Thrombin-Activatable Fibrinolysis Inhibitor Protects against Acute Lung Injury by Inhibiting the Complement System

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Acute lung injury (ALI) is a devastating disease with an overall mortality rate of 30 to 40%. The coagulation/fibrinolysis system is implicated in the pathogenesis of ALI. Thrombin-activatable fibrinolysis inhibitor (TAFI) is an important component of the fibrinolysis system. Recent studies have shown that the active form of TAFI can also regulate inflammatory responses by its ability to inhibit complement C3a, C5a, and osteopontin. We hypothesized that TAFI might have a protective role in ALI. To demonstrate this hypothesis, the development of ALI was compared between wild-type (WT) and TAFI-deficient mice. ALI was induced by intratracheal instillation of LPS. Control mice were treated with saline. Animals were killed 24 hours after LPS. The number of inflammatory cells and the concentration of total protein and inflammatory cytokines were significantly increased in bronchoalveolar lavage fluid from LPS-treated, TAFI-deficient mice compared with their WT counterparts. Significantly higher concentrations of C5a were found in bronchoalveolar lavage fluid and plasma in LPS-treated TAFI knockout mice compared with WT mice. Pretreatment with inhaled C5a receptor antagonist blocked the detrimental effects of TAFI deficiency to levels found in WT mice. Our results show that TAFI protects against ALI, at least in part, by inhibiting the complement system.

Keywords: coagulation; lung injury; inflammation; fibrinolysis

Acute respiratory distress syndrome (ARDS) is a devastating disease with a mortality rate ranging between 24 and 48% depending on the severity of hypoxemia (1–3). The incidence of the disease shows geographical variations; there are 65 to 79 cases/100,000 persons annually in Northern Europe (3, 4). The lungs of patients with ARDS are pathologically characterized by an initial exudative phase with increased release of inflammatory cytokines, neutrophil infiltration, and vascular endothelial/alveolar epithelial injury, a proliferative phase with enhanced proliferation of alveolar epithelial cells and extracellular matrix deposition, and a terminal fibrotic phase with collagen deposition and lung functional failure (5, 6).

There is no curative therapy for patients with ARDS (3, 6). Several clinical trials with anti-inflammatory agents, including corticosteroids and pulmonary vasodilators, have failed to reduce mortality (3, 6). The present standard therapy includes mechanical ventilation with strategic adjustment to avoid ventilator-associated lung injury and general supportive care to attenuate multiorgan system failure (3). Thus, the development of novel drugs could improve the clinical outcome of the disease.

Thrombin-activatable fibrinolysis inhibitor (TAFI; procarboxypeptidase B2, procarboxypeptidase U) is a procarboxypeptidase produced by several cells, including hepatocytes, macrophages, and megakaryocytes. Its active form (TAFIa) induces the inactivation of peptides and proteins by removing the arginine and lysine residues from their carboxy-terminus (7, 8). Activation of TAFI can be induced by the thrombin–thrombomodulin complex, plasmin, or thrombin (8). TAFIa inhibits fibrinolysis by cleaving the lysine residues of degraded fibrin and thus abrogating tissue plasminogen activator–mediated activation of plasminogen to plasmin (9). In addition to its antifibrinolytic activity, TAFIa exerts its anti-inflammatory effects by inactivating complement factors C5a and C3a and bradykinin (8, 10). The proof-of-concept regarding the anti-inflammatory activity of TAFIa was given by several in vivo studies performed in animal models of human disease; TAFI deficiency exacerbated airway hyper-responsiveness and allergic bronchial inflammation in a mouse model of bronchial asthma and inflammatory arthritis in a model of autoimmune arthritis (11, 12).

However, the role of TAFIa in the pathogenesis of acute lung injury (ALI)/ARDS has not been investigated.
In the present study, we hypothesized that TAFI may play a role in the pathogenesis of ALI by inactivating complement factor C5a. To demonstrate this hypothesis, we compared the development of LPS-induced ALI in wild-type (WT) and TAFI knockout (KO) mice.

**MATERIALS AND METHODS**

LPS was purchased from Sigma-Aldrich (St. Louis, MO). Complement C5a receptor antagonist (C5aRA) was prepared by Biomatik Corporation (Cambridge, ON, Canada) (13, 14).

