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REVIEW ARTICLE

Antigen-Specific Immunotherapy against Allergic Rhinitis: The State of the Art

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ABSTRACT

Allergic rhinitis is the most prevalent type I allergy in industrialized countries. Pollen scattering from trees or grasses often induces seasonal allergic rhinitis, which is known as pollinosis or hay fever. The causative pollen differs across different areas and times of the year. Impaired performance due to pollinosis and/or medication used for treating pollinosis is considered to be an important reason for the loss of concentration and productivity in the workplace. Antigen-specific immunotherapy is an only available curative treatment against allergic rhinitis. Subcutaneous injection of allergens with or without adjuvant has been commonly used as an immunotherapy; however, recently, sublingual administration has come to be considered a safer and convenient alternative administration route of allergens. In this review, we focus on the safety and protocol of subcutaneous and sublingual immunotherapy against seasonal allergic rhinitis. We also describe an approach to selecting allergens for the vaccine so as to avoid secondary sensitization and adverse events. The biomarkers and therapeutic mechanisms for immunotherapy are not fully understood. We discuss the therapeutic biomarkers that are correlated with the improvement of clinical symptoms brought about by immunotherapy as well as the involvement of Tr1 and regulatory T cells in the therapeutic mechanisms. Finally, we focus on the current immunotherapeutic approach to treating Japanese cedar pollinosis, the most prevalent pollinosis in Japan, including sublingual immunotherapy with standardized extract, a transgenic rice-based edible vaccine, and an immunoregulatory liposome encapsulating recombinant fusion protein.

KEY WORDS

allergic rhinitis, biomarker, immunotherapy, pollinosis, regulatory T cell

INTRODUCTION

Allergic rhinitis is the most prevalent type I allergy, and pollen grains are one of the most common causes of respiratory allergies. In western Europe, the prevalence of clinically confirmable allergic rhinitis was estimated to be 23%, with more than 50% of the allergic subjects possessing specific IgE against grass pollen.[1] In Japan, the prevalence of allergic rhinitis was estimated to be 39.4% and that of pollinosis was 29.8%.[2]

Pollinosis is induced by the invasion of pollen grains onto the ocular and nasal mucosa. Pollen grains easily access internal binding sites on contact with the aqueous phases of nasal and ocular mucosal membranes. After pollens are hydrated on aqueous membranes, they swell, rupture, and release their cytoplasmic components. It has been reported that grass pollen grains rupture in water and release large amounts of respirable particles, such as starch granules containing allergens.[3] Although pollinosis patients have a low rate of asthma attacks during pollen season, the attacks that do occur may be attributable to these respirable particles bearing allergens from pollen grains.[4] Pollen grains release not only allergen-bearing particles but also immunomodulatory mediators such as pollen-associated lipid mediators (PALMs) and NADPH oxidases. Proinflammatory PALMs such as leukotriene B4-like substances attract and activate human peripheral blood eosino-

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phils and polymorphonuclear granulocytes from both allergic and non-allergic donors.[5,6] Immunomodulatory PALMs, such as phytoprastanes, inhibit IL12 production in dendritic cells and Th1-type cytokine production in antigen-specific T cells, while inducing antigen-specific Th2 responses.[7] NADPH oxidase rapidly increases the level of reactive oxygen species (ROS) in lung epithelium and induces neutrophil recruitment to the airway independent of the adaptive immune responses.[8,9] These reports strongly suggest that pollen grains themselves act primarily as adjuvants to induce pollen-antigen-specific Th2 responses and to enhance inflammatory processes during the elicitation phase of allergic responses.

The most common treatments against pollinosis are medications like antihistamines, leukotriene inhibitors, and corticosteroids. However, these treatments are not curative and sometimes induce impaired performance as a results of their side effects.[10,11] Antigen-specific immunotherapy can change the natural course of allergic rhinitis and is recognized as a curative treatment against type I allergy without impaired performance. In this century, since the first report on subcutaneous immunotherapy (SCIT), SCIT has been developed and improved and has become safer and more effective.[12,13] Recently, sublingual immunotherapy (SLIT) has been developed and has become a safer and more beneficial immunotherapy for patients.

This review focuses on the recent approach of using antigen-specific immunotherapy to treat allergic rhinitis, and focuses especially on the use of SLIT against pollinosis using standardized extract or recombinant allergens. We also discuss the therapeutic mechanisms and therapeutic biomarkers for SLIT. Finally, we discuss the recent immunotherapeutic approach to treat Japanese cedar (*Cryptomeria japonica*) pollinosis, which is the most common pollinosis in Japan.

ANTIGENS FOR IMMUNOTHERAPY

For immunotherapy, extracts from an allergen source, i.e. pollen extract, are widely used after the concentration of their major allergen is adjusted so as to be standardized. To standardize such extracts, it is important to analyze their component allergens and establish a quantification system for major allergens.[14] The World Allergy Organization (WAO) recommends that standardized vaccines be used for immunotherapy if they are available.[15] However, the protocols and methods for the standardization of allergen extract are different among different suppliers, which use their own in-house reference materials and their own unique allergen units. This made it difficult to compare the therapeutic effects and safety among clinical trials involving different products. It has been proposed that vaccines be standardized using a protocol based on mass units of major allergens and that the active ingredients of the treatment be quantified. The CREATE project has been working to select major allergens for use in the standardization of vaccines and to establish a quantification system and recombinant allergens for the standardization.[16]

To improve the safety and clinical therapeutic effects of a vaccine, the selection of allergens for vaccination is an important issue. Extract from pollen may contain many allergens that cross-react with those from fruit, vegetables, and latex. These allergens may cause minor local side effects, especially in SLIT, among patients who suffer from oral allergies and/or latex-fruit syndrome. Latex-fruit syndrome sometimes induces severe systematic reactions such as anaphylactic shock in response to natural rubber and some latex fruits.[17] The cross-reactive allergens may have to be removed from vaccines in order to avoid severe systematic adverse reactions caused by cross-reactivity with latex allergens for safer SLIT. For the elucidation of reactive allergens, protein microarray techniques have recently been applied to allergy diagnosis. Microarray-chip technology using a glass slide with the immobilization of large numbers of proteins on the surface enable us to simultaneously test IgE-binding reactivity against large numbers of allergens from various sources.[18,19] This diagnostic technique is applicable to the diagnosis of allergens from a single allergen source. This component-resolved diagnosis is a powerful tool for selecting components of allergens for immunotherapy vaccines and may improve the safety and clinical therapeutic efficacy of the vaccines in comparison to traditional immunotherapy using crude extract.[20] Such an allergen diagnosis enables us to choose only IgE-binding allergens that are individually sensitized for antigen-specific immunotherapy. This approach, in which only sensitized allergens are used for immunotherapy, avoids secondary additional sensitization against nonreactive proteins that can occur with the use of crude extracts or a mixture of allergens (Fig. 1).

Recombinant technology has been used to construct vaccines for immunotherapy.[21] Immunotherapy clinical trials were performed using a mixture of five recombinant grass allergens (rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, and rPhl p 6), and the results suggested that a recombinant allergen vaccine can be an effective and safe treatment to ameliorate the symptoms of allergic rhinitis.[22] Immunotherapy using recombinant Bet v 1 was also recently reported to show clinical efficacy, and its therapeutic effects were comparable with those obtained using native Bet v 1 against birch pollen allergy.[23]

Vaccines using allergoids and modified allergens, such as T cell-epitopes, pathogen-related molecular pattern molecule-conjugated allergens, and others, are under development, and some of them are considered to be promising for use as therapeutic vaccines.[13,24]

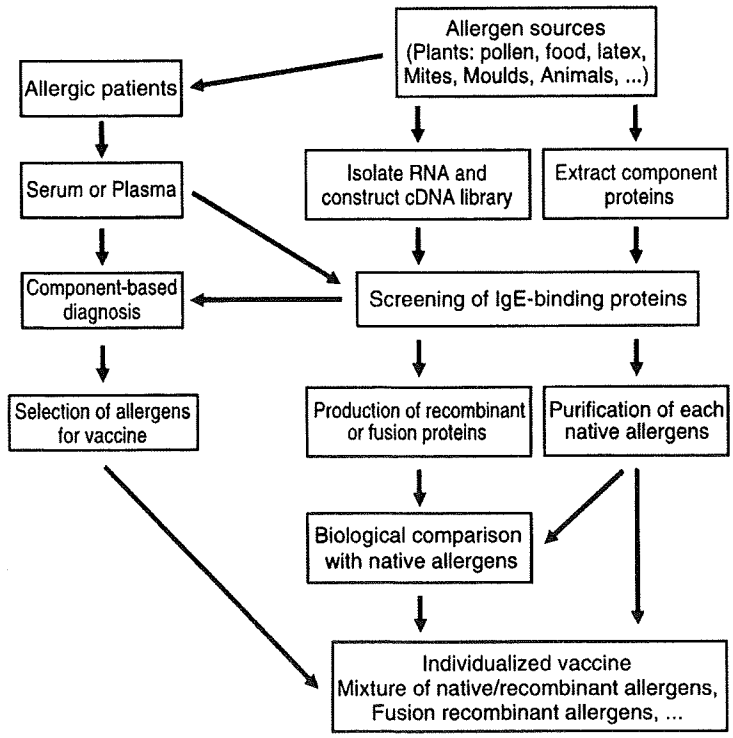

Fig. 1 Schematic procedure of the steps involved in the identification and development of an individualized vaccine using only sensitized antigens for immunotherapy. To identify component allergens which have the capacity to react with serum IgE from allergic patients, it is important to establish individualized vaccines to avoid secondary sensitization. Allergens with which an individual patient reacted can be elucidated by a component-based diagnosis, and an individualized vaccine can be established using a mixture of the purified native or the standardized recombinant allergens to which the patient is sensitized.

