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Thrombin-Activatable Fibrinolysis Inhibitor Deficiency Attenuates Bleomycin-Induced Lung Fibrosis

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Decreased fibrinolytic function favors the development of pulmonary fibrosis. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a strong suppressor of fibrinolysis, but its role in lung fibrosis is unknown. Therefore, we compared bleomycin-induced lung fibrosis in TAFI-deficient, heterozygous, and wild-type mice. The animals were sacrificed 21 days after bleomycin administration, and markers of lung fibrosis and inflammation were measured. The bronchoalveolar lavage fluid levels of total protein, neutrophil proteases (elastase, myeloperoxidase), cytokines (tumor necrosis factor- α , interleukin-13), chemokine (monocyte chemoattractant protein-1), coagulation activation marker (thrombin-antithrombin complex), total soluble collagen, and growth factors (platelet-derived growth factor, transforming growth factor- β 1, granulocyte-macrophage growth factor) were significantly decreased in knockout mice compared to wild-type mice. Further, histological findings of fibrosis, fibrin deposition, and hydroxyproline and collagen content in the lung were significantly decreased in knockout mice compared to wild-type mice. Depletion of fibrinogen by anicrod treatment led to equalization in the amount of fibrosis and collagen deposition in the lungs of knockout and wild-type mice. No difference was detected in body temperature or arterial pressure between the different mouse pheno-

types. These results suggest that the anti-fibrinolytic activity of TAFI promotes lung fibrosis by hindering the rate at which fibrin is degraded. (*Am J Pathol* 2006, 168:1086–1096; DOI: 10.2353/ajpath.2006.050610)

Lung fibrosis is the end-stage of a heterogeneous group of respiratory disorders caused by injury of the lung parenchyma, increased proliferation of mesenchymal cells, and excessive accumulation of extracellular matrix in the lung.^{1,2} Decreased degradation of extracellular matrix because of deficient function of alveolar fibrinolysis plays a fundamental role in driving the fibrotic response in the lung.^{2,3} The effector enzyme of the fibrinolytic system is plasmin, which results from the activation of plasminogen by urokinase or tissue plasminogen activator. Plasmin promotes extracellular matrix degradation by directly degrading a number of extracellular matrix macromolecules or by activating several promet-alloproteinases and prostromelysins.⁴ Plasmin can also rapidly degrade fibrin formed after leakage of proteins and activation of the coagulation cascade in the alveolar space.⁴ Under physiological conditions, the alveolar space of the lung has potent fibrinolytic activity. However, patients with lung injury such as acute respiratory dis-

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tress syndrome and interstitial lung diseases have low alveolar fibrinolytic activity.⁵ Animal models of lung injury such as that induced by bleomycin or lipopolysaccharide also show deficient activation of the intra-alveolar plasminogen-plasmin system.⁶ The protective role of plasmin against lung fibrosis has been recently demonstrated in experiments using animals expressing either no plasminogen activator inhibitor (PAI)-1 or high concentration of it, the main inhibitor of plasmin generation.⁷ The results of these studies have shown that bleomycin-induced lung fibrosis is more severe in transgenic mice overexpressing PAI-1 than in PAI-1-deficient mice, and bleomycin-treated PAI-1-deficient mice have less lung fibrosis and a better outcome than mice that overexpress PAI-1.⁷ Underlining the importance of fibrinolysis in this model, inhibition of plasmin in PAI-1-deficient mice after treatment with bleomycin increased fibrin and collagen deposition in the lung.⁸

The cause of the reduced fibrinolysis after lung injury is not clear but several lines of evidence indicate PAI-1 as a potential candidate. PAI-1 is a member of the serine protease inhibitor gene family that rapidly and potently inhibits both urokinase plasminogen activator (PA) and tissue PA.⁴ Mice that are deficient in PAI-1 display enhanced fibrinolytic activity. High concentrations of PAI-1 in the bronchoalveolar lavage fluid (BALF) from patients with acute respiratory distress syndrome and idiopathic pulmonary fibrosis reduce the fibrinolytic activity in the fluid.⁶ Similar findings have been reported in animal models of lung injury induced by bleomycin or lipopolysaccharides.^{9,10} In addition, patients with systemic disorders including diabetes mellitus and arterial hypertension with glomerulosclerosis also have decreased fibrinolytic activity because of an increased circulating level of PAI-1.¹¹

Another candidate to explain the decreased plasmin generation in lung injury is thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI is a glycoprotein with a molecular weight of 55 kd. TAFI is secreted from hepatocytes in zymogen form and it is activated by thrombin-, thrombin-thrombomodulin complex-, plasmin- or trypsin-catalyzed proteolysis to a carboxypeptidase B-like enzyme that inhibits fibrinolysis.¹² Activated TAFI reduces generation of plasmin because it cleaves the carboxy-terminal lysine residues from partially degraded fibrin and thereby abrogates the fibrin co-factor function in tPA-mediated catalysis of plasminogen to plasmin. In addition, activated TAFI may also directly inactivate plasmin, further impairing fibrinolysis.¹² Patients with lung injury including those with idiopathic pulmonary fibrosis have increased intra-alveolar levels of TAFI, and this abnormality has been linked to decreased plasminogen activator activity in the lung.¹³ This observation implicates TAFI in the fibrinolytic dysfunction of lung injury. In addition, the high concentration of TAFI in patients with lung injury has been found to be significantly associated with activation of the coagulation system and with markers of inflammation and collagen deposition in the lung, supporting the role of TAFI in the pathogenesis of pulmonary fibrosis.¹³ In the present study, we hypothesized that inhibition of fibrinolysis by TAFI augments lung fibrosis. To demonstrate this hypothesis, in this study we evaluated and compared the

development of lung fibrosis in wild-type (TAFI^{+/+}), heterozygous TAFI (TAFI^{+/-}), and TAFI-deficient (TAFI^{-/-}) mice.

