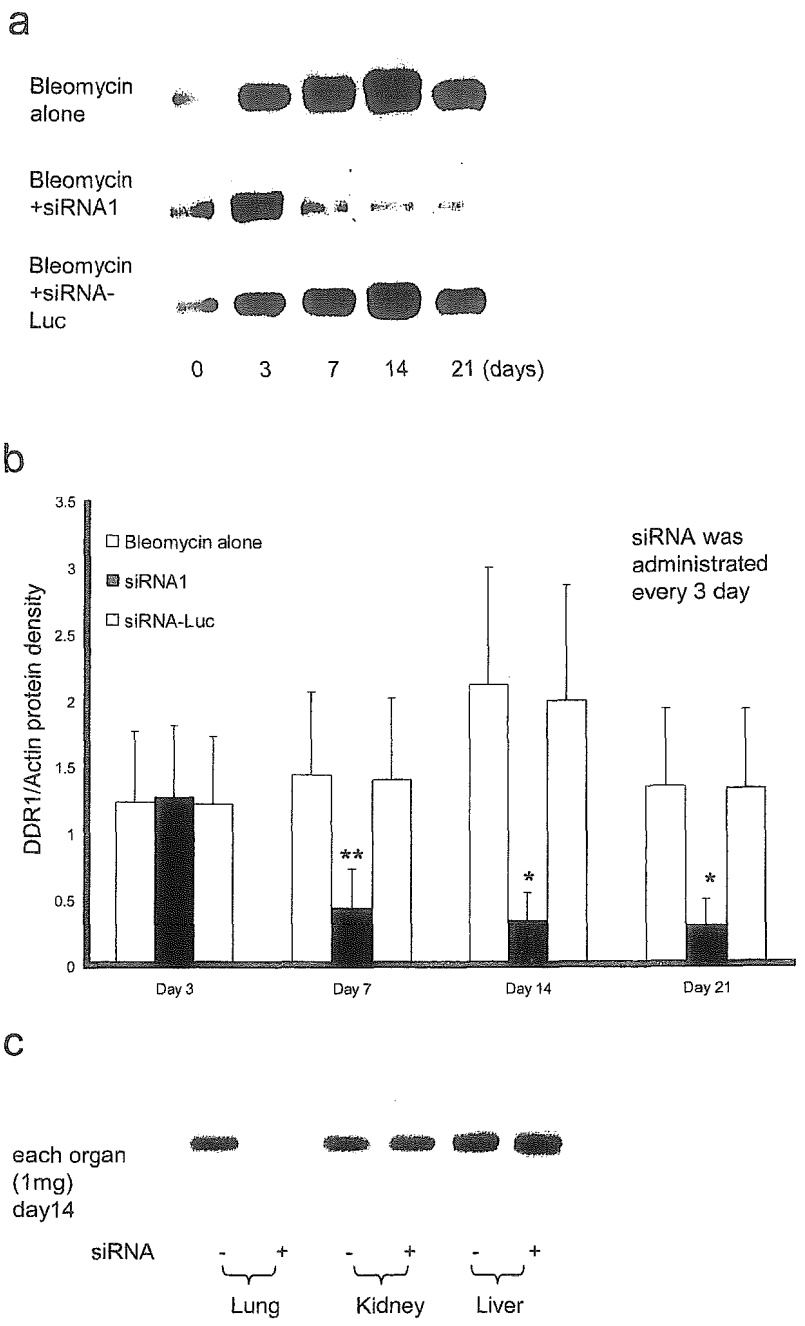

FIGURE 3. The effect of transnasal every 3 days administration of siRNA1 on lung DDR1 expression (*a* and *b*). The transnasal administration of 5 nM siRNA1 every 3 days continuously inhibited lung DDR1 expression, while the siRNA-Luc did not affect it (*a* shows representative data obtained from Western blot analysis; *b* shows the statistical result of DDR1/actin protein ratio in the lungs; *, $p < 0.01$, compared with bleomycin alone, Bonferroni-Dunn test with one-way factorial ANOVA, $n = 5$ in each group). Bleomycin treatment induced lung DDR1 expression that peaked on day 14 and then showed a decrease (*a* and *b*). *c*, Shows the amount of DDR1 expression in each organ on day 14. The inhibitory effect of siRNA1 was specific to the lungs.

the histological changes on day 3 were almost similar across the three groups, bleomycin-treated mice (without siRNA administration), DDR1 siRNA1-administered bleomycin-treated mice, and siRNA-Luc-administered bleomycin-treated mice, the infiltration of the inflammatory cells decreased after the administration of siRNA1. On day 21, the lung tissue showed slight infiltration of inflammatory cells in the DDR1 siRNA1-administered bleomycin-treated mice. In contrast, in the bleomycin-treated mice and the siRNA-Luc-administered bleomycin-treated mice, the bleomycin-induced inflammation progressed and resulted in severe pulmonary fibrosis on day 21 (Fig. 4*a*). After day 14, the pulmonary fibrosis score of DDR1 siRNA1-administered bleomycin-treated mice was significantly lower than that of siRNA-Luc-administered bleomycin-treated mice and that of bleomycin-treated mice ($p < 0.01$; Fig. 4*b*). When half the dose of bleomycin (45 μg) was administered on day 0, the pulmonary fibrosis score of DDR1 siRNA1-administered half dose bleomycin-treated mice on day 7 was significantly lower than that of half dose bleomycin-treated mice. On day 21, the amount of hydroxyproline was significantly lower in the DDR1 siRNA1-administered bleomycin-treated mice than that in the siRNA-Luc-administered bleomycin-treated mice and in the bleomycin-treated mice ($p < 0.01$; Fig. 4*c*). The administration of siRNA1 alone did not affect the amount of hydroxyproline present in the lungs. Starting the siRNA1 administration after day 7 did not attenuate the bleomycin-induced lung inflammation (data not shown). The body weight on day 3 was almost the same across the three groups. After day 14, the body weight of DDR1 siRNA1-administered bleomycin-treated mice was significantly higher than those of siRNA-Luc-administered bleomycin-treated mice and the bleomycin-treated mice (Fig. 4*d*). No mice died in the DDR1 siRNA1-administered bleomycin-treated mice group, while seven mice died in the siRNA-Luc-administered bleomycin-treated group and six mice died in the bleomycin-treated group. One mouse died in the half dose bleomycin (45 μg)-treated group,

