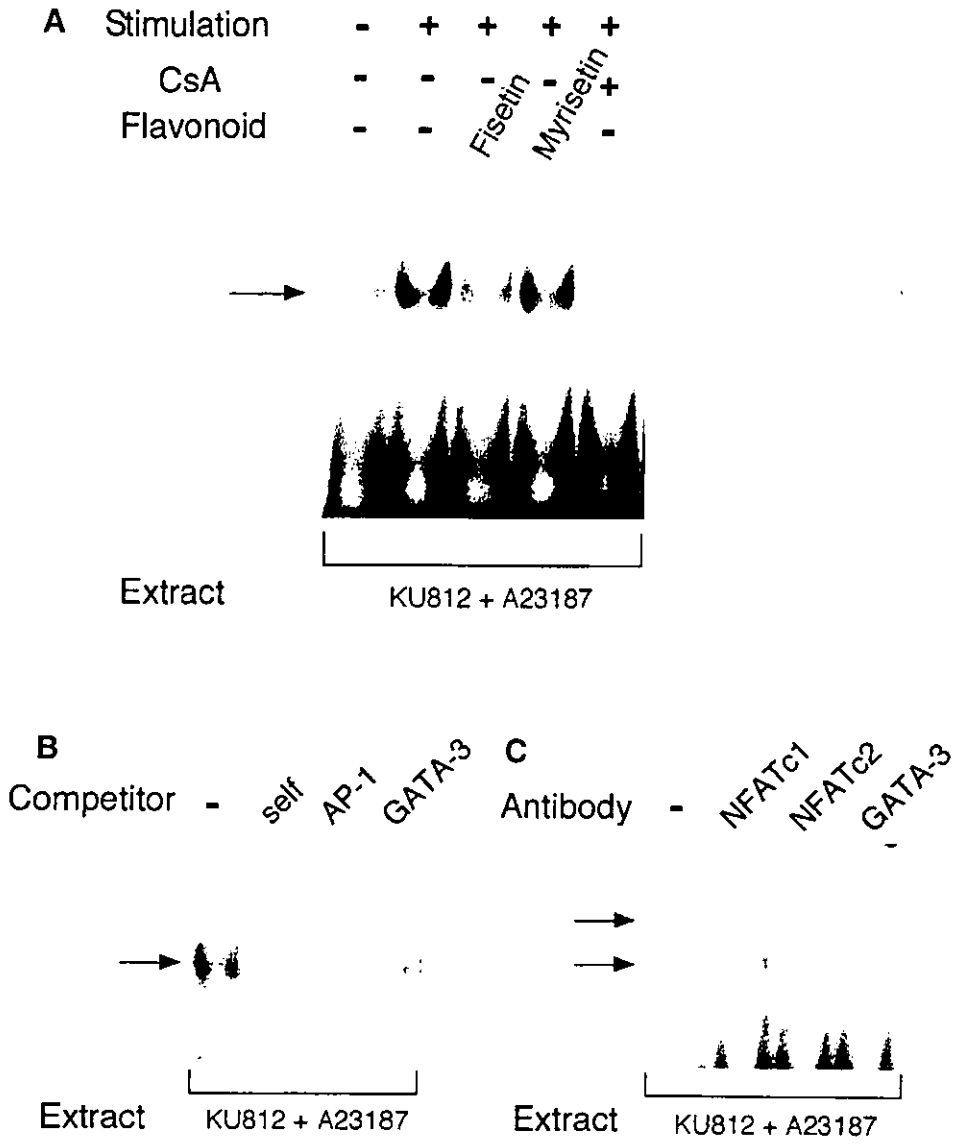

FIG 4. EMSA showing the inducibility of NFAT in KU812 cells by A23187 and the inhibitory effect of fisetin on NFAT-DNA binding activity. **A,** EMSA was performed with a ^{32}P-labeled NFAT probe and nuclear proteins (10 μg) from KU812 cells unstimulated or stimulated with A23187 for 2 hours in the presence of fisetin or myricetin or 1000 ng/mL cyclosporin A. **B,** Competition (50 times molar excess of the labeled oligonucleotide) was performed by adding the unlabeled NFAT probe or AP-1 probe or unrelated cold GATA-3 probe. **C,** Supershift EMSA was performed with A23187-stimulated nuclear proteins isolated from KU812 cells. Antibodies (4 μg) specific for NFATc1, NFATc2, and GATA-3 proteins were added to the reaction mixture and incubated for 30 minutes on ice before the addition of a labeled oligonucleotide. Data are representative of 3 independent experiments.

identified kaempferol, quercetin, rutin, and fisetin as inhibiting histamine release or hexosaminidase by human basophils or the rat basophilic cell line RBL.[18,19] We confirmed that flavonoids used in this study substantially inhibited histamine release by peripheral blood basophils stimulated through FcεRI (data not shown). Its biologic properties of inhibiting these cytokines and chemical mediators make fisetin a potential candidate for a novel antiallergic substance targeting basophils.

Among the cytokines examined, fisetin specifically inhibited IL-4, IL-5, and IL-13 expression but not IL-6 and IL-8 by KU812 cells, suggesting that fisetin regulates the transcriptional activation of these cytokines. Cytokine gene transcription is regulated by cis elements and transcriptional DNA binding factors. The observations that fisetin reduces the expression of IL-4, IL-5, and IL-13 without any effect on IL-6 and IL-8 expression suggests that fisetin suppresses the activation of certain

common specific nuclear factors that positively regulate *IL4*, *IL5*, and *IL13* gene activation or induce repressor proteins. The NFAT family plays a key role in inducing many cytokine genes in T cells, including *IL2*, *IL4*, *IL5*, *IL13*, *IFNG*, *TNFA*, and *GMCSF*, and many studies have also identified NFAT-dependent cytokine gene expression in cells other than T cells, including B cells, hematopoietic cells, and rodent mast cells.[31,32] With regard to cytokine production, recent observations that immunosuppressive drugs, such as cyclosporin A or FK506, inhibit both anti-IgE antibody and calcium ionophore–induced IL-4 and IL-13 production from basophils or mast cells suggest that these stimuli use a calcium-dependent calcineurin pathway for the generation of these cytokines.[31,32] In our study we were able to show that, in fact, an NFAT-DNA complex was formed with nuclear lysates from KU812 cells and was detected with greater binding intensity in the lysates of activated cells. Fisetin suppressed the NFAT-DNA binding activity markedly, whereas myricetin did not, suggesting that fisetin might partly affect the calcium/calmodulin-calcineurin-NFAT pathway for the generation of these cytokines by KU812 cells. Nishino et al[33] reported that quercetin could interact directly with a calmodulin, the Ca^{2+}-sensing protein, and we confirmed that fisetin could also bind with it (data not shown). Another explanation of the inhibitory effects of fisetin is that it might modulate the function of calmodulin and affect some Ca/calmodulin-dependent enzymes other than calcineurin, which regulate transcriptional activation of these cytokines in addition to NFAT inhibition. Schroeder et al[34] have recently demonstrated the involvement of NFAT in the generation of IL-4 by anti-IgE antibody–stimulated normal basophils. Among 3 isoforms of NFAT checked, NFAT2 (NFATc1) was abundantly involved in EMSA assay by human basophils. However, in our assay NFAT1 (NFATc2) appeared to a predominant isoform in KU812 cells. These results might come from the difference of cell type (cell line vs normal cell), but further study will be needed to clarify this point. In addition, whether flavonoids could affect other FcεRI-mediated signaling pathways or show similar inhibition on NFAT activation in normal basophils is yet to be determined.

