Vascular endothelial growth factor produced in nasal glands of perennial allergic rhinitis

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ABSTRACT

Background: *Vascular endothelial growth factor (VEGF), a pleiotropic polypeptide that mediates endothelial cell–specific responses such as induction of angiogenesis and vascular leakage, is hyperproduced in a variety of inflammatory disorders. In asthma, VEGF hyperproduction promotes mucosal edema by enhancing vascular leakage. However, in allergic rhinitis, details of the pathophysiological importance remain unclear. This study was designed to investigate and discuss the pathophysiological significance of VEGF in nasal secretions from perennial allergic rhinitis sufferers.*

Methods: *Seven allergic rhinitis patients sensitized with house-dust mites and 12 chronic rhinosinusitis patients were enrolled. Nasal secretion VEGF was quantified and compared between groups. In allergic rhinitis cases, nasal lavage VEGF was estimated before and after the antigen provocation. Nasal gland VEGF was immunohistochemically investigated. VEGF messenger RNA (mRNA) levels in serous and mucous acini were analyzed by laser microdissection and light cycler-polymerase chain reaction.*

Results: *VEGF levels in nasal secretions and nasal lavage from allergic rhinitis were higher than in nonallergic rhinosinusitis, after rather than before antigen provocation. VEGF mRNA expression was higher in serous versus mucous acini. These results are consistent with the immunohistochemistry results.*

Conclusion: *In allergic rhinitis, there was significant VEGF production in serous acini, which was hypersecreted after antigen provocation. VEGF may play an important role in pathophysiology of allergic rhinitis.*

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Key words: Acute phase reaction, allergic rhinitis, house-dust mite, laser microdissection, nasal glands, serous acini, vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a pleiotropic polypeptide that mediates endothelial cell–specific responses, such as induction of angiogenesis and vascular leakage in tumor growth, as well as chronic inflammation.[1–3] Recently, isoforms of VEGF and its receptors (VEGFRs), along with their mutual specific binding patterns, have been clarified. The biologically predominant isoforms of VEGF are able to bind to VEGF-specific tyrosine-kinase receptors (VEGFR-1/flt-1 and VEGFR-2/KDR) and induce angiogenesis and vascular leakage.[4,5]

VEGF is hyperproduced in a variety of inflammatory disorders, including arthritis and retinopathy.[6,7] In upper and lower respiratory tract inflammation, increased secretory levels of VEGF are reportedly present.[8–11] In individuals with asthma, VEGF overproduction promotes not only mucosal edema but also antigen sensitization and type 2 helper T cell–associated inflammatory response.[12] We found increased VEGF levels in the effusion of paranasal sinuses in chronic rhinosinusitis (CRS), which indicates VEGF hyperproduction in the sinus mucosa is in response to hypoxia, bacterial endotoxin, and inflammatory cytokines in paranasal sinuses, during upper respiratory tract inflammation.[13,14]

To date, there are only a few reports of VEGF in allergic rhinitis, in which increased VEGF production has been identified in nasal mucosa or in secretions from seasonal allergic rhinitis cases.[15,16] Its source and pathophysiological importance in allergic rhinitis are unclear.

To study the pathophysiological importance of VEGF in allergic rhinitis, we compared VEGF levels in the nasal secretions of perennial allergic rhinitis and nonallergic rhinosinusitis. Additionally, increased VEGF levels in nasal lavage were confirmed after antigen provocation in perennial allergic rhinitis. Subsequently, VEGF was studied immunohistochemically in nasal mucosa, and its production was quantitatively analyzed in serous and mucous acini by enzyme-linked immunosorbent assay (ELISA), and at the messenger level including VEGF isoforms by reverse transcriptase–polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Patients

Subjects, enrolled at random from the outpatients clinic of Kagoshima University Hospital, consisted of 12 CRS patients without complicating allergic rhinitis or asthma, 7 perennial allergic rhinitis patients sensitized with house-dust mites without complicating CRS or asthma, and 8 cases free from allergic rhinitis or CRS used as controls. Control cases were eventually diagnosed as moderate acute or chronic rhinitis without type 1 allergic etiology (Table 1).

CRS was diagnosed based on clinical symptoms, such as

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Table 1 **Demographic characters of patients and controls**

Characteristic	CRS	Allergic Rhinitis	Control
No. of subjects	12	7	8
Age Average ± SD (yr)	39 ± 3.1	28 ± 2.5	47 ± 3.5
Sex (No.)			
male	6	4	5
female	6	3	3
Duration of illness	>5 yr	>5 yr	Unknown
Diseases (No.)			
Bronchial asthma	0	0	0
Allergic rhinitis	0	7	0
Paranasal sinusitis	12	0	0
Positive subjects (No.)			
After provocation by H.D.	0	7	0
Medication			
Topical steroids	0	2	0
Immunotherapy	0	0	0
Macrolides	7	2	2
Other antibiotics	2	0	2
Antihistamine	0	3	4

CRS = chronic rhinosinusitis.

nasal discharge, postnasal drip, headache, hyposmia and nasal obstruction, endonasal findings of mucopurulent secretions, and nasal polyps with paranasal sinus shadow observed by CT examination. Perennial allergic rhinitis sensitized with house-dust mites was diagnosed based on clinical symptoms such as sneezing, watery nasal discharge, and nasal obstruction and verified as house-dust mite–specific IgE followed by the antigen provocation test by the commercial paper disk (Torii Pharmacy, Tokyo, Japan) based on the *Practical Guidelines for the Management of Allergic Rhinitis* in Japan.[17]

Duration of illness was clarified by interviewing each patient at his/her first visit to our clinic. Some patients did not remember the precise duration of illness, but all of the patients suffered from CRS or allergic rhinitis for a minimum 5 years. The duration of acute or chronic rhinitis in control cases was unclear. With regards to medication history, some patients had been prescribed at least one of the following: macrolides, other antibiotics, topical steroids, and/or antihistamines, at other ear, nose, and throat clinics. However, each had quit their medication at least 2 weeks before visiting our clinic. The precise duration of each medication in each case was unclear.

Nasal mucosa was harvested from surgical cases on the partial resection of the inferior turbinate mucosa with/without septoplasty, to reduce severe nasal obstruction, after obtaining informed consent in accordance with the policies of the Review Board of Kagoshima University, Graduate School of Medical and Dental Sciences.

VEGF Analysis in Nasal Secretions from Patients with Allergic Rhinitis and Paranasal Sinusitis by ELISA

Nasal secretions (200 μL) from the middle meatus of the nasal cavity were collected by JUHN TYM-TUP (Medtronic Xomed, Inc., Jacksonville, FL) from CRS and allergic rhinitis patients as well as control cases in the outpatients clinic of Kagoshima University Hospital. Collected nasal secretions were diluted with 2 mL phosphate-buffered saline (PBS) and centrifuged at 350 × g for 10 minutes. The supernatant was then harvested. In addition, peripheral blood samples were taken from allergic rhinitis patients. All of the prepared samples were stored at −80°C, and VEGF levels in the harvested supernatants and peripheral blood were measured by ELISA (BioSource, Camarillo, CA). The minimum detectable dose of VEGF by this commercial kit is <5 pg/mL.

VEGF Analysis in Nasal Lavage from Patients with Allergic Rhinitis before and after the Antigen Provocation Test

All of the cases enrolled in this study underwent the provocation test by house-dust mites. No positive symptomatic reactions were confirmed by 15 minutes in CRS and control cases. In the seven allergic rhinitis cases, nasal lavage was obtained before and after intranasal provocation by the sensitized antigen, a commercial paper disk of house-dust mites (Torii Pharmacy). In every case, before the provocation by house-dust mites, it was confirmed that the symptomatic changes such as increasing nasal discharge, nasal obstruction, and sneezing were not observed after the control paper disk was inserted into the nasal cavity. In reference to a similar study on inflammatory cytokines in nasal secretions,[18] nasal lavage samples were collected before and 15 minutes after the provocation as follows: 5 mL of normal saline was delivered into each nostril and wash fluid from the nasal cavity was collected 10 seconds later. After centrifugation at 350 × g for 10 minutes, supernatants were transferred to other tubes and stored at −20°C. VEGF levels were then measured by ELISA.

Immunohistochemistry of VEGF in Nasal Glands

Inferior turbinate mucosa of allergic rhinitis patients was obtained from surgical cases with informed consent. The mucosa was fixed in formalin and embedded in paraffin blocks for preparation of 4-μm sections. These sections were immunohistochemically stained with VEGF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using commercial ABC kits (Vector Laboratories, Inc., Burlingame, CA).

Laser Microdissection (LMD) for Harvesting of Total RNA from Mucous or Serous Acini of Inferior Turbinate Mucosa

Inferior turbinate mucosa obtained from surgical cases was embedded in OCT compound (Sakura Finetech, Tokyo, Japan) and stored at −20°C until sectioning. Cryosections (5 μm) were cut with a cryostat and placed on glass slides designed for the LMD microscope (Leica Microsystems, Japan, Tokyo).[19] Twenty sections were necessary to obtain sufficient RNA for analysis. Samples were briefly rinsed with RNase-free water, stained with hematoxylin, air-dried, and

cut for LMD to obtain mucous or serous acini of the inferior turbinate mucosa, followed by purification of total RNA from each type of acini using commercial kits (High Pure RNA Isolation Kit; Roche Diagnostics GmbH, Mannheim, Germany). Under light microscopy, serous acini were distinguished from mucous acini in histology sections stained with hematoxylin, based on the morphological features as described in a basic histology textbook.[20]

Light Cycler–PCR Conditions

The cDNA used for light cycler-PCR was produced using a First-Strand cDNA Synthesis Kit (Roche Diagnostics GmbH), according to the manufacturer's protocol.[21] The same RNA was reverse transcribed with 2 μL of 10× reaction buffer, 4 μL of 25-mM $MgCl_2$, 2 μl of deoxynucleotide mix, 2 μL of Rando PrimerP(dN)6, 1 μL of RNase inhibitor, 0.8 μL of avian myeloblastosis virus RT and 0.4 μL of gelatin in a total volume of 20 μL. The reaction mixture was incubated at 25°C for 10 minutes and then at 42°C for 60 minutes. After the 42°C incubation, avian myeloblastosis virus RT was denatured by incubating the reaction mixture at 99°C for 5 minutes, followed by cooling to 4°C for 5 minutes. VEGF and β-actin were analyzed by real-time PCR using the Light Cycler Human VEGF and Human β-actin kits (Roche Diagnostics GmbH), according to the manufacturer's instructions. Denaturation at 95°C was followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 68°C for 10 seconds, and extension at 72°C for 10 seconds. Light Cycler version 3.5 software (Roche Molecular Biochemicals, Mannhaim, Germany) was used throughout this study.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from glandular cells was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics GmbH) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed with 200 U of RT (SuperScript II; Invitrogen Corp., Carlsbad, CA), 500 μg of oligo(dT), 0.5 M of 2′-deoxyribonucleoside-5′-triphosphates (Pharmacia, Piscataway, NJ), and 10 mM dichlorodiphenyltrichloroethane in a total volume of 20 μL according to the manufacturer's instructions. Reverse transcription was performed at 42°C for 50 minutes and terminated by heating at 70°C for 15 minutes. cDNA was amplified using the Accupower PCR PreMix kit (Bioneer Corp., Chungbuk, Korea) containing 1 U of Taq polymerase, 250 μM of 2′-deoxyribonucleoside-5′-triphosphates, 10 mM of Tris-HCl (pH 9.0), 40 mM of KCl, and 1.5 mM of $MgCl_2$ with 1.25 M of oligomer primers in a reaction volume of 20 μL. Amplification was performed as follows: 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute in an automatic thermocycler (Perkin-Elmer Cetus, Scientific Support, Inc., Hayward, CA). The PCR products were resolved by electrophoresis on 2% agarose gels and visualized with ethidium bromide. The internal control was β-actin. Primer sequences were VEGF subtype forward, 5′-GTCTATCAGCGCAGCTACTG-3′; VEGF subtype reverse, 5′-CCGCCTCGGCTTGTCACA-3′; β-actin sense, 5′-GTGGGGCGCCCCAGGCACCA-3′; β-actin antisense, 5′-CTCCTTAATGTCACGCACGATTTC-3′.[13,14]

Statistics

For the analysis and comparison between groups consisting of a small number of samples, the Mann-Whitney *U* test was used to calculate the differences among clinical samples (Figs. 1 and 2) and experimental groups (see Fig. 4). A value of $p < 0.05$ was considered statistically significant.

