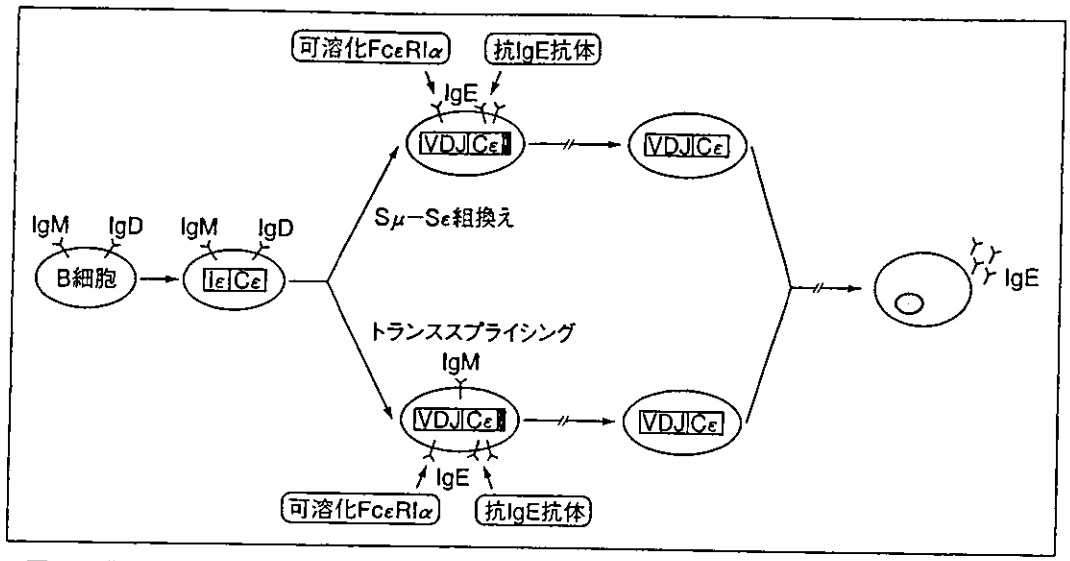

図4　膜型Cε mRNAあるいはε鎖trans-mRNAの産物に特異的に結合する可溶化FcεRIαと抗IgE抗体

受けることによってIε exonとCε遺伝子が直結したgermline Cε mRNAが発現される．このステライル転写産物の発現により，IgEクラススイッチの方向性が決定される．

さらに，CD40の活性化を介してgermline Cε mRNAの発現が増強されるとともにSμとSεの領域間でDNA組換えが起こり，これらの領域間に位置している他のC_H遺伝子は環状DNAとしてループアウトされる．その結果，V_HDJ_H遺伝子群がEμエンハンサーとともにCε遺伝子のすぐ上流に転座し，またRNA転写とスプライシングにより可変領域を含む成熟Cε mRNAが発現される．可溶化FcεRIαは，IL-4刺激によるgermline Cε mRNAの発現や抗CD40抗体との共刺激によるその発現増強には影響を与えないが，IL-4と抗CD40抗体による膜型や分泌型の成熟Cε mRNAの発現は可溶化FcεRIαによって抑制される[3-6]．したがって，可溶化FcεRIαはgermline Cε mRNAの発現以降の過程を抑制することによりIgE産生の抑制作用を示す．以下，成熟Cε mRNAの発現機序を踏まえて考察する．

IgEクラススイッチにはSμ→Sεの直接的な組換えが関与していることについては，IL-4と抗CD40抗体で共刺激したB細胞由来の染色体DNAをnested PCR法を用いて解析した多くの実験結果から支持されている．また，IL-4やIL-13はIgEクラススイッチのみならずIgG4クラススイッチに対しても促進的に作用するので，Sμ→Sγ4→Sεの連続的な組換えも一部関与している．Sγ4を介する連続的な組換えに際して2度目の組換えがSμ/Sγ4領域内のSμとSεとの間で起こると，最終的には染色体DNAからはSγ4が欠失するので，Sμ→Sεの直接的な組換えと区別することはできない．これに対して，ループアウトされた環状DNAにおける各S領域断片の塩基配列を調べれば両者の判別は可能である．実際，この解析結果によれば[14]，IgEクラススイッチの大部分はSμ→Sεの直接的な組換えに依存しているが，一部Sμ→Sγ4→Sεの連続的な組換えも認められる．したがって，IgEクラススイッチにはSμ，Sγ4およびSεの各領域におけるさまざまな組換えが関与していると推測される．いずれにしても，可溶化FcεRIαはSμ−Sε組換えを介して誘導されるIgE$^+$B細胞の1分子の膜型IgEと特異的に結合することによってIL-6産生を抑制し，その結果IgE産生細胞への分化に必要なステップである膜型Cε mRNAから分泌型Cε mRNAへの変換を抑制すると考えられる．これに対して，抗IgE抗体のF(ab′)$_2$フラグメントによる成熟Cε mRNAの発現抑制にはIgE$^+$B細胞のアポトーシスが関与している．

一方，膜型Cμ mRNAとgermline Cε mRNAとの間でトランススプライシングが起これば，DNA組換え非依存的に成熟Cε mRNA（ε鎖

trans-mRNA）が発現される[15]．たとえば，ヒト膜型μ鎖遺伝子を導入したマウスB細胞をLPSとIL-4で共刺激すると，ε鎖trans-mRNAの産生を介して膜型IgEが発現される．このようなε鎖trans-mRNAの産生は可溶化FcεRIαの添加により抑制されるが，LPS単独刺激によるγ2b鎖とγ3鎖の両trans-mRNAの産生には影響は認められない．したがって，可溶化FcεRIαはε鎖trans-mRNAの産生を特異的に抑制する．

以上の結果から，膜型Cε mRNAやε鎖trans-mRNAの発現はこれらの産物である膜型IgEと特異的に結合する可溶化FcεRIαや抗IgE抗体などのIgE結合分子によって抑制されることは明らかである（図4）．しかし，膜型IgEとの結合様式の相違に起因する細胞内シグナル伝達系の違いについては不明な点が多いので，膜型IgEと会合する各種分子の同定を含めてさらに詳細に検討する必要がある．また，IgEのCε3ドメインには，アミノ酸置換を伴う遺伝的多型が存在するので[16,17]，この多型と可溶化FcεRIαの相互作用についても今後の課題である．

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IL-13 and its signal pathway are thought to be promising targets to develop a therapeutic reagent for bronchial asthma.

Signal Transduction of IL-13 and Its Role in the Pathogenesis of Bronchial Asthma

by K. Izuhara and K. Arima

Summary

Interleukin-13 (IL-13) is a Th2-type cytokine, secreted from $CD4^+$ T cells, mast cells, basophils and eosinophils. The human IL-13 gene locates at 5q31, generating a cluster with other Th2-type cytokines such as IL-4 and IL-5. Although the homology between IL-13 and IL-4 at the amino acid level is only about 25%, the IL-13 structure determined by NMR is very similar to that of IL-4. Both cytokines share their receptors and signal pathways, giving these two cytokines similar biological properties. However, the important role of IL-13 in the pathogenesis of bronchial asthma has recently been recognized, based mainly on analyses of mouse models. IL-13 and its signal pathway are thought to be promising targets to develop a therapeutic reagent for bronchial asthma. In this article, we summarize the signal transduction pathway of IL-13, the pathological roles of IL-13 in bronchial asthma and the reagents to inhibit IL-13 signals that are now under development.

Bronchial asthma is a complex disorder involving a combination of genetic and environmental factors.[1] Improved hygiene, having such results as a decrease in childhood infections, has been thought to be an important environmental factor.[2,3] This would explain the dramatic increase in prevalence of allergic diseases, including bronchial asthma, in developed areas in the last few decades. Genetic predisposition to allergic diseases is thought to be polygenic and to exist among nucleoside polymorphisms.[4] Extensive efforts have been made to identify such factors by genome-wide search and candidate-gene study.[5] A combination of genetic and environmental factors results in expansion of Th2 cells and a predisposition to allergic diseases. Th2 cells, mast cells and eosinophils infiltrate the bronchial tissue of asthma patients, where downstream mediators are released, so that airway function is disordered.[1] This means that Th2 cytokines would take the lead in generating the asthmatic phenotype. Although the paradigmatic Th2 cytokines, interleukin-4 (IL-4) and IL-5, had been thought to be the primary regulators of allergic responses, recent studies based mainly on analyses of mouse models suggest that IL-13 is the central mediator of the effector phase of allergic responses.[6-8] IL-13 is a protein weighing about 10 kDa. Although the homology between IL-13 and IL-4 at the amino acid level is only about 25%, the structure determined by NMR is very similar to that of IL-4, and both cytokines share their receptors and signal pathways, so that these two cytokines have common biological properties, as described later. In this review, we summarize the signal transduction pathway of IL-13, the pathological roles of IL-13 in bronchial asthma and the reagents to inhibit IL-13 signals that are now under development.

