Review

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2005;137:319–325
DOI: 10.1159/000086464

Published online: June 20, 2005

Much Atopy about the Skin: Genome-Wide Molecular Analysis of Atopic Eczema

Hirohisa Saito

Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan

Key Words
Atopic eczema · Chemokines · Microarray · Transcriptome

Abstract
Background: Atopic eczema (AE) is a chronic inflammatory skin disorder with an increasing prevalence in industrialized countries. ***Methods:*** Genox Research Incorporation was founded in 1996 to identify new genes involved in allergic diseases in collaboration with the National Children's Hospital in Tokyo. In the AE project, they have discovered several hundred new genes and partial DNA sequences by mainly using microarrays. Here, I review the results obtained using transcriptome analysis, performed by Genox and other investigators. ***Results:*** Transcriptome analysis using skin lesion, CD4+ T cells, monocytes and eosinophils derived from AE patients identified some differentially expressed genes which became biologically relevant in the following studies. Missing linkages between these genes have been found due to the recent development of genomics. ***Conclusion:*** Many AE-related genes found in the genome-wide studies still remain to be determined regarding their functions and to be systemically organized. After the comprehensive characterization of these genes by further studies, we will identify the precise molecular mechanisms involved in AE and other diseases.

Introduction

Atopic eczema (AE) is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers [1, 2]. The diagnosis of AE is based on the following constellation of clinical findings: pruritus, facial and extensor eczema in infants and children, flexural eczema in adults and chronicity of the dermatitis [3]. AE usually presents during early infancy and childhood, but it can persist or start in adulthood [4]. The lifetime prevalence of AE is 10–20% in children and 1–3% in adults. Its prevalence has increased two- to threefold during the past three decades in industrialized countries. Various investigators reported that AE has a complex etiology, with an activation of multiple immunologic and inflammatory pathways [1, 2, 5]. The clinical phenotype that characterizes AE is the product of complex interactions among susceptibility genes, the host's environment, defects in skin barrier function and systemic and local immunologic responses. An understanding of the relative role of these factors in the pathogenesis of AE has been made possible by a variety of approaches, including the analysis of cellular and cytokine gene expression in AE skin lesions in humans as well as various AE model mice.

By a complete reading of the genome sequence, we will be able to determine the role of genomic DNA sequence variation among individuals, such as single nucleotide polymorphism, in the pathogenesis of diseases. The com-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/05/1374–0319$22.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Hirohisa Saito, MD, PhD
Department of Allergy and Immunology
National Research Institute for Child Health and Development
2-10-1 Okura, Setagaya-ku 157-8535 Tokyo (Japan)
Tel. +81 3 5494 7027, Fax +81 3 5494 7028, E-Mail hsaito@nch.go.jp

prehension of the genome will also accelerate an understanding of the transcriptome, evaluating all the transcripts present in a cell [6]. Messages induced by a new therapy, such as unexpected adverse effects, will not be missed by using such a comprehensive assay. Allergic diseases will be investigated by understanding the total functional elements comprising the immune system, not just a single molecule present in an immunocyte working in our immune system [7]. The current review will summarize the progress in our understanding of the molecular biology of AE, especially by focusing on the recent transcriptome analyses.

Differentially Expressed Genes in T Cells

The key role of immune effector T cells in AE is supported by the observation that individuals with primary T cell immunodeficiency disorders frequently have elevated serum IgE levels and eczematous skin lesions following successful bone marrow transplantation. Furthermore, in animal models of AE, the eczematous rash does not occur in the absence of T cells.

In AE, memory T cells expressing the skin-homing receptor, a cutaneous lymphocyte-associated antigen (CLA) [8], produce increased levels of Th2 cytokines. These include interleukin (IL)-4 and IL-13, which are known to induce isotype switching to IgE synthesis, as well as IL-5, which plays an important role in eosinophil development and survival. These CLA+ T cells produce abnormally low levels of interferon (IFN)γ, a Th1 cytokine known to inhibit Th2 cell function [1, 2, 9]. Genox Research Incorporation (Genox) was founded in 1996 to identify new genes involved in allergic diseases in collaboration with the National Children's Hospital (renamed in 2002 to National Center for Child Health and Development) in Tokyo. In the AE project, they first examined differentially expressed genes present in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells derived from severe AE patients. They also used quantitative methods such as real-time PCR to determine the expression levels of differentially expressed genes. At first, these investigators found markedly decreased PHA-induced IFNγ production in AE patients with hyper IgE (>10,000 IU/ml) who were insensitive to topical glucocorticoid therapy compared with those who were sensitive to the same treatment and with normal controls [10]. Using an oligonucleotide microarray (GeneChip, Affymetrix), IFNγ was found in the 4 most differentially expressed genes among 5,600 genes [11]. Since upregulation of transcriptional levels in crude cells is often simply caused by an increase in cell number of a certain cell type, they purified CD4+ T cells from AE patients and examined differentially expressed genes. A number of genes, including CC chemokine receptor (CCR)4, an IL-2-inducible T cell-specific tyrosine kinase, a heterodimer receptor for laminin, very late antigen 6 consisting of integrin β_1 and integrin α_6 molecules and a T cell-specific global gene regulator, as well as special AT-rich sequence-binding protein 1 were shown to be highly expressed in patients with moderate and/or severe AE in comparison with controls or patients with mild AE. Because the products of these upregulated genes influence chemotaxis, adhesion, migration and Th2 polarization, the investigators suggested that circulating T cells may function differently in severer AE [12]. In the same series of the experiment, they have found a strong correlation between a transcription factor, a suppressor of cytokine signaling (SOCS)3 expression and the clinical severity of AE, as well as serum IgE levels [13]. SOCS3 is a negative signal transducer specific for IL-12 as well as a signal transducer and activator of transcription 4 signaling, and thereby downregulates IFNγ production and Th1 immunity. SOCS3 was later found to be upregulated in an AE-like skin lesion in an AE model NC/Nga mouse [14]. IFNγ and IL-12 production by peripheral blood mononuclear cells increased significantly after successful treatment with topical glucocorticoids, while the production of IL-1, IL-4 and IL-13 was not significantly changed [15]. Therefore, according to the findings obtained by Genox's genomic analysis, downregulation of Th1 cytokines, but not upregulation of Th2 cytokines, seems to be an important marker for evaluating AE disease activity. However, in contrast to the above observation, Wohlfahrt et al. [16] used nylon membrane-based cDNA arrays to find several genes for IL-4, IL-5 and IL-13, macrophage-derived chemokine (MDC) CC chemokine ligand (CCL)22 and monocyte chemotactic protein (MCP)-4 (CCL13) markedly upregulated in freshly isolated and restimulated CD4+ T cells from patients with allergic asthma or AE compared with healthy subjects. They also found that fractalkine (CX3CL1), I-309 (CCL1) and IL-8 were expressed at a higher level in AE patients compared with other subjects. It would be interesting to determine the absolute expression levels of these genes among the same AE population by using more quantitative methods.

Differentially Expressed Genes in Monocytes and Eosinophils

The role of IL-4 and IL-13 in the pathogenesis of skin inflammation was recently addressed by treating NC/Nga mice with an IL-4/IL-13 receptor inhibitor, kept under conventional conditions. Surprisingly, treated mice displayed an increased eczema severity and IgE levels, suggesting a downmodulatory role for IL-4 and IL-13 in the skin inflammatory immune response [for a review, see ref. 17]. This raised the possibility that IL-4 and IL-13 may regulate the production of proinflammatory cytokines by monocytes/macrophages in AE etiology, as well as in autoimmune diseases [18]. Thus, the Genox group examined the transcriptome information in peripheral blood monocytes derived from AE patients. They found that genes for the Toll-like receptors (TLR)2, TLR4 and TLR6, a signal transducer for TLR stimulation called 'ATP-binding cassette transporter' (TAP2), CD36 and the γ-chain of the high-affinity IgE receptor Fcε receptor I (FcεRIγ) were markedly upregulated in monocytes derived from AE patients [19].

CD36 has been recently found to be a selective and nonredundant sensor of microbial diacylglycerides that signal via the TLR2/6 heterodimer [20]. TLR2 is essentially involved in the protection against *Staphylococcus aureus* [21], which often colonizes on the AE skin. Unlike other TLRs, TLR2 stimulation does not induce the expression of IFNα/β [22]. IFNα/β stimulates the production of IFNγ via the signal transducer and activator of transcription 4 in human T cells but not in mouse T cells [23]. A bacterial lipoprotein abundant in *S. aureus* can modulate allergen-specific Th2 effector cells in an allergic response in vivo for a prolonged period via stimulation of the TLR2 signaling pathway [24]. Taken together, monocytes derived from AE patients can induce strong inflammation by producing more proinflammatory cytokines including TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-18 [25] in response to *S. aureus* via upregulation of the TLR2 signaling pathway. Furthermore, antigen-presenting cells derived from these TLR2-upregulated monocytes are considered to be more sensitive to allergen challenge.

Interestingly, only TLR2 among all TLRs is upregulated in response to glucocorticoid treatment in respiratory epithelial cells. Homma et al. [26] claimed that the TLR2 upregulation results in the production of β-defensin, and thus works for protection from bacteria. It should be determined whether TLR2 is upregulated in AE skin and whether the upregulated TLR2 accelerates allergen sensitization. Regarding the upregulation of the FcεRIγ chain in AE monocytes, it is known that AE skin, compared with nonatopic skin, contains an increased number of IgE-bearing dendritic cells, which are sometimes derived from peripheral blood monocytes, expressing FcεRI. The increased expression of FcεRI on dendritic cells in atopic skin is due to the enhanced expression of the FcεRIγ chain and is preserved by increased IgE levels [27].

Next, the Genox investigators determined the transcriptome expression in eosinophils derived from AE patients. Several genes, such as those for cytokine receptors, e.g., GM-CSF receptor (GM-CSFR) α- and β-chain and IL-3 receptor (IL-3R) α-chain, CD44 and platelet-activating factor receptor were expressed at significantly higher levels in AE patients than in healthy volunteers [28]. It is known that eosinophils in inflammatory tissues are activated and express more GM-CSFR and IL-3R than peripheral blood eosinophils [29].

Differentially Expressed Genes in Skin Lesion

Upregulation of transcriptional levels in a crude tissue is often caused by an increase in recruitment of a certain inflammatory cell type. Indeed, Genox faced the difficulty in determining whether the upregulated genes in the skin are truly upregulated in the skin-dwelling cells or artifactually upregulated by the contamination of inflammatory cells. Nomura et al. [30] have succeeded in solving this problem in their microarray study on AE skin specimens by comparing the tissues derived from AE and psoriasis. They identified several genes for chemoattractant cytokines and chemokines, which play a central role in defining the nature of the inflammatory infiltrate in AE, to be differentially expressed. For example, cutaneous T cell-attracting chemokine (CCL27) is highly upregulated in AE and preferentially attracts CLA+ T cells into the skin. Compared with psoriasis, several CC chemokines such as I-309 (CCL1), RANTES (CCL5), MCP-4 (CCL13) and eotaxin (CCL11) are increased in AE skin lesions and likely contribute to the chemotaxis of CCR3-expressing eosinophils, macrophages and Th2 lymphocytes in AE skin. Selective recruitment of CCR4-expressing Th2 cells into AE skin may also be mediated by MDC (CCL22) and thymus and activation-regulated cytokine (CCL17), which are increased in AE. It would be concluded that skin-dwelling cells in AE patients preferentially produce several CC chemokines such as I-309 (CCL1), MCP-4 (CCL13) and MDC (CCL22). However, as reported by other investigators [16], purified CD4+ cells from AE pa-

tients preferentially express CC chemokines. Therefore, in a future study, it should be determined whether T cells or tissue-dwelling cells can predominantly produce these CC chemokines after obtaining highly purified cells from AE patients.