ROUTE OF VACCINE ADMINISTRATION FOR IMMUNOTHERAPY AND ITS SAFETY

Immunotherapy vaccines against allergies were originally injected subcutaneously without an adjuvant.[12] However, subcutaneous injection of allergens often induces severe adverse reactions like local allergic reactions, urticaria, asthma, and frequent anaphylaxis. To increase the safety and therapeutic efficacy of immunotherapy vaccines, aqueous allergen extracts absorbed into adjuvants such as aluminum hydroxide have been used in SCIT.[25] Pretreatment with antihistamine or anti-IgE antibody has been used to prevent the adverse events that can be induced after subcutaneous vaccine injection, and the pretreatments also enhance the therapeutic efficacy of SCIT.[26,27]

In this decade, SLIT has been developed as a safer method for immunotherapy and has been used with increasing frequency, especially in Europe and the US. SLIT is noted to be a very safe method without fetal adverse reactions. In most cases, adverse reactions to SLIT have been mild local reactions such as oral pruritus, edema of the mouth, throat irritation, and sneezing.[28] However, a few cases of anaphylaxis have been reported after SLIT using a crude or standardized vaccine.[29-33] These reports suggest that SLIT is not always safe for patients, especially those with severe asthma or who have experienced severe adverse reactions to SCIT. It has been recommended that the first dose of the vaccine is to be administered in a doctor's office under observation.[32]

The administration regimens for SLIT, including dosing, the build-up phase, duration of the treatment, and frequency of the maintenance dose, differ greatly among the clinical trials.[34] The sublingual and supralingual administration methods of oral drops were evaluated by a double-blind, placebo-controlled study using mixed standardized extract in patients allergic to grass pollen. In this report, sublingual administration significantly reduced the nasal, ocular, and bronchial symptoms, as well as the intake of symptom-reducing drugs compared to the placebo. Supralin-

Table 1 Comparison between SLIT and SCIT

	SLIT	SCIT
Administration	Sublingual spitting or sublingual swallowing	Subcutaneous injection with or without adjuvant
Pre-treatment	None	Medication or anti-IgE
Build-up phase	A few weeks, one day for rush protocol, or no up-dosing phase	A few weeks or a few days for rush protocol
Vaccination	Once daily or a few times weekly	A few times weekly or monthly
Adverse event	Local mild reaction in most cases, a few reports of fetal adverse reactions	Sometimes induces fetal adverse reactions

gual treatment also attenuated the symptoms and symptom-reducing drugs intake; however, only the nasal symptom score showed a significant reduction compared to the placebo-control group.[35] Thus, holding the vaccine under the tongue may be an important way to achieve better therapeutic effects with SLIT.

Vaccines for SLIT can also be delivered by two methods: sublingual spitting, in which the vaccine is spat out after being held under the tongue, and sublingual swallowing, in which the vaccine is swallowed after being kept under the tongue. In studies using radiolabeled allergens, most of the allergens remained in the mouth after the vaccine was spat out. However, plasma radioactivity began to increase only after swallowing.[36-38] The author concluded that contact between the allergens and the oral mucosa is a crucial step in the mechanisms of SLIT, and suggested that the more appropriate and advantageous way to administer the allergen sublingually is via the sublingual swallowing procedure.[38]

It has been recommended that the administration of SLIT vaccine be started at least 8 weeks before pollen season for better therapeutic effects.[39] However, an ultra-rush scheme of SLIT treatment for children allergic to grass pollen was reported to significantly improve the symptoms and the medication score compared to the placebo group. In this 2-year randomized, double-blind, placebo-control trial, the authors administered standardized extract of five grass pollen (*Dactylis glomerata*, *Anthoxanthum odoratum*, *Lolium perenne*, *Poa prantensis*, and *Phleum pretense*) beginning 2 weeks before the pollen season started with one day for ultra-rush induction, and followed by daily treatment (120 IR, 10 μg major allergen) for 6 months. It has been reported that SLIT significantly improved the asthma symptom score and reduced the nasal symptom score and the use of rescue medication score compared to the placebo group.[40] The starting point and duration of treatment varied among the clinical trials, and the best procedure for administration remains unclear.[41] (Table 1)

As a novel route to enhance the therapeutic efficacy of the vaccine, direct intralymphatic injection was proposed for the administration of peptide vaccine against viral infection and tumor in the mouse. This paper reported that the direct administration of peptide vaccine into a lymph node induced enhanced immunogenicity compared to subcutaneous and intradermal vaccination.[42] This novel technique was recently applied to patients with hay fever in an open-label, randomized control trial.[43] The authors injected 1,000 SQ-U of aluminum hydroxide-adsorbed grass pollen extract into a superficial inguinal lymph node under ultrasonic guidance. Three intralymphatic injections over 2 months resulted in long-lasting tolerance with the amelioration of hay favor symptoms, reduced skin prick test reactivity, and decreased serum allergen-specific IgE comparable with conventional SCIT. Furthermore, the author reported that there were fewer adverse events than in SCIT, even without premedication with antihistamines, and the injection was less painful than venous puncture.[43] Further clinical trials with a larger population are needed to evaluate the safety, therapeutic efficacy, and duration of tolerance of this treatment.

BIOMARKERS FOR SLIT

The therapeutic effects obtained by antigen-specific immunotherapy are commonly judged on the basis of clinical symptoms according to quality-of-life (QOL) score, symptom diary, and symptom-reducing drugs intake. The biomarkers correlated with the therapeutic effects are still controversial, especially for SLIT.

Antigen-specific IgG4 is considered to be a biomarker for antigen-specific immunotherapy; however, the correlation between the induction of IgG4 production and clinical symptoms is controversial.[44] In a report about the use of SLIT against timothy pollinosis, antigen-specific IgG4 was significantly upregulated in the SLIT group compared to the placebo group, and the authors concluded that the upregulation of IgG4 was correlated with the improvement of symptoms compared with the previous year. However, the clinical score and medication score were not significantly different between the SLIT group and the placebo group.[45] A recent study of dairy administration of grass allergen tablets showed dose-dependent efficacy of the SLIT and the induction of blocking IgG. This report showed that the administration of 75,000 SQ-T (15 μg Phl p 5) dose significantly reduced the symptom and medication

scores, and up-regulated specific IgG; however, a 2,500 SQ-T (0.5 μg Phl p 5) dose did not result in amelioration of the symptom and medication scores nor in the induction of IgG.[46] We previously reported that specific IgG4 was significantly increased in pollen season concomitant with improvement of the symptom medication score in the SLIT group compared to the placebo group.[47] The disagreement in results related to the induction of blocking IgG or IgG4 and the improvement of clinical symptoms may depend on the dose and/or the method of administration of the SLIT vaccine.

Other serological parameters have been recently reported to be useful as therapeutic biomarkers for SLIT. A 3-month course of pre-seasonal treatment of patients with grass pollen allergic rhinitis induced a reduction of the serum level of soluble human leukocyte antigen (sHLA)-G. The authors reported a significant relationship among the decrease of the sHLA-G serum level, the increase of interferon (IFN)-γ producing cells, and the decrease of sHLA-A, -B, and -C after SLIT.[48] Furthermore, the changes of serum sHLA levels were significantly correlated with the clinical symptom score measured using a visual analogue scale (VAS) after SLIT.[49] In this preliminary open-labeled study, the authors suggested that sHLA molecules might be considered as possible biomarkers of the response to SLIT.

Recently, two reports investigated the change of serum reptin levels after SLIT. Leptin is primarily produced by adipocytes and has been reported to protect T lymphocytes from apoptosis, regulate T cell activation, and up-regulate adhesion molecules in endothelial cells.[50] Furthermore, leptin was reported to modulate the hyporesponsiveness and proliferation of human naturally occurring $Foxp3^+CD25^+CD4^+$ regulatory T (nTreg) cells.[51] After a 3-month course of SLIT against pollinosis, serum leptin levels were reported to significantly correlate with symptom severity as assessed by VAS of nasal symptoms in women, the number of peripheral eosinophils in men, the allergen threshold dose for allergen-specific nasal challenge in both men and women, and the medication score in women. This 3-month course of SLIT showed a tendency to increase serum leptin levels compared to the levels before the SLIT, albeit the increase was not significant.[52] After a 2-year course of SLIT, the serum leptin level was significantly increased in men.[53] The relationship between the up-regulation of leptin by SLIT and clinical symptoms remains unclear; however, the difference of the clinical therapeutic efficacy may depend on gender and the presence or absence of obesity.

The reduction of antigen-specific Th2 responses is considered to be an important biomarker for antigen-specific immunotherapy. The increase in the size of the specific Th2 clone, which produces IL4 after being stimulated with Cry j 1 (a major allergen of the Japanese cedar pollen), after pollen season was reported to be significantly reduced in the SLIT group compared with the placebo group in a double-blind, placebo-controlled study of Japanese cedar pollinosis. The increase of specific IL5-producing cells after pollen season was also reduced in the SLIT group, but the reduction was not statistically significant.[47] It has also been reported that after a 2-year course of SCIT against Japanese cedar pollinosis, B and T lymphocyte attenuator (BTLA) expression on $CD4^+$ T cells was down-regulated in untreated patients after Cry j 1 stimulation and up-regulated in SCIT-treated patients. Furthermore, the change of BTLA expression was negatively correlated with IL5 production. The authors concluded that BTLA-mediated coinhibition of IL5 production may contribute to the regulation of allergen-specific T cell responses by antigen-specific immunotherapy.[54]