Materials and Methods

Animals

TAFI^{+/+}, TAFI^{+/-}, and TAFI^{-/-} mice in a mixed background of C57BL/6 and 129/Sv strains were previously characterized.¹⁴ Wild-type littermates were used as controls. Female mice weighing 18 to 22 g between 8 and 12 weeks of age were used. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Animal Model

Lung injury was induced by bleomycin (Nihon Kayaku, Tokyo, Japan) dissolved in saline at a dose of 100 mg/kg by constant (7 days) subcutaneous infusion through osmotic minipumps (Alza, Palo Alto, CA) as described.⁹ For control animals, the minipumps were loaded with saline in a similar manner.

Experimental Design

There were six groups of animals: wild-type mice treated with saline (wt/sal, $n = 29$) or bleomycin (wt/blm, $n = 23$), heterozygous mice treated with saline (ht/sal, $n = 4$) or bleomycin (ht/blm, $n = 30$), and knockout mice treated with saline (ko/sal, $n = 9$) or bleomycin (ko/blm, $n = 30$). To evaluate changes in the grade of inflammation during the progression of disease, sampling was performed on days 7 and 14 in the groups of mice ($n = 3 \sim 4$) after completion of subcutaneous bleomycin administration.

BALF Biochemical Analysis and Cell Count

Mice were sacrificed on day 21 by pentobarbital overdose. BALF was sampled as described.⁹ Total protein was measured by dye-binding assay (Bio-Rad Laboratories, Hercules, CA). The plasma level of lactate dehydrogenase (LDH) was measured using a commercial kit (LDH ICII kit; Wako Pure Chemical Industry, Osaka, Japan) following the manufacturer's instructions. Thrombin-antithrombin complexes (TAT) were measured using an enzyme immunoassay kit from Cedarlane Laboratories (Ontario, Canada). Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β 1, and granulocytic-macrophage colony-stimulating factor (GM-CSF) in BALF were measured using enzyme immunoassay kits from BD Biosciences Pharmingen (San Diego, CA) and interleukin (IL)-13 using enzyme immunoassay kits from Genzyme (Minneapolis, MN). The level of tissue plasminogen activator (tPA) was measured by enzyme immunoassay using monoclonal antibodies specific for tPA (Oxford Biomedical Research, Oxford, MI). The level of urokinase

Table 1. Cell Count in Bronchoalveolar Lavage Fluid

	wt/sal	wt/blm	ht/sal	ht/blm	ko/sal	ko/blm
Total cell count ($\times 10^5/\text{ml}$)	4.27 ± 0.57	$28.3 \pm 7.39^*$	4.30 ± 2.30	$18.33 \pm 1.20^*$	3.60 ± 0.36	$15.67 \pm 2.03^{*\dagger}$
Macrophages ($\times 10^5/\text{ml}$)	4.16 ± 0.57 (97.2%)	$11.14 \pm 4.08^*$ (37.6%)	4.20 ± 2.26 (97.5%)	$8.22 \pm 0.65^*$ (45.0%)	3.49 ± 0.34 (97.0%)	$8.93 \pm 0.61^*$ (58.0%)
Lymphocytes ($\times 10^5/\text{ml}$)	0.08 ± 0.01 (2.0%)	$12.63 \pm 2.49^*$ (46.0%)	0.08 ± 0.05 (2.0%)	$8.02 \pm 0.98^*$ (43.6%)	0.08 ± 0.02 (2.3%)	$5.28 \pm 1.11^{*\dagger}$ (33.0%)
Neutrophils ($\times 10^5/\text{ml}$)	0.03 ± 0.01 (0.7%)	$4.5 \pm 0.97^*$ (16.3%)	0.01 ± 0.01 (0.5%)	$2.09 \pm 0.25^*$ (11.3%)	0.02 ± 0.01 (0.6%)	$1.45 \pm 0.32^{*\dagger}$ (9.0%)

wt/sal, wild type/saline; wt/blm, wild type/bleomycin; ht/sal, heterozygous/saline; ht/blm, heterozygous/bleomycin; ko/sal, knockout/saline; ko/blm, knockout/bleomycin.

* $P < 0.05$, compared to wt/sal, ht/sal, and ko/sal groups.

$^\dagger P < 0.05$, compared to wt/blm group.