Persistent skin inflammation in chronic lesions may be due to elevated IL-5 and GM-CSF expression in the skin leading to enhanced survival of eosinophils, monocyte/macrophages and dendritic cells. In addition, extracellular matrix molecules deposited in chronic skin lesions have been found to enhance the survival of memory T cells [31]. When compared with normal skin or uninvolved skin of AE patients, acute skin lesions have a significantly greater number of IL-4, IL-5 and IL-13 mRNA-expressing cells, but few IFNγ or IL-12 mRNA-expressing cells. Chronic AE skin lesions have significantly fewer IL-4 and IL-13 mRNA-expressing cells, but a greater number of IL-5, GM-CSF, IL-12 and IFNγ mRNA-expressing cells than acute AE skin lesions. However, compared with psoriasis, AE skin produces more Th2 cell-attracting CC chemokines, even in the chronic skin lesion [31].

Epidermal keratinocytes from AE patients produce a unique profile of chemokines and cytokines following mechanical stimulation, e.g. scratching, or exposure to proinflammatory cytokines such as TNFα. Keratinocytes in AE are also reported to be deficient in their ability to synthesize antimicrobial peptides needed for innate immune responses against microbes, and thus, this allows the colonization of *S. aureus* on the skin [32]. The same authors reported that IL-13 is a key role cytokine downregulating the production of antimicrobial peptides by keratinocytes [33]. Interestingly, another group has recently identified that staphylococcal enterotoxin-B stimulated T cells to produce IL-13 [34].

Apoptosis-Related Genes

AE is usually thought to be an allergen-driven disease with prominent roles played by antigen-presenting cells and effector Th2 cells. But keratinocytes provide an important window to the environment and are also thought to contribute to the development of AE. Here, I introduce several genomic findings regarding the interaction between keratinocytes and T cells. Keratinocytes produce inflammatory mediators that promote or maintain allergic inflammation, exert apoptosis that is induced by activated T cells [35] and respond to staphylococcal toxins and various pathogen-associated molecular patterns bearing microbial agents [for a review, see ref. 1, 2]. Clearly, these findings have highlighted a more active role of the epithelium than was previously recognized. Indeed, therapeutic effects of topical glucocorticoid or FK506 are considered to be mediated by two different mechanisms, i.e. inducing apoptosis of T cells and eosinophils and blocking apoptosis of keratinocytes [36]. Genomic studies performed by Genox identified several known genes such as bcl-2 and bcl-xL [28] as well as newly found genes [37, 38] regulating apoptosis of T cells and eosinophils differentially regulated in AE patients.

Genetic Analysis of AE-Susceptible Genes

Many attempts have been made to identify the genes susceptible to the onset of AE. Since AE is an atopic disorder, candidate genes involving IgE and Th2 cytokines have been extensively examined. There has been particular focus on chromosome 5q31–33, as it contains a clustered family of Th2 cytokine genes, i.e. IL-3, IL-4, IL-5, IL-13 and GM-CSF [39]. Case-control comparisons have suggested a genotypic association between the IL-4 gene promoter region and AE, as patients with AE tend to have an increased IL-4 gene promoter activity. Similarly, this is the case with IL-13 coding region variants, a gain-of-function polymorphism in the α-subunit of the IL-4 receptor. These genes are also found among the candidate genes for 'atopy' asthma, since most of these studies included patients with elevated IgE levels. Genes unrelated to IgE or Th2 cytokines should also be involved in the pathogenesis of AE. NC/Nga mice undoubtedly represent the best characterized mouse model of AE currently available. Subcutaneous injection of TGF-β_1, a regulatory cytokine, into these mice led to significant clinical improvement of skin lesions. Additionally, another regulatory cytokine, the IL-10Rα gene, was found among a candidate locus of these mice by reciprocally crossing and subsequently backcrossing NC/Nga with BALB/c mice [for a review, see ref. 17]. Indeed, patients with IPEX syndrome lacking the FOXP3 gene responsible for regulatory T cells exert systemic skin eczema and hyper IgE as well [40].

Human genome screens have been performed and identified susceptibility loci for AE on chromosome 3q21 [41], which have not been linked to asthma candidate genes. This region encodes the costimulatory molecules CD80 and CD86 and may therefore modulate T cell responses. Another human genome screen reported a linkage of AE to loci on chromosomes 1q21, 17q25 and 20p. Interestingly, these same regions are known to contain

Table 1. Differentially expressed genes in the AE patients obtained by transcriptome analyses

Transcripts[a]	CD4+ T cells	Monocytes	Eosinophils	Skin
IL-4, IL-13	↑ or →	→	→	↑ (cell number)
IL-12	→	↓	→	↑ (chronic AE)
IFNγ	↓ (severe AE)	→	→	↑ (chronic AE)
IL-3R	→	→	↑	→
GM-CSFR	→	→	↑	→
CCL1[b]	↑	→	→	↑
CCL5	→	→	→	↑
CCL11	→	→	→	↑
CCL13	↑	→	→	↑
CCL17	→	→	→	↑
CCL22	↑	→	→	↑
CCL27	→	→	→	↑
CCR4	↑	→	→	↑ (cell number)
TLR2, TLR6	→	↑	→	→
TLR4	→	↑	→	→
CD36	→	↑	→	→
TAP2	→	↑	→	→
SOCS3	↑	→	→	↑
VLA6	↑	→	→	→
ITK	↑	→	→	→
SATB1	↑ (moderate AE)	→	→	→

VLA = Very late antigen; ITK = inducible T cell-specific tyrosine kinase; SATB1 = special AT-rich sequence-binding protein 1.

[a] Transcripts differentially expressed in skin lesion [30], peripheral blood monocytes [19], CD4+ T cells [10–13, 16] and eosinophils [28] derived from AE patients are summarized in this figure.

[b] CC chemokines: CCL1 = I-309; CCL5 = RANTES; CCL11 = eotaxin; CCL13 = MCP-4; CCL17 = TARC = thymus and activation-regulated cytokine; CCL22 = MDC; CCL27 = CTACK = cutaneous T cell-attracting chemokine.

psoriasis susceptibility genes, which suggests common candidate genes involved in the control of skin inflammation. Although AE and psoriasis are distinct skin diseases, both conditions involve dry, scaly skin and disrupted epidermal differentiation.

AE is characterized by dry skin and even involves nonlesional skin and increased transepidermal water loss. This impairment of the skin barrier function in AE leads to increased antigen absorption contributing to the cutaneous hyperreactivity characteristic of AE. In particular, ceramides serve as the major water-retaining molecules in the extracellular space of the cornified envelope, and the barrier function of these complex structures is provided by a matrix of structural proteins that are bound to ceramides [42, 43].

A Glu420Lys polymorphism variant in the SPINK5 gene, which encodes serine proteinase inhibitors, has shown a significant association with AE in two independent cohorts [44]. This gene is expressed in the outermost layers of the skin and has been implicated in Netherton disease, an autosomal recessive disorder characterized by ichthyosis and atopy. Recently, SPINK5–/– mice faithfully replicating key features of the Netherton syndrome were established. In these mice, abnormal desmosome cleavage is seen in the skin through degradation of desmoglein 1 due to stratum corneum tryptic enzyme and stratum corneum chymotryptic enzyme-like hyperactivity. This leads to defective stratum corneum adhesion and a resultant loss of the skin barrier function [45]. Although the human SPINK5 gene is located on chromosome 5q31–33, its function is primarily unrelated to IgE production and is involved in an AE-unique function, the skin barrier dysfunction.

Conclusion

AE-related genes identified by transcriptome analysis using clinical samples are summarized in table 1 and figure 1. Genome-wide transcriptome analysis is often per-

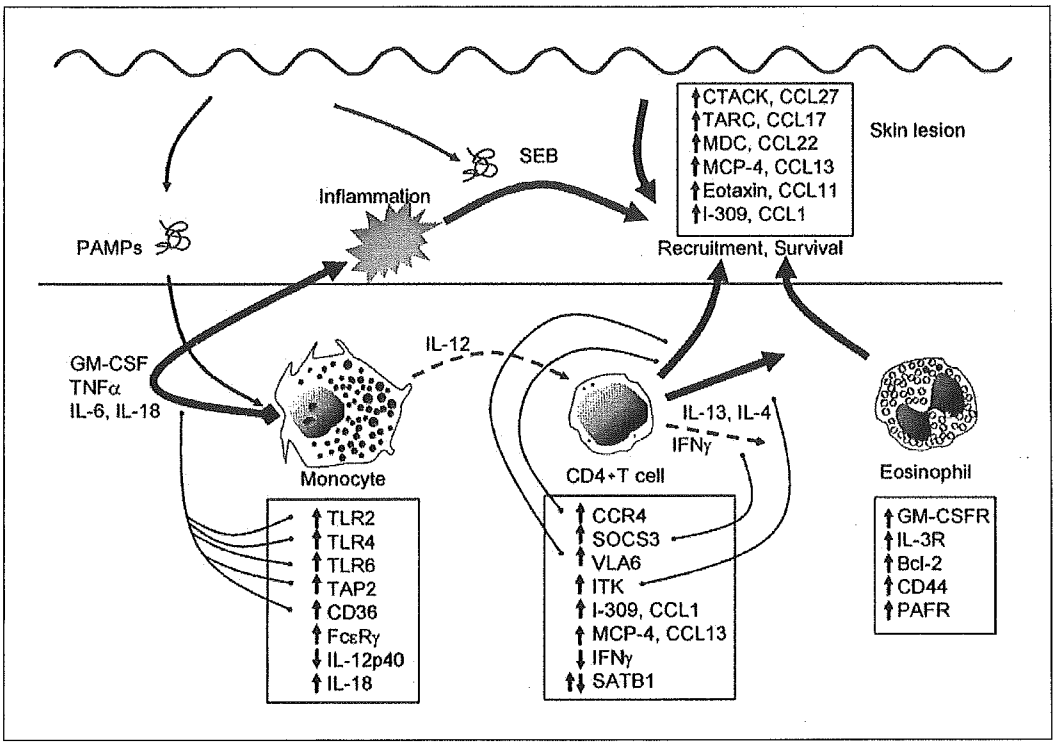

Fig. 1. Differentially expressed molecules in AE patients. Representative molecules which are differentially expressed in AE patients found in the transcriptome research are shown in the boxes. Transcripts differentially expressed in skin lesion [30], peripheral blood monocytes [19], CD4+ T cells [10–13, 16] and eosinophils [28] derived from AE patients are summarized. PAMPs = Pathogen-associated molecular patterns; SEB = staphylococcal enterotoxin-B; PAFR = platelet-activating factor receptor; explanations of other abbreviations are provided in table 1.

formed independent of hypothesis. Therefore, until a few years ago, such newly found genes sometimes seemed irrelevant to clinical aspects or even to biological significance. Some missing linkages between these genes have now been found and the biological importance of these genes is increasing due to the recent development of genomics. However, many AE-related genes found in these genome-wide studies still remain to be determined regarding their functions and to be systemically organized. As summarized in figure 1, immunological aspects of AE have been extensively investigated so far. Now, it appears necessary to focus on the role of skin lesion in AE patients as well as the interaction between skin and inflammatory cells. After comprehensive characterization of these genes by further studies, we will identify the precise molecular mechanisms involved in AE and other diseases.

