MATRIX PATHOBIOLOGY

Inhibition of Cell Apoptosis and Amelioration of Pulmonary Fibrosis by Thrombomodulin

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Pulmonary fibrosis is the terminal stage of a group of idiopathic interstitial pneumonias, of which idiopathic pulmonary fibrosis is the most frequent and fatal form. Recent studies have shown that recombinant human thrombomodulin (rhTM) improves exacerbation and clinical outcome of idiopathic pulmonary fibrosis, but the mechanism remains unknown. This study evaluated the mechanistic pathways of the inhibitory activity of rhTM in pulmonary fibrosis. Transgenic mice overexpressing human transforming growth factor- β 1 that develop spontaneously pulmonary fibrosis, and wild-type mice treated with bleomycin were used as models of lung fibrosis. rhTM was administered to mice by i.p. injection or by the intranasal route. Therapy with rhTM significantly decreased the concentration of high mobility group box1, interferon- γ , and fibrinolytic markers, the expression of growth factors including transforming growth factor- β 1, and the degree of lung fibrosis. rhTM significantly suppressed apoptosis of lung epithelial cells *in vivo* and *in vitro* experiments. The results of the present study demonstrated that rhTM can inhibit bleomycin-induced pulmonary fibrosis and transforming growth factor- β 1-driven exacerbation and progression of pulmonary fibrosis, and that apart from its well-recognized anticoagulant and anti-inflammatory properties, rhTM can also suppress apoptosis of lung epithelial cells. (*Am J Pathol* 2017, 187: 2312–2322; <http://dx.doi.org/10.1016/j.ajpath.2017.06.013>)

Pulmonary fibrosis is the end stage of chronic interstitial pneumonitis characterized by abnormal accumulation and activation of myofibroblasts leading to excessive production and secretion of extracellular matrix proteins and by decreased degradation of fibrous components of the lung connective tissue.¹ There are several factors that may cause interstitial pneumonitis, including infectious agents, environmental pollutants, pharmaceutical products, and tumors, but the cause of idiopathic interstitial pneumonitis is still unknown.² Some forms of idiopathic interstitial pneumonitis evolve to idiopathic pulmonary fibrosis (IPF), which is the most frequent and fatal form of the disease.³ Chronic and repetitive injury of alveolar epithelial cells associated with increased lung concentrations of growth factors and profibrotic cytokines, including transforming growth factor- β 1 (TGF- β 1), connective tissue growth factor (CTGF), tumor necrosis factor- α (TNF- α), and platelet-derived

growth factor, with increased epithelial cell apoptosis and concomitant enhanced differentiation of epithelial cells to myofibroblasts are implicated in the progression of IPF.⁴ The median survival of patients with IPF is only 2 to 3 years after diagnosis.³ Pirfenidone and nintedanib are antifibrotic drugs presently used to treat IPF patients; although these antifibrotic agents can delay the decline in lung function, they have no beneficial effects on survival.^{5,6} Therefore, novel pharmaceutical agents are currently under intense experimental scrutiny.

Thrombomodulin (TM) is a transmembrane glycoprotein that structurally has a lectin-like domain, six epidermal

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growth factor—like domains, a serine/threonine-rich domain, a transmembrane portion, and a cytoplasmic tail.⁷ TM plays an essential role in the regulation of blood coagulation, fibrinolysis, and hemostasis.⁸ TM binds to the procoagulant protease thrombin formed during activation of the coagulation cascade, converting it to an anticoagulant and antifibrinolytic factor.⁹ Thrombin bound to TM can cleave protein C to activated protein C, an anticoagulant and anti-inflammatory protein and thrombin-activatable fibrinolysis inhibitor (TAFI) to activated TAFI, an antifibrinolytic and anti-inflammatory factor.^{10,11} Recent studies have demonstrated that TM can also suppress the inflammatory response by promoting the thrombin-mediated degradation of high mobility group protein B-1 (HMGB-1), by modulating the proinflammatory activity of dendritic cells and by regulating the complement system.^{7,12} The TM lectin domain is capable of suppressing the activation of the classical, lectin, and alternative pathways of the complement system.^{7,13,14} Recombinant human soluble (rh) TM (rhTM) is being used in clinical practice for the treatment of patients with disseminated intravascular coagulation.¹⁵

Recent studies have shown that rhTM improves exacerbation and survival in patients with idiopathic pulmonary fibrosis, but the mechanistic pathways are unknown.^{16–18} To clarify the mechanism in the present study, we evaluated the therapeutic efficacy of rhTM in wild-type mice with bleomycin-induced lung fibrosis and in TGF- β 1 transgenic (TG) mice with spontaneous lung fibrosis.