The therapeutic biomarkers of SLIT in children also remain unclear. In a study of the administration of the SLIT treatment to children with seasonal allergic rhinoconjunctivitis to grass pollen, the authors reported that a 2-year course of SLIT using a standardized 5-grass mixture (1.5 μg/week) did not alter the systemic immunologic reaction of IL4, IL5, and IFN-γ cytokine production, nor the proliferation of PBMC after stimulation with allergens in the SLIT group compared to the placebo group, although a positive effect on rescue medication use was achieved by SLIT treatment.[55] However, another study reported the up-regulation of mRNA expression in PBMC during SLIT in children using SQ-standardized tree pollen extracts. The authors reported that after the stimulation of PBMC with allergen *in vitro*, the mRNA expression of signaling lymphocytic activation molecule (SLAM) was significantly increased from baseline after 1 year in the SLIT group receiving a high-dose (weekly dose of 200,000 SQ-U) treatment. This up-regulation was reported to be correlated with IL10 and transforming growth factor-β (TGF-β) mRNA expression. The IL18 mRNA expression was also increased in the high-dose group over a 1-year treatment compared to the placebo group and was reported to be inversely correlated with the late-phase skin reaction after the second study year. The authors reported that this up-regulation of SLAM and IL18 mRNA expression suggested the down-regulation of Th2-type inflammatory responses by increased Th1-type responses.[56] Another study of SLIT in children using SQ-standardized tree pollen extract (weekly dose of 200,000 SQ-T, 30 μg major allergen containing Bet v 1, Aln g 1, and Cor a 1) reported that specific allergen-induced Foxp3 mRNA expression after a 2-year course of SLIT treatment was significantly increased in PBMCs compared to the placebo group and compared to the level before treatment. Changes in allergen-induced Foxp3 expression that significantly correlated with IL10 mRNA expression

were reported in the whole study group, including the low-dose (weekly dose of 24,000 SQ-T) group and the placebo group, after 1- and 2-year courses of treatment, and correlated with TGF-β1 mRNA after 1 year of treatment. Furthermore, IL17A mRNA expression was significantly correlated with symptom-medication score (SMS) in the whole study group and especially in the high-dose treated group. The authors concluded that IL17 expression may be associated with a poor therapeutic outcome of SLIT.[57]

MECHANISMS OF ANTIGEN-SPECIFIC IMMUNOTHERAPY

Numerous data showing that antigen-specific Th2-type responses are down-regulated and, in contrast, Th1-type and/or regulatory T cell (Treg) responses are up-regulated by immunotherapy have been accumulated. The imbalance of the population among the antigen-specific Th1, dominant Th2, and Treg is considered to induce sensitization and subsequent allergic inflammation in response to invading allergens, and immunotherapy may correct the imbalance of these cells. Actually, the high frequency of IL4-secreting Th2 cells was reported in allergic individuals, as was, in contrast, the dominance of IL10-secreting Tr1 cells in healthy subjects.[58] These authors suggested that the balance between allergen-specific Tr1 cells and Th2 cells causes the development of the allergy.

IL10-producing regulatory cells are considered to play a crucial role in clinical therapeutic mechanisms in immunotherapy. In a study of SCIT using house dust mite (HDM) extract in patients allergic to HDM, SCIT induced the suppression of PBMC proliferation and the suppression of IFN-γ, IL5, and IL13 production in PBMC stimulated with Der p 1 (a major allergen of HDM) at 70 days after treatment compared to the levels before treatment. In contrast to the suppression of Th1 and Th2 cytokines, the production of both IL10 and TGF-β was significantly increased. The report also showed that the suppression of proliferation was dependent on IL10 and TGF-β and that the source of IL10 is $CD25^+CD4^+$ T cells.[59] It has also been reported that IL10 production was induced by SLIT against HDM. The authors also reported the suppression of the proliferation of PBMC stimulated with extract of mite (*Dermatophagoids farinae*) and the increase of IL10 production compared to non-treated subjects.[60] The IL10 production after 3 years of SLIT treatment was significantly correlated with the improvement of clinical symptoms as assessed by forced expiratory flow between 25% and 75% (FEF_{25-75}).[61]

In a report about the use of SLIT to treat birch pollinosis, the authors investigated the antigen-specific proliferation and mRNA levels of cytokines and Foxp3. They reported that 4 weeks of SLIT induced a reduction in Bet v 1-specific proliferation and induced mRNA expression of IL10 and Foxp3 in $CD3^+$ cells compared to the levels before SLIT. These up-regulations of IL10 and Foxp3 mRNA expression were not seen after 52 weeks after SLIT; however, IFN-γ mRNA expression was significantly induced at 52 weeks after SLIT. The reduced Bet v 1-specific proliferation was significant after both 4 and 52 weeks, and this down-regulation was dependent on IL10 at 4 weeks. It has also been reported that neither TGF-β levels nor cell-cell contact-mediated suppression of $CD25^+CD4^+$ cells were changed during the course of SLIT.[62] Another report shows the significant reduction of IL5 mRNA expression and increased IL10 expression compared to the placebo group after 1 and 2 years of SLIT at a weekly dose of 200,000 SQ-U (30 μg major allergen) in children with tree pollinosis. It has been reported that TGF-β expression remained low after 1 and 2 years of SLIT; however, TGF-β expression was inversely correlated with IL5 and positively correlated with IL10 expression after 1 year of SLIT.[63]

In addition to IL10-secreting Tr1 cells, $Foxp3^+$ Treg cells are also considered to play a crucial role in the therapeutic effects achieved by immunotherapy (Fig. 2). It has been reported that 2 years of SCIT against hay fever significantly induced an increase in the number of $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cells in the nasal mucosa compared to the number before SCIT and the number in untreated patients out of season. Twenty per cent of $CD3^+CD25^+$ cells were reported to also be Foxp3-positive, and 18% of CD3+IL10-expressing cells were Foxp3-positive in the nasal mucosa after immunotherapy. This report suggested that the increase of $Foxp3^+CD25^+CD3^+$ cells in the nasal mucosa was associated with the clinical efficacy and suppression of seasonal allergic inflammation. This report also suggested the involvement of different types of regulatory T cells, namely IL10-secreting Tr1 cells and adaptive or induced Foxp3-positive Treg, in the therapeutic mechanisms of immunotherapy.[64] The involvement of Treg cells in immunotherapy was also reported in SCIT against hymenoptera venom allergy. In this report, the authors showed that the numbers of peripheral Treg cells defined as $Foxp3^+CD25^{bright}CD4^+$ T cells were significantly increased by venom immunotherapy, and the increase of circulating Treg cells was significantly correlated with the venom specific IgG4/IgE ratio.[65]

Antigen-specific Tr1 and Treg cells are considered to be involved not only in the suppression of Th2 cells but also, directly or indirectly, in the suppression of peripheral allergic inflammation[24] (Fig. 3). It has been reported that $CD25^+CD4^+$ Treg cells, more than 90% of which are $Foxp3^+$, directly inhibited the FcεR1-dependent mast cell degranulation after crosslinking of IgE, and this inhibition was dependent on cell-cell contact involving OX40-OX40L interactions between Treg and mast cells in the mouse.[66] Furthermore, al-

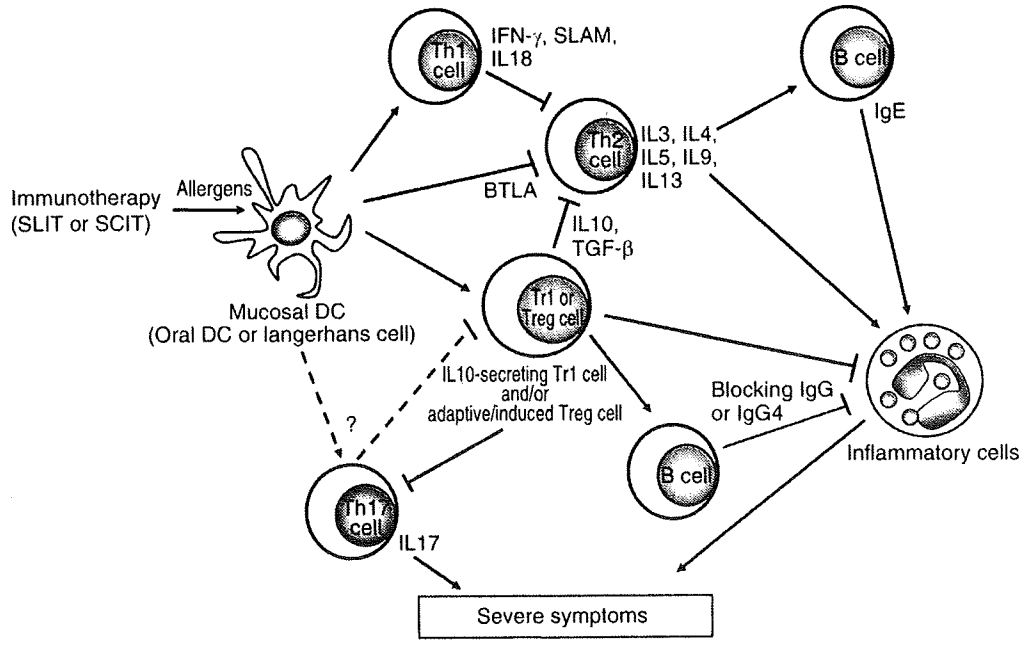

Fig. 2 T cells in antigen-specific immunotherapy. Antigen-specific immunotherapy induces regulatory T cells and Th1 cells via antigen-presentation by mucosal dendritic cells (DC). Th17 cells may be induced in a non-responder population by immunotherapy. The induced Th1 cells and/or regulatory T cells down-regulate the activation of Th2 cells and subsequently the activation of inflammatory cells such as eosinophils and mast cells. The regulatory T cells also activate B cells to produce blocking IgG or IgG4, and the blocking antibody inhibits binding between allergen and surface IgE on inflammatory cells to prevent the secretion of inflammatory chemical mediators.

lergic human eosinophils in peripheral blood and chronically inflamed nasal tissues were reported to express CD40, and the cross-linking of CD40 and CD40L enhanced the survival of eosinophils and induced the release of granulocyte/macrophage colony-stimulating factor (GM-CSF). In this report, IL10 down-regulated the constitutive expression of CD40 mRNA expression in eosinophils.[67] The induction of IL10-producing Tr1 or Treg cells in the nasal mucosa may play an important role in the reduction of nasal symptoms via cross-talk down-regulation of mast cells and eosinophils.