**Animals**

TAFI-deficient mice have been previously well characterized. The WT and TAFI-deficient mice used in the experiments were C57BL/6 (15). Male mice (8 wk old) weighing 19 to 22 g were used in the experiments; WT mice used as controls were purchased from Nihon SLC and maintained in the animal house of Mie University. All animals were kept in the standard mouse unit on a constant 12-hour light/12-hour dark cycle and in a temperature- and humidity-controlled room and were given ad libitum access to food and water. The Committee of Mie University for Animal Investigation approved the experimental research protocol.

**Ali in TAFI-Deficient Mice**

The animals were categorized into the following groups: WT/LPS (n = 5) and TAFI KO/LPS (n = 5) groups received intratracheal instillation of LPS (150 μg/mouse) dissolved in physiological saline (SAL) (75 μl) under anesthesia (62.5 mg/kg intraperitoneal sodium pentobarbital), and the WT/SAL (n = 5) and TAFI KO/SAL (n = 5) received intratracheal instillation of saline (75 μl) under anesthesia. These experiments were performed independently three times in a similar fashion, but representative results from one individual experiment are shown in the figures. Mice of each group were killed at 24 hours for sampling.

In addition to the above groups, in two separate experiments, a group of WT (WT/C5aRA/LPS [n = 4]) and a group of TAFI KO (TAFI KO/C5aRA/LPS [n = 3]) mice were pretreated with C5aRA (1,000 μg/mouse) by inhalation using a nebulizer (MIPS, Osaka, Japan) 24 and 2 hours before intratracheal instillation of LPS (150 μg/mouse) to evaluate the role of complement activation in TAFI KO-associated ALI. Mice receiving intratracheal saline alone (WT/SAL [n = 3], TAFI KO/SAL [n = 4 ~ 5]) or intratracheal saline 24 and 2 hours before intratracheal LPS instillation (WT/SAL/LPS [n = 4 or 5], TAFI KO/SAL/LPS [n = 6]) were used as controls. Mice in all the above groups were killed at 24 hours for sampling.

**Biochemical Analysis**

The total concentration of protein in BALF was measured using the dye-binding assay (Bio-Rad Laboratories, Hercules, CA). Concentrations of inflammatory cytokines, including IL-6, IL-1β, and TNF-α, were measured using ELISA kits from BD Biosciences Pharmingen (San Diego, CA) following the manufacturer’s instructions. Mouse-specific thrombin-antithrombin (TAT) complexes and D-dimers were measured using enzyme immunoassay kits from Cusabio Biotech (Wuhan, China) following the manufacturer’s instructions. The concentration of plasminogen activator inhibitor (PAI)-1 was measured by enzyme immunoassay using murine anti–PAI-1 (Molecular Innovations, Southfield, MI) antibody with biotin-labeled antiserum antibody.

**Statistical Analysis**

Data were expressed as mean ± SEM. The statistical difference between variables was calculated by ANOVA with post hoc analysis using Fisher’s predicted least significant difference test. The distribution of the data was calculated using the Kolmogorov-Smirnov test. Statistical analyses were performed using the StatView 4.5 package for Macintosh (Abacus Concepts, Berkeley, CA). A P value < 0.05 was considered to be significant.

**RESULTS**

**Dose-Dependent Effect of LPS Instillation in the Lung of WT Mice**

Previous reports of low-dose LPS instillation into the lungs of mice had failed to detect complement system activation (16, 17). We investigated if higher doses of LPS could trigger complement activation. Mice were instilled with saline (WT/SAL) or with 100 (WT/LPS 100 μg), 150 (WT/LPS 150 μg), or 200 μg (WT/LPS 200 μg) of LPS, and the number of total cells and neutrophils in BALF and the lung tissue concentrations of C5a and Factor D were evaluated. Six hours after LPS instillation, the total number of BALF cells in the WT/150 μg group and the neutrophil count in WT/150 μg and WT/200 μg groups were significantly increased compared with the WT/SAL group. Twenty-four hours after LPS instillation, the total number of cells in the WT/LPS 100 μg, WT/LPS 150 μg, and WT/LPS 200 μg groups and the neutrophil count in the WT/LPS 150 μg and WT/LPS 200 μg groups were significantly increased compared with the WT/SAL group (see Figures E1A–E1C in the online supplement).