Materials and Methods

Animals

C57BL/6 wild-type (WT) mice used for induction of lung fibrosis with bleomycin (BLM) were purchased from Nihon SLC (Hamamatsu, Japan). Human TGF- β 1 TG (TGF β 1-TG) mice backcrossed to C57BL/6J mice for more than 10 generations were previously characterized.¹⁹ Nine-week-old female WT mice weighing 19 to 21 g were used for induction of lung fibrosis with BLM. Ten-week-old male TGF β 1-TG mice weighing 20 to 25 g were used to assess the efficacy of rhTM on TGF- β 1-induced lung fibrosis. All mice were maintained in a specific pathogen-free environment under a 12-hour light/dark cycle in the animal house of Mie University. The Committee for Animal Investigation of Mie University approved the experimental protocols (Approval No. 24-50), and all procedures were performed in accordance with approved institutional guidelines.

Blood and Lung Tissue Concentrations after rhTM Administration

C57BL/6 WT mice ($n = 4$) received 3 mg/kg intravenous rhTM injection and blood and tissue lung samples were collected after several hours for measurement of human TM levels.

Treatment Allocation

BLM (Nihon Kayaku, Tokyo, Japan) was used to induce lung fibrosis in WT mice. BLM was dissolved in saline and infused at a dose of 100 mg/kg through subcutaneously implanted osmotic minipumps (Alza, Palo Alto, CA) as previously described.²⁰ rhTM (ART-123) was provided by Asahi Kasei Pharma (Tokyo, Japan). Mice receiving infusions of saline through subcutaneous osmotic minipumps were used as controls. The therapeutic efficacy of intranasal or i.p. rhTM on BLM-induced lung fibrosis was evaluated. rhTM (3 mg/kg mouse weight) or saline was administered to mice by the i.p. route from day 11 to day 21 after BLM infusion. Mice were allocated to three treatment groups: WT mice receiving saline through osmotic minipumps and by the i.p. route (SAL/ip-SAL), and mice receiving BLM through minipumps and saline (BLM/ip-SAL) or rhTM (BLM/ip-rhTM) by the i.p. route. In a separate experiment, rhTM (1 mg/kg mouse weight) or saline was administered by the intranasal route on days 2, 4, 7, 9, and 18 after BLM infusion. Mice were allocated to four treatment groups: WT mice receiving saline through minipumps and treated with saline (SAL/in-SAL) or rhTM (SAL/in-rhTM) by the intranasal route, and mice receiving BLM through minipumps and treated with saline (BLM/in-SAL) or rhTM (BLM/in-rhTM) by the intranasal route.

The TGF β 1-TG mice were used to evaluate the efficacy of rhTM on acute exacerbation and on spontaneous progression of lung fibrosis. There were four treatment groups in the acute exacerbation experimental model: i) a group of WT mice (WT/ip-SAL) and ii) a group of TGF β 1-TG mice (TGF β 1-TG/ip-SAL) receiving i.p. injection of saline during 4 consecutive days and intratracheal instillation of saline on day 4, 6 hours after the last i.p. injection of saline, iii) a group of TGF β 1-TG mice (TGF β 1-TG/ip-SAL/it-LPS) receiving i.p. injection of saline during 4 consecutive days and 100 μ g of intratracheal lipopolysaccharide (LPS; 100 μ g per mouse) on day 4, 6 hours after the last i.p. injection of saline, and iv) a group of TGF β 1-TG mice (TGF β 1-TG/ip-rhTM/it-LPS) receiving i.p. injection of rhTM (3 mg/kg mouse weight) during 4 consecutive days and intratracheal LPS on day 4, 6 hours after the last i.p. injection of rhTM.

There were three treatment groups in the spontaneous disease progression experimental model: i) a group of WT mice (WT/ip-SAL) and ii) a group of TGF β 1-TG mice (TGF β 1-TG/ip-SAL) receiving daily i.p. injections of saline for 21 consecutive days, and iii) a group of TGF β 1-TG mice (TGF β 1-TG/ip-rhTM) receiving daily i.p. injections of rhTM (3 mg/kg mouse weight) for 21 consecutive days.

Sample Collection and Biochemical Analysis

Mice were euthanized on day 22 by pentobarbital overdose. Blood was sampled by cardiac puncture and placed into tubes containing heparin; plasma was collected after centrifugation and stored at -80°C until analysis.