RESULTS

VEGF Analysis in Nasal Secretions, Nasal Lavage, and Peripheral Blood by ELISA

VEGF levels were significantly higher in nasal secretions (average ± SD; 14.20 ± 1.34 pg/mL) than in peripheral blood (1.31 ± 1.29 pg/mL) of perennial allergic rhinitis patients. In addition, VEGF levels in nasal secretions were significantly higher in perennial allergic rhinitis than in CRS (5.65 ± 2.61 pg/mL) or control (1.39 ± 1.30 pg/mL) subjects (Fig. 1), showing VEGF production is increased pathologically in allergic rhinitis more so than CRS without type 1 allergic etiology. VEGF levels were significantly higher in nasal lavage before (5.26 ± 4.91 pg/mL) rather than after (78.94 ± 17.54 pg/mL) the antigen provocation test with the house-dust mite allergen (Fig. 2), showing that VEGF production was increased in the acute phase of a type 1 allergic reaction.

Immunohistochemistry of VEGF in Nasal Glands

Nasal glands were mixed-type glands consisting of serous and mucous glandular components, as previously described.[20] Distinction between serous and mucous acini was easily viewed morphologically under the light microscope after immunohistochemistry with the counter stain by hematoxylin; nuclei were rounded and located near the apex of cells in serous acini cells, while nuclei of mucous acini cells were flattened and located near the base of the cells.[22] VEGF staining was strongly positive in serous acini in the nasal inferior turbinate mucosa of allergic rhinitis. In contrast,

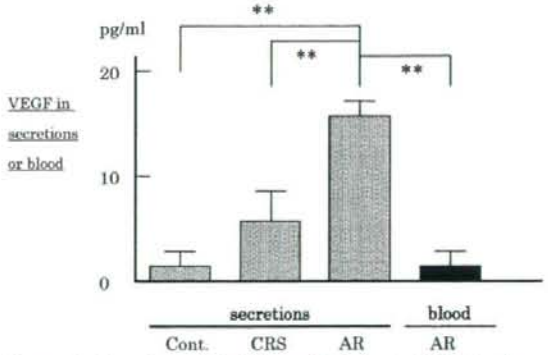


*Figure 1. Vascular endothelial growth factor (VEGF) levels are significantly higher in nasal secretions than peripheral blood in allergic rhinitis patients. Additionally, VEGF levels in nasal secretions are significantly higher in allergic rhinitis than in control or chronic rhinosinusitis patients. Results represent the average ± SD. **$p < 0.01$. AR, allergic rhinitis, Cont., nasal secretions from control cases.*

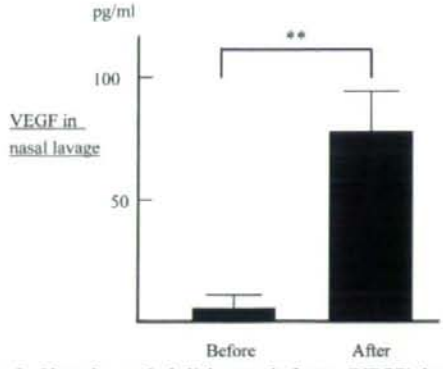


*Figure 2. Vascular endothelial growth factor (VEGF) levels are significantly higher in nasal lavage after the provocation test with house dust. Results represent the average ± SD. **$p < 0.01$. After, after provocation test; Before, before provocation test.*

VEGF staining was very faint in the cytoplasm in mucous acini of the same mucosa (Fig. 3).

LMD and PCR

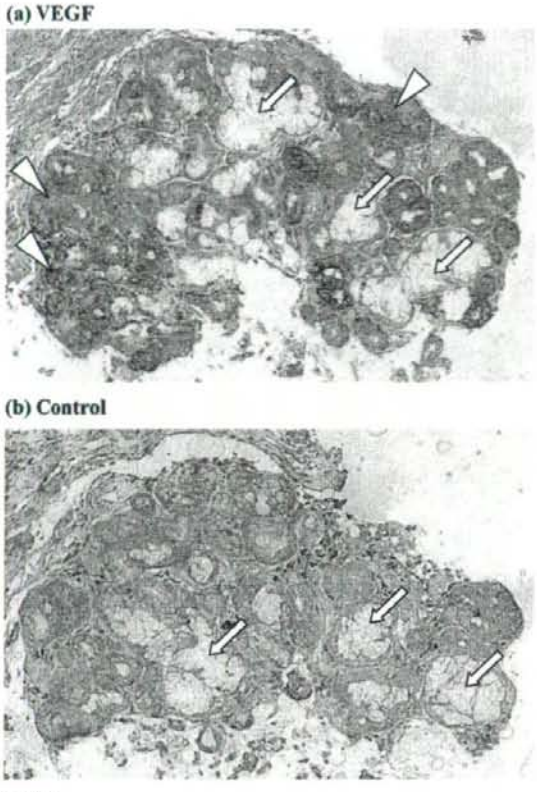
Under the light microscope, serous and mucous acini were confirmed clearly in cryosections of the inferior turbinate mucosa of allergic rhinitis after staining by hematoxylin, as seen by the immunohistochemistry results. With laser beam sectioning, serous and mucous acini cells could be collected separately to harvest each messenger RNA (mRNA). By Light Cycler-PCR, it was confirmed that total VEGF mRNA levels (expressed as VEGF/β-actin) were significantly higher in serous (1.56 ± 0.21) rather than mucous (0.85 ± 0.10) acini (Fig. 4). The pattern of VEGF isoforms was similar in serous and mucous acini, and the major isoforms were VEGF121 and VEGF165 (Fig. 5).

DISCUSSION

Nasal secretion VEGF levels are significantly higher in allergic rhinitis than in control or CRS. It is well known that a remarkable increase in watery nasal discharge in the acute phase of allergic rhinitis originates from increased glandular secretions and increased exudates from mucosal microvascular leakage.[22,23] However, because peripheral blood VEGF is quite low, as shown in Fig. 1, the source of increased nasal secretion VEGF is thought to be mainly from nasal mucosal components, including nasal glands, and not from systemic circulation exudates.[24] We have previously reported VEGF production from nasal fibroblasts in a culture,[13] but there has been no report of VEGF production in nasal mucosa *in vivo* to date. This is the first study where nasal glands, especially serous acini, have been established as potential key sources of hypersecreted VEGF in nasal secretion of allergic rhinitis sufferers.

Although there are previous reports on VEGF production in seasonal allergic rhinitis,[15,16] this is the first showing more VEGF hyperproduction in allergic rhinitis versus nonallergic rhinosinusitis. Nasal lavage VEGF levels significantly increased after provocation in pollinosis cases, in a prior study by Benson *et al.*[15] In the present study, such an increase of VEGF was confirmed not only in pollinosis but also in perennial allergic rhinitis. VEGF is thought to be hypersecreted in the acute phase of type 1 allergic reaction irrespective of seasonal or perennial allergic rhinitis. Before analyzing VEGF production in serous and mucous acini at the messenger level, including the isoform patterns, it was imperative to confirm the increase in nasal lavage VEGF after provocation in perennial allergic rhinitis.

Based on the characteristic findings of nuclei and cytoplasm of serous and mucous acini stained by hematoxylin as seen in a standard histology textbook,[20] $VEGF^+$ cells were predominantly found immunohistochemically in serous acini in nasal glands and not in mucous nasal glands. LMDs of cryosections enabled us to harvest mRNA of serous or mucous acini, separately. VEGF mRNA was detected more quantitatively in serous than mucous acini by the analysis of the light cycler technique. This is the first study to show the importance of serous acini as the source of VEGF in nasal secretion of allergic rhinitis. In the present immunohistochem-



(x200)

Figure 3. (a) Representative immunohistological vascular endothelial growth factor (VEGF) section is shown. VEGF is positively stained in serous (arrow head) but negative in mucous (arrow) acini of mucosal glands in nasal inferior turbinate mucosa of an allergic rhinitis patient. (b) Control without primary antibody (×200).

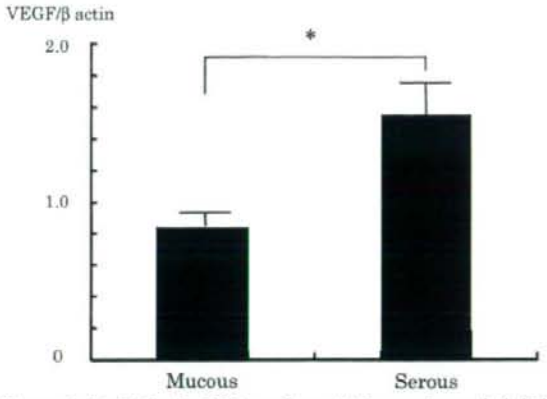


*Figure 4. Light Cycler-PCR confirms total vascular endothelial growth factor (VEGF) mRNA levels (expressed as VEGF/β-actin) is significantly higher in serous versus mucous glands in nasal inferior turbinate mucosa of allergic rhinitis patients. Results represent the average ± SD, *p < 0.05.*

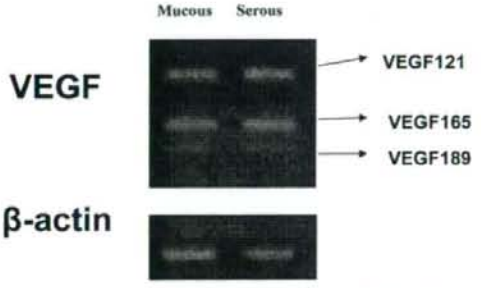


Figure 5. Vascular endothelial growth factor (VEGF) isoform distribution is similar in serous and mucous acini of mucosal glands. The major VEGF subtypes are VEGF121 and VEGF165.

ical study, unremarkable $VEGF^+$ stain was diffused from serous acini toward the surrounding periglandular tissue. As a functional role of VEGF in serous acini, however, further study of VEGF produced in serous acini may elicit its effect on periglandular tissue and other mucosal components including microvessels on vasodilation.