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom,[16,17] but daily intake of flavonoids remains controversial. It is thought that the average daily US diet contains approximately 1 g of flavonoids. Quercetin is usually included at a high concentration in these materials, and it has been suggested that it is consumed at a rate of at least 50 mg/d.[16] In contrast, the average intake of flavonoids was found to be only 23 mg/d in The Netherlands.[35] The fisetin content of most food items and its daily consumption, however, are still unknown as far as we know. Oral intake of substances, including higher amounts of fisetin or other flavonoids, might result in improvement of allergic symptoms or prevent development of allergic diseases.[36] We have, in fact, demonstrated that oral administration of astragalin, which is absorbed after being converted into kaempferol, suppresses the onset of dermatitis in NC/Nga mice.[21,22] More extensive screening of this class of compounds and preparation of synthetic analogues might thus yield molecules with increased potency and efficacy that could be useful for patients with allergic disease. We hope that the evidence presented here might contribute to the clinical application of these flavonoids as alternative and complementary therapeutic agents for allergic diseases.

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IL-4 and IL-13: Their Pathological Roles in Allergic Diseases and their Potential in Developing New Therapies

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Abstract: The incidence of allergic diseases has dramatically increased in recent decades, and it is socially and medically important to establish more useful strategies to overcome allergic disorders. Various kinds of drugs are utilized for allergic patients; however, some cases are unresponsive to these drugs and in others there are undesired adverse effects. On the other hand, a substantial body of evidence has accumulated pointing to the pivotal role of Th2-cytokines, interleukin (IL)-4, and IL-13, in the pathogenesis of bronchial asthma. The evidence is categorized as (1) expression of these cytokines in the bronchial lesions, (2) genetic association of the signaling molecules of these cytokines, (3) analyses of mouse models. In addition, the molecular mechanism of the signal transduction of these cytokines has also been well characterized. Based on such information, IL-4 and IL-13 have emerged as promising means of improving allergic states, and several IL-4/IL-13 antagonists have been developed, among which soluble IL-4 receptor is now in human trials. Identifying the structure of the IL-13 variant and of the IL-4/IL-13–inducing genes would be of great use. It is expected that in the near future, several drugs will emerge based on these strategies, which will give us wider choice in treating patients, depending on the pathogenesis of the diseases.

Keywords: IL-4, IL-13, IL-4 receptor, IL-13 receptor, allergic disease, bronchial asthma, soluble IL-4 receptor, soluble IL-13 receptor

INTRODUCTION

The incidence of allergic diseases has dramatically increased in recent decades, especially in urban and industrialized areas. It has been reported that, at present, up to half of the population in Japan suffers from bronchial asthma, atopic dermatitis, or allergic rhinitis [1]. The medical cost for treating such patients is huge and on the increase. Thus, it is important socially as well as medically to establish more useful strategies to overcome allergic disorders.

Various kinds of drugs–such as glucocorticosteroids, antihistamines, antileukotrienes, adrenergic agents, and theophyllines–are used for allergic patients [2]. However, cases that can not be controlled by these drugs still remain. Inhalation or oral administration of steroids has been widely used because of their potent anti-inflammatory effects; however, these have a disadvantage of various adverse effects, including broad immune suppression. To avoid this, a new agent to act on narrower targets, which would benefit allergic patients, has been awaited.

On the other hand, a substantial body of evidence stands behind the pivotal role of Th2-cytokines, interleukin (IL)-4 and IL-13, in the pathogenesis of allergic diseases [3-7]. Based on such information, IL-4 and IL-13 have emerged as promising means of improving allergic states. In this review, we summarize the pathophysiological roles of IL-4 and IL-13 in allergic disease and the strategies to inhibit IL-4 and/or IL-13 signals as a therapy for allergic diseases.

1. Signal Transduction Mechanism of IL-4 and IL-13 and Tissue Distribution of Their Receptors

IL-4 and IL-13 exert their biological activities by binding to their receptors on the cell surface as well as other cytokines. Two types of the IL-4 receptor (IL-4R) exist (**Fig. (1)**)[3, 8]. One is composed of two components, the IL-4R α chain (IL-4Rα) and the IL-2R γ chain (γc), denoted type I IL-4R, whereas the other is composed of IL-4Rα and the IL-13R α1 chain (IL-13Rα1), called type II IL-4R. As the IL-13R also consists of IL-13Rα1 and IL-4Rα, the IL-13R is identical to type II IL-4R [9-12]. However, IL-13Rα1 and IL-4Rα are important binding units for IL-13 and IL-4, respectively [9, 10, 13-16], meaning that the binding mechanism of IL-13 and IL-4 to the IL-13R/type II IL-4R is likely to be different. There exists another IL-13–binding unit, IL-13Rα2 [17, 18]. As the cytoplasmic domain of IL-13Rα2 is short, IL-13Rα2 is thought not to transduce the IL-13 signal, but to block it, acting as a 'decoy receptor'.

As γc is expressed on most hematopoietic and immune cells, IL-4 is assumed to act on these cells through type I IL-

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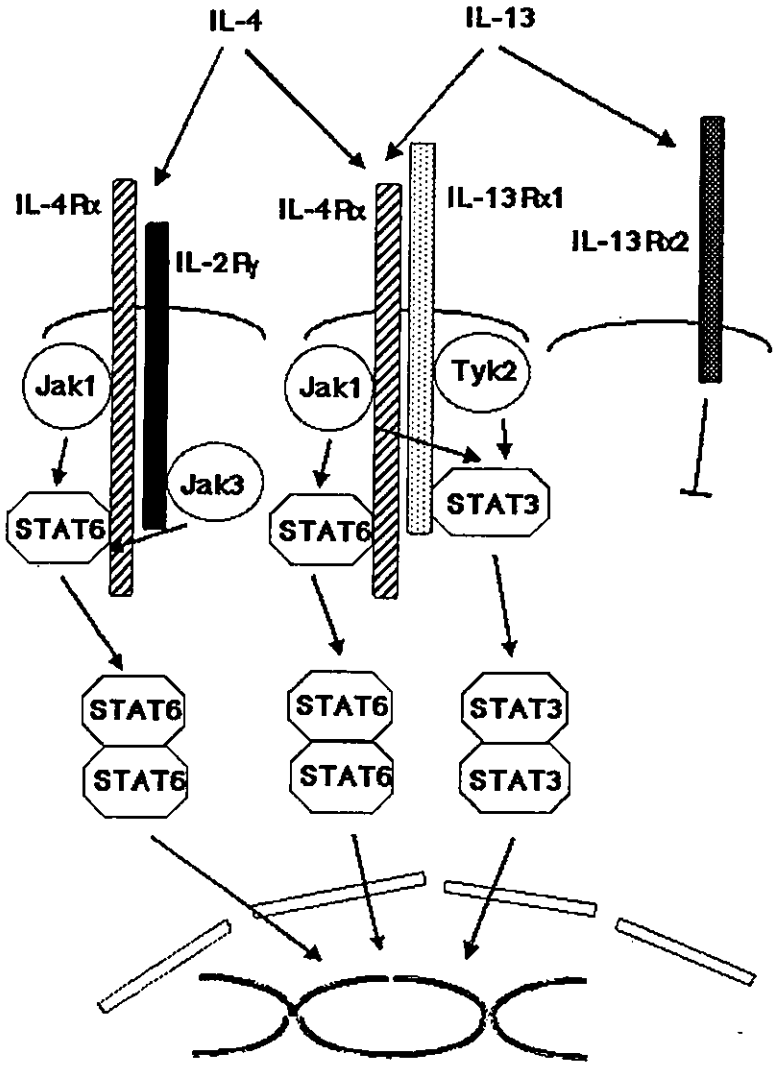

Fig. (1). The receptor structure and signal transduction mechanism of IL-4 and IL-13

IL-4 binds to type I or type II IL-4R, whereas IL-13 binds to IL-13R. Engagement of these receptors causes signal transduction, mainly through the JAK-STAT pathway.

4R [19, 20]. In contrast, expression of IL-13Rα1 is limited to some lineages such as B cells in hematopoietic and immune cells, but is ubiquitously detected on non-immune cells [10, 21-24]. Thus it is thought that IL-4 and IL-13 act on non-immune cells through type II IL-4R/IL-13R. Particularly, it has been shown that IL-13Rα1 is constitutively expressed on bronchial epithelial cells and bronchial smooth muscle cells in the bronchial tissue [23]. Tissue distribution and expression regulation of IL-13Rα2 remain obscure.