References

1 Leung DY, Bieber T: Atopic dermatitis. Lancet 2003;361:151–160.
2 Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA: New insights into atopic dermatitis. J Clin Invest 2004;113:651–657.
3 Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. Acta Derm Venerol 1980; 92:44–47.
4 Spergel JM, Paller AS: Atopic dermatitis and the atopic march. J Allergy Clin Immunol 2003;112:S118–S127.
5 Novak N, Bieber T, Leung DYM: Immune mechanisms leading to atopic dermatitis. J Allergy Clin Immunol 2003;112:S128–S139.
6 Saito H: Translation of the human genome into clinical allergy. Allergol Int 2003;52:65–70.
7 Saito H: Translation of the human genome into clinical allergy. Part 2. Allergol Int 2004;53: 87–92.

8 Santamaria Babi LF, Picker LJ, Perez Soler MT, et al: Circulating allergen-reactive T cells from patients with atopic dermatitis and allergic contact dermatitis express the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen. J Exp Med 1995;181: 1935–1940.

9 Akdis M, Akdis CA, Weigl L, et al: Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated cytokine pattern: IgG4 counter-regulation by CLA-memory T cells. J Immunol 1997;159:4611–4619.

10 Katsunuma T, Kawahara H, Yuki K, et al: Impaired interferon-γ production in a subset population of severe atopic dermatitis. Int Arch Allergy Immunol 2004;134:240–247.

11 Heishi M, Kagaya S, Katsunuma T, et al: High-density oligonucleotide array analysis of mRNA transcripts in peripheral blood cells of severe atopic dermatitis patients. Int Arch Allergy Immunol 2002;129:57–66.

12 Matsumoto Y, Oshida T, Obayashi I, et al: Identification of highly expressed genes in peripheral blood T cells from patients with atopic dermatitis. Int Arch Allergy Immunol 2002; 129:327–340.

13 Seki Y, Inoue H, Nagata N, et al: Suppressor of cytokine signaling (SOCS)-3 regulates onset and maintenance of T(H)2-mediated allergic responses. Nat Med 2003;9:1047–1054.

14 Horiuchi Y, Bae SJ, Katayama I, Mori Y, Yamanishi K: Effects of ultraviolet-inactivated herpes simplex virus type I on atopic dermatitis-like lesions in NC/Nga mice: role of the suppressor of cytokine signaling in the skin. Allergol Int 2004;53:331–340.

15 Kaneko S, Furutani K, Koro O, et al: Transient shift toward T helper 1 cytokine production by peripheral blood mononuclear cells following successful treatment of patients with atopic dermatitis. Allergol Int 2003;52:21–29.

16 Wohlfahrt JG, Kunzmann S, Menz G, et al: T cell phenotype in allergic asthma and atopic dermatitis. Int Arch Allergy Immunol 2003; 131:272–282.

17 Gutermuth J, Ollert M, Ring J, et al: Mouse models of atopic eczema critically evaluated. Int Arch Allergy Immunol 2004;135:262–276.

18 Bessis N, Boissier MC, Ferrara P, et al: Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13. Eur J Immunol 1996;26: 2399–2403.

19 Nagata N, Oshida T, Yoshida NL, et al: Analysis of highly expressed genes in monocytes from atopic dermatitis patients. Int Arch Allergy Immunol 2003;132:156–167.

20 Hoebe K, Georgel P, Rutschmann S, et al: CD36 is a sensor of diacylglycerides. Nature 2005;433:523–527.

21 Yoshimura A, Lien E, Ingalls RR, et al: Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1–5.

22 Toshchakov V, Jones BW, Perera PY, et al: TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. Nat Immunol 2002;3:392–398

23 Rogge L, D'Ambrosio D, Biffi M, et al: The role of STAT4 in species-specific regulation of Th cell development by type I IFNs. J Immunol 1998;161:6567–6574.

24 Revets H, Pynaert G, Grooten J, et al: Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses. J Immunol 2005;174:1097–1103.

25 Ohnishi H, Kato Z, Watanabe M, et al: Interleukin-18 is associated with the severity of atopic dermatitis. Allergol Int 2003;52:123–130.

26 Homma T, Kato A, Hashimoto N, et al: Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. Am J Respir Cell Mol Biol 2004;31:463–469.

27 Novak N, Tepel C, Koch S, et al: Evidence for a differential expression of the FcεRIγ chain in dendritic cells of atopic and nonatopic donors. J Clin Invest 2003;111:1047–1056.

28 Ogawa K, Hashida R, Miyagawa M, et al: Analysis of gene expression in peripheral blood eosinophils from patients with atopic dermatitis and in vitro cytokine-stimulated blood eosinophils. Clin Exp Immunol 2003;131:436–445.

29 Gregory B, Kirchem A, Phipps S, et al: Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor α-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor α expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor α expression. J Immunol 2003;170: 5359–5366.

30 Nomura I, Gao B, Boguniewicz M, et al: Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. J Allergy Clin Immunol 2003;112:1195–1202.

31 Akdis M, Trautmann A, Klunker S, et al: T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. FASEB J 2003;17: 1026–1035.

32 Ong PY, Ohtake T, Brandt C, et al: Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 2002; 347:1151–1160.

33 Nomura I, Goleva E, Howell MD, et al: Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. J Immunol 2003;171: 3262–3269.

34 Lehmann HS, Heaton T, Mallon D, et al: Staphylococcal enterotoxin-B-mediated stimulation of interleukin-13 production as a potential aetiologic factor in eczema in infants. Int Arch Allergy Immunol 2004;135:306–312.

35 Trautmann A, Akdis M, Kleemann D, et al: T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. J Clin Invest 2000;106:25–35.

36 Trautmann A, Akdis M, Schmid-Grendelmeier P, et al: Targeting keratinocyte apoptosis in the treatment of atopic dermatitis and allergic contact dermatitis. J Allergy Clin Immunol 2001;108:839–846.

37 Imai Y, Nakada A, Hashida R, et al: Cloning and characterization of the highly expressed ETEA gene from blood cells of atopic dermatitis patients. Biochem Biophys Res Commun 2002;297:1282–1290.

38 Ogawa K, Itoh M, Miyagawa M, et al: Expression of a human SOCS protein, HSOCP-1, in peripheral blood eosinophils from patients with atopic dermatitis. Int Arch Allergy Immunol 2004;134:S2–S6.

39 Liu X, Beaty TH, Deindl P, et al: Associations between total serum IgE levels and the 6 potentially functional variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study. J Allergy Clin Immunol 2003;112:382–388.

40 Gambineri E, Torgerson TR, Ochs HD: Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. Curr Opin Rheumatol 2003; 15:430–435.

41 Cookson WO, Moffatt MF: The genetics of atopic dermatitis. Curr Opin Allergy Clin Immunol 2002;2:383–387.

42 Sator PG, Schmidt JB, Honigsmann H: Comparison of epidermal hydration and skin surface lipids in healthy individuals and in patients with atopic dermatitis. J Am Acad Dermatol 2003;48:352–358.

43 Melnik B, Hollmann J, Plewig G: Decreased stratum corneum ceramides in atopic individuals – A pathobiochemical factor in xerosis? Br J Dermatol 1988;119:547–549.

44 Kato A, Fukai K, Oiso N, et al: Association of SPINK5 gene polymorphisms with atopic dermatitis in the Japanese population. Br J Dermatol 2003;148:665–669.

45 Descargues P, Deraison C, Bonnart C, et al: SPINK5-deficient mice mimic Netherton syndrome through degradation of desmoglein 1 by epidermal protease hyperactivity. Nat Genet 2005;37:56–65.

Current perspectives

Allergy-related genes in microarray: An update review

Hirohisa Saito, MD, PhD, Jun Abe, MD, PhD, and Kenji Matsumoto, MD, PhD
Tokyo, Japan

Microarrays have attracted tremendous interest among biologists. However, questions have been raised regarding the reproducibility between experiments performed in different laboratories using different platforms. Here, we discuss these problems and reach the following conclusions. First, the reproducibility between different platforms of arrays is low, but bioinformatics may permit compensation at least among oligonucleotide microarrays. Second, it is hard to interpret microarray results generated using mixed cell populations. Hierarchical clustering may be applied to identify whether upregulated transcripts in an inflammatory tissue were caused simply by an increased number of inflammatory cells. (J Allergy Clin Immunol 2005;116:56-9.)

Key words: *Microarray, DNA chip, transcriptome, bioinformatics*

Microarrays, also called *genome arrays* or *DNA chips*, were lauded by former President Clinton in his State of the Union address on January 27, 1998, and have been expected to be a powerful research tool.[1] They have attracted tremendous interest among biologists. However, questions were raised in 2002 regarding the reproducibility of studies performed using microarrays.[2] Thus, a document was generated outlining the minimal information that should be reported about a microarray experiment to enable its unambiguous interpretation and reproduction when submitting a microarray study to most journals.[3]

Microarray technologies can be classified into 2 types. One consists of oligonucleotide microarrays that use synthesized oligonucleotide as probes, whereas the other consists of cDNA microarrays that use whole cDNA molecules with irregular lengths as probes. The microarray guidelines[3] aim mainly at reproducing the results obtained bay using cDNA microarrays handmade in individual laboratories. Compared with oligonucleotide microarrays, the results of cDNA microarray assays are less reproducible because both the target RNA and probe sizes are irregular, resulting in variable binding capacity.[4] However, questions were raised again regarding the reproducibility between results obtained even when using 3 different oligonucleotide microarray platforms.[5] Here, we discuss the pros and cons regarding microarray technology by focusing on studies related to allergy and asthma.

Abbreviation used
T7-primer: Oligo (dT) primer containing a T7 RNA polymerase promoter

PROS OF MICROARRAY

Most successful microarray-based results, especially in allergy-related topics, have been obtained in studies dealing with well-controlled animal model experiments. For example, Karp et al[6] obtained 8 new strains of mice by crossing A/J mice, which tend to have antigen-specific airway hypersensitivity, with C3H/HeJ mice. They used GeneChip (Affymetrix, Santa Clara, Calif), an oligonucleotide microarray platform, to examine >7000 genes expressed in the lungs after sensitization and challenge with allergens. They found that a genetic polymorphism of complement C5a, known to induce mast cell degranulation, was associated with the allergen-induced airway hypersensitivity. Zimmerman et al[7] created an asthma model by sensitizing Balb/c mice with ovalbumin and *Aspergillus fumigatus* and used GeneChip to analyze the lungs of these mice and controls after allergen challenge. As a result, they found 291 genes whose expression levels were commonly increased by challenge with both antigens. Among those genes, arginase I, arginase II, and cationic acid transporter 2 were found to be dramatically

From the Department of Allergy and Immunology, National Research Institute for Child Health and Development.
Disclosure of potential conflict of interest: H. Saito received grants/research support in part from the Pharmaceutical and Medical Devices Agency with the Ministry of Health, Labour and Welfare (Millennium Genome Project, MPJ-5) and is employed by the Japanese Ministry of Health, Labour and Welfare only. No commercial association.
Received for publication January 28, 2005; revised March 6, 2005; accepted for publication March 11, 2005.
Available online May 24, 2005.
Reprint requests: Hirohisa Saito, MD, PhD, Department of Allergy and Immunology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku 157-8535 Tokyo, Japan. E-mail: hsaito@nch.go.jp.
0091-6749/$30.00

doi:10.1016/j.jaci.2005.03.048

increased. They suggested the importance of these molecules in the pathology of asthma by showing that they were also highly expressed in human asthmatic airway epithelium. It should be noted that these 2 groups have confirmed their results to be highly reproducible by comparing 2 samples after allergen challenge from each experimental condition.

In a recent issue of the Journal, Wang et al[8] and Okumura et al[9] used GeneChip to analyze IgE receptor–activated mast cell transcripts *in vitro*. These 2 groups separately identified amphiregulin as a transcript that is markedly increased following aggregation of FcεRI. Amphiregulin induced tissue remodeling, ie, proliferation of lung fibroblasts and marked induction of *MUC5AC* transcripts in a human respiratory epithelial cell line. Both groups showed that amphiregulin-positive mast cells are increased in the airways of patients with asthma. These investigators employed culture-derived human mast cells for microarray as the first screening and confirmed the *in vivo* expression and function of this upregulated gene using human tissue samples. Thus, microarray experiments seem to be highly reproducible when well-controlled animal models or pure cells are used.