The concentrations of C5a and Factor D were significantly increased in the WT/LPS 150 μg and WT/LPS 200 μg groups compared with the WT/SAL group 6 hours after LPS instillation (Figures E1D and E1E). Similarly, the concentrations of C5a and Factor D were significantly increased in all LPS-treated groups compared with the WT/SAL group 24 hours after LPS instillation (Figures E1F and E1G), showing that high-dose LPS could lead to complement activation. Based on these data, 150 μg/mouse of LPS was selected as the dose for the rest of the investigation.

**Lung Cell Infiltration by Neutrophils and TAFI Deficiency**

Because TAFIa has been shown to inactivate C5a, we investigated if mice that were deficient in C5a would have altered outcomes after instillation of LPS. The total number of cells and neutrophils counted after Giemsa staining of lung sections was increased after treatment with intratracheal LPS (WT/LPS and TAFI KO/LPS) compared with mice receiving intratracheal saline (WT/SAL and TAFI KO/SAL). TAFI was shown to play a role in this model because the number of total cells and neutrophil infiltrating the lung was higher in the TAFI KO/LPS group than in the WT/LPS group (Figures 1A and 1B). Histological evaluation of the number of infiltrating cells in the lungs disclosed similar results; inflammatory cells were increased in the mice treated with LPS compared with saline-treated mice, but they were remarkably increased in the TAFI KO/LPS group compared with the WT/LPS group (Figures E2A and E2B).

**Lung Inflammation and Coagulation System**

To confirm these data, an alternate general marker of lung inflammation—the amount of protein leakage into the alveolar space—was measured. As expected, the concentration of total protein in BALF was significantly increased in the WT/LPS and TAFI KO/LPS groups compared with the WT/SAL and TAFI KO/SAL groups, but it was remarkably increased in the TAFI KO/LPS group compared with the WT/LPS group (Figure 2A).

TAT was measured as a marker of coagulation system activation. The BALF concentrations of TAT complexes were significantly increased in the WT/LPS group, but not in the TAFI KO/LPS group, compared with the saline control group (Figure 2B). The concentration of TAT in lung tissue was significantly increased in mice treated with LPS (WT/LPS and TAFI KO/LPS) compared with those treated with saline (WT/SAL and TAFI KO/SAL), but it was not significantly different between the TAFI KO/LPS and WT/LPS groups (Figure 2C). The level of D-dimer, a marker of fibrinolysis activity, was lower in the WT/LPS group compared with the WT/SAL group but did not reach significance; there was no significant difference in the level of D-dimer between the TAFI KO/LPS and TAFI KO/SAL groups, but a significant difference was found between the TAFI KO/LPS and WT/LPS groups (Figure 2D).
PAI-1 is an inhibitor of plasminogen activators that has a different mechanism than TAFI for inhibition of fibrinolysis and is frequently induced in inflammatory situations (18). The concentration of PAI-1 in plasma, BALF, and lung tissue homogenates was significantly increased in the TAFI KO/LPS group compared with the WT/LPS groups, but both groups had raised levels compared with the saline groups (Figure E3).

**Inflammatory Cytokines**

IL-1β, TNF-α, and IL-6 play key roles in the pathogenesis of lung injury (6). The concentrations of IL-6 and TNF-α were significantly elevated in BALF from mice in the WT/LPS and TAFI KO/LPS groups compared with the saline-treated groups. The concentration of IL-6 in BALF was significantly elevated in the TAFI KO/LPS group compared with the WT/LPS group. All of these inflammatory cytokines were significantly increased in the TAFI KO/LPS group compared with the WT/LPS group in BALF (Figure 3). The plasma IL-6 levels were not elevated in the WT/LPS mice, but they were significantly increased in the TAFI KO/LPS groups compared with the WT/LPS, WT/SAL, and TAFI KO/SAL groups (Figure 3), whereas there was no detectable change in the levels of IL-1β and TNF-α in plasma (data not shown).