Signal transduction mechanism of IL-13

IL-13 exerts its biological activities by binding to its receptor on the cell surface as well as other cytokines.[8] The IL-13 receptor (IL-13R) is composed

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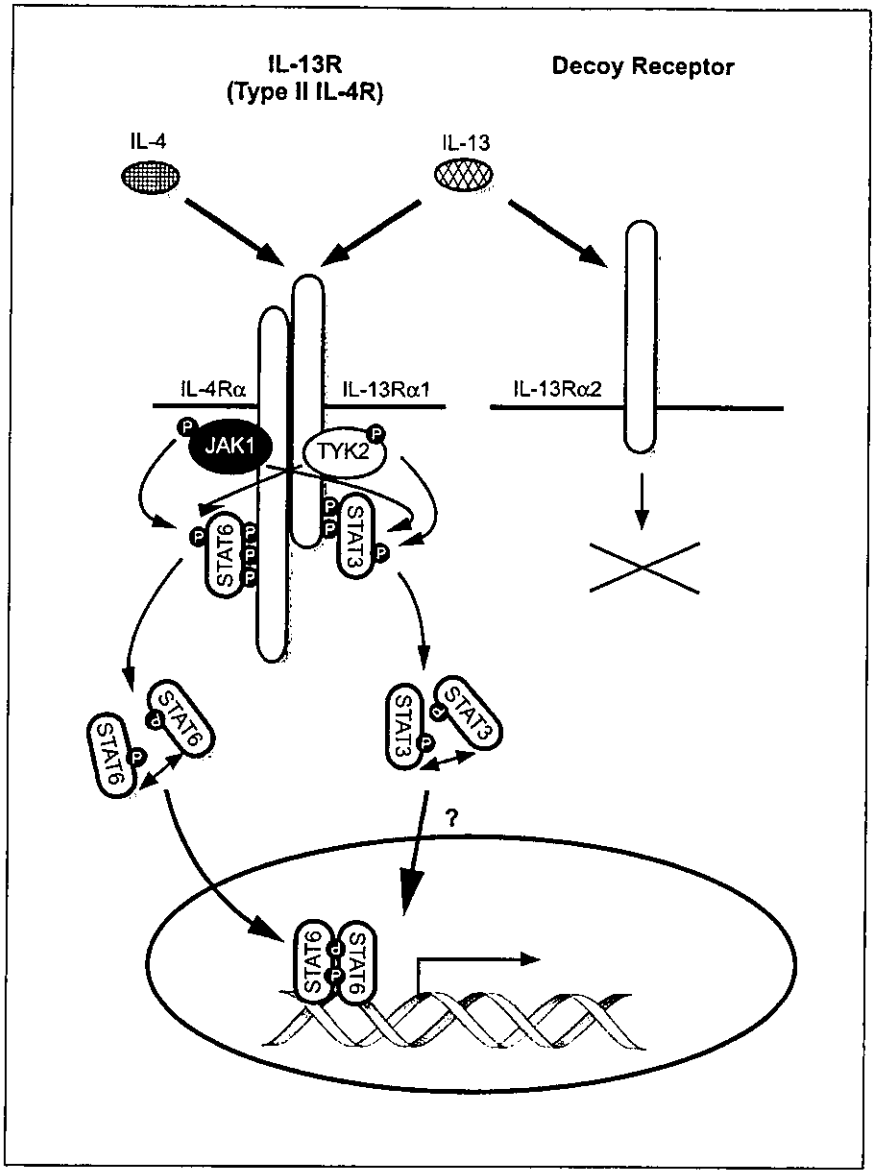

Fig. 1. The receptor structure and signal transduction mechanism of interleukin-13 (IL-13). IL-13 binds to IL-13 receptor (IL-13R), which is composed of IL-4Rα and IL-13Rα1. IL-13R also acts as type II IL-4R. Engagement of the receptor causes signal transduction, mainly through the JAK–STAT pathway. IL-13 also binds to IL-13Rα2, although this receptor does not transduce the signal, acting as a decoy receptor.

of the IL-4Rα chain (IL-4Rα) and the IL-13Rα1 chain (IL-13Rα$_1$). As IL-4 is able to bind to this receptor, it is also called type II IL-4R (Fig. 1).[9–12] However, as IL-13Rα1 and IL-4Rα are important binding units for IL-13 and IL-4, respectively,[9,10,13–16] the binding mechanism of IL-13 and IL-4 to the IL-13R/type II IL-4R is likely to be different.

Both IL-4Rα and IL-13Rα1 belong to the cytokine receptor superfamily, and they contain the conserved cysteine residues and the WSXWS motif in their extracellular domains.[9,10,13–16] Box 1 motifs exist in their cytoplasmic domains close to the transmembrane domains through which JAK1 and TYK2 bind to IL-4Rα and IL-13Rα1, respectively.[12,17,18] In addition, the cytoplasmic domain of IL-4Rα contains the ID-1 portion important for the signal, the I4R motif to which insulin receptor substrate 1/2 (IRS-1/2) binds, the tyrosine residues to which STAT6 binds and the immunoreceptor tyrosine-based inhibitory motif (ITIM) with which tyrosine phosphatases associate, downregulating the signal (Fig. 2).[19–21] IL-13Rα1 contains the tyrosine residues to which STAT3 binds in its cytoplasmic domain (Fig. 2).[12,18]

Upon engagement of the receptor by IL-13, its signal is transduced intracellularly, mainly via the JAK–STAT and the phosphatidylinositol 3-kinase/IRS-1/2 pathways (Fig. 1).[19–21] In the JAK–STAT pathway, engagement of the receptor by IL-13 activates JAK1 and TYK2, followed by activation of both STAT6 and STAT3.[12,18] STAT6 is a critical transcription factor for IL-13, as well as IL-4, to exert its biological activities.[22,23] In contrast, thus far, the biological role of STAT3 in the IL-13 signals remains to be clarified.

There exists another IL-13–binding unit, the IL-13Rα2 chain (IL-13Rα2).[24,25] IL-13Rα2 also belongs to the cytokine receptor superfamily, containing the conserved cysteine residues and the WSXWS motif in its extracellular domain. However, its cytoplasmic domain is short and does not have a Box1 motif. When IL-13Rα2 is expressed on some cell lines, STAT6 activation by IL-13 is blocked,[26,27] meaning that IL-13Rα2 acts as a "decoy receptor."

It is known that SOCS molecules act as negative regulators for JAK–STAT pathways of cytokine signals.[28] Although it has not been shown that any SOCS molecule inhibits the IL-13 signal, SOCS-1 induced by interferon β or interferon γ in monocytes or a B lymphoma cell line inhibits STAT6 activation by IL-4, which at least partially explains the inhibitory effects of interferon β or interferon γ on the IL-4 signal.[29–31] Taking into account that IL-13 and IL-4 receptors share IL-4Rα, followed by activation of STAT6, it would be possible that SOCS-1 inhibits the signal pathway of IL-13 as well as IL-4.

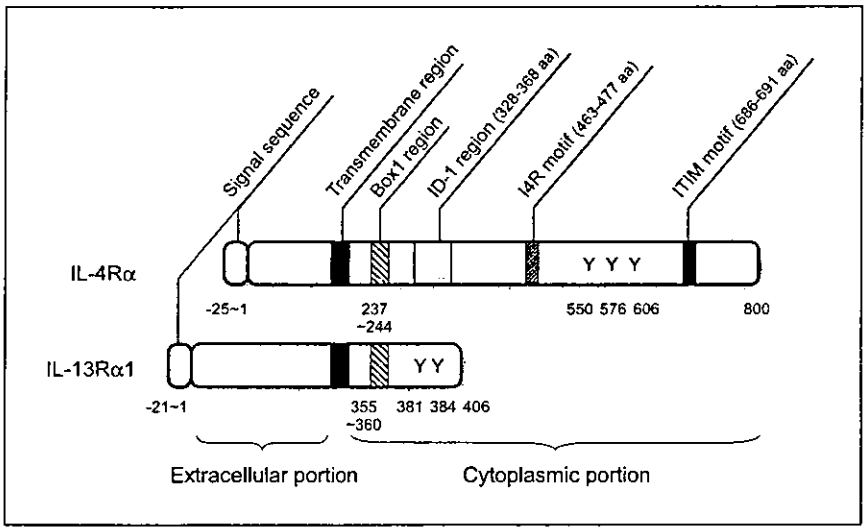

Fig. 2. A schematic model of the functional mapping of interleukin-4 receptor α (IL-4Rα) and IL-13Rα1. Functional domains of IL-4Rα and IL-13Rα1 are depicted. Four regions in the intracellular domain of IL-4Rα and one region in the intracellular domain of IL-13Rα1 are critical for the signal transduction. The figure is numbered from the start of the mature protein. Y represents tyrosine residues binding sites for STAT6 and STAT3.

Expression of the IL-13 receptor and its regulation

IL-4Rα and IL-13Rα1 are ubiquitously expressed in both immune and nonimmune cells, with the exception that T cells do not express IL-13Rα1.[10,32–35] However, the regulation of expression of these receptors is variable among the cells. In the resting stage of B cells, only a small amount of IL-13Rα1 is expressed. On stimulation of anti-IgM antibody and/or anti-CD40 antibody, expression of IL-13Rα1 is significantly augmented, which enables IL-13 signals to be transduced, whereas expression of IL-4Rα is not affected as much.[12,36] In contrast, expression of IL-13Rα1 is not influenced by the IL-13 stimulation or the asthmatic status; therefore, it is thought that expression of IL-13Rα1 is invariable in bronchial tissues.[37]

The regulation of IL-13Rα2 expression has been recently revealed. In hepatocytes or bronchial epithelial cells, only a low amount of IL-13Rα2 is expressed at the constitutive state; however, the stimulation of IL-4 or IL-13, or some pathogenic status such as parasite infection or bronchial asthma, upregulates expression of IL-13Rα2, inhibiting the IL-13 signal.[37–39] These results suggest that induction of IL-13Rα2 by IL-13 acts as a negative-feedback system for the IL-13 signal (Fig. 3). Thus far, expression of IL-13Rα2 has not been detected on lymphocytes.

Biological activities of IL-13

IL-13 has many biological activities in common with IL-4; for example, IL-13 induces class switching toward IgE and IgG4 (IgG1 in the case of mice), and expression of CD23 and MHC class II in B cells, and anti-inflammatory actions in monocytes as well as IL-4.[32] In contrast, IL-13 does not show any action on T cells such as induction of differentiation toward Th2 cells and proliferation. Activation of mast cells by IL-13 is not as clear as that by IL-4. Furthermore, mouse IL-13 does not have an effect on B cells, in contrast to human IL-13.

