Induction of Pulmonary Thromboembolism by Neutrophil Elastase in Collagen-Induced Arthritis Mice and Effect of Recombinant Human Soluble Thrombomodulin

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Key Words

Collagen-induced arthritis • Thrombosis • Pulmonary thromboembolism • Thrombin • Recombinant human soluble thrombomodulin

Abstract

We previously reported that during total knee arthroplasty in rheumatoid arthritis (RA) patients, the use of tourniquet might promote local release of neutrophil elastase (NE) from neutrophils, which may contribute to the development of pulmonary thromboembolism (PTE) and tissue injury. The aim of this study was to develop PTE by the use of NE in a mouse model of collagen-induced arthritis (CIA) and investigate the relationship between thrombus and endothelial cells as well as the effect of recombinant human soluble thrombomodulin (rhs-TM) in reducing the risk of PTE. Male DBA/1J mice were injected intracutaneously at several sites with an emulsion containing bovine collagen and later a booster shot to produce CIA mice. Subsequently, NE was injected intravenously 2 times a day for 3 days and after a further 4 days, mice were sacrificed. A group of mice received rhs-TM injections prior to NE injections. We divided the mice into four groups of normal, CIA control, CIA + NE, and CIA + rhs-TM + NE mice and evaluated thrombus formation status. All CIA + NE mice developed PTE. In contrast, no thrombosis

was found in normal control, CIA control and CIA + rhs-TM + NE mice. Plasma thrombin level, fibrinogen expression and neutrophil count were increased in CIA + NE mice. Double staining for anticoagulant TM and procoagulant von Willebrand factor (vWF) in pulmonary endothelial cells in normal mice showed a TM-dominant expression while in both CIA control and CIA + NE mice a vWF-dominant expression compatible with coagulant status was observed. Injection of rhs-TM into CIA + NE mice resulted in a phenotypic conversion of endothelial cells from vWF-dominant to TM-dominant expression and a reduction in fibrinogen deposition. These findings demonstrate that by repeated use of NE in CIA mice, it is feasible to produce PTE and to study its pathogenesis and that rhs-TM reduces the risk of PTE. We suggest that in surgical operations of upper and lower extremities in RA patients, the use of a tourniquet should be avoided as it may trigger NE release.

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Introduction

Pulmonary thromboembolism (PTE) is an extremely common medical problem, occurring either alone or as a postoperative complication of several diseases. Particularly, PTE is a serious complication after total knee ar-

throplasty (TKA) in orthopedic surgery, sometimes leading to patients' death. Clinically detected PTE occurs in 2–10% of patients with deep venous thrombosis who have had knee or hip arthroplasty and the incidence of fatal pulmonary embolism is 1–2% [1, 2]. Asymptomatic pulmonary embolism may occur in as many as 10% of patients [1, 3, 4].

Recently, many researchers have studied the causes and treatments of PTE using different animal models. The methods of producing animal models of PTE are diverse [5, 6], for example, injections of thrombin and autologous blood clot embolization [3, 7], different types of knockout mice [5], bone marrow transplantation [4, 8], or vascular injury models by different chemicals. But all these animal models represented acute PTE, or in other words they were not organized thrombosis models. Commonly neutrophils, monocytes, and endothelial cells enter the thrombus as it is organized.

PTE may result from an imbalance between procoagulant, anticoagulant, and fibrinolytic activity. In this context, inflammation and hemostasis are coupled through common activation and regulatory systems. During inflammation, the hemostatic balance may be disturbed, resulting in the increased production of procoagulant factors and decreased regulation of anticoagulant responses. Thus, thrombosis promotes inflammation, which in turn stimulates a prothrombotic tendency.

Thrombomodulin (TM) is a potent anticoagulant protein expressed mainly on the surface of vascular endothelial cells and prevents blood clotting on the internal surface of vessels. Endothelial TM is a key component of the protein C anticoagulant pathway that facilitates the activation of protein C by thrombin [9]. Therefore, TM acts as an intrinsic anticoagulant barrier between the blood and the endothelium. The plasma level of TM is elevated in disseminated intravascular coagulation and atherosclerotic arterial disease [5, 10]. Moreover, several studies have shown that reduced TM enhances thrombus formation [11, 12]. Furthermore, recombinant human soluble TM (rhs-TM), a soluble derivative of human TM, has been developed and shown to bind directly to thrombin, showing a potent anticoagulant activity [13]. Previous studies have shown that rhs-TM acts as a potent anti-inflammatory molecule such as in endotoxin-induced tissue damage [14] and by inhibiting pulmonary accumulation of leukocytes through thrombin binding and the subsequent protein C activation [15] or by suppressing leukocyte/macrophage infiltration in the glomeruli in a rat model of thrombotic glomerulonephritis [16], and thus may have some effect on chemotaxis.