**Complement System**

Components of the complement system have been reported to play an important role in the pathogenesis of ARDS (19). To confirm this in this model, we measured the concentration of the anaphylatoxin C5a in the different groups of mice in BALF and plasma. The results showed increased concentrations of C5a in BALF and plasma from WT/LPS and TAFI KO/LPS mice compared with saline-treated mice with much more significantly increased levels in the TAFI KO/LPS group than in the WT/LPS group (Figure 4).

**Pathogenic Role of the Complement System**

Because TAFI KO mice have exacerbated disease that suggests that TAFI or TAFIa is protective, but there are several possible substrates. To clarify if the protective role of TAFI in the mechanism of ALI is via inactivation of C5a rather than an alternative TAFIa substrate, we pretreated the mice with an antagonist of the C5a receptor two times by inhalation before inducing lung injury by LPS instillation. Before performing this experiment, the C5a receptor antagonist was confirmed to bind to C5a receptor on lung cells by in vitro (Figures E4A and EB) and in vivo (Figure E4C–E4E) experiments. The group of TAFI KO mice pretreated with the C5a receptor antagonist (TAFI KO/C5aRA/LPS) before LPS intratracheal instillation showed significantly decreased infiltration of inflammatory cells in the lungs (Figure 5); decreased lung mRNA expression of the inflammatory cytokines IL-6, monocyte chemotactic protein (MCP)-1, and IL-1β (Figures 6A and 6B); and decreased lung tissue concentration of C5a peptides in the TAFI KO/C5aRA/LPS mice compared with the untreated TAFI KO/LPS group.
KO/LPS group (Figure 6C). However, there was no significant difference in the number of lung infiltrating cells (Figure 5), in lung RNA expression of MCP-1 and IL-1β, or in the lung tissue level of C5a (Figures 6A–6C) between WT mice pretreated with C5a receptor antagonist (WT/LPS/C5aRA) and those pretreated with saline (WT/LPS/SAL) before LPS instillation. To clarify this lack of response in the WT mice, we performed another experiment in which WT mice were pretreated four times by inhalation with the C5a receptor antagonist. WT mice receiving more frequent inhalations of the C5a receptor antagonist had decreased lung cell infiltration and reduced concentrations of total protein in BALF and reduced levels of C5a, TAT, and PAI-1 in lung tissue homogenates (Figures E5A–E5E). The BALF concentrations and the lung mRNA expression level of TNF-α, MCP-1, and IL-1β were also significantly reduced in the WT/LPS/C5aRA group compared with the WT/LPS/SAL group (Figures 7A and 7B).

Figure 2. Protein leakage and coagulation system. Samples were harvested 24 hours after LPS instillation. (A) The concentration of total protein was significantly increased in the TAFI KO/LPS group compared with the other groups. The concentrations of thrombin–antithrombin (TAT) complex in BALF (B) and lung tissue (C) were not significantly different between the WT/LPS and TAFI KO/LPS groups, but in BALF and lung tissue there was a significant increase in both groups of LPS-treated mice (WT/LPS and TAFI KO/LPS) compared with their respective saline controls (WT/SAL and TAFI KO/SAL). (D) BALF D-dimer was significantly different between the WT/LPS and TAFI KO/LPS groups; it was decreased in WT/LPS compared with control mice but not at significant level (n = 5 mice in each group). The figure shows representative results from one of three independent experiments. Bars indicate means ± SEM. Statistical analysis by ANOVA with post hoc analysis using Fisher’s predicted least significant difference test. *P < 0.05 versus the respective saline groups; ¤P < 0.05 versus WT/LPS.

Figure 3. Inflammatory cytokines. Samples were harvested 24 hours after LPS instillation. The BALF and plasma concentration of IL-6 and the BALF concentration of TNF-α and IL-1β were significantly elevated in the TAFI KO/LPS groups compared with all other groups (n = 5 mice in each group). The figure shows representative results from one of three independent experiments. Bars indicate means ± SEM. Statistical analysis by ANOVA with post hoc analysis using Fisher’s predicted least significant difference test. *P < 0.05 versus the respective saline groups; ¤P < 0.05 versus WT/LPS.
DISCUSSION

The results of this study show that the TAFI KO animals have worse disease than WT and therefore TAFI plays a protective role in the mechanism of LPS-induced ALI. Because the difference between the TAFI KO mice and WT is abrogated by a pharmacological intervention preventing C5a signaling through its cognate receptor, it is likely that the protective effect of TAFI is due to inactivation of the complement anaphalotoxins C3a and C5a.