In a reports on the rush protocol of SCIT against Japanese cedar pollinosis using standardized pollen extract, the percentage of $CD203c^{high}$ cells in $CD3^-$ $CRTH2^+$ basophils after allergen stimulation was reported to be down-regulated after rush immunotherapy without a decrease of the serum specific IgE titer. Furthermore, the percentage of $CD203c^{high}$ on basophils after *in vitro* stimulation was reported to be significantly correlated with symptom score.[68] The mechanisms which attenuate the sensitivity of peripheral basophils without a change in serum specific IgE remain unclear; however, this attenuation may be partially due to the up-regulation of inhibitory blocking antibody on the surface of basophils.

ANTIGEN-SPECIFIC IMMUNOTHERAPY AGAINST JAPANESE CEDAR POLLINOSIS

In Japan, Japanese cedar pollinosis is one of the most prevalent types of seasonal allergic rhinitis, with a prevalence estimated to be 26.5%.[2] Two clinical trials described the therapeutic effects of SLIT against Japanese cedar pollinosis.[47,69] In both trials, standardized Japanese cedar pollen extract was used at a monthly cumulative dose of 8,000 JAU, which contains approximately 10 μg of Cry j 1. This dosage is less than that reported in Europe, where a dose of 75,000 SQ-T (15 μg of a major grass allergen Phl p 5) was administered once daily for 18 weeks.[46] Unless the monthly cumulative dose is approximately $1/40^{th}$ of the amount required to be considered a major allergen (10/450 μg as a major allergen) in Japan, SLIT with an active treatment group against Japanese cedar pollinosis is still effective for improving quality of life and significantly ameliorates patients' SMS and symptom score during the pollen season. The up-regulation of the IL4-producing clone size specific to epitopes from Cry j 1 and Cry j 2[70] was reported to be significantly attenuated, and Cry j 1-specific IgG4 production was also significantly induced by active SLIT.[47] Furthermore, IL10-producing Tr1 cells were

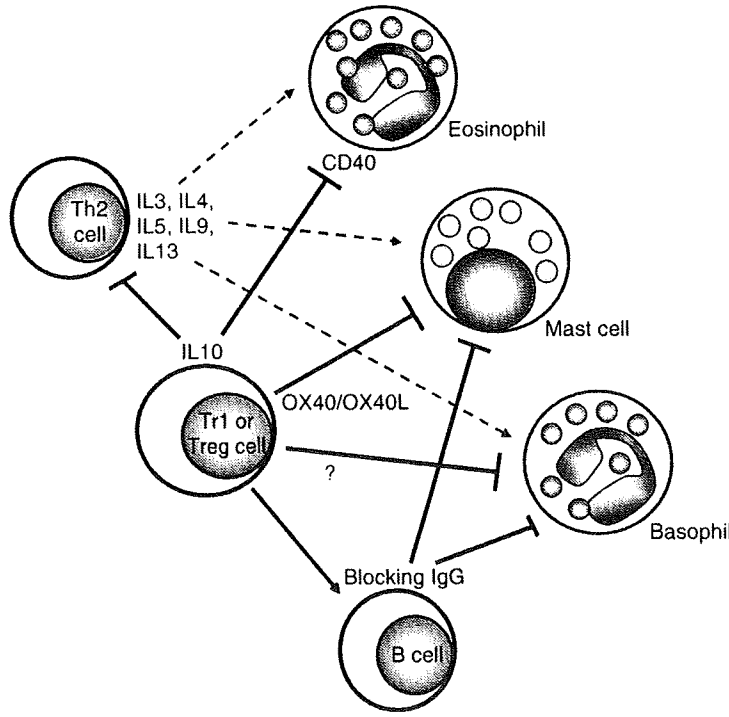

Fig. 3 Proposed roles of regulatory T cells on inflammatory cells in allergen-specific immunotherapy. Regulatory T cells, namely IL10-secreting Tr1 cells or adaptive/induced Treg cells, down-regulate inflammatory cells, directly or indirectly. Regulatory T cells down-regulate the activation of Th2 cells and subsequently Th2-type cytokine secretion. Regulatory T cells suppress the activation of inflammatory cells directly via their surface molecules and by secreting cytokines, and indirectly via the down-regulation of cytokine production in Th2 cells and by the activation of B cells to produce blocking IgG.

reported to be significantly increased in patients treated with SLIT compared with the levels in untreated patients and healthy subjects, and the proliferation of $CD4^+$ leukocytes stimulated with Cry j 1 and Cry j 2 was significantly suppressed by SLIT treatment in an IL10-dependent manner.[71] Supplementation with recombinant or native Cry j-allergens and/or updosing of the extract by bio-engineering may lead to more effective SLIT for treating pollinosis.

Another approach to safer immunotherapy is the use of oral immunotherapy using transgenic rice seed accumulating Cry j 1.[72] The generated transgenic rice plants expressed recombinant, structurally disrupted Cry j 1 peptides but spanned the entire Cry j 1 region as fusion proteins with the major rice storage protein glutenin. These fusion proteins aggregated with cysteine-rich prolamin and were deposited in endoplasmic reticulum-derived protein body I in rice seed. Transgenic rice expressing T cell epitopes from Cry j 1 and Cry j 2 successfully suppressed antigen-specific Th2-mediated IgE responses in a mouse model of allergic rhinitis.[73] Further clinical trials are needed to develop a rice-based edible vaccine as a tool for oral immunotherapy to control allergies.

An immunoregulatory liposome encapsulating the recombinant fusion protein of Cry j 1-Cry j 2 was manufactured as a novel vaccine for Japanese cedar pollinosis without risk of anaphylaxis.[74] The hybrid fusion allergen is expected to provide safer and more effective vaccines for immunotherapy. Vaccines using only T cell epitopes are also safer than native allergens, but there is wide variation among individual T cell epitopes. The fusion protein of major allergens covers all sequential T cell epitopes but is expected to have less IgE-binding capacity because its three-dimensional structure is disrupted in some B cell epitopes. Recombinant hybrid molecules using major allergens of timothy grass pollen induced stronger proliferation of PBMC in timothy-allergic patients than did mixtures of corresponding allergens, but still possess IgE-binding capacity and induce IgG production in sensitized mice.[75] In a mouse model sensitized with native Cry j 1 and Cry j 2, the vaccine that con-

tained Cry j 1-Cry j 2 fusion protein in the immunoregulatory liposome showed suppression of IgE and IgG antibody responses after being challenged with the allergens. Furthermore, oral administration of the vaccine showed efficient suppression of IgE antibody production.[74]

CONCLUSIONS

The standardization of a vaccine enables us to compare the results from varied clinical trials with respect to dose, clinical effects, and changes in biological parameters. Many reports have shown positive clinical therapeutic effects and suppressed effector/inflammatory responses. It is considered that IL10-producing Tr1 and/or adaptive or induced Treg cells may be involved in the suppression of the antigen-specific Th2-responses and local inflammation. However, how immunotherapy induces suppressor cells like Tr1 and Treg cells remains unclear, although the involvement of mucosal dendritic cells has been proposed. High-quality clinical studies are indispensable to clarify the therapeutic biomarkers and the mechanisms of induction of suppressor cells, and the resultant data from the studies may enable us to develop safer and more effective immunotherapy through the modification of the allergens, optimum dose, or administration regimen of a vaccine.

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Poly(I:C) induces BLyS-expression of airway fibroblasts through phosphatidylinositol 3-kinase

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ABSTRACT

B lymphocyte stimulator (BLyS), B cell activating factor (BAFF), a member of the tumor necrosis factor ligand superfamily has potent co-stimulatory activity on B cells, and BLyS-production in the airway mucosa is of potential importance as it triggers innate and adaptive immune responses. To investigate whether airway fibroblast could express BLyS, we examined BLyS-expression in human nasal airway fibroblasts and compared to its expression in tonsillar and skin fibroblasts as well as the effect of the Toll-like receptor (TLR) ligands on that in human nasal airway fibroblasts. The expression of BLyS by nasal fibroblasts in the presence of polyinocinic–polycytidykic acid (poly(I:C)) was markedly induced, to a level of more than 100 times higher than that observed in the absence of poly(I:C). In order to demonstrate the intracellular pathways involved in poly(I:C)-induced BLyS-expression, we used specific inhibitors of phosphatidylinositol 3-kinase (PI3-kinase), spleen tyrosine kinase (Syk), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular-signal related kinase (ERK)-signaling in these events. Pre-incubation with the PI3-kinase inhibitor LY294002 or Wortmanin reversed the poly(I:C)-induced production and expression of BLyS. Syk kinase inhibitor Piceatannol partially reduced its production and expression. Thus, we were able to show that PI3-kinase signaling is directly involved in poly(I:C)-induced BLyS-expression in nasal airway fibroblasts. These results indicate that human nasal airway fibroblasts strongly induce BLyS-expression and production by poly(I:C) through PI3-K signaling during airway immune responses.

1. Introduction

TNF ligand superfamily member 13B, BLyS,[1] BAFF, plays critical roles in respiratory mucosal defense, because it is a potent co-activator of B cells in vitro or in vivo. BLyS induces B cell proliferation, human Ig class switch recombination, and Ig secretion [1–3]. Activation of B cells in the airways is now believed to be of great importance in immunity to pathogens, and it also participates in the pathogenesis of airway diseases. The expression of BLyS was detected in TLR ligand-treated BEAS-2B cells and primary human bronchial epithelial cells [4]. In the nasal mucosa, the expression of BLyS for in sinonasal tissue was found to be significantly correlated with CD20, and overproduction of BLyS contributes to the pathogenesis of chronic rhinosinusitis via the local induction of IgA and the activation of eosinophils [5]. Although nasal airway fibroblasts are a rich source of cytokines, chemokines, and growth factors, it is unknown whether airway fibroblast could express BLyS.

As 'a mucosal guardian' in the upper airway, the inferior turbinate of the human nose is easily exposed to a variety of stimulus such as viral and bacterial infection during the common cold. Most of the viruses that cause upper respiratory infection are RNA viruses including rhinoviruses, coxsackievirus, echovirus, and influenza viruses. RNA viruses synthesize double-stranded RNA (dsRNA) during replication [6], and this is a strong stimulus for innate anti-viral responses through the secretion of cytokines. TLRs play key roles in innate immunity by recognizing microbial conserved pathogen-associated molecular patterns, and TLR3 is involved in the recognition of the synthetic dsRNA analogue, polyinocinic–polycytidykic acid (polyI:C) [7]. It is not clear which TLR ligand induces BLyS-expression or production of human airway fibroblasts.

