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Original Article

Nattokinase, profibrinolytic enzyme, effectively shrinks the nasal polyp tissue and decreases viscosity of mucus

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CRS, Chronic rhinosinusitis; CRSwNP, CRS with nasal polyps; CRSsNP, CRS without nasal polyps; NK, Nattokinase; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; FU, fibrin degradation units; UFH, unfractionated heparin; LMWH, low molecular weight heparin; FXIII-A, factor XIII-A; FDPs, fibrin degradation products

ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is often comorbid with asthma and resistant to therapeutic interventions. We recently reported that excessive fibrin deposition caused by impairment of fibrinolysis might play pivotal role in forming nasal polyp. Nattokinase (NK), a serine protease produced by *Bacillus subtilis*, has been reported to be a strong fibrinolytic enzyme. NK could be a promising drug candidate for use in the treatment of both CRSwNP and asthma. The objective of this study was to investigate the effects of NK on nasal polyp tissues from patients with CRSwNP. The nasal discharge from patients with CRSwNP and sputum from subjects with asthma were also used to investigate whether NK influences the viscosity of mucus.

Methods: To examine the effects on NK on nasal polyp tissues, pieces of nasal polyps were incubated either with saline or NK (10–1000 FU/ml) at 37 °C for 24 h. We assessed the presence of fibrin in nasal polyp tissue incubated with NK by means of immunohistochemistry. To examine the effects of NK on nasal discharge and sputum from patients with CRSwNP and asthma, respectively, were incubated with NK solution at 37 °C for 1 h.

Results: NK effectively shrinks the nasal polyp tissue through fibrin degradation. We also found that the viscosity of the nasal discharge and sputum from patients with CRSwNP and asthma, respectively, was significantly reduced by incubation with NK solution.

Conclusions: NK may be an effective alternative therapeutic option in patients with CRSwNP and comorbid asthma by causing fibrin degradation.

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Introduction

Chronic rhinosinusitis (CRS) is characterized by persistent symptomatic inflammation of the nasal mucosa and is one of the most common chronic diseases in adults.^{1–3} Although the etiology and pathogenesis of CRS remain elusive, allergies, bacterial and fungal infections, and structural abnormalities have all been theorized to play a role.⁴ CRS is generally divided into two types: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps

(CRSsNP). CRSwNP, in particular, is often comorbid with asthma and resistant to therapeutic interventions.¹ Previous studies suggested that CRSwNP is characterized by a Th2-skewed eosinophilic inflammation characterized by significantly elevated levels of IL-5, IL-13, eotaxin, and eosinophilic cationic protein.^{5–7} Many patients with CRSwNP suffer from nasal polyps and surgical intervention is frequently necessary to clear the nasal passages. Therefore, efforts to control nasal polyps have particular significance in improving the effects of CRSwNP therapy. Although oral and topical nasal steroid administration is effective in treating CRSwNP to some extent, side effects of long-term steroid use are a significant concern. Therefore, an alternative therapeutic approach would greatly aid in the treatment of CRSwNP.

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Nasal polyps usually arise from in and around the middle nasal meatus or paranasal sinuses. Major histopathological features of nasal polyps are intense infiltration of inflammatory cells, prominent edematous stroma, and formation of pseudocysts filled with plasma proteins, mainly albumin.⁸ We recently reported that excessive fibrin deposition in nasal polyps caused by impairment of fibrinolysis and acceleration of extrinsic coagulation cascade might play pivotal roles in forming edema or pseudocysts.^{9,10} These findings imply that degrading excessive fibrin in nasal polyps might therefore be of therapeutic value for treating patients with CRSwNP. Sputum from patients with asthma has been reported to be characterized by excessive fibrin formation that can lead to mucus plug formation, airway narrowing, and bronchial hyper-reactivity.¹¹ Excessive fibrin formation was also observed in the nasal discharge from patients with CRSwNP.¹²

Various fibrinolytic enzymes produced by microorganisms have been reported.^{13,14} Nattokinase (NK, also known as subtilisin NAT), a serine protease produced by *Bacillus subtilis*, is composed of 275 amino acid residues (molecular weight 27,724) and was originally discovered in natto, a cheese-like food made of fermented soybeans.^{15,16} The fibrinolytic mechanisms of NK have been examined more extensively than those of other microbial fibrinolytic enzymes.¹⁷ It has been reported to have an approximately four-fold stronger fibrinolytic activity than plasmin in clot lysis assays.¹⁸ Furthermore, NK not only degrades fibrin directly but also activates other fibrinolytic enzymes, such as pro-urokinase and tissue plasminogen activator (t-PA).^{17,19} NK also inactivates plasminogen activator inhibitor-1 (PAI-1) *in vitro*, the primary inhibitor of t-PA, resulting in the enhancement of fibrinolysis.²⁰ Because excessive fibrin deposition in the nasal mucosa plays a pivotal role in forming nasal polyps, the fibrinolytic effects of NK might be a new therapeutic approach for patients with CRSwNP.

Here we investigated the effect of NK on nasal polyp tissues obtained during routine functional sinus surgery in patients with CRSwNP. The nasal discharge from patients with CRSwNP and sputum from subjects with asthma were also used to investigate whether NK influences the viscosity of mucus. The results raise the important new therapeutic possibility of using NK to treat patients with CRSwNP and asthma.