Following the analyses of biological activities of IL-13 on immune cells, studies on nonimmune cells have been performed, revealing that biological activities of IL-13 are very diverse. Particularly, biological activities of IL-13 on resident cells in the bronchial tissue have attracted a lot of attention, in correlation with the pathogenesis of bronchial asthma. The following activities on the resident cells in the bronchial tissues have been reported: 1) production of TGF-β[40,41] and eotaxin-3,[42] expression of a chloride channel, human CLCA1 (mouse CLCA3)[43] and production of mucin[44] in bronchial epithelial cells; 2) production of eotaxin,[45] expression of integrin[46] and proliferation[47] in fibroblasts; and 3) production of eotaxin[48,49] and enhancement of contraction[50–52] in smooth muscle cells.

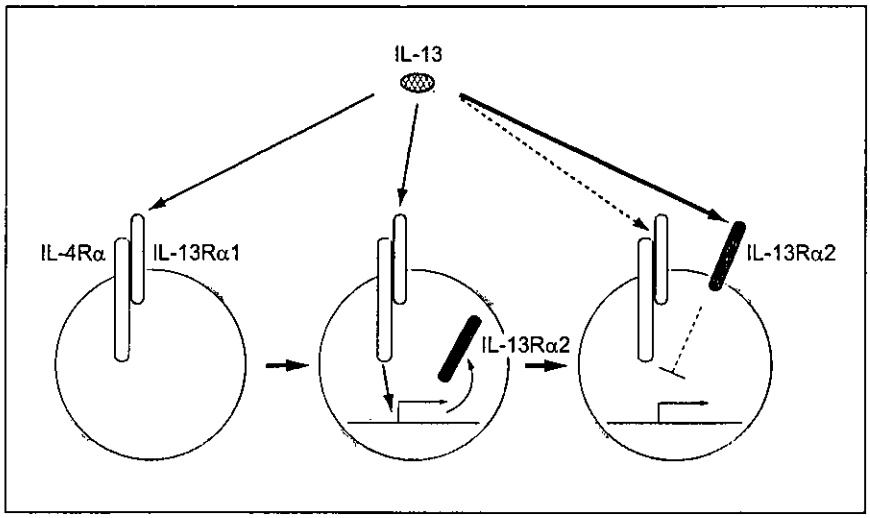

Fig. 3. Negative feedback system of interleukin-13 (IL-13) signal by interleukin-13 receptor α2 (IL-13Rα2). The negative feedback system of IL-13 signal by induction of IL-13Rα2 is depicted. In this system, IL-13 induces expression of IL-13Rα2, which downregulates IL-13 signal.

Significance of IL-13 in the pathogenesis of bronchial asthma

Analyses of mouse models

The significance of IL-13 in the pathogenesis of bronchial asthma was first suggested based on 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 (AHR), a characteristic feature of asthma.[53–56] Webb et al. described how inhibition of both IL-13 and IL-4 signals impair AHR induced by ovalbumin compared with each inhibition, indicating the redundant role of IL-13 and IL-4 in generating AHR.[56] In contrast, Grünig et al. and Wills-Karp et al. reported that specific inhibition of IL-13 signals completely abolished AHR and mucous production without change of eosinophilic infiltration,[57,58] which indicates that IL-13 is the central mediator of the effector phase of bronchial asthma. Analyses using recombination-activating gene 1-disrupted mice suggest that this effect of IL-13 is independent of lymphocytes, indicating that the effects of IL-13 on nonimmune resident cells in the bronchial tissue are important.[57] The findings that epithelial overexpression of an IL-13 transgene induces an asthma-like phenotype,[59] and that reconstitution of STAT6 only in bronchial epithelial cells into STAT6-disrupted mice restores IL-13–induced AHR and mucous production, but not inflammation or fibrosis,[60] support the importance of IL-13's direct action on bronchial epithelial cells.

Analyses of asthma patients

To confirm that results from analyses of mice might be relevant to human patients, the following analyses would be important: 1) expression of IL-13 in the bronchial lesions; 2) genetic association of the IL-13 signaling molecules; and 3) effects of IL-13 antagonists for asthma patients. It could happen that the pathological roles of some molecules in mice and human patients would differ. For example, it was shown that IL-5–disrupted mice proved to have decreased AHR induced by ovalbumin,[61] which indicated that IL-5 had a critical role in the pathogenesis of bronchial asthma. However, anti–IL-5 antibody improved eosinophilia, but not AHR of asthma patients,[62] which brought into question the role of IL-5. Therefore, to analyze effects of the antagonists for asthma patients, the most important among the indicated analyses would be the evaluation of the significance in the pathogenesis of bronchial asthma. Such analyses are now underway, so we will await the results.

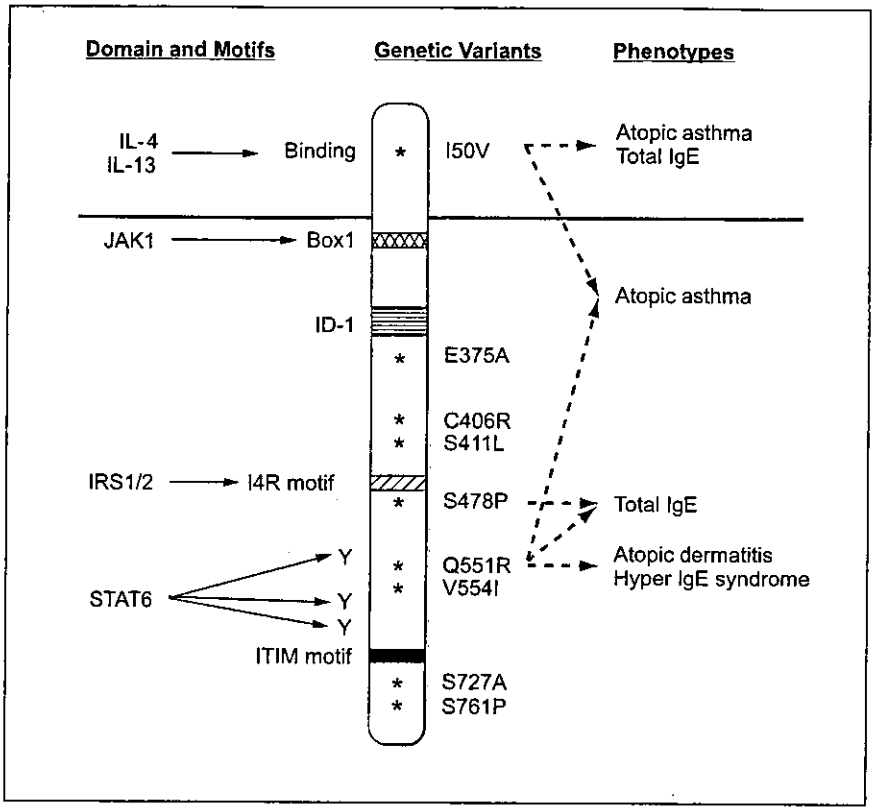

Fig. 4. The localization of the polymorphisms of IL-4Rα that substitute amino acids. The polymorphisms of IL-4Rα that substitute amino acids and phenotypes correlated with those are depicted. The figure is numbered from the start of the mature protein. Abbreviations: IRS1/2, insulin receptor substrate 1/2; ITIM, immunoreceptor tyrosine-based inhibitory motif.

Expression of IL-13 in bronchial lesions

Expression of IL-13 is higher at the baseline and is greatly upregulated by allergen challenge in bronchial tissues or bronchoalveolar lavage fluids derived from asthma patients, much higher than with IL-4.[63,64]

Genetic association of the IL-13 signaling molecules

On the basis of candidate gene study, it has been reported that several single nucleotide polymorphisms (SNPs) of genes encoding IL-13 signaling molecules—such as IL-13, IL-4Rα, IL-13Rα1, STAT6 and BCL6—are genetically associated with asthma or atopy.[65] On the IL-4Rα genes, three SNPs that substitute amino acids—Ile50Val, Ser478Pro and Gln551Arg—are reported to be associated with several allergic phenotypes such as atopic bronchial asthma, total IgE, atopic dermatitis and hyper IgE syndrome (Fig. 4).[66–68] We demonstrated that the Ile50Val polymorphism enhances the IL-4 signal without change of the affinity with IL-4,[67] and it has been shown that Gln551Arg decreases association with a phosphotyrosine phosphatase, SHP-1;[66] how-

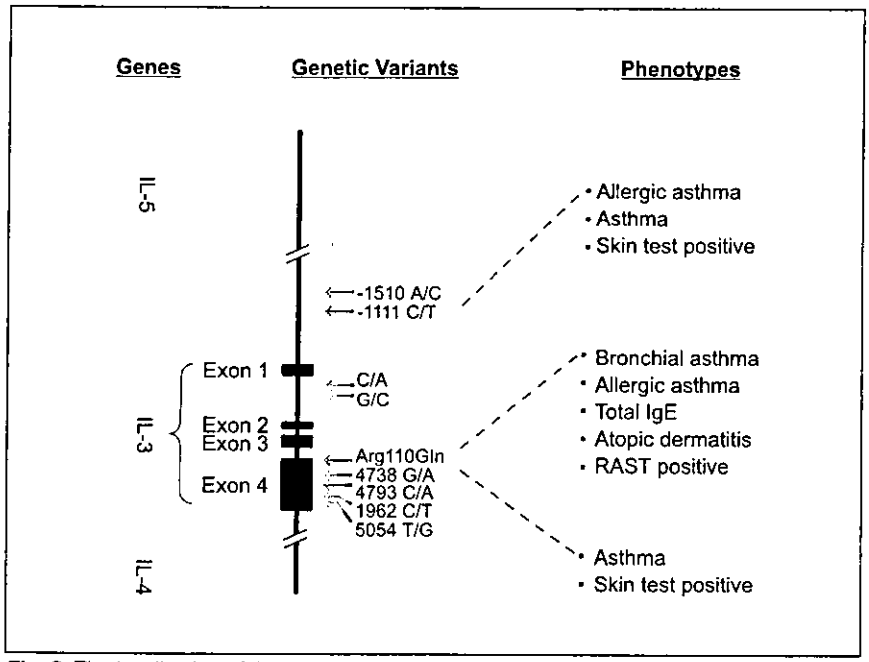

Fig. 5. The localization of the polymorphisms of interleukin-13 gene. The polymorphisms of interleukin-13 and phenotypes correlated with those are depicted.