(uPA) was measured spectrophotometrically using the synthetic chromogenic substrate S-2444 (Chromogenix, Molndal, Sweden). The level of D-dimer was measured using the Imuclone D-dimer enzyme-linked immunosorbent assay kit from American Diagnostica (Stamford, CT). Platelet-derived growth factor (PDGF) was measured using anti-PDGF (Genzyme, Boston, MA) and biotin-labeled anti-PDGF antibodies.¹⁰ The total number of cells in BALF was measured using a nucleocounter from ChemoMetec (Allerød, Denmark). For differential cell counting BALF was centrifuged using a cytospin and the cells were stained with May-Grunwald-Giemsa (Merck, Darmstadt, Germany).

BALF Enzymes

Activity of neutrophil elastase and myeloperoxidase (MPO) was spectrophotometrically measured as previously described using the synthetic substrates methoxysuccinyl-alal-pro-val-paranitroanilide (MeOSAAPVpNa; Sigma Chemical Co., St Louis, MO) and 2,2'-azino-bis(ethylbenzylthiazoline-6-sulfonic acid) diammonium salt, respectively.¹⁵

Collagen and Hydroxyproline

Total collagen was measured using the Sircol collagen assay kit (Biocolor, Belfast, UK) following the manufacturer's instructions, and hydroxyproline content by a colorimetric method as described.¹⁶

Histological Study

Lung samples were drawn on day 21 after bleomycin or saline infusion before being fixed in formalin, embedded in paraffin, and prepared for hematoxylin and eosin (H&E) and Mallory-Azan staining.

Immunohistochemistry

Lung tissues were sampled, fixed in 10% formalin solution, and then embedded in paraffin. Histological sections with a width of 5 μm were prepared, deparaffinized, and washed several times with phosphate-buffered saline. Immunohistochemical staining of collagen type I and fibrin was then performed by treating the samples with rabbit anti-mouse collagen I antibody (Bethyl Laborato-

ries, Montgomery, TX) or rabbit anti-fibrin(ogen) (DAKO, Glostrup, Denmark) antibody and using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as described.^{17,18} The samples were then treated with biotin-labeled rabbit anti-mouse IgG, peroxidase-labeled streptavidin, and peroxidase substrate by using the Catalyzed Signal Amplification System from DAKO (Kyoto, Japan). The degree of collagen and fibrin deposition in five areas per lung sample was scored using computer software (WinRoof; Mitani Corp., Fukui, Japan).

Fibrinogen Depletion

Ancrod (Sigma Chemical Co.) was administered at 2 U per mouse once a day by subcutaneous injection from day 0 to day 14. Four groups of mice (each $n = 4$) received ancrod: wild-type (wt)/sal, wt/blm, heterozygous (ht)/blm, and knockout (ko)/blm. Bleomycin was administered as described above.

Blood Pressure and Body Temperature

The systolic and diastolic blood pressures and body temperature were measured once every 3 days. Blood pressures in conscious mice were determined by the tail-cuff method (BA-98A System; Softron Co., Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean $\pm$ SE. Statistical analyses were performed using the StatView 4.5 package for Macintosh (Abacus Concepts, Berkeley, CA). Differences between variables were calculated by the non-parametric Kruskal-Wallis analysis of variance and posthoc test using the Wilcoxon sum-rank test. Relationship between variables was assessed by the Spearman's correlation. A $P < 0.05$ was considered as significant.

Results

Inflammatory Response and Lung Injury

The total number of cells and the number of macrophages, lymphocytes, and neutrophils in BALF were sig-

Figure 1. BALF concentration of inflammatory mediators. Total protein was measured by colorimetric assay and elastase and MPO by amidolytic assay as described in Materials and Methods. Total protein (**A**), elastase (**B**), and MPO (**C**) were significantly increased in wt/blm and ht/blm as compared to wt/sal and ht/sal, respectively. Total protein, elastase, and MPO were significantly decreased in ko/blm compared with wt/blm and ht/blm groups. * $P < 0.04$ compared to wt/sal, ht/sal, and ko/sal. ‡ $P < 0.01$ compared to wt/blm and ht/blm. Number of mice in each group: wt/sal, 29; wt/blm, 23; ht/sal, 4; ht/blm, 30; ko/sal, 9; ko/blm, 30.

nificantly increased in all groups of mice treated with BLM as compared to those treated with saline (Table 1). The total number of cells and the number of lymphocytes and neutrophils were significantly decreased in the ko/blm group as compared to the wt/blm group. The BALF concentrations of total protein, elastase, and MPO were significantly increased in wt/blm, ht/blm, and ko/blm mice compared to wt/sal, ht/sal, and ko/sal groups, respectively. The concentrations of total protein, elastase and MPO in BALF were significantly decreased in ko/blm mice as compared to wt/blm and ht/blm mice (Figure 1, A–C). No statistical difference was observed in the concentrations of total protein, elastase, or MPO between ht/blm and wt/blm groups. Overall, these findings sug-