Methods

Patients and sample preparation

All subjects signed an informed consent, and the protocol and consent forms governing procedures for the study were approved by the institutional review board of the University of Fukui, in accordance with the ethical principles contained in the Declaration of Helsinki. Patients with CRSwNP were recruited from the Department of Otorhinolaryngology Head & Neck Surgery of the University of Fukui. Nasal polyp tissues were obtained during routine functional endoscopic sinus surgery, and the nasal discharge was also collected. All patients met the criteria for CRS, as defined by the guidelines of the European position paper on rhinosinusitis and nasal polyps.²¹ The inferior turbinate tissue, used for the control nasal mucosa, was obtained from 12 patients undergoing nasal surgery, who either had a septal deviation and conchal hypertrophy. Patients with an established immunodeficiency, pregnancy, coagulation disorder, or a diagnosis of classic allergic fungal sinusitis, Samter's triad, Churg–Strauss syndrome, or cystic fibrosis were excluded from the study. Our study excluded patients treated with systemic or topical corticosteroids within the 4 weeks prior to surgery. Other than corticosteroids, subjects were on a variety of medications, including nonsteroidal anti-inflammatory drugs and antihistamines. The subjects' characteristics

are shown in Table 1, 2. Patients diagnosed with moderate-to-severe asthma by a medical specialist (certified by either the Japanese Respiratory Society or Japanese Allergy Society) underwent sputum induction by inhaling nebulized hypertonic saline (3%). Details of these subjects' characteristics are shown in Table 3.

Nattokinase

NK (NSK-SD; Japan Bio Science Laboratory, Osaka, Japan) is a spray-dried powder of a compound including an extract of fermentation products prepared by *B. subtilis natto* and dextrin as a stabilizer. The fibrinolytic activity of NK is measured in fibrin degradation units (FU), and NK powder was dissolved in saline to achieve the desired concentrations. Normal saline without NK was used as a control.

Effects of NK on nasal polyps

Surgically obtained nasal polyps were divided into approximately 5-cubic millimeter pieces, and the weight of each was measured. Pieces of nasal polyp were incubated either with 1 ml of saline or NK (10–1000 FU/ml) at 37 °C for approximately 24 h with gentle agitation, after which the weights were measured again.

Western blotting

Western blotting of supernatants was performed as follows. Approximately 100 mg of nasal polyp tissues were incubated with 1 ml of NK solution or saline at 37 °C for 24 h with gentle agitation. After incubation of the nasal polyp with NK solution or saline, the supernatant was collected. Supernatants were boiled for 5 min in an equal volume of 2× Laemmli running buffer (Bio-Rad, Hercules, CA, USA) containing 5% v/v β-mercaptoethanol (Bio-Rad), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels (Bio-Rad); the protein was transblotted to Hybond-P (GE Healthcare Life Sciences, Uppsala, Sweden) in transfer buffer (192 mM glycine, 25 mM Tris, 2.5 mM SDS, and 10% methanol). The blots were blocked with 3% nonfat dry milk in pH 7.4 tris-buffered saline (TBS) with 0.25% Tween-20. Then, they were incubated with anti-d-dimer antibody (1: 100) (Santa Cruz Biotechnology, Dallas, TX, USA). The blots were incubated secondarily with horse radish peroxidase-conjugated anti-mouse IgG antibody (Dako, Agilent Technologies, Santa Clara, CA, USA). Subsequently, the blots were developed with chemiluminescence Western blot detection reagents (Dako) according to the manufacturer's instructions.

Mouse experiment

This study was approved by the Animal Research Committee, University of Fukui (Permission number: 27076) and carried out according to the Regulations for Animal Research at University of Fukui, and every effort was made to minimize the suffering of the animals. The euthanasia of all experimental mice was achieved by cervical dislocation.

Wild-type BALB/c mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Mice were nasally administrated NK, 50 µl of NK solution, or 50 µl saline alone for one day or 7 consecutive days. At 24 h or 7 days after injection, the mice were sacrificed. Histological examinations of mouse nose specimens were performed as previously described.²⁷ In brief, the facial skin was stripped, heads were severed between the upper and lower jaws, and the nose was removed; thereafter, it was fixed in 4% paraformaldehyde for 3 days and decalcified in 0.12 mol/L EDTA solution (pH 6.5) for 7 days at room temperature. After

Table 1

Subject characteristics (NP tissues from patients with CRSwNP).

No	Age	Sex	Asthma	Aspirin intolerance	Blood eosinophils, (%)	Tissue eosinophils, (/HPF)	CT score	Total IgE
1	71	M	N	N	4.1	2	10	111
2	39	F	N	N	2.4	11	U	15.7
3	63	M	N	N	3.2	85	15	356
4	31	M	Y	N	4.2	3	22	160
5	57	M	U	U	U	U	U	U
6	29	F	Y	Y	13.2	121	10	97
7	64	M	Y	Y	5.7	156	22	517
8	74	M	N	N	5.8	15	22	1000
9	76	M	N	N	2.6	76	12	78.4
10	55	M	N	N	2.5	35	9	137
11	70	F	N	N	1.4	5	17	U
12	67	M	N	N	3.3	3	14	258
13	54	M	N	N	8.5	112	20	100
14	55	M	N	N	1.7	23	6	505

F, female; M, male; Y, yes; N, No; U, unknown; HPF, high power field.

Table 2

Subject characteristics (Nasal discharges from patients with CRSwNP).

No	Age	Sex	CRSwNP	Asthma	Aspirin intolerance	Blood eosinophils, (%)	Total IgE
1	64	M	Y	Y	Y	5.7	517
2	31	F	Y	Y	N	5.6	37
3	55	M	Y	Y	Y	6.4	510
4	42	M	Y	N	N	5.6	137
5	29	F	Y	N	N	0.7	17
6	72	F	Y	N	N	8.5	136

F, female; M, male; Y, yes; N, No; U, unknown; CRSwNP, chronic rhinosinusitis with nasal polyp.

decalcification, the specimens were embedded in paraffin, and 4 μ m coronal sections were sectioned and stained with hematoxylin and eosin.