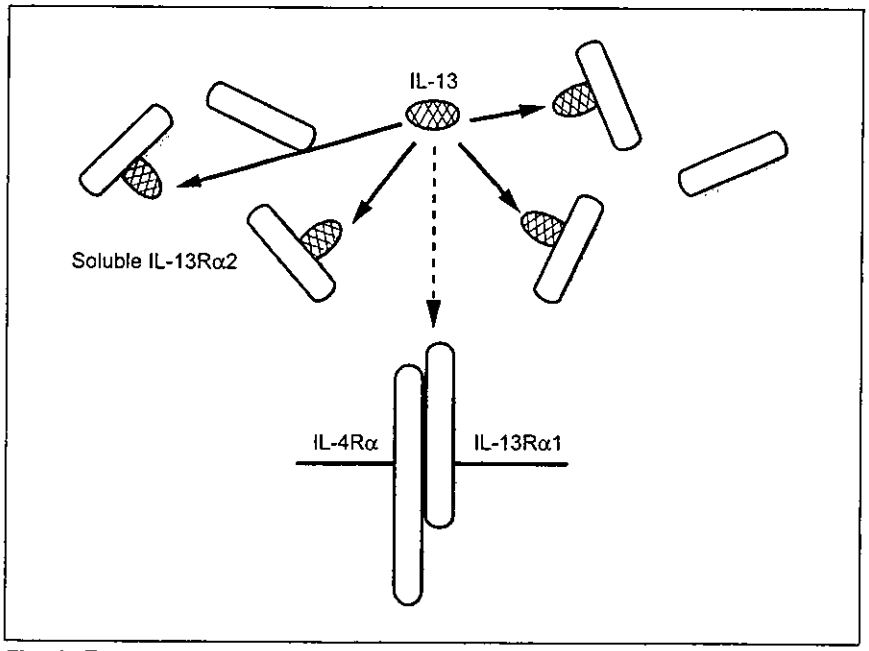

Fig. 6. The molecular mechanism of soluble interleukin-13 receptor (IL-13R). Soluble IL-13Rα2 inhibits the binding of IL-13 to IL-13R on the cell surface by trapping IL-13.

TABLE I: LIST OF IL-13 ANTAGONISTS

AGENTS	PRODUCING COMPANY	IL-4 BLOCK	IL-13 BLOCK
Soluble IL-13 Receptor	Wyeth Genetics Institute	–	+
IL-4/IL-13 Trap	Regeneron	–	+
IL-4 mutein	Bayer	+	+
Anti–IL-13Rα1 Ab	AMRAD	?	+

ever, some reports conflict with these results.[69–71] In the IL-13 gene, three SNPs, -1111C/T, Arg110Gln and 4738G/ A, are reported to be associated with several allergic phenotypes (Fig. 5).[38,72–76] It has been demonstrated that the -1111C/T polymorphism affects IL-13 expression, probably by increasing association of the transcription factor NFAT with this region.[73] We showed that Gln110Arg polymorphism decreased the affinity with IL-13Rα2 and enhanced stability as a protein, causing upregulation of the IL-13 concentration *in vivo*.[77]

Targeting IL-13 to develop a new agent for bronchial asthma

Because IL-13 is thought to be the central mediator of the effector phase of bronchial asthma, it is reasoned that blocking the biological activities of IL-13 should be a good strategy to improve asthmatic status. The following reagents targeting IL-13 are currently under development (Table I).

Soluble IL-13R

The strategy to improve bronchial asthma by a soluble cytokine receptor was first applied to soluble IL-4Rα.[78,79] Such a strategy has the advantage of avoiding immunogenicity, compared with a neutralizing antibody or a variant protein. Two candidates of soluble IL-13R exist: soluble IL-13Rα1 and IL-13Rα2. Because the affinity of IL-13Rα2 with IL-13 (K_d; 50–500 pM) is more than 10-fold higher than that of IL-13Rα1 (K_d; ~4 nM), soluble IL-13Rα2 has superior potency as a blocking agent (Fig. 6).[24,77,80] The Wyeth Genetics Institute is developing soluble IL-13Rα2 as a novel product, and they have reported that it is effective for decreasing AHR in bronchial asthma-induced mice.[57,58] Further studies conducted with bronchial asthma patients are awaited. On the other hand, Regeneron Pharmaceuticals Inc. is developing a chimera protein in which soluble IL-4Rα and soluble IL-13Rα1 are tandem lined, denoted IL-4/IL-13 trap.[81]

IL-4 mutein

IL-4 mutein is listed as another IL-13 antagonist. IL-4 muteins in this context 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).[82–84] These muteins act as antagonists for both IL-4 and IL-13, because they are able to bind to the IL-4R/IL-13R, but not able to transduce the signal. Bayer Corp. is now developing this reagent as a therapy for allergic diseases.

Other IL-13 antagonists

AMRAD Inc. is now developing an anti-IL-13Rα1 antibody as a therapeutic reagent for allergic diseases, but its details are unclear.

Strategy for applying the findings of IL-13–inducing genes to developing a new reagent

Once the effector molecules downstream of the IL-13 signals are identified, we may apply such a finding to develop a new reagent against bronchial asthma instead of blocking the binding of IL-13 to the IL-13R. To identify such a promising molecule, we and others identified IL-13–inducing genes in bronchial epithelial cells by microarray analysis.[85,86] In addition to these studies, gene expression profiles in asthma patients and a monkey or a mouse model of bronchial asthma by microarray analysis have also been reported.[87–89] Genomic information based on these investigations would give us a hint in developing a new reagent for bronchial asthma.

Conclusion

In this review article, we summarized the signal transduction pathway of IL-13, the pathological roles of IL-13 in bronchial asthma and the reagents to inhibit IL-13 signals now under development. 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 disease.

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ICOS REPORTS 2003 HIGHLIGHTS

On February 3, 2004, Icos Corp. announced product and financial highlights for the fourth quarter and year ended December 31, 2003. Throughout 2003, Icos continued developing early-stage research and preclinical candidates in order to move one or two candidates into phase I clinical development in 2004. The most advanced preclinical candidates include a potent oral LFA-1 antagonist (leukocyte function-associated antigen one) for the treatment of psoriasis, a phosphodiesterase inhibitor for inflammatory disease, a cell cycle checkpoint modulator for cancer and an oral modulator of B lymphocyte function for an autoimmune disease.

In addition, by the end of the year, Icos had completed patient follow-up in a phase II clinical study evaluating *RTX*™ (**resiniferatoxin**) for the treatment of interstitial cystitis. In late January 2004, it was determined that *RTX* was not effective in relieving patients' symptoms; thus the company decided not to pursue additional studies of interstitial cystitis. Also, in December 2003, a phase II clinical study began with **IC-485** in patients with chronic obstructive pulmonary disease. This study is scheduled to be completed in 2005.

Moreover, during 2003, *Cialis*® (**tadalafil**) was launched in 55 countries worldwide for the treatment of erectile dysfunction. Commercial launches began in Europe, New Zealand and Australia in February, and finished in the United States and Canada in November. *Cialis* is being marketed by Lilly Icos, a 50/50 joint venture between Icos and Eli Lilly and Co., in North America and Europe. Elsewhere, Lilly has rights to market *Cialis*, and pays a royalty, to Lilly Icos, for product sales in those territories.

Application of Functional Genomics to Bronchial Asthma

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Abstract: Bronchial asthma is a complicated and diverse disorder affected by genetic and environmental factors. It is widely accepted that it is a Th2-type inflammation originating in lung and caused by inhalation of ubiquitous allergens. The complicated and diverse pathogenesis of this disease is yet to be clarified. Functional genomics is the analysis of whole gene expression profiling under given condition, and microarray technology is now the most powerful tool for functional genomics. Several attempts to clarify the pathogenesis of bronchial asthma have been carried out using microarray technology, providing us some novel pathogenic mechanisms of bronchial asthma as well as the information of gene expression profiling. In this article, we review the outcomes of these analyses by the microarray approach as applied to bronchial asthma.

INTRODUCTION

The incidence of allergic diseases has dramatically increased in recent decades, particularly in developed 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 [Mao 2000]. The medical cost for treating such patients is huge and on the increase. Thus, it is important socially as well as medically to clarify the pathogenesis of allergic diseases and to establish more useful strategies to overcome allergic disorders. Recent advances in the technology of functional genomics, such as massive parallel gene expression profiling using microarrays, are revolutionizing, the approaches to these complex scientific questions [Butte 2002, Churchill 2002, Xiang 2003]. Several trials to dissect the pathogenesis of bronchial asthma using microarray technology have been performed, providing some novel pathogenic mechanisms of bronchial asthma as well as the information of gene expression profiling. The information of gene expression profiling would be relevant for finding drug targets or biomarkers for bronchial asthma. This article describes the pathogenesis of bronchial asthma; some fundamental aspects of functional genomics, particularly validation and design of the microarray analysis; and lastly, the outcomes of microarray analyses applied to bronchial asthma.