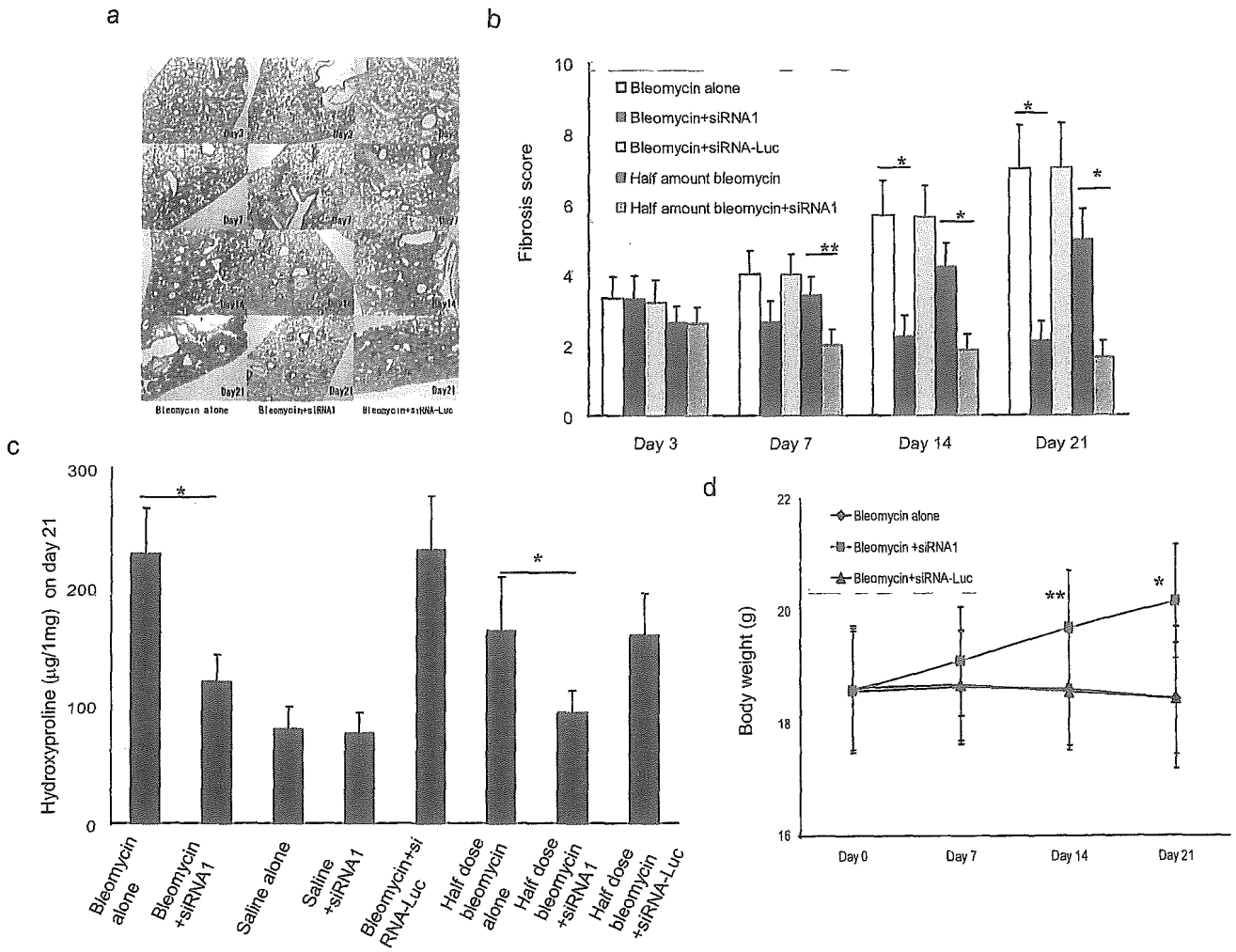

FIGURE 4. Pathological changes after the administration of siRNA1 or siRNA-Luc (*a*). We started the administration of each siRNA on day 3, and it was administrated every 3 days. Bleomycin treatment induced intense infiltration of inflammatory cells on day 3 in all groups. The inflammation progressed and inflammatory cells infiltrated almost the entire lungs on day 14; this finally led to pulmonary fibrosis on day 21 (*left panels*). The administration of siRNA1 reduced the amount of inflammatory cell infiltration on day 7. The area of inflammatory cell infiltration was apparently smaller in the siRNA1-administered bleomycin-treated mice (*center panels*) than in the bleomycin-treated mice (*left panels*) and the siRNA-Luc-administrated bleomycin-treated mice (*right panels*). In the siRNA1-administrated bleomycin-treated mice, the area of inflammatory cell infiltration was smaller on day 21 than on day 7 (arrows). The administration of siRNA-Luc did not decrease the infiltration of inflammatory cells, and pulmonary fibrosis occurred on day 21 (*right panels*). The pulmonary fibrosis score of siRNA1-administered bleomycin-treated mice was significantly lower than those of the bleomycin-treated mice and the siRNA-Luc-administered bleomycin-treated mice (*b*, average ± SDs of 16 mice; *, $p < 0.01$; **, $p < 0.05$, Bonferroni-Dunn test with one-way factorial ANOVA). The amount of hydroxyproline on day 21 was significantly lower in the siRNA1-administered bleomycin-treated mice (*c*; $n = 12$ in each group; *, $p < 0.01$, Mann-Whitney U test). The body weight on day 3 was almost the same among all of the three groups. After day 14, the body weight of the DDR1 siRNA1-administered bleomycin-treated mice was significantly higher than that of the siRNA-Luc-administered bleomycin-treated mice and the bleomycin-treated mice (*, $p < 0.01$; **, $p < 0.05$, Bonferroni-Dunn test with one-way factorial ANOVA; *d*, $n = 21$ on day 3 in each group).

whereas no deaths were recorded in the siRNA1-administered half dose bleomycin-treated group.

Immunohistochemical analysis of DDR1 expression

Immunohistological examinations revealed that infiltrating inflammatory cells, alveolar macrophages, and bronchoepithelial cells of bleomycin-treated mice expressed endogenous DDR1. DDR1 siRNA1 apparently suppressed DDR1 expression of these cells on day 7 (Fig. 5*a*). The administration of siRNA-Luc did not affect DDR1 expression, and the suppressive effect of DDR1 siRNA1 remained for 21 days (Fig. 5*b*).

BALF analysis and cytokine expression in lung tissue

The total BALF cell count along with the macrophage, lymphocyte, and neutrophil counts were significantly lower in the DDR1 siRNA1-administered bleomycin-treated mice than those in the siRNA-Luc-administered bleomycin-treated mice and the bleomycin-treated mice (Fig. 6*a*). MCP-1, MIP-2, and MIP-1α mRNA expression and protein production were significantly inhibited by the DDR1 siRNA1 administration (Fig. 6, *b* and *c*). In all groups, TGF-β mRNA expression and protein production were almost the same on day 7; however, these were significantly lower on days 14 and 21 in the DDR1 siRNA1-administered bleomycin-treated mice than those in the siRNA-Luc-administered bleomycin-treated mice and the bleomycin-treated mice (Fig. 6, *b* and *c*).