Upon engagement of the receptors by IL-4 or IL-13, their signals are transduced intracellularly mainly via the JAK-STAT and the phosphatidylinositol (PI)-3 kinase/insulin receptor substrate (IRS)-1/2 pathways (**Fig. (1)**) [3, 8, 25]. In the case of type I IL-4R, activation of JAK3 and JAK1 is followed by activation of STAT6, a critical transcription factor for IL-4 and IL-13 to exert their biological activities [26-31]. In contrast, when type II IL-4R/IL-13R is engaged, activation of JAK1 and TYK2 is followed by activation of not only STAT6 but also STAT3 [12, 32]. Thus far, the biological role of STAT3 in the IL-4 and IL-13 signals remains to be clarified. The PI-3 kinase/IRS-1/2 pathway is important for cell proliferation induced by IL-4 [8]. In addition, The C terminus of IL-4Rα contains an ITIM (immunoreceptor tyrosine-based inhibitory motif), with which tyrosine phosphatases associate, down-regulating the IL-4 signal [8, 25].

2. Significance of IL-4 and IL-13 in the Pathogenesis of Allergic Diseases

Allergic diseases are complex disorders involving a combination of genetic and environmental factors [33]. Particularly, in the case of bronchial asthma among allergic diseases, these factors result in infiltration of Th-2 lymphocytes, mast cells, and eosinophils into the bronchial

lesions, with downstream mediator release and disordered airway function. Cytokines derived from the Th-2 lymphocytes are considered to orchestrate the asthmatic phenotype [33]. Among the Th-2 cytokines, considerable evidence supports key roles for IL-4 and IL-13 in the pathogenesis of bronchial asthma. Such evidence is classified into the following categories.

a. Expression of IL-4 and IL-13 in the Bronchial Lesions

Expression of IL-4 and IL-13 is higher at the baseline and greatly up-regulated by allergen challenge in bronchial tissues or bronchoalveolar lavage fluids derived from asthma patients [34-37]. Particularly, IL-13 is predominantly expressed, compared to IL-4 [35, 37].

b. Genetic Association of the IL-4 and IL-13 Signaling Molecules

Genetic predisposition to allergic diseases is thought to be polygenic, and the genetic factors are thought to exist among polymorphisms [38]. Extensive efforts have been made to identify such factors by genome-wide search and candidate-gene study [39]. Based on candidate-gene study, it has been reported that several polymorphisms of genes encoding IL-4 and IL-13 signaling molecules such as *IL4, IL13, IL2RG, IL4RA, IL13RA1, STAT6,* and *BCL6* are genetically associated with asthma or atopy [40]. Among the single nuclear polymorphisms (SNPs) that are demonstrated to be genetically associated with bronchial asthma or atopy, we have analyzed the functional properties of variants of *IL4RA* (Ile50Val) and *IL13* (Gln110Arg). The variant of IL-4Rα whose amino acid at 50 is isoleucine up-regulates receptor responses to IL-4, leading to increased STAT6 activation and IgE synthesis, compared to the valine type [41, 42]. The variant of IL-13 whose amino acid at 110 is glutamine has less affinity with IL-13Rα2, a decoy receptor, and an enhanced stability, compared to the arginine type, causing up-regulation of the IL-13 concentration *in vivo* [23, 43].

c. Analyses of Mouse Models

Analyses of mice null for components of the IL-4 and IL-13 signal transduction pathways, including IL-4, IL-13, IL-4Rα and STAT6, have revealed that both cytokines play a pivotal role in induction of airway hyperresponsiveness, a characteristic feature of asthma [44-46]. Furthermore, epithelial overexpression of an IL-13 transgene or administration of IL-4 or IL-13 in mice has shown that these cytokines induce an asthma-like phenotype independent of lymphocytes [47-50]. Thus IL-4 and IL-13 are able to act directly on non-immune cells in bronchial tissue and induce goblet cell hyperplasia, sub-epithelial fibrosis, and bronchial hyperresponsiveness.

As we mentioned above, biological activities of IL-4 and IL-13 are very similar, because both cytokines share the same receptor; however, a remarkable difference between these cytokines is that IL-4 is able to expand Th-2 type cells, whereas IL-13 can not [21, 51]. This is ascribed to the fact that these cells do not express IL-13Rα1. In contrast, actions in bronchial tissue such as mucus secretion, thickness of basement membrane, and hypertrophy of bronchial smooth muscle are possessed by both IL-4 and IL-13, or rather IL-13 is thought to have more potency to induce these phenotypic changes [51]. It is uncertain whether there exists an IL-13–specific signal in non-immune cells. The predominant activity of IL-13 in bronchial tissue may be attributed by the difference of expression of IL-4 and IL-13 as ligands in the tissue [35, 37]. Thus, even though IL-4 and IL-13 have common activities, the biological activities of IL-4 and IL-13 are skewed towards immune cells and peripheral tissues, respectively, *in vivo* (**Table 1**).

Table 1. Biological Activities of IL-4 and IL-13

	IL-4	IL-13
Th2 Induction	+	-
T Cell Proliferation	+	-
IgE Synthesis	+	+
Anti-inflammatory Actions against Monocyte	+	+
Mast Cell Activation	+	−±
Mucus Production	+	++
Basement Membrane Thickness	+	++
Smooth Muscle Hypertrophy	+	++

3. Targeting IL-4 and IL-13 to Develop a New Agent for Allergic Diseases

Because Th-2 cytokines have a crucial role in the pathogenesis of allergic diseases, particularly of bronchial asthma, it is reasoned that blocking the biological activities of IL-4 and IL-13 should be a good strategy to improve allergic status. Another Th-2 cytokine, IL-5, had been thought to be a promising candidate as means of developing a new agent for bronchial asthma, because of its potency to activate eosinophils [52]. However, administration of anti–IL-5 antibody did not improve airway hyperresponsiveness of bronchial asthma patients, although it decreased eosinophil count in the peripheral blood [53]. From such reasons, more attention has recently been paid to IL-4 and IL-13 as having potential in the search for a new medication for allergic diseases (**Table 2**).

a. Soluble IL-4Rα

Blocking actions of cytokines by administration of their soluble receptors is widely accepted as a promising strategy for treating various diseases. For example, soluble TNF receptor (Enbrel™) has been shown to be effective for rheumatoid arthritis [54]. Soluble IL-4Rα physiologically exists in peripheral blood as an alternative splicing product or a shedding product [55, 56]. For this reason, soluble IL-4Rα has an advantage of not having immunogenicity, compared to anti–IL-4 antibody or an IL-4 variant (IL-4 mutein). Thus, trials to apply soluble recombinant IL-4Rα to bronchial asthma patients have begun (**Fig. (2)**).

Table 2. The List of IL-4/IL-13 Antagonists

Agents	Producing Company	IL-4 Block	IL-13 Block
Soluble IL-4 Receptor	Immunex●Nuvance™●	+	-
Soluble IL-13 Receptor	Genetic Institute	-	+
IL-4/IL-13 Trap	Regeneron	+	+
IL-4 mutein	Bayer	+	+
Anti-IL-13Rα1 Ab	AMRAD	?	+

Soluble IL-4Rα was expressed in Chinese hamster ovary cells and subsequently purified (Nuvance™; Immunex). As the first trial, a single nebulization of soluble IL-4Rα was administered to moderate asthma patients, on day after inhaled corticosteroids were stopped, and then the patients were observed for 12 days [57]. Subsequently, the patients who received 1.5 mg of nebulization showed improvement in symptoms, β_2-agonist use, and FEV_1, compared to the placebo group. In a further trial, when nebulization once a week was continued for 12 weeks, symptoms and FEV_1 were improved in the group receiving 3.0 mg of administration [58]. The results of the trials thus far demonstrated the utility of soluble IL-4Rα for improving asthmatic status. No serious adverse event was observed. The half-life of soluble IL-4Rα in the body was estimated to be five to seven days.