PATHOGENESIS OF BRONCHIAL ASTHMA

Bronchial asthma is a complicated and diverse disorder affected by genetic and environmental factors; however, it is widely accepted that it is a Th2-type inflammation originating in lung caused by inhalation of ubiquitous allergens [Umetsu 2003, Wills-Karp 2001]. High expression of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 in the lesions is the cardinal feature of bronchial asthma [Bodey 1999, Huang 1995, Humbert 1997, Kotsimbos 1996, Robinson 1992]. Inhalation of allergens results in infiltration of Th2 cells, mast cells, and eosinophils into asthmatic airways, and Th2 cytokines together with other mediators released from these cells generate asthmatic phenotypes such as mucous hypersecretion, infiltration of inflammatory cells, and airway hyperresponsiveness (AHR) (Fig. 1). It is thought that a combination of genetic and environmental factors contributes to activation of Th2-type immune responses [Holgate 1999]. The hygiene hypothesis, described below, may explain how Th2-type immune responses are activated by allergens and why the incidence of asthma is increasing in developed countries [Umetsu 2002, Wills-Karp 2001, Yazdanbakhsh 2002]. This hypothesis suggests that the improvement of hygiene–including low exposure to bacteria, vaccination, and high use of antibiotics–has developed allergic diseases. This hypothesis was explained earlier by the notion that insufficient activation of Th1 cells, which are normally stimulated by infection, cannot counterbalance the expansion of Th2 cells, resulting in the development of allergic diseases. However, recently, another explanation is preferred: insufficient induction of regulatory T cells by high hygiene status leads to the failure to suppress activation of Th2 cells [Umetsu 2002, Wills-Karp 2001, Yazdanbakhsh 2002].

FUNDAMENTAL ASPECTS OF FUNCTIONAL GENOMICS

Functional genomics is defined as the analysis of whole gene expression of a cell, tissue, or organ under given conditions [Butte 2002, Joos 2003]. The most common tools used to carry out gene expression include complementary DNA (cDNA) microarrays, oligonucleotide microarrays, and serial analysis of gene expression (SAGE) [Butte 2002]. Particularly, microarray technoiogy is now the most powerful tool for functional genomics. Oligonucleotide microarrays provide direct information about mRNA expression levels [Yang 2002]. By contrast, the data of cDNA microarrays are relative. The findings of functional genomics can be applied to various uses: (1) Identification of drug targets correlating with the pathogenesis of diseases. (2) Biomarker determination to find genes correlated with

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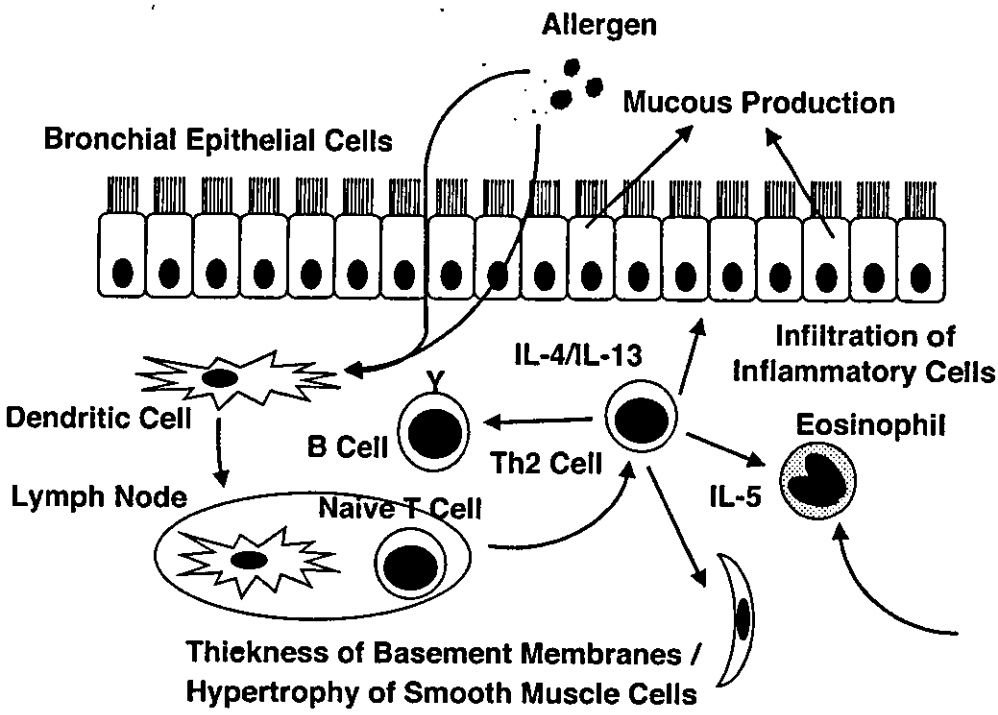

Fig. (1). Pathogenic mechanism of bronchial asthma. Pathogenic mechanism of bronchial asthma is depicted. Invaded allergen is recognized by dendritic cells, which induce Th2 cells. Secreted cytokines from Th2 cells generate asthma phenotypes, such as mucous production, infiltration of inflammatory cells, thickness of basement membranes, and hypertrophy of smooth muscle cells.

disease-subclass determination, disease stage, or prognosis. (3) Drug discovery process for assessment of drug actions and patient responses [Butte 2002, Petricoin 2002]. Microarray technology has been applied to various diseases such as malignancies, neurological disorders, and cardiovascular diseases as well as bronchial asthma [Guo 2003, Marcotte 2003, Napoli 2003].

Although microarray technology is powerful and relevant, we need to improve the accuracy and precision of each analysis [Moreau 2003]. Many factors affecting the accuracy and precision of microarray analysis can be categorized into three groups: (1) Biological variation intrinsic to each cell, tissue, or organ and influenced by genetic or environmental factors. (2) Technical variation introduced during the extraction, labeling, and hybridization of samples. (3) Measurement error associated with reading fluorescent signals [Churchill 2002]. The problems of these variations could be resolved, at least, in part, by the following three types of replications: (1) Biological replication, an experiment replicated by producing a new biological sample. (2) Technical replication, an experiment replicated by producing a new sample of labeled nucleic acids from the same biological samples. (3) Technical repetition, hybridization of the same labeled sample to another array [Moreau 2003]. However, these replications have limitations to improve the accuracy and precision. Particularly, biological variation is an inevitable factor, irrespective of the handling or the assay system. This problem is apparent from the result that the correlation between samples obtained from individual inbred mice will be as low as 30% [Churchill 2002]. To confirm microarray data and to make it more useful for the researchers' primary purposes, it is important to validate the data and design the experiments well.

VALIDATION AND DESIGN OF MICROARRAY ANALYSIS

To confirm microarray data, particularly derived from oligonucleotide microarrays, there are two ways of validation, *in silico* analysis and laboratory-based analysis [Chuaqui 2002]. *In silico* analysis means comparison of the microarray data with information available in the literature and databases. Laboratory-based analysis indicates independent experimental analysis of gene-expression using other methods. For the analysis of expression profiling at mRNA level, semi-quantitative reverse transcription PCR (RT-PCR), real-time RT-PCR, Northern blot, ribonuclease protection assay, or *in situ* hybridization can be used [Chuaqui 2002]. Particularly, the real-time RT-PCR method is superior in quantification of the samples and rapidity of the analysis, once it is established. It has been reported that the majority of array results were highly correlated with the results using real-time RT-PCR [Chuaqui 2002, Petricoin 2002]. On the other hand, the expression of approximately 7% of genes appeared significantly altered in opposite directions using the two methods [Petricoin 2002], suggesting that a careful examination of the array data based on real-time RT-PCR is important. Following analysis at the mRNA level, analysis at the protein level using immunoblot or immunohistochemistry would be relevant for validation [Chuaqui 2002]. The coincidence ratio between transcription level and protein expression is unclear; however, it is estimated at less than 50% [Chuaqui 2002]. For example, it

pathogenesis of bronchial asthma [Corry 1999, Izuhara 2003, Wills-Karp 2003]. Epithelial overexpression of an IL-13 transgene or administration of IL-13 in mice has shown that IL-13 induces asthma-like phenotypes independent of lymphocytes [Grünig 1998, Wills-Karp 1998, Zhu 1999]. Particularly, the IL-13 action on epithelial cells is crucial for generation of AHR and mucous production [Kuperman 2002]. Furthermore, in human, the *IL13* variant Gln110Arg is genetically associated with bronchial asthma [Arima 2002, Heinzmann 2000]. Sheppard's group listed a variety of genes induced by IL-13 in bronchial epithelial cells, smooth muscle cells, and lung fibroblasts, among which expression of some genes was validated by real-time RT-PCR, and so was expression of MCP-1 and a proinflammatory cytokine, IL-6, in fibroblasts by ELISA [Lee 2001]. MCP-1 is a chemokine recruiting lymphocytes, basophils, macrophages, and dendritic cells [Rothenberg 1999]. Very few genes overlapped in expression profiling of three kinds of cells [Sheppard 2002].