Chemical mediators from mast cells, such as histamine, are known to induce watery nasal secretion from nasal glands *via* irritating sensory nerves in nasal mucosa followed by activating parasympathetic nerves.[25] VEGF produced and stored in serous acini of nasal glands is released into the nasal cavity as a component of this watery nasal secretion in the acute phase of type 1 allergic reaction. Additionally, major isoforms of the increased VEGF in nasal secretion were shown to be two, VEGF121 and VEGF165, of the five potential isoforms. Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids (VEGF121–206) are produced by alternative splicing of the VEGF mRNA. An important biological property that distinguishes the different VEGF isoforms is their heparin and heparan-sulfate binding ability. Although the three secreted VEGF splice forms, VEGF121, VEGF145, and VEGF165, induce physiological activities among five VEGF isoforms, VEGF121 and VEGF165 usually predominate. VEGF145 production is quite restricted and expressed in cells derived from the reproductive organs. VEGF189 contains the peptides inducing a higher affinity to heparin and heparan-sulfate than VEGF145 or VEGF165 and is sequestered on heparan-sulfate proteoglycans of cell surfaces and in the extracellular matrix without secretion into the medium of VEGF189-producing cells. Among the five VEGF isoforms, VEGF121 and VEGF165 are the dominant secretary forms and are known to have the strongest biological activity in the field of inflammation and tumor growth in literature.[27,28] Because there was no difference in VEGF isoforms between serous and mucous acini cells, the functional rolls of mucous acini VEGF are thought to be same as in the serous acini.

Although functional roles of this increased VEGF in nasal secretion in allergic rhinitis remain unclear, secreted VEGF in the bronchial lumen of asthma reportedly activated mucosal epithelium *via* VEGFRs on the epithelium and induced VEGF and other cytokine production diffusing into subepithelial layer. In addition, in the research field of asthma, VEGF produced from the activated mucosal epithelium is understood to induce inflammatory and immunologic events under the epithelial layer including vasodilation *via* VEGFRs expressed on vascular endothelial cells.[12] VEGF released into nasal secretion in the acute phase is likely to stimulate nasal epithelium *via* VEGFRs and induce VEGF production in epithelium and other mucosal components such as fibroblasts, secondarily, in the positive feedback manner in the late-phase allergic reaction, although the distribution of VEGFRs have not been analyzed in nasal mucosa to date. VEGF in nasal secretion is finally supposed to elevate VEGF levels in mucosal lamina propria around the microvasculature and increase the mucosal vascular permeability and edematous change in allergic rhinitis. Additional studies are necessary to understand the effects of VEGF on the mucosal epithelium in allergic rhinitis.

It is true that histamine causes albumin leak *via* plasma extravasation and this leak is blocked 100% by H_1-receptor antagonists in the acute allergic reaction. [22] However, nasal obstruction in allergic rhinitis is not always resolved clinically only by H_1-receptor antagonists. According to the assessment by guinea pig skin, VEGF increases vascular permeability >50,000 times as strong as histamine on a molar basis. [28] This very powerful vasodilator VEGF hypersecreted in the acute phase is supposed to cause persistent nasal obstruction in the late-phase allergic reaction.

Histamine and other mediators stimulate afferent neurons that recruit central, parasympathetic cholinergic reflexes. The reflex-mediated glandular secretion is blocked 100% by atropine. However, because the positive feedback cycle of VEGF hyperproduction from mucosal components such as epithelium, fibroblasts, and infiltrating cells cannot be stopped by H_1-receptor antagonists or atropine, anti-VEGF or anti-VEGFR antibodies or blocking intracellular signaling downstream to the coupling of VEGF and VEGFR at the effecter sites are deemed necessary to inhibit the effects of VEGF. By further elucidating the pathophysiological roles of VEGF in allergic rhinitis, regulation of VEGF production may prove to be a new type of pharmacologic therapy, which proves quite

effective as a therapeutic strategy on allergic rhinitis as discussed in the field of asthma.[29]

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REFERENCES

1. Senger D, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor which promotes ascites fluid accumulation. Science 219:983–985, 1983.
2. Ferrara N, and Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858, 1989.
3. Ferrara N, Houck K, Jakeman L, and Leung DW. Molecular and biological properties of the vascular endothelial growth factor of proteins. Endocr Rev 13:18–32, 1992.
4. Devries C, Escobedo JA, Ueno H, et al. The fms-like tyrosinekinase, receptor for vascular endothelial growth factor. Science 255:989–991, 1992.
5. Terman BI, Dougher-Vermazen M, Carrion ME, et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 187:1579–1586, 1992.
6. Nagashima M, Yoshino S, Ishiwata T, and Asano G. Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. J Rheumatol 22:1624–1630, 1995.
7. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331:1480–1487, 1994.
8. Jung HH, Kim MW, Lee JH, et al. Expression of vascular endothelial growth factor in Otitis Media. Acta Otolaryngol 119:801–808, 1999.
9. Wittekindt C, Hess A, Bloch W, et al. Immunohistochemical expression of VEGF and VEGF receptors in nasal polyps as compared to normal turbinate mucosa. Eur Arch Otorhinolaryngol 259:294–298, 2002.
10. Hoshino M, Takahashi M, and Aoike N. Expression of vascular endothelial growth factor, basic fibroblasts growth factor and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. J Allergy Clin Immunol 107:295–301, 2001.
11. Hoshino M, Nakamura Y, and Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. J Allergy Clin Immunol 107:1034–1038, 2001.
12. Lee CG, Link H, Baluk P, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhance TH2-mediated sensitization and inflammation in the lung. Nat Med 10:1095–1103, 2004.
13. Matsune S, Sun D, Ohori J, et al. Inhibition of VEGF by macrolides in cultured fibroblasts from nasal polyps. Laryngoscope 115:1953–1956, 2005.
14. Sun D, Matsune S, Ohori J, et al. TNF-α and endotoxin increase hypoxia-induced VEGF production by cultured human nasal fibroblasts in synergistic fashion. Auris Nasus Larynx 32:243–249, 2005.
15. Benson M, Carlsson B, Carlsson LMS, et al. Increased expression of vascular endonasal growth factor-A in seasonal allergic rhinitis. Cytokine 20:268–273, 2002.
16. Kirmaz C, Ozbligin K, Yuksei H, et al. Increased expression of angiogenic markers in patients with seasonal allergic rhinitis. Eur Cytokine Netw 15:317–322, 2004.
17. Baba K, Konno A, and Takenaka H. (eds.) Examination and diagnosis. In Practical Guideline for the Management of Allergic Rhinitis in Japan, 5th ed. Tokyo: Life Science, 2005. 18–30.
18. Yamada T, Fjujieda S, Mori S, et al. Macrolide treatment decreased the size of nasal polyps and IL-8 levels in nasal lavage. Am J Rhinol 14:143–148, 2000.
19. Kobayashi K, Utsumi H, Okada M, et al. One step RT-PCR without initial RNA isolation step for laser-microdissected tissue sample. J Vet Med Sci 65:917–919, 2003.
20. Junquerira LC, and Carneiro J. (eds.). Respiratory system. In Basic Histology, 4th ed. Singapore: Maruzen Asia Press, 1983. 363–382.
21. Wellmann S, Taube T, Paal K, et al. Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by light cycler technology. Clin Chem 47:654–660, 2001.
22. Raphael GD, Meredith SD, Baraniuk JN, et al. The pathophysiology of rhinitis. II. Assessment of the sources of protein in histamine-induced nasal secretions. Am Rev Respir Dis 139:791–800, 1989.
23. Ichikawa K, Okuda M, Naka F, and Yago H. The sources of chemical substances in allergic nasal. Rhinology 29:143–149, 1991.
24. Konno A, Terada N, Okamoto Y, and Togawa K. The role of chemical mediators and mucosal hyperreactivity in nasal hypersecretion in nasal allergy. J Allergy Clin Immunol 79:620–607, 1987.
25. Wagenmann M, Baroody FM, Cheng CC, et al. Bilateral increases in histamine after unilateral nasal allergen challenge. Am J Respir Crit Care Med 155:426–431, 1997.
26. Pufe T, Petersen W, Tillmann B, and Mentlein R. Splice variants VEGF121 and VEGF165 of angiogenic peptide vascular endothelial cell growth factor are expressed in the synovial tissue of patients with rheumatoid arthritis. J Rheumatol 28:1482–1485, 2001.
27. Basic M, Edward NA, and Merrill MJ. Differential expression of vascular endothelial growth factor (vascular permeability factor) forms in rat tissue-short communication. Growth Factors 12:11–15, 1995.
28. Senger DR, Connolly DT, Van De Water L, et al. Purification and NH2-terminal amio sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 50:1774–1778, 1990.
29. Rothenberg ME. VEGF obstructs the lung. Nat Med 10:1041–1042, 2004. □

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ORIGINAL ARTICLE

A Randomized Double-Blind Comparative Study of Sublingual Immunotherapy for Cedar Pollinosis

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ABSTRACT

Background: Seasonal allergic rhinitis (SAR) induced by Japanese cedar pollen is a substantial problem in Japan. Sublingual immuno-therapy (SLIT) is safer than conventional antigen-specific immunotherapy, the only treatment modality by which complete cure of the disease can be expected. We investigated the safety and efficacy of SLIT in the treatment of cedar pollinosis patients compared to placebo.

Methods: A randomized, placebo-controlled, double-blind study was conducted in 61 cedar pollinosis patients. Increasing doses of standardized Japanese cedar extract or placebo were administered sublingually in intervals ranging from daily to once a week after six weeks. The primary efficacy variable was the mean of the daily total symptom scores (TSS) during the pollen dispersing period. Secondary efficacy variables included the QOL scores and related variables.

Results: Primary efficacy variable scores were significantly lower for some days in the SLIT group than in the placebo group ($P < .01$ or $P < .05$). Secondary efficacy for the QOL score in SLIT group was almost of half of placebo group. There was no significant difference in the overall incidence of side effects between the SLIT group and the placebo group.

Conclusions: SLIT was effective and safe in the treatment of cedar pollinosis.

KEY WORDS

Japanese cedar, placebo-controlled study, QOL, seasonal allergic rhinitis

INTRODUCTION

In agreement with the results of worldwide epidemiological assessments, the number of patients with allergic rhinitis such as Japanese cedar (JC) pollinosis in Japan is increasing.[1] Okuda considers that the current prevalence of allergic rhinitis is 16%, but many researchers predict that the rate will still increase.[2] Pollinosis is a typical type I allergy in which allergic conjunctivitis and allergic rhinitis develop. In spite of its refractory nature, pollinosis deteriorates patient QOL only in severe cases; however, it greatly affects the patient's life in general in that they must keep working even if the condition is severe.[3] Many of the patients with cedar pollinosis have also been sensitized to cypress pollen which disperses after cedar pollen. Consequently, symptoms of cedar pollinosis are followed by those of cypress pollinosis; patient symptoms last, though they are seasonal, for as long as 4 months (from February to May).

