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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.

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 hyperreactivity.[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 antiinflammatory 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 Allergology 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 b-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