On the other hand, we identified the genes induced by either IL-13 or IL-4, another Th2-type cytokine sharing the receptor with IL-13, by the microarray approach, and we validated expression of 12 genes among the identified genes induced by both IL-13 and IL-4 by real-time RT-PCR [Yuyama 2002]. We furthermore compared expression of 12 genes with the expression profiling of the samples derived from bronchial biopsies from atopic asthma patients, finding that expression of four genes–*SERPINB3*, *SERPINB4*, *KAL-1*, and *MAP17*–was up-regulated or present in the asthma samples. We furthermore validated the expression of *SERPINB3* and *SERPINB4* at their protein levels by ELISA and immunostaining [Yuyama 2002]. We found that the squamous cell carcinoma antigen 2 (SCCA2) coded by the *SERPINB4* gene, a member of the ovalubumin serpin family, inhibited the cysteine proteinase activity of a major mite allergen, Der p 1 [Sakata 2004]. These results indicated that SCCA2 produced by IL-13 or IL-4 in bronchial epithelial cells has a protective role against an extrinsic proteinase activity. Among 12 genes, induction of IL-13Rα2 by IL-13 or IL-4 was confirmed by immunostaining [Yasunaga 2003]. It has been shown that IL-13Rα2 acts as a decoy receptor, inhibiting the IL-13 signal [Arima 2002, Bernard 2001, Chiaramonte 2003, Kawakami 2001, Wood 2003, Yasunaga 2003], indicating that there is a negative feed-back regulation for the IL-13 signal in bronchial epithelial cells by induction of IL-13Rα2.

2. Inducible Genes in Activated Human Mast Cells

Using microarray technology, Cho and collaborators tried to identify inducible genes in a human mast cell line, HMC-1, activated by phorbol ester and calcium ionophore, finding that expression of the plasminogen activator inhibitor type-1 (PAI-1) was up-regulated [Cho 2000]. They confirmed by ELISA that activated HMC-1 cells and primary cultured human mast cells secreted PAI-1. PAI-1 inhibits the plasminogen activator converting plasminogen to plasmin, which enhances proteolytic degradation of the extracellular matrix. These results indicated that activated mast cells could play an important role in airway remodeling by secreting PAI-1.

3. Inducible Genes in Lung Tissues Derived from Ovalubumin- or *Aspergillus*-inducible Asthmatic Mice

Zimmermann and collaborators tried to identify inducible genes in the lung tissues derived from ovalubumin- or *Aspergillus*-inducible asthmatic mice [Zimmermann 2003]. Ovalubumin and *Aspergillus* induced 496 and 527 genes, respectively, among 12,422 genes, and only 291 genes overlapped among the identified genes, indicating that there exist pathogenic differences related to the nature of the allergen and/or the immunization route. Among the overlapping genes, expression of arginase I, arginase II, and cationic amino acid transporter 2 (CAT) was significantly augmented. These three molecules are involved in uptake and metabolism of arginine. The precise role of these molecules in the pathogenesis of bronchial asthma is unclear. However, because arginine is metabolized to nitric oxide (NO) by NO synthase, induction of arginase I, arginase II, and CAT may affect the NO synthesis. Alternatively, induction of these molecules may enhance collagen synthesis. Furthermore, it turned out that the cells predominantly expressing arginase are macrophages, and that expression of arginase I and arginase II is enhanced by IL-4 and IL-13, abundantly expressed in the lung tissues of asthmatic mice. These results are consistent with the microarray data demonstrating that thioglycolate-elicited macrophages stimulated by IL-4 showed up-regulation of expression of arginase as well as Ym1 [Welch 2002].

4. A Susceptibility Factor to Allergen-induced AHR

It is known that A/J mice and C3H/HeJ mice are highly susceptible and highly resistant to allergen-inducing AHR, respectively. Karp and collaborators designed a unique experiment using microarray technology to try to identify a susceptibility factor to dissect this difference [Karp 2000]. They found that 227 genes exhibited great change in expression among 7350 genes, and paid an attention to C5 among the listed genes, because *C5* was situated near a locus correlated with allergen-induced bronchial hyperresponsiveness. It turned out that A/J mice, but not C3H/HeJ mice, have a 2-bp deletion in a 5' exon of the *C5* gene that renders them deficient in C5 mRNA and protein production. Furthermore, they found that C5 induced IL-12 production in monocytes. These results indicated that C5 is involved in determining susceptibility to bronchial asthma by inducing IL-12 production, which counterbalances the Th2-type immune responses.

5. *Ascaris*– or IL-4–inducible Genes in Lung Tissues Derived from Asthmatic Monkeys

Zou *et al.* tried to identify *Ascaris*– or IL-4–inducible genes in lung tissues derived from asthmatic monkeys by the microarray approach [Zou 2002]. They found that 169 cDNAs among 40,000 changed their expression levels in either pair-wise comparison. Expression of some listed genes was validated by real-time RT-PCR, and several chemokines–such as MCP-1, MCP-3, and eotaxin–showed significant changes in expression. MCP-1, MCP-3, and eotaxin are known to recruit Th2 cells, eosinophils, basophils, macrophages, and dendritic cells [Homey 1999,

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Characterization of novel squamous cell carcinoma antigen-related molecules in mice

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Abstract

The squamous cell carcinoma antigen 1 (SCCA1) and SCCA2 are unique serpins that can inhibit cysteine proteinases. SQN-5, their mouse ortholog, has already been identified, and its inhibitory property has been characterized; however, its biological role has remained undefined. Furthermore, no other mouse homolog of SQN-5 has been known. We characterize three mouse members of SCCA-related molecules including SQN-5 in this article. Serpinb3a (SQN-5) and Serpinb3b, but not Serpinb3c, were functional, inhibiting both serine and cysteine proteinases with different inhibitory profiles due to the difference of two amino acids in their reactive site loops. Serpinb3a was ubiquitously expressed in most tissues, whereas expression of Serpinb3b was limited to keratinocytes. Keratinocytes secreted both SCCA-related proteins, Serpinb3a and Serpinb3b. These results indicate that Serpinb3a and Serpinb3b may play different roles by inhibiting intrinsic or extrinsic proteinases with different expression distributions and different inhibitory profiles.

Keywords: Serpin; Proteinase; SCCA1; SCCA2; SQN-5; Serpinb3a; Serpinb3b; Keratinocyte; Reactive site loop

The squamous cell carcinoma antigen 1 (SCCA1: SERPINB3) and SCCA2 (SERPINB4) belong to the ovalbumin-serpin (serine proteinase inhibitors) family, and these proteins are 91% identical at the amino acid level [1]. Both genes locate very closely at human chromosome 18q21.3, suggesting that either gene could arise from the other by gene duplication [2]. Target proteinases for most serpins are the chymotrypsin family of serine proteinases, so a serpin inhibiting cysteine proteinase is defined as a cross-class serpin. Both SCCA1 and SCCA2 inhibit cysteine proteinases such as cathepsin K, L, and S, or Der p 1 and Der f 1, respectively [3–5]. Therefore, SCCA1 and SCCA2 are defined as cross-class serpins, indicating their unique properties.

The biological roles of SCCA1 and SCCA2 remain obscure. It is thought that SCCA1 and SCCA2 play a role in limiting injury by inhibiting lysosomal cysteine proteinases such as cathepsin K, L, and S, or a cationic neutral serine proteinase synthesized by neutrophils and mast cells, cathepsin G [1,4]. We recently found that expression of SCCA1 and SCCA2 was upregulated by two Th2-type cytokines, IL-4 and IL-13, in human bronchial epithelial cells (HBECs) and keratinocytes [6] (K. Mitsuishi et al., unpublished data).

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We furthermore demonstrated that SCCA2 inhibited the cysteine proteinase activities of Der p 1 and Der f 1, group I allergens derived from house dust mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively [5]. Taking these results together, we assume that SCCA1 and SCCA2 may protect against extrinsic proteinases derived from microbes or parasites involved in the defense mechanism of IL-4 and IL-13.

In contrast to SCCA1 and SCCA2, only one mouse SCCA-related molecule, SQN-5, now denoted as Serpinb3a, has been identified [7,8]. Serpinb3a was 60% and 61% identical with SCCA1 and SCCA2, respectively, and it inhibited both cysteine proteinases such as cathepsin S, K, L, and V, and serine proteinases such as human mast cell chymase (HMC) and cathepsin G. These findings suggested that Serpinb3a had mixed properties of SCCA1 and SCCA2, and that Serpinb3a was also a cross-class serpin. It was reported that Serpinb3a was broadly expressed in most organs at the mRNA level [7]. However, the biological roles of Serpinb3a remained undefined. Furthermore, no other mouse homolog of Serpinb3a was known.

To address these questions, we tried to identify novel mouse homologs of Serpinb3a in silico. It turned out that there existed two novel homologous cDNAs, and we analyzed the inhibitory properties as serpins of their deduced proteins, the expression profiles of these genes, and their induction by IL-4 and IL-13. During the preparation of this manuscript, Askew et al. [9] presented identification of novel genes homologous to Serpinb3a. In this article, we named our three molecules following their nomenclature.

Materials and methods

Materials. Papain, cathepsin G, cathepsin L, and HMC were purchased from Sigma (St. Louis, MO), Calbiochem (San Diego, CA), Athens Research and Technology (Athens, GA), and Cortex Biochem (San Leandro, CA), respectively.

Generation of the proteins of the Serpinb3 members and the Der p 1 protein. Serpinb3a cDNA was prepared by PCR using cDNA derived from mouse lung according to a previous report [8]. *Serpinb3b* and *Serpinb3c* cDNAs were prepared from two RIKEN FANTOM clones, AK003220 and AK003650, from K.K. Danaform (Ibaraki, Japan). These three cDNAs were incorporated into pGEX(-KG)-4T (Amersham Biosciences, Piscataway, NJ). Mutants of Serpinb3a and Serpin b3b were generated by oligonucleotide-directed mutagenesis using two complemented primers with mutations, as previously described [5,10]. Purification of glutathione *S* transferase (GST)-fused proteins of wild and mutated types of the Serpinb3 members was performed as described before [10].

Recombinant Der p 1 protein was generated as described before [11]. Der p 1-N52Q, in which Asn52 was replaced with Gln, diminishing the N-glycosylation site, was used in all experiments.