Bronchoalveolar lavage fluid (BALF) was sampled under deep anesthesia as previously described.²⁰ Tissue homogenates were prepared by homogenizing the lung in a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) with a Tomy Micro Smash MS-100R (Tomy Digital Biology, Tokyo, Japan) as previously described.²⁰

For measurement of total protein, a commercial dye-binding assay (Bio-Rad Laboratories, Hercules, CA) was used. TNF- α , IL-6, and TGF- β 1 were measured with enzyme immunoassay kits from BD Biosciences Pharmingen (San Diego, CA) and IL-13 and interferon- γ (IFN- γ) using commercial immunoassay kits from R&D Systems (Minneapolis, MN). Thrombin–antithrombin complex (TAT; Affinity Biologicals, ON, Canada) and D-dimer (Uscn Life Science Inc., Wuhan, China) were measured using commercial immunoassays kits following the instructions of the manufacturers. HMGB-1 (SHINO-TEST, Tokyo, Japan) and soluble TM (R&D Systems) were measured by enzyme immunoassays using commercial kits.

The total number of cells in BALF was measured using a nucleocounter from ChemoMetec (Allerød, Denmark), and for differential cell counting, the BALF samples were centrifuged with a cytospin, and cells were stained with May–Grunwald–Giemsa (Merck, Darmstadt, Germany). Hydroxyproline content was measured using a colorimetric assay kit (Biovision Inc., Mountain View, CA).

Histologic Study

Mice were sacrificed and lung samples were collected on day 22 after starting the experiment. The resected lungs were fixed in formalin, embedded in paraffin, and then prepared for hematoxylin and eosin staining. The severity of lung fibrosis was quantitated based on the Ashcroft criteria as previously described²⁰; for this purpose, photographs of lung fields were taken at random using an Olympus BX50 microscope combined with an Olympus DP70 digital camera (Olympus, Tokyo, Japan) and scored blindly by four independent observers.

Table 1 Primers for RT-PCR

cDNA	Sequence	Accession no.*	Product size
GAPDH			
Sense	5'-TGGCCTTCCGTGTTCTAC-3'	NM_008084	178 bp
Antisense	5'-GAGTTGCTGTTGAAGTCGCA-3'		
mTGF- β 1			
Sense	5'-CTCCCGTGGCTTCTAGTGC-3'	NM_011577	133 bp
Antisense	5'-GCCTTAGTTTGGACAGGATCTG-3'		
hTGF- β 1			
Sense	5'-CTAATGGTGGAAACCCACAACG-3'	NM_000660	209 bp
Antisense	5'-TATCGCCAGGAATTGTTGCTG-3'		
Col1a1			
Sense	5'-TAAGGGTCCCAATGGTGAGA-3'	NM_007742	203 bp
Antisense	5'-GGGTCCCTCGACTCCTACAT-3'		
HMGB-1			
Sense	5'-GGCGAGCATCTGGCTTATC-3'	NM_010439	86 bp
Antisense	5'-GGCTGCTTGTCATCTGCTG-3'		
IFN- γ			
Sense	5'-GCTCTGACACAATGAACGCT-3'	NM_008337	227 bp
Antisense	5'-AAAGAGATAATCTGGCTCTGC-3'		
PDGF- α			
Sense	5'-CCATTGCGCAGGAAGAGAAGTA-3'	NM_008808	434 bp
Antisense	5'-GGCAATGAAGCACCATACATA-3'		
IL-6			
Sense	5'-CTGGTCTTCTGGAGTACCATAG-3'	NM_031168	374 bp
Antisense	5'-AAGTCAGATACCTGACAACAGG-3'		
IL-13			
Sense	5'-GACCCAGAGGATATTGCATG-3'	NM_008355	214 bp
Antisense	5'-CCAGCAAAGTCTGATGTGAG-3'		
TNF- α			
Sense	5'-ACGTGGAACGGCAGAAGAG-3'	NM_013693	284 bp
Antisense	5'-CTCCTCCACTTGGTGGTTTG-3'		
CTGF			
Sense	5'-GAGGAAAACATTAAGAAGGGCAA-3'	NM_010217	362 bp
Antisense	5'-ACTCAGTTCAAGTTATAGTCTAA-3'		

*Accession numbers available through the NCBI-Nucleotide database: <https://www.ncbi.nlm.nih.gov/nucleotide>.

CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGB-1, high mobility group box protein 1; hTGF- β 1, human transforming growth factor- β 1; mTGF- β 1, mouse transforming growth factor- β 1; PDGF- α , platelet-derived growth factor- α ; TNF- α , tumor necrosis factor- α .