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[1] *Abbreviations used:* BLyS, B lymphocyte stimulator; BAFF, B cell activating factor; TLR, Toll-like receptor; dsRNA, double-stranded RNA; IRF, interferon regulatory factor; PGN, peptidoglycan; poly(I:C), polyinocinic-polycytidykic acid; LPS, Lipopolysaccharide; PI-3K, phosphatidylinositol 3-kinase; Syk, spleen tyrosine kinase; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal related kinase; TACI, transmembrane activator and CAML interactor.

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The presence of dsRNA during viral infections is a key step in the activation of several signaling pathways, including mitogen-activated protein kinase (MAPK), activator protein-1, and interferon regulatory factors (IRFs). Poly(I:C) induces the rapid activation of the mitogen-activated protein kinases (MAPKs) (p38 MAPK, c-Jun N-terminal kinase (JNK), ERK) and MAPK-dependent expression of proinflammatory cytokines, chemokines, and adhesion molecules [8,9]. In accordance with the expression of TLR3, poly(I:C) stimulation induces the activation of interferon regulatory factor-3 (IRF-3) transcription factor and p38 MAPK [10]. PI3-kinase plays an essential role in IRF-3-binding to the promoter of the target gene and TLR3-mediated gene induction by dsRNA-treated cells [11]. Spleen tyrosine kinase (Syk) regulates PI3K activation and RNA virus endocytosis in the airway mucosae [12]. The potential role of dsRNA-induced BLyS-expression in signaling is poorly understood.

Although the ability of BLyS-production from airway mucosa is of potential importance as it provides innate and adaptive immune responses, the details of BLyS-expresion in airway fibroblasts remains unexplored. In this study, we established fibroblast lines from the human inferior turbinate and other tissues, and it has been confirmed whether BLyS is expressed in human nasal airway fibroblasts, tonsillar, and skin fibroblasts. We examined the effect on BLyS-expression of TLR ligands including peptidoglycan (PGN); poly(I:C); lipopolysaccharide (LPS); and CpG in human nasal airway fibroblasts. In order to demonstrate the intracellular pathways involved in dsRNA-induced BLyS-expression, we used specific inhibitors of PI3-kinase, Syk, p38 MAPK, JNK, and extracellular-signal related kinase (ERK)-signaling in these events.

2. Materials and methods

2.1. Reagents

The following reagents were used: poly(I:C) (Amersham Bioscience, Piscataway, NJ); PGN (Sigma); LPS (MERCK bioscience, Germany); CpG, a synthetic oligodeoxynucleotide that contains CpG motifs that mimicks bacterial DNA (5′-ACCGATCGTTCGGCCGGTGACGGCACCA-3′); SP600125 as a specific inhibitor of JNK (BIOMOL); SB203580 as a specific inhibitor of p38 MAP kinase (Promega); PD98059 as a specific inhibitor of MEK-1 (Promega); LY294002 as a specific inhibitor of PI3-kinase (Promega); Wortmannin (Sigma); AKT inhibitor (CALBIOCHEM); anti-human BLyS monoclonal Ab (R&D system); p44/42 MAP Kinase rabbit polyclonal antibody (Ab) (Cell Signaling, Beverly, MA); SAPK/JNK rabbit polyclonal Ab (Cell Signaling); phospho-p44/42 MAPK (E10) mouse monoclonal Ab (Cell Signaling); phospho-JNK (G9) mouse monoclonal Ab (Cell Signaling); phospho-p38 MAPK (28B10) mouse monoclonal Ab (Cell Signaling); phospho-AKT (587F11) mouse monoclonal Ab (Cell Signaling); AKT Rabbit polyclonal Ab (Cell Signaling); and p38 (A12) mouse monoclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

2.2. Cells, cell lines, and cell culture

Human primary nasal fibroblast lines were established from human nasal biopsy tissues of inferior turbinates removed during the operation ($n = 6$). All the nasal specimens had been taken from patients with allergic rhinitis. Five males and one female aged 30.5 ± 6.6 year (mean ± SEM) were atopic, diagnosed on the basis of elevation of at least one of the capsulated hydrophobic carrier polymer-radioallergosorbent tests against 8 common aeroallergens. All of the patients had a house dust or a cedar pollen CAP-RAST score of 2 or more. The subjects had given written informed consent, and its study protocol was approved by the Ethics Committee at University of Fukui. The patients had no smoking and no special background including pollution, without any medication at least 14 days before operation. Only fibroblast lines between the sixth and tenth passages were used in this study. No contamination of epithelial cells was confirmed by immunohistochemical examination using cytokeratin markers. The fibroblasts were stimulated by TLR ligands in RPMI-1640 medium supplemented with 10% FCS and in humidified atmosphere of 10% CO_2 in air at 37 °C.

2.3. Real time PCR

Total RNA was extracted using a total RNA isolation NucleoSpin™ RNA II Kit (MACHERY-NAGEL, Düren Germany). The reverse transcription reaction was performed with TaqMan® RT Reagents (Applied Biosystems Japan, Tokyo, Japan) using random hexamer primers. The amplification of TLRs, BLyS, and β_2-microglobulin-cDNA was performed in a MicroAmp optical 96-well reaction plate (Applied Biosystems). All TaqMan® probe/primer combinations used in this study were TaqMan® Gene Expression Assay products purchased from Applied Biosystems. β2-Microglobulin was chosen as the reference housekeeping gene because it is convenient to assay and highly expressed. Furthermore, in order to select the housekeeping gene, we evaluated it using a TaqMan® Human Endogenous Control Plate, which was most suitable. TaqMan® PCR was performed in a 20-μl volume using TaqMan® Universal PCR master mix (Applied Biosystems). The reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The reaction mixtures were pre-incubated for 2 min at 50 °C. The PCR program involved 10 min of Taq Gold activation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C (maximum ramping speed between temperatures). Human cDNA equivalent to 50 ng of total RNA from each sample was assayed in each tube. The threshold cycle number was determined with sequence Detector Software (version 1.1: Applied Biosystems) and transformed using comparative methods as described by the manufacturer with β_2-microglobulin as the reference gene.

2.4. Cytokine assay

The cells were cultured in the presence of poly(I:C) for appropriate periods, and then the culture supernatants were harvested and stored at −80 °C. The amounts of BLyS in the cell culture supernatant were measured with an ELISA kit that was purchased from R&D system.

2.5. Immunoblot analysis

The samples were added to a 2-fold volume of sample buffer [95% laemmli sample buffer (BIORAD) and 5% 2-mercaptoethanol]. After heating the mixture at 95 °C for 5 min, the samples were electrophoresed. The proteins were then transferred electrophoretically onto polyvinylamidedifluoride membranes (Amersham Bioscience). The blotted membranes were rinsed with 5% non-fat-dried milk diluted in PBS containing 0.1% Tween 20 for 60 min at room temperature, and then incubated with the antibodies for 16 h at 4 °C. After being washed, the membranes were treated with HRP-conjugated anti-mouse immunoglobulin (Ig) Ab or HRP anti-rabbit Ig Ab (DAKO, Carpinteria, CA) for 60 min at room temperature. Peroxidase color visualization was achieved with TMB membrane peroxidase substrate (KPL, Gaithersburg, MD).

2.6. Antibody array

Signal Transduction AntibodyArray™ which contains 400 high quality antibodies against well-studied signaling proteins, was purchased from Proteomics Company. Nasal fibroblasts were stim-

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ulated with 10 μg/ml poly(I:C) for 30 min, washed twice with ice cold Tris saline (50 mM Tris pH 7.5, 150 mM NaCl, 1.5 mM PMSF), and lysed using Triton Extraction buffer containing 15 mM Tris pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.5% triton X-100, 10 μg/ml leupeptin, and 0.5 mM PMSF. Pelleted cellular debris was removed by centrifugation at maximum speed (14,000 rpm). The supernatant was collected, and the membrane of Signal Transduction Antibody Array™ was incubated with the whole cell extracts in 5 ml extraction solution containing 1% BSA for 2 h at room temperature with slow shaking. After washing the membrane, HRP-conjugated anti phosphotyrosine antibody was applied for 2 h at room temperature. Peroxidase substrate was used and the membrane was washed and then exposed to X-ray film.

2.7. Data and statistical analysis

Statistical analysis was performed using the Wilcoxon signed-ranks test to assess the significance of differences.

3. Results

3.1. BLyS-expression in human fibroblasts

To determine whether BLyS is expressed in human fibroblasts, we established fibroblast lines from small pieces of human inferior turbinate, tonsil, and skin respectively from six individuals and then examined BLyS-expression in stimulated fibroblasts. As shown in Fig. 1, the expression of BLyS in nasal fibroblasts was markedly induced in the presence of poly(I:C), to a level more than 100 times higher than that observed in the absence of poly(I:C). In skin fibroblasts, we could not detect any induction of BLyS-expression in the presence of poly(I:C). Although poly(I:C) induced BLyS-expression by tonsillar fibroblasts, its induction was lower than that induced in nasal stimulated fibroblasts .