Decreased P38 MAPK phosphorylation in DDR1 siRNA1-administered mice

DDR1 activation induces P38 MAPK phosphorylation via a unique pathway (4, 13), and the activation of P38 MAPK occurs in

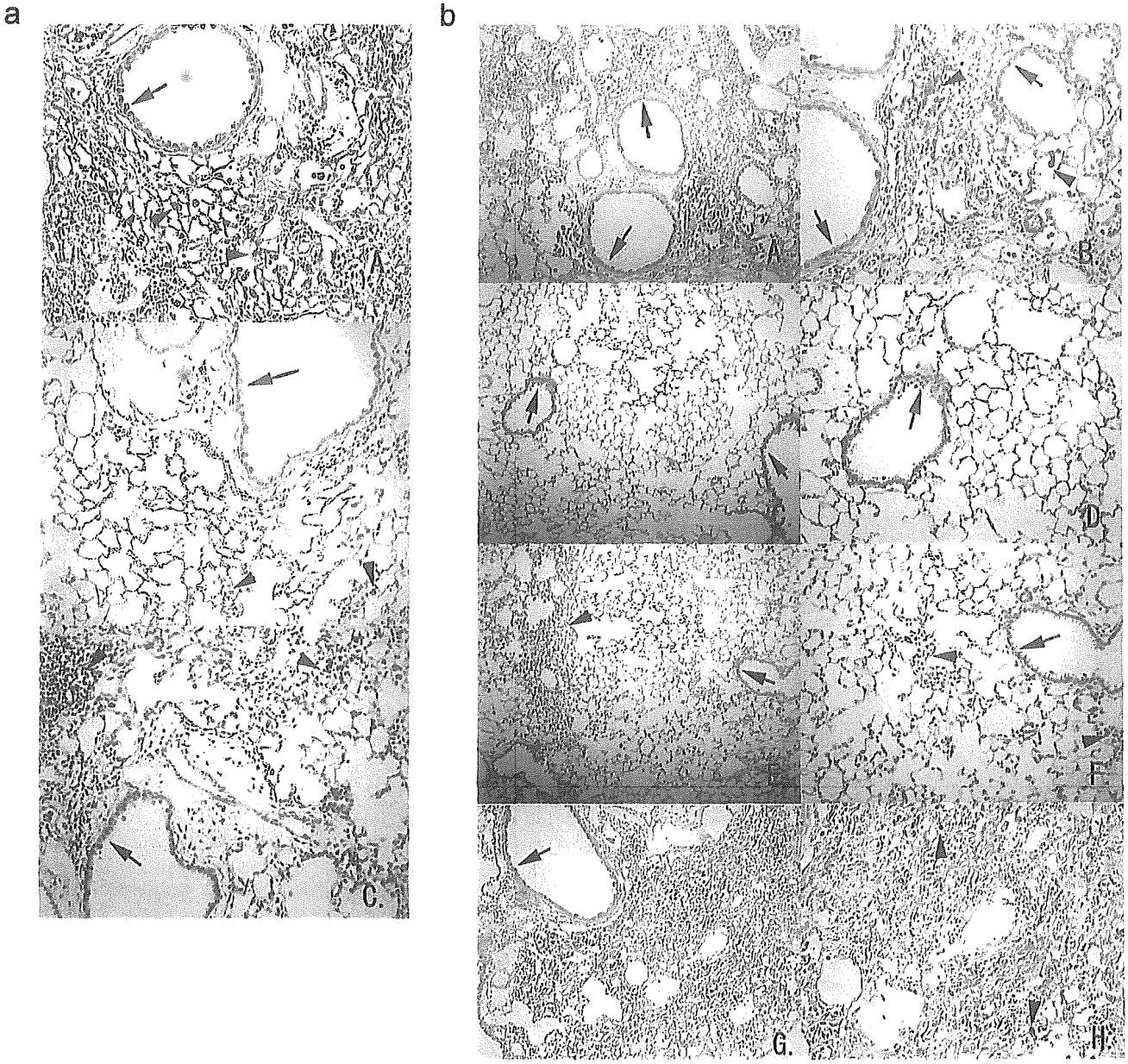

FIGURE 5. Immunohistochemical analysis of lung DDR1 expression. *a*, Shows the immunohistochemical analyses of DDR1 expression on day 7 (*A*, bleomycin alone; *B*, negative control; *C*, bleomycin + siRNA1); *b*, shows those on day 21 (*A* and *B*, bleomycin alone; *C* and *D*, negative control; *E* and *F*, bleomycin + siRNA1; *G* and *H*, bleomycin + siRNA-Luc). We started the administration of each siRNA on day 3, and it was repeated every 3 days. In the bleomycin-treated mice, alveolar macrophages (arrowheads), infiltrating inflammatory cells (arrowheads), and bronchoepithelial cells (arrows) expressed endogenous DDR1 on days 7 and 21 (arrows, *a*, *A* and *b*, *A* and *B*). The administration of the negative control alone did not affect endogenous DDR1 expression and did not induce pulmonary inflammation on day 7 (*a*, *B*) and on day 21 (*b*, *C* and *D*). The administration of siRNA1 inhibited the DDR1 expression in alveolar macrophages (arrowheads), infiltrating inflammatory cells (arrowheads), and bronchoepithelial cells (arrows) on day 7 (*a*, *C*). This inhibitory effect continued until day 21 (*b*, *E* and *F*). The administration of siRNA-Luc did not inhibit the endogenous DDR1 expression on day 21 (*b*, *G* and *H*). Representative data of 10 different mice of each group (*a*, ×400 original magnification; *b*, *left panels*, ×150 original magnification, and *right panels*, ×400 original magnification).

the bleomycin-treated mouse model (15). Therefore, to evaluate whether P38 MAPK activation was affected by DDR1 suppression, we examined P38 MAPK phosphorylation. On day 3, P38 MAPK was activated. On day 7, P38 MAPK phosphorylation in the lungs was significantly down-regulated in the DDR1 siRNA1-administered bleomycin-treated mice than in the siRNA-Luc-administered mice and bleomycin-treated mice. This phenomenon continued for 21 days (Fig. 7*a*). DDR1 siRNA1 did not have any effect on the amount of P38 MAPK (Fig. 7*a*) and β_1 integrin, another collagen receptor (16) (Fig. 7*c*). During the observation period, the amount of β_1 integrin did not show any difference across the three groups (data not shown).

Discussion

This study showed that DDR1 suppression by appropriately designed transnasal administration of siRNA has an anti-inflammatory effect specifically in the lung. There are several reports that show the involvement of DDR1 in malignant tumor proliferation and migration (17–19). Regarding nonmalignant diseases, DDR1 is involved in ECM remodeling in atherosclerosis