Pharmacological therapy prescribed by general practitioners is common for the treatment of the disease. Both oral medications and topical medication, however, are symptomatic treatment; they do not cure the disease or remain effective until the following year.[4] Antigen-specific subcutaneous immuno-

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therapy (SCIT) is the only treatment modality by which complete cure of the disease can be expected.[5] WHO position paper stipulates the use of standardized antigen and the concentration of the antigen to be maintained.[6] The efficacy of the therapy has been proven in placebo-controlled, double-blind comparative studies using pollen, house dust mite, and animal protein.[6,7] In Japan, it is customary to start the administration of causative antigen extract by subcutaneous injection at the threshold of skin reaction or its 10-fold diluted concentration, and to increase the dose gradually.[4] Treatment with SCIT requires special attention because it may cause, as a side effect, anaphylactic shock, which prevents the therapy from becoming popular in Japan.[8] In order to reduce the possibility of this side effect, immunotherapy is administered by other routes (sublingual, intranasal, oral, and transbronchial) in Europe and the United States, and has achieved desired outcomes.[9-11] Especially, sublingual immunotherapy (SLIT) has become popular in Europe considerably, and there are many reports supporting the effectiveness of the therapy.[11-13] As for side effects due to SLIT, there are no reports of anaphylactic shock, but oral itching, skin reaction (such as urticaria), and mild asthma-like attacks have been reported.[13] Since cedar pollinosis greatly deteriorates patient QOL, many physicians and patients will opt for immunotherapy if it is proven to be safe. We conducted a randomized, placebo-controlled double-blind comparative study to investigate whether SLIT reported in Europe and the United States is effective for the treatment of JC pollinosis and whether it can be performed safely.

STUDY DESIGN

This multi-centre, double-blind, randomized, placebo-controlled, parallel-group study was conducted in six centers across Japan between October 2004 and April 2005. The study protocol was approved by the appropriate local ethics committees, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent prior to participation.

SUBJECTS

Patients with JC pollinosis were enrolled in this study if they had a RAST score of 2 against JC or above and pollinosis symptoms during the cedar pollen dispersal period at least in the past 2 years and if they had visited any of the following medical institutions: Department of Otorhinolaryngology, Nippon Medical School; Department of Otorhinolaryngology, University of Fukui; Department of Otorhinolaryngology, Head and Neck Surgery, Okayama University; Department of Otorhinolaryngology, Dokkyo University School of Medicine; Department of Otorhinolaryngology, University of Yamanashi; and Department of Otorhinolaryngology, Head and Neck Surgery, Chiba University. Patients who had nose diseases (perennial allergic rhinitis, nasal septum deviation, or sinusitis) which may interfere with accurate symptom assessment were excluded from the study. Patients receiving treatment for conditions such as severe cardiac disease and malignant tumor were also excluded. As a result, a total of 61 patients were blindly randomized either to the active group or the placebo group in the ratio of 2 active to 1 placebo.

METHODS

The study was initiated in October, 2004. Patients were assigned and randomized to either the active group or the placebo group. Cedar antigen extract (active group) at concentrations of 2 to 2000 JAU/ml diluted with diluent (made by Torii Pharmaceutical Co., Ltd.) and diluent alone (placebo group) were used in eye drop containers (made by Hirakata Plastic).

Administration of the antigen extract was started at 2 JAU/ml, which is considered a sufficiently safe level, and was increased to the final maintenance concentration of 2000 JAU/ml. Active drug was administered as follows: 1 drop (about 50 μl) to 20 drops (about 1 ml) of prepared extract was dropped onto bits of bread (about 1.5 cm × 1.5 cm × 1.5 cm), which were held sublingually for 2 minutes and then expectorated. The treatment schedule was as follows: antigen extract was administered sublingually daily from Week 1 to Week 4; 20 drops of the antigen extract 2000 JAU/ml were administered two days per week in Week 5, once per week in Week 6 and thereafter throughout the season (Table 1).

Patients experiencing pollinosis symptoms in cedar and cypress pollen dispersal periods received symptomatic treatment with medications such as antihistamines on an as needed basis; such patients were asked to record the date of treatment in their allergy diary.

ENDPOINTS

The patients were instructed to fill in their allergy diary from February 22, 2005 to April 6, 2005, the period when cedar and cypress pollen dispersed in 2005, and they were also asked to fill in QOL questionnaire once a month during the same period. Symptoms recorded in the allergy diary (sneezing, runny nose, nasal congestion, and interference with daily life), the total nasal symptom scores calculated based on each symptom, sneezing, runny nose, nasal congestion (none; 0, mild; 1, moderate; 2, severe; 3), and symptom medication scores (antihistamine; 1, topical steroid; 2, general steroid; 3) were calculated. The Japanese Allergic Rhinitis QOL Standard Questionnaire No.1 (JRQLQ No1) was used for the assessment of the QOL of patients with allergic rhinitis (Fig. 1). Nasal and Ocular symptom scores, QOL-

Table 1 Allergen administration schedule (Increasing dosing)

	Week 1 (2 JAU/ml)	Week 2 (20 JAU/ml)	Week 3 (200 JAU/ml)	Week 4 (2000 JAU/ml)	Week 5 (2000 JAU/ml)
Day 1	1 drop	1 drop	1 drop	1 drop	20 drops
Day 2	2 drops	2 drops	2 drops	2 drops	
Day 3	3 drops	3 drops	3 drops	4 drops	
Day 4	4 drops	4 drops	4 drops	8 drops	
Day 5	6 drops	6 drops	6 drops	12 drops	20 drops
Day 6	8 drops	8 drops	8 drops	18 drops	
Day 7	10 drops	10 drops	10 drops	20 drops	

Initial dose of SLIT for JC pollinosis was 1 drop of 2 JAU/ml of standardized JC allergen, and the administrating dose is increased up to 20 drops of 2000 JAU/ml at 4th week, the maintenance dose.

Japanese Rhino-conjunctivitis Quality of Life Questionnaire (JRQLQ No.1)

To patients with allergic rhinitis (including pollinosis)
These days, the aim of medical treatment is not just to cure disease but also to give patients a better quality of life. The purpose of this survey is to determine to what extent your rhinitis interferes with your life and whether it would be improved by treatment. As with all medical treatment, the information you provide in this survey will remain strictly confidential.

You may find some of the following questions difficult to answer, but just answer to the best of your ability.

/ Tick the box that best describes the severity of the worst nasal and eye symptoms you have experienced in the past 1–2weeks.

Nasal and eye symptoms	0. No	1. Mild	2. Moderate	3. Severe	4.Very severe
Runny nose	☐	☐	☐	☐	☐
Sneezing	☐	☐	☐	☐	☐
Blocked nose (nasal congestion)	☐	☐	☐	☐	☐
Itchy nose	☐	☐	☐	☐	☐
Itchy eyes	☐	☐	☐	☐	☐
Watery eyes	☐	☐	☐	☐	☐

// Tick the box that best describes the worst extent to which the symptoms in / above have interfered with your quality of life in the past 1–2 weeks. If any of the items listed under Quality of life below definitely do not relate to the symptoms in / (nose, eyes), then there is no need to tick a box for that particular item.

Quality of life	0. No	1.Yes, slightly	2.Yes, moder-ately	3.Yes, severe	4.Yes, very severe
1. Reduced at work/home	☐	☐	☐	☐	☐
2. Poor moral concentration	☐	☐	☐	☐	☐
3. Reduced thinking power	☐	☐	☐	☐	☐
4. Impaired reading book/newspaper	☐	☐	☐	☐	☐
5. Reduced memory loss	☐	☐	☐	☐	☐
6. Limitation of out of life (e.g. sport, picnics)	☐	☐	☐	☐	☐
7. Limitation going out	☐	☐	☐	☐	☐
8. Hesitation friends or relatives	☐	☐	☐	☐	☐
9. Reduced contact with friends or others by telephone or conversation	☐	☐	☐	☐	☐
10. Not an easy to be around	☐	☐	☐	☐	☐
11. Impaired sleeping	☐	☐	☐	☐	☐
12. Tiredness	☐	☐	☐	☐	☐
13. Fatigue	☐	☐	☐	☐	☐
14. Frustration	☐	☐	☐	☐	☐
15. Irritability	☐	☐	☐	☐	☐
16. Depression	☐	☐	☐	☐	☐
17. Unhappiness	☐	☐	☐	☐	☐

/// Please circle the number of the face that best describes your general state (including your symptoms, life and emotion) in the past 1–2 weeks.

Do not fill out the following

To be completed by physician

Patient's name: Medical record to: Age: yr Sex: M F
Name of medical institution: Physician's name: Date:
Diagnosis:
SAR (Antigen) Treatment [prevention,drug,immunology,therapy,operation]
PAR (Antigen) Treatment [prevention,drug,immunology,therapy,operation]
Non-Allergy: Disease: ( ) Treatment: ( )
QOL score: None 0 , Mild 1 , Moderate 2 , Severe 3 , Very severe 4
Total QOL score
Score by QOL category 1–5 points daily life 6–7 points out-door
8–10 points social 11 points sleep
12–13 points body 14–17 points psyco-life
Please write the names of drugs used it possible
Score: None: 0 points Mild: 1 point Moderate: 2 points
Severe: 3 points Very Severe: 4 points

Fig. 1 Japanese Allergic Rhinitis QOL Standard Questionnaire No.1 (JRQLQ No1).

related questionnaire scores, and the overall face scale were calculated and statistically analyzed. In other words, the QOL deterioration score was calculated by subtracting QOL-related questionnaire scores recorded in February (i.e. at baseline) from the scores recorded in the middle of March to April, when the largest amount of pollen dispersal was observed.