Evaluation of viscosity measurement of the nasal discharge or sputum

The viscosity of the nasal discharge or sputum was measured using a disposable dropper (Nippon Genetics, Tokyo, Japan). The higher the viscosity of the mucus, the larger the lump formed. The volume of one drop of each specimen of the nasal discharge or sputum was measured. Purulent nasal discharge obtained from patients with CRSwNP was divided into 150–500 μ l aliquots, which were placed in 24-well plates containing 1000 μ l of saline or NK solution (1000 FU/ml) and then incubated at 37 °C for 1 h with gentle agitation. Sputum obtained from patients with asthma were divided into 500 μ l aliquots and placed in 24-well plates containing 500 μ l of saline or NK solution (1000 FU/ml) and incubated at 37 °C for 1 h with gentle agitation.

Effect of heparin on nasal polyp tissue

Surgically obtained nasal polyps were divided into approximately 5 mm³ pieces, and the weight of each was measured. Pieces of tissue were incubated with 10 IU/ml of UFH or 10 IU/ml of LMWH at 37 °C for approximately 24 h with gentle agitation, after which the weights were measured again.

Histological analysis

After treatment with saline or NK, the pieces of nasal polyp were weighed and then immediately fixed in 10% formalin, embedded in

Table 3

Subject characteristics (Sputum from patients with asthma).

No	Age	Sex	CRSwNP	Aspirin intolerance	Blood eosinophils (%)	Total IgE
1	71	F	Y	N	3.8	305
2	61	F	N	N	3.3	320
3	84	F	U	N	6.7	378
4	65	F	Y	N	3.7	1208
5	79	F	U	N	8.1	79.7
6	28	F	U	N	12.6	423
7	72	F	Y	N	9.7	405
8	68	M	U	N	0	U
9	52	F	Y	N	U	U
10	33	F	N	N	4.9	154
11	77	F	U	N	8.2	U
12	77	F	U	N	2.8	783
13	43	F	U	N	1.2	53
14	50	F	U	N	10.6	155
15	67	F	U	N	3.6	26
16	67	F	N	N	1.2	157
17	21	F	N	N	7.2	168
18	39	F	Y	N	6.1	147
19	59	F	Y	Y	4	758
20	48	F	Y	Y	6.2	183.6

F, female; M, male; Y, yes; N, No; U, unknown; CRSwNP, chronic rhinosinusitis with nasal polyp.

paraffin, and sectioned in 3- μ m slices using a Retortome REM-710 (Yamato Kohki, Saitama, Japan). Blocked sections were incubated with anti-human fibrin antibody (Sekisui Diagnostics, Stamford, CT, USA) at 4 °C overnight. After washing, sections were incubated with ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 1 h. Sections were rinsed and incubated in DAB reagent (Invitrogen, Carlsbad, CA, USA) and then counterstained with hematoxylin. Sections were dehydrated, cleaned, mounted, and coverslipped using PARAmount-N (Falma, Osaka, Japan). Microscopic analysis was performed with an Olympus BX53 upright research microscope using a 40 $\times$ objective lens, and images were collected with cellSens (Olympus, Tokyo, Japan).

Statistical analysis

All data are reported as mean $\pm$ SEM unless otherwise noted. Differences between groups were analyzed using a paired Student's t test. A P value of less than 0.05 was considered statistically significant.

Results

NK shrinks nasal polyps through fibrin degradation

Our recent findings suggested that excessive fibrin deposition was observed in the submucosa of nasal polyp tissue.⁹ NK is well known to degrade fibrin clot directly and indirectly.¹⁷ Nasal polyp tissues were collected from 14 subjects with CRSwNP to determine the effect of NK on nasal polyp tissue. Subjects' characteristics are shown in Table 1. To evaluate the effects of NK on nasal polyp tissue, pieces of nasal polyp were incubated either with saline or NK (10–1000 FU/ml) at 37 °C for approximately 24 h with gentle agitation, after which the weights changes were observed. The weight of the nasal polyp tissue incubated with saline was not altered (188.1 $\pm$ 29.2 to 192.6 $\pm$ 28.1 mg; mean $\pm$ SD), but the weights of tissues incubated with NK (100 and 1000 FU/ml) decreased significantly (100 FU/ml: 162.7 $\pm$ 25.1 to 67.8 $\pm$ 15.8 mg; P < 0.001, 1000 FU/ml: 199.3 $\pm$ 32.3 to 67.3 $\pm$ 14.29 mg; P < 0.001) (Fig. 1A) in a time- and dose-dependent manner (Fig. 1B, C). The nasal polyp tissue incubated with NK appeared to become thin