CONS OF MICROARRAY REPRODUCIBILITY BETWEEN DIFFERENT PLATFORMS

Because cDNA microarray analysis has many difficulties in yielding reproducible results among arrays made in different laboratories, we discuss the subsequent issues by focusing only on oligonucleotide microarray systems such as GeneChip.

In the GeneChip standard protocol, the mRNA contained in more than 5 μg total RNA is reverse-transcribed into cDNA and amplified using an oligo (dT) primer containing a T7 RNA polymerase promoter (T7-primer), followed by transcription into cRNA. In a newly developed small sample protocol, this amplification step using a T7-primer is repeated. Thus, only 50 ng total RNA is sufficient to reproduce the results with this protocol. The correlation between the 2 different protocols using the same sample is not very high ($r^2 < 0.7$). This is because the T7-primer binds to the 3′ flanking region of mRNA and sometimes fails to amplify a sufficient amount of 5′ sequences in the small-sample protocol. Microarray data are usually normalized against the mRNA concentration of certain housekeeping genes, total mRNA quantity, or other empirical baselines considered universal among samples. However, such relative measurements can not be applied beyond different sample protocols or different microarray platforms. To overcome this problem, the Toxicogenomics Projects at National Institute of Health Sciences[10] established a bioinformatics system called Percellome that will normalize the gene expression values on a per 1 cell basis. Briefly, they have established a system using internal standards and a compensation program for each transcriptional level. Once normalized, the data from all samples and studies can be expressed as a copy number per genomic DNA level. This system is primarily for use with the Affymetrix GeneChip but can be expanded to other platforms. However, degenerated RNA, found, for example, in human allergic clinical samples rich in eosinophils that contain RNAse-rich granules, has less 5′ sequence complementary RNA and causes irreproducibility even when using the same sample protocol. This is one of the problems facing clinical application of microarrays. Fresh RNA samples are definitely preferred whenever possible.

USE OF BIOINFORMATICS FOR INTERSPECIES COMPARISON

Comparison of the gene expression levels between different microarray platforms may be somewhat similar to interspecies comparison of orthologous genes. It is theoretically possible to compare orthologous genes by using a suitable correction formula and by using similar high-quality RNA from both species.

Animal disease models have long been used as surrogates for human diseases and have been informative. Controversy does exist, however, about the relevance of these models for allergic diseases such as asthma. Rodent mast cells are also commonly used experimental tools but are often different from their human counterparts in such things as their responses to certain cytokines and anti-allergic drugs.[11] Nakajima et al[12] have comparatively examined the genome-wide gene expression in human and mouse mast cells. The expression levels of several cytokine and chemokine genes (eg, *CCL5, IL-5, CSF1, TNF, SERPINE1, CCL4, IL-3*, and *CCL1*) were markedly upregulated both in human and mouse mast cells after stimulation via FcεRI. They concluded that the regulatory mechanisms of these genes are highly conserved between these species. To facilitate interspecies comparisons, an interspecies comparison database has recently been constructed online (bio.mki.co.jp/en/results/_notes/comparativeDB_index.html), which includes human and mouse mast cell transcriptomes for orthologous genes. Studies on the function of molecules that are highly expressed only in mouse cells must be interpreted carefully with regard to their relevance to potential function in humans. Another implication of the interspecies comparison database for gene expression is that we may be able to correct the information obtained for the mouse transcriptome so that it can be extrapolated to the human transcriptome by using the Percellome bioinformatics system. Mouse disease models are often still required to investigate the pathogenesis of human diseases. Interspecies comparison of functional genomics might then become particularly useful.

CONS OF MICROARRAY EXPERIMENTS USING MIXED CELL POPULATION

The gene expression levels obtained using GeneChip are usually expressed between 1 and approximately

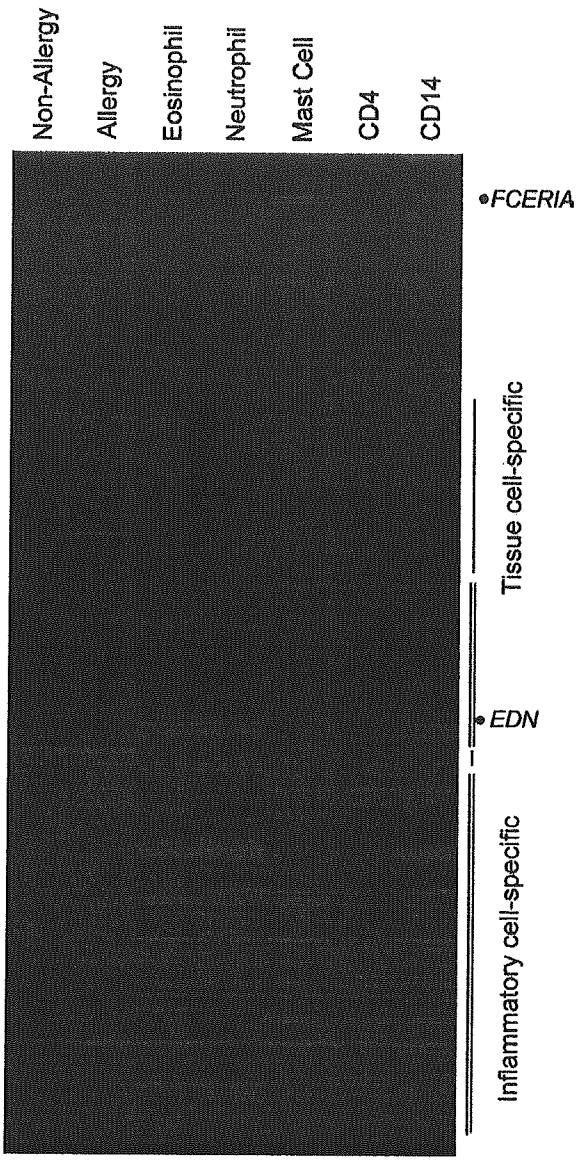

FIG 1. Hierarchical clustering analysis using public database: gene expression profiles of nasal mucosa tissues with or without inflammation and various inflammatory cells. All data were obtained from our Web site (www.nch.go.jp/imal/GeneChip/public.htm). Hierarchical clustering was applied based on Eisen's Gene Cluster and Tree View (rana.lbl.gov/EisenSoftware.htm) to identify whether 774 upregulated (2-fold) transcripts in the inflammatory tissue (nasal mucosa from allergic rhinitis vs normal control) were caused by the increased number of inflammatory cells (eosinophils, neutrophils, mast cells, CD4 cells, and CD14 cells). The normalized expression index for each transcript sequence *(rows)* in each sample *(columns)* is indicated by a color code (*red,* highest; *blue,* lowest). Computations were clearly separated into normal tissue–derived transcripts and inflammatory tissue–derived transcripts. Normalization of copy number of transcripts by using the bioinformatics system Percellome in the future should help clarify whether upregulated transcripts are caused by increased expression or simply by the increased number of inflammatory cells.

25,000. However, the expression levels of certain abundantly or scantly expressed genes do not show linear relationships, so that the actual dynamic range is considered to be only 10^2. In our early GeneChip study using human clinical samples, we identified several increased transcripts in PBMCs obtained from severe atopic patients by calculating the ratio of their expression levels.[13] However, the ratio should have been more carefully calculated when the values were small even when accompanied by the presence call, which means an analysis that is statistically significant. Increased transcripts in PBMC included defensin-1 and ribosomal protein L37. However, these genes may have been derived from contamination of the PBMC population by basophils, because our recent results regarding cell type-specific transcriptome data[14] found these genes to be highly expressed by basophils. Thus, contamination by a very small population of a different cell type having a certain highly expressed transcript may cause an artifactual presence of the transcript in the whole population even where the major cell type lacks it.

Changes in cell populations are especially crucial in microarray analysis of tissues characterized by recruitment of inflammatory cells. Indeed, arginase I is highly expressed by neutrophils among the human leukocyte types. Upregulation of transcriptional levels in a crude tissue is often caused simply by an increase in recruitment of a certain inflammatory cell type. Therefore, to avoid difficulty in interpreting the results, it is important, for comparison, to purify the target cell population as much as possible. However, mRNA is unstable, meaning that complicated procedures for purification should be avoided. Guajardo et al[15] have recently succeeded in identifying nasal epithelial–specific upregulated genes by differentiating the cell types obtained by scraping the nasal membranes of patients with allergic rhinitis. However, computational identification of the cell type specificity may be preferable when a certain important transcript is found in crude tissue samples when cell types can not be identified. For this purpose, a cell type–specific transcriptome database has been developed.[14]

CELL TYPE–SPECIFIC GENE EXPRESSION

A cell type–specific transcriptome database is useful for determining whether an increase of a certain transcript in inflammatory tissues reflects an increase in its expression level or an increase in the number of inflammatory cells that express the transcript at a high level (Fig 1). This database may be also applied efficiently to select safer drug targets.[14]

Mast cells, eosinophils, and basophils are crucially involved in allergic reactions and inflammation. Genes specific for mast cell, basophil, and/or eosinophil could be potential therapeutic targets for allergic diseases because these cells play crucial roles in allergic inflammation. A certain gene that is highly expressed in mast cells, for example, can be easily found by searching the mast cell transcriptome. Comparison with the transcriptomes ex-

pressed by other cell types, however, may reveal that the said gene is not truly mast cell–specific. Thus, it is especially important to elucidate information regarding the cell type specificity when developing a new drug. Such information may help to predict drug-related adverse events caused by interaction of a drug with responsible molecules present in important organs. In the future, the safety of candidate drugs could be evaluated by comparing their efficacy on these granulocytes with their toxicity to vital organs.

FUTURE MICROARRAY APPLICATIONS

The increasing prevalence of allergic diseases in developed countries is considered by many to be caused, at least in part, by rapid improvement of hygiene. In human beings, the immune system developed as an ingenious device for defending against frequent attacks by microbes. Therefore, our immune system seems to have become deranged in our recent, unprecedented hygienic environment. It is now necessary to understand the total functional elements making up the immune system, not just a single molecule present in an immunocyte working in our immune system. Here, we have mainly discussed pros and cons of microarrays as a high-throughput assay method. However, it should be stressed that microarrays can detect whole transcripts present in a cell and can be used for understanding system biology.[16] It is anticipated that continued advances in this and related technologies will help our understanding of deranged human immunity as a system.