STATISTICAL ANALYSIS

Symptom scores, total symptom scores, and symptom medication scores calculated from the allergy diary in the placebo group and the active group were analyzed by non-paired t-test and the Chi-squared test

Table 2 The background of the subjects

Items	Placebo $n = 22$	Active $n = 37$	p value (*)
Age	40.14±15.30	40.65±15.14	0.901
Sex			
Male	7 (31.8%)	18 (48.6%)	
Female	15 (68.2%)	19 (51.4%)	
Nasal and eye symptoms	0.62±0.54	0.43±0.35	0.169
QOL-related questionnaire	0.25±0.29	0.21±0.25	0.568
Usual daily activities	0.13±0.25	0.11±0.32	0.762
Outdoor activities	0.14±0.48	0.21±0.46	0.630
Social functioning	0.05±0.22	0.08±0.23	0.648
Sleep disturbance	0.14±0.36	0.09±0.29	0.537
Physical problems	0.17±0.33	0.24±0.46	0.510
Emotional function	0.12±0.23	0.11±0.32	0.953
Overall face scale	1.14±0.73	1.09±0.74	0.780

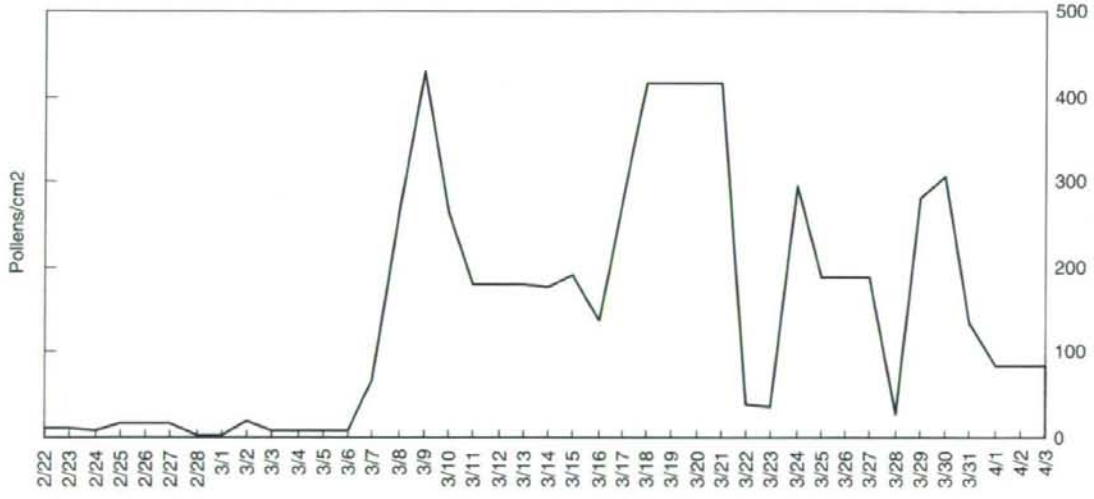


Fig. 2 The changing the number of cedar and cypress pollen dispersals in 2005.

using SPSS 11.0J. QOL-related questionnaire score of the 2 groups were compared using analysis of covariance (ANCOVA).

RESULTS

Of the 61 randomized patients, there were 2 dropouts for those whose treatment was unknown; there were 37 patients in the active group and 22 patients in the placebo group. In the analysis of allergic symptoms, 2 patients whose outcome was available only in the form of a diary were excluded, and the results of 36 patients in the active group and 21 patients in the placebo group were analyzed. In the analysis of QOL, 3 patients were excluded because baseline assessment was unavailable, and the results of 35 patients in the active group and 21 patients in the placebo group were analyzed.

As shown in Table 2, no difference was observed between the two groups in terms of patient characteristics (sex was analyzed by the Chi-squared test, and other items were analyzed by t-test).

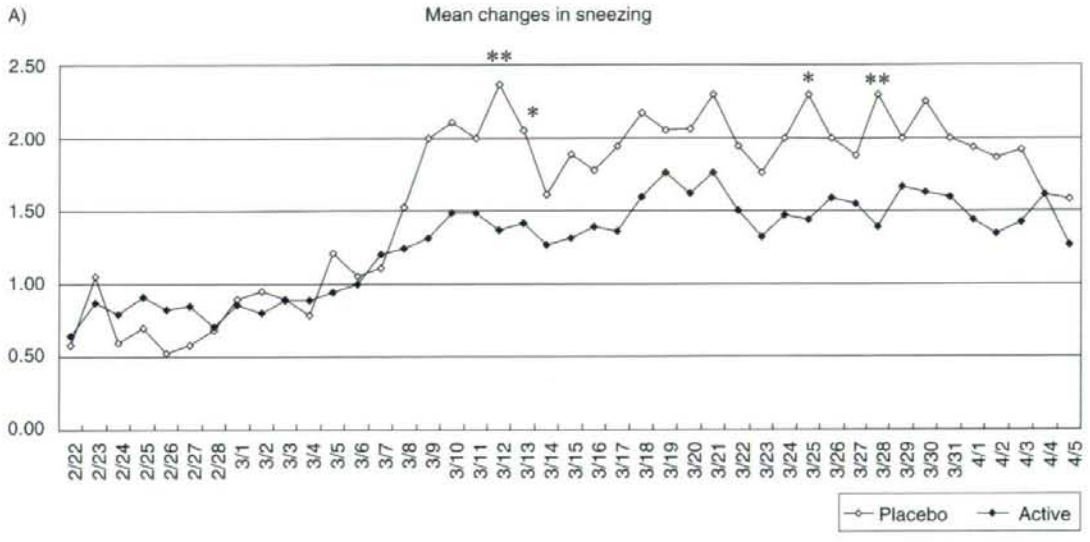
In 2005, the number of cedar and cypress pollen dispersals observed was the largest during the 10-year period since 1995. According to the data of the Chiyoda ward—the area nearest to the Nippon Medical School—announced by the Tokyo Metropolitan Government, the first pollen dispersal was observed on February 22, which was about the same time as in the past years, and an average of 10,625 pollens per square centimeter by the Durham method were ob-

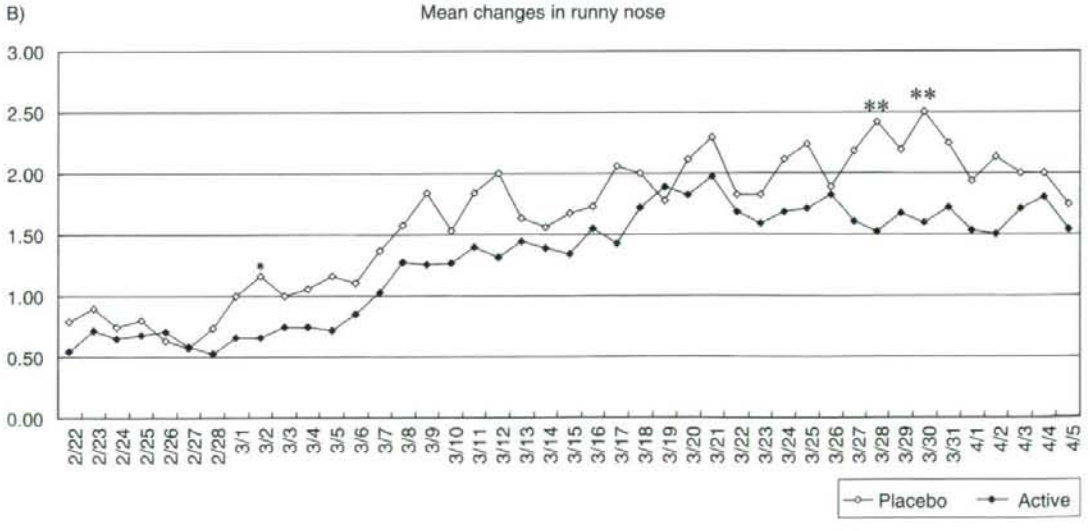
served during the season (Fig. 2). The number of the cedar and cypress pollens by the same method observed in each institution was 3424, 2383, 16002, 5859 and 7752 for University of Fukui, Okayama University, Dokkyo University, University of Yamanashi and Chiba University respectively, and these pollen numbers were also largest dispersing during the last ten years at any place.

Symptom scores for sneezing (Fig. 3A) and runny nose (Fig. 3B) in the active group were significantly better than those in the placebo group on 4 days and 2 days, respectively, but no difference was observed between the active group and the placebo group in terms of nasal congestion (Fig. 3C). Between the 2 groups, there was no difference in the number of medications used during the season (Fig. 3D).

The active group had a significantly lower total symptom score (Fig. 4A) and symptom medication score (Fig. 4B) on 4 days during the season. Overall, better outcomes were observed in the active group during the latter half of the season (i.e. from the end of March to the beginning of April), which roughly overlaps the period when the largest amount of cedar and cypress pollen was dispersed.

In the placebo group, the nasal and ocular symptom score was 1.15, the QOL-related questionnaire





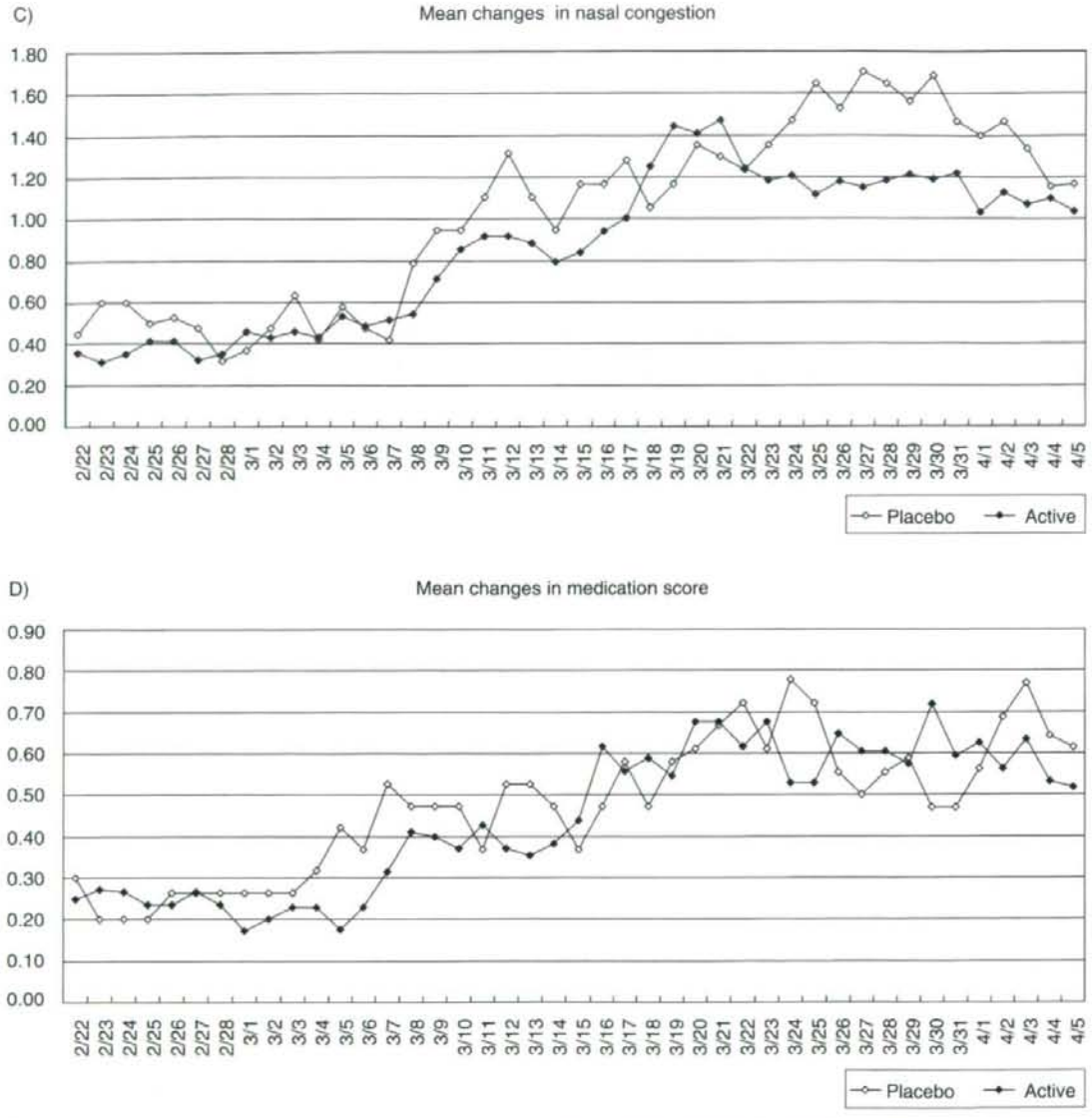


Fig. 3 The mean changes in each symptom in the season of 2005. **A**) Mean changes in sneezing. **B**) Mean changes in runny nose. **C**) Mean changes in nasal congestion. **D**) Mean changes in medication score. The open square indicates the placebo group, the filled square indicates the active group. Significant difference was evaluated as * $p < 0.05$; ** $p < 0.01$.

score was 1.10, and the overall face scale score was 1.24; in the active group, the nasal and ocular symptom score was 0.92, the QOL-related questionnaire score was 0.58, and the overall face scale score was 1.03: the deterioration score in the QOL-related questionnaire in the active group was only about half the score in the placebo group (Fig. 5A). In each domain of QOL question items, deterioration in usual daily activities, outdoor activities, social functioning, sleep problems, general physical problems, and emotional function in the active group was only about half the score in the placebo group as well. The *p*-values for the above domains were 0.089, 0.086, 0.067, 0.060, 0.083 and 0.046; a significant difference was observed only in emotional function (Fig. 5B).