b. Soluble IL-13R

As mentioned, it has been shown that IL-13 is more important in bronchial tissue than IL-4 in causing asthmatic phenotype. Based on these findings, soluble recombinant IL-13R has also been tested as well as soluble IL-4Rα. Two candidates of soluble IL-13R exist; one is soluble IL-13Rα1 and the other is IL-13Rα2. Because the affinity of IL-13Rα2 with IL-13 (Kd; 50-500 pM) is more than ten-fold higher than that of IL-13Rα1 (Kd; ~4 nM), soluble IL-13Rα2 has a superior potency as a blocking agent (**Fig. (3)**)[17, 43, 59]. The Genetic Institute is developing soluble IL-13Rα2 as a novel product, and they report that it was effective for decreasing airway hyperresponsiveness in bronchial-asthma-inducing mice [47, 49]. Further studies conducted with bronchial asthma patients are awaited. On the other hand, Regeneron is developing a complex compound in which soluble IL-4Rα and soluble IL-13Rα2 are tandem lined, denoted as IL-4/IL-13 trap.

c. Other IL-4/IL-13 Antagonists

As IL-4/IL-13 antagonists other than soluble IL-4 and IL-13 receptors, IL-4 mutein, IL-4δ2, and anti-IL-13Rα1 antibody are listed. IL-4 muteins indicate two types of IL-4 variants whose tyrosine at 124 is replaced with aspartate (Y124D), and arginine at 121 is furthermore replaced with aspartate (R121D/Y124D) [60-62]. These muteins act as an IL-4 antagonist, because those are able to bind to the IL-4R, but not able to transduce the signal. As type II IL-4R is identical to the IL-13R, these IL-4 muteins also act as an IL-

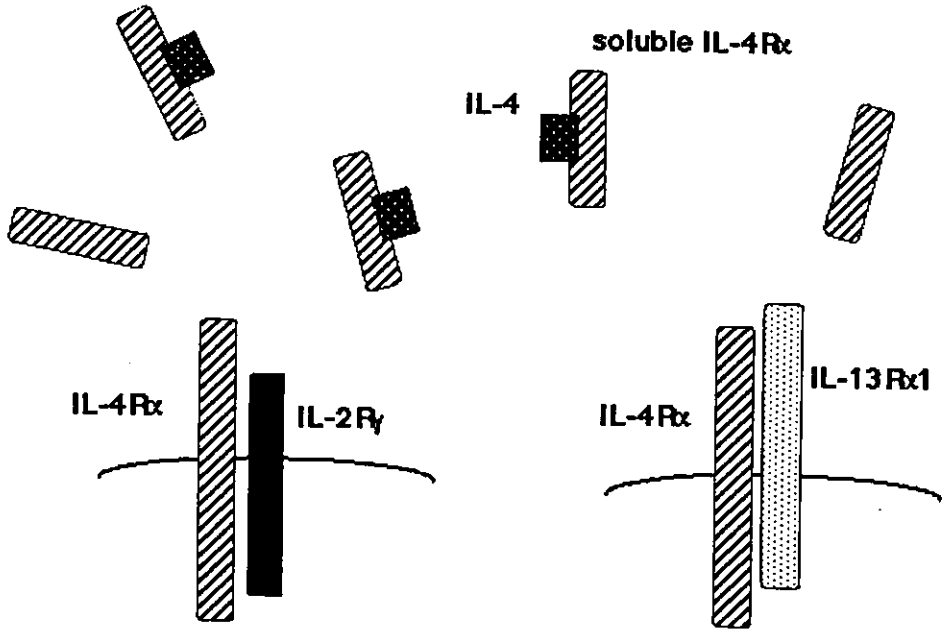

Fig. (2). The molecular mechanism of soluble IL-4R

Soluble IL-4R inhibits the binding of IL-4 to IL-4R on the cell surface by trapping IL-4.

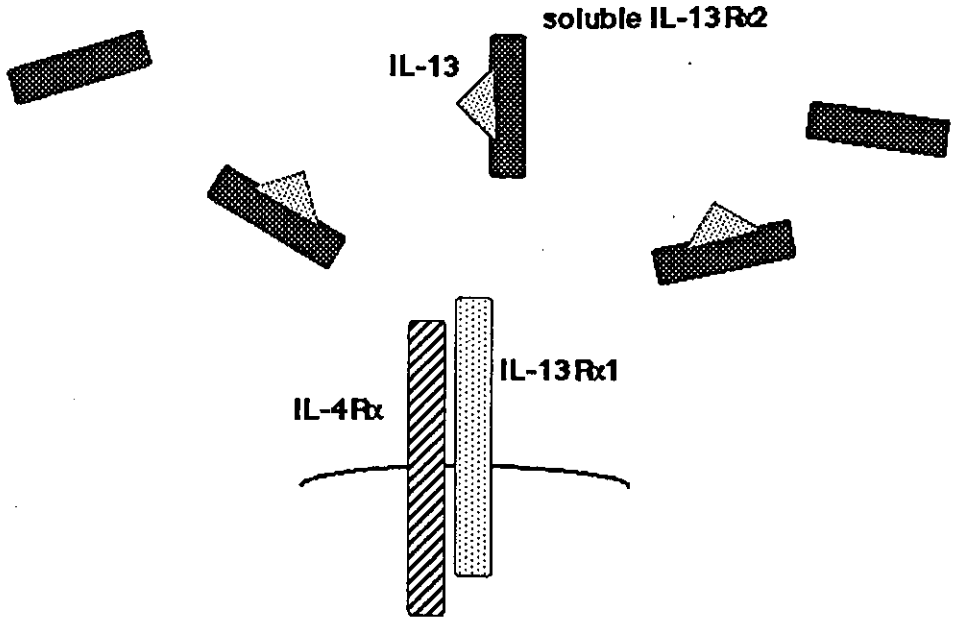

Fig. (3). The molecular mechanism of soluble IL-13R.

Soluble IL-13Rα2 inhibits the binding of IL-13 to IL-13R on the cell surface by trapping IL-13.

13 antagonist by occupying the receptor. IL-4δ2 is an alternative splicing variant of IL-4, and acts as an antagonist on activated T cells, monocytes, and B cells [63, 64]. It also has a potential for adopting to allergic diseases. The details of the anti–IL-13Rα1 antibody are unclear.

d. A Strategy for Applying the Findings about the IL-13 Variant to Developing a New Agent

As we described before, the affinity of IL-13 with IL-13Rα2 is much higher than IL-13Rα1 [17, 43, 59]. And the analyses of the IL-13 variant have demonstrated that the variant which bears glutamine at amino acid 110 (Gln110) has a lower affinity with IL-13Rα2 than the arginine type (Arg110), whereas both IL-13 proteins show the same affinity with IL-13R [43]. To explain such properties of the IL-13/IL-13 receptor binding, a homology modeling of the IL-13/IL-13 receptor complex was proposed by Dr. Matsuo and his colleagues (RIKEN, unpublished data). According to this computer modeling, it is predicted that there exist five ionic bonds between IL-13 and IL-13Rα2 (Asp^{97}-Lys^{208}, Lys^{103}-Asp^{319}, Lys^{104}-Asp^{206}, Arg^{107}-Asp^{318}, Arg^{110}-Asp^{271}; the amino acid numbers of IL-13 and IL-13Rα2 are depicted, respectively). In contrast, only three ionic bonds among these five pairs are predicted to be conserved between IL-13 and IL-13Rα1 (Lys^{103}-Asp^{324}, Lys^{104}-Asp^{194}, Arg^{107}-Asp^{323}; the amino acid numbers of IL-13 and IL-13Rα1 are depicted, respectively). The loss of two ionic bonds could explain why the affinity of IL-13 with IL-13Rα1 is lower than IL-13Rα2. In this context, the IL-13 variant (Gln110) is assumed to lose one ionic bond compared with Arg110, which might weaken the affinity with IL-13Rα2, whereas it does not affect the affinity with IL-13Rα1. We are now validating this model by incorporating the mutagenesis into the predicted ionic binding sites.