Figure 2. BALF concentration of cytokines. Cytokines were measured by enzyme-linked immunosorbent assay. **A:** IL-13 was significantly increased in wt/blm, ht/blm, and ko/blm as compared to wt/sal, ht/sal, and ko/sal, respectively. There was no difference between the wt/blm, ht/blm, and ko/blm groups. **B:** MCP-1 was significantly elevated in wt/blm compared with wt/sal. The MCP-1 level was significantly different between ko/blm and wt/blm. **C:** TNF-α was significantly increased in wt/blm and ht/blm compared with wt/sal and ht/sal, respectively. TNF-α was significantly decreased in ko/blm as compared to wt/blm and ht/blm. * $P < 0.05$ compared to wt/sal, ht/sal, and ko/sal. ‡ $P < 0.05$ compared to wt/blm. § $P < 0.02$ compared to ht/blm. Number of mice in each group: wt/sal, 29; wt/blm, 23; ht/sal, 4; ht/blm, 30; ko/sal, 9; ko/blm, 30.

gest that TAFI-deficient mice are protected from lung injury as compared to their wild-type and heterozygous counterparts.

Cytokine Expression

The cytokine profile of the immune/inflammatory response may determine the disease phenotype responsible for either resolution or progression to end-stage fibrosis. Th1 cytokines such as interferon- γ exert suppressive effects on production of extracellular matrix, whereas Th2 cytokines such as IL-13 stimulate the secretion of collagen type-I and type III from fibroblasts.¹⁹ In the present

study, the level of IL-13 was markedly increased in BALF from wt/blm, ht/blm, and ko/blm mice as compared to wt/sal mice (Figure 2A). The BALF level of IL-13 was not significantly different among the ko/blm, wt/blm, and ht/blm mice or among ko/sal, wt/sal, and ht/sal groups. The chemokine MCP-1 and the proinflammatory cytokine TNF- α have been also implicated in the pathogenesis of lung fibrosis by promoting the infiltration of monocytes and macrophages as well as stimulating the proliferation of mesenchymal cells.¹⁹ In the present study, the BALF concentration of MCP-1 was significantly elevated in wt/blm compared to wt/sal group but no significant difference was observed between the ht/sal and ht/blm or between the ko/sal and ko/blm groups. BALF concentration of MCP-1 was significantly decreased in ko/blm as compared to wt/blm group (Figure 2B). It is worth noting that BALF MCP-1 level was significantly decreased in wt/sal as compared to ht/sal and ko/sal groups. BALF level of TNF- α was significantly increased in wt/blm and ht/blm as compared to wt/sal and ht/sal, respectively; no difference was observed between ko/sal and ko/blm groups. The BALF level of TNF- α was significantly decreased in ko/blm as compared to wt/blm and ht/blm groups (Figure 2C). These observations suggest that TAFI deficiency is associated with suppression of MCP-1 and TNF- α in bleomycin-induced lung fibrosis.

Activation of Coagulation and Fibrinolysis Systems

Activation of the coagulation system and hypofibrinolysis in the intra-alveolar space play a fundamental role in the establishment of lung fibrosis.³ We measured the level of TAT as a marker of coagulation system activation and the level of tPA activity and also D-dimer levels as markers of fibrinolytic activity (Figure 3, A and B). The BALF level of TAT was significantly increased in mice treated with bleomycin (wt/blm, ht/blm, and ko/blm) as compared to those treated with saline (wt/sal, ht/sal, ko/sal). TAT was significantly elevated in wt/blm compared with ko/blm mice. On the other hand, tPA activity in BALF was significantly decreased in wt/blm as compared to wt/sal group. tPA was remarkably increased in ko/blm groups as compared with the wt/blm and ht/blm groups. Differences between ht/sal and ht/blm or between ko/sal and ko/blm groups were not statistically significant. The level of uPA was not significantly different between wt/blm (149 ± 20 ng/ml), ht/blm (122 ± 14 ng/ml), and ko/blm (151 ± 22 ng/ml) groups, but it was significantly ($P < 0.05$) increased in bleomycin-treated groups as compared to wt/sal (26 ± 6 ng/ml), ht/sal (71 ± 16 ng/ml), and ko/sal (17 ± 233 ng/ml) groups, respectively. Similarly, no statistical differences were found in the level of D-dimer in BALF between wt/blm (616 ± 120 pg/ml), ht/blm (442 ± 78 pg/ml), and ko/blm (675 ± 192 pg/ml) groups, but it was significantly ($P < 0.05$) increased in bleomycin-treated groups as compared to wt/sal (96 ± 51 pg/ml), ht/sal (130 ± 18 pg/ml), and ko/sal (96 ± 34 pg/ml) groups, respectively. The ratio of D-dimer to TAT in BALF was calculated to assess the balance between coagula-

Figure 3. BALF concentration of markers of coagulation and fibrinolysis. TAT and tPA were measured by enzyme-linked immunosorbent assay. TAT (A) was significantly increased in wt/blm, ht/blm, and ko/blm as compared to wt/sal, ht/sal, and ko/sal, respectively. TAT level was markedly decreased in ko/blm mice compared with wt/blm. The level of tPA (B) was significantly lower in wt/blm than in wt/sal mice. * $P < 0.02$ compared to wt/sal, ht/sal, and ko/sal mice. † $P < 0.05$ compared to wt/blm mice. ‡ $P < 0.04$ compared to wt/blm. Number of mice in each group: wt/sal, 29; wt/blm, 23; ht/sal, 4; ht/blm, 30; ko/sal, 9; ko/blm, 30.

tion and fibrinolysis. The D-dimer/TAT ratio in BALF was significantly increased in the ko/blm group (0.9 ± 0.2) as compared to the wt/blm (0.2 ± 0.0) and ht/blm (0.1 ± 0.0) groups, and in the wt/sal (1.2 ± 0.8), ht/sal (3.6 ± 0.8), and ko/sal (4.3 ± 0.8) groups as compared to bleomycin-treated mice.

