and reduced their severity throughout the season. Furthermore, there were neither local nor systemic side effects, as reported elsewhere for other antigens. SLIT for cedar pollinosis is a new therapy and in the future SLIT may by indicated for patients with nasal allergy caused by other allergens such as house dust mites or animal dander through improvement of the administration schedule and establishing the dose at which the most potent effects are achieved. This study may contribute to the methodology for the future immunotherapy in Japan.

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

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ORIGINAL ARTICLE Asthma and Rhinitis

Gene-expression profiles in human nasal polyp tissues and identification of genetic susceptibility in aspirin-intolerant asthma

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Clinical & Experimental Allergy

Summary

Background Aspirin-intolerant asthma (AIA) is a subtype of asthma induced by non-steroidal anti-inflammatory drugs and characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). The lower airway lesion and the nasal polyp in AIA are postulated to have common pathogenic features involving aspirin sensitivity that would be reflected in the gene expression profile of AIA polyps.

Objective This study was conducted to clarify the pathogenesis of AIA using gene expression analysis in nasal polyps, and identify genetic susceptibilities underlying AIA in a case–control association study.

Methods Global gene expression of nasal polyps from nine AIA patients was examined using microarray technology in comparison with nasal polyps from five eosinophilic sinusitis (ES) patients, a related disease lacking aspirin sensitivity. Based on the AIA-specific gene expression profile of nasal polyp, candidate genes for AIA susceptibility were selected and screened by a case–control design of 219 AIA patients, 374 non-asthmatic control (CTR), and 282 aspirin-tolerant asthmatic (ATA) subjects.

Results One hundred and forty-three elevated and three decreased genes were identified as AIA-specific genes that were enriched in immune response according to Gene Ontology analysis. In addition, a *k*-means-based algorithm was applied to cluster the genes, and a subclass characteristic of AIA comprising 18 genes that were also enriched in immune response was identified. By examining the allelic associations of single nucleotide polymorphisms (SNPs) of AIA candidate genes relevant to an immune response with AIA, two SNPs, one each of *INDO* and *IL1R2*, showed significant associations with AIA ($P = 0.011$ and 0.026 after Bonferroni's correction, respectively, in AIA vs. CTR). In AIA–ATA association analysis, modest associations of the two SNPs with AIA were observed.

Conclusion These results indicate that *INDO* and *IL1R2*, which were identified from gene expression analyses of nasal polyps in AIA, represent susceptibility genes for AIA.

Keywords aspirin-intolerant asthma, candidate genes, genetic association, genome-wide gene expression, single nucleotide polymorphism

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Introduction

In some asthmatic patients, aspirin and several other non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase enzymes (COXs) induce a severe asthmatic attack, a disease known as aspirin-intolerant asthma (AIA) [1, 2]. Several large surveys have concluded that the incidence of AIA in adult asthmatic patients is 5–15% based on patients' histories alone, but the frequency becomes two to three times higher when adult asthmatic

patients are challenged with aspirin. In women, AIA is overrepresented in a ratio of 2.3 : 1 and is more severe and has an earlier onset. AIA patients have typical clinical features including asthma, aspirin sensitivity, and bilateral nasal polyps, known as Samter's triad. Despite the well-defined pharmacological trigger, the molecular pathogenesis of AIA is still unclear. The usual hypothesis is a disturbance in the metabolism of arachidonic acid, because aspirin and NSAIDs target COXs, key enzymes of the prostaglandin biosynthetic pathway. However, the precise pathogenesis requires further investigation.

There is a moderate genetic background in AIA: the European Network on Aspirin-Induced Asthma found that 5.8% of 500 AIA patients had a family history of aspirin sensitivity [3]. First, a polymorphism in the promoter of leukotriene C_4 synthase, A-444C, was reported to be associated with AIA in Polish patients [4, 5]. A recent report showed that a haplotype of the 5-lipoxygenase gene was weakly associated with AIA in a Korean population [6]. With an extensive candidate gene analysis related to arachidonic acid metabolism, our group reported that single nucleotide polymorphisms (SNPs) in the prostaglandin E_2 receptor subtype 2 gene were significantly associated with AIA, and the functional impact of a promoter variant was further demonstrated [7]. Most recently, SNPs in prostaglandin E_2 receptor subtype 3 gene were associated in Korean population [8].

In the past few years, microarray techniques for gene expression profiling have been applied to a wide range of biological problems and have contributed to the discoveries of complex networks of biochemical processes underlying complex diseases. Microarray techniques have also helped to identify novel biomarkers, disease subtypes, and discrepancies of gene expression in human populations. Despite the advances in microarray techniques, application of the technology to identify susceptibility genes underlying complex diseases appears to be unsuccessful so far, with some exceptions [9, 10].

AIA is characterized by an aggressive mucosal inflammation of the lower airway (asthma) and the upper airways (rhinitis and nasal polyp). Rhinitis symptoms first occur in most AIA patients before the development of asthmatic intolerance to aspirin and other NSAIDs, whereas nasal polyps in AIA patients are first diagnosed at almost the same time aspirin intolerance appears [3]. We postulated that the lower airway lesion and the polyp in AIA have a common pathophysiology of aspirin intolerance, suggesting the nasal polyp as a pleiotropic genetic model of the bