

Probable Roles of Coagulation Cascade and Fibrinolysis System in the Development of Allergic Rhinitis

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Abstract

Background: Dysregulation of the coagulation cascade and fibrinolysis system may play an etiologic role in many diseases. Allergic diseases such as bronchial asthma, atopic dermatitis, and conjunctivitis are also associated with fibrin accumulation caused by a change in hemostasis. However, only a few studies have dealt with the relationship between allergic rhinitis (AR) and the coagulation system.

Objective: We investigated the difference of coagulation and fibrinolysis cascade components between an AR mouse model and a control mice.

Methods: BALB/c mice were sensitized and challenged with ovalbumin. Multiple parameters of coagulation cascade and fibrinolysis system such as factors II, V, VII, X, and XIII; tissue-type plasminogen activator; urokinase-type plasminogen activator (u-PA); plasminogen activator inhibitor-I (PAI-I); and fibrin were compared between the AR model group and the control group.

Results: The symptom scores and eosinophil counts were higher in the AR group than in the control group ($P < .01$). The mRNA expression level of u-PA ($P = .040$) was significantly lower, and the expression levels of factor II ($P = .038$) and factor X ($P = .036$) were significantly higher, in the AR group. Immunohistochemical staining revealed that most of the fibrinolysis system and coagulation cascade components were localized to the epithelium, endothelium, and submucosal glands of the nasal mucosa. u-PA was downregulated in the AR group, whereas fibrin deposition was more prominent in the AR group than in the control group.

Conclusion: In AR, particular components of the coagulation cascade were increased and fibrinolysis system was decreased compared to normal control. This difference may be associated with the fibrin deposition in the mucosa of AR mouse model.

Keywords

allergic rhinitis, coagulation factor, fibrinolysis, mouse model, pathogenesis

Introduction

Allergic rhinitis (AR), which is one of the most common chronic allergic diseases, represents a substantial socio-economic burden. It is becoming increasingly prevalent in industrialized societies.¹ Moreover, in spite of advances in medical science with regard to diagnosis and symptom control, the current treatment strategies for AR do not provide a complete cure. Therefore, efforts investigating for a novel efficient approach to manage the pathophysiology of AR are required.

Hemostasis, which involves a coagulation cascade and fibrinolysis system, is related to many inflammation diseases such as asthma, rheumatoid arthritis, delayed-type hypersensitivity, and chronic rhinosinusitis (CRS). It has been reported that the dysregulation of the

coagulation pathway and the deposition of fibrin—which is the end product of hemostasis—in the distal airways of the lung contribute significantly to airway

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hyperresponsiveness and closure in asthma.² A decrease in the levels of a fibrinolysis system component called tissue-type plasminogen activator (t-PA) has been reported in CRS patients, and the consequent deposition of fibrin in the submucosa of nasal polyps is also thought to play a critical role in the pathogenesis of CRS.³

AR shares many clinical characteristics with asthma, in accordance with the unified airway concept. Moreover, AR has many common pathophysiologic similarities to CRS, which is also an upper airway mucosal disease. However, only a few studies have dealt with the role of the coagulation cascade and fibrinolysis system with regard to AR,^{4,5} and the pathogenic mechanism underlying the correlation between inflammatory diseases and coagulation remains unclear.

Therefore, in the present study, we investigated the expression and localization of coagulation and fibrinolysis cascade components in a murine model of AR.

Material and Methods

Murine Model of AR

We used 4-week-old female BALB/c mice (18–20 g each) in the experiments. The mice were divided into 2 groups—an AR group and a control group—each consisting of 20 mice. The negative control group and the AR group were sensitized and challenged with phosphate-buffered saline (PBS) and ovalbumin (OVA), respectively. The procedures for allergen sensitization and treatment are summarized in Figure 1. Briefly, the mice were sensitized with an intraperitoneal injection of 25 µg of OVA (grade V; Sigma, St. Louis, MO) and 1 mg of aluminum hydroxide gel (Sigma) on days 0, 7, and 14. From day 22, a droplet of OVA (100 µg) was administered to the nasal cavity on 7 consecutive days. This animal study was approved by the Institutional Animal Care Committee of the Clinical Research Institute of Seoul National University Hospital (15-0076-S1A0).

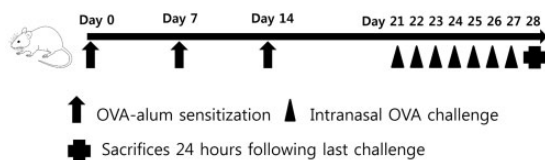


Figure 1. Experimental protocol for allergen sensitization and challenge. BALB/c mice were sensitized on days 0, 7, and 14 by intraperitoneal injection of OVA and 1 mg of alum. On days 22 through 29 after the initial general sensitization, the mice were challenged with OVA intranasally. The control group mice were sensitized and challenged with PBS. AR, allergic rhinitis; PBS, phosphate-buffered saline; OVA, ovalbumin; alum, aluminum hydroxide gel.

Evaluation of Allergic Responses

Nasal symptom scores were evaluated after the final OVA challenge with 200 µg of OVA on day 27. The number of sneezes and episodes of rubbing were counted for 15 min by a blinded observer. Mice were then sacrificed 24 h after the last OVA challenge. After perfusion with 4% paraformaldehyde, the heads of 10 mice from each group were removed en bloc and fixed with 4% paraformaldehyde. Nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4-µm-thick sections) for the evaluation of nasal histology. Each section was stained with hematoxylin and eosin (H&E), and the eosinophils on both sides of the septal mucosa were counted. The eosinophils in the submucosal area of the whole nasal septum were counted under a light microscope (400× magnification).

Real-time Reverse Transcription Polymerase Chain Reaction

We carried out reverse transcription polymerase chain reaction (qRT-PCR) on the nasal mucosa homogenates of five mice from each group. Total RNA was prepared from the nasal mucosa using TRIzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using superscript reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) primers (Fermentas, Burlington, ON). The sequences of the specific PCR primers used to amplify the fibrinolysis system components—including urokinase-type plasminogen activator (u-PA), t-PA, and plasminogen activator inhibitor-1 (PAI-1)—and the coagulation cascade components—including factors II, V, VII, X, and XIIIa—are given in Supplemental Table 1. We carried out the qRT-PCR using a SYBR® Green RT-PCR kit (Applied Biosystems, Foster City, CA) and a QuantStudio 3 system (Applied Biosystems) according to manufacturer's instructions. RNA integrity and the success of the RT reaction were monitored by PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The amplification of the cDNA of each component and that of *GAPDH* was carried out in MicroAmp 96-well optical reaction plates (Applied Biosystems). The average transcript levels of the genes were then normalized. Each PCR run included the 5 points of the standard curve, a no-template control, and unknown patient cDNAs. The fluorescence intensity of each target gene was normalized to that of *GAPDH*. The amplified PCR products were also resolved in 2% agarose gels and photographed under ultraviolet light.

Immunohistochemical Staining

We cut the paraffin-embedded specimens into 4-µm-thick sections and floated them onto silane-coated

micro slides (Muto-Glass, Tokyo, Japan). After deparaffinization in xylene, the sections were rehydrated with ethanol. The sections were then microwaved for 15 min with an antigen-unmasking solution (Vector Laboratories, CA). After microwave treatment, the sections were treated with 0.3% hydrogen peroxide in methanol for 15 min to inhibit the endogenous peroxidase activity of the blood cells. Normal horse serum (2.5%) was used as the blocking reagent. We purchased appropriate primary and secondary antibodies against factor V and factor X from Santa Cruz Biotechnology (Santa Cruz, CA); against factors VII, t-PA, u-PA, and PAI-1 from Abcam (Boston, MA); and against fibrin from Sekisui (Stamford, CT). The treated sections were incubated with the primary antibodies for t-PA (1:500, ab157469), u-PA (1:500, ab133563), PAI-1 (1:1000, ab28207), factor V (1:500, sc292858), factor VII (1:500, ab97614), factor X (1:500, sc20673), and fibrin (1:50, a350) at the appropriate concentrations in blocking reagent overnight at 4°C. After washing thoroughly with PBS, each sample was incubated with the secondary antibodies at the appropriate concentrations (impress anti-rabbit IgG, vector) in blocking reagent for 30 min at 25°C (or room temperature). After washing again, the immunoreactive sites were visualized with peroxide substrate kit 3, 3'-diaminobenzidine (DAB; Vector Laboratories, CA) and counterstained with H&E. The control sections were incubated with non-immune rabbit IgG (impress) at the same concentration as the primary antibody. Image J software (US National Institutes of Health, Bethesda, MD) was used to quantify the strength of the immunohistochemical staining in each section.

Measurement of Expression of Fibrinolysis System Components in Nasal Lavage Fluids

We attempted to figure out whether there was a significant difference of protein secretion, same as PCR and immunohistochemistry (IHC) result, in the nasal lavage fluids. On day 28, the nasal lavage fluids were collected 24 h after the last intranasal challenge in 10 mice from

each group. After partial tracheal resection, a plastic catheter was inserted into the tracheal opening of each mouse toward the upper airway, and saline wash was collected from the nostril. The nasal lavage fluids were centrifuged, and the supernatants were assayed by enzyme-linked immunosorbent assay (ELISA). The concentrations of t-PA, u-PA, and PAI-1 in the lavage fluid were measured with an ELISA kit (Molecular Innovations Inc., Novi, MI) according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a microplate reader. The sensitivities of t-PA, u-PA, and PAI-1 were 0.035, 0.00998, and 0.031 ng/mL, respectively.

Statistical Analysis

The results are reported as the mean $\pm$ standard deviation of each independent experiment. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). A Mann-Whitney *U* test was used to compare the results between the AR and control groups. A *P* value of less than .05 was deemed to indicate statistical significance.

Results

Preparation of the AR Model

There was a statistically significant difference in the nasal symptom score between the AR and control groups, and the number of rubbing (*P* = .029) and sneezing (*P* = .001) episodes was higher in the AR group. H&E staining revealed pronounced eosinophil infiltration of the nasal mucosa in the AR group, which was significantly different from that in the control group (*P* < .001; Figure 2).

Measurement of mRNA Expression Levels of Fibrinolysis System and Coagulation Cascade Components in the Nasal Mucosa

We carried out qRT-PCRs on the fibrinolysis system and coagulation cascade components. The expression of

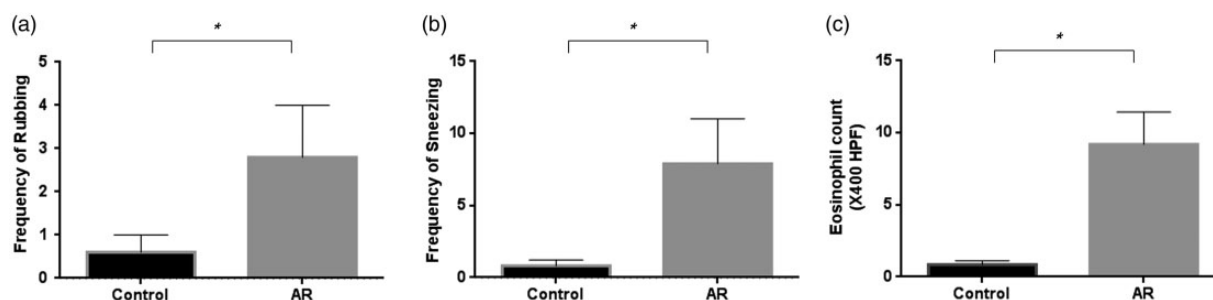


Figure 2. Symptom score and eosinophil infiltration: (a) Rubbing symptom score, (b) sneezing symptom score, and (c) the number of eosinophils in the nasal mucosa of each group at a magnification of 400 $\times$. Values are expressed as mean $\pm$ SEM. **P* < .05. AR, allergic rhinitis group.

mRNA increased in most of the coagulation cascade components. The mRNA expression levels of factor II ($P = .038$) and factor X ($P = .036$) increased significantly in the AR group, whereas those of factor V ($P = .645$), factor VII ($P = .121$), and factor XIII ($P = .878$) did not (Figure 3). With regard to the fibrinolysis system components, the mRNA expression levels of u-PA decreased significantly in the AR group compared with the control group ($P = .040$), whereas PAI-1 mRNA expression showed a tendency to increase in the AR group ($P = .054$). There was no significant difference in the level of t-PA mRNA expression between the groups ($P = .888$; Figure 4).

Immunohistochemical Localization in Murine Nasal Mucosa

Immunohistochemical staining revealed that the components of the fibrinolysis system and coagulation cascade were mainly located in the epithelium, submucosal glands, and endothelial lining of the vessels of the

nasal mucosa. Fibrin-immunoreactive material was localized predominantly in the interstitial area of the submucosa and the epithelium of the nasal mucosa (Figure 5(G)).

Among the components, there was significantly less u-PA staining in the AR group than in the control group ($P = .041$), in agreement with the mRNA expression pattern (Figure 5(E)). There were no statistically significant differences with regard to the other components. There was significantly greater fibrin staining in the AR group than in the control group ($P = .026$; Figure 5(H)).

Fibrinolysis System Components in Nasal Lavage Fluid

There was no statistically significant difference in t-PA concentration in the nasal lavage fluid between the AR and control groups. However, there was a statistically significant difference in the level of u-PA ($P = .029$) between the 2 groups. The average level of u-PA was significantly lower in the AR group (0.064 ± 0.029 ng/mL) than in the control group (0.093

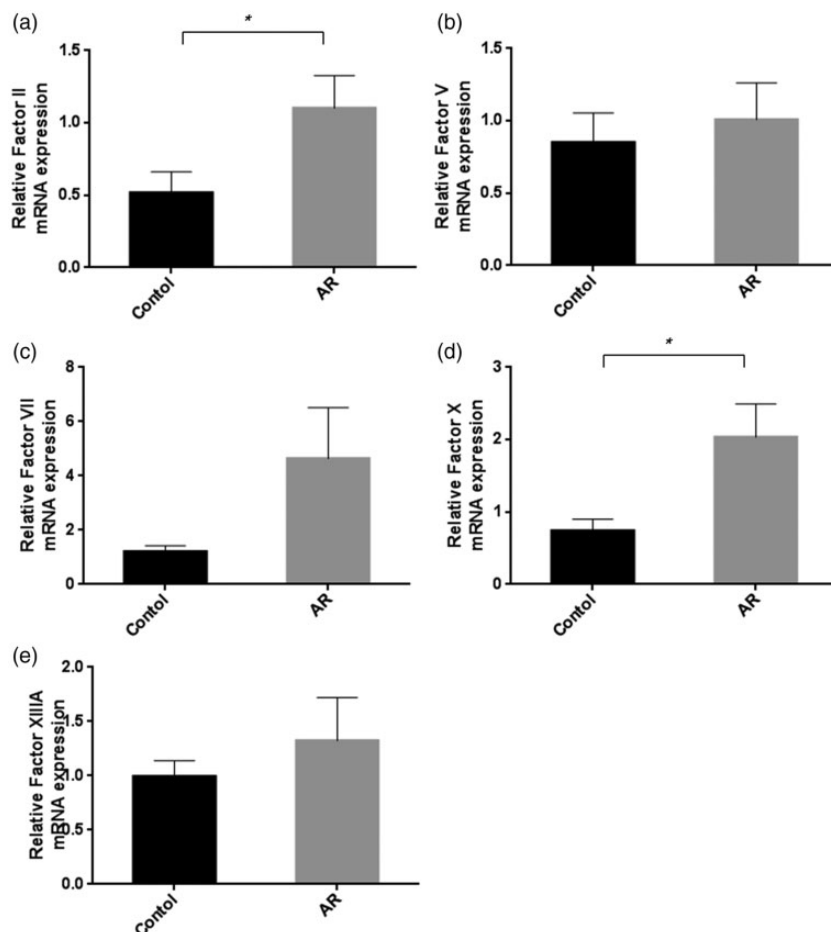


Figure 3. The mRNA expression of coagulation cascade components in mouse nasal tissues determined by real-time reverse transcription polymerase chain reaction (qRT-PCR): (a) factor II, (b) factor V, (c) factor VII, (d) factor X, and (e) factor XIII. Values are expressed as mean $\pm$ SEM. * $P < .05$.

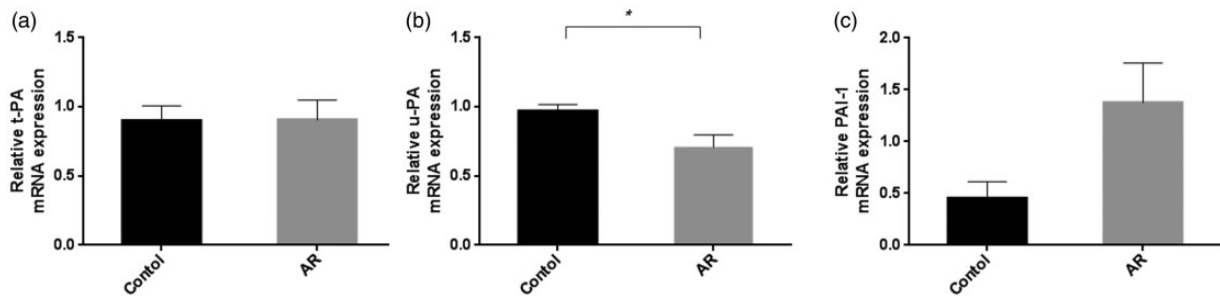


Figure 4. The mRNA expression of fibrinolysis system components in mouse nasal tissues determined by real-time reverse transcription polymerase chain reaction (qRT-PCR): (a) tissue-type plasminogen activator (t-PA), (b) urokinase-type plasminogen activator (u-PA), and (c) plasminogen activator inhibitor-1 (PAI-1). Values are expressed as mean $\pm$ SEM. * $P < .05$.

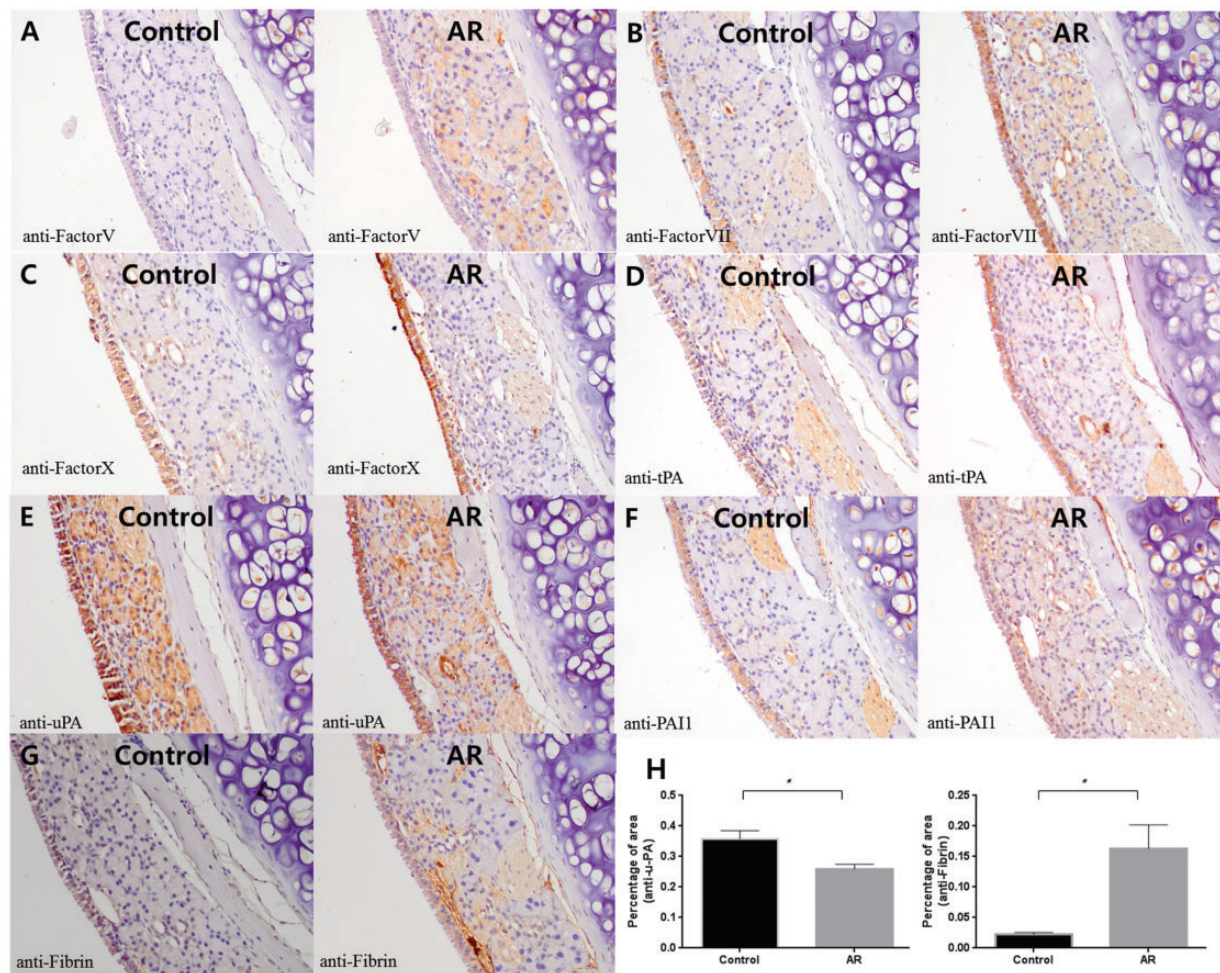


Figure 5. Immunohistochemical staining of fibrinolysis system components. Sections were immunostained with (A) anti-factor V, (B) anti-factor VII, (C) anti-factor X, (D) anti-tissue-type plasminogen activator, (E) anti-urokinase-type plasminogen activator, (F) anti-plasminogen activator inhibitor-1, and (G) anti-fibrin. H, Quantification by means of image analysis of urokinase-type plasminogen activator and fibrin. Values are expressed as mean $\pm$ SEM. * $P < .05$.

± 0.029 ng/mL). The ELISAs failed to detect PAI-1 in most of the control group lavage samples, whereas it was detected in more than half of the AR group samples (0.084 ± 0.052 ng/mL; Figure 6).

Discussion

It is known that a significant cross-talk exists between the coagulation system and the inflammatory process.^{6,7} Although AR is one of the most common allergic

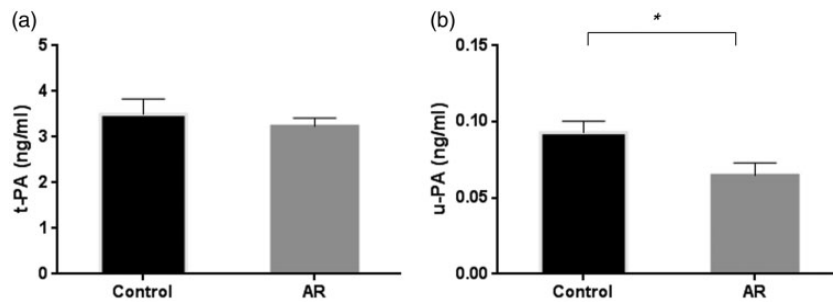


Figure 6. Enzyme-linked immunosorbent assay of fibrinolysis system components in nasal lavage fluid. Comparison of the concentrations of (a) tissue-type plasminogen activator (t-PA) and (b) urokinase-type plasminogen activator (u-PA) between the control and allergic rhinitis groups. Values are expressed as mean $\pm$ SEM. * $P < .05$.