Figure 1 I.P. administration of recombinant human thrombomodulin (rhTM) reduces lung fibrosis. Mice were infused bleomycin (BLM) or saline (SAL), treated with i.p. rhTM from day 11 to day 21 and sacrificed on day 22. **A** and **B**: Lung histological sections were stained with hematoxylin and eosin (**A**) or Masson's trichrome (**B**). The grade of fibrosis was quantified by Ashcroft score (**A**) or using an image analyzer (**B**). **C**: Hydroxyproline was measured by a colorimetric assay, transforming growth factor-β1 (TGF-β1) by immunoassay, and TGF-β1 mRNA expression by RT-PCR. SAL/ip-SAL and BLM/ip-SAL groups were treated with i.p. saline. BLM/ip-rhTM group was treated with i.p. rhTM. Data are expressed as means ± SEM. $n = 7$ SAL/ip-SAL mice; $n = 11$ BLM/ip-SAL mice; $n = 8$ BLM/ip-rhTM group mice. * $P < 0.05$ versus SAL/ip-SAL; † $P < 0.05$ versus BLM/ip-SAL. Scale bars = 100 μm.

(K.F., T.K., H.F., T.Y.). The fibrotic scores of all lung fields given by each observer were averaged, and the fibrotic score of an individual mouse was then calculated. Lung histological samples were also stained with Masson trichrome, and the stained fibrotic area was quantitated using ImageJ software version 1.48 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Computed Tomography

Lung fibrotic findings were also assessed using a micro-computed tomography scanner (Latheta LCT-200, Hitachi Aloka Medical, Tokyo, Japan). For computed tomography scanning, the mice were placed in a prone position for data acquisition under isoflurane inhalation anesthesia as

previously described.¹⁹ Computed tomography findings from each mouse were scored by six specialists (K.F., T.K., H.F., H.N., Y.T., T.Y.) in respiratory diseases blinded to the treatment groups, according to the following criteria: score 1, normal lung findings; 2, intermediate findings; 3, slight lung fibrosis; 4, intermediate findings; 5, moderate lung fibrosis; 6, intermediate findings; and 7, advanced lung fibrosis. The fibrotic scores of all specialists were averaged, and the fibrotic score of an individual mouse was then calculated.

Apoptosis Evaluation

Cell apoptosis in lung histological samples was assayed by the terminal deoxynucleotidyl transferase-mediated dUTP nick

Figure 2 Intranasal administration of recombinant human thrombomodulin (rhTM) reduces inflammatory response and fibrosis in the lungs. Mice were infused bleomycin (BLM) or saline (SAL), treated with intranasal (in) rhTM on days 2, 4, 7, 9, and 18, and sacrificed on day 22. **A:** The number of cells in bronchoalveolar lavage fluid (BALF) was measured using a nucleocounter. **B:** The grade of fibrosis was quantified by Ashcroft score. SAL/in-SAL and SAL/in-rhTM groups were treated with intranasal saline; BLM/in-SAL and BLM/in-rhTM groups were treated with intranasal rhTM. Data are expressed as means $\pm$ SEM. $n = 4$ SAL/in-SAL, SAL/in-rhTM, and BLM/in-rhTM mice; $n = 3$ BLM/in-SAL mice. * $P < 0.05$ versus SAL/in-SAL; † $P < 0.05$ versus BLM/in-SAL. Scale bars: 20 μ m (A); 50 μ m (B).

end-labeling method, using a commercially available kit (Chemicon International, Temecula, CA). The number of deoxynucleotidyl transferase-mediated dUTP nick end-labeling—positive cells within the lungs was counted in at least 10 fields per mouse by an investigator (T.Y.) in a blinded fashion.

Cell Culture and *in Vitro* Study

Human lung adenocarcinoma cell line A549 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and sodium pyruvate, and in an atmosphere containing 5% CO₂ at 37°C. Subconfluent cells were treated with 50 μ g/mL BLM in the presence of rhTM at concentrations of 0 to 200 nmol/L. Flow cytometry was used to assess the effect of rhTM on apoptosis after staining the cells with annexin V and propidium iodide. In brief, the cells were suspended in annexin V-binding buffer at 0.5×10^6 cells/mL, and 100 μ L of the cell suspension were incubated with 5 μ L of annexin V-fluorescein isothiocyanate (1 μ g/mL), and 10 μ L of propidium iodide (2.5 μ g/mL) for 15 minutes in the dark. Analysis was performed with a FACScan

(Becton Dickinson, San Jose, CA) and CellQuest Pro software version 5.2.1 (Becton Dickinson) after adding 400 μ L of binding buffer to each sample. Cells positive for annexin V were defined as apoptotic.