The Complement System and Lung Injury

The complement system plays a critical role in innate host defense (5, 6). Components of the complement system can directly lyse bacteria, enhance bacterial phagocytosis by opsonization, stimulate the recruitment of immune cells, and increase vascular leakage of inflammatory mediators from the bloodstream (5, 6). Excessive activation of the complement system may contribute to the pathogenesis of ALI/ARDS (19–23). Previous studies have shown that increased levels of complement proteins in the lung of patients with ARDS were associated with worse clinical outcomes (20, 22, 24). Enhanced concentration of complement factors has been also reported in ALI induced in mice by intratracheal instillation of LPS, and these observations are consistent with the increased lung levels of C5a in our LPS-treated mice (25, 26). In addition, we demonstrated that the lung level of C5a is dose dependent, being insignificantly increased when the intratracheal dose of LPS is 100 μg but significantly high at doses of 150 or 200 μg of intratracheal LPS compared with control mice. The use of relatively low doses of LPS (50 or 100 μg) for inducing ALI may explain why previous studies found no increase in the levels of complement factors in the lungs of LPS-treated mice (16, 17). We have also shown in this study that pretreatment with inhaled C5a receptor antagonist ameliorates LPS-induced ALI in WT mice, further supporting the role of the complement system in the pathogenesis of the disease.

In addition to the complement system, other mechanisms are involved in LPS-induced ALI. For example, LPS can enhance the inflammatory response in ALI by increasing the surface expression of tissue factor, the initiator of coagulation, leading to enhanced generation of thrombin, which binds to its receptor (protease-activated receptor [PAR]-1, -3, and 4) and enhances the secretion of proinflammatory cytokines and the recruitment of leukocytes (6). LPS can also bind to Toll-like receptor 4, with a consequent inflammatory response.

Figure 4. The complement system. Samples were harvested 24 hours after LPS instillation. The BALF and plasma levels of C5a were significantly increased in the WT/LPS and TAFI KO/LPS mice compared with all saline-treated groups. The concentrations of C5a in BALF and plasma were markedly elevated in the TAFI KO/LPS groups compared with the WT/LPS group (n = 5 mice in each group). The figure shows representative results from one of three independent experiments. Bars indicate means ± SEM. Statistical analysis by ANOVA with post hoc analysis using Fisher’s predicted least significant difference test. *P < 0.05 versus the respective saline groups; $P < 0.05 versus WT/LPS.

Figure 5. C5a receptor antagonist inhibits lung cell infiltration in TAFI KO mice with acute lung injury. WT and TAFI KO mice were pretreated with complement C5a receptor antagonist (C5aRA) (WT/C5aRA/LPS [n = 4]; TAFI KO/C5aRA/LPS [n = 5]) or saline (WT/SAL/LPS [n = 4]; TAFI KO/SAL/LPS [n = 6]) by inhalation using a nebulizer 24 hours and 2 hours before intratracheal instillation of LPS. WT/SAL (n = 3) and TAFI KO/SAL (n = 5) groups received saline instillation instead of LPS. Samples were harvested 24 hours after LPS instillation. Lung tissues were stained by H&E, and the number of cell nuclei per area of lung field was determined as described in Materials and Methods. (A and B) Cell infiltration was significantly decreased in mice (TAFI KO/C5aRA/LPS) treated with the C5a receptor antagonist compared with untreated TAFI KO mice with acute lung injury (TAFI KO/SAL/LPS). The figure shows representative results from one of two independent experiments. Bars indicate means ± SEM. Scale bars indicate 50 μm. Statistical analysis by ANOVA with post hoc analysis using Fisher’s predicted least significant difference test. *P < 0.05 versus TAFI KO/SAL/LPS.
activating intracellular pathways that increase the release of proinflammatory factors and chemotaxis of inflammatory cells into the lungs (6). In the present study, the inflammatory response and the level of C5a in the lung tissue were significantly reduced in TAFI-deficient mice with lung injury pretreated with C5aRA, supporting the critical role of TAFI-mediated regulation of CR5a in ALI. However, the fact that lung injury was not completely inhibited by the C5aRA suggests that, in addition to C5a, other mechanistic pathways are involved in the pathogenesis of LPS-induced ALI.