Total Lung Content of Collagen and Growth Factors

Abnormalities in lung collagen metabolism were assessed by measuring the total collagen content and hydroxyproline in lung tissue and soluble collagen concentration in BALF from each group of animals. The BALF level of soluble collagen was significantly increased in all groups of mice treated with bleomycin (wt/blm, ht/blm, ko/blm) as compared to those treated with saline. The BALF level of collagen was significantly lower in ko/blm than in both wt/blm and ht/blm groups (Figure 4A). The total collagen and hydroxyproline content of lung tissue was significantly elevated in the wt/blm group as compared to wt/sal group; no difference was observed between the ko/sal and ko/blm groups. In addition, the lung content of collagen and hydroxyproline was significantly reduced in ko/blm as compared to wt/blm and ht/blm mice (Figure 4, B and C). These findings suggest the

Figure 4. Collagen markers in the lung. Soluble collagen in BALF (A) was significantly increased in wt/blm, ht/blm, and ko/blm as compared to wt/sal, ht/sal, and ko/sal mice, respectively. BALF level of collagen was significantly lower in ko/blm than in wt/blm and ht/blm groups. The hydroxyproline content (B) and total collagen (C) of lung tissue were significantly elevated in wt/blm as compared to wt/sal and ko/blm groups. * $P < 0.01$ compared to wt/sal, ht/sal, and ko/sal mice. [‡] $P < 0.05$ compared to wt/blm mice. [§] $P < 0.05$ compared to ht/blm. Number of mice in each group: wt/sal, 29; wt/blm, 23; ht/sal, 4; ht/blm, 30; ko/sal, 9; ko/blm, 30.

protective role of TAFI deficiency against collagen deposition in the lung.

TGF- β 1 promotes extracellular matrix deposition by enhancing the synthesis and secretion of collagens and of tissue-type metalloproteinase inhibitors.²⁰ PDGF is a potent mitogen and chemoattractant for mesenchymal cells and also, it is able to induce expression of genes encoding cell matrix-related molecules such as collagen, fibronectin, and glycosaminoglycans.²⁰ GM-CSF is also profibrotic. In the present study, the BALF concentrations of TGF- β 1 (0.9 ± 0.2 versus 0.4 ± 0.1 ng/ml), PDGF (69.9 ± 19.5 versus 2.8 ± 0.8 pg/ml), and GM-CSF (10.2 ± 0.7 versus 4.1 ± 0.4 pg/ml) were significantly increased in wt/blm group as compared to wt/sal mice, but no difference was observed between ko/sal (data not shown) and ko/blm groups. The BALF concentrations of

TGF- β 1 (0.7 ± 0.1 versus 0.2 ± 0.1 ng/ml) and PDGF (70.2 ± 27.1 versus 14.6 ± 14.6 pg/ml) were significantly higher in ht/blm than in ht/sal group, but no difference was observed in the concentration of GM-CSF between ht/sal and ht/blm groups (data not shown). TGF- β 1 (0.9 ± 0.2 versus 0.3 ± 0.1 ng/ml), PDGF (69.9 ± 19.5 versus 23.1 ± 13.7 pg/ml), and GM-CSF (10.2 ± 0.7 versus 4.2 ± 0.9 pg/ml) were significantly decreased in ko/blm group as compared to wt/blm group. The BALF PDGF level was also significantly decreased in the ko/blm group as compared to ht/blm mice. Statistically, there were no significant differences in the BALF concentrations of PDGF and TGF- β 1 among ko/sal, ht/sal, and wt/sal mice. Overall, these results suggest that TAFI deficiency is associated with a lower secretion of growth factors in the lung.

Correlation between Variables in BALF from BLM-Treated Mice

To uncover factors involved in the process of lung fibrosis in our animal model, the relationship between variables was evaluated in BALF from animals treated with bleomycin. The concentration of soluble collagen, a marker of collagen deposition and synthesis was proportionally and significantly correlated with the concentrations of IL-13 ($r = +0.4$, $P = 0.004$), elastase ($r = +0.7$, $P < 0.0001$), MPO ($r = +0.8$, $P < 0.0001$), TGF- β 1 ($r = +0.6$, $P = 0.001$), TAT ($r = +0.8$, $P < 0.0001$), and MCP-1 ($r = +0.3$, $P = 0.04$) suggesting the involvement of growth factors, Th2 cytokines, and activation of the coagulation cascade in the mechanism of bleomycin-induced lung fibrosis.