How can we apply these findings to developing a new therapeutic agent? If this model is confirmed, we may be able to manipulate the affinity between IL-13 and the IL-13 receptor by changing the numbers of ionic bonds. If we could find a mutated type of IL-13Rα2 that forms more ionic bonds with IL-13, causing a higher affinity with IL-13 than the wild type of IL-13Rα2, we would be able to subject such a 'super receptor' to synthesizing a remodeled soluble IL-13Rα2 that would trap IL-13 more efficiently. Thus, there is a possibility that the functional findings based on the polymorphism associated with some diseases are of great use for developing new medications.

e. A Strategy for Applying the Findings about IL-4/IL-13–Inducing Genes to Developing a New Agent

In order to clarify the molecular mechanism of bronchial asthma induced by IL-4 and IL-13, we and others identified IL-4/IL-13–inducing genes in bronchial epithelial cells by microarray analysis [65](Yuyama N. *et al.*, unpublished data). These genes contain proteases, protease inhibitors, cytokine receptors, and matrix proteins. Thus far, it is unclear how these molecules are involved in the pathogenesis of bronchial asthma. However, we expect that these molecules would contribute to phenotypic changes in bronchial asthma, whether it deteriorates or improves the status. Elucidating the functional roles of these molecules would give us a hint to develop a new therapeutic strategy.

CONCLUSION

In this review article, we summarized the mechanism of signal transduction of IL-4 and IL-13, the pathological roles of IL-4 and IL-13 in bronchial asthma, and the possibilities to develop novel therapeutic methods for allergic diseases

targeting the IL-4/IL-13 signals. It is expected that in the near future, several drugs will emerge based on these strategies, giving us a wider choice of treatments, depending on the pathogenesis of the diseases.

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The importance of interleukin-13 in the pathogenesis of bronchial asthma

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Abstract

Bronchial asthma is a complex disorder involving a combination of genetic and environmental factors. It is widely known that Th2 cytokines have a pivotal role in the pathogenesis of bronchial asthma. Among Th2 cytokines, the importance of interleukin-13 has recently emerged. In this article, we describe recent progress in understanding the relationship between IL-13 and bronchial asthma and current problems to be resolved.

Introduction

The genetic and environmental factors involved in bronchial asthma (1) result in infiltration of Th2 lymphocytes, mast cells, and eosinophils into asthmatic airways with

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downstream mediator release and disordered airway function. Cytokines derived from the Th-2 lymphocytes are considered to orchestrate the asthmatic phenotype, irrespective of atopy (1). The significance of Th2 cytokines is confirmed by the findings that $CD4^+$ cells secreting Th2 cytokines infiltrate in bronchial lesions and that administration of Th2-type $CD4^+$ cells into SCID mice restores airway hyperresponsiveness (AHR) (2). Furthermore, among the Th2 cytokines, considerable evidence supports a key role for IL-13 in the pathogenesis of bronchial asthma (3, 4). Here, we describe our work in understanding the relationship between IL-13 and bronchial asthma and describe problems still to be solved.

1. The structure of the IL-13 receptor (IL-13R) and signal transduction of IL-13

IL-13 must bind to its receptor on the surface of the target cells to exert its biological activities. IL-13R is a heterodimer composed of the IL-13R α1 chain (IL-13Rα1) and the IL-4R α chain (IL-4Rα) (5, 6)(Fig. 1). In addition to IL-13Rα1, the IL-13R α2 chain (IL-13Rα2) has been identified as another IL-13–binding unit (7). However, as the cytoplasmic domain of IL-13Rα2 is short, it is assumed that certain signal-transducing molecules do not associate with it (Fig. 1). The biological function of IL-13Rα2 is thought to act as a decoy receptor by inhibiting the IL-13 signal through IL-13Rα1 in the cells on which both IL-13Rα1 and IL-13Rα2 are expressed or to clear local IL-13 into the cells on which IL-13Rα2 is expressed.

Engagement of IL-13R by IL-13 evokes activation of the cascade of the signal-transducing molecules in the cells (4). Although it is known that various molecules are involved in this pathway, most of these molecules converge into two pathways: the Jak/STAT pathway and the insulin receptor substrate (IRS)-1/2/phosphatydilinositol (PI)3-kinase pathway (Fig. 1). With respect to the Jak/STAT pathway, two Jak molecules, Tyk2 and Jak1, which constitutively associate with the box-1 region of IL-13Rα1 and IL-4Rα, respectively are first activated upon stimulation of IL-13. Then two members of the STAT family, STAT6 and STAT3, are activated (6). The tyrosine residues of the cytoplasmic portion in IL-4Rα and IL-13Rα1 are crucial for binding to STAT6 and STAT3, respectively (6, 8). By this pathway, certain genes such as *germline ε, CD23, MHC class II,* and *STAT6* itself are induced (4). Activation of STAT6 induced by both IL-13 and IL-4 confer common biological activities on both cytokines. It remains unclear how STAT3 is involved in the biological actions of IL-13. IRS-1/2 is phosphorylated on tyrosine residues by Jak1 (9), and then PI3-kinase associates with IRS-1/2 (10). This pathway is thought to be important for the proliferation activity of IL-13 (11).

In immune cells, IL-13Rα1 is expressed on B cells, but not on T cells (12). Although expression level of IL-13Rα1 is very low in non-activating B cells, B cell activation by anti-IgM antibody (Ab) and/or anti-CD40 Ab augments IL-13Rα1 expression (6, 13). On the other hand, we have shown that IL-13Rα1 is constitutively expressed on bronchial epithelial cells and bronchial smooth muscle cells by immunohistochemistry (14). It is known that some tumor cells and fibroblasts express IL-13Rα2 (15, 16); however, the expression of IL-13Rα2 in other cells is still not fully understood. Its expression is undetectable on immune cells (6, 13). Analysis is required of the expression mechanism of IL-13Rα1 and IL-13Rα2 in each cell and the expression level of these receptor molecules in certain diseases.

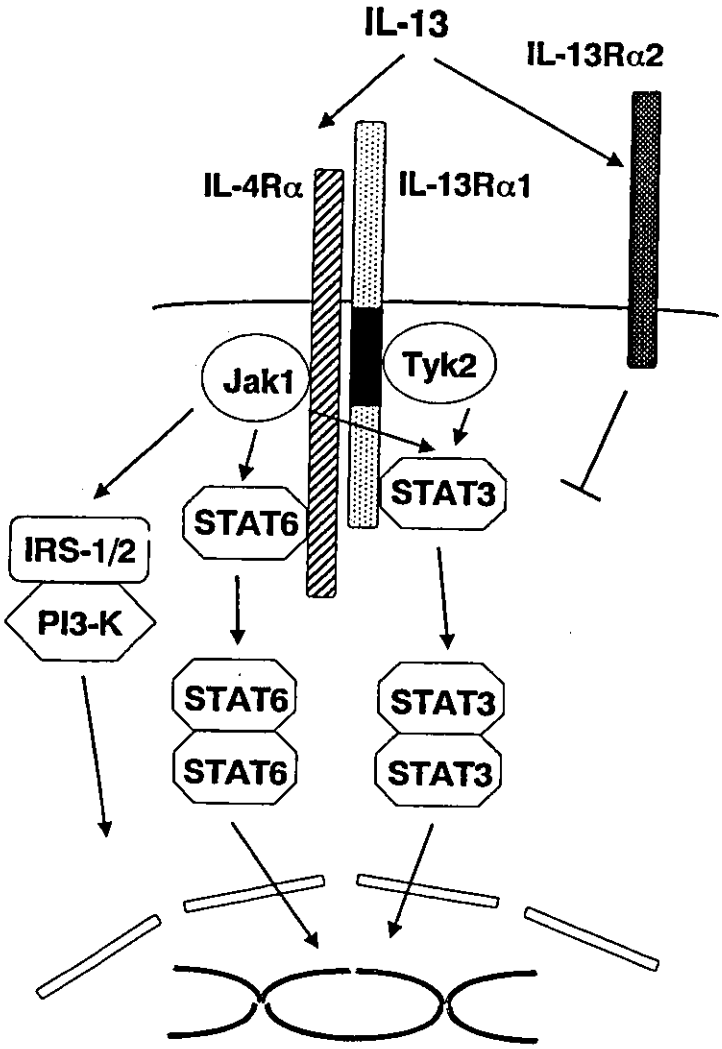

Figure 1. The structure of IL-13R and signal transduction of IL-13

The two types of the IL-13R structure and the signal pathways focusing the Jak/STAT and the IRS-1/2/PI3-kinase pathway are depicted.