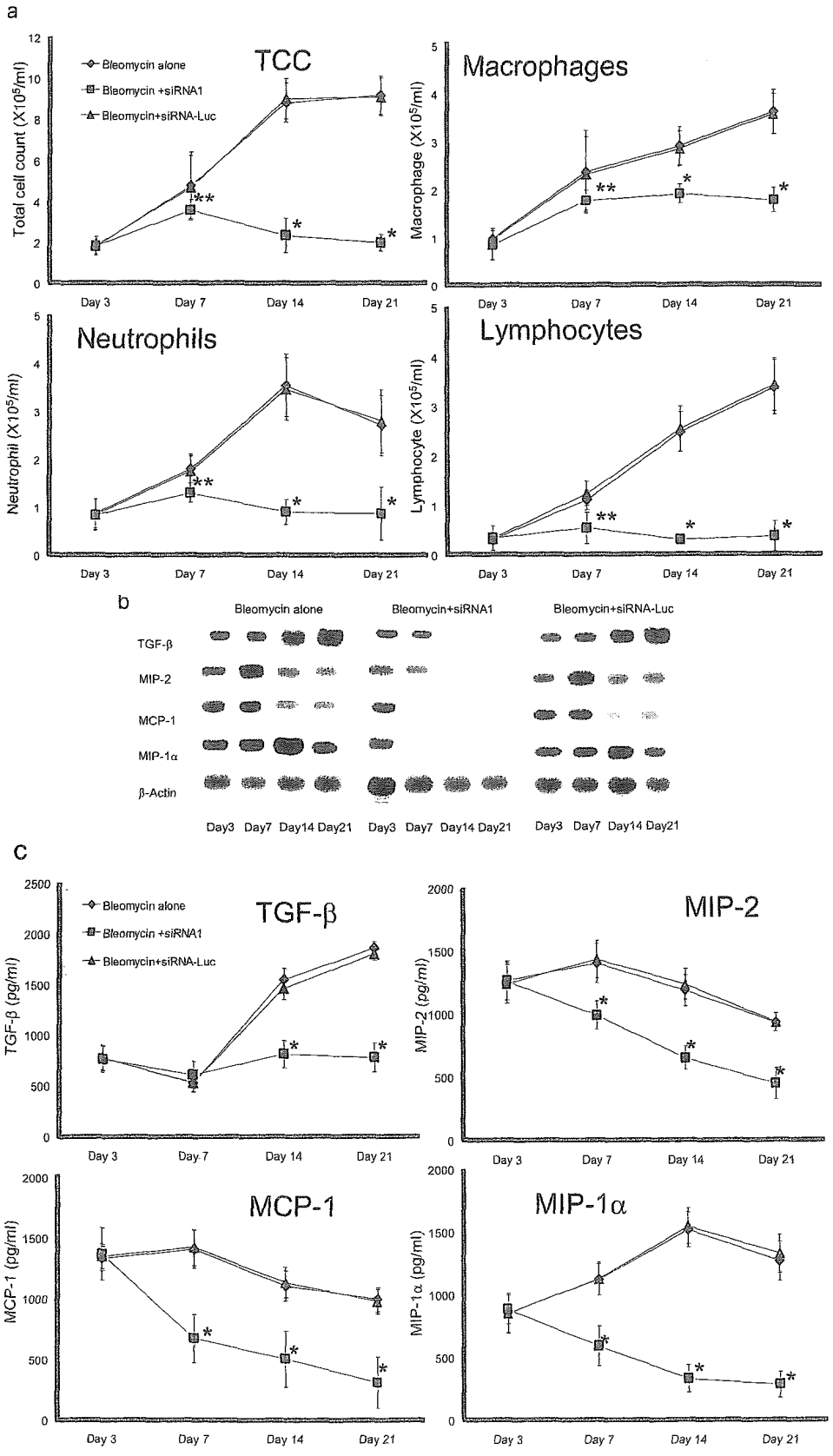

FIGURE 6. BALF cell analysis (*a*), Northern blot analysis of cytokine *mRNA* expression in the lungs (*b*), and cytokine concentration in 1 mg of lung tissue (*c*) in each group. In BALF analysis, total cell counts, macrophage counts, neutrophil counts, and lymphocyte counts significantly decreased after the administration of siRNA1 (*a*, $n = 12$ in each group; *, $p < 0.01$; **, $p < 0.05$, Bonferroni-Dunn test with one-way factorial ANOVA). Bleomycin treatment induced intense expression of *TGF-β*, *MIP-2*, *MCP-1*, and *MIP-1α mRNA* in the lung (*b*). We started administration of each siRNA on day 3, and it was repeated every 3 days. Administration of siRNA1 inhibited the expression of these cytokine mRNAs. The concentrations of MIP-2, MCP-1, and MIP-1α in 1 mg of lung tissue were significantly inhibited by the administration of siRNA1 from days 7 to 21 (*c*, $n = 12$ in each group; *, $p < 0.01$, Bonferroni-Dunn test with one-way factorial ANOVA). In all groups, the concentration of TGF-β was approximately the same on day 7; however, it was significantly inhibited on days 14 and 21 in the siRNA1-administered bleomycin-treated mice (*c*, $n = 12$ in each group; *, $p < 0.01$, Bonferroni-Dunn test with one-way factorial ANOVA).

and lymphangiomyomatosis (20), as well as in the proliferative stage of renal disorders (21). In the lungs, bronchoepithelial cells (22) as well as alveolar macrophages (7) express DDR1. Our recent study showed that infiltrating inflammatory cells in IPF express endogenous DDR1, and its interaction with collagen contributes to the secretion of chemokines, including MCP-1 (7). In the bleomycin-induced pulmonary fibrosis model, the DDR1 ligand (collagen) shows an increase from an early time point after the initial alveolar wall injury and then its level plateaus (23). In this process, the inflammatory cells infiltrate the lesion through the

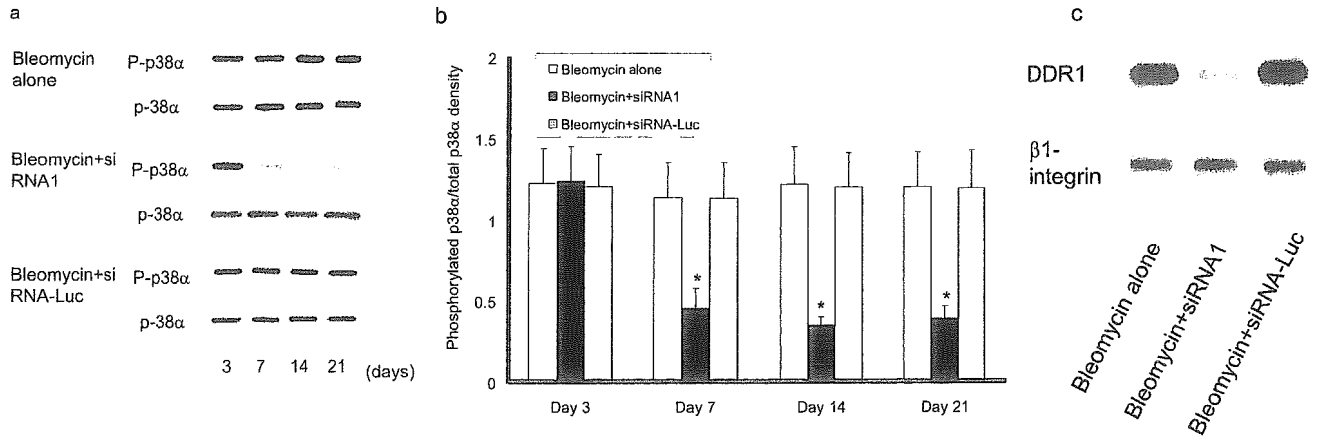

FIGURE 7. The effect of transnasal administration of siRNA1 against phosphorylation of P38 MAPK in the lungs (*a* and *b*). Bleomycin treatment resulted in P38 MAPK phosphorylation, and administration of siRNA1 down-regulated the P38 MAPK phosphorylation (*a*, representative data of six different mice in each group). We started administration of each siRNA on day 3, and it was repeated every 3 days. *b*, Shows the statistical result of P38 MAPK phosphorylation in the lungs from each group. The total amount of P38α from 1 mg of lung tissue did not differ between the groups. The phosphorylation of P38α was significantly down-regulated in siRNA1-administered bleomycin-treated mice than in siRNA-Luc-administered bleomycin-treated mice and the bleomycin-treated mice (*b*, *, $p < 0.01$, compared with bleomycin alone, Bonferroni-Dunn test with one-way factorial ANOVA). *c*, Shows the effect of DDR1 siRNA1 on the expression of β_1 integrin in the lung. DDR1 siRNA1 did not affect the amount of β_1 integrin (*c*, 1 mg of lung tissue on day 7; representative data of six different mice in each group).