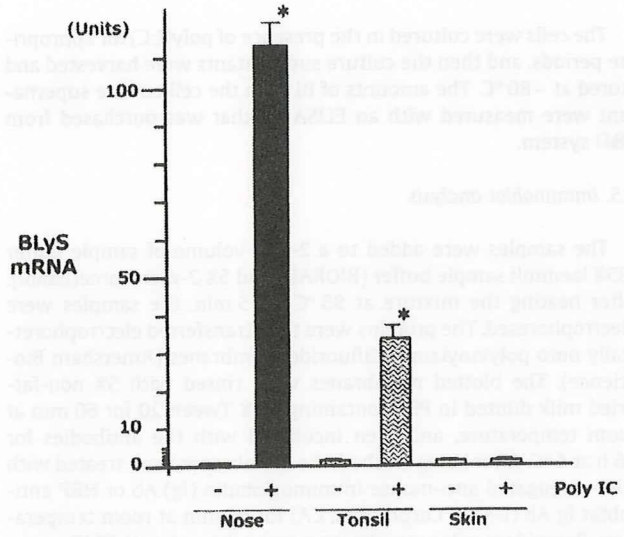

Fig. 1. BLyS-expression in human fibroblasts. After the cells has been treated with poly(I:C) (20 μg/ml) for 6 h, total RNA was isolated from human nasal (closed bar), tonsillar (waved bar), and skin (open bar) fibroblasts. The expression levels of BLyS-mRNA were assayed by real time RT-PCR. The RNA was reverse transcribed to cDNA, which was then used for real time PCR. Reactions were performed in three wells, and results are expressed relative to the expression levels of β_2-microglobulin. Data are expressed as the mean ± SEM of the fold increase relative to the control ($n = 6$). *$P < 0.05$ compared with control using Wilcoxon's signed-ranks test.

3.2. Toll-like receptor ligands and BLyS-expression

Since TLR 3 ligand strongly induced the expression of BLyS especially in human nasal fibroblasts, next we observed the expression of TLRs on human nasal fibroblasts. The mRNA expression of TLRs on fibroblasts was confirmed by real time RT-PCR. Fig. 2A shows the relative expression levels of TLR mRNAs on the cells. TLR 3 and 4 were highly expressed, while TLR 2 and 9 were moderately expressed. TLR1, 5, and 6 were also detected, but their expression levels were lower than those of TLR2, 3, 4, and 9. We could not detect the expression of TLR 7, 8, and 10 in human nasal fibroblasts. In order to look at which TLR ligand induces BLyS-expression in human nasal fibroblasts, we examined the effect on BLyS-expression of TLR ligands (PGN, poly(I:C), LPS, and CpG). Fibroblasts were treated with the agonists for 6 h, and BLyS-mRNA expression was assessed by real time PCR. The BLyS-expression was induced 100-fold by poly(I:C) and 10-fold ($P < 0.05$) by LPS in nasal fibroblasts, while it was hard to find any effecet by PGN or CpG on the level of mRNA for BLyS.

3.3. Dose-dependence and time-course of poly(I:C)-induced BLyS-expression

Having shown that TLR3 ligands strongly induce BLyS-expression in human nasal fibroblasts, next we have investigated its expression precisely. Poly(I:C) induced BLyS-mRNA expression in a dose-dependent manner with the maximal stimulation generally being at 10 μg/ml or higher, and its expression was detected 10-fold ($P < 0.05$) at 1 μg/ml in nasal fibroblasts (Fig. 3A). The exposure of nasal fibroblasts to TLR3 ligand triggered a rapid expression of BLyS-mRNA at 6 h and decreased thereafter. The expression was sustained at 80-fold at 24 h and 15-fold ($P < 0.05$) at 48 h (Fig. 3B).

3.4. Poly(I:C) induces BLyS-production from human nasal fibroblasts

Similar to other TNF family members, BLyS is generally expressed as a transmembrane protein and is cleaved from the surface to release its active soluble form. Production of soluble BLyS-proteins was detected using ELISA. Poly(I:C) increased BLyS-production at 1 μg/ml and higher ($P < 0.05$) in a dose-dependent manner (Fig. 4A) . Its production was 100 times higher than that detected in the absence of poly(I:C) at 10 μg/ml. Although the BLyS gene encodes a putative 285 amino acid (aa) type II transmembrane protein, the 152 aa form can also be shed from the membrane because the N-terminal side contains a furin cleavage site. We also examined the supernatants from human nasal fibroblasts using Western blotting. Fig. 4B shows that the soluble form of BLyS from human nasal fibroblast weighs 18 kDa, and its production occurred in a dose-dependent manner with the maximal stimulation observed at 10 μg/ml.

In the presence of IL-4, Ig class switch recombination in human B cells was always detected by BLyS-treatment at 100 ng/ml or higher [2]. The human nasal fibroblasts produce enough amounts of BLyS-protein to cause Ig class switch recombination, as shown in Fig. 4.

3.5. Suppression of poly(I:C)-induced and BLyS-production and expression by PI3-kinase inhibitor

Poly(I:C) is a TLR3 ligand that induces TLR3-signaling. We screened the intracellular signal transduction molecules of poly(I:C)-stimulated human nasal fibroblasts using *AntibodyArray*™ which contains 400 high quality antibodies, and found that poly(I:C)-induced signaling involved Syk, Rho, or TRAF6. Also, we demonstrated that the exposure of cells to poly(I:C) triggered phosphorylation and activation of p38 MAPK, JNK, and AKT by

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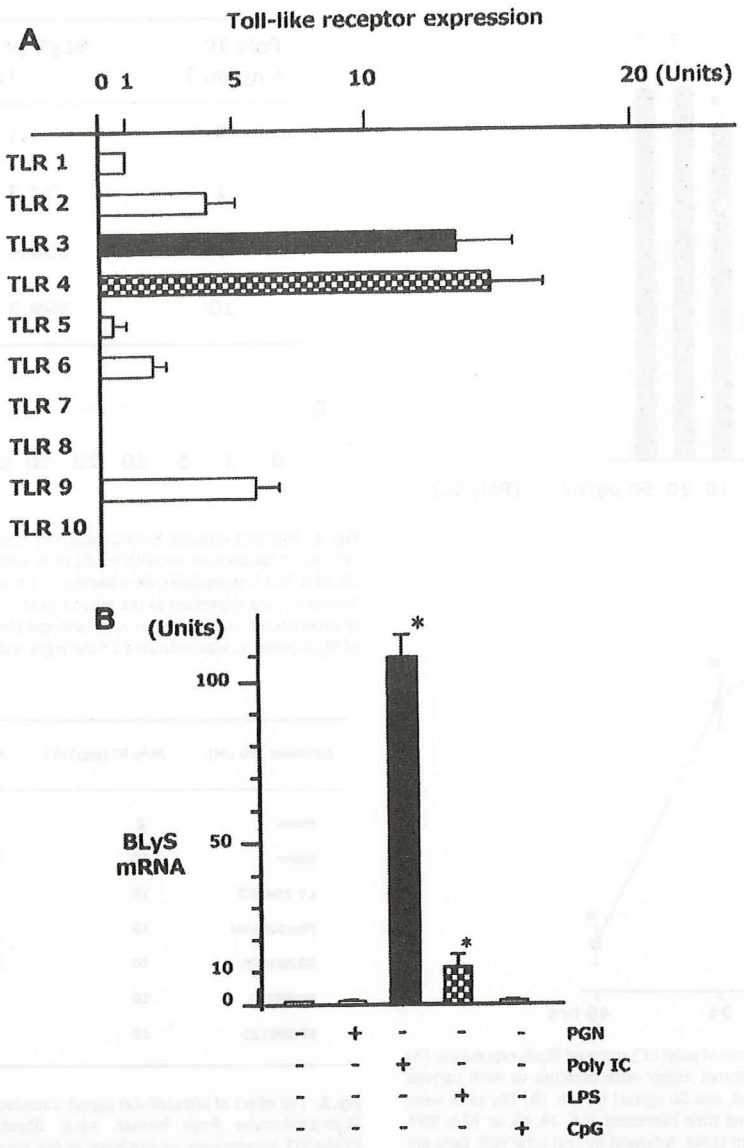

Fig. 2. Toll-like receptor ligands and BLyS-expression. (A) TLR1–9 mRNA expression levels were assayed by real time RT-PCR using a cDNA template derived from human nasal fibroblasts. The results are expressed relative to the expression levels of β_2-microglobulin ($n = 6$). (B) Analysis of the expression of BLyS-mRNA induced by Toll-like receptor ligands using real time PCR. Fibroblasts were treated with PGN (20 μg/ml), poly(I:C) (20 μg/ml), LPS (1 μg/ml), or CpG (1 μM) for 6 h. The RNA was isolated from the cells and BLyS-expression was measured. Data are expressed as the mean ± SEM of the fold increase relative to the control ($n = 6$). *$P < 0.05$.

Western blotting with specific antibodies to phosphorylated p38 MAPK, JNK, and AKT. Since PI3-kinase participates in TLR3-signaling [11], we sought to determine whether MAPKs, Syk kinase, or PI3-kinase signaling is directly involved in BLyS-expression. To accomplish this, we tested the ability of SB203580 (a specific inhibitor of p38 MAPK signaling), SP600125 (JNK inhibitor), PD98059 (a specific inhibitor of ERK signaling), Piceatannol (an inhibitor of Syk kinase), LY294002 and Wortmanin (PI3-kinase inhibitor) to affect the expression of BLyS in nasal fibroblasts stimulated with poly(I:C) (Figs. 5 and 6A).

Pre-incubation with the PI3-kinase inhibitor LY294002 and Wortmanin markedly suppressed the poly(I:C)-induced production and expression of BLyS, although inhibition of p38 MAP, JNK, and ERK did not have any effect. The Syk kinase inhibitor Piceatannol reduced poly(I:C)-induced production and expression of BLyS by 40% (Figs. 5 and 6A). The specific PI3-kinase inhibitor LY294002 reversed its expression in a dose-dependent manner (Fig. 6B). LY294002 decreased the expression of BLyS in poly(I:C)-stimulated nasal fibroblasts by 90% compared to the control level ($P < 0.05$). There were no differences in cell shape or viability among the four inhibitors.