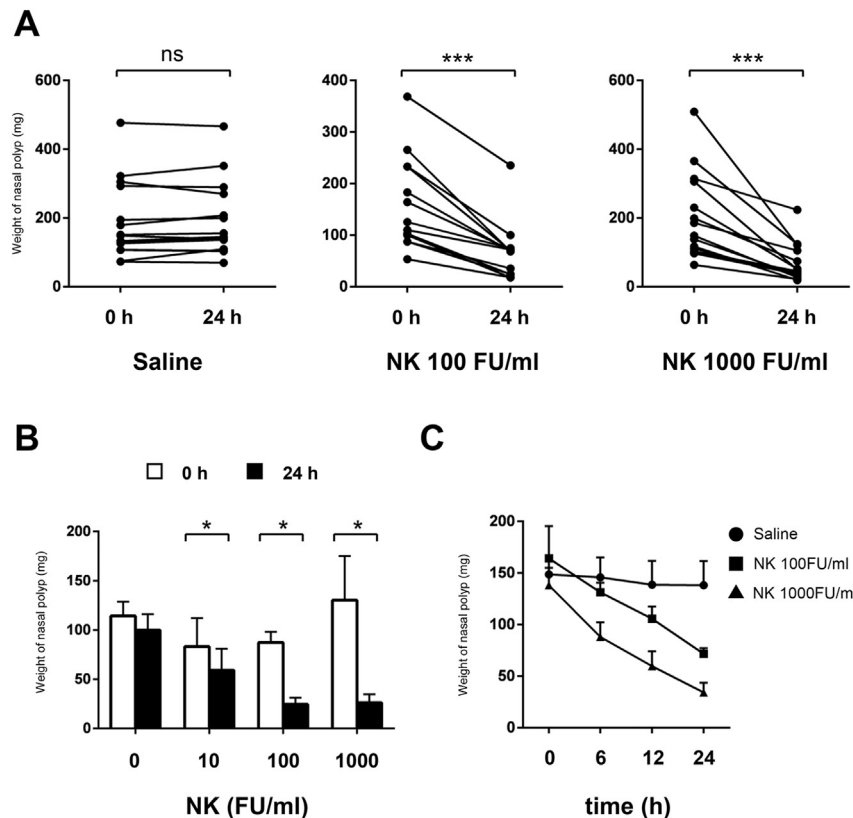


Fig. 1. NK effectively dissolved the nasal polyp tissue from patients with CRSwNP. (A) Nasal polyp tissues were weighed and then incubated for 24 h with saline or 100 or 1000 FU/ml of NK. After incubation, the tissue was cleaned and weighed. (B) Nasal polyp tissues were incubated for 24 h with 0–1000 FU/ml of NK. (C) Nasal polyp tissues were incubated with 100 or 1000 FU/ml of NK for approximately 24 h. The results shown are expressed as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

(Fig. 2A). Therefore, we wanted to assess whether digestion of the nasal polyp tissue by NK depends on fibrin degradation. To observe the presence of fibrin in the nasal polyp tissue incubated with NK, we performed immunohistochemical analysis using anti-human fibrin antibody. Decreased fibrin staining was observed in the nasal polyp tissue incubated with NK (Fig. 2B). Since NK has strong fibrinolytic activity, we assessed the levels of fibrin degradation products (FDPs) in the supernatant of the nasal polyp tissue after incubation with the NK solution. We measured the levels of d-dimer, which is an important FDP. d-Dimer protein levels were increased in a dose-dependent manner (Fig. 2C). These results suggest that NK digests the nasal polyp tissue through fibrin degradation.

NK shrinks nasal polyps through fibrinolytic activity

To verify that digestion of nasal polyp tissue was caused by fibrinolytic activity, nasal polyp tissues were incubated with plasmin (10 and 100 mU/ml), the major fibrinolytic enzyme. Likewise, NK, the weight of nasal polyp tissue was decreased significantly (10 mU/ml: 121.0 ± 10.2 to 101.8 ± 8.9 mg; $P < 0.05$, 100 mU/ml: 131.7 ± 9.4 to 88.8 ± 6.6 mg; $P < 0.001$) by incubation with plasmin (Fig. 3A).

Since heparin possesses anti-inflammatory effects and several studies suggested that heparin is a therapeutic candidate for treating allergic diseases, including CRSwNP,¹² we wanted to see the effects of unfractionated heparin (UFH) or low molecular weight heparin (LMWH) on nasal polyp tissue. Incubation of the nasal polyp tissue with heparin did not result in any weight change

(10 IU/ml UFH: 181.9 ± 31.7 to 186.5 ± 33.2 mg, 10 IU/ml LMWH: 228.0 ± 14.7 to 228.8 ± 8.8 mg) (Fig. 3B).

We also evaluated the effect of papain (10 μ M and 100 μ M), a well-known representative of cysteine protease family, on nasal polyp tissue. Incubation of the nasal polyp tissue with papain did not result in any weight change (10 μ M: 103.9 ± 8.8 – 101.7 ± 9.0 mg, 100 μ M: 105.6 ± 11.0 – 99.3 ± 10.9 (Fig. 3C)). These results suggest that digestion of nasal polyp tissue is caused by fibrinolytic activity instead of by anti-coagulant effects or proteolytic activity.

NK does not affect normal nasal mucosa

To see if NK affects normal nasal mucosa, we used the inferior turbinate as control nasal mucosa. Although, control nasal mucosa incubated with either saline or NK solution appeared to become slightly swollen (Fig. 4B), we could not find any significant weight changes in the inferior turbinate tissue on incubation with NK (Saline: 71.1 ± 8.5 – 74.3 ± 8.9 mg, 100 FU/ml: 73.1 ± 8.3 – 72.4 ± 8.3 mg, 1000 FU/ml: 75.3 ± 7.9 – 71.9 ± 8.3 mg) (Fig. 4A). We next examined the direct effects of NK on nasal mucosa *in vivo*. In mice, nasal administration of saline or NK solution (1000 FU/ml) and histological examination of the nasal cavity thereafter revealed that the NK solution does not affect the nasal mucosa of mice (Fig. 4C).