ECM that is composed of collagen and reach the lesion (24). In addition, infiltrating fibroblasts produce procollagen, and this leads to collagen deposition (25). Thus, the bleomycin model simplifies the activation of DDR1. The siRNA against DDR1 apparently inhibited the endogenous DDR1 expression in bronchoepithelial cells, alveolar macrophages, and infiltrating inflammatory cells on day 7, the limiting point of DDR1 siRNA effectiveness. In addition, siRNA did not affect the expression of β_1 integrin, which is another important collagen receptor in inflammatory responses (26). Therefore, we believe that DDR1 suppression in infiltrating cells and bronchoepithelial cells is responsible for the successful attenuation of bleomycin-induced lung inflammation. Once DDR1 is activated, it induces intense inflammatory cytokine production (6). In our study, starting siRNA administration after day 7, when the destruction of lung structure begins (23), did not attenuate the bleomycin-induced lung inflammation. These results indicate that DDR1 contribution might occur during the early stages of bleomycin-induced lung injury.

DDR1 suppression down-regulated the production of MCP-1, MIP-2, MIP-1α, and TGF-β in the lungs. Activation of DDR1 in human macrophages induced MCP-1 and MIP-2 production in a P38 MAPK-dependent manner (6). MCP-1 is a major chemoattractant for monocytes in inflammation and immune responses (27). MCP-1 can be detected in the BALF of IPF patients (28) and has been suggested to be associated with the pathogenesis of IPF (29). Alveolar macrophages and epithelial cells are the main cellular sources of MCP-1 production in IPF (30). Anti-MCP-1 gene therapy attenuates bleomycin-induced pulmonary fibrosis in mice (31). MIP-2 is a murine functional homologue of IL-8, and is therefore a potent chemoattractant for neutrophils and plays a pivotal role in acute inflammation by recruiting and activating neutrophils (32). In the bleomycin model, infiltrating inflammatory cells are a cellular source of MIP-2, and neutralization of MIP-2 attenuates bleomycin-induced pulmonary fibrosis (33). MIP-1α regulates the trafficking and activation of select subgroups of inflammatory cells, modulates the adhesion of leukocytes to the endothelium, and contributes to leukocyte recruitment into the lungs (34). Through these functions, MIP-1α contributes to the development of bleomycin-induced pulmonary fibrosis (35). TGF-β is a critical mediator in lung injury (36). Bleomycin can induce significant TGF-β production by bronchoepithelial cells, fibroblasts, and alveolar macrophages (37, 38). The mechanism by which DDR1 suppression led to the down-regulation of TGF-β production is unclear; however, we believe that this may be an indirect effect of DDR1 suppression because TGF-β inhibition occurred on day 14, and not on day 7. DDR1 is also associated with leukocyte migration (5). Therefore, we believe that decreased DDR1 activation and inhibition of the inflammatory cell infiltrate are responsible for the decreased cytokine production, an important factor in the development of bleomycin-induced lung inflammation.

In our study, siRNA against DDR1 suppressed the phosphorylation of the P38 MAPK pathway. P38 MAPK is activated in the lung tissue of a murine IPF model (15). In addition, the inhibition of P38 MAPK can ameliorate murine bleomycin-induced pulmonary fibrosis (39). Monocyte-derived macrophages secrete inflammatory chemokines such as MCP-1 in a P38 MAPK-dependent manner (6). Thus, the suppression of P38 MAPK phosphorylation that was observed in our study may contribute to the attenuation of bleomycin-induced lung inflammation. Although we cannot deny the possibility that the blunting of cytokine responses may secondarily affect this response, we believe that DDR1 might be one of the upstream signaling molecules of P38 MAPK in bleomycin-induced fibrosis because DDR1 activation can induce P38 MAPK phosphorylation via a unique signaling pathway (4, 6, 13).

In conclusion, we have shown that DDR1 suppression could decrease chemokine and cytokine production in bleomycin-induced pulmonary fibrosis. DDR1 has been reported to play an important role in regulating the attachment to collagen, chemotaxis, proliferation, and matrix metalloproteinase production by smooth muscle cells; DDR1 also contributes to collagen deposition in an arterial wound repair model (40). It is possible that DDR1 has other functions and contributes to collagen deposition in pulmonary fibrosis. In our study, lung epithelial cells expressed DDR1. The lung epithelial cells closely attached to ECM, which is abundant in collagen, and play a role in regulating the immunity in the lung through monocyte chemoattractant activity and by releasing several chemokines such as IL-8, MCP-1, and RANTES (41–43). Although we did not examine the biological function of DDR1 in

lung epithelial cells in this study, we believe that there is a possibility that DDR1 in lung epithelial cells contributes to pulmonary fibrosis. Because the distribution of ECM is known to be associated with the activation of inflammatory cells, this interaction is considered to be a key event that results in intraluminal fibrosis (44). Further studies on the role of DDR1 might provide an insight into the clarification of the pathogenesis of pulmonary fibrosis.