On the other hand, tissue factor (TF) is a cell surface protein that is expressed constitutively by monocytes, macrophages and fibroblasts [17, 18]. The main function of TF is to form a complex with factor VII/VIIa that converts factors IX and X to their active forms. TF exposure is a potent prothrombotic trigger that initiates activation of both the intrinsic and extrinsic blood coagulation cascade [19]. TF is also involved in the pathophysiology of systemic inflammatory disorders, coagulopathies, and atherosclerotic disease [20]. Thus TF plays a pivotal role in the initiation of thrombotic complications.

We previously suggested that during TKA in rheumatoid arthritis (RA) patients, the use of a tourniquet might promote local release of neutrophil elastase (NE) from neutrophils, which can contribute to the development of PTE and tissue injury [21]. NE is a mediator derived from activated neutrophils and has a very strong tissue-damaging effect in spite of its original role in host defense [22–25]. Moreover, it is known that in RA patients, the general event is hypercoagulability based on inflammation status [26]. Thus we hypothesized that with repeated injections of NE in collagen-induced arthritis (CIA) mice, it will be feasible to develop thrombus formation and PTE. To test this hypothesis, we compared two groups of mice, one with and the other without CIA, in terms of thrombus formation. We could not verify thrombus formation in normal control and CIA control mice. Moreover, a single injection of NE was not sufficient to produce thrombus, as these mice did not show microvessel thrombi even 4 days after the last injection. This indicated that fibrinolysis events had been taken place. From our preliminary experimental data, we determined that NE injection of 2 times per day for 3 days was the most effective regimen to induce thrombus formation.

In the present study, we succeeded in generating organized thrombus and developing PTE in a mouse model of CIA and investigated the relationship between thrombus and endothelial cells as well as the effect of rhs-TM in reducing the risk of PTE in this model.

Materials and Methods

Animals

Male DBA/1J mice, 6 weeks old, were purchased from Saitama Experimental Animal Supply Co. (Saitama, Japan). The study protocol was approved by the Nippon Medical School Animal Care and Use Committee, and the care and handling of the animals were in accordance with the National Institutes of Health guideline.

Reagents

Elastase (Athens Research & Technology Inc., Athens, Ga., USA) was from human neutrophils with an activity of 20–22 units/mg protein. One unit is defined as the amount of enzyme that hydrolyzes 1 μ mol of Meo-suc-ala-ala-pro-val-pNA/min at 25°C in 100 mM Tris-HCl, pH 7.5, with 500 mM NaCl. Elastase was used after dissolving in a buffer containing 50 mM Na acetate, pH 5.5, with 150 mM NaCl to be added to the salt-free lyophilized solid. rhs-TM was kindly provided by Asahi Chemical Industry (Tokyo, Japan); the method used for preparation of rhs-TM has been described previously [12].

Collagen-Induced Arthritis

Complete Freund's adjuvant (CFA) was prepared by grinding 100 mg heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories, Detroit, Mich., USA) in 20 ml incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo., USA). An emulsion was formed dissolving 2 mg/ml bovine collagen (Gijyutsu Kensehukai Co., Tokyo, Japan) overnight at 4°C in 0.05 N acetic acid and combining it with an equal volume of CFA. CFA solution and the emulsion with CFA were always freshly prepared. Mice were injected intracutaneously at several sites into the base of the tail with a total of 100 μ l emulsion containing 100 μ g CFA and 250 μ g *M. tuberculosis*. The same injection was repeated at day 21; however, due to toughening of the skin at the base of the tail, booster injections were proximal to the primary injection site. In experiments to test for the requirement for CFA, CFA was omitted from the emulsion of CFA [27–29].