Gene Expression

Total RNA was extracted from cells or lung tissue using Sepasol RNA-I Super G reagent (Nacalai Tesque Inc., Kyoto, Japan) following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μ g of total RNA with oligo-dT primer and ReverTra Ace Reverse Transcriptase (Toyobo Life Science Department, Osaka, Japan). PCR was performed with gene-specific primers (Table 1), and the expression of each gene was normalized by the transcription level of glyceraldehyde-3-phosphate dehydrogenase.

Statistics

Data are expressed as means $\pm$ SEM. The statistical difference between treatment groups was calculated by

Figure 3 I.P. administration of recombinant human thrombomodulin (rhTM) decreases inflammation, coagulation activation in TGF- β 1 with lung fibrosis and acute exacerbation. TGF β 1 mice with matched lung fibrosis based on computed tomography findings were used. Mice were treated with rhTM or saline by the i.p. route for 4 days before induction of acute exacerbation with intratracheal LPS, and sacrificed after 24 hours. All parameters were measured by immunoassays. WT/ip-SAL, WT mice treated with i.p. saline; TGF β 1-TG/ip-SAL TG mice, with i.p. saline; TGF β 1-TG/ip-SAL/it-LPS TG mice, with i.p. saline and intratracheal lipopolysaccharide (LPS); TGF β 1-TG/ip-rhTM/it-LPS TG mice, with i.p. rhTM and intratracheal LPS. Data are expressed as means $\pm$ SEM. $n = 5$ WT/ip-SAL mice; $n = 6$ TGF β 1-TG/ip-SAL mice; $n = 10$ TGF β 1-TG/ip-SAL/it-LPS mice; $n = 9$ TGF β 1-TG/ip-rhTM/it-LPS mice. * $P < 0.05$ versus TGF β 1-TG/ip-SAL; † $P < 0.05$ versus TGF β 1-TG/ip-SAL/it-LPS. BALF, bronchoalveolar lavage fluid; TG, transgenic; WT, wild-type.

analysis of variance with *post hoc* analysis using the Fisher predicted least significant difference test. The StatView package version 4.5 for Macintosh (Abacus Concepts, Berkeley, CA) was used for statistical analyses.

Results

Blood and Lung Tissue Concentrations of Human TM after Intravenous Administration of rhTM

Mice received intravenous injection of rhTM and the concentration of TM was measured by immunoassays. Compared to the concentration 1 hour after rhTM injection, the blood and lung tissue concentration of human TM decreased to about half after 24 hours of injection (Supplemental Figure S1).

I.P. Administration of rhTM Suppresses BLM-Induced Lung Inflammation and Fibrosis

Pulmonary fibrosis was induced in C57BL/6 WT mice by BLM infusion, and rhTM was administrated daily by i.p. injection from day 11 to 21 after BLM. Radiological study showed significantly decreased fibrotic changes in the lungs from mice treated with rhTM compared to mice treated with saline alone (Supplemental Figure S2). General examination disclosed no signs of bleeding in peripheral tissues

(eg, skin) or in the surface of resected lungs of any mouse treated or nontreated with rhTM. As expected, high concentrations of TM were detected in BALF and lung tissue from mice treated with rhTM (Supplemental Figure S3A). Analysis of inflammatory cells in BALF disclosed significantly decreased number of total cells and lymphocytes in mice treated with rhTM compared with control mice (Supplemental Figure S3B). The BALF and plasma concentrations of D-dimer and the lung tissue mRNA level of IFN- γ were significantly decreased in mice with lung fibrosis treated with rhTM compared to mice counterparts treated with saline (Supplemental Figure S3C). The lung fibrosis score and the degree of collagen deposition were significantly lower in mice treated with rhTM than in untreated control mice (Figure 1, A and B). The lung hydroxyproline content, the protein concentration, and mRNA expression of TGF- β 1 in the lung were significantly decreased in the BLM/ip-rhTM group compared to the BLM/ip-SAL group (Figure 1C).

Intranasal Administration of rhTM Suppresses BLM-Induced Lung Inflammation and Fibrosis

Because of the easy accessibility of the lungs by airway delivery, rhTM was administered by the intranasal route to mice with BLM-induced lung fibrosis. There was no sign of hemorrhage in the skin or in the resected lung surface of any