REFERENCES

1. Editorial: getting hip to the chip. Nat Genet 1998;18:195-7.
2. Editorial: microarray standards at last. Nature 2002;419:323.
3. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001;29: 365-71.
4. Southern E, Mir K, Shchepinov M. Molecular interactions on microarrays. Nat Genet 1999;21:5-9.
5. Marshall E. Getting the noise out of gene arrays. Science 2004;306: 630-1.
6. Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. Nat Immunol 2000;1:221-6.
7. Zimmermann N, King NE, Laporte J, Yang M, Mishra A, Pope SM, et al. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. J Clin Invest 2003;111: 1863-74.
8. Wang SW, Oh CK, Cho SH, Hu G, Martin R, Demissie-Sanders S, et al. Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts. J Allergy Clin Immunol 2005;115: 287-94.
9. Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y. FcεRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. J Allergy Clin Immunol 2005;115: 272-9.
10. Kanno J, Aisaki K, Ono A, Igarashi K. Toxicogenomics using "Percellome" and "Millefeuille" data system [abstract]. Toxicol Appl Pharmacol 2004;197:265.
11. Okayama Y, Church MK. Comparison of the modulatory effect of ketotifen, sodium cromoglycate, procaterol and salbutamol in human skin, lung and tonsil mast cells. Int Arch Allergy Immunol 1992;97: 216-25.
12. Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, et al. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcε receptor I cross-linking: an interspecies comparison. Blood 2002;100:3861-8.
13. Heishi M, Kagaya S, Katsunuma T, Nakajima T, Yuki K, Akasawa A, et al. High-density oligonucleotide array analysis of mRNA transcripts in peripheral blood cells of severe atopic dermatitis patients. Int Arch Allergy Immunol 2002;129:57-66.
14. Nakajima T, Iikura M, Okayama Y, Matsumoto K, Uchiyama C, Shirakawa T, et al. Identification of granulocyte subtype-selective receptors and ion channels by high-density oligonucleotide probe array. J Allergy Clin Immunol 2004;113:528-35.
15. Guajardo JR, Schleifer KW, Daines MO, Ruddy RM, Aronow BJ, Wills-Karp M, et al. Altered gene expression profiles in nasal respiratory epithelium reflect stable versus acute childhood asthma. J Allergy Clin Immunol 2005;115:243-51.
16. Kitano H. Systems biology: a brief overview. Science 2002;295:1662-4.

FcεRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells

Shigeru Okumura, DDS, PhD,[a,b,c] Hironori Sagara, MD, PhD,[d] Takeshi Fukuda, MD, PhD,[d] Hirohisa Saito, MD, PhD,[a,b] and Yoshimichi Okayama, MD, PhD[a] *Yokohama, Tokyo, Chiba, and Tochigi, Japan*

Background: Topical application of a glucocorticoid is now widely recognized as the first-line therapy for bronchial asthma. However, glucocorticoid treatment is largely ineffective in relation to overproduction of sputum and lung tissue remodeling.

Objective: The purpose of the current study was to identify human mast cell (MC) products that are related to goblet cell hyperplasia.

Methods: The FcεRI-mediated gene expression profile of MCs was examined by using high-density oligonucleotide probe arrays and RT-PCR. Secretion of a protein, amphiregulin, by the MCs was measured by ELISA. Upregulation of mucin genes in NCI-H292 cells by amphiregulin was evaluated by real-time RT-PCR. The expression levels of amphiregulin on histological sections obtained from 40 subjects with asthma and 6 healthy control subjects were estimated by immunohistochemical staining, and the correlation with the number of goblet cells was studied.

Results: Amphiregulin was secreted by human MCs after aggregation of FcεRI, and its expression was not inhibited by a glucocorticoid (dexamethasone). Amphiregulin upregulated mucin gene expression in airway epithelial cells. Upregulation of amphiregulin expression was observed in MCs of patients with asthma, but not normal control subjects. Furthermore, upregulation of amphiregulin in MCs significantly correlated with the extent of goblet cell hyperplasia in the mucosa of patients with bronchial asthma.

Conclusion: These results suggest that after exposure to antigens, human MCs may induce sputum production via release of amphiregulin. Therefore, amphiregulin may be a new target molecule for treatment of overproduction of sputum in bronchial asthma. (J Allergy Clin Immunol 2005;115: 272-9.)

Key words: Mast cells, amphiregulin, bronchial asthma, goblet cell hyperplasia, dexamethasone

Bronchial asthma is characterized physiologically by variable airflow obstruction and airway hyperresponsiveness. Goblet cell hyperplasia has been established as a pathologic characteristic of mild, moderate, and severe asthma.[1] Abnormalities in goblet cell numbers are accompanied by changes in stored and secreted mucin.[1] Mucus hypersecretion is often a marked feature, particularly in status asthmaticus. The presence of mucus hypersecretion was associated with a significantly greater decline in FEV_1.[2] Topical application of a glucocorticoid is now widely recognized as the first-line therapy for bronchial asthma. Although treatment with steroids has been reported to prevent the development of allergen-induced goblet cell hyperplasia in animal models,[3] it has less effect once goblet cell hyperplasia has been established.[4] Furthermore, in human beings, treatment with a corticosteroid is largely ineffective in relation to tissue remodeling and mucus production, both pathologically[5] and clinically. Currently, there are no drugs that exert a specific action on mucus production.

Sensitized and allergen-exposed animals develop marked goblet cell hyperplasia.[4,6] The pathogenesis underlying allergen-induced goblet cell hyperplasia in mice is thought to involve a variety of mediators, including IL-4,[7] IL-13,[8,9] IL-9,[10] the epidermal growth factor (EGF) system,[11] a disintegrin and metalloprotease family,[12] and ion channels such as gob-5[13] (hCLCA1 in human beings[14]), probably by upregulating the expression of mucin genes. The major effector cell is thought to be the T_H2 subtype of $CD4^+$ lymphocytes. However, allergen-challenge high-affinity receptors for IgE (FcεRI)–knockout mice showed less airway inflammation, less goblet cell hyperplasia, and lower levels of IL-13 in lung homogenates compared with the controls.[15] Furthermore, IL-9 but not IL-4 or IL-13 increased mucin gene expression in a human airway epithelial cell culture system.[16] We

From [a]the Research Unit for Allergy Transcriptome, Research Center for Allergy and Immunology, RIKEN Yokohama Institute; [b]Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo; [c]Department of Microbiology, Tokyo Dental College, Chiba; and [d]Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi.

Supported in part by grants from the RIKEN Yokohama Institute, the Grants-in-Aid for Scientific Research (C) program of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (project number 14570402, to Dr Okayama), the Hokuriku Seiyaku Co, Ltd, Japanese Allergy Foundation (Dr Okayama), the AstraZeneca Asthma Research Award 2002 (Dr Okayama), the Kyowa-Hakko Co, Ltd, Foundation (Dr Okayama), and the Pharmaceutical and Medical Devices Agency with the Ministry of Health, Labour and Welfare (Millennium Genome Project, MPJ-5, to Dr Saito).

Dr Okumura and Dr Sagara contributed equally to this work.

Received for publication August 18, 2004; revised September 27, 2004; accepted for publication October 1, 2004.

Available online January 4, 2005.

Reprint requests: Yoshimichi Okayama, MD, Research Unit for Allergy Transcriptome, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan. E-mail: yokayama@rcai.riken.go.jp.

0091-6749/$30.00

doi:10.1016/j.jaci.2004.10.004

Abbreviations used
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
MC: Mast cell
MNC: Mononuclear cell
rh: Recombinant human
SCF: Stem cell factor

therefore hypothesized that after aggregation of FcεRI, human mast cells (MCs) produce molecules that induce goblet cell hyperplasia, and that the expression of these molecules is not inhibited by glucocorticoids.

Mast cells play pivotal roles in immediate-type and inflammatory allergic reactions that can result in asthma. Cross-linking of the FcεRI on MCs activates a signaling pathway that leads to degranulation, *de novo* synthesis of arachidonic acid metabolites, and production of various cytokines/chemokines.[17] We recently reported that human MCs express epiregulin as one FcεRI-mediated specific gene.[18] The EGF family consists of EGF, amphiregulin, heparin-binding–EGF, TGF-α, betacellulin, epiregulin, and neuregulins. Amphiregulin was originally purified from conditioned medium of 12-*O*-tetradecanoylphorbol-13-acetate–treated MCF-7 human breast carcinoma cells.[19] The carboxyl terminal half of the amphiregulin molecule exhibits striking homology of with EGF, and it can therefore be classified as a member of the EGF family. Like EGF and TGF-α, amphiregulin plays important roles in cell proliferation,[20,21] survival,[22] and differentiation.[21] Amphiregulin is synthesized in the form of a transmembrane precursor, with the secreted final protein released by proteolytic cleavage.

We examined the FcεRI-mediated gene expression profile by using high-density oligonucleotide probe arrays and performed clustering analysis depending on the effect of a glucocorticoid on FcεRI-mediated gene expression in human MCs. We found remodeling-related molecules in the cluster genes whose expression was not inhibited by dexamethasone. Furthermore, MC-specific molecules were selected by comparison with the gene expression profiles of human blood mononuclear cells, eosinophils, and neutrophils, and amphiregulin was included in the subset of genes upregulated by aggregation of FcεRI but not downregulated by pretreatment with dexamethasone. Thus, we examined the effect of amphiregulin on mucin gene expression in human epithelial cells *in vitro* and *in vivo*.

METHODS

Cytokines and antibodies

Recombinant human (rh) IL-3 was purchased from Intergen (Purchase, NY). RhIL-6 and rh stem cell factor (SCF) were purchased from PeproTech EC Ltd (London, England). Rh amphiregulin, rhEGF, and mouse antihuman amphiregulin mAb were purchased from Genzyme Techne (Minneapolis, Minn). Mouse antihuman tryptase mAb (clone AA1) was purchased from Dako Ltd (Carpinteria, Calif).

Generation of cord blood–derived MCs and adult peripheral blood–derived MCs

All human subjects in this study provided written informed consent, and the study was approved by the ethical review board of each hospital. Human cord blood mononuclear cells (MNCs) and peripheral blood MNCs were isolated by centrifugation on a Ficoll-Isopaque density gradient (Nycomed, Oslo, Norway). Lineage-negative MNCs were selected from the cord blood MNCs and peripheral blood MNCs and then cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and Iscove modified Dulbecco medium containing SCF at 200 ng/mL, IL-6 at 50 ng/mL, and IL-3 at 1 ng/mL, as previously described.[18] On day 42 of culture, methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in Iscove modified Dulbecco medium containing SCF at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS.

Purification of leukocytes

Granulocytes and mononuclear cells were separated from venous blood of normal volunteers. Eosinophils were isolated by Percoll (1.090 g/mL) density centrifugation and then further purified by negative selection with anti-CD16–bound micromagnetic beads, as described previously.[23] After this negative selection, neutrophils were isolated by Percoll (1.085 g/mL) density centrifugation. The neutrophils were further purified by negative selection with anti-CD81 antibody for eliminating contaminating eosinophils.

Activation of human MCs

For activation of human MCs by aggregation of their surface FcεRI, MCs were first sensitized with 1 μg/mL human myeloma IgE (CosmoBio Tokyo, Japan) at 37°C for 24 hours. After washing, the cells were challenged with either rabbit antihuman IgE Ab (Dako Ltd) or the culture medium alone at 37°C for the indicated period. To investigate the effect of dexamethasone (a glucocorticoid; PeproTech EC Ltd) on the IgE-mediated gene expression profile and amphiregulin production by MCs, MCs were pretreated with 10^{-6} mol/L dexamethasone for 24 hours before activation. In all conditions, the cells were suspended in the complete Iscove modified Dulbecco medium containing SCF and IL-6. The treatment of MCs with 10^{-6} mol/L dexamethasone and/or IgE/anti-IgE did not significantly change the cell viability or cell number.

Isolation of RNA, RT-PCR, real-time quantitative RT-PCR, and GeneChip expression analysis

This information can be found at http://www.nch.go.jp/imal/GeneChip/AREG.htm and in the Journal's Online Repository at www.mosby.com/jaci.

Effect of amphiregulin or MC supernatants on mucin gene expression in NCI-H292 cells

Mast cells were sensitized with myeloma IgE, washed, and then challenged with or without 1.5 μg/mL anti-IgE for 24 hours. The supernatants were harvested. The confluent serum-depleted NCI-H292 cells (American Type Culture Collection, Rockville, Md) were treated with either 10 μg/mL neutralizing antiamphiregulin mAb or isotype control mIgG1 for 20 minutes before MC supernatants were added. Total RNA was then extracted for quantitative real-time PCR analysis of *MUC2* and *MUC5A* expression.

ELISA for amphiregulin

Human amphiregulin was measured with an ELISA kit purchased from R&D Systems (Minneapolis, Minn). The sensitivity of the assays of human amphiregulin was 5 pg/mL.