PTE Model

Normal male DBA/1J mice ($n = 13$) were injected intravenously with Na acetate buffer containing dissolved NE into the lateral tail vein 2 times a day for 3 days. After 4 days from the last injection, mice were sacrificed. CIA + NE mice ($n = 28$) were injected with NE (5 U/kg, 2 times a day) into the tail vein for 3 days, and were sacrificed 4 days after the last injection. CIA + TM + NE mice ($n = 13$) were injected with rhs-TM (1.5 mg/kg, 2 times a day, 5 min before NE) and NE (5 U/kg, 2 times a day) for 3 days, and were sacrificed 4 days after the last injection. These CIA model mice were injected with one more booster (collagen type II and CFA). We induced hyperarthritis that is considered as hypercoagulability [30]. Overall, model groups consisted of normal mice ($n = 10$), CIA control mice ($n = 10$), CIA + NE mice ($n = 28$), and CIA + rhs-TM + NE mice ($n = 10$).

Surgical Procedure

Mice (20–24 g) were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital. After obtaining blood or bronchoalveolar lavage (BAL), fresh lung tissues were cut into pieces for different preparations. For example, frozen sections with brief fixation in 4% buffered paraformaldehyde were used for immunofluorescence staining, and fresh tissues with fixation in 4% buffered paraformaldehyde were used for paraffin embedding. For immunohistochemical studies, the frozen or paraffin sections reacted with primary antibodies as described below.

Thrombin-Antithrombin Complex III

To evaluate coagulability status, blood was collected from anesthetized mice by cardiac puncture and anticoagulated with 3.8% trisodium citrate (1:10 vol). Anticoagulated blood samples

were centrifuged at 3,000 rpm for 5 min at 4°C and plasma samples stored at –20°C until use. Thrombin-antithrombin III (TAT) complex concentration in plasma was measured by a commercially available sandwich enzyme immunoassay kit designed for human TAT, which also cross-reacts with murine TAT. Each new thrombin molecule formed in plasma will immediately be coupled to ATIII-TAT complexes and is measured as a surrogate for the circulating thrombin levels [31].

BAL Fluid

In brief, after intraperitoneal administration of pentobarbital sodium, an incision was made in the anterior neck, and a catheter was secured in the trachea with surgical suture. BAL was performed with 3 ml of saline and 2–2.5 ml of lavage fluid was gently withdrawn. Cell differentiation of BAL was analyzed using a smear stained by the Wright-Giemsa method.

Light Microscopy and Immunoperoxidase Staining

Deparaffinized tissue sections were stained with hematoxylin and eosin for routine examination. Serial sections were stained by an avidin-biotin complex immunoperoxidase method [32–34]. The sections were treated with 0.3% hydrogen peroxidase for 30 min at 20°C to suppress endogenous peroxidase activity and incubated for 20 min in 10% normal goat serum to prevent nonspecific binding of the primary antibody. Incubation with the primary antibody for fibrinogen (1:600 rabbit polyclonal IgG; DAKO, Glostrup, Denmark) was performed overnight at 4°C. The sections were reacted with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

Immunofluorescence Staining

Fresh tissues were embedded in OCT compound, snap frozen in acetone dry ice, and stored at –80°C until use. An indirect single or double immunofluorescence method was applied on 6- μ m-thick sections as described previously [32–34]. Briefly, sections were incubated overnight at 4°C with either a combination of mouse monoclonal IgG and rabbit polyclonal IgG antibodies against TM (1:100; DAKO, Carpinteria, Calif., USA) or von Willebrand factor (vWF; 1:500; Glostrup, Denmark). After washing, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif., USA) for a single stain. For double staining, FITC-labeled goat anti-rabbit and Texas red-labeled horse anti-mouse IgG were applied as secondary antibodies for 60 min at room temperature in the dark. Nuclear counterstaining was done at room temperature with 0.01% TOTO-3 iodide (Molecular Probes, Eugene, Oreg., USA). The sections were evaluated by a confocal laser scanning microscope (model TC-SP, Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources. In the resulting preparations, red fluorescence represented TM and green fluorescence showed vWF. Nuclei appeared as blue.

Immunohistochemical Control Procedures

Negative control preparations consisted of (1) omission of the primary antibody from the staining procedure and (2) substitution of corresponding amounts of normal immunoglobulin for the primary antibodies. Both control procedures consistently gave negative results.

Fig. 1. Serum TAT complex levels. Enzyme immune assay for serum concentrations of TAT in mice shows significant increases in CIA + NE and CIA + rhs-TM + NE mice as compared to the normal control. Data are expressed as mean $\pm$ SEM of three independent measurements. * $p < 0.01$; ** $p < 0.05$.

Semiquantitative Evaluation of Fibrinogen

Each lung sample was fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, embedded in paraffin blocks, and serially sectioned at 3- to 4- μ m thickness. Tissue sections were deparaffinized, rehydrated, and stained for fibrinogen (1:600; DAKO, Carpinteria, Calif., USA) to complete the analysis of the histopathology [30]. The staining for fibrinogen was semiquantitated with the observer blinded to treatment, using a Nikon microscope (Tokyo, Japan) with a $\times 20$ objective lens. The fibrinogen stains excluding nuclear stains were assessed by the Scion image analysis system (Beta 4.02 for Windows, Frederick, Md., USA). We evaluated fibrinogen densities in 20 microscopic fields and compared the mean values in each group [35].

Apoptosis and Proliferation Assessment

A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay that relied on DNA fragmentation, the hallmark of apoptosis, was used as a primary method to investigate apoptosis. Tissue sections were processed for the TUNEL. Briefly, sections were rehydrated through xylene and graded alcohols. Endogenous peroxidase activity was quenched in peroxide/methanol prior to incubation for 30 min with proteinase K (10 μ g/ml in 10 mM Tris/HCl, pH 7.6) at 37°C. Sections were then incubated for 1 h with terminal deoxynucleotidyl transferase (Tdt; In Situ Cell Death Detection Kit, Roche Inc., Germany) which catalyzes the labeling of DNA strand breaks with deoxyuridine triphosphates. During this step, slides were coverslipped. After washing and subsequent incubation with antidigoxigenin peroxidase conjugate for 30 min, slides were developed with DAB substrate, counterstained with methyl green, dehydrated, coverslipped, and then examined for evidence of apoptosis. We also investigated whether endothelial cell apoptosis was accompanied by a compensatory proliferative or reparative response. We selected Ki-67 immunohistochemical staining (rabbit polyclonal IgG, Abcam, Cambridge, UK, 1:25) as a marker for the cell proliferation.

Semiquantitative evaluation of TUNEL assay and Ki-67 staining were performed in each group. At first, each section was observed at $\times 200$ magnification, and then 20 middle (40–100 μ m in diameter)- or small (10–40 μ m in diameter)-sized vessels were randomly selected by three observers. Next, in each vessel positive endothelial cells were counted and a ratio was represented as the number of positive endothelial cells/vessel total endothelial cells according to a method described previously [36].

Immunohistochemical Staining for Caspase 3 (Active Form)

ABC immunostaining was applied to identify the active form of caspase 3 (rabbit polyclonal IgG, Abcam). The sections were pretreated with proteinase K (20 μ g/ml) for 30 min at 37°C, and then endogenous peroxidase was inhibited as above. The primary antibody for caspase 3 was applied for 1 h at 37°C. Biotin-labeled goat anti-rabbit IgG was applied as the secondary antibody and the chromogenic reaction was evaluated by DAB. Semiquantitative evaluation was done as described above.

Statistical Analysis

All values were expressed as mean $\pm$ SEM. Significance was determined by a Mann-Whitney's U test to determine differences among groups. These tests were performed using SPSS statistical software, and $p < 0.05$ was considered significant.

Results

Development of Thrombosis

In all CIA + NE mice ($n = 28$) thromboemboli were observed in the lung which was partly organized thrombosis surrounded by endothelial cells. Formation of fibrin-rich thromboemboli was distributed in small (10–40 μ m)- and medium (40–100 μ m)-size microvessels in the lung. On the other hand, in normal mice ($n = 10$), CIA control mice ($n = 10$), and CIA + rhs-TM + NE mice ($n = 10$) no thrombosis was found.