inflammatory diseases, studies dealing with the relationship between the coagulation cascade and the fibrinolysis system are rare compared with those on other inflammatory diseases such as asthma and chronic rhinosinusitis. Moreover, most studies deal with either the components of the coagulation cascade or those of fibrinolysis, but not with both. In the present study, we reported changes in the expression of coagulation and fibrinolysis system components, skewing toward fibrin accumulation in the condition of AR.

Several studies have demonstrated the role of coagulation cascade components in inflammatory diseases. They suggest that the activation of the coagulation system occurs during the allergic response, and significant concentrations of thrombin (factor II) have been found in nasal secretions after allergic provocation in allergy patients.⁸ Thrombin may play a role in nasal polyp formation by stimulating vascular endothelial growth factor production in airway epithelial cells.⁹ Factor X transcript levels and factor X activity increase in the lungs of OVA-challenged asthmatic mice, and factor X may play a role in airway remodeling.¹⁰ In the present study using an AR mouse model, there was a significant increase in thrombin and factor X mRNA levels, which was consistent with previous reports, and other factors were also activated.

Plasmin, which dissolves fibrin clots, is activated by plasminogen activators. Plasminogen activators are inhibited by plasminogen activator inhibitors (PAIs), which belong to the protease inhibitor superfamily.¹¹ Previous studies have demonstrated a close relationship between fibrinolysis system components and inflammatory disorders. In one study of chronic rhinosinusitis, the t-PA levels were downregulated in the patient group, inhibiting the conversion of plasminogen to plasmin in the presence of Th2 cytokines.³ Increased synthesis of PAI-1 in the airways of asthma patients, which seemed to contribute to the development of airway hyperresponsiveness, was found in another study.¹² In the present study, the differences in the expression of t-PA mRNA and immunohistochemical staining intensity between the

AR and control groups were not pronounced. Instead, there were statistically significant differences in the mRNA levels of u-PA. Although statistically not significant, there was a strong tendency of increased expression in the mRNA level of PAI-1 in AR group. These results share similar aspects with other studies. u-PA-mediated fibrinolysis normally fulfils a protective function in inflammatory disease, and a decrease in the level of u-PA can cause sustained inflammation.¹³ Furthermore, the accumulation of fibrin, resulting from the altered balance between coagulation and fibrinolysis, is a feature of chronic inflammatory disease.¹⁴ The authors of one study reported an increased level of PAI-1 mRNA in allergic nasal tissues, whereas the level of t-PA mRNA did not change significantly in human nasal mucosa.¹⁵ On the other hand, t-PA gene depletion was associated with increased AR related remodeling process by comparing the nasal mucosa of a t-PA knock out AR mice with a wild-type AR mice.⁴ Alicja et al. demonstrated no difference in the u-PA level between AR and healthy human subjects. However, they assessed the circulating plasma level of u-PA and not the local reaction activity.⁵ Our immunohistochemical research revealed that the plasminogen activators were mostly localized in the epithelium and submucosal glands, which agreed with the findings reported in the literature.¹⁵ Plasminogen activators and their cellular receptors provide functional units that control inflammation by regulating the plasma contact system.¹⁶ Therefore, the downregulation of u-PA in the present study reflects a dysregulation of the inflammatory process, which results in the allergic condition.