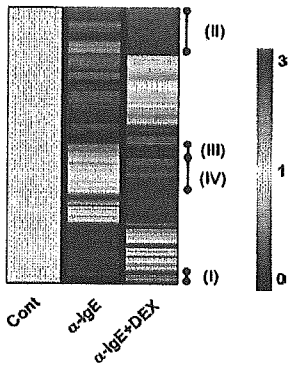

FIG 1. Representation of mRNA expression levels in control MCs, anti-IgE–stimulated MCs, and anti-IgE plus dexamethasone *(DEX)*–stimulated MCs. Human MCs were precultured with IgE in the presence or absence of dexamethasone and then activated with anti-IgE for 6 hours. Each *row* of colored bars represents 1 gene, and each *column* represents 1 stimulus. *Colored bars* show the magnitude of the response for each gene, according to the scale shown. *I* indicates a set of genes that were upregulated by anti-IgE stimulation but decreased by pretreatment with dexamethasone after activation with anti-IgE. *II* indicates a set of genes that were upregulated by anti-IgE-stimulation but not affected by dexamethasone pretreatment after activation with anti-IgE. *III* indicates a set of genes that were not affected by anti-IgE stimulation but were downregulated by dexamethasone treatment before activation with anti-IgE. *IV* indicates a set of genes were not affected by anti-IgE stimulation but were upregulated by dexamethasone pretreatment. *Cont,* Control.

Subjects

Forty subjects with asthma whose disease severity was defined by using a combination of the asthma symptom grade and the frequency of symptoms on the basis of the criteria of the Japanese Society of Allergology[24] and 6 normal control subjects without asthma were studied (Table E1 in the Journal's Online Repository at www.mosby.com/jaci). None of the subjects was a current smoker, and none had smoked during the previous 2 years. No subjects had any bronchial or respiratory tract infections during the month preceding the test. The study was approved by the ethics committee of Dokkyo University School of Medicine, and all subjects provided written informed consent. The thickness of the total basement membrane in each asthmatic and control subject was assessed as previously described.[25] Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.[26]

Bronchial biopsy

Tissue samples of patients with asthma were taken from the subcarina between the right lower lobe and middle lower lobe bronchi (the origin of right B6 bronchus) by using a standard forceps during fiberoptic bronchoscopic examination, as previously described.[25] Each biopsy specimen was immediately placed in OCT medium, snap-frozen in liquid nitrogen, and stored at −80°C until cryostat sectioning.

Immunohistochemistry

Three-micrometer consecutive serial sections of respiratory mucosa from patients with asthma and control subjects were stained with monoclonal antiamphiregulin mAb and antitryptase mAb by using ABC kits (Vector Laboratories, Burlingame, Calif). Briefly, slides were quenched in 3% H_2O_2 for 10 minutes to block endogenous peroxidase and washed in PBS. Sections were next incubated with the primary antibody for 1 hour and then with biotinylated secondary antibody, followed by the ABC reagents. Color development was achieved by incubating with diaminobenzidine as a substrate. Slides were counterstained with Mayer's hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Amphiregulin$^+$ cells were counted in at least 6 high-power fields in each sample by 3 independent observers. Hansel's stain (Torii Pharmaceutical Co, Ltd, Tokyo, Japan) was used to identify eosinophils. Sequential Alcian blue and periodic acid–Schiff staining of airway tissue sections allowed clear visualization of mucins in secretory cells. The intracellular mucus glycoproteins of the epithelial secretory cells were recognized as purple oval disks of varying size. To analyze secretory responses of goblet cells, a mucus score was determined from the histologic sections by grading the amount of mucus in each secretory cell as follows.[27] For grade 1, the vertical distance of the stained area was within 1/3 of the epithelial layer, measured from basement membrane to cell apices. For grade 2, the vertical distance of the stained area exceeded 1/3 of the epithelial layer.

Stained areas were graded in 20 consecutive high-power fields along the 2 walls of the trachea. In each donor, mucus score was calculated as $n_1 + 2n_2$, where n_1 and n_2 were the total number of cells for grade 1 and grade 2, respectively.

The average score assigned to each sample by each investigator was first calculated, and then the average score for each sample by 3 investigators was calculated and recorded as data.

Statistical analysis

Differences between 2 paired groups were analyzed by the unpaired Student t test and considered significant at $P < .05$. Values are expressed as the means ± SEMs.

RESULTS

Clustering analysis of dexamethasone-regulated gene expression by human MCs

To identify genes upregulated by aggregation of FcεRI but not downregulated by pretreatment with dexamethasone, the gene expression profile in human MCs was explored by using high-density oligonucleotide probe arrays (GeneChip; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). We first divided the genes into the following 4 sets. The first sets contains genes (1) whose expression changed by at least 2-fold (activation program) after aggregation of FcεRI and (2) whose increased gene expression changed by less than 0.5-fold after dexamethasone pretreatment (Fig 1, *I*; referred to as set I). The second set includes genes (1) whose expression changed by at least 2-fold after aggregation of FcεRI and (2) whose increased gene expression changed by more than 0.5-fold after dexamethasone pretreatment (Fig 1, *II*; set II). The third set consists of genes (1) whose expression changed by less than 2-fold after aggregation of FcεRI and (2) whose

TABLE I. Complete list of anti-IgE–upregulated, glucocorticoid-insensitive, MC-specific transcripts

GeneBank	Product	MC1C		MC1a		MC1da		MC2C		MC2a		MC2da		Eo	MNC	Ne	Specif
HG1437-HT1437	Trk oncogene	7.4	P	20.3	P	15.0	P	12.1	P	33.8	P	14.3	P	0.0	0.1	0.0	248.3
AL050090	Hypothetical protein	1.3	P	3.6	P	2.5	P	1.5	P	3.5	P	1.9	P	0.0	0.1	0.0	55.3
U51694	Phosphodiesterase 4D interacting protein	0.4	P	1.1	P	3.5	P	0.7	P	2.1	P	4.3	P	0.1	0.0	0.0	48.9
X03541	Trk oncogene	6.8	P	18.6	P	15.7	P	13.8	P	35.4	P	14.6	P	0.7	0.4	0.8	43.4
AB002341	Neuronal cell adhesion molecule	0.5	P	1.4	P	1.3	P	0.6	P	4.2	P	2.7	P	0.0	0.2	0.0	22.3
X60957	Receptor tyrosine kinase	1.6	P	5.0	P	4.3	P	1.3	P	5.1	P	1.8	P	0.1	0.2	0.3	14.7
M30704	Amphiregulin	0.0	A	0.5	P	3.7	P	0.1	A	8.0	P	7.1	P	0.8	0.0	0.0	10.1
U88629	RNA polymerase II elongation factor ELL2	2.0	P	3.6	P	2.8	P	0.8	P	3.2	P	1.4	P	0.4	0.2	0.4	8.0
X66363	Serine/threonine protein kinase	0.5	P	0.5	P	0.0	P	1.0	P	2.5	P	2.0	P	0.3	0.0	0.0	7.9
X54232	Glypican	0.0	A	0.8	P	1.4	P	0.5	A	1.9	P	1.7	P	0.0	0.2	0.0	7.8
AF102803	αE-catenin	1.2	P	4.3	P	2.5	P	1.8	P	4.5	P	2.0	P	0.0	0.6	0.3	7.5
D31887	KIAA0062 protein	1.5	P	3.3	P	6.8	P	3.0	P	7.1	P	8.2	P	0.4	1.2	0.7	7.1
AF038660	β-1,4- Galactosyltransferase	1.0	P	3.0	P	2.3	P	1.6	P	2.6	P	2.2	P	0.1	0.5	0.4	6.1
AB011105	KIAA0533 protein	0.4	P	0.9	P	1.5	P	0.9	P	2.0	P	2.3	P	0.0	0.2	0.4	5.5
X52015	IL-1R antagonist	7.5	P	18.4	P	14.4	P	33.1	P	80.0	P	37.6	P	5.8	3.5	14.9	5.4
L23805	α1(E)-catenin	11.5	P	26.9	P	16.6	P	13.9	P	27.8	P	12.7	P	0.5	5.4	3.6	5.2
AL022310	OX40L	2.0	P	5.8	P	5.7	P	1.0	A	5.5	P	2.5	P	0.3	1.2	0.0	5.0

MC1(2)c, Untreated control MCs used in experiment 1(2); *MC1(2)a*, anti-IgE–stimulated MCs used in experiment 1(2); *MC1(2)d*, MCs treated with anti-IgE and dexamethasone used in experiment 1(2); *Eo*, eosinophils; *Ne*, neutrophils; *Specif*, MC specificity (ratio to other leukocytes).

increased gene expression changed by less than 0.5-fold after dexamethasone pretreatment (Fig 1, *III*; set III). The last group includes genes (1) whose expression changed by less than 2-fold after aggregation of FcεRI and (2) whose increased gene expression changed by more than 0.5-fold after dexamethasone pretreatment (Fig 1, *IV*; set IV). Furthermore, we selected MC-specific genes on the basis of comparison with the gene expression profiles of human PBMCs, eosinophils, and neutrophils. Data were considered MC-specific when the expression level in MCs was at least 5 times higher than the maximal expression levels of human PBMCs, eosinophils, and neutrophils, as described in the Methods section. Twenty-four genes were thus identified as MC-specific in set I, and these were NFκB pathway members such as cytokines (IL-5 and GM-CSF) and chemokines (MCP-1 and I-309; Table E2 in the Online Repository at www.mosby.com/jaci). Seventeen MC-specific genes identified in set II include amphiregulin and adhesion molecules such as α1-(E) catenin and neuronal cell adhesion molecule (Table I). In set III, 22 genes were identified as MC-specific and included cathepsin G, chymase, and metalloproteinase 9 (Table E3 in the Online Repository at www.mosby.com/jaci; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). Last, 236 genes were selected as MC-specific in set IV, and they included tryptase, major basic protein, PGD2 synthase, and c-kit (Table E4 in the Online Repository at www.mosby.com/jaci; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). Because amphiregulin is a cytokine of the EGF family, we focused on amphiregulin among the MC-specific genes that are upregulated by aggregation of FcεRI but not downregulated by dexamethasone pretreatment.

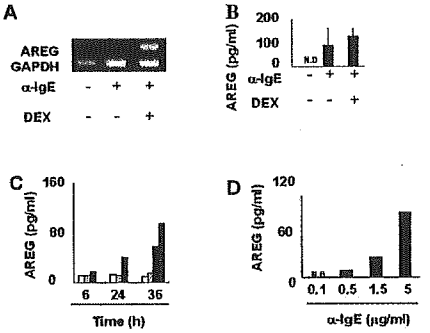

FIG 2. Expression of amphiregulin (AREG) by human MCs. **A,** Upregulation of AREG expression in human MCs by FcεRI-mediated activation. Human MCs with (+) and without (−) pretreatment with dexamethasone *(DEX)* were cultured with IgE then activated with 1.5 μg/mL anti-IgE. Intracellular mRNA for AREG and GAPDH was amplified by RT-PCR. **B,** AREG secretion from MCs after anti-IgE (1.5 μg/mL) stimulation with (+) or without (−) dexamethasone pretreatment. Cell supernatants were harvested at 24 hours for ELISA of AREG (n = 3 donors). **C,** Time course of AREG production by anti-IgE (1.5 μg/mL)-stimulated human MCs with *(gray bar)* or without *(closed bars)* dexamethasone pretreatment. Control cells were incubated with IgE in the presence *(hatched bars)* or absence *(open bars)* of dexamethasone, but anti-IgE was omitted. **D,** Concentration-response study of anti-IgE–induced AREG production by human MCs. MCs were preincubated with IgE and then activated with 0.1, 0.5, 1.5 or 5 μg/mL anti-IgE for 24 hours. *ND,* Not detected.