Effects of NK on the nasal discharge and sputum

Given that NK effectively degrades fibrin clot, we hypothesized that NK can also reduce the viscosity of the nasal discharge and sputum from patients with CRSwNP and asthma, respectively. Mucus was collected from 6 subjects with CRSwNP and 20 subjects

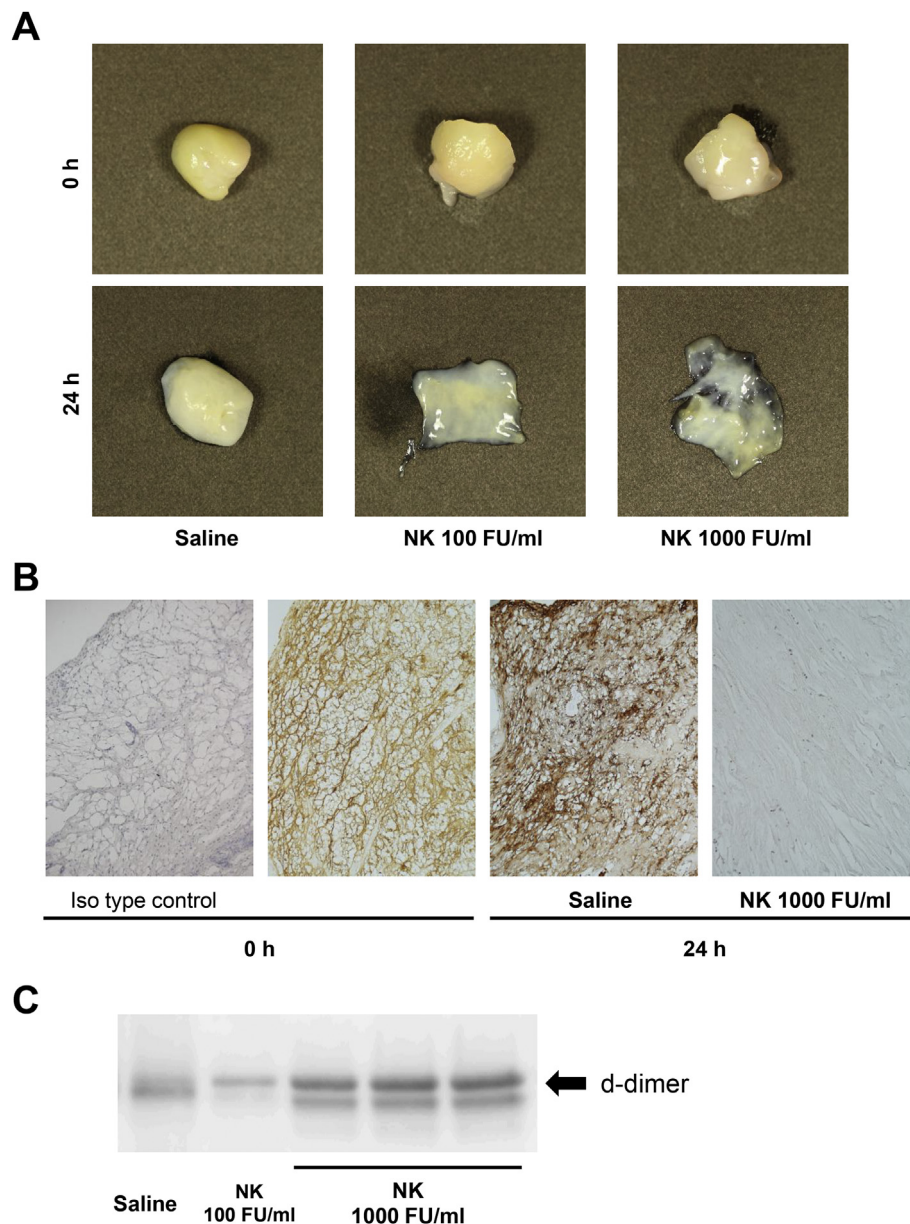


Fig. 2. Effect of NK on the nasal polyp tissue. **(A)** NP tissues were incubated for 24 h with saline or 100 or 1000 FU/ml of NK. **(B)** Immunohistochemical detection of fibrin was performed using anti-human fibrin mAb. Representative immunostaining for fibrin in the NP tissue from a control (no incubation) and the tissue was incubated for 24 h with saline or 1000 FU/ml of NK. Negative control antibody staining in nasal polyp tissue is shown (left panel). **(C)** Western blotting. The levels of d-dimer in the supernatant of the nasal polyp tissue after incubation with NK solution (0, 100, 1000 FU/ml).

with asthma to determine the effect of NK on mucus. Subjects' characteristics are shown in Table 2, 3. To examine the effect of NK on nasal discharge or sputum, nasal discharge and sputum from patients with CRSwNP and asthma, respectively, were incubated with NK solution at 37 °C for 1 h. The viscosity of the nasal discharge from patients with CRSwNP was not altered by incubation with saline (345.2 ± 65.3 – 354.2 ± 74.7 ml), whereas incubation with NK solution significantly reduced the viscosity (371.7 ± 69.1 – 45.0 ± 9.2 ml, $P < 0.01$) (Fig. 5A). The gross appearance of the nasal discharge was ameliorated by incubation with NK solution (Fig. 5B). We also observed that incubation with NK solution reduced the viscosity of sputum from patients with asthma (234.5 ± 35.1 – 26.5 ± 1.2 ml; $P < 0.01$) (Fig. 5C), and the gross appearance of the sputum was also improved by incubation with NK solution (Fig. 5D).

Discussion

NK has been reported to be a strong fibrinolytic enzyme *in vitro* and *in vivo*.¹⁶ The current study demonstrated that NK effectively shrinks the nasal polyp tissue through fibrin degradation. We also found that the viscosity of the nasal discharge and sputum from patients with CRSwNP and asthma, respectively, was significantly reduced by incubation with NK solution.