Histological and Collagen Deposition in the Lung

On H&E staining, animals from the wt/blm and ht/blm groups showed severe fibrotic changes in the central regions of the lung involving the perivascular and peribronchiolar areas, and with areas of consolidation in the subpleural regions (data not shown). In comparison, the ko/blm group showed less parenchymal consolidation, reduced subpleural thickening, and less vascular or bronchial wall remodeling. The lungs from wt/sal, ht/sal, and ko/sal groups showed normal findings.

Mallory-Azan staining was performed and the degree of collagen deposition in the lung was scored. Deposition of collagen was more pronounced in mice from the wt/blm and ht/blm groups than those from the ko/blm group (data not shown). The wt/sal, ht/sal, and ko/sal groups showed normal findings. Immunostaining of lung tissue from mice of the wt/blm and ht/blm groups showed more remarkable deposition of extracellular collagen type I in thickened alveolar walls as compared to the ko/blm group (Figure 5). The degree of collagen type I deposition was not different between wt/sal, ht/sal, and ko/sal groups.

Figure 5. Immunological staining of collagen. Tissue preparations were stained with anti-collagen I antibody and immunoreactive areas (brown) were quantified using Winroof software. Quantification is shown in histograms. Lung tissue of the wt/blm ($n = 6$) and ht/blm ($n = 6$) groups showed more significant deposition of extracellular collagen type I in thickened alveolar walls as compared to ko/blm group ($n = 6$). Mice of the wt/sal ($n = 6$), ht/sal ($n = 4$), and ko/sal ($n = 4$) groups showed normal lung findings. * $P < 0.01$ compared to wt/sal and ht/sal groups. [‡] $P < 0.05$ compared to wt/blm and ht/blm mice. Original magnifications, $\times 200$.

Fibrin Deposition in the Lung

Fibrin formation was more significantly observed in the interstitial and alveolar spaces in wt/blm and ht/blm mice than in ko/blm mice (Figure 6). Fibrin deposition was almost completely absent in wt/sal, ht/sal, and ko/sal mice.

Effect of Fibrinogen Depletion on Lung Fibrosis

Ancrod is an enzyme that removes fibrinogen that allows the testing of the hypothesis that the protective effect of TAFI deficiency on bleomycin-induced lung fibrosis is because of the action of TAFI in reducing plasminogen activation and hence fibrinolysis. In the absence of fibrinogen and hence fibrin, if the effect of TAFI is mediated by

Figure 6. Immunological staining of fibrin deposits. Tissue preparations were stained with anti-fibrin/fibrinogen antibody and immunoreactive areas (brown) were quantified using Winroof software. Quantification is shown in histograms. Significant immunoreactivity of fibrin deposits was observed in the interstitial and alveolar spaces of wt/blm ($n = 6$) and ht/blm ($n = 6$) mice compared to wt/sal ($n = 6$) and ht/sal ($n = 4$) groups, respectively. Mice of the wt/blm showed more significant fibrin deposition than ht/blm and ko/blm ($n = 6$) groups. * $P < 0.01$ compared to wt/sal and ht/sal groups. [‡] $P < 0.05$ compared to wt/blm. Original magnifications, $\times 400$.

fibrin, then there should be no difference between mice with TAFI and mice lacking TAFI in response to bleomycin. Each group of animals received 14 days of ancrod treatment after the 7-day infusion of bleomycin. In mice treated with ancrod, the BALF concentrations of total protein, soluble collagen, and the total content of collagen in lung tissue were significantly higher in wt/blm, ht/blm, and ko/blm groups as compared to wt/sal group but no difference was found among wt/blm, ht/blm, and ko/blm groups (Figure 7). These findings suggest that fibrin is involved in the protective effect of TAFI deficiency from lung fibrosis.

Changes during Progression of Disease

The concentrations of total protein and MCP-1 were measured in BALF as markers of lung inflammation and LDH in plasma as a marker of systemic injury. Soluble collagen was measured in BALF as an index of collagen

Figure 7. Effect of fibrinogen depletion on lung fibrosis. In mice treated with ancoed, the BALF concentrations of total protein (**A, black bars**) and soluble collagen (**B, black bars**) and the total content of collagen (**B, white bars**) in lung tissue were significantly higher in wt/blm, ht/blm, and ko/blm groups as compared to wt/sal group but no differences were observed among wt/blm, ht/blm, and ko/blm mice. Number of mice in each group was four.

metabolism. The BALF level of total protein and collagen and the plasma level of LDH were significantly increased in wt/blm as compared to wt/sal on 7 and 14 days after completion of BLM infusion (Figure 8). There were significant differences between wt/blm and ko/blm in the levels of total protein, LDH, and soluble collagen on days 7 and 14 after BLM infusion. There was a significant difference between wt/blm and ko/blm in the level of MCP-1 on day 21 but not on 7 day after BLM infusion (Figure 8).

Changes in Blood Pressures and Body Temperatures

TAFI has been shown to inhibit the bradykinin system, which is known to regulate body temperature and systemic blood pressures. Body temperature and systolic and diastolic blood pressures were also followed during our experimental protocol. Neither body temperature nor blood pressure was significantly affected in our chronic model of lung fibrosis (data not shown).