Evaluation of TAT Complex Levels

Each new thrombin molecule formed in plasma will immediately be coupled to ATIII-TAT complexes which were measured as a surrogate for the circulating thrombin levels. We compared the plasma TAT level of the normal mice group with other CIA mice groups (fig. 1). There were significant increases in plasma TAT levels in CIA + NE and CIA + rhs-TM + NE mice versus normal mice (9.998 ± 1.297 and 9.038 ± 1.553 vs. 4.150 ± 0.832 ng/ml; $p < 0.01$ and $p < 0.05$, respectively). CIA control mice also showed an increase. There was no statistically significant difference between the CIA + NE group and the CIA + rhs-TM + NE group. However, CIA + rhs-TM + NE mice did not show pulmonary embolism or fibrin deposition in the lung despite a high level of serum TAT.

Fig. 2. Evaluation of BAL fluids. **a** Total cell number in BAL from CIA control and CIA + NE mice increased significantly as compared to normal control mice (* $p < 0.05$) and administration of rhs-TM could decrease the total cell number. **b** Differential cell count in BAL showed significantly increased neutrophils in CIA + NE mice (* $p < 0.05$), increase in lymphocytes in all CIA mice groups and no change in macrophage counts.

Evaluation of BAL Fluids

Total cell number in BAL was increased significantly in CIA control and CIA + NE mice ($p < 0.05$) but not in CIA + rhs-TM + NE mice (fig. 2a). Cell differential counts showed a significantly increased neutrophil count in CIA + NE mice compared to the other groups ($p < 0.05$), and no change in macrophage counts (fig. 2b). Lymphocyte counts were increased in all CIA mice groups compared to the normal control group. As BAL in RA patients usually show increased lymphocytes, CIA mice in general simulated the same pulmonary internal environment. Moreover, multiple injections of NE intravenously affected endothelium and epithelium (alveolar cells) and interalveolar cells; therefore, total cell number and neutrophil count were increased in BAL from CIA + NE mice. Treatment with rhs-TM could reduce injuries exerted by NE administrations, as neutrophils in BAL from CIA + rhs-TM + NE mice were not increased (fig. 2b).

Immunohistochemical and Confocal Microscopic Observations

Hematoxylin and eosin stains showed organized thrombosis surrounded by endothelial cells in CIA + NE mice.

Immunohistochemical examination for fibrinogen in CIA + NE mice ($n = 28$) at 4 days from the last injection showed many microthrombi composed of platelet aggregates and fibrin and thrombi in vessels ($>40 \mu\text{m}$ in diameter) in all 28 mice. CIA control mice showed a slight exudation of fibrin in alveolar microvessels. CIA +

rhs-TM + NE mice ($n = 10$) did not show thrombus formation and fibrin exudation (fig. 3).

A coagulatory protein, vWF or factor VIII-related antigen is considered as one of the biological markers of endothelial cells, and is stored in the cytoplasmic inclusions (Weibel-Palade bodies) of endothelial cells. In contrast, TM which is an anticoagulant protein is expressed along the endothelial cell plasma membrane and plays a pivotal role in maintaining physiological functions of the endothelial cells in balance with procoagulant vWF protein. Confocal laser scanning immunofluorescence microscopy demonstrated a positive reaction for TM apparently in both luminal and abluminal plasma membranes of alveolar capillary endothelium in normal mice (fig. 4a). A group of microvessels ($>10 \mu\text{m}$ in diameter) in connective tissue, including vasa vasorum of large vessels, arterioles (of the pulmonary circulation), and venules of the bronchial circulation, was exclusively reactive for vWF in CIA mice (fig. 4b), but mostly nonreactive for TM similar to that in human lung. TM expression of endothelial cells appeared to be suppressed by NE injections in CIA mice (fig. 4c) while vWF expression especially in the juxta-alveolar zone was increased. CIA + NE mice treated with rhs-TM slightly lost TM expression and did not show increased vWF expression (fig. 4d).

Semiquantitative Evaluation of Fibrinogen

Fibrinogen intensity measurements showed that CIA control and CIA + NE mice had increased fibrinogen and the increase in fibrinogen in CIA + NE mice was more

Fig. 3. Immunohistochemical observations. Organized thrombosis developed in lung from CIA + NE mice. Hematoxylin and eosin-stained section (**a**) and comparative fibrinogen-stained section (**c**) showing a thrombus surrounded by endothelium. **b, d** Higher magnifications. Original magnifications: $\times 200$ (**a, c**) and $\times 400$ (**b, d**).

than that in CIA control mice ($p < 0.05$) (fig. 5). In addition, administration of rhs-TM revealed reduced fibrinogen depositions. The rationale for these events may be that inflammation itself renders coagulate dominant status and repeated administration of NE adds to this by causing fibrin deposition and thrombosis.