Because patients with nasal polyps typically complain of nasal obstruction and loss of smell, causing markedly impaired quality of life, treatment of CRSwNP primarily aims to shrink or eliminate nasal polyps. In the nasal polyp tissue, activation of mast cells and eosinophils facilitates plasma exudation, intense edema, or pseudocyst formation, which are the major histopathologic characteristics of nasal polyps.^{8,22} New pharmacological approaches

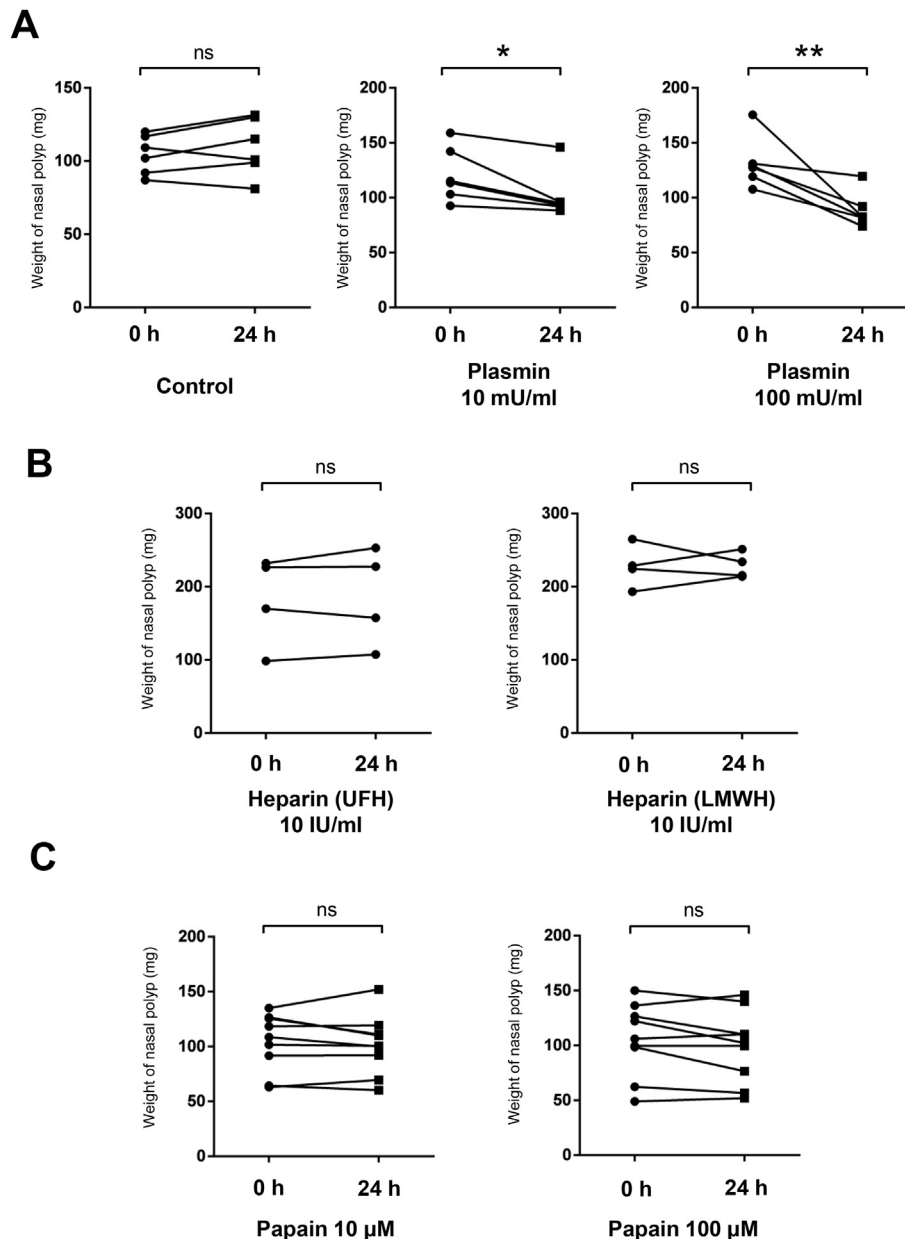


Fig. 3. Fibrinolytic activity dissolves the nasal polyp tissue. **(A)** Nasal polyp tissues were weighed and then incubated for 24 h with saline or 10 or 100 mU/ml of plasmin. After incubation, the tissue was cleaned and weighed. **(B)** Nasal polyp tissues were incubated for 24 h with UFH (left panel) or LMWH (right panel). **(C)** Nasal polyp tissues were incubated for 24 h with 10 or 100 μM of papain. * $p < 0.05$, ** $p < 0.01$.

targeting these effector cells have recently been suggested in addition to the conventional therapies, including topical corticosteroids and antihistamines.²³ However, management of CRSwNP is still unsatisfactory. We have demonstrated that profound fibrin deposition plays a pivotal role in the retention of exuded plasma proteins through tissue inflammation and the formation of intense edema and pseudocysts in nasal polyp tissues.⁹ On the basis of this pathophysiologic observation, degradation of fibrin in nasal polyp tissues is a reasonable therapeutic approach to treat patients with CRSwNP.