Figure 4 I.P. administration of recombinant human thrombomodulin (rhTM) decreases inflammation, coagulation activation, and profibrotic cytokines in TGF-β1 TG mice with progressive lung fibrosis. TGF-β1 mice with matched lung fibrosis based on computed tomography (CT) findings were used. Mice were treated by i.p. administration of rhTM or saline for 21 consecutive days before sacrifice on day 22. WT/ip-SAL, WT mice were treated with i.p. (ip) saline; TGFβ1-TG/ip-SAL TG mice, with i.p. saline; TGFβ1-TG/ip-rhTM TG mice, with i.p. rhTM. Inflammatory and coagulation markers were measured by immunoassays and mRNA expression by RT-PCR. Data are expressed as means ± SEM. $n = 3$ WT/ip-SAL mice; $n = 9$ TGFβ1-TG/ip-SAL mice; $n = 10$ TGFβ1-TG/ip-rhTM mice. * $P < 0.05$ versus WT/ip-SAL; † $P < 0.05$ versus TGFβ1-TG/ip-SAL. BALF, bronchoalveolar lavage fluid; PDGF, platelet-derived growth factor; TG, transgenic; WT, wild-type.

mouse. The number of total cells and lymphocytes was significantly reduced in mice treated with intranasal rhTM (BLM/in-rhTM) compared to mice treated with intranasal saline (Figure 2A). The grade of lung fibrosis was also evaluated by histological staining with hematoxylin and eosin and Ashcroft score, and the results showed a significant reduction in fibrosis score in the BLM/in-rhTM group compared to mice of the BLM/in-SAL group (Figure 2B).

Comparative Analysis of Efficacy after Intranasal and i.p. Administration of rhTM

The BALF total cell count and the number of total lymphocytes are low in BLM/SAL mice treated with intranasal rhTM compared to BLM/SAL mice treated with i.p. rhTM, but are not statistically significant. There was no significant difference between the relative mRNA expression of collagen I and the Ashcroft fibrosis score between both groups (Supplemental Figure S4).

Suppressive Activity of rhTM on Acute Exacerbation of TGFβ1-Associated Lung Fibrosis

TGFβ1-TG mice were treated with i.p. injection of rhTM for 4 days before receiving an intratracheal instillation of LPS. Signs of easy bleeding related to rhTM administration were not detected. The number of infiltrated neutrophils (Supplemental Figure S5) and the concentration of IFN-γ in BALF (Figure 3) were significantly reduced in the group of mice treated with rhTM (TGFβ1-TG/ip-rhTM/it-LPS) compared to the group treated with saline alone (TGFβ1-TG/SAL/LPS). The plasma concentrations of IFN-γ and HMGB-1, and the lung tissue concentrations of HMGB-1, TAT, and fibrinogen were significantly decreased in the TGFβ1-TG/ip-rhTM/it-LPS group compared with the TGFβ1-TG/ip-SAL/it-LPS group (Figure 3). The plasma concentration of TAT was not significantly changed by LPS instillation but rhTM decreased the basal level of plasma TAT. As expected i.p. injection of rhTM yielded high lung tissue levels of TM protein (Figure 3).

Figure 5 I.P. administration of recombinant human thrombomodulin (rhTM) inhibits the progression of lung fibrosis in TGF- β 1 TG mice. TGF- β 1 mice with matched lung fibrosis based on computed tomography findings were used. Mice were treated by i.p. administration of rhTM or saline (SAL) for 21 consecutive days before sacrifice on day 22. Lung tissue was stained with hematoxylin and eosin (A) or Masson trichrome (B). Collagen deposition (C) was quantitated using an image analyzer, (Ashcroft) lung fibrosis was scored blindly by five experts (K.F., T.K., H.F., T.Y., Y.T.) and hydroxyproline was measured by a colorimetric assay. WT/ip-SAL, WT mice were treated with i.p. saline; TGF β 1-TG/ip-SAL TG mice, with i.p. saline; TGF β 1-TG/ip-rhTM TG mice, with i.p. rhTM. Data are expressed as mean $\pm$ SEM. $n = 3$ WT/ip-SAL mice; $n = 9$ TGF β 1-TG/ip-SAL mice; $n = 10$ TGF β 1-TG/ip-rhTM mice. * $P < 0.05$ versus WT/ip-SAL, Scale bars = 100 μ m. TG, transgenic; WT, wild-type.

The mRNA expressions of IFN- γ , HMGB-1, TGF- β 1, and collagen 1 were significantly decreased in mice treated with rhTM compared to those treated with saline (Supplemental Figure S6).