2. Relationship between IL-13 and bronchial asthma

Several lines of evidence indicate that IL-13 has an important role in the pathogenesis of bronchial asthma.

A. Analyses of cytokine production in lesions of bronchial asthma

Expression of Th2 cytokines such as IL-4, IL-5, and IL-13 is augmented constitutively or by allergen challenge in bronchial tissues or bronchial alveolar lavage cells derived from asthma patients (17-20). Particularly, expression of IL-13 is dominant, compared to that of IL-4 (18, 20). These results indicate that IL-13 plays some role in the lesions of bronchial asthma.

B. Analyses of model mice

Several analyses using knock-out mice disrupting certain IL-13–signaling molecules indicate the involvement of IL-13 in the pathogenesis of bronchial asthma. Knock-out mice disrupting IL-13 do not show impairment of AHR induced by ovalbumin; however, administration of anti–IL-4 Ab into these mice aiming at inhibiting both the IL-4 and IL-13 signals completely blocks the induction of AHR (21). In the knock-out mice disrupting IL-13, mucous production is impaired, differing from AHR.

It has been shown that although administration of Th2 cells derived from knock-out mice disrupting IL-4 into wild mice induces mucous production, administration of Th2 cells derived from knock-out mice disrupting IL-4Rα does not show such a phenotype (22). Furthermore, in knock-out mice disrupting STAT6, a common signal transducer of IL-13 and IL-4, induction of AHR is impaired (23). These findings suggest the importance of IL-13 in the pathogenesis of bronchial asthma, and also that there exists a redundancy between IL-13 and IL-4 actions, meaning that both IL-13 and IL-4 have an important role for bronchial asthma, and that when both signals are inhibited, the onset of bronchial asthma is significantly impaired.

In addition to such studies, it has been demonstrated that administration of IL-13 in knock-out mice disrupting RAG-1 in which lymphocytes are defective still causes enhancement of AHR, enrichment of goblet cells and eosinophil infiltration (24). Furthermore, it has been demonstrated that transgenic mice expressing IL-13 specifically in bronchial epithelial cells show asthmatic phenotypes such as enhancement of mucous production, hyperplasia of goblet cells, fibrosis in the subcutaneous bronchial tissue, and up-regulation of AHR (25). Considering these findings, IL-13 has a pivotal role in the pathogenesis, probably by acting directly on bronchial tissues.

C. Genetic analyses

Several genome-wide searches for quantitative traits underlying bronchial asthma have been performed, yielding linkages to diverse chromosomal loci (26), explainable by differences of ethnicity and/or definition for subjects. However, chromosome 5q was confirmed as the linkage to bronchial asthma in most of the studies (27-29). As Th2 cytokine genes are clustered in 5q23-31, responsible gene(s) should exist among them. To date, as a candidate gene existing in this region, it has been reported that a variant of the promoter region of the IL-4 gene, -590C/T is related to higher IgE levels (30), and that the IL-9 gene associates with atopy (31). In addition to these genes, we have recently found a variant on the IL-13 gene, in which arginine residue at amino acid 110 is substituted with glutamine, genetically associates with bronchial asthma (32).

When we analyzed the incidence of the arginine type at amino acid 110 (R110) and the glutamine type (Q110) in bronchial asthma patients, that of Q110 was higher in asthma patients than normal donors in both Japanese and British populations. It should be noted that the genetic association of this variant did not depend on the existence of atopy (32). The same variant was thereafter reported to be correlated with high IgE level and atopic dermatitis (33, 34). These results solidified the notion that this variant is a newly appreciable genetic factor for allergic diseases, irrespective of ethnic difference. Based on our functional analyses of this variant, it is assumed that Q110 augments local concentration of IL-13 by lowering affinity with IL-13Rα2 compared to R110, although Q110 and R110 have almost the same affinity with IL-13Rα1, and that Q110 also

up-regulates systemic concentration of IL-13 by enhancing its stability in plasma (Arima K, submitted). Taken together, it is confirmed that this variant could act as a functional genetic factor of bronchial asthma.

3. Involvement of IL-13 in the pathogenesis of bronchial asthma

The mechanism to cause AHR had been explained as follows (35). In the sensitization phase, allergen is processed by professional antigen-presenting cells (APC) such as dendritic cells. Presentation of certain peptides derived from allergen by APC causes expansion of Th2 cells that secrete IL-4, IL-13, IL-3, IL-5, and GM-CSF. IL-4 and IL-13 induces IgE switching in B cells, and IL-4, but not IL-13 also drives expansion of Th2 cells. Secreted IgE binds to the high affinity IgE receptor on surface of mast cells. In the activation phase, allergen binds to IgE on mast cells, and then it triggers secretion of various chemical modulators such as tryptase and leukotrienes from mast cells, modifying AHR. This pathway is called the immediate reaction. On the other hand, IL-3, IL-5, and GM-CSF secreted from Th2 cells act on eosinophils, expanding and activating the cells. Eosinophil-derived granule contents such as major basic protein (MBP) and eosinophil cationic protein (ECP) are also involved in forming the asthmatic phenotype. This pathway is called the delayed reaction. In this scenario, IL-13 is thought to be involved in the pathogenesis of bronchial asthma by inducing IgE production in B cells as well as IL-4 (Fig. 2).

In addition to such mechanisms, it is predicted that IL-13 acts directly on bronchial tissues, causing bronchial asthma, based on analyses of model mice (Fig. 2). If this is the case, how does IL-13 cause bronchial asthma, acting on non-immune cells? As we have already shown that bronchial epithelial cells and bronchial smooth muscle cells highly express IL-13R (32), it is important to clarify the biological activity of IL-13 on such cells.

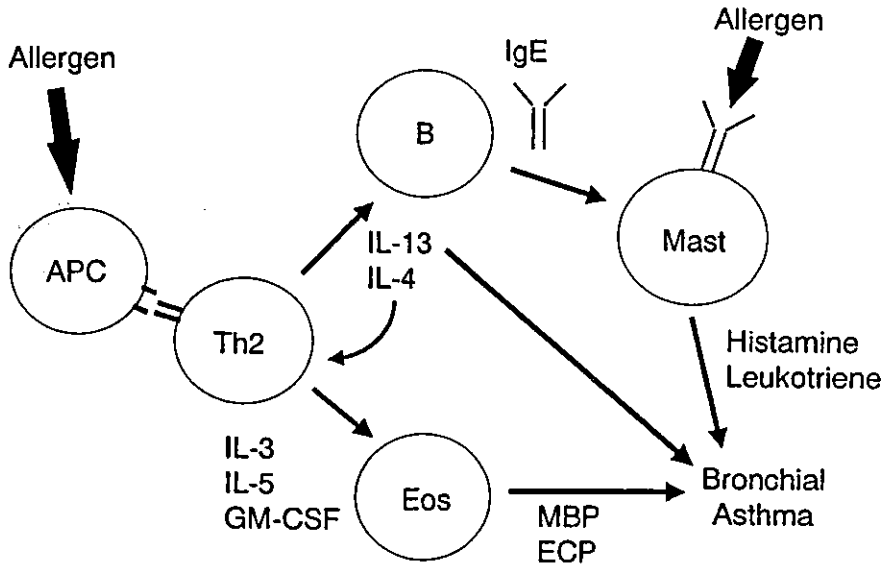

Figure 2. Involvement of IL-13 in the pathogenesis of bronchial asthma (modified from ref. 35)

In addition to IgE synthesis in B cells, it is assumed that IL-13 acts directly on bronchial tissues, causing bronchial asthma.