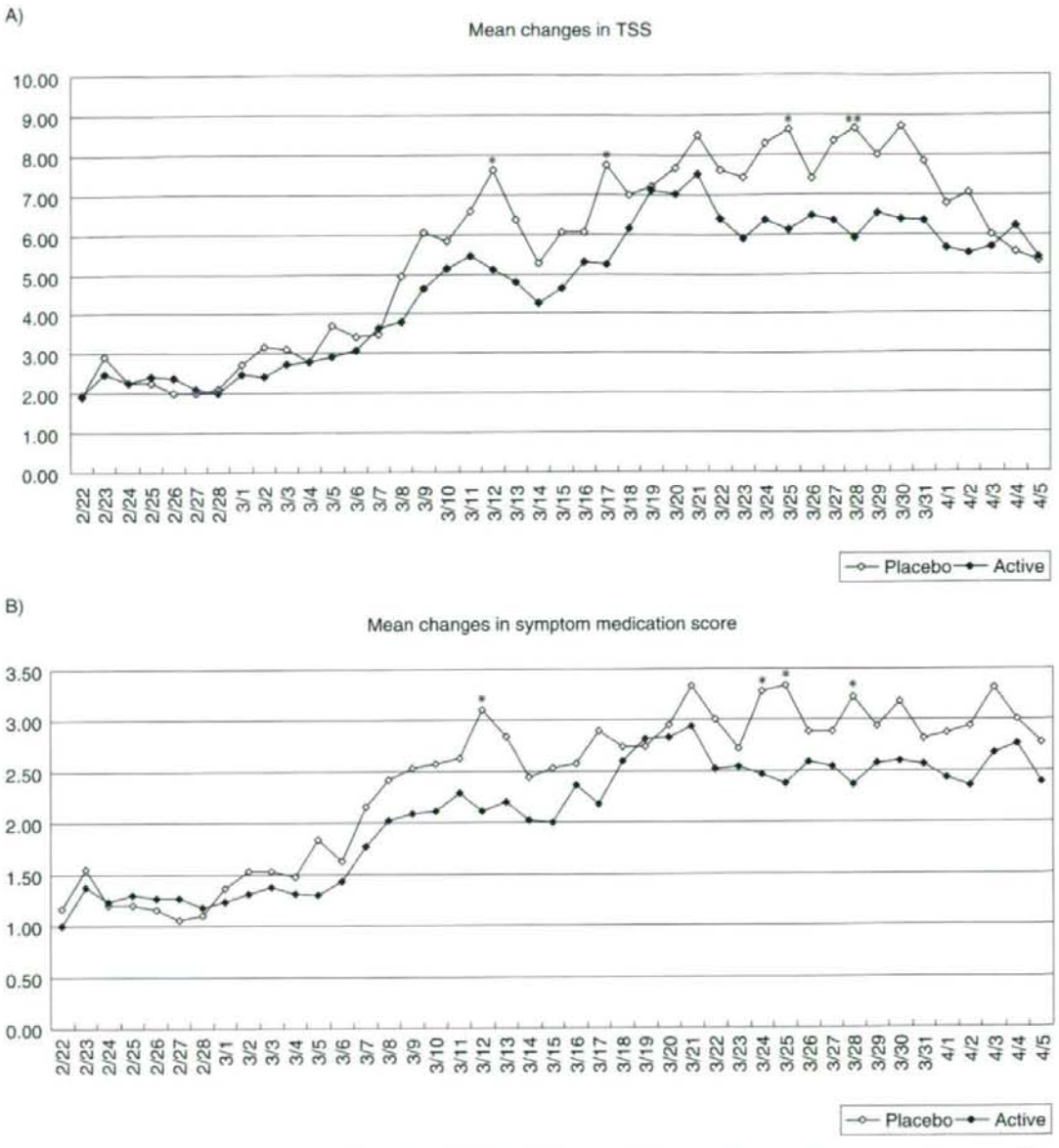


Fig. 4 The mean changes in **A**) the total symptom score (TSS) and **B**) symptom medication score in the season of 2005. Significant difference was evaluated as * $p < 0.05$; ** $p < 0.01$.

SIDE EFFECTS

No systemic side effect occurred during SLIT. Local side effects occurred in six volunteers in the active group. Mild mouth itching was exhibited in all six volunteers in increasing dose up to 2000 JAU 1 ml, however this itching was diminished for two or three times just after allergen administration. All six volunteers finished this study totally without any change of this protocol.

DISCUSSION

Approximately 16% of the Japanese population are affected by Japanese cedar pollinosis[2] and the proportion of severe status patients is higher than with

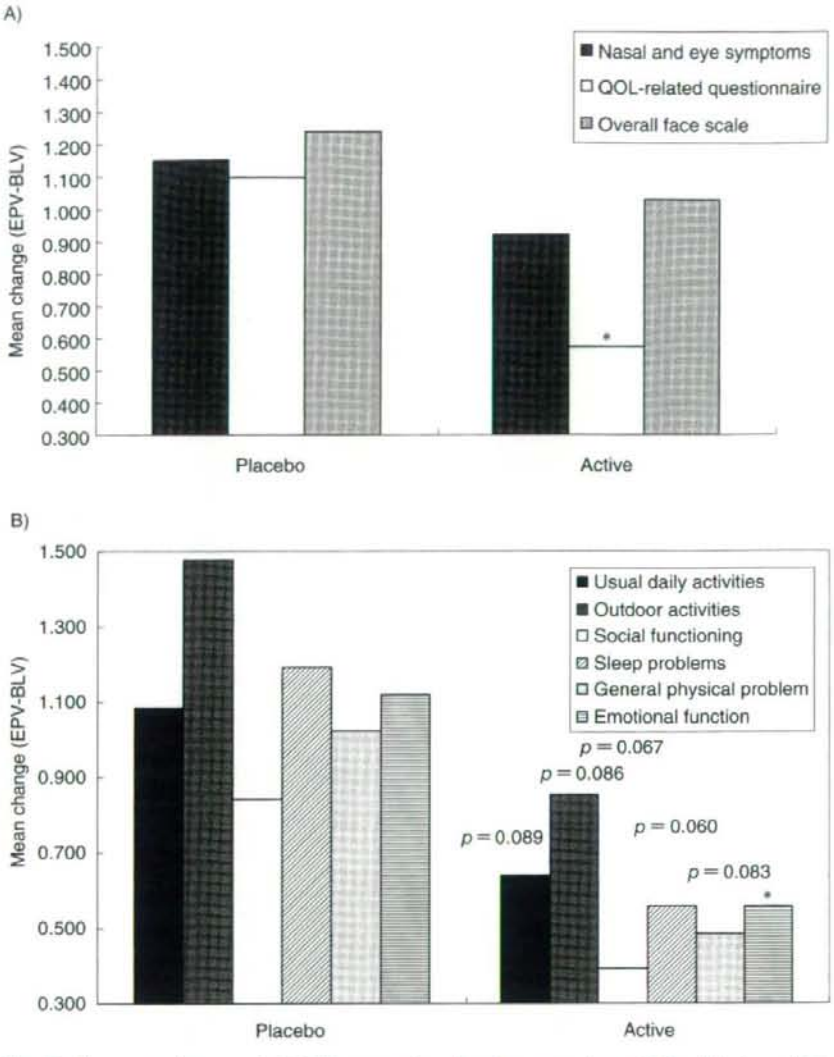


Fig. 5 The mean changes of **A**) QOL scores (nasal and eye symptoms, QOL related questionnaire, overall face scale) and **B**) each domain of QOL question items deterioration (in usual daily activities, outdoor activities, social functioning, sleep problems, general physical problems, and emotional function) from baseline data of February to peak data of peak pollen scattering period. Difference between placebo and active indicates * $p < 0.05$ (analysis of covariance, ANCOVA). Placebo: $n = 21$, Active: $n = 35$.

grass or ragweed pollinosis, which are the representative conditions in other countries, and the symptoms persist for about 3 months, becoming a social issue. When the amount of pollen increases, patients show more severe symptoms, and the number of severe status patients is greatest in mid-March (late season) when the pollen count reaches its peak. Substantial antigen exposure enhances the antigen-antibody reaction in the airways (airway hypersensitivity), which is the mechanism involved in severe pollinosis, and SCIT may control the exacerbation of the symptoms in the latter half of the cedar pollen season by inhibiting antigen-related enhancement of nasal mucosal hypersensitivity.

As shown in the WHO position paper, the effects of immunotherapy in the treatment of pollinosis have

been substantiated in many double-blind comparative studies.[6] However, the therapy tends to be avoided in Japan because of factors such as the current high cost, the complicated procedure involved, and possible side effects. In Japan, owing to these disadvantages and the fact that the department of allergy has not been widely established in medical institutions, pharmacological therapy is the mainstream modality for the treatment of pollinosis. Still, immunotherapy is an important modality for the complete cure of allergic diseases.