Evaluation of Apoptosis and Proliferation in Endothelial Cells in Vessels

In TUNEL stains, positive endothelial cells in vessels were more increased in CIA control and CIA + NE mice than normal mice ($p < 0.01$). On the other hand, administration of rhs-TM could reduce apoptosis ($p < 0.05$) (fig. 6). In caspase-3 stains, we could observe significant

differences between normal mice and CIA + NE mice (fig. 7). But among other groups, although caspase-3 staining markedly decreased in CIA + rhs-TM + NE mice, there were no significant differences which could be attributed to the differences in the methodology. Staining for Ki-67 cell proliferation marker was similar with apoptosis staining as mentioned above. Ki-67-positive endothelial cells were increased in CIA control and CIA + NE mice as compared to normal mice but not in CIA + rhs-TM + NE mice (fig. 8). These results show that endothelial cells injured by inflammation and NE were committing apoptosis and many Ki-67-positive cells were generated to preserve homeostasis.

Fig. 4. Confocal microscopic observations. Confocal laser scanning immunofluorescence microscopy shows a positive reaction for TM apparently in both luminal and abluminal plasma membranes of alveolar capillary endothelium in normal mice (a). A group of microvessels ($>10\ \mu\text{m}$ in diameter) in connective tissue, including vasa vasorum of large vessels, arterioles (of the pulmonary circulation), and venules of the bronchial circulation, was exclusively reactive for vWF in CIA mice (b), but mostly unreactive for TM similar to the human lung. TM expression of endothelial cells was suppressed in CIA/NE mice (c) while vWF expression especially in the juxta-alveolar zone was increased. In CIA + rhs-TM + NE mice (d) TM expression was slightly lost and vWF expression did not increase.

Fig. 5. Semiquantitative evaluation of fibrinogen. Assessment of fibrinogen level shows a significant increase in CIA + NE mice compared to CIA control mice, which was suppressed in CIA + rhs-TM + NE mice indicating the effectiveness of rhs-TM administration. Data are expressed as mean $\pm$ SEM of three independent measurements. * $p < 0.05$.

Fig. 6. Evaluation of apoptosis by the TUNEL method. **a** TUNEL staining for apoptosis cells in the lungs from different groups of mice. Original magnification: $\times 200$. **b** Endothelial cell (EC) apoptosis was markedly increased in CIA control and CIA + NE mice and was suppressed in CIA + rhs-TM + NE mice showing the effectiveness of rhs-TM administration. Data are expressed as mean $\pm$ SEM. * $p < 0.01$; ** $p < 0.05$.

Discussion

In the present study, we succeeded in generating thrombus by repeated use of NE in a model of CIA mice that subsequently developed PTE. Previously we demonstrated that during TKA, the use of a tourniquet increased the NE level 8-fold and suggested that this event may promote the risk of developing PTE, particularly in RA patients [21, 37]. On the other hand, the absence of PTE has been associated with a general status of decline in hyper-

coagulability [26, 37]. We attributed this to the so-called 'reperfusion injury', that is to say the release of NE by deflating tourniquet was the most important factor [25, 38]. Therefore, we focused our attention on the role of NE in promoting PTE.

NE is known as a cause of acute lung injury and is the most destructive enzyme. It possesses various activities including, for example, degradation of almost all extracellular matrix and key plasma proteins, degradation of various cytokines such as proinflammatory cytokines

Fig. 7. Evaluation of apoptosis by caspase staining. **a** Caspase-3 (active form) staining for apoptosis cells in the lungs from different groups of mice. Original magnification: $\times 200$. **b** Endothelial cell (EC) apoptosis was markedly increased in CIA + NE mice but not in CIA + rhs-TM + NE mice showing the effectiveness of rhs-TM administration. Data are expressed as mean $\pm$ SEM. * $p < 0.05$.