Suppressive Activity of rhTM on Progression of Lung Fibrosis in TGF β 1-TG Mice

TGF β 1 TG mice were treated with i.p. injection of rhTM or saline for 21 days before euthanasia on day 22. The computed tomography lung fibrosis scores of the TGF β 1-TG/ip-SAL and TGF β 1-TG/ip-rhTM groups were similar at day 0 before the start of rhTM therapy (Figure 4). No sign of hemorrhage in the skin or resected lungs related to rhTM administration was detected. The BALF concentration of D-dimer, the plasma and lung tissue concentration of HMGB-1, the lung tissue concentration of collagen I, and the mRNA expressions of IL-6, IL-13, TNF- α , platelet-derived growth factor-A, CTGF, and mouse and human TGF- β 1 were significantly reduced in TGF β 1-TG/ip-rhTM compared with TGF- β 1-TG/ip-SAL mice (Figure 4). The relative high plasma level of HMGB-1 in the WT/ip-SAL group compared to that observed in the same (WT/ip-SAL) group of the exacerbation experiment described in Figure 3 may be explained by the skewed data and the fewer number of mice in the experiment described in Figure 4. Evaluation of lung tissue disclosed less collagen deposition, lower Ashcroft scores, and hydroxyproline content in

mice treated with rhTM compared to those treated with saline (Figure 5).

Inhibition of Alveolar Epithelial Cell Apoptosis by rhTM

Apoptosis of alveolar epithelial cells plays a critical role in the pathogenesis of pulmonary fibrosis.^{21–23} The degree of apoptosis in lung tissue was compared between the WT/ip-SAL, TGF β 1-TG/ip-SAL, and TGF β 1-TG/ip-rhTM groups. The number of apoptotic cells expressed as the total area of apoptosis in lung tissue was significantly reduced in the lung from TGF β 1-TG mice compared to untreated TG mice (Figure 6A). To confirm this antiapoptotic activity of rhTM, apoptosis of the alveolar epithelial cell line A549 was induced with BLM, and the effect of rhTM was evaluated. Apoptosis of A549 cells induced by BLM was significantly suppressed in the presence of rhTM (Figure 6B). Dose-dependent evaluation of the apoptotic activity of rhTM showed that rhTM can inhibit apoptosis of alveolar epithelial cells even at low concentrations (Supplemental Figure S7).

Discussion

The results of this study showed that parenteral or airway delivery of rhTM ameliorates acute exacerbation and the progressive course of pulmonary fibrosis, and that rhTM can

Figure 6 Recombinant human thrombomodulin (rhTM) reduces apoptosis of lung epithelial cells. For the *in vivo* study, mice were treated by i.p. administration of rhTM or saline for 21 consecutive days before sacrifice on day 22. **A:** Apoptosis in lung specimens was evaluated by the deoxynucleotidyl transferase-mediated dUTP nick end labeling method. **Arrow** shows an apoptotic cell. **B:** For the *in vitro* study, A549 cells were cultured and treated with 50 μ g/mL BLM in the presence or absence of 200 nmol/L rhTM for 48 hours. Experiments were performed in triplicate. The cell viability of A549 cells was measured by flow cytometry (**B**). Wild type (WT)/ip-SAL WT mice treated with i.p. (ip) saline; TGF β 1-TG/ip-SAL TG mice, with i.p. saline; TGF β 1-TG/ip-rhTM TG mice, with i.p. rhTM. Data are expressed as means $\pm$ SEM. $n = 3$ WT/ip-SAL mice; $n = 9$ TGF β 1-TG/ip-SAL mice; $n = 10$ TGF β 1-TG/ip-rhTM mice. * $P < 0.05$ versus WT/ip-SAL or control group; † $P < 0.05$ versus TGF β 1-TG/ip-SAL or bleomycin group. Scale bars indicate 20 μ m. BLM, bleomycin; TG, transgenic.

suppress apoptosis of lung epithelial cells apart from its anticoagulant and anti-inflammatory properties.

Acute Fibrosis Exacerbation and Coagulation Activation

Acute exacerbation of IPF is the rapid worsening of symptoms, lung function, and radiological findings without identifiable causes such as infection, pulmonary thromboembolism, or ischemic heart disease.²⁴ There is also an increased number of active fibroblastic foci, indicating concomitant acceleration of the underlying fibrotic process.²⁴ IPF patients with acute exacerbation of IPC have a very poor prognosis with a mortality rate of more than 70%.^{3,25} The underlying causative factor is unknown. Alteration in the coagulation system in the lungs with enhanced procoagulant activity and low fibrinolytic function have been reported during acute exacerbation,

suggesting a role for coagulation abnormalities in the pathogenesis of the disease.^{26,27} Previous studies showing that IPF patients that survived after acute exacerbation have significantly less intravascular coagulation activation than nonsurvivors support the involvement of the coagulation cascade in the process.^{16,28} The most well-known property of TM is the anticoagulant function.¹⁰ TM can directly inhibit the procoagulant activity of thrombin and promote activation of the anticoagulant protein C pathway.¹⁰ Therapy with rhTM improved survival in IPF patients with acute exacerbation.^{17,18} In the present study, we evaluated the therapeutic efficacy of rhTM on acute exacerbation and coagulation disturbance in TGF β 1-TG mice with pulmonary fibrosis. The results revealed a significantly decreased level of coagulation activation markers in association with less collagen deposition in the lungs from mice treated with rhTM, further supporting the detrimental effect of coagulation activation and the

beneficial effect of rhTM therapy in acute exacerbation of pulmonary fibrosis.