The efficacy of our SLIT was not demonstrated based on patient allergy diaries. However, the quality of life (QOL) score was approximately 1/2 of that in the placebo group, with a significant difference. In addition, a *P*-value corresponding to a significant difference was obtained in each QOL domain. In the mental health domain, there was a significant difference. Assessment using the Japanese guidelines differs from that in other countries; even a single sneeze is regarded as (+). In other countries, 4 grades (none, mild, moderate, and severe) are employed for assessment, and the presence or absence of symptoms is not evaluated. For this reason, the usefulness of SLIT may not have been demonstrated based on diaries. However, the QOL is evaluated via self-assessment, which is consistent with the system for the self-reporting of symptoms in other countries (none to severe). Therefore, QOL assessment of SLIT was favorable, and was consistent with the reduction rates in other countries. According to the JRQLQ criteria, the reduction rate for nasal/ocular symptoms was 22%, consistent with the evaluation of SLIT in other countries. In the future, the JRQLQ criteria, which were designed in reference to overseas self-assessment, may be essential for evaluating drug efficacy and such a novel treatment. This finding is suggestive of the fact that the QOL questionnaire developed in Japan is of good quality,[14] and that SLIT is effective for preventing QOL deterioration in patients with pollinosis rather than for lowering their symptom score. Placebo effects of SLIT may be present. However, it was evaluated in 2005, when the amount of scattered pollen was highest over the past 10 years. In addition, considering that the study involved a placebo-controlled design, we can conclude that SLIT was effective for cedar pollinosis in Japan. In evaluating the treatment response, we cannot rule out the influence of Japanese cypress pollen scattering. However, in a study excluding Japanese cypress pollen-positive reacting patients, the efficacy of SLIT and reduction rate for symptoms were also similar (unpublished data). This maybe caused by the combination of a large amount of JC and a small amount of cypress that was dispersed in 2005. These types of pollinosis should be regarded as JC/Japanese cypress pollinosis, as their seasons are sequential in the near future. In addition, a Japanese cypress pollen antigen for immunotherapy must be prepared. It should be considered that symptoms of cedar/Japanese cypress pollinosis in April are associated with cedar pollen scattering-related nasal mucosal/conjunctival inflammation, not with Japanese cypress pollen scattering alone.

Less side effects including problematic anaphylaxis are noted in SLIT although the side effects observed cannot be theoretically complete anaphylactic shock when comparing the therapy administered via injection with sublingual route.[15] Similar to the oral allergy syndrome (OAS), which is the focus of public attention, the development of symptoms such as strange feelings, oral itching, and swelling were feared because the antigen remains in the oral cavity; however, itching was the only reaction observed so far. The results obtained from the study of tentative SLIT, which was performed exclusively in the Department of Otorhinolaryngology, Nippon Medical School, were roughly consistent with the results of similar studies conducted every year thereafter, including the results of the study in 2005,[16] In our study of SLIT for the treatment of cedar pollinosis, symptom medication score was consistently lower than that of the pharmacological therapy group throughout the pollen dispersal season. The finding indicates that patients receiving SLIT tend to use fewer drugs, which is consistent with the results of a double-blind comparative study using a placebo,[17] SLIT, which is as effective as pharmacological therapy and decreases the amount of drug use, is considered advantageous also in the current medical economy in Japan.

The mechanism of action for SLIT, or for conventional SCIT, is still unclear, but for SCIT, reduction of effector cells[18,19] and blocking antibody[20-23] have been the conventional theories. Recently, however, it has become widely accepted that immunotherapy may modify the T cell response to natural allergen because of T cell anergy and/or immune deviation.[24-27] For SLIT in particular, allergen administered to the oral mucosa accumulates in the submandibular lymph node, in which the immune response occurs[28] and peaks at approximately 2 hours after administration.[29] In our investigation, an increase in the Stimulatory Index in PBMC during the early phase of SLIT conducted in 1999 shows at least that systemic immune induction was caused by sublingually administered antigen.[30] In SLIT, it is intended to cause fewer side effects than SCIT injection by decreasing systemic effects. However, it has become clear that the therapy also leads to systemic immune induction, which is greatly different from conventional topical immunotherapy administered intranasally or orally.

In the present study, SLIT both inhibited the exacerbation of symptoms in the latter half of the season and reduced their severity throughout the season. Furthermore, there were neither local nor systemic

side effects, as reported elsewhere for other antigens. SLIT for cedar pollinosis is a new therapy and in the future SLIT may by indicated for patients with nasal allergy caused by other allergens such as house dust mites or animal dander through improvement of the administration schedule and establishing the dose at which the most potent effects are achieved. This study may contribute to the methodology for the future immunotherapy in Japan.

The development of this SLIT in Japan is in progress as a multi-center study conducted as part of the research project on the prevention and treatment of immunological and allergic diseases (H17-immunology-common-001) entitled "Evaluation research of the relationship between the number of dispersed pollen observed by real-time monitoring and QOL achieved by the current treatment modalities, and the development of definitive treatment for pollinosis", which is supported by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare.

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REFERENCES

1. Strachan DP, Sibbald B, Weiland SK *et al.* Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr Allergy Immunol* 1997;**8**:161-76.
2. Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. *Ann Allergy Asthma Immunol* 2003;**91**: 288-96.
3. Okubo K, Gotoh M, Shimada K, Ritsu M, Kobayashi M, Okuda M. Effect of fexofenadine on the quality of life of Japanese cedar pollinosis patients. *Allergol Int* 2004;**53**: 245-54.
4. *Hana Arerugi Shinryo Gaidorain [Practical Guideline for the Management of Allergic Rhinitis in Japan]*, 5th edn. Tokyo: Life Science, 2005 (in Japanese).
5. Durham SR, Walker SM, Varga EM *et al.* Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;**341**:468-75.
6. Lockey RF. ARIA: Global guidelines and new forms of allergen immunotherapy. *J Allergy Clin Immunol* 2001;**108**: 497-9.
7. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;**102**:558-62.
8. Onishi M, Ikeda M, Okuda M *et al.* [Side effects due to specific hyposensitization observed in our department]. *Jibi to Rinsho [Otologia Fukuoka]* 1991;**37**:1073-8 (in Japanese).
9. Andri L, Senna G, Betteli C *et al.* Local nasal immunotherapy with extract in powder form is effective and safe in grass pollen rhinitis: a double-blind study. *J Allergy Clin Immunol* 1996;**97**:34-41.
10. Passalacqua G, Albano M, Fregonese L *et al.* Randomised controlled trial of local allergoid immunotherapy on allergic inflammation in mite-induced rhinoconjunctivitis. *Lancet* 1998;**351**:629-32.
11. Sabbah A, Hassoun S, Le Sellin J *et al.* A double-blind, placebo-controlled trial by the sublingual route of immunotherapy with a standardized grass pollen extract. *Allergy* 1994;**49**:309-13.
12. Horak F, Stubner P, Berger UE *et al.* Immunotherapy with sublingual birch pollen extract. A short-term double-blind placebo study. *J Investig Allergol Clin Immunol* 1998; **8**:165-71.
13. Tari MG, Mancino M, Monti G. Efficacy of sublingual immunotherapy in patients with rhinitis and asthma due to house dust mite. A double-blind study. *Allergol Immunopathol* 1990;**18**:277-84.
14. Okuda M, Ohkubo K, Goto M *et al.* Comparative study of two Japanese rhinoconjunctivitis quality-of-life questionnaires. *Acta Oto-Laryngologica* 2005;**25**:736-44.
15. Mungan D, Misirligil Z, Gurbuz L. Comparison of the efficacy of subcutaneous and sublingual immunotherapy in mite-sensitive patients with rhinitis and asthma: a placebo controlled study. *Ann Allergy Asthma Immunol* 1999;**82**: 485-90.
16. Gotoh M, Okubo K. Sublingual immunotherapy for Japanese cedar pollinosis. *Allergol Int* 2005;**54**:167-71.
17. Pradalier A, Basset D, Claudel A *et al.* Sublingual-swallow immunotherapy (SLIT) with a standardized five-grass-pollen extract (drops and sublingual tablets) versus placebo in seasonal rhinitis. *Allergy* 1999;**54**:819-28.
18. Kimura I, Tanizaki Y, Goda Y, Komagoe H, Kitani H. Decrease in reactivity of basophils by immunotherapy with housedust extract. *Clin Allergy* 1985;**15**:1-7.
19. Otsuka H, Mezawa A, Ohnishi M, Okubo K, Seki H, Okuda M. Changes in nasal metachromatic cells during allergen immunotherapy. *Clin Exp Allergy* 1991;**21**:115-9.
20. Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998;**102**:157-64.
21. Van Neerven RJ, Wikborg T, Lund G *et al.* Blocking antibodies induced by specific allergy vaccination prevent the activation of CD41 T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999;**163**: 2944-52.
22. Golden DB, Meyers DA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. *J Allergy Clin Immunol* 1982;**69**:489-93.
23. Van-der-Zee JS, Aalberse RC. The role of IgG in immediate-type hypersensitivity. *Eur Respir J Suppl* 1991; **13**:91s-6s.
24. Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med* 1993;**178**:2123-30.
25. Lamb JR, Skidmore BJ, Green N, Chiller JM, Feldmann M. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med* 1983;**157**:1434-47.
26. Fasler S, Aversa G, Terr A, Thestrup-Pedersen K, De Vries JE, Yssel H. Peptide-induced anergy in allergen-specific human Th2 cells results in lack of cytokine production and B cell for IgE synthesis: reversal by IL-2, not by IL-4 or IL-13. *J Immunol* 1995;**155**:4199-206.
27. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in de-

crease of IL-4 and IL-5 and increase of IFN-γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;**154**:4187-94.

28. Muller D, Ruitenberg EJ, Elgersma A. The influence of different immunization pathways on the immunological response in the oral mucosa. *Br J Exp Pathol* 1983;**64**:367-72.

29. Bagnasco M, Passalacqua G, Villa G *et al*. Pharmacokinetics of an allergen and a monomeric allergoid for oromucosal immunotherapy in allergic volunteers. *Clin Exp Allergy* 2001;**31**:54-60.

30. Okubo K, Gotoh M, Shimada K, Okuda M, Yagi T, Dairiki K. [Sublingual immuno-therapy for Japanese cedar pollinosis: pilot study]. *Nihon Bika Gakkai Kaishi [Jpn J Rhinol]* 2002;**41**:30-5 (in Japanese).

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Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis

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Epithelial cell-derived thymic stromal lymphopoietin (TSLP) is a master switch for asthma or atopic dermatitis by inducing a dendritic cell-mediated Th2-type allergic inflammation. Allergic rhinitis is also pathologically characterized by Th2-type allergic inflammation. This study demonstrates that mast cells regulate the epithelial TSLP expression in allergic rhinitis. TSLP expression was found to be up-regulated predominantly in the nasal epithelium in the ovalbumin (OVA)-sensitized and -nasally challenged mouse model of allergic rhinitis, which was abolished in mast cell-deficient WBB6F1-W/W^v in comparison with control WBB6F1-+/+ mice. Similarly, the epithelial TSLP expression was reduced in Fc receptor γ chain (FcγR)-deficient mice, where the high-affinity IgE receptor (FcεRI) is not expressed on mast cells, in comparison with control C57BL/6 mice. Furthermore, the administration of neutralizing TSLP antibody during the challenge phase of OVA inhibited the development of allergic rhinitis. These results suggest that the direct stimulation of epithelial cells by antigens alone may not be sufficient to induce TSLP expression in the nasal epithelium, and that mast cell regulation of epithelial TSLP expression, possibly *via* FcεRI, plays an important role in the development of allergic rhinitis.