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Disclosures

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References

1. Shrivastava, A., C. Radziejewski, E. Campbell, L. Kovac, M. McGlynn, T. E. Ryan, S. Davis, M. P. Goldfarb, D. J. Glass, G. Lemke, and G. D. Yancopoulos. 1997. An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol. Cell* 1: 25–34.
2. Vogel, W., G. D. Gish, F. Alves, and T. Pawson. 1997. The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol. Cell* 1: 13–23.
3. Vogel, W. 1999. Discoidin domain receptors: structural relations and functional implications. *FASEB J.* 13 Suppl: S77–S82.
4. Matsuyama, W., H. Kamohara, C. Galligan, M. Faure, and T. Yoshimura. 2003. Interaction of discoidin domain receptor 1 isoform b (DDR1b) with collagen activates p38 mitogen-activated protein kinase and promotes differentiation of macrophages. *FASEB J.* 17: 1286–1288.
5. Kamohara, H., S. Yamashiro, C. Galligan, and T. Yoshimura. 2001. Discoidin domain receptor 1 isoform-a (DDR1α) promotes migration of leukocytes in three-dimensional collagen lattices. *FASEB J.* 15: 2724–2726.
6. Matsuyama, W., L. Wang, W. L. Farrar, M. Faure, and T. Yoshimura. 2004. Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-κB. *J. Immunol.* 172: 2332–2340.
7. Matsuyama, W., M. Watanabe, Y. Shirahama, K. Oonakahara, I. Higashimoto, T. Yoshimura, M. Osame, and K. Arimura. 2005. Activation of discoidin domain receptor 1 on CD14-positive bronchoalveolar lavage fluid cells induces chemokine production in idiopathic pulmonary fibrosis. *J. Immunol.* 174: 6490–6498.
8. McManus, M. T., and P. A. Sharp. 2002. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 3: 737–747.
9. Brummelkamp, T. R., R. Bernards, and R. Agami. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296: 550–553.
10. Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik. 2005. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* 11: 50–55.
11. Ashcroft, T., J. M. Simpson, and V. Timbrell. 1988. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J. Clin. Pathol.* 41: 467–470.
12. Matsuyama, W., T. Hashiguchi, K. Matsumuro, F. Iwami, Y. Hirotsu, M. Kawabata, K. Arimura, and M. Osame. 2000. Increased serum level of vascular endothelial growth factor in pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* 162: 1120–1122.
13. Matsuyama, W., M. Faure, and T. Yoshimura. 2003. Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-β-activated protein kinase 1 binding protein 1β/p38α mitogen-activated protein kinase signaling cascade. *J. Immunol.* 171: 3520–3532.
14. Matsuyama, W., M. Yamamoto, I. Higashimoto, K. Oonakahara, M. Watanabe, K. Machida, T. Yoshimura, N. Eiraku, M. Kawabata, M. Osame, and K. Arimura. 2004. TNF-related apoptosis-inducing ligand is involved in neutropenia of systemic lupus erythematosus. *Blood* 104: 184–191.
15. Nick, J. A., S. K. Young, K. K. Brown, N. J. Avdi, P. G. Arndt, B. T. Suratt, M. S. Janes, P. M. Henson, and G. S. Worthen. 2000. Role of p38 mitogen-activated protein kinase in a murine model of pulmonary inflammation. *J. Immunol.* 164: 2151–2159.
16. Marra, F., S. Pastacaldi, R. G. Romanelli, M. Pinzani, P. Ticali, V. Carloni, G. Laffi, and P. Gentilini. 1997. Integrin-mediated stimulation of monocyte chemotactic protein-1 expression. *FEBS Lett.* 414: 221–225.
17. Dejmek, J., K. Leandersson, J. Manjer, A. Bjartell, S. O. Emdin, W. F. Vogel, G. Landberg, and T. Andersson. 2005. Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clin. Cancer Res.* 11: 520–528.
18. Heinzelmann-Schwarz, V. A., M. Gardiner-Garden, S. M. Henshall, J. Scurry, R. A. Scolyer, M. J. Davies, M. Heinzelmann, L. H. Kalish, A. Bali, J. G. Kench, et al. 2004. Overexpression of the cell adhesion molecules DDR1, Claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer. *Clin. Cancer Res.* 10: 4427–4436.
19. Ongusaha, P. P., J. I. Kim, L. Fang, T. W. Wong, G. D. Yancopoulos, S. A. Aaronson, and S. W. Lee. 2003. p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop. *EMBO J.* 22: 1289–1301.
20. Ferri, N., N. O. Carragher, and E. W. Raines. 2004. Role of discoidin domain receptors 1 and 2 in human smooth muscle cell-mediated collagen remodeling: potential implications in atherosclerosis and lymphangioleiomyomatosis. *Am. J. Pathol.* 164: 1575–1585.
21. Curat, C. A., and W. F. Vogel. 2002. Discoidin domain receptor 1 controls growth and adhesion of mesangial cells. *J. Am. Soc. Nephrol.* 13: 2648–2656.
22. Sakamoto, O., M. Suga, T. Suda, and M. Ando. 2001. Expression of discoidin domain receptor 1 tyrosine kinase on the human bronchial epithelium. *Eur. Respir. J.* 17: 969–974.
23. Raghow, R., S. Lurie, J. M. Seyer, and A. H. Kang. 1985. Profiles of steady state levels of messenger RNAs coding for type I procollagen, elastin, and fibronectin in hamster lungs undergoing bleomycin-induced interstitial pulmonary fibrosis. *J. Clin. Invest.* 76: 1733–1739.
24. Lemjabbar, H., P. Gosset, E. Lechapt-Zalcman, M. L. Franco-Montoya, B. Wallaert, A. Harf, and C. Lafuma. 1999. Overexpression of alveolar macrophage gelatinase B (MMP-9) in patients with idiopathic pulmonary fibrosis: effects of steroid and immunosuppressive treatment. *Am. J. Respir. Cell Mol. Biol.* 20: 903–913.
25. Coker, R. K., G. J. Laurent, S. Shahzeidi, P. A. Lympany, R. M. du Bois, P. K. Jeffery, and R. J. McAnulty. 1997. Transforming growth factors-β1, -β2, and -β3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. *Am. J. Pathol.* 150: 981–991.
26. De Fougerolles, A. R., A. G. Sprague, C. L. Nickerson-Nutter, G. Chi-Rosso, P. D. Rennert, H. Gardner, P. J. Gotwals, R. R. Lobb, and V. E. Koteliansky. 2000. Regulation of inflammation by collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in models of hypersensitivity and arthritis. *J. Clin. Invest.* 105: 721–729.
27. Rollins, B. J. 1997. Chemokines. *Blood* 90: 909–904.
28. Suga, M., K. Iyonaga, H. Ichiyasu, N. Saita, H. Yamasaki, and M. Ando. 1999. Clinical significance of MCP-1 levels in BALF and serum in patients with interstitial lung diseases. *Eur. Respir. J.* 14: 376–382.
29. Iyonaga, K., M. Takeya, N. Saita, O. Sakamoto, T. Yoshimura, M. Ando, and K. Takahashi. 1994. Monocyte chemoattractant protein-1 in idiopathic pulmonary fibrosis and other interstitial lung diseases. *Hum. Pathol.* 25: 455–463.
30. Antoniades, H. N., J. Neville-Golden, T. Galanopoulos, R. L. Kradin, A. J. Valente, and D. T. Graves. 1992. Expression of monocyte chemoattractant protein 1 mRNA in human idiopathic pulmonary fibrosis. *Proc. Natl. Acad. Sci. USA* 89: 5371–5375.
31. Inoshima, I., K. Kuwano, N. Hamada, N. Hagimoto, M. Yoshimi, T. Maeyama, A. Takeshita, S. Kitamoto, K. Egashira, and N. Hara. 2004. Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 286: L1038–L1044.
32. McColl, S. R., and I. Clark-Lewis. 1999. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J. Immunol.* 163: 2829–2835.
33. Keane, M. P., J. A. Belperio, T. A. Moore, B. B. Moore, D. A. Arenberg, R. E. Smith, M. D. Burdick, S. L. Kunkel, and R. M. Strieter. 1999. Neutralization of the CXC chemokine, macrophage inflammatory protein-2, attenuates bleomycin-induced pulmonary fibrosis. *J. Immunol.* 162: 5511–5518.
34. Cook, D. N., M. A. Beck, T. M. Coffman, S. L. Kirby, J. F. Sheridan, I. B. Pragnell, and O. Smithies. 1995. Requirement of MIP-1α for an inflammatory response to viral infection. *Science* 269: 1583–1585.
35. Smith, R. E., R. M. Strieter, S. H. Phan, N. Lukacs, and S. L. Kunkel. 1998. TNF and IL-6 mediate MIP-1α expression in bleomycin-induced lung injury. *J. Leukocyte Biol.* 64: 528–536.
36. Pittet, J. F., M. J. Griffiths, T. Geiser, N. Kaminski, S. L. Dalton, X. Huang, L. A. Brown, P. J. Gotwals, V. E. Koteliansky, M. A. Matthay, and D. Sheppard. 2001. TGF-β is a critical mediator of acute lung injury. *J. Clin. Invest.* 107: 1537–1544.
37. Khalil, N., and A. H. Greenberg. 1991. The role of TGF-β in pulmonary fibrosis. *Ciba Found. Symp.* 157: 194–207.
38. Zhang, K., K. C. Flanders, and S. H. Phan. 1995. Cellular localization of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. *Am. J. Pathol.* 147: 352–361.
39. Matsuoka, H., T. Arai, M. Mori, S. Goya, H. Kida, H. Morishita, H. Fujiwara, I. Tachibana, T. Osaki, and S. Hayashi. 2002. A p38 MAPK inhibitor, FR-167653, ameliorates murine bleomycin-induced pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283: L103–L112.
40. Hou, G., W. Vogel, and M. P. Bendeck. 2001. The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. *J. Clin. Invest.* 107: 727–735.
41. Koyama, S., E. Sato, H. Nomura, K. Kubo, S. Nagai, and T. Izumi. 1997. Type II pneumocytes release chemoattractant activity for monocytes constitutively. *Am. J. Physiol.* 272: L830–L837.
42. Standiford, T. J., S. L. Kunkel, M. A. Basha, S. W. Chensue, J. P. Lynch III, G. B. Toews, J. Westwick, and R. M. Strieter. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line: a model for cytokine networks in the lung. *J. Clin. Invest.* 86: 1945–1953.
43. Standiford, T. J., S. L. Kunkel, S. H. Phan, B. J. Rollins, and R. M. Strieter. 1991. Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J. Biol. Chem.* 266: 9912–9918.
44. Sires, U. I., G. L. Griffin, T. J. Broekelmann, R. P. Mecham, G. Murphy, A. E. Chung, H. G. Welgus, and R. M. Senior. 1993. Degradation of entactin by matrix metalloproteinases: susceptibility to matrilysin and identification of cleavage sites. *J. Biol. Chem.* 268: 2069–2074.