IHC revealed that the expression patterns of the coagulation and fibrinolysis component proteins and mRNAs were similar. The degree of staining of most coagulation components and PAI-1 showed a tendency to increase, and u-PA staining was significantly less pronounced in the AR group. We performed ELISA of nasal lavage for the components that showed significant difference in both PCR and IHC analysis. Same expression patterns were found that so the u-PA concentration

was significantly lower in the AR group. However, there was no significant difference in the t-PA concentration between the 2 groups. We could not detect a significant concentration of PAI-1 in most of the control group samples, but ELISA revealed the presence of PAI-1 in some of the AR samples. We assume that this finding reflects the difference in PAI-1 concentration between the 2 groups.

The upregulation and downregulation of certain components in coagulation cascade and fibrinolysis system may be associated with the fibrin accumulation. Fibrin, which is the end product of the coagulation cascade, is essential for blood clotting and the repair of vessel injuries. However, it is also crucial for host defense and the modulation of the inflammatory response, because it affects leukocyte migration and cytokine production under inflammatory conditions.¹⁷ Previous studies have shown that the accumulation of fibrin can cause diverse chronic inflammatory diseases such as rheumatoid arthritis, severe asthma, glomerulonephritis, delayed-type hypersensitivity, and Crohn's diseases.¹⁸ Accumulated fibrins are assumed to retain plasma protein, resulting in mucosal edema, which is a histopathological characteristic of AR.^{3,19} Moreover, fibrin has its own proinflammatory properties; it can directly induce the production of chemokines by affecting endothelial cells and fibroblasts, thereby promoting the migration of leukocytes and macrophages.^{16,20} In the present study, as in previous studies, we detected fibrin deposition in the epithelial and submucosal regions, with more pronounced staining in the AR group than in the control group.

In terms of investigating a novel treatment target for AR, alterations in hemostatic components have great clinical significance. An aerosolized fibrinolytic agent, t-PA, significantly reduced mucosal hyperresponsiveness in mice with allergic airway inflammation.² The attenuated effect of fondaparinux, a factor X inhibitor, was proved in airway remodeling.¹⁰ Furthermore, the authors of previous studies have reported that the removal of fibrin can reduce the development of inflammatory diseases and symptoms.^{21,22}

The present study had several inherent limitations. First, even though the murine model is well established, it cannot fully reproduce human AR. Second, we only assessed the association between each component and allergic status; the causal relationship was not established. Future studies should focus on investigating the human sinonasal mucosa in a large number of individuals. Analyzing the clinical effects of agents that reduce fibrin formation would provide useful insight into the etiology of AR.

Conclusion

The present study reports the difference in coagulation cascade and fibrinolysis system components expression

in the nasal mucosa between a normal and AR murine model. Overall, the coagulation cascade components were upregulated and the fibrinolysis system components were downregulated, leading to fibrin deposition in AR condition. The dysregulation of hemostatic components in the nasal mucosa may be related to the pathogenesis of AR.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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E-SUPPLEMENT TABLE 1 Reverse transcription polymerase chain reaction primer sequences for coagulation cascade, fibrinolysis system components and glyceraldehyde-3-phosphate dehydrogenase.

Primer	Forward	Reverse	size
GAPDH	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG	74bp
F II	TGACCGGAAGGGGAAATACG	CCACCCACACACCTATCCAAA	97bp
F V	CCTGGTCAGCGCAACATCTA	GCCTGCATCCCAGCTTGATA	105bp
F VII	GACCATGTAGGGACCAAGCG	TTCTCCCACACGGGTACTCA	103bp
F X	CATCTGACCTGACACCCATGC	GCTACCCTTATCCAGAACTGCCA	129bp
F XIIIa	GAAGTACCCAGAGGCACACAG	TGGAGTTATTGGGCGGGAC	137bp
t-PA	AGAGATGAGCCAACGCAGAC	CAACTTCGGACAGGCACTGA	134bp
u-PA	AAATTCCAGGGGGAGCACTG	GTGTTGGCCTTTCCTCGGTA	86bp
PAI-1	CAAGGGGCAACGGATAGACA	AAGCAAGCTGTGTCAAGGGA	81bp

Abbreviations: t-PA, Tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; F, Factor