4. Discussion

In the present study, we demonstrated that the expression of BLyS was strongly induced by nasal airway fibroblasts in the presence of poly(I:C), while we could not detect any induction of BLyS-expression in skin fibroblasts. Consistent with the high expression of TLR 3 and 4 mRNA on nasal airway fibroblasts, poly(I:C) and LPS induced BLyS-expression. Poly(I:C) induced BLyS-mRNA expression and protein-production in a dose-dependent manner. BLyS-expression and production from airway fibroblasts has not been reported previously, although BLyS is expressed in proinflammatory-cytokine-stimulated fibroblast-like synoviocytes from the inflamed

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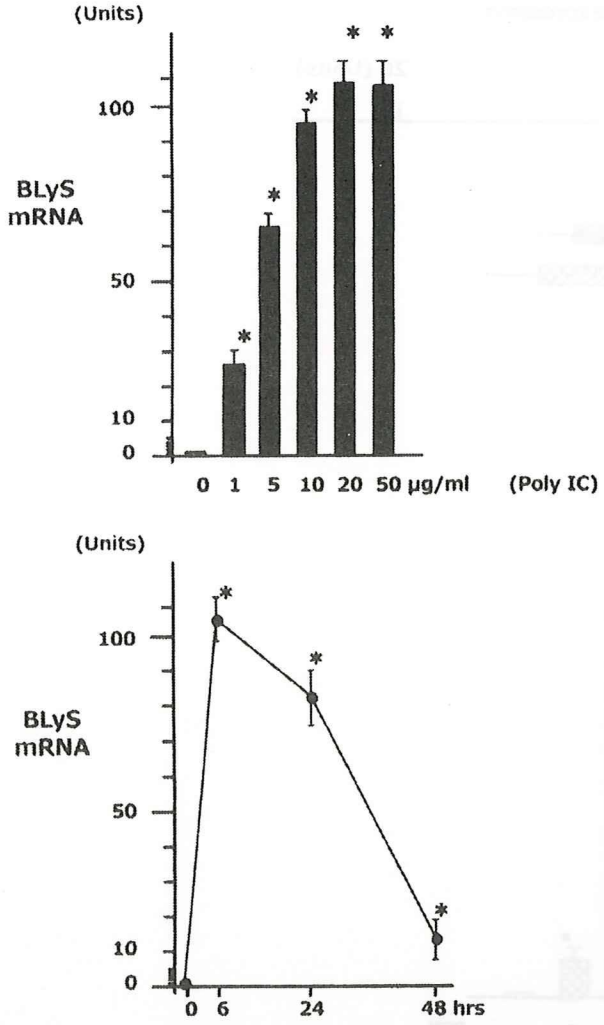

Fig. 3. Dose-dependence and time-course of poly(I:C)-induced BLyS-expression. (A) Human nasal fibroblast cells were cultured either with medium or with various concentrations of poly(I:C) (1, 5, 10, 20, and 50 μg/ml) for 6 h. (B) The cells were stimulated with poly(I:C) (10 μg/ml) and then harvested at 6, 24, 48, or 72 h. RNA was prepared and reverse transcribed to cDNA, followed by real time PCR. Data are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$.

A

Poly IC (μg/ml)	BLyS production in 24 hrs (pg/ml)
0	4.1 ± 1.5
1	34.3 ± 15.6
5	116.4 ± 50.0
10	399.2 ±100.7

B

Fig. 4. Poly(I:C) induces BLyS-production from human nasal fibroblasts. (A) The cells were cultured either with medium or with various concentrations of poly(I:C) for 24 h. The supernatants were harvested for analysis of BLyS-production by ELISA. The results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$. (B) The same amount of supernatant was applied to each lane and blotted with anti-BLyS Ab. The position of BLyS-proteins was indicated to the right with the arrow.

Inhibitor (10 μM)	Poly IC (μg/ml)	BLyS production in 24 hrs (pg/ml)
None	0	4.1 ± 4.4
None	10	391.8 ± 72.7
LY 294002	10	13.3 ± 5.9
Piceatannol	10	245.1 ± 52.8
SB203580	10	279.9 ± 51.2
PD98059	10	322.9 ± 64.9
SP600125	10	296.5 ± 82.3

Fig. 5. The effect of intracellular signal transduction inhibitors on Poly(I:C)-induced BLyS-production from human nasal fibroblasts. After pre-incubation with LY294002, Wortmanin, an inhibitor of PI3-kinase; Piceatannol, an inhibitor of Syk kinase; SB203580, an inhibitor of p38 MAP kinase; PD98059, an inhibitor of MEK; or SP600125, an inhibitor of JNK, the cells were stimulated with poly(I:C) (1, 5, and 10 μg/ml) for 24 h. The supernatants were harvested for analysis of BLyS-production by ELISA. The results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$.

joints of patients with rheumatoid arthritis [13]. Pre-incubation with the PI3-kinase inhibitor LY294002 or Wortmanin reversed the poly(I:C)-induced production and expression of BLyS. The Syk kinase inhibitor Piceatannol also partially reduced its production and expression. Thus, we were able to show that PI3-kinase signaling is directly involved in poly(I:C)-induced BLyS-expression in nasal airway fibroblasts.

Airway submucosal fibroblasts play important roles, in the innate anti-viral response to human rhinovirus infections and dsRNA [14], while airway fibroblasts support the proliferation of bronchial epithelial cells, which is an important biological process in physiological conditions and various human airway diseases [15]. The inferior turbinate of the human nose is a 'mucosal protector' on the upper airway that acts against viral or bacterial infection and antigen exposure. Nasal fibroblasts of the inferior turbinate produce BLyS strongly when stimulated by dsRNA, leading to the innate anti-viral response including B cell proliferation and Ig secretion. Here, we have demonstrated the high BLyS-expression of nasal fibroblasts originating from nasal mucosa of the inferior turbinate.

Upper airways characteristically consist of a periosteum and bone covered by nasal respiratory mucosa. We also examined BLyS-expression of nasal fibroblasts originating from the periosteum of the inferior turbinate, and poly(I:C) strongly also induced BLyS-expression in these cells (data not shown). Fibroblasts also play key roles in airway remodeling process [16,17] and in nasal polyps, which are products of nasal airway remodeling, BLyS-mRNA was significantly increased in nasal polyps of patients with chronic rhinosinusitis, and its protein was present in mucosal epithelial cells in the nasal polyps along with unidentified cells in the lamina propria[5]. We also found that nasal fibroblasts originating from nasal polyps express BLyS by stimulation with dsRNA and LPS (data not shown).

Due to the high expression of TLR3 and TKR4, nasal fibroblasts can respond to their ligands. Through TLR3 and adaptor molecules, poly(I:C) stimulation induces the activation of IRF-3 transcription factor [10]. When PI3-kinase is not recruited to TLR3 or its activity

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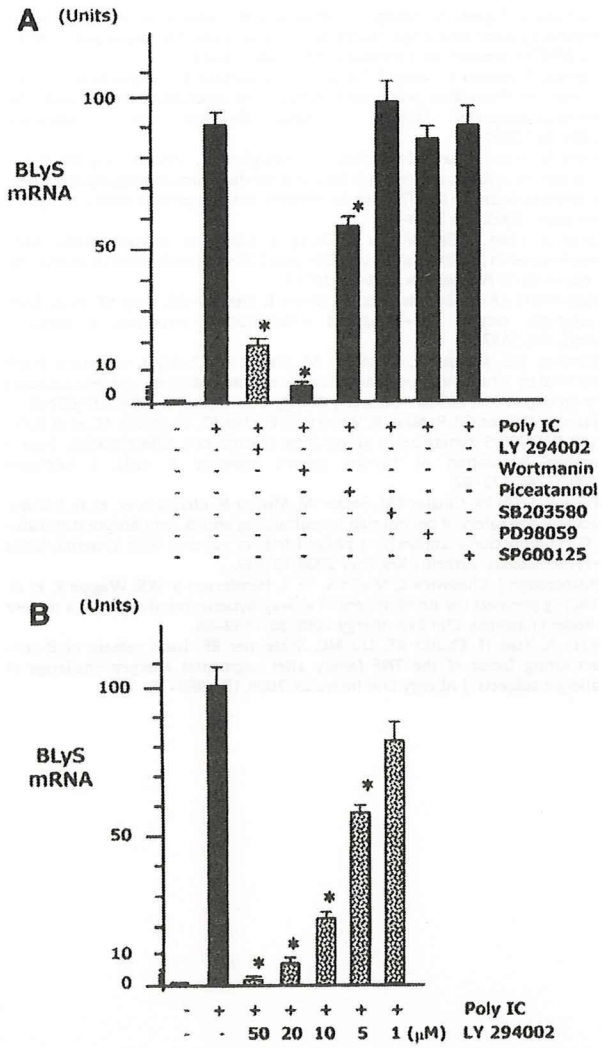

Fig. 6. Suppression of poly(I:C)-induced BLyS-expression by a PI3-kinase inhibitor. (A) Human nasal fibroblasts were pre-incubated with LY294002, Wortmanin, Piceatannol, SB203580, PD98059, or SP600125. The cells were harvested 6 h after stimulation with poly(I:C) (10 μg/ml), and the RNA was prepared for analysis of BLyS-expression by RT-PCR. Data are presented as mean ± SEM compared with the levels without inhibitor using Wilcoxon's signed-ranks test ($n = 6$). *$P < 0.05$. (B) Dose response of PI3-kinase inhibition of poly(I:C)-induced BLyS-expression. The culture conditions were the same as those for the experiment shown in Fig. 5A. except that the concentration of LY294002 was varied as indicated.

is blocked, IRF-3 is only partially phosphorylated and fails to bind to the promoter of its target gene in dsRNA-treated cells [11]. The PI3-kinase pathway plays an essential role in TLR3-mediated gene induction. As a specific PI3-kinase inhibitor reversed the expression of BLyS in dsRNA-stimulated nasal fibroblasts, PI3-kinase is considered to play a pivotal role in dsRNA-induced BLyS-expression in human nasal fibroblasts. PI3-kinase inhibitor also decreased TLR3-experssion in nasal fibroblasts (data not shown). A frequent byproduct of virus infection, dsRNA, is recognized by TLR3 as a method for mediating the innate immune response to virus infection. The most common acute infection in humans, human rhinovirus is a leading cause of exacerbations of airway inflammation. We previously reported that the protein tyrosine kinase Syk is expresses in nasal fibroblasts [18,19] and regulates PI3K activation and human rhinovirus endocytosis [20]. Although the full intracellular signaling pathway for dsRNA-induced BLyS-expression remains to be elucidated, we did find that, a PI3-kinase inhibitor and a Syk kinase inhibitor significantly reduced dsRNA-induced production and expression of BLyS in nasal fibroblasts.