The biological activity of IL-13 in such cells has not been fully understood, although there are some reports that IL-13 induces expression of eotaxin and 15-lipooxygenase in bronchial epithelial cells (36, 37). We have identified the IL-13–inducing genes in these cells using the microarray method (Yuyama N, submitted). This is expected to lead to figuring out the molecular mechanism of bronchial asthma by analyzing the action of IL-13 on non-immune cells in the bronchial tissues.

Conclusion

Recently, the importance of IL-13 in the pathogenesis of bronchial asthma has emerged as described in this article. Clarification of the molecular mechanism of IL-13 activities in non-immune cells is now the goal. Such an analysis would reveal the pathogenesis of bronchial asthma at the molecular level. Based on the results obtained by these analyses, we can hope that a new method to diagnose bronchial asthma or a new target to improve the asthmatic state will be developed in the near future.

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Upregulation of IL-13 concentration in vivo by the *IL13* variant associated with bronchial asthma

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Background: A substantial body of evidence exists to support the pivotal role of IL-13 in the pathogenesis of bronchial asthma. We recently found that a variant of the *IL13* gene (Arg110Gln) is genetically associated with bronchial asthma, which is concordant with animal experiments using IL-13 in the development of asthma.

Objective: To address whether the Gln110 variant of *IL13* influences IL-13 function, contributing to the pathogenesis of bronchial asthma, we studied the functional properties of the variant.

Methods: We generated 2 types of recombinant IL-13 proteins, the amino acids of which at 110 were arginine or glutamine, and analyzed the binding affinities with the IL-13 receptors, as well as the stability of the proteins. We further compared the relationship between the genotype and serum levels of IL-13.

Results: The variant showed a lower affinity with the IL-13 receptor α2 chain, a decoy receptor, causing less clearance. The variant also demonstrated an enhanced stability in both human and mouse plasma. We further identified that asthmatic patients homozygous for the Gln110 variant have higher serum levels of IL-13 than those without the variant.

Conclusion: These results suggested that the variant might act as a functional genetic factor of bronchial asthma with a unique mechanism to upregulate local and systemic IL-13 concentration in vivo. (J Allergy Clin Immunol 2002;109:980-7.)

Key words: IL-13, polymorphism, IL-13 receptor α2 chain, allergy, bronchial asthma

Bronchial asthma is, like other common human diseases, a complex disease in that it is multifactorial, exhibits genetic heterogeneity, or both.[1] The increasing incidence of allergic diseases in the last few decades, including bronchial asthma, has been attributed to environmental changes, particularly in developed areas. However, genetic factors involved in the pathogenesis of bronchial asthma have been identified on the basis of analyses of inheritance patterns in families and twins.[1,2]

Several genome-wide searches for quantitative traits underlying bronchial asthma have been performed, yielding linkages to diverse chromosomal loci,[3] which is explainable by differences of ethnicity, definition for subjects, or both. However, importantly, most of the studies confirmed linkage to chromosome 5q.[4-6] T_H2 cytokine genes are clustered in 5q31-33, presenting obvious candidates. A variant of the promoter region of the *IL4* gene, −590C/T, has been shown to be related to higher IgE levels.[3] This variant associated in vitro with IL-4 expression and showed higher DNA binding affinity. However, no direct link to cellular IgE synthesis has been shown, and some other groups argued against this association.[7] Significant association of atopy with the *IL9* gene was observed,[8] although this also remains controversial.[9] We recently have found a variant of the *IL13* gene, in which

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arginine residue at amino acid 110 is substituted with glutamine (Arg110Gln). This variant was associated with bronchial asthma in both Japanese and British populations.[10] The same variant was thereafter reported to be positively correlated with high IgE levels and atopic dermatitis.[11,12] These results confirmed the candidacy of this variant as a novel genetic factor for allergic diseases across ethnicity, although it remains unanswered whether this variant itself or other polymorphisms that are in linkage disequilibrium affect the IL-13 signal. Such findings have led us to determine what role this variant may play in the pathogenesis of allergic diseases.

Cytokines derived from the T_H2 lymphocytes are considered to orchestrate the asthmatic phenotype.[13-15] Among T_H2 cytokines, several lines of evidence exist, implicating IL-13 in the pathogenesis of bronchial asthma. Expression of T_H2 cytokines, including IL-13, was augmented constitutively or induced by means of allergen challenge in bronchial tissues or bronchoalveolar lavage fluids derived from asthmatic patients, and the expression of IL-13 was dominant compared with that of IL-4.[16,17] Analyses of mice with disrupted IL-13 signaling molecules, such as IL-13, IL-4R α chain (IL-4Rα), and signal transducer and activator of transcription 6 (STAT6) showed that IL-13 has a pivotal role in the induction of airway hyperreactivity.[18-20] It had been assumed that IL-13's role in the pathogenesis of bronchial asthma would be an action on immune cells, such as induction of IgE switching in B cells, as well as IL-4.[21] Nevertheless, it was recently revealed that IL-13 induces asthmatic phenotype in mice independent of lymphocytes.[22-24] This result indicated that IL-13 acts directly on nonimmune cells in bronchial tissue, which may have an important role in the pathogenesis of bronchial asthma.[25,26] In fact, we have recently shown that bronchial epithelial cells (BECs) and bronchial smooth muscle cells express high levels of IL-4R and IL-13R.[10] Genetic analysis showing that the IL-13 variant was correlated with bronchial asthma rather than with atopy also supports this idea.[10]

To date, 2 IL-13–binding molecules are known to exist.[26,27] An IL-13–binding molecule, the IL-13R α1 chain (IL-13Rα1), alone shows a low affinity with IL-13; however, it forms a high-affinity receptor and transduces the IL-13 signal together with IL-4Rα.[28-33] Another IL-13–binding unit, the IL-13R α2 chain (IL-13Rα2), generates a high-affinity receptor by itself, although it is thought not to transduce the IL-13 signal,[31] acting as a decoy receptor because of its short cytoplasmic domain.[30] Although IL-13Rα2 is expressed on a variety of epithelial tumor and human fibroblast cell lines,[34-36] it is not completely clear which other cell types express IL-13Rα2 and how expression of IL-13Rα1 and IL-13Rα2 is regulated.

In this study, to address whether the IL-13 variant influences IL-13 function, thereby contributing to the pathogenesis of bronchial asthma, we generated 2 types of recombinant IL-13 proteins for this analysis: one with arginine (R110) and the other with glutamine (Q110) at position 110. It turned out that the affinity of R110 and Q110 to IL-13Rα1 was almost equal, whereas Q110 showed slightly lower affinity with IL-13Rα2, a decoy receptor, than with R110. Consequently, Q110 was slightly less cleared from the extracellular environment by IL-13Rα2 than R110. Furthermore, Q110 showed an enhanced stability in vitro and in vivo compared with R110. These results indicated that this variant could influence local and systemic concentrations of IL-13 in vivo. As expected, the serum level of IL-13 was higher in Q110-bearing individuals. Taken together, we could conclude that this variant may be a functional genetic factor in bronchial asthma.