Apoptosis and Acute Exacerbation of Lung Fibrosis

Analysis of the gene expression profile of lung tissues from IPF patients with acute exacerbation has shown an increased expression of genes associated with epithelial proliferation and apoptosis compared to lungs from patients with stable disease, suggesting the relevance of alveolar epithelial apoptosis in the mechanism of pulmonary fibrosis.²² In support of this, increased levels of HMGB-1 have been described in the lung from IPF patients with acute exacerbation.²⁹ HMGB-1 is a nuclear nonhistone protein that can be released passively during cell apoptosis or necrosis, or actively secreted by several cells at sites of tissue injury.³⁰ HMGB-1 may promote both the inflammatory response and the process of cell apoptosis by activating the transcription factor nuclear factor- κ B after binding to its receptors.^{30,31} IPF patients that survived after an acute exacerbation have lower serum levels of HMGB-1 than nonsurvivors.¹⁶ The N-terminal lectin domain of TM can sequester and inactivate HMGB-1.³² The good response of IPF patients with acute exacerbation to therapy with rhTM may be explained by direct inactivation of HMGB-1 by rhTM. Here, we showed that TGF β 1-TG mice with acute exacerbation receiving rhTM therapy have lower plasma and lung tissue concentrations and less lung mRNA expression of HMGB-1 than their counterpart TG mice treated with saline alone, supporting the inhibitory activity of rhTM on HMGB-1 in lung fibrosis. In addition, the decrease of HMGB-1 during rhTM therapy may also result from direct inhibitory activity of rhTM on apoptosis of alveolar epithelial cells.^{33,34} We evaluated this possibility and found that TGF β 1-TG mice treated with rhTM have significantly less lung apoptosis and that *in vitro* rhTM can inhibit apoptosis of lung epithelial cells. Overall, these findings suggest that therapy with rhTM improves acute exacerbation of pulmonary fibrosis by inactivation of HMGB-1, by decreasing HMGB-1 mRNA expression and by inhibition of lung epithelial apoptosis.

Progressive Course of Lung Fibrosis and TM

Although there are several clinical variants of IPF, the disease generally has a progressive and fatal course.⁴ Therefore, development of a pharmaceutical drug that can stop or at least retard the progression of fibrosis may positively impact the clinical outcome of the patients. To address the potential beneficial effect of rhTM therapy on the chronic and gradual progression of pulmonary fibrosis, in the present study, we used two experimental models of pulmonary fibrosis: in one, WT mice were treated with rhTM or saline during the chronic (fibrotic) phase of BLM-induced lung fibrosis, and in a second model, TGF β 1-TG mice that have spontaneous lung fibrosis were treated with rhTM or saline

and then the progression of lung fibrosis was compared. The results were less inflammatory response, coagulation markers, fibrosis score, hydroxyproline content, collagen deposition, and TGF β 1 expression in the lungs from WT mice with BLM-induced lung fibrosis and from TGF β 1-TG mice with lung fibrosis treated with rhTM compared to their counterpart mice treated with saline. These observations suggest that administration of rhTM to patients with IPF may retard the progressive course of the disease. Apart from IPF, it is also conceivable that rhTM may be effective for the treatment of other TGF β 1-driven fibrosis including drug-induced pulmonary fibrosis.³⁵

Delivery System

An important question that needs to be addressed is the delivery system of rhTM in patients with pulmonary fibrosis. In the clinics, rhTM is presently administered by intravenous route to patients with disseminated intravascular coagulation¹⁵; in this case, a high concentration of the drug is generally required because of its dilution in the systemic circulation. Airway delivery system (eg, inhalation) is an alternative route of administration that would allow easy accessibility to the lungs. Here, we found that the grade of lung fibrosis inhibition achieved with intranasal administration of rhTM used at low dose was similar to that achieved by systemic administration of a high dose of rhTM.

Conclusions

In brief, the results of the present study demonstrated that rhTM can inhibit BLM-induced pulmonary fibrosis and TGF- β 1-driven exacerbation and progression of pulmonary fibrosis, and that apart from its well-recognized anticoagulant and anti-inflammatory property, rhTM can also suppress apoptosis of lung epithelial cells.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.06.013>.

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