Analysis of amphiregulin expression in human MCs

By using mRNA extracted from resting and IgE/anti-IgE–activated human MCs with or without dexamethasone pretreatment, we examined the expression of

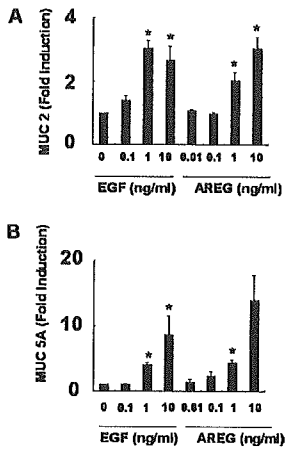

FIG 3. Effect of amphiregulin (AREG) on *MUC2* and *MUC5AC* expression in NCI-H292 cells. NCI-H292 cells were incubated with 0.01 to 10 ng/mL rhAREG or 0.1 to 10 ng/mL rhEGF for 24 horus. Total RNA was extracted from the cells, and quantitative real-time RT-PCR analysis was used to determine the amounts of *MUC2* (**A**) and *MUC5AC* (**B**) mRNA. The results were expressed as the fold change (mean ± SEM) in the mucin mRNA level of AREG-treated or EGF-treated cells compared with AREG-untreated or EGF-untreated cells (n = 3). *$P < .05$ compared with cells not treated with AREG or EGF.

amphiregulin. Amphiregulin mRNA was clearly detected in the IgE/anti-IgE activated MCs (Fig 2, *A*). Amphiregulin mRNA appeared to be upregulated by dexamethasone pretreatment of MCs after FcεRI aggregation (Fig 2, *A*). To demonstrate the secretion of amphiregulin, we used ELISA kit to supernatants of activated MCs and confirmed the presence of amphiregulin. Dexamethasone pretreatment appeared to upregulate IgE-mediated release of amphiregulin, but it was not significant (Fig 2, *B*). Fig 2, *C*, shows the time course of amphiregulin production by MCs after FcεRI aggregation. The production continued to increase until at least 36 hours after cross-linking of FcεRI. Dexamethasone alone resulted in almost the same level as with the medium alone. Amphiregulin production appeared to be upregulated by dexamethasone pretreatment of MCs 36 hours after FcεRI cross-linking (Fig 2, *C*). As can be seen from Fig 2, *D*, amphiregulin was released by anti-IgE in a concentration-dependent manner.

Amphiregulin upregulates *MUC2* and *MUC5AC* expression

It was reported that *MUC2* and *MUC5AC* were 2 target genes of EGF receptor (EGFR) ligands in a human pulmonary mucoepidermoid carcinoma cell line, NCI-H292 cells. We hypothesized that amphiregulin might upregulate expression of mRNA for *MUC2* and *MUC5AC* in NCI-H292 cells. As can be seen in Fig 3, amphiregulin increased *MUC2* and *MUC5AC* gene expression in NCI-H292 cells dose-dependently. At 10 ng/mL, amphiregulin increased *MUC2* and *MUC5AC* gene expression 3-fold and 13-fold, respectively. The increase in *MUC2* and *MUC5AC* induced by amphiregulin is almost same as that by EGF. Next, we confirmed that the anti-IgE–activated MC supernatant increased both *MUC2* and *MUC5AC* gene expression in NCI-H292 cells. These increases were partially but significantly blocked by neutralizing antibody against amphiregulin (Fig 4).

Amphiregulin expression in bronchial MCs of patients with asthma

To determine whether amphiregulin is expressed in bronchial MCs of patients with asthma, we performed immunohistochemical analysis by using bronchial mucosal biopsy specimens obtained from 40 patients with asthma and 6 healthy control subjects (Table EI in the Online Repository at www.mosby.com/jaci). To identify amphiregulin-positive (amphiregulin$^+$) cells as being MCs, we used sequential sections and stained one section with antitryptase mAb and the other section with anti-amphiregulin mAb. Bronchial biopsy samples derived from the healthy control subjects showed little immunoreactivity for amphiregulin (data not shown). In contrast, biopsy samples derived from the subjects with asthma showed clearly positive immunoreactivity for amphiregulin in bronchial MCs (Fig 5, *A*). We next counted the number of amphiregulin$^+$ cells in tryptase-positive (tryptase$^+$) cells. The mean percentages of amphiregulin$^+$ MCs were 35%, 52.8%, and 52% in mild, moderate, and severe asthma, respectively (data not shown). We counted the number of amphiregulin$^+$ cells in square millimeters of the bronchial mucosa (amphiregulin$^+$ cells/mm^2) of subjects with asthma and control subjects (Fig 5, *B*). The number of amphiregulin$^+$ cells/mm^2 was significantly increased in subjects with asthma compared with control subjects ($P < .01$). Airway epithelial cells of both subjects with asthma and normal subjects exhibited little immunoreactivity for amphiregulin. To clarify this, we next counted the number of amphiregulin$^+$tryptase$^+$/mm^2 (Fig 5, *C*). Furthermore, the percentages of amphiregulin$^+$ epithelial cells, eosinophils, and others among total amphiregulin$^+$ cells in normal and asthmatic lung samples were counted. The results showed that less than 10% of the total amphiregulin$^+$ cells were epithelial cells and eosinophils (Fig 5, *D*). Other cells accounted for less than 1 % (data not shown). Thus, the number of amphiregulin$^+$ cells was almost the same as the total number of amphiregulin$^+$ MCs. We next investigated the relationship between the number of amphiregulin$^+$ MCs and the mucus score (see Methods). The results revealed a significant correlation between these 2 numbers (Fig 5, *E*; $P < .005$; $r = 0.54$), suggesting that amphiregulin induces goblet cell hyperplasia.

DISCUSSION

In this article, we identified 17 MC-specific, IgE/anti-IgE–inducible but dexamethasone-insensitive genes in human MCs by using GeneChip, and we found amphiregulin in 1 subset of those genes (Fig 1 and Table I). Secretion of amphiregulin was upregulated by FcεRI

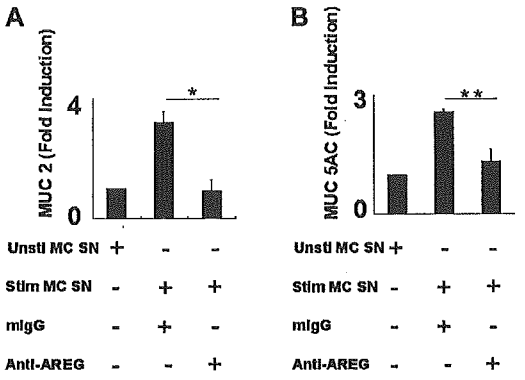

FIG 4. Activated MC supernatants increase *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (AREG). Confluent, serum-depleted NCI-H292 cells were pretreated with 1 μg/mL anti-AREG neutralizing antibody or 1 μg/mL mouse IgG1, and then incubated with unstimulated MC supernatant (Unsti MC SN) or activated MC supernatant (Stim MC SN) for 24 hours. Total RNA was extracted from the NCI-H292 cells, and quantitative real-time RT-PCR analysis was used to determine the amounts of *MUC2* **(A)** and *MUC5AC* **(B)** mRNA. The results were expressed as the fold change (mean ± SEM) in the mucin mRNA level of anti-AREG mAb or $mIgG_1$-treated cells incubated with activated MC supernatant compared with cells incubated with unstimulated MC supernatant (n = 3). $*P < .05$, $**P < .01$ compared between cells treated with anti-AREG neutralizing antibody and treated with mIgG1.

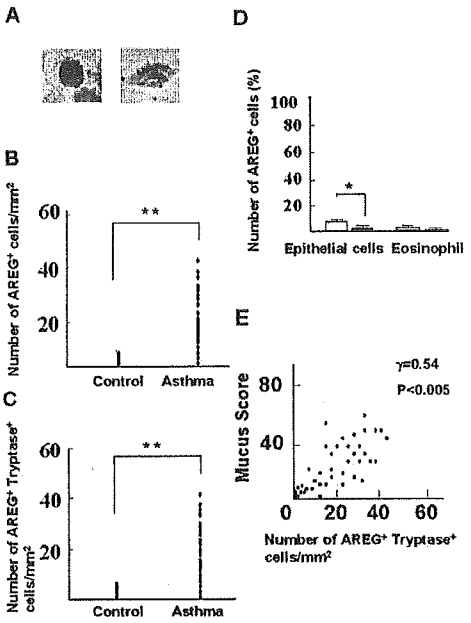

FIG 5. Correlation of the number of amphiregulin (AREG)$^+$ tryptase$^+$ cells with the extent of goblet cell hyperplasia in the airways of asthmatic subjects. **A,** Colocalization of AREG in tryptase$^+$ MCs. Two sequential 3-μm sections of bronchial biopsy specimens from subjects with asthma were immunostained for tryptase *(left panel)* and AREG *(right panel)*. **B,** The number of AREG$^+$ cells in 1 mm^2 bronchial mucosa of normal control subjects *(Control)* and subjects with asthma *(Asthma)*. AREG$^+$ cells were counted in at least 6 high-power fields in each sample by three independent observers. $**P < .01$ compared between the number of AREG$^+$ cells in control subjects and subjects with asthma. **C,** The number of AREG$^+$tryptase$^+$ cells in 1 mm^2 bronchial mucosa of normal control subjects *(Control)* and subjects with asthma *(Asthma)*. $**P < .01$ compared between the number of AREG$^+$ tryptase$^+$cells in control subjects and subjects with asthma. **D,** Percentages of AREG$^+$ epithelial cells and eosinophils among total AREG$^+$ cells in normal *(open bar)* and asthmatic lung *(closed bar)* samples. $*P < .05$ for the percentages of AREG$^+$ epithelial cells in control subjects and subjects with asthma. **E,** Correlation of expression of AREG in MCs with the extent of goblet cell hyperplasia in patients with asthma. The extent of goblet cell hyperplasia was scored as described in the Methods section; its correlation with the number of AREG$^+$tryptase$^+$ cells in the airways of patients with asthma was analyzed.

cross-linking (Fig 2). We further demonstrated that amphiregulin induces *MUC2* and *MUC5AC* expression by NCI-H292 cells (Fig 3). Activated MC supernatants further increased the *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (Fig 4). We compared amphiregulin expression in bronchial MCs from 40 subjects with asthma and 6 normal subjects by immunohistochemical analysis using bronchial mucosal biopsy specimens (Table EI in the Online Repository at www.mosby.com/jaci). MCs from the subjects with asthma expressed amphiregulin, but MCs from the normal donors showed minimal expression (Fig 5, *C*). We further demonstrated that upregulation of amphiregulin in the MCs significantly correlated with the incidence of goblet cell hyperplasia in the mucosa of patients with bronchial asthma (Fig 5, *E*). Because amphiregulin seems to induce goblet cell hyperplasia, it can be surmised that in asthma MCs function not only to induce inflammation by production of proinflammatory cytokines such as TNF-α but also to regulate remodeling by production of amphiregulin.