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ORIGINAL PAPER

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Genetic variability in the extracellular matrix protein as a determinant of risk for developing HTLV-I-associated neurological disease

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Abstract Aggrecan, which is a well-known proteoglycan in joint cartilage, also exists in the spinal cord and plays an important role in maintaining water content in the extracellular matrix structure. In this study, we first examined the variable number of tandem repeat (VNTR) polymorphism of the *aggrecan* gene in 227 HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, in 217 HTLV-I-infected healthy carriers (HCs), and in 85 normal controls. The VNTR allele 28 (1,630 bp) was more frequently observed in HAM/TSP patients than in HCs (χ^2=12.02, p=0.0005, odds ratio 1.79, 95% C.I. 1.29–2.50) and in controls (χ^2=13.43, p=0.0002, odds ratio 2.54, 95% C.I. 1.52–4.25), although this allele was not related to disease progression or to HTLV-I provirus load. We also found that the aggrecan concentration in cerebrospinal fluid (CSF) from rapidly progressive HAM/TSP patients was significantly higher than in slowly progressive patients (corrected p=0.0145) but not in infected non-inflammatory neurological other disease controls (OND) (corrected p=0.078). We then analyzed this aggrecan VNTR polymorphism in the different set of patients with HAM/TSP (n=58) and healthy carriers (n=70). This analysis, again, revealed that allele 28 was detected more frequently in HAM/TSP group than in HCs (χ^2=11.03, p=0.0009, odd ratio 3.04, 95% C.I. 1.55–5.97). The reproducibility of our study was regarded as a second- or third-class association by comparing combined p values and the Better Associations for Disease and GEnes (BADGE) system. Our results suggest that aggrecan polymorphism can be a novel genetic risk factor for developing HAM/TSP.

Keywords Aggrecan · Extracellular matrix · HTLV-I · VNTR · HAM/TSP

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Introduction

HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive inflammatory disease of the spinal cord, which occurs in only 1–2% of HTLV-I-infected individuals (Gessain et al. 1985; Osame et al. 1986). As we have previously reported, that the main HTLV-I-harboring cells in the spinal cord of HAM/TSP patients are not neuronal cells but $CD4^+$ T cells (Moritoyo et al. 1996), a T-cell-mediated immunologic process initiated by HTLV-I infection can be a possible pathological process of HAM/TSP. Although the factors that cause different manifestations of HTLV-I infection are not fully understood, our recent population association studies of more than 200 cases each of HAM/TSP and HTLV-I-infected healthy carriers (HCs) in Kagoshima, an endemic area of HTLV-I infection in Japan, have revealed several important risk factors (Jeffery et al. 1999, 2000; Nagai et al. 1998; Vine et al. 2002). One of the major risk factors for developing HAM/TSP is the provirus load. The median provirus load was approximately 16 times higher in HAM/TSP patients than in HCs, and a high provirus load is also associated with an increased risk for

progression to HAM/TSP (Nagai et al. 1998). We have also reported that *HLA-A*02* and *Cw*08* genes were associated with a lower HTLV-I provirus load and protection from HAM/TSP, whereas *HLA-DRB1*0101* and *B*5401* were associated with susceptibility to HAM/TSP (Jeffery et al. 1999, 2000). Moreover, we have revealed non-HLA genetic risk factors such as TNF−α, SDF-1, and IL-15 (Vine et al. 2002), as well as the association between *HTLV-I Tax* gene variation and the risk for HAM/TSP (Furukawa et al. 2000). From these observations, we now can identify approximately 88% of cases of HAM/TSP in the Kagoshima cohort.

Our detailed clinical analysis of 213 patients with HAM/TSP has revealed that 17% showed arthropathy (Nishioka et al. 1989) characterized by erythema, swelling, and severe pain on moving which mainly occur in large joints (Nakagawa et al. 1995). As the recent study by Levin et al. identified an autoantibody against heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1) which cross-reacts with HTLV-I Tax protein in IgG isolated from HAM/TSP patients (Levin et al. 2002), it is possible that host recognition of 'self' molecules that mimic HTLV-I contributes to the tissue damage seen in HAM/TSP and its accompanying arthropathy. If this is the case, an immune reaction against a protein that exists in both the spinal cord and joint may be a good candidate for autoantigen.

Human aggrecan is a major extracellular matrix protein expressed in both joint cartilage and the spinal cord, and consists of a core protein and attached glycosaminoglycan (GAG) side chains (Asher et al. 1995; Doege et al. 1991; Milev et al. 1998; Moon et al. 2003; Takahashi-Iwanaga et al. 1998; Watanabe et al. 1998). The reported functions of aggrecan are, first, to maintain the high water content in the extracellular matrix, and second, to act as a barrier against cell migration and a guide for axonal growth in the central nervous system (CNS) along with other chondroitin sulfate (CS) proteoglycans such as phosphocan, neurocan, and versican (Adams et al. 1993; Ang et al. 1999; Asher et al. 2000; Grumet et al. 1993; Moon et al. 2003; Oohira et al. 2000; Perris and Perissinotto 2000). Some reports provide evidence that aggrecan is produced by astrocytes in the perineurial region of the CNS (Matthews et al. 2002; Takahashi-Iwanaga et al. 1998).

Interest in aggrecan function has been increasing as a result of recent research on autoimmune and inflammatory arthritis (Glant et al. 1998; Poole 1998; Zhang et al. 1998b). There are reports showing that aggrecan may act as an immunogenic epitope of T and B cells both in vivo and in vitro. Once the G1 domain has been removed from the core protein of aggrecan by the enzyme aggrecanase (Feng et al. 1998; Zhang et al. 1998a), the molecule discloses a T-cell epitope. It has also been reported that a decrease of CS content elicits a T-cell immune response, whereas a decrease of keratan sulfate (KS) content elicits a B-cell response (Glant et al. 1998).