While BLyS is an important survival factor for B lymphocytes, it can enhance immune responses not only by increasing the number of B cells but also by elevating CD4-positive T lymphocyte function and NK cell activity [21]. In BLyS-transgenic mice, the delayed-type hypersensitivity scores were found to correlate directly with BLyS levels in serum [22]. BLyS also provides a co-stimulatory signal to T cells and T cell activation, and bronchial structural cells including fibroblasts might play a critical role in the regulation of inflammation in asthma by increasing the survival of T lymphocytes [23]. Human post-switched IgG-positive B cells respond specifically and exclusively to BLyS by differentiating into IgG-secreting plasma cells [24]. On the contrary, in the presence of IL-4, BLyS induced immunoglobulin class switch recombination to epsilon in a CD40-independent manner [1,2].

BLyS participates in a variety of disorders, and interruption of the BLyS pathway is a candidate for therapeutic targeting of some diseases. In patients treated with belimumab, a fully human monoclonal antibody that inhibits the biological activity of the soluble form of BLyS in patients with systemic lupus erythematosus, significant reductions in the median percentage of CD20 positive B cells were observed versus placebo [25]. In nasal polyps, the expression of BLyS-mRNA in sinonasal tissue was significantly correlated with CD20 and transmembrane activator and CAML interactor (TACI) in sinus tissue [5]. TACI has been identified as a BLyS receptor. In a murine model of airway hyperresponsiveness, using soluble mTACI-Ig, a receptor for BLyS, it was revealed that mTACI-Ig treatment reduced the levels of total and allergen-specific IgE in serum and it was more effective than anti-IgE treatment in reducing airway hyperresponsiveness to inhaled antigens [26]. In human airways, the levels of BLyS-protein were significantly increased in bronchoalveolar lavage fluid after allergen challenge and its level was also correlated with IL-13 [27]. These in vitro and in vivo studies of BLyS and our analysis of BLyS-production reinforce the idea of BLyS being a possible therapeutic modality and its signaling pathway being potential targets for drug interventions against airway diseases.

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RHINOLOGY

Bax-gene transfer enhances apoptosis by steroid treatment in human nasal fibroblasts

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Abstract Gene therapy has become a focus not only in the study of cancer but also lifestyle-related diseases. In case of chronic rhinosinusitis with nasal polyps and aspirin-induced asthma, nasal polyps poorly respond to a local administration of steroid. The Bax and Bcl-2 proteins play important roles in the regulation of apoptosis. The treatment of steroid (prednisone) induced apoptosis in the fibroblast. The Bax accelerates apoptosis. Apoptosis is very important in the anti-inflammatory mechanism. In this study, we investigated whether the overexpression of Bax in human fibroblasts influences apoptosis by treatment with a steroid (prednisolone) in vitro. Human nasal fibroblasts were isolated from small pieces of nasal polyp and were transfected with a *bax* gene-bearing mammalian expression vector. Human nasal fibroblasts were transiently transfected with the expression vector hBaxpcDNA3 (Bax-NF) or native pcDNA3 (Neo-NF). Both transfectants (Bax-NF, Neo-NF) and wild-type-nasal fibroblast (wt-NF) were cultured in conditioning medium and treated with each concentration of prednisolone for 72 h. Prednisolone at a concentration of 10 ng/ml decreased the viability of Bax-NF compared to that of Bax-NF in the absence of prednisolone. The cytotoxicity of prednisolone to Bax-NF was significantly higher than that to Neo-NF or wt-NF ($p < 0.01$) and the susceptibility of Bax-NF to prednisolone was about 1,000 times that of Neo-NF or wt-NF. We found that the transfer of the exogenous *bax* gene enhanced the induction of apoptosis by steroid-treatment in human nasal fibroblasts. Therefore, we suggest that exogenous Bax protein expression by gene transfer might be useful for the treatment of nasal polyps. We will further the preclinical study in improving steroids dose and in adopting to transfer bax gene to the nasal polyps by intranasal injection, thus providing a more effective and safer way for the nasal polyps that poorly respond to a local administration of steroids.

Keywords Bax overexpression · Nasal polyp · Prednisolone · Gene therapy · Chronic sinusitis · Glucocorticoid

Introduction

Nasal polyps are a chronic inflammatory disease of the upper airway, leading to recurrent protrusions of benign edematous nasal mucosae from the nasal sinuses into the nasal cavities. Nasal polyps are not a simple edema of the mucous membrane. They are characterized by the infiltration of inflammatory cells, such as eosinophils, basophiles, and lymphocytes and structural abnormalities including stromal fibrosis. Fibroblasts are resident cells thought to effect the development of fibrosis. The proliferation of stromal and epithelial elements is thought to account for the growth of nasal polyps [1]. It is proposed that fibroblasts play a critical role in the switch from acute inflammation to adaptive immunity and tissue repair. Fibroblasts actively define the structure of tissue microenvironments and modulate immune cell behavior by conditioning the local cellular

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and cytokine microenvironment so that the kinetics and nature of the inflammatory infiltrate are appropriate to the cause of the damage [2]. Nasal polyp-derived fibroblasts produce a large amount of RANTES by IFN γ (Th1 cytokine) [3] and eotaxin by IL-4 (Th2 cytokine) to attract and activate eosinophils [4]. Nasal fibroblasts also spontaneously release IL-8 and GM-CSF, and enhanced IL-8 production by the inflammatory cytokine IL-1 band TNFa induces neutrophils to move into the infected area [5].

Apoptosis, which is regulated both by cell survival and by death signals, is important for the swift clearance of unwanted cells. The treatment of nasal polyps involves an important role for apoptosis in the removal of inflammatory cells and resolution of inflammatory processes. Glucocorticoids have been shown to induce apoptosis in most nucleated cells, such as thymocytes, myeloma cells, and peripheral blood monocytes and play a major role in the attenuation of inflammatory responses [6, 7]. Topical steroids have been demonstrated to be effective in the treatment of nasal polyps [8]. The administration of topical steroids in vivo increased apoptotic index of eosinophils and T-lymphocytes in nasal polyps [9]. After oral prednisolone treatment, eosinophilic apoptosis was accompanied by a significant decrease in the number of EG2 and IL-5 double positive cells [10]. Treatment with glucocorticoids in vitro increased the apoptosis of both eosinophils and T-lymphocytes derived from nasal polyps in a specific culture system [9]. Also, glucocorticoids induced the apoptosis of nasal polyp-derived fibroblasts [11] and stromal cells [12].

Induction of apoptosis by gene transfer into appropriate target cells and tissues has become a focus of study as a therapeutic approach of cancer [13, 14]. The Bcl-2/Bax system and Fas/Fas-ligand system play a crucial role in the regulation of the apoptotic process. Bcl-2 gene was the first to be identified because of its involvement in the *t*(14, 18) chromosomal translocation found in many B-cell lymphomas. Bax is a member of an apoptosis-promoting protein and has an antagonistic role against the action of Bcl-2. Both Bcl-2 and Bax proteins would therefore be important in the regulation of apoptosis. Bcl-2 is an intercellular protein that inhibits apoptosis while Bax counteracts the anti-apoptotic function of Bcl-2 by binding to this molecule [15].

Although glucocorticoids have been widely employed for the treatment of nasal polyps and allergic sinusitis, serious problems include poor response or resistance to steroids in patients with aspirin-induced asthma. In this study, we investigated whether a *bax* gene transfer to human fibroblasts treated with prednisolone would influence the induction of apoptosis to establish a more effective steroid therapy for nasal polyps.

Materials and methods

Cell lines and culture conditions

Nasal polyps were obtained from patients with chronic rhinosinusitis with nasal polyp during endonasal sinus surgery. Aspirin-induced asthma was excluded. Nasal specimens were cultured in 10-cm dishes containing RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Gibco, Grabd Island, NY), 0.29 mg/ml glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, at 37°C in 5% CO_2 humidified air. After a period of 3 to 4 weeks, when the growth of nasal fibroblasts was established, nasal fragments were removed and the first passage was performed [3, 16]. The cells were used at passage number 3 to 5. There was no contamination by epithelial cells as confirmed by immunohistochemical examination using a cytokeratin marker.

Informed consent for surgery and participation in the study was obtained from all patients and their families.

Transfection

The expression vector hBaxpcDNA3 was used with the permission of Drs. J.-C. Martinou and R. Brown (Glaxo Wellcome, Bedsurham, UK). This vector containing a full-length human *bax* cDNA tagged at the 3′ with 57 bps was subcloned into the mammalian expression pcDNA3 (Invitrogen, Carlsbad, CA) to distinguish the transfected tagged-Bax protein from intrinsic Bax protein [14]. Bax-nasal fibroblasts (Bax-NF) were transiently transfected with hBaxpcDNA3. Neo-NF was transiently transfected with native pcDNA3. Both vectors were introduced into nasal fibroblasts using the effectene transfection reagent (QIAGEN, Valencia, CA) under the conditions recommended by the manufacturer.

MTT assay

Prednisolone (1 mg) was dissolved in DMSO (5 ml, Wako, Osaka, Japan) and diluted with phosphate-buffered saline. Approximately 5×10^3 cells were seeded in each well of a 96-well plate in triplicate and cultured in 200 µl of medium. After 24 h, 1 µl of each concentration of prednisolone solution was added to the well, and the cells were cultured for 72 h. At that stage, the medium was removed, 10 µl of 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) in PBS was administered and the cells were incubated at 37°C for 4 h. DMSO (200 µl) was added and the absorbance of each well was measured at 540 nm (reference absorbance at 630 nm). All values represent the mean ± standard deviation (SD) from triplicate cultures. The test was performed independently 6

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