METHODS

Generation of the IL-13 proteins

IL-13 cDNA was cloned from a human T_H2 cell clone, SM4.6.[37] Because the cloned IL-13 was the R110 type, to replace an arginine residue with a glutamine residue, we used a PCR method with the primer 5′-GCGAGGGACAGTTCAACTGAAACTTC-3′ and its complement. Two types of the *IL13* gene were incorporated into pMAL-cX vector (New England Biolabs, Beverly, Mass), with a TEV protease cleavage site (Gln-Asn-Leu-Tyr-Phe-Gln-Gly) attached to the N terminus of *IL13*. As a result, the N terminus of the cleaved product got glycine (underlined), as previously reported.[38]

Maltose-binding protein–fused IL-13 proteins were expressed in *Escherichia coli* and isolated by using an amylose affinity column (New England Biolabs), followed by cleavage by TEV protease (Life Technologies, Rockville, Md) under reducing conditions. The cleaved protein mixtures were denatured with a solution containing 5 mol/L guanidine hydrochloride, 5 mmol/L dithiothreitol, 50 mmol/L Tris/HCl (pH 8.0), 50 mmol/L NaCl, and 1 mmol/L EDTA. Then the proteins were refolded by means of sequential dialyses against the solutions containing lower concentrations (1.5, 0.9, and 0 mol/L) of guanidine hydrochloride and 2 mmol/L–0.2 mmol/L reduced-oxidized glutathione. The IL-13 proteins were purified by using a Q-Sepharose Fast Flow column, followed by a Superdex 75 HR10/30 column (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Purity of the generated proteins was greater than 98% and estimated by means of silver staining of SDS-PAGE gel. Molecular weights of R110 and Q110 were 12341.2 and 12309.3, respectively, as evaluated with Voyager RP MALDI-TOF mass spectrometry (PerSeptive Biosystems, Framingham, Mass), and these weights were consistent with their theoretic values on the basis of cDNA sequences. The concentration of R110 was assessed by means of Western blotting with anti–IL-13 antibody (AF-213-NA; R&D Systems, Minneapolis, Minn), with the commercial IL-13 (PeproTech, Rocky Hill, NJ) used as a standard. The moles of the 2 IL-13 proteins were adjusted on the basis of the values calculated with an amino acid analyzer (L-8500; Hitachi, Hitachinaka, Japan).

Cells and plasmids

DND-39 cells cotransfected with IL-13Rα1 and pGL3-enhancer vector (Promega, Madison, Wis), into which the promoter region from −187 to +6 of the human Iε gene (Gε) was inserted, were constructed as described previously.[29] The cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin containing 6 μg/mL blasticidin S hydrochloride (Funakoshi, Tokyo, Japan) and 250 μg/mL hygromycin B (Wako, Osaka, Japan). A neomycin-resistant gene was inserted into the plasmid coding IL-13Rα2 in pME18S mammalian expression vector, and this plasmid was incorporated into DND-39 cells pretransfected with Gε alone by means of electroporation. Expression of IL-13Rα2 was confirmed by means of flow

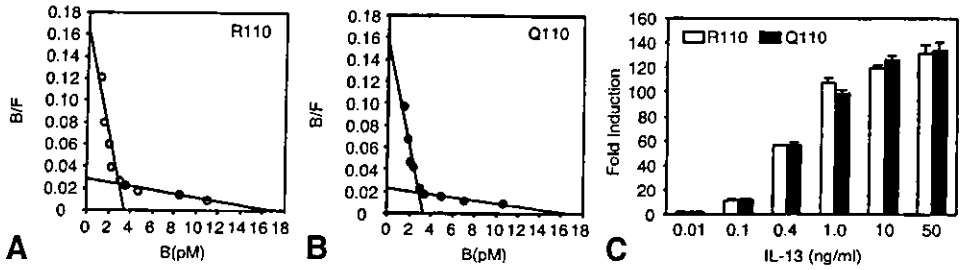

FIG 1. Binding assay of R110 and Q110 to IL-13Rα1. **A** and **B**, The Scatchard plot with IL-13Rα1–expressing DND-39 cells incubated with 2 types of ^{125}I-labeled recombinant IL-13 (**A**, R110; **B**, Q110) is depicted. The same experiments were performed 3 times, and the representative data are depicted. **C**, Luciferase activity of IL-13Rα1–transfected DND-39 cells incubated with either R110 or Q110 is shown. Fold induction was estimated comparing the value without the stimulus. Each experiment was done with 3 samples, and the mean values are shown.

cytometry with anti–IL-13Rα2 antibody (Diaclone, Besançon, France). The transfectants were maintained with a medium containing 1.25 mg/mL geneticin (Sigma, Saint Louis, Mo).

Harvested cells were stimulated with the indicated concentration of R110 or Q110 for the indicated period. In some experiments IL-13 was incubated with either 100% of human serum derived from healthy donors or PBS containing 120 mg/mL BSA for the indicated period.

Binding assay

Iodine 125–labeled IL-13 was generated and used in a binding assay for Scatchard plot analysis, as described before.[39] The concentration of the labeled IL-13 was determined by means of self-displacement binding to the IL-13Rα2–expressing DND-39 cells with nonradiolabeled IL-13 at a known concentration. After cells were incubated with various concentrations of ^{125}I-labeled IL-13 for 2 hours at 4°C, bound and free ligands were separated by means of centrifugation through an oil gradient. Nonspecific binding was measured by adding 100-fold or more molar excess of nonradiolabeled IL-13.

Kinetic studies to measure k_{on} rate and k_{off} rate were performed, as described earlier.[40] An association kinetic study was performed under a pseudo first-order condition with respect to both the free ligand and the unoccupied receptor. We incubated 5.0×10^6/mL cells with 15 pmol/L ^{125}I-labeled IL-13 for 2 minutes to 4 hours at 4°C. Specifically bound radioactivity at various intervals up to the equilibrium binding was plotted versus time. To yield the k_{on} rate, the initial slope of the plotted curve was divided by the initial concentrations of free ligand (15 pmol/L), and the expressed receptor was determined by means of Scatchard analysis. In the dissociation kinetic study 5.0×10^6/mL cells were first incubated with 250 pmol/L ^{125}I-labeled IL-13 for 90 minutes at 4°C to let the receptors become fully occupied by the radiolabeled IL-13. After the cells were washed once and resuspended in binding buffer at 4°C, aliquots of cells were taken at various time intervals, and the bound IL-13 was measured. Specifically bound radioactivity at various intervals was plotted versus time.

Luciferase assay

Luciferase activity assay was carried out as described previously.[29] The cells were incubated with the indicated concentrations of IL-13 for 24 hours. After the cells were washed once in PBS and lysed with reporter lysis buffer (Toyoink, Tokyo, Japan), cell lysates were mixed with luciferase assay reagent (Toyoink).

Pharmacokinetics of IL-13 in vivo

One microgram of IL-13 was intraperitoneally administered into 8- to 9-week–old female BALB/c mice, and blood samples were collected at the indicated times. Four mice were analyzed for both R110 and Q110. The procedures were conducted according to the "Guide for the Care and Use of Laboratory Animals," and the study was approved by Saga Medical School's Research Committee.

Measurement of IL-13 levels in plasma

IL-13 levels in mouse and human plasma were immunoassayed in the Mitsubishi Kagaku BCL laboratories by means of a commercial kit.[41] The minimal detectable level was 3.1 pg/mL. Because of the different sensitivities of R110 and Q110 to this assay, the values of R110 and Q110 obtained by the kit were adjusted on the basis of the known proteins as a standard. As for human serum level, blood samples were obtained between 9 and 10 AM to limit circadian variation in cytokine production, and data were presented as medians with 95% confidence intervals and were analyzed with the median χ^2 test to compare 2 groups.

Genotyping of IL-13

Genotyping of IL-13 was studied in a general population of Japanese children from Wakayama Prefecture, as described previously.[41] All the asthmatic subjects had been given diagnoses by asthma specialists of (1) recurrent breathlessness and chest tightness requiring ongoing treatment, (2) physician-documented wheeze, and (3) documented labile airflow obstruction with variability in serial peak expiratory flow rates of greater than 30%.

Genotyping of IL-13 was conducted by using the PCR method as follows.[10] DNA samples were extracted with a commercial kit (IsoQuick; Microprobe, Garden Grove, Wash). PCR primers were 5′-TGGCGTTCTACTCACGTGCT-3′ and 5′-TTTCGAAGTTTC-AGTAGTAC-3′. The underlined sequences were mutated to incorporate a restriction site for *ScaI*. The study was approved by Kyoto University's Ethics Committee.

RESULTS

Binding activity of the IL-13 variant with IL-13Rα1

It has been already demonstrated that IL-13R consisting of IL-13Rα1 and IL-4Rα forms a high-affinity recep-