Based on these findings, we wished to consider the possibility that genetically determined characteristics of extracellular matrix proteins and their degradation are related to the pathogenesis of HAM/TSP. To test this possibility, we analyzed the variable number of tandem repeat (VNTR) polymorphism that was recently identified in the second exon of the *aggrecan* gene, and which encodes a CS attachment site (Doege et al. 1997), in 227 HAM/TSP patients, 217 HCs, and 85 normal controls, and in 58 HAM/TSP patients and 70 HCs. We also examined the protein level of *aggrecan* in both serum and CSF from HTLV-I-infected individuals.

Finally, we have employed a special criterion proposed as the Better Association for Disease and GEnes (BADGE) system (Manly 2005) to assure the reproducibility of our genetic association study. This is because some genetic association studies have problems on reproducibility. In fact, several studies have shown poor reproducibility (Becker et al. 2004; Cardon and Bell 2001; Colhoun et al. 2003; Hirschhorn et al. 2002; Ioannidis et al. 2001; Redden and Allison 2003). This novel system is simple to use and is useful when one needs to estimate reproducibility in the absence of direct experimental replication.

Materials and methods

Study population

The genomic DNA sequences of the *aggrecan* gene were compared among 227 HAM/TSP patients, randomly selected 217 HCs, and 85 normal controls. All cases, HCs, and controls were Japanese and resided in Kagoshima Prefecture, which is an endemic area of HTLV-I infection in Japan. All HCs and normal controls were blood donors and were not related to the patients. The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (Osame 1990). Sex and ages of subjects were as follows: HAM/TSP group, 69 males and 158 females, 23–76 (mean 57) years old; HC group, 101 males and 116 females, 20–74 (mean 50) years old; control group, 35 males and 50 females, 35–55 (mean 48) years old. The second set of DNA samples were derived from 58 patients with HAM/TSP and 70 HCs from our area. Sex and ages of subjects of this second group were as follows: HAM/TSP group, 20 males and 38 females, 40–65 (mean 50) years old; HC group, 30 males and 40 females, 35–50 (mean 42) years old.

To measure the level of aggrecan in serum and CSF, we used serum samples from 33 HAM/TSP patients and from 11 HCs and CSF samples from 52 HAM/TSP patients, CSF samples from 18 HTLV-I-infected non-inflammatory other disease controls (OND) (five motor neuron disease, four spinocereberal degenaration, two Parkinson's disease, two quadriceps myopathy, one thyroid dysfunction, one essential tremor, one hemifacial spasm, one arrhythmia, and one leg fracture). There was no paired sample of serum and CSF.

We defined rapidly progressive HAM/TSP patients as those who became unable to walk within 3 years after onset of the disease. Sex and ages of rapidly progressive HAM/TSP patients were seven males and 11 females, 48–65 (mean 55) years old, and those of chronic HAM/TSP patients were 11 males and 23 females, 40–64 (mean 54) years old. All samples were taken under written informed consent. The

Ethical Committee of Kagoshima University Faculty of Medicine approved this study.

Serum, CSF, and genomic DNA preparation

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by Histopaque-1077 (Sigma, Tokyo, Japan) density gradient centrifugation, and washed three times with phosphate buffered saline (PBS) with 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PBMCs using a QIAamp blood kit (Qiagen Ltd, Tokyo, Japan) according to the manufacturer's instructions. The CSF and serum samples were also collected, and stored at −70°C until use.

Determination of polymorphism and provirus load measurement

The *aggrecan* gene contains a large exon (exon 12) of 3.5 kb, which encodes the entire glycosaminoglycan (GAG) attachment regions of its core protein (Doege et al. 1991). This region consists of numerous repeated sequences, including a particularly highly conserved set of repeats in the CS attachment site. The VNTR polymorphism of the *aggrecan* gene in exon 10 has already been reported (Doege et al. 1997) This VNTR can be detected by PCR as different lengths of PCR products. A genomic PCR was performed with 20 ng of genomic DNA as template, 50 pmol of each primer (forward: 5′-TAG AGG GCT CTG CCT CTG GAG TTG-3′ and reverse: 5′-AGG TCC CCT ACC GCA GAG GTA GAA-3′), 20-mM deoxynucleotide triphosphates (dNTPs), 15-mM $MgCl_2$, reaction buffer provided by the manufacturer, and one unit of Takara-Taq DNA polymerase (Takara, Tokyo, Japan) in a final volume of 10 μl. PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and elongation at 72°C for 60 s with a final extension at 72°C for 10 min. PCR products were separated on 1% agarose gels, visualized by ethidium bromide staining, after which the products were determined. Several of the alleles differ by only one repeat in size (59 bp), and care was taken to identify these alleles using appropriate gels and size markers. Two independent readers scored the alleles.

Provirus load of the samples was measured by a quantitative PCR method using an ABI Prism 7700 (PE-Applied Biosystems) (Nagai et al. 1998).

Quantification of aggrecan in serum and CSF

Serum, as well as CSF aggrecan concentration, was measured in duplicate using a commercial ELISA kit (BiSource Europe S.A., Nivelles, Belgium). According to the manufacturer's instruction, the kit detects aggrecan and aggrecan fragments, and the assay system used is sensitive to detect 0.9 ng/ml of aggrecan in samples. Serum aggrecan levels in normal adults ranged between 1 and 4.4 μg/ml, whereas no information was available regarding CSF levels. When we needed to separate CSF aggrecan amounts into two groups, we selected 0.9 ng/ml as the cut-off level, as this value was the lowest value of the cut-off range and there was no previous report measuring CSF aggrecan concentration. Optical density at 450 nm was measured on the ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and aggrecan concentration was determined by linear regression from a standard curve using the aggrecan supplied with the kit as standard.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows release 7.0, run on an IBM-compatible computer (Analytical software, Version 7, Tallahassee, FL, USA). Comparison of whole-allele distribution between patients with HAM/TSP and HCs was performed using a chi-square test for 2×9 contingency table with a significance level $p<0.05$. The distribution of each allele and genotype of the VNTR polymorphism of the *aggrecan* gene in patients with HAM/TSP patients was compared with those in HCs using a chi-square test for a 2×92 and 2×3 contingency table. Bonferroni multiple adjustments (Motulsky 1995) were made to the level of significance because of the multiple comparisons for VNTR allele frequencies. This level was set at $p<0.0057$ ($p=1-0.95^{(1/9)}$).

To assure reproducibility of our study, we have combined p values from the analysis on two sets of populations and have compared the combined p values to the BADGE (Manly 2005).

Serum and CSF aggrecan levels in patients and controls in three different groups were compared using either ANOVA or Kruskal–Wallis test. A p value less than 0.05 was considered statistically significant. When aggrecan levels in three groups were different, multiple comparisons were done by Scheffe's test. We also performed multiple-hypotheses testing when it was needed, and the level was set at $p<0.017$ ($p=1-0.95^{(1/3)}$).

Results

Frequency of aggrecan VNTR allele 28 was significantly higher in HAM/TSP than HCs and normal control

We applied two-step analysis on our cohort. We first typed 100 samples from each group observing nine aggrecan VNTR alleles, and found the difference between the groups [$\chi^2=18.18$ ($df=8$), $p=0.019$]. We then proceeded to analyze whole samples in this study (227 HAM/TSP patients and 217 HCs) (Table 1).

Comparison of whole allele distribution among patients with HAM/TSP, in HCs and normal controls was performed using a chi-square test for 3×9 contingency table with a significance level $p<0.05$. This analysis has revealed