Key words: Allergic rhinitis · Epithelial cells · Mast cells · TSLP

Introduction

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine, which binds to a TSLP receptor (TSLPR) consisting of the IL-7 receptor α-chain (IL-7Rα) and a common γ receptor-like chain (TSLPR-γ) [1, 2]. TSLP was originally identified as a factor derived from a thymic stromal cell line that could support the growth of a mouse B cell line[3]. However, recently, TSLP, derived from epithelial cells, has been shown to be capable of activating CD11c$^+$ myeloid DC to up-regulate costimulatory molecules, leading to the differentiation of CD4$^+$ T cells into Th2 cells. Therefore, it plays a key role in the development of allergic diseases such as asthma or atopic dermatitis [1, 2, 4]. For instance, transgenic mice expressing TSLP in keratinocytes or in lung epithelial cells have been shown to develop atopic dermatitis- or asthma-like inflammation in the skin or the lung, respectively, while TSLPR null mice failed to develop an inflammatory lung response to inhaled antigen[5–7]. In humans, TSLP has been shown to be expressed by keratinocytes in atopic dermatitis and by bronchial epithelial cells in asthmatic airways[8, 9].

In contrast to the pro-allergic action of TSLP, regulation of TSLP expression in epithelial cells has not been fully elucidated. Previous studies suggest that TSLP expression in mouse keratinocytes is regulated by vitamin D and retinoic acid signaling [10]. Most recently, TNF-α, IL-1β, and ligands for Toll-like receptors

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such as LPS were shown to up-regulate TSLP expression *via* NF-κB pathway in cultured human bronchial epithelial cells and keratinocytes[11–13].

Mast cells play a critical role in allergic inflammation including asthma and allergic rhinitis *via* multiple mechanisms [14–16]. Mast cells in patients with allergic rhinitis and asthma produce Th2-type cytokines, induce IgE synthesis in B cells, and can autoactivate themselves *via* the mast cell-IgE-high affinity receptor (FcεRI) cascade [17]. In addition, mast cells up-regulate the production of a variety of cytokines/chemokines in epithelial cells and fibroblasts, and induce the recruitment of basophils, T cells, and eosinophils into sites of allergic inflammation as well as their own intraepithelial accumulation. However, it remains unclear whether mast cells contribute to the epithelial TSLP expression at the site of allergic inflammation.

In this study, a direct assessment of the roles of mast cells on epithelial TSLP expression was conducted using a mouse model of allergic rhinitis and two types of genetically mast cell-deficient mice (WBB6F$_1$-*KitW/W-v* mice and C57BL/6-*KitW-sh/W-sh* mice).

Results and discussion

TSLP expression in nasal epithelium requires mast cells and FcγR in a mouse model of allergic rhinitis

WBB6F1-+/+ mice with a mutation of c-kit are deficient for mast cells [18] and FcγR-deficient mice showed no expression of FcR (FcγRI, FcγRIII, and FcεRI) [19] because the expression of FcR requires a homodimer of the subunit FcγR for surface expression and signal transduction in the mouse [20]. C57BL/6 and WBB6F1-+/+ mice challenged with OVA showed an infiltration of leukocytes in the nasal submucosa, whereas WBB6F1-W/W^v mice and FcγR-deficient mice challenged with OVA did not show any infiltration of leukocytes into the nasal submucosa, as previously described [21, 22] (data not shown). Immunohistochemical staining with anti-TSLP antibody revealed little positive immunoreactivity of TSLP in the nasal mucosa obtained from the PBS-challenged C57BL/6 mice, but the positive immunoreactivity of TSLP increased predominantly in the nasal epithelium after the induction of allergic rhinitis in C57BL/6 and WBB6F1-+/+ mice (Fig. 1 and 2). In contrast, the increase of TSLP-positive cells in the nasal epithelium after the induction of allergic rhinitis was absent in WBB6F1-W/W^v mice and FcγR-deficient mice (Fig. 2). Immunohistochemical staining with control IgG antibody showed negative staining in the nasal mucosa after the induction of allergic rhinitis in C57BL/6 mice (Fig. 1). Therefore, TSLP expression is induced in the nasal epithelium in a mouse model of allergic rhinitis and the presence of mast cells and FcγR is necessary for the allergen-induced epithelial TSLP expression.

To further clarify the roles of mast cells and FcγR in mast cells in epithelial TSLP expression in a mouse model of allergic rhinitis, mast cell-deficient C57BL/6-*KitW-sh/W-sh* mice [23, 24] were reconstituted with BM-derived mast cells (BMMC) derived from wild-type C57BL/6 mice or FcγR-deficient mice with C57BL/6 genetic background. As shown in Fig. 2, an increase in the number of TSLP-positive cells in the nasal epithelium after the induction of allergic rhinitis was observed in the C57BL/6-*KitW-sh/W-sh* mice reconstituted with wild-type BMMC, but not with FcγR-deficient BMMC. Therefore, mast cells and FcγR in mast cells plays a critical role in the epithelial TSLP expression in a mouse model of allergic rhinitis.

TSLP is critical for the development of allergic rhinitis

To determine whether TSLP, expressed in the nasal epithelium, plays a critical role for the development of the mouse model of allergic rhinitis, the effects of TSLP neutralizing antibody on the development of allergic rhinitis were clinically and pathologically evaluated by periodic acid-Schiff (PAS) staining to demonstrate goblet cells.

C57BL/6 mice treated with control IgG antibody during the challenge phase with OVA showed a significant increase in the nasal rub counts (Fig. 3). The mice also showed an infiltration o leukocytes in the nasal submucosa, and the number of PAS positive cells in the nasal epithelium (goblet cells) and submucos (gland cells) also increased in these mice (Fig. 3 and data nc shown). In contrast, the mice treated with anti-TSLP neutralizin antibody showed a significant reduction in the frequency of nas rubs with little infiltration of leukocytes in the nasal submucos and a decreased number of PAS-positive cells in the nas epithelium and submuosa (Fig. 3 and data not shown Interestingly, immunohistochemical staining revealed the TSI levels in the nasal epithelium to be relatively up-regulated aft treatment with anti-TSLP antibody (Fig. 3). To exclude t possibility that a trivial deposition of anti-TSLP antibody on t tissue may affect the TSLP staining, the nasal mucosa specime obtained from C57BL/6 mice treated with anti-TSLP antibo during PBS challenge were stained with anti-TSLP antibody a only slight immunoreactivity for TSLP was confirmed in th tissue specimens (data not shown). These results indicated that blockade of TSLP activity inhibited the development of the mo model of allergic rhinitis.

The current results suggest that the direct stimulation epithelial cells by antigens alone may not be sufficient to ind TSLP expression in the nasal epithelium because the deficienc mast cells and FcγR in mast cells abolished epithelial T expression regardless of the stimulation with the antigen (OVA the nose. This is consistent with recent *in vitro* studies, w suggest that TSLP expression in epithelial cells requires pr flammatory cytokines such as TNF-α [11–13]. Therefore, possible that mast cell-derived inflammatory cytokines inclu TNF-α generated at the site of allergic rhinitis may pla important role for the induction of TSLP expression in the epithelium. Because the mast cell release of TNF-α or proinflammatory cytokines can occur immediately followin antigen stimulation in the nose of patients with allergic rh [25] and FcγR was also shown to be critical for the epithelial expression (Fig. 2), we therefore speculate that mast cells (po

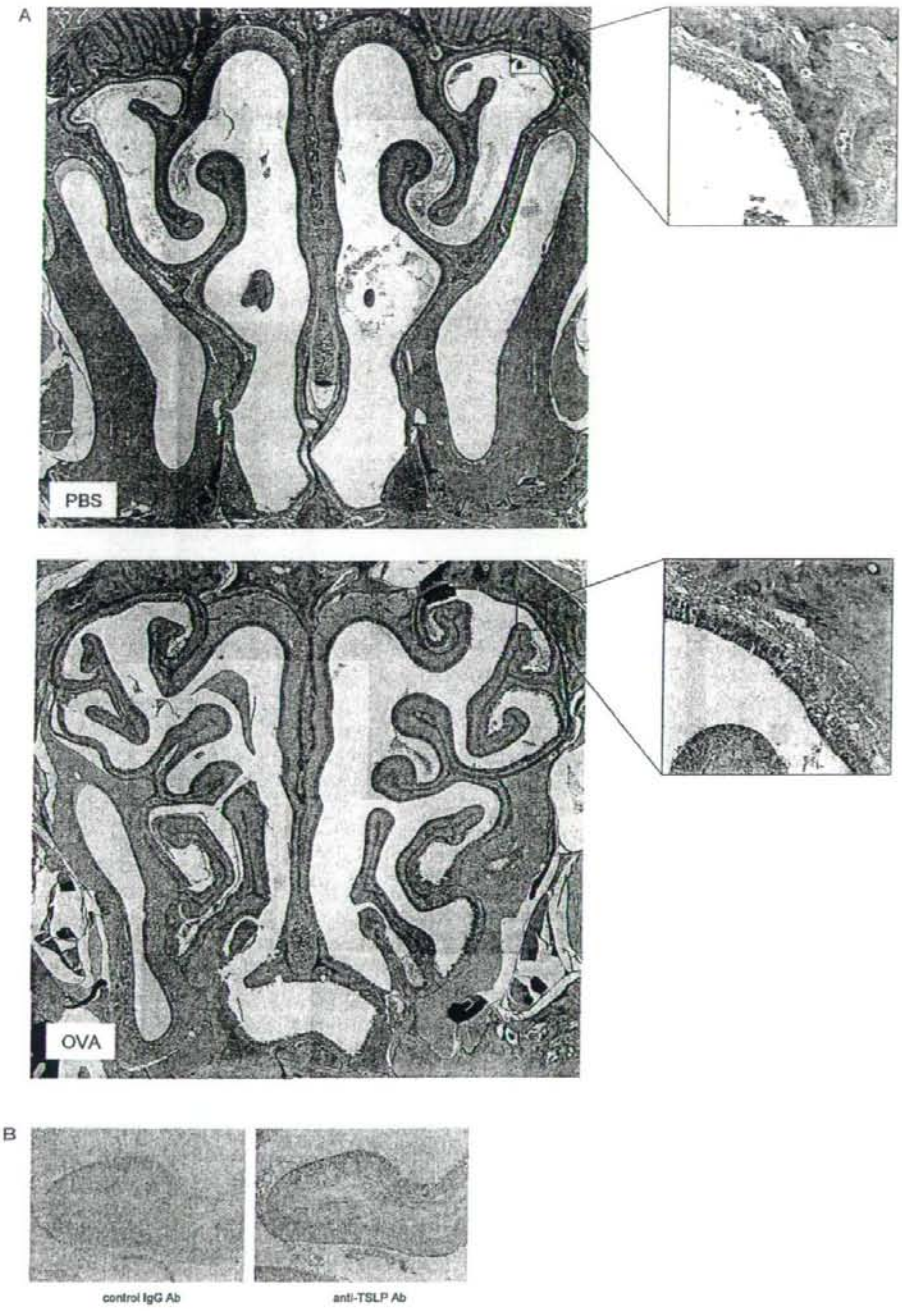

Figure 1. Repeated nasal exposure to OVA in OVA-sensitized C57BL/6 mice induces TSLP expression in the nasal epithelium. (A) Representative pictures of TSLP expression in the nasal mucosa of OVA-sensitized C57BL/6 mice repeatedly challenged with OVA (OVA) or control PBS (PBS) are shown. The curved lines indicate positive staining area. (B) Representative pictures of the nasal mucosa specimens stained with control IgG antibody (control Ab) or anti-TSLP antibody (anti-TSLP Ab) after the induction of allergic rhinitis.