Discussion

The protective role of the plasminogen-plasmin activation system against lung fibrosis has been well documented. The effector enzyme of the fibrinolysis system is plasmin,

Figure 8. Longitudinal changes in inflammatory and collagen markers. Total protein soluble collagen and MCP-1 were measured in BALF and LDH in plasma. The levels of total protein (**top, white bars**), collagen (**bottom, black bars**), and LDH (**top, black bars**) were significantly increased in wt/blm as compared to wt/sal on 7 and 14 days. There were significant differences between wt/blm and ko/blm in the levels of total protein, LDH, and soluble collagen on days 7 and 14. There was a significant difference between wt/blm and ko/blm in the level of MCP-1 (**bottom, white bars**) on day 21 but not on day 7 after BLM infusion. * $P < 0.05$ compared to wt/sal group. † $P < 0.05$ compared to wt/blm group. § $P < 0.05$ compared to ht/blm. Each group included approximately three to four mice.

which results from the activation of plasminogen by tPA or uPA.⁴ Plasmin is the key enzyme for preventing the development of pulmonary fibrosis because it lyses intra-alveolar deposits of fibrin, degrades matrix components, and activates the precursors of several metalloproteinases. Previous studies have clearly demonstrated that low intra-alveolar plasmin activity is associated with the occurrence of pulmonary fibrosis.^{2,7,8,16,17} Inhibition of fibrinolytic activity may occur because of suppression of plasminogen activators by specific plasminogen activator inhibitors (PAI-1, PAI-2), direct inhibition of plasmin by its specific inhibitor α_2 -antiplasmin, or because of decreased generation of the ternary complex formed by binding of plasminogen and tPA on the fibrin surface. This latter mechanism depends on TAFI activity.¹² TAFI may also inhibit fibrinolysis by blocking the activity of uPA.²¹

On activation, TAFI decreases the binding of plasminogen to fibrin surfaces by removing the carboxy-terminal arginine and lysine residues from partially degraded fibrin, thereby reducing the formation of the ternary complex of t-PA, plasminogen, and fibrin and subsequently the production of plasmin.¹² Lung fibrosis-associated hypofibrinolysis is induced by plasminogen activator inhibitors and α_2 -antiplasmin but the role of TAFI was previously untested. In the present study, we hypothesized that activation of TAFI reduces plasmin generation leading to lung fibrosis. To demonstrate this hypothesis, we induced lung fibrosis in TAFI^{+/+}, TAFI^{+/-}, and TAFI^{-/-} mice by chronic subcutaneous administration of bleomy-

cin. This model was chosen because low fibrinolytic activity has been implicated in the pathogenesis of lung fibrosis induced by bleomycin in the mouse.

Inflammation is the initial response to lung injury. The inflammatory response is characterized by the recruitment of macrophages, neutrophils, lymphocytes, and eosinophils within the alveolar and interstitial compartment of the lung.¹⁹ C-C chemokines such as MCP-1 released from injured lung tissue play a fundamental role in triggering the recruitment of leukocyte cells into the lung.²² Other inflammatory mediators including the proinflammatory cytokine TNF- α , the procoagulant factor thrombin, and the Th2 cytokine IL-13 may also favor the migration of inflammatory cells into the lungs by stimulating the secretion of chemoattractant proteins from injured lung resident cells.^{22,23} In addition, exaggerated release of enzymes such as elastase and MPO from activated leukocytes may further exacerbate the lung inflammatory response.¹⁹ Lung injury causes increased permeability of alveolar epithelium and vascular endothelium resulting in extravasation of plasma proteins, activation of the coagulation system, and deposition of fibrin clots in the alveolar spaces. In the present study, the BALF levels of total protein, MCP-1, TNF- α , and neutrophil-derived enzymes were significantly decreased in TAFI^{-/-} mice as compared to TAFI^{+/+} mice. These observations suggest that TAFI deficiency protects the lung from inflammation. Fibrin and derivatives have been reported to exacerbate lung inflammation by stimulating the migration of neutrophils, the expression of leukocyte adhesion molecules and chemokines from endothelial cells, and proinflammatory cytokines from monocytic cells, by increasing the permeability of endothelial cells and by promoting angiogenesis.²⁴⁻²⁹ In the present study, histochemical studies disclosed decreased lung deposition of fibrin in TAFI^{-/-} mice as compared to TAFI^{+/+} mice. This finding may be because of lower thrombin generation and higher fibrinolytic activity in lungs from mice with TAFI deficiency as demonstrated by the decreased level of TAT and elevated values of tPA activity in BALF from TAFI^{-/-} mice as compared to that from their wild-type counterparts. Thus, the reduced fibrin formation is the most probable explanation for the lower inflammatory reaction in lungs from TAFI-deficient mice. The fact that fibrinogen depletion by anicrod treatment abrogated the difference in BALF levels of total protein between TAFI^{-/-} and TAFI^{+/+} and TAFI^{+/-} mice supports the role of fibrin in the induction of inflammation during bleomycin-induced lung injury in the mouse.