Fig. 8. Assessment of cell proliferation. **a** Staining for Ki-67 proliferative cell marker in the lungs from different groups of mice. Original magnification: $\times 200$. **b** Endothelial cell (EC) proliferation was markedly increased in CIA control and CIA + NE mice and was suppressed in CIA + rhs-TM + NE mice showing the effectiveness of rhs-TM administration. Data are expressed as mean $\pm$ SEM. * $p < 0.05$.

or inflammatory cytokines like interleukin-6, interleukin-8, granulocyte-macrophage colony-stimulating factor, and inactivation of various coagulation-fibrinolysis factors in vitro [13–15]. One of the important coagulation-fibrinolysis factors is TM that is regarded as the endothelial receptor for the thrombin-catalyzed conversion of protein C to activated protein C. It acts as a potent membrane-bound anticoagulant cofactor and is regarded as a reliable marker of endothelial cell injury in vitro [39, 40].

In our study, the frequency of TUNEL-positive, caspase-3-positive, and Ki-67-positive endothelial cells although increased in CIA + NE mice was not significantly different from CIA control mice. However, neutrophils in BAL fluid and fibrinogen staining were significantly increased in CIA + NE mice. These findings may suggest that NE did not significantly contribute to apoptosis and proliferation of endothelial cells but played an important role in endothelial cell injury in combination with inflammation due to CIA leading to thrombosis.

The plasma level of TAT in the CIA + NE model increased significantly compared to normal controls. This represented a hypercoagulability status. There was no significant difference between the TAT plasma levels of CIA + NE and CIA + rhs-TM + NE mice, suggesting that preadministration of rhs-TM had no significant effect on the TAT level. However, its phenotypic reversal effect, i.e. changes from vWF dominance to TM dominance, could facilitate precondition in CIA + rhs-TM + NE mice against developing pulmonary embolism or fibrin deposition in the lung.

To study pulmonary internal environments, we examined BAL fluids. The results revealed that neutrophil differentiation was significantly increased in CIA + NE mice but not in CIA mice, indicating endothelial and epithelial cell injuries. Thus in CIA + NE mice vascular endothelial cells were injured and were in a condition that seemed to be acute respiratory distress syndrome. Double staining for TM and vWF in endothelial cells in both CIA control and CIA + NE mice showed vWF-dominant expression. In general, inflammation by arthritis is known to cause hypercoagulability. Moreover, TM is cleaved to its soluble form by other substances produced during chronic inflammatory responses, immunologic reactions and complement activation in addition to NE in order to protect against hypercoagulopathy. Therefore, in CIA control and CIA + NE mice groups, endothelial cells in the lung changed their phenotypic character from anticoagulant-dominant expression to coagulant-dominant expression. In other words, pulmonary internal environ-

ment was rich in vWF instead of TM. But by this change per se PTE could not be developed. Multiple injections of NE were needed to injure both endothelial and epithelial cells. Fibrinogen was also elevated in CIA control and CIA + NE mice and administration of rhs-TM showed reduction in fibrinogen deposition. Haubitz et al. [41] showed that NE increases TF activity that is known as the primary cellular initiator of blood coagulation. TF exposure is a potent prothrombotic trigger that initiates activation of the coagulation cascade, while TM is a potent anticoagulant protein that inhibits the extent of activation. Both TF-neutralizing antibodies and soluble TM are considered effective anticoagulants. Thus, a multifactorial combination was involved in the generation of PTE in our model.

We found that rhs-TM reduced PTE. Administration of rhs-TM to CIA + NE mice revealed a conversion in the phenotype of endothelial cells from vWF-dominant expression to TM-dominant expression, which may contribute to the anticoagulant function. In a previous study, Ikeguchi et al. [16] showed that rhs-TM is a potential novel therapeutic tool for thrombosis. In fact, rhs-TM is a new anticoagulant that was well tolerated in a phase I study in human volunteers [42]. These results were in agreement with our findings. In addition, rhs-TM prevented apoptosis and suppressed proliferation of endothelial cells, which may indicate a synchronized regulation for stabilization of endothelial cell function by diminishing endothelial cell injury.

In conclusion, we developed organized PTE by repeated use of NE in CIA mice. We also showed that rhs-TM reduced PTE. Taken together, our observations suggest that the use of a tourniquet in RA patients should be considered as one of the risk factors for postoperative development of PTE as it may cause NE release. We suggest, therefore, that where possible the use of a tourniquet should be avoided in surgical operations of upper and lower extremities in RA patients.

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