Enzyme assays. Enzyme assays were performed as described previously [5,10]. The substrates used for enzyme assays of papain, cathepsin L, and Der p 1 were benzoyl-Arg-7-amino-4-methylcoumarin (Bz-R-MCA), benzyloxycarbonyl-Phe-Arg-methylcoumarin (Z-FR-MCA), and butyloxycarbonyl-Gln-Arg-methylcoumarin (Boc-QAR-MCA), respectively. Succinyl-Ala-Ala-Pro-Phe-methylcoumarin (Suc-AAPF-MCA) was used for cathepsin G and HMC. All were purchased from Peptide Institute.

Generation of anti-Serpinb3a serum. Purified GST-fused Serpinb3a protein was used together with complete Freund's adjuvant (Sigma) for immunization of New Zealand White rabbits. Anti-Serpinb3a serum was cross-reacted with Serpinb3b and Serpinb3c.

Reverse transcription-PCR. Total RNA was extracted from each mouse organ by ISOGEN (Nippongene, Tokyo, Japan), followed by the phenol:chloroform extraction. The reverse transcription (RT) reaction primed with random hexamer was performed using a GeneAmp RNA PCR Kit (Applied Biosystems Japan, Tokyo, Japan). The PCR was performed with cDNA as a template using the indicated primers after an initial 5 min denaturation at 95 °C, followed by the indicated cycles of 95 °C for 1 min, the indicated annealing temperature for 1 min, and 72 °C for 1 min. The cycles used were 40, 40, and 23, for Serpinb3a, Serpinb3b, and GAPDH, respectively. The annealing temperatures used were 60, 56, and 55 °C for Serpinb3a, Serpinb3b, and GAPDH, respectively. The PCR primers used were 5′-CATTTGTTTGCTGAAGCCACTAC-3′ and 5′-CATGTTCGAAATCCAGTGATTCC-3′ for Serpinb3a, 5′-ATTCGTTTTCATGCAGCTGATGT-3′ and 5′-GAAAGCTGAAGTTAAATTTGTTTCG-3′ for Serpinb3b, and 5′-GCACCACCAACTGCTTAGCC-3′, and 5′-GATGCAGGGATGATGTTCTGG-3′ for GAPDH, respectively.

Culture of mouse primary keratinocytes. Mouse keratinocytes were obtained and cultured by modifying the previous method [12]. Back skin removed from 2-day newborn C57/BL6 mice or tail skin removed from adult C57/BL6 mice was washed with Modified Eagle's Medium (MEM, Invitrogen, Carlsbad, CA) and cut down. Then the skin was incubated with MEM containing 2 U/ml dispase (Invitrogen), 100 U/ml penicillin G (BANYU Pharmaceutical, Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), and 250 ng/ml amphotericin B (Invitrogen) at 4 °C for 10 h. After the skin was washed by MEM, epidermis and dermis were peeled from it. Keratinocytes were obtained from the epidermis incubated with 0.25% trypsin and 0.02% EDTA in PBS at 37 °C for several minutes, followed by filtration. Fibroblasts were obtained from the dermis cultured with F-12 nutrient mixture (Ham's F-12, Invitrogen) containing 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Keratinocytes were cultured by a double-dish culture system. In this system, mouse fibroblasts were cultured as a feeder layer on the bottom of the outer dish, and keratinocytes were cultured with F-12 Nutrient Mixture containing 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin, on type I collagen gel (Nitta Gelatin, Osaka, Japan), on the bottom of the inner dish.

Immunostaining. Mouse keratinocytes were fixed on slides by 4% paraformaldehyde (Wako, Osaka, Japan). The sections were pretreated with 0.3% Triton X-100 (Wako) at 4 °C for 5 min. The sections were probed with 50-fold diluted anti-Serpinb3a serum, followed by incubation with FITC-labeled gout anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). The section was mounted with VECTASHIELD H-100 (Vector Laboratories, Burlingame, CA) and the localization of SCCA-related protein was analyzed by Carl Zeiss Axiophoto (Carl Zeiss, Oberkochen, Germany).

Transfection. HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Transient expression of Serpinb3a or Serpinb3b into HEK293T was performed by TransFast transfection reagent (Promega, Madison, WI).

Immunoprecipitation and Western blotting. Procedures of immunoprecipitation and Western blotting were carried out as previously described [13]. The proteins were immunoprecipitated from the culture medium of mouse primary keratinocytes from C57/BL6 mice by anti-Serpinb3a serum. The immunoprecipitates were applied to SDS–PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blotted by 100-fold diluted anti-Serpinb3a serum.

The proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Results and discussion

Localization and organization of the genes of Serpinb3 members

We first surveyed the SQN-5 homolog by BLAST search, and found that two novel cDNAs homologous with SQN-5 were inserted into two RIKEN FANTOM clones, AK003220 and AK003650. The homologies of the deduced amino acids of AK003220 and AK003650 with SQN-5 were 87% and 82%, respectively. Askew et al. have very recently demonstrated that there exist four Serpinb3 members including SQN-5, two novel homologs that we had found, and another homolog that we had not recognized, named Serpinb3a, b, c, and d, respectively [9]. The localization and organization of these four genes were verified by a survey using BLAST search. The genes of *Serpinb3a*, *d*, *b*, and *c* were localized sequentially in this turn at 60 cM on chromosome 1 (Fig. 1A). The locations of *Serpinb3c* and *Serpinb3a* genes were adjacent to *Serpinb7* and *Serpinb5* genes, respectively, and together with *Serpinb8* and *Serpinb2* genes, these genes made a cluster of Serpin clade b genes. All *Serpinb3b*, *Serpinb3c*, and *Serpinb3c* genes were composed of 8 exons, as was the *Serpinb3a* gene [7] (Fig. 1B). These results indicated that the four genes encoding Serpinb3 members would have arisen by gene multiplication, as the two genes encoding SERPINB3 and SERPINB4 are assumed to be evoked by gene duplication.

Functional analyses of Serpinb3 members

To perform functional analyses of Serpinb3 members, we expressed and purified recombinant proteins of GST-fused Serpinb3a, b, and c. The purity of these three proteins was greater than 95%, as estimated by Coomassie brilliant blue staining (data not shown). We examined the inhibitory effects of these three proteins on two cysteine proteinases, cathepsin L and Der p 1, and two serine proteinases, cathepsin G and HMC. Serpinb3a inhibited all proteinase activities, whereas Serpinb3b inhibited proteinase activities of cathepsin L and cathepsin G, but not Der p 1 and HMC (Fig. 2). Serpinb3c did not inhibit any activity of these four proteinases. None of the three Serpinb3 members inhibited papain. These results suggested that both Serpinb3a and Serpinb3b were able to inhibit both serine and cysteine proteinases, and that Serpinb3a and Serpinb3b showed different inhibitory profiles. It meant that both Serpinb3a and Serpinb3b belonged to cross-class serpins as well as SCCA1 and SCCA2. Askew et al. [9] have reported that neither Serpinb3c nor Serpinb3d performed any inhibitory activity. Together with our present finding, there is no evidence thus far that Serpinb3c is a functional serpin.

Preference of amino acids in the reactive site loop sequences of Serpinb3a and Serpinb3b

The inhibitory mechanism of the serpin is well characterized [14]. The exposed reactive site loop (RSL) of the serpin is recognized by the proteinase, and a 'bait' peptide bond (P1–P1′) that mimics the normal substrate of the proteinase is attacked by the active serine residue of the proteinase. By either forming an acyl-enzyme intermediate linked by an oxy-ester bond or cleaved by the proteinase just as the substrate of the proteinase, the serpin inhibits the target proteinases. It is well known that different inhibitory profiles of SCCA1 and SCCA2 are due to the different amino acid sequences of their RSLs [5,10,15]. Although only two amino acids were different among the 13 amino acids of the RSL sequences of Serpinb3a and Serpinb3b (Table 1), it

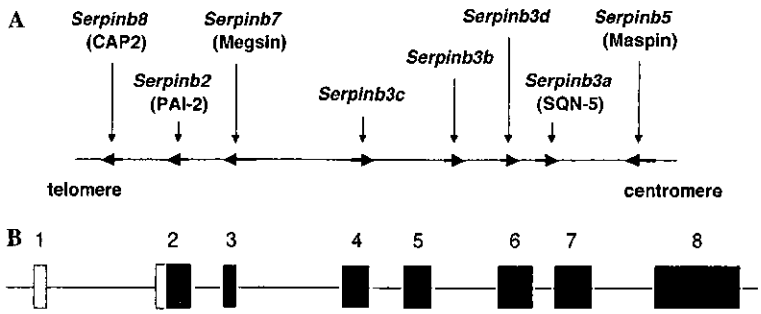

Fig. 1. Mapping and organization of the genes of the Serpinb3 family. (A) Mapping of the genes of the Serpinb3 family and the adjacent genes on chromosome 1 is depicted. CAP2 and PAI-2 represent cytoplasmic antiproteinase 2 and plasminogen activator inhibitor-2, respectively. (B) Organization of the exon/intron of the Serpinb3 family genes is shown. The exon numbers are depicted, and the closed boxes represent the portion of the open reading frame.