In the current study, we used surgically obtained nasal polyp tissues. It seems likely that the mechanisms by which NK shrinks nasal polyps might be due to cleavage of cross-linked fibrin. Besides direct fibrinolytic activity, NK influences the expression of other fibrinolytic agents, including t-PA, plasmin, and pro-urokinase, to

degrade fibrin.¹⁷ Furthermore, NK inactivates PAI-1, a down-regulator of the fibrinolysis cascade, resulting in increased fibrinolytic activity.²⁰ Among the effects of NK, the enhancement of t-PA release from cells is of particular interest. We recently found that expression of t-PA was significantly decreased in the nasal polyp tissue. Stimulation by Th2 cytokines downregulated the expression of t-PA in cultured nasal epithelial cells, suggesting a potential role of Th2-related mechanisms in nasal polyps.⁹ Furthermore, we have also demonstrated that tissue levels of coagulation factor XIII-A (FXIII-A) are profoundly increased in the nasal polyp tissue and that M2 macrophages are major FXIII-A-producing cells in nasal polyps.¹⁰ FXIII-A is a transglutaminase that participates in the final stage of the coagulation cascade. Activated FXIII-A catalyzes the formation of covalent cross-links between γ -glutamyl and ϵ -lysyl residues on adjacent fibrin chains in polymerized fibrin to yield a

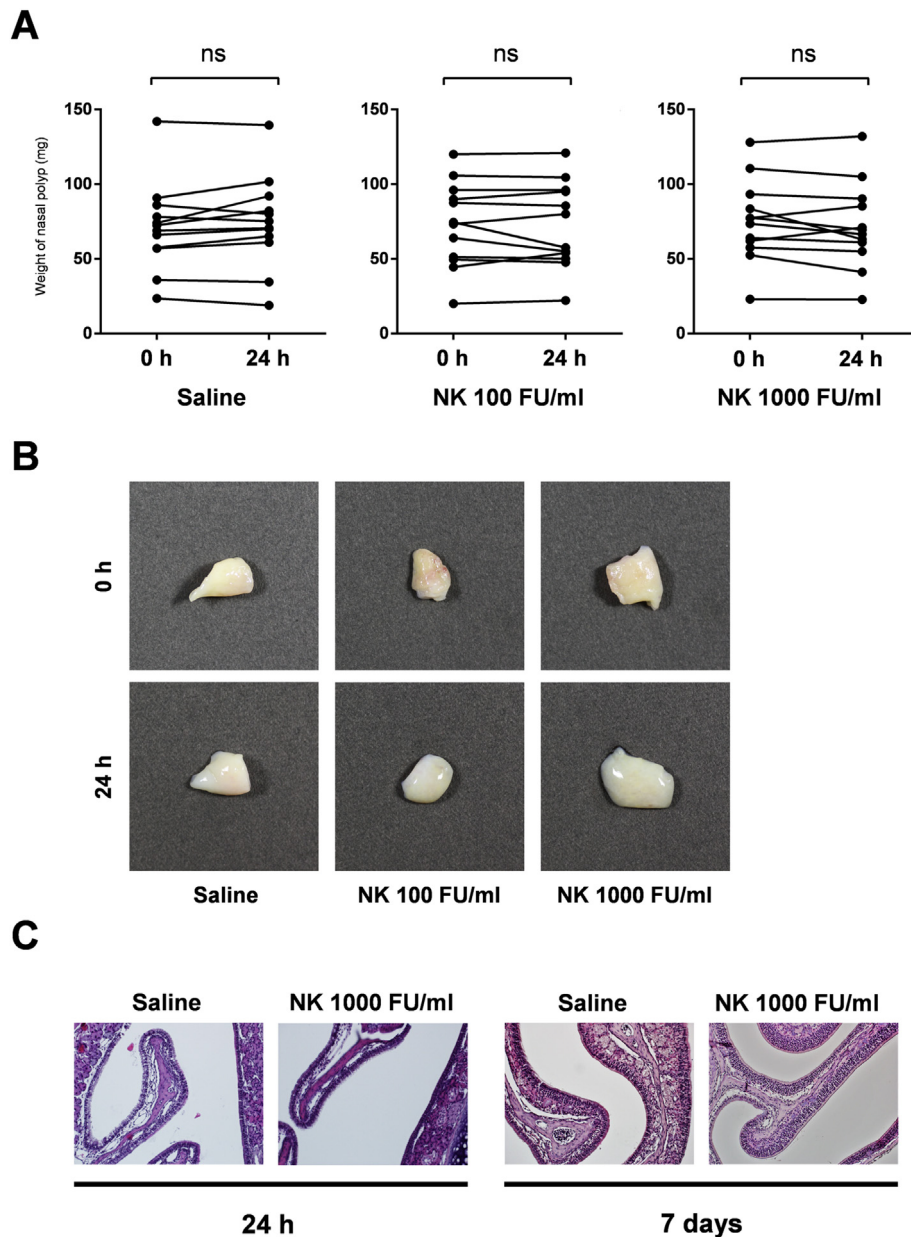


Fig. 4. Effects of NK on normal nasal mucosa. **(A)** Inferior turbinate tissues were weighed and then incubated for 24 h with saline or 100 or 1000 FU/ml of NK. After incubation, the tissue was cleaned and weighed. **(B)** Mice histological examination of nasal cavity after being nasally administrated with saline or NK solution (1000 FU/ml) **(C)**.

mature clot. The cross-linking of fibrin enhances its stiffness and rigidity, allowing it to retain exuded plasma proteins, resulting in intense edema and pseudocysts in nasal polyps.²⁴ NK potentiates not only fibrinolytic activity but also anticoagulation. NK administration in human subjects has been reported to decrease plasma levels of fibrinogen, factor VII, and factor VIII. The authors of that study argued that administration of NK could be considered as a cardiovascular disease nutraceutical, acting through down-regulation of the coagulation cascade.²⁵ Therefore, the actions of NK make it an attractive therapeutic candidate for protection against recurrence of nasal polyps after surgery by inhibition of the coagulation cascade.