By using GeneChip, we identified MC-specific transcripts by comparing the gene expression levels with the criteria described in the Methods section. We found that 17 genes were MC-specific, IgE/anti-IgE–inducible, and dexamethasone-insensitive. Amphiregulin is included in that subset of genes. We focused on amphiregulin for the following reasons: (1) amphiregulin is involved in the process of lung branching morphogenesis in mice,[28] (2) MUC2 and MUC5AC proteins were induced by EGF in mucoepidermoid NCI-H292 cells,[29] (3) Human airway trypsin-like protease increased mucin expression in NCI-H292 cells through release of amphiregulin,[30] and (4) amphiregulin acted as a potent mitogen for a vascular smooth muscle cell line.[20] These findings strongly suggest that amphiregulin produced by human MCs is involved in lung tissue remodeling. As can be seen from Figs 2, 3, and 4, after aggregation of FcεRI, MCs secrete amphiregulin, which induces upregulation of mucin gene expression. In human cell culture studies, increased *MUC2* and *MUC5AC* mRNA levels also accompanied cell differentiation, with increased mucin secretion coinciding with altered morphology of human airway epithelial cells.[31] In our current study, bronchial MCs from patients with asthma expressed amphiregulin, and upregulation of amphiregulin correlated with an increase in goblet cell hyperplasia. In the bronchial mucosa of patients with asthma, amphiregulin-immunopositve cells were MCs

(Fig 5, *B and C*) and airway epithelial cells, but epithelial cells showed very weak immunoreactivity (Fig 5, *D*). This is in agreement with the recent finding[32] that airway epithelial cells of naive animals exhibited little immunoreactivity for amphiregulin, whereas staining of MCs in the peritracheal connective tissues showed prominent amphiregulin immunoreactivity. After antigenic challenge of sensitized, chronically exposed mice, there was transiently increased expression of amphiregulin in the cytoplasm of epithelial cells, but no evidence of staining in other cells such as eosinophils. *In vitro*, the levels of amphiregulin produced by MCs were much higher than those produced by epithelial cells.[30] These results indicate that MCs store amphiregulin in their cytoplasm, and IgE-mediated activation of MCs directly induces mucus production in the human airway.

Antiamphiregulin mAb partially but significantly inhibited mucin gene expression induced by MC supernatants. This may be a result of contributions of other molecules that induce mucin gene expression. Exposure of NCI-H292 cells to TNF-α[33] or IL-1β increased MUC2 expression. In our limited investigation of the expression of the EGF family by human MCs by using GeneChip, MCs expressed epiregulin after cross-linking of FcϵRI. Ectodomain shedding of EGFR ligands and EGFR phosphorylation by metalloproteinases such as ADAM17 are implicated in mucin production in airway epithelial cells.[12,34,35] MCs upregulated the expression of TNF-α, IL-1β, and ADAM17 after aggregation of FcϵRI (data not shown). These data further support our hypothesis that IgE-mediated MC activation induces mucin production by epithelial cells.

The effect of glucocorticoids on *MUC5AC* expression in human epithelial cells *in vitro* is controversial. Dexamethasone was reported to attenuate steady-state mRNA levels of *MUC5AC*,[36] whereas it was reported to upregulate *MUC5AC* expression.[37] Thus, we examined the effect of 10^{-6} mol/L and 10^{-7} mol/L dexamethasone on steady-state mRNA for *MUC5AC* in NCI-H292 cells by using real-time PCR. We found that these concentrations of dexamethasone did not have a significant effect on steady-state mRNA in *MUC5AC* (data not shown). Furthermore, IL-13–induced MUC5AC overexpression and goblet cell hyperplasia are resistant to glucocorticoid.[38] Treatment of patients with asthma with glucocorticoids has not been significantly effective in relation to overproduction of sputum. Because mucus hypersecretion is an important cause of morbidity and mortality in patients with asthma and no specific treatments are available, further clinical targets and therapeutic strategies are urgently needed. Elucidation of the molecular mechanisms of goblet cell hyperplasia induced by human MC amphiregulin should provide new targets for novel therapeutic interventions.

REFERENCES

1. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. Chest 2002;122:320S-6S.
2. Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. N Engl J Med 1998; 339:1194-200.
3. Kamachi A, Munakata M, Nasuhara Y, Nishimura M, Ohtsuka Y, Amishima M, et al. Enhancement of goblet cell hyperplasia and airway hyperresponsiveness by salbutamol in a rat model of atopic asthma. Thorax 2001;56:19-24.
4. Vanacker NJ, Palmans E, Kips JC, Pauwels RA. Fluticasone inhibits but does not reverse allergen-induced structural airway changes. Am J Respir Crit Care Med 2001;163:674-9.
5. Jenkins HA, Cool C, Szefler SJ, Covar R, Brugman S, Gelfand EW, et al. Histopathology of severe childhood asthma: a case series. Chest 2003; 124:32-41.
6. Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med 2000;161:627-35.
7. Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. J Immunol 1999;162:6233-7.
8. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261-3.
9. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, et al. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. Am J Physiol Lung Cell Mol Physiol 2001;280:L134-40.
10. Louahed J, Toda M, Jen J, Hamid Q, Renauld JC, Levitt RC, et al. Interleukin-9 upregulates mucus expression in the airways. Am J Respir Cell Mol Biol 2000;22:649-56.
11. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci U S A 1999;96:3081-6.
12. Shao MX, Ueki IF, Nadel JA. Tumor necrosis factor alpha--converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci U S A 2003;100: 11618-23.
13. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, et al. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A 2001;98:5175-80.
14. Zhou Y, Dong Q, Louahed J, Dragwa C, Savio D, Huang M, et al. Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. Am J Respir Cell Mol Biol 2001;25:486-91.
15. Taube C, Wei X, Swasey CH, Joetham A, Zarini S, Lively T, et al. Mast cells, FcepsilonRI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. J Immunol 2004;172:6398-406.
16. Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T, et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. J Clin Invest 1999;104:1375-82.
17. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997;77: 1033-79.
18. Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, et al. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. Blood 2003;102: 2547-54.
19. Shoyab M, McDonald VL, Bradley JG, Todaro GJ. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. Proc Natl Acad Sci U S A 1988;85:6528-32.
20. Kato M, Inazu T, Kawai Y, Masamura K, Yoshida M, Tanaka N, et al. Amphiregulin is a potent mitogen for the vascular smooth muscle cell line, A7r5. Biochem Biophys Res Commun 2003;301:1109-15.
21. Falk A, Frisen J. Amphiregulin is a mitogen for adult neural stem cells. J Neurosci Res 2002;69:757-62.
22. Hurbin A, Dubrez L, Coll JL, Favrot MC. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines. J Biol Chem 2002;277: 49127-33.
23. Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment in vitro. Blood 1995;86:1437-43.

24. Makino S, Furusho K, Ohta K, Mukoyama T. A survey on awareness and utilization of new asthma management guidelines in Japan. J Asthma 2003;40:701-8.
25. Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, Olivieri D. Airways remodeling is a distinctive feature of asthma and is related to severity of disease. Chest 1997;111:852-7.
26. Makino S, Ikemori R, Fukuda T, Motojima S, Namai S, Toda M, et al. [Clinical evaluation of standard method of acetylcholine inhalation test in bronchial asthma]. Arerugi 1984;33:167-75.
27. Tokuyama K, Kuo HP, Rohde JA, Barnes PJ, Rogers DF. Neural control of goblet cell secretion in guinea pig airways. Am J Physiol 1990;259: L108-15.
28. Schuger L, Johnson GR, Gilbride K, Plowman GD, Mandel R. Amphiregulin in lung branching morphogenesis: interaction with heparan sulfate proteoglycan modulates cell proliferation. Development 1996;122:1759-67.
29. Perrais M, Pigny P, Copin MC, Aubert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J Biol Chem 2002;277: 32258-67.
30. Chokki M, Yamamura S, Eguchi H, Masegi T, Horiuchi H, Tanabe H, et al. Human airway trypsin-like protease increases mucin gene expression in airway epithelial cells. Am J Respir Cell Mol Biol 2004;30:470-8.
31. Guzman K, Gray TE, Yoon JH, Nettesheim P. Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5AC mRNA levels by retinoids. Am J Physiol 1996;271:L1023-8.
32. Kumar RK, Herbert C, Foster PS. Expression of growth factors by airway epithelial cells in a model of chronic asthma: regulation and relationship to subepithelial fibrosis. Clin Exp Allergy 2004;34:567-75.
33. Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. Am J Physiol 1999;276:L549-55.
34. Le Gall SM, Auger R, Dreux C, Mauduit P. Regulated cell surface pro-EGF ectodomain shedding is a zinc metalloprotease-dependent process. J Biol Chem 2003;278:45255-68.
35. Lemjabbar H, Li D, Gallup M, Sidhu S, Drori E, Basbaum C. Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. J Biol Chem 2003;278: 26202-7.
36. Kai H, Yoshitake K, Hisatsune A, Kido T, Isohama Y, Takahama K, et al. Dexamethasone suppresses mucus production and MUC-2 and MUC-5AC gene expression by NCI-H292 cells. Am J Physiol 1996;271:L484-8.
37. Gollub EG, Waksman H, Goswami S, Marom Z. Mucin genes are regulated by estrogen and dexamethasone. Biochem Biophys Res Commun 1995;217:1006-14.
38. Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, et al. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. Am J Respir Crit Care Med 2003;167:50-6.

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2006;139:25–30
DOI: 10.1159/000089519

Received: May 23, 2005
Accepted after revision: August 18, 2005
Published online: November 3, 2005

Lack of Association between the *IL13* Variant Arg110Gln and Susceptibility to Cedar Pollinosis in a Japanese Population

Lei Cheng[a, b] Tomomitsu Hirota[c] Tadao Enomoto[d] Mayumi Tamari[c] Mitsuteru Akahoshi[c] Akira Matsuda[c] Makiko Shimizu[c] Naomi Takahashi[c] Keisuke Enomoto[e] Akiko Yamasaki[b] Xiao-Quan Mao[b] Julian M. Hopkin[f] Taro Shirakawa[b]

[a]International Research Center for Nasal Allergy, Nanjing Medical University, and Department of Otorhinolaryngology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China; [b]Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, [c]Laboratory for Genetics of Allergic Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, [d]Department of Otolaryngology, Japanese Red Cross Society Wakayama Medical Center, Wakayama, and [e]Department of Otolaryngology and Sensory Organ Surgery, Osaka University Medical School, Osaka, Japan; [f]Asthma and Allergy Research Group, University of Wales Swansea School of Medicine, Swansea, UK

Key Words
Allergic rhinitis · Candidate gene · Hay fever · Interleukin-13 · Japanese cedar pollinosis · Single nucleotide polymorphism

Abstract
Background: Interleukin (IL)-13 has come to be appreciated as a molecule critically involved in allergic inflammatory responses. Recent studies revealed that a common variant in the coding region of the *IL13* gene, Arg110Gln, has been implicated in the development of asthma and atopy. ***Methods:*** To assess whether the *IL13* variant Arg110Gln is associated with cedar pollinosis, one of the most common atopic diseases in the Japanese population, we examined the Arg110Gln variant using PCR-RFLP to compare the genotype and allele frequencies between 95 patients with cedar pollinosis and 95 healthy control subjects. Relationships between the Arg110Gln variant and the pollinosis-related traits, e.g. rhinitis severity, eosinophil counts in nasal secretion and serum total and allergen-specific IgE levels, were also investigated. ***Results:*** The frequencies of the minor allele Gln110 were 25.8% in patients with cedar pollinosis and 30.9% in healthy control subjects ($p > 0.05$). There was also no significant difference in the genotype frequencies between cases and controls ($p > 0.05$). In addition, we found no significant association of the Arg110Gln variant with any of the pollinosis-related phenotypes ($p > 0.05$). ***Conclusions:*** Our data suggest lack of evidence for identifying the variant Arg110Gln at the *IL13* locus as a genetic risk factor involved in the development of Japanese cedar pollinosis.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/06/1391–0025$23.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Lei Cheng
Department of Otorhinolaryngology
The First Affiliated Hospital of Nanjing Medical University
300 Guangzhou Road, Nanjing 210029 (China)
Tel. +86 25 8373 3043, Fax +86 25 8372 4440, E-Mail teirai@hotmail.com