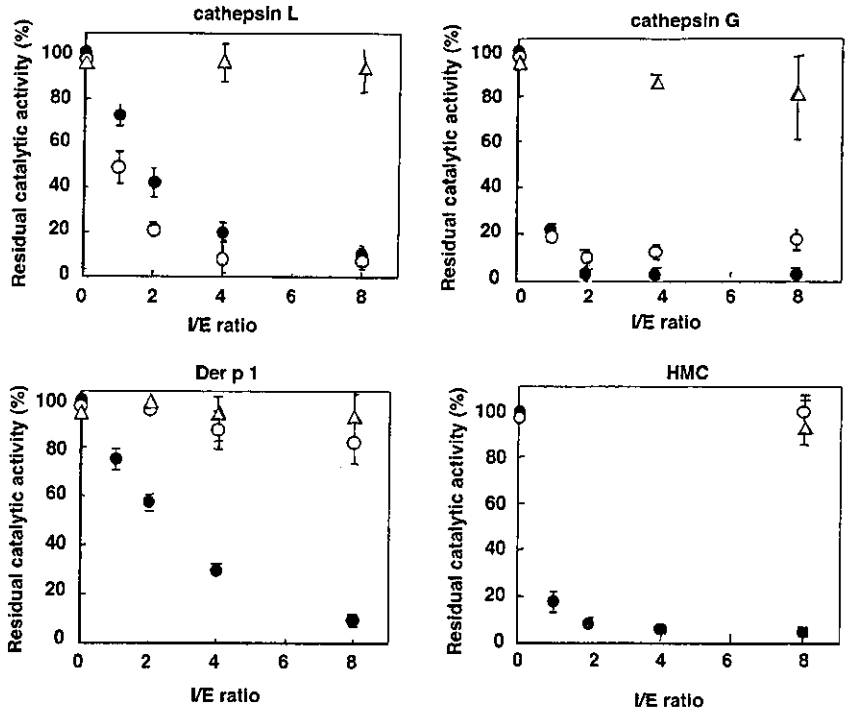

Fig. 2. Inhibitory activity of Serpinb3a, Serpinb3b, and Serpinb3c. Cathepsin L (5 nM), cathepsin G (40 nM), Der p 1 (10 nM), and HMC (40 nM) were incubated with either Serpinb3a (closed circles) or Serpinb3b (open circles) or Serpinb3c (open triangles) at the indicated I/E ratio. The residual enzyme activities are depicted.

Table 1
Alignment of RSLs of the mutants and their inhibitory activities

Position	Proximal hinge							Reactive site loop													Distal hinge	CatL	CatG	DerpI	HMC
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1′	2′	3′	4′	5′	6′					
Human																									
SCCA1	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	T	S	T	H	+++	–	–	–
SCCA2	.	V	.	.	.	.	.	.	.	.	.	V	V	E	L	.	S	P	.	.	C	–	+++	++	+++
Mouse																									
Serpinb3a	G	T	E	A	A	A	A	T	G	V	E	V	S	L	T	S	A	Q	I	A	C	+++	+++	++	+++
Serpinb3aL351V	.	.	.	.	.	.	.	.	.	.	.	.	.	V	T	.	.	.	.	.	.	ND	ND	++	+++
Serpinb3aT352R	.	.	.	.	.	.	.	.	.	.	.	.	.	L	R	.	.	.	.	.	.	ND	ND	+	+
Serpinb3b	.	.	.	.	.	.	.	.	.	.	.	.	.	V	R	.	.	.	.	.	.	+++	+++	–	–
Serpinb3bV351L	.	.	.	.	.	.	.	.	.	.	.	.	.	L	R	.	.	.	.	.	.	ND	ND	–	–
Serpinb3bR352T	.	.	.	.	.	.	.	.	.	.	.	.	.	V	T	.	.	.	.	.	.	ND	ND	+	+
Serpinb3c	.	.	.	.	D	P	.	S	.	E	.	.	I	L	R	L	.	.	V	.	R	–	–	–	–

Bold letter, mutated amino acid; ND, not determined.

was assumed that the different properties of Serpinb3a and Serpinb3b on HMC and Der p 1 were due to the difference of these two amino acids in their RSL sequences. To explore this possibility, we exchanged either Leu351 or Thr352 corresponding to Serpin b3a with Val or Arg corresponding to Serpinb3b, or vice versa, and analyzed their inhibitory effects on HMC and Der p 1 (Table 1). When Thr352 in Serpinb3a was replaced with Arg, the mutated type drastically decreased its inhibitory activity, whereas replacement of Leu351 with Val did not affect it. In contrast, when Arg352 in Serpinb3b was replaced with Thr, the mutated type partially recovered the inhibitory effect, whereas no recovery was observed when exchanging Val351 with Leu. These results demonstrated that Thr352 in the RSL of Serpinb3a was critical for its inhibitory activity on HMC and Der p 1.

Expression of Serpinb3a and Serpinb3b

It is known that SCCA1 and SCCA2 were co-expressed broadly in normal tissues: the epithelium of tongue, tonsil, esophagus, uterine cervix, vagina, and the

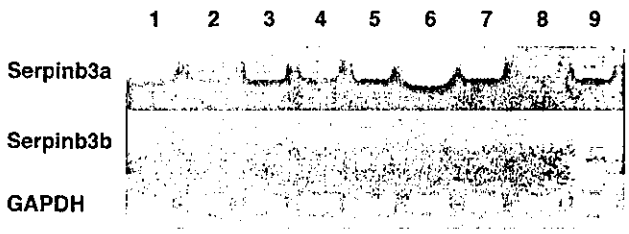

Fig. 3. Expression of Serpinb3a and Serpinb3b. Total RNA was extracted from each organ, and then RT-PCR for Serpinb3a, Serpinb3b, and GAPDH was performed. The numbers represent (1) liver, (2) kidney, (3) spleen, (4) heart, (5) lung, (6) thymus, (7) intestine, (8) muscle, and (9) keratinocytes.

conducting airways; Hassall's corpuscles of the thymus; and some areas of the skin [16]. It has been reported that Serpinb3a is also expressed broadly at the mRNA level [7]. We next analyzed the expression of Serpinb3a and Serpinb3b in each organ at the mRNA level. The homology between Serpinb3a and Serpinb3b was as high as 93%, so we developed a RT-PCR method that could distinguish expression of Serpinb3a and Serpinb3b (data not shown). When we analyzed liver, kidney, spleen, heart, lung, thymus, intestine, muscle, and keratinocytes, expression of Serpinb3a was observed in all investigated organs or cells, most strongly in thymus (Fig. 3). In contrast, among the examined samples Serpinb3b was expressed only in keratinocytes. These results demonstrated that although Serpinb3a and Serpinb3b were highly homologous, the transcriptional regulations of Serpnb3a and Serpinb3b were different.

Secretion of SCCA-related proteins in keratinocytes

We recently found that expression of SCCA1 and SCCA2 was upregulated in skin lesion of atopic dermatitis patients and that epidermal keratinocytes were the main source of SCCA production in skin lesions (K. Mitsuishi, unpublished data). The present finding that both Serpinb3a and Serpinb3b were co-expressed in keratinocytes at the mRNA level prompted us to investigate their protein production in mouse keratinocytes. When keratinocytes derived from infant and adult mice were cultured in vitro, keratinocytes showed positive staining for anti-Serpinb3a serum, but not pre-immune serum (Fig. 4A). We previously indicated the possibility that HBECs secreted the SCCA proteins [6]. To address whether mouse keratinocytes also secrete SCCA-related proteins, we analyzed the existence of SCCA-related proteins in the cultured medium of mouse keratinocytes (Fig. 4B). The mobility of Serpinb3a was slightly faster than that of Serpinb3b. A doublet-band corresponding to the sizes of Serpinb3a and Serpinb3b was detected in the cultured medium of mouse keratinocytes. These results suggested that mouse keratinocytes generated and secreted Serpinb3a and Serpinb3b proteins at almost equal levels.

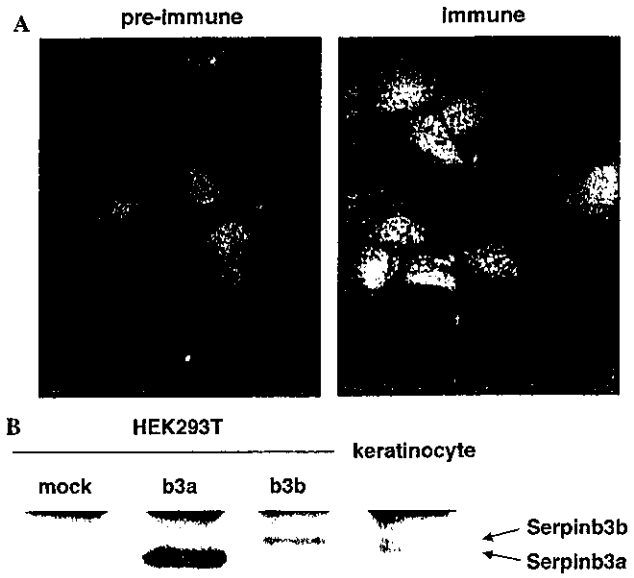

Fig. 4. Production and secretion of Serpinb3a and Serpinb3b from mouse keratinocytes. (A) Immunostaining of SCCA-related molecules in mouse keratinocytes by anti-Serpinb3a serum or pre-immune serum is shown. The fluorescence images are depicted. (B) Culture media were prepared from Serpinb3a- or Serpinb3b-transfected HEK293T cells or mouse keratinocytes, and then immunoprecipitates of anti-Serpinb3a serum were subjected to Western blotting using anti-Serpinb3a serum.

Recently, we showed that IL-4 and IL-13 had an ability to induce expression of SCCA1 and SCCA2 in human keratinocytes and HBECs [6] (K. Mitsuishi, unpublished data). Thus, we explored the possibility that expression of Serpinb3a or Serpinb3b was also augmented by IL-4 and IL-13 in mouse keratinocytes; however, IL-4 and IL-13 did not affect their expression (data not shown). These results demonstrated that expression mechanism of SCCA-related genes was different in mice and humans; *SERPINB3* and *SERPINB4* genes were induced by IL-4 and IL-13 in humans, whereas *Serpinb3* genes were constitutively expressed in mice. It may indicate that biological roles of IL-4 and IL-13 are different in mice and humans in the expression of proteinase inhibitors.

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