Patients with CRSwNP often have comorbid asthma, with a poor therapeutic response and high rate of recurrence, making their disease more recalcitrant.²¹ In both sinusitis and asthma, the high viscosity of mucus aggravates symptoms. Sticky, viscous mucus worsens bronchial obstruction in asthma. Here we found that NK

solution effectively dissolved and reduced the viscosity of mucus from patients with CRSwNP and asthma within a short period (Fig. 5). It has been reported that excessive fibrin formation is observed in the sputum from patients with asthma. Physiologically undesirable, excess fibrin may lead to mucus plug formation, airway narrowing, and bronchial hyper-reactivity.¹¹ Profound fibrin formation has also been observed in the nasal discharge from patients with CRSwNP.¹² Therefore, the effect of NK on mucus is most likely due to cleavage of cross-linked fibrin. Thus, efforts to limit fibrin deposition in the course of CRSwNP and asthma have particular significance for improving the effects of treatment.

It is to be noted that although proteins are usually digested into peptides or amino acids in the gastrointestinal tract by the effects of digestive enzymes, NK has pH and temperature stability, retaining its protease activity after intestinal absorption.¹⁶ Several studies have reported that oral administration of NK appears to enhance fibrinolysis and anticoagulation simultaneously by several different

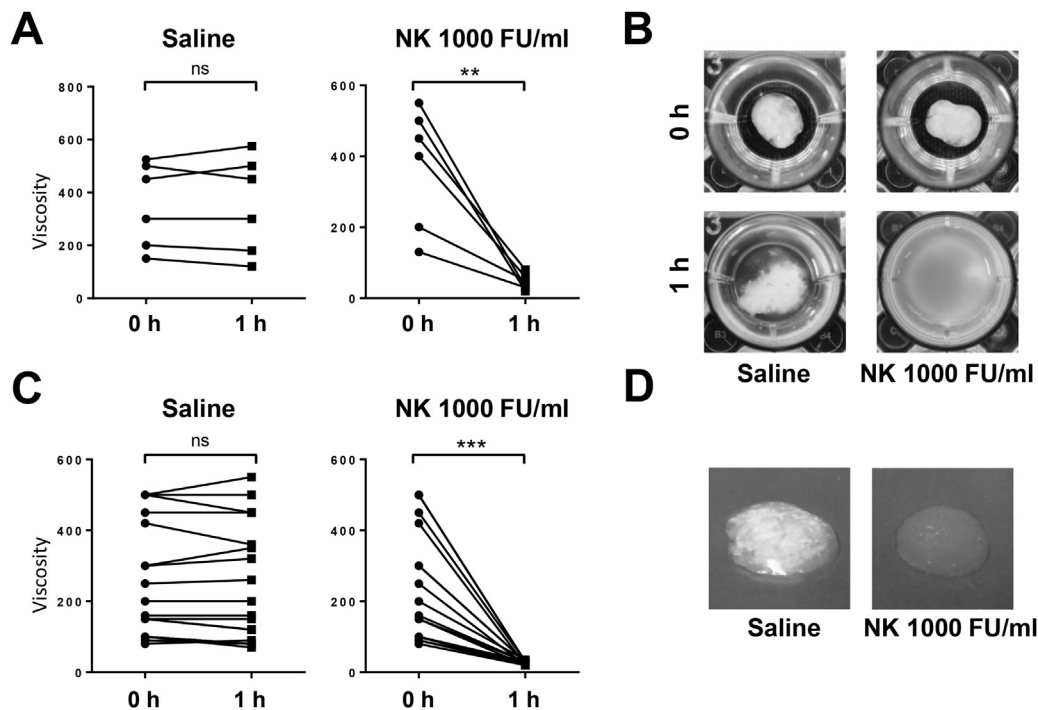


Fig. 5. NK decreased the viscosity of mucus from patients with CRSwNP or asthma. (A, B) Viscosity of the nasal discharge at baseline and after 1 h of incubation with saline or 1000 FU/ml of NK. Viscosity of the sputum at baseline and after 1 h of incubation with saline or 1000 FU/ml of NK (C, D). ** $p < 0.01$, *** $p < 0.001$.

pathways in animal and human experiments.²⁶ NK is purified from natto, a traditional Japanese food that has been consumed daily for more than 1000 years. This provides strong reassurance of the safety of oral administration of NK.¹⁹ The possibility of both local and oral administration of NK suggests it is a promising drug candidate for use in the treatment of both CRSwNP and asthma as well as prevention of nasal polyp recurrence after surgery.

Since NK possesses both fibrinolytic and anti-coagulant activity, it should be cause of concern that NK administration might lead to hemorrhagic diathesis, and it is important to address the potential toxicities and comprehensive safety profile of the ingredient. Several studies have reported that the oral administration of NK (2000–4000 FU/day) did not cause any side effects, including bleeding tendency.^{25,28} These results support the safety of NK intake. In an animal model, intravenous administration of NK did not show any cytotoxic effect.¹⁸ We also observed that the nasal administration of NK in mice did not affect the histological integrity of the nasal mucosa (Fig. 4C). However, because we also observed cytotoxic effects of NK on normal human bronchial epithelial cell in a dose dependent manner (data not shown), additional studies are needed to further develop a more informed safety profile of NK *in vivo*.

We showed that NK effectively shrinks surgically removed the nasal polyp tissue by causing fibrin degradation. In addition, the viscosity of the nasal discharge and sputum was significantly reduced by incubation with NK solution. Our results provide proof of the concept that NK may be an effective alternative therapeutic option in patients with CRSwNP and comorbid asthma. Further studies on NK are needed to examine the details of its metabolism, appropriate dosing and duration, and safety for the treatment of humans.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TTa and SF designed the research. TTa, YI, MS, TTo and NN collected nasal polyp tissues and nasal discharge from patients with CRSwNP. TI collected sputum from patient with asthma. TTa and YI evaluated the effects of nattokinase on nasal polyp tissue, nasal discharge, and sputum. TTa performed immunohistochemistry. YK and KY performed mouse experiment. All authors revised the manuscript and approved the final version.

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