The Inflammatory Response and TAFI

The inflammatory response in ALI may be triggered by an ongoing lung tissue insult or by a systemic infectious process leading to increased lung and plasma concentration of cytokines, enhanced endothelial/epithelial barrier permeability with leakage of fluids, and plasma proteins into interstitial and alveolar spaces and subsequent recruitment of leukocytes, particularly of neutrophils, into the lungs (5, 6). IL-1β and TNF-α are proinflammatory cytokines secreted by alveolar macrophages during the early stages of the disease and thus are believed to be responsible for the initial pathophysiological changes in the lungs (6). Both of these proinflammatory cytokines can stimulate the secretion of chemokines, including MCP-1, macrophage inflammatory protein-1α, and IL-8, and thus they can further promote the accumulation of monocytes, lymphocytes, and neutrophils at sites of lung injury (6). High concentrations of proinflammatory cytokines in the lungs have been associated with increased mortality in patients with ARDS (5, 27, 28). Consistent with these observations, in the present study we observed significantly higher concentrations of IL-1β, TNF-β, and IL-6, along with increased lung infiltration of neutrophils in WT mice treated with LPS compared with control mice. These inflammatory changes were significantly much worse in LPS-treated, TAFI-deficient mice than in their WT counterparts, suggesting a protective role of TAFI in ALI.

The Coagulation/Fibrinolysis Cascade and TAFI

Imbalance between the coagulation and fibrinolysis systems occurs in patients with ALI (5). There is an increased activation of the coagulation cascade with accelerated thrombin generation and impairment of fibrinolysis (5). This imbalance results in excessive cleavage of fibrinogen by thrombin and increased deposition of fibrin, which may further promote inflammation in the injured lungs (5). In our present study, we found a low level of D-dimer in WT mice treated with LPS compared with control mice, confirming impaired fibrinolytic activity in this lung injury. The high level of PAI-1 may explain the impairment of fibrinolysis in the Wt mice with LPS-induced lung injury (5, 29). Interestingly, despite the higher level of PAI-1 in the TAFI KO/LPS group than in the WT/LPS group, the BALF level of D-dimer remained the same as in control mice. This finding may...
be explained by the lack of TAFI, which is also an inhibitor of plasmin generation (8). It could be that the TAFI-deficient mice have a lower lung fibrin burden, muting the detection of increased fibrinolysis anticipated in these animals.

The Complement System and TAFI

Experimental in vivo models of allergic bronchial asthma and autoimmune arthritis have demonstrated that TAFIa exerts its anti-inflammatory effect by inactivating C5a (11, 12), whereas in some other models its effects are via inhibition of fibrinolysis (8). Previously it had been shown that instillation of C5a directly into the lungs of TAFI KO mice caused worse alveolitis than in WT mice (30). In this study, we hypothesized that the stronger inflammatory response in LPS-treated, TAFI-deficient mice than in their WT counterparts is due to increased C5a activity generated endogenously. To demonstrate this thesis, we pretreated mice with C5a receptor antagonist before LPS intratracheal instillation and compared the results with untreated mice. The results showed a reduction in the expression of inflammatory cytokines and in the level of C5a in the lungs of LPS-treated, TAFI-deficient mice treated with inhaled C5aRA compared with untreated mice. Unfortunately, the available ELISAs for C5a measure total C5a without distinguishing between active C5a and inactive C5adesArg produced by TAFIa cleavage of C5a. We speculate that the reduction in C5a level by inhaled C5aRA therapy in TAFI-deficient mice is due to mitigation of the vicious cycle of C5a-mediated cell receptor activation, inflammatory response, and complement activation with C5a generation. Taken together these data suggest that the anti-inflammatory activity of TAFI is mediated by inactivation of C5a.

Conclusions

The results of the present investigation showed that TAFI is protective in ALI, suggesting that TAFI plays a critical role in the pathogenesis of the disease via its regulation of the activity of complement C5a.

Author disclosures are available with the text of this article at www.atsjournals.org.

References