Current knowledge suggests that cytokines released from T-helper cells may play a critical role in the pathogenesis of lung injury and fibrosis.³⁰ Two functionally distinct subsets of helper T cells have been defined depending on their cytokine expression profile: Th1 cells, which mainly secrete IFN- γ and IL-2, and Th2 cells, which produce IL-4, IL-5, IL-13, IL-6, and IL-10.³¹ An imbalance between Th1/Th2 cells occurs in lung fibrosis, with the balance tipped away from the normally predominant Th1 cells in favor of Th2 cells. A growing body of evidence suggests that a localized Th2 response by the host leads to excessive fibrosis, whereas a predominant Th1 re-

sponse protects the host from an exuberant fibrotic response.³¹ In accord with this concept, the present study showed significantly increased levels of IL-13 in BALF from mice treated with bleomycin as compared to control mice. However, unlike other inflammatory mediators described above, IL-13 was not affected by TAFI deficiency, suggesting TAFI does not influence the Th2/Th1 balance.

Previous studies have attributed an anti-inflammatory activity to TAFI because of its inhibitory activity on some active components of the complement and bradykinin systems.^{32,33} The effect on the bradykinin system was evaluated by determining the body temperature and blood pressure in the different groups of mice during the entire course of the study. However, no significant difference was noted among TAFI^{+/+}, TAFI^{+/-}, and TAFI^{-/-} mice in any of these parameters, suggesting that in our chronic model of lung fibrosis these anti-inflammatory activities of TAFI are not the dominant effects of TAFI for modulating the course of disease compared to its effect on fibrinolysis. Also, it is of interest to note that under these circumstances, there was no evidence of TAFI modulation of the bradykinin system.

The hallmark of bleomycin-induced lung fibrosis is the extensive deposition of collagen in the interstitial and alveolar spaces of the lung.²⁰ In this study, although all groups of bleomycin-treated mice developed lung fibrosis when compared to saline-treated mice, enhanced fibrosis was observed in bleomycin-treated TAFI^{+/+} and TAFI^{+/-} mice compared to TAFI^{-/-} mice. In addition, the lung content of collagen and hydroxyproline and the BALF level of soluble collagen were significantly increased in bleomycin-treated TAFI^{+/+} mice as compared to bleomycin-treated TAFI^{-/-} mice. Excessive collagen deposition in the lung may result from disruption of the balance between processes of synthesis and degradation that is regulated by a complex network of cytokines such as IL-13 and growth factors such as TGF- β 1, PDGF, and GM-CSF.³¹ PDGF favors lung fibrosis by promoting the proliferation of fibroblasts, and IL-13 and TGF- β 1 by stimulating the secretion of extracellular matrix proteins including collagens. TGF- β 1 may also promote collagen deposition in the lung by stimulating the secretion of tissue-type metalloproteinase inhibitors. Several inflammatory mediators including TNF- α and thrombin up-regulate the expression of growth factors.³¹ In our study, the BALF concentrations of TAT, a marker of thrombin generation, total protein, a marker of lung inflammation, and TNF- α were significantly lower in bleomycin-treated TAFI^{-/-} than in bleomycin-treated TAFI^{+/+} mice. Thus, the relative protection of TAFI-deficient mice from lung fibrosis may be due to an attenuated inflammatory response with subsequent reduced expression of profibrotic factors in the lung. In agreement with this theory, the BALF concentration of PDGF and TGF- β 1 was significantly decreased in bleomycin-treated TAFI^{-/-} mice as compared to that observed in their wild-type counterparts.

Another explanation for the protection from lung fibrosis of TAFI deficiency may be the high fibrinolytic activity

in bleomycin-treated TAFI^{-/-} mice. Although intra-alveolar fibrin is not required for lung fibrosis it can promote it by providing a provisional matrix onto which fibroblasts migrate and produce collagens.³⁴ The similarity of collagen deposition in the lung between different mouse genotypes after treatment with anacard supports the hypothesis that low fibrin is the protective mechanism of TAFI deficiency from lung fibrosis. In addition, TAFI deficiency may also protect from collagen deposition independently of fibrin by indirectly promoting plasmin-mediated activation of prometalloproteinases and secretion of hepatocyte growth factor. Metalloproteinases increase the degradation of extracellular matrix proteins and reduce fibrosis.³⁵ Hepatocyte growth factor exerts potent mitogenic and motogenic effect on lung epithelial cells; it may inhibit lung fibrosis by favoring the repair of epithelial cells during lung injury.³⁶ Increased plasmin generation has been associated with enhanced bioavailability of hepatocyte growth factor in the lung, probably as a result of its increased release from matrix-bound stores by the proteolytic action of plasmin.³⁶ Thus, TAFI deficiency-associated high plasmin generation may be an important mechanism for the decreased development of lung fibrosis in TAFI-deficient mice observed in our study. The fact that the D-dimer/TAT ratio in BALF was higher in the ko/blm group than in the wt/blm group suggests the occurrence of increased plasmin generation in mice with TAFI deficiency. The almost complete inhibition of collagen deposition in TAFI^{-/-} mice treated with BLM without anacard also suggests that TAFI deficiency may protect from lung fibrosis via a fibrin-independent mechanism.

In conclusion, the results reported in this study showed that TAFI deficiency is associated with an attenuated inflammatory response and collagen deposition in the lung, suggesting that the anti-fibrinolytic activity of TAFI is relevant and may play a role in the pathogenesis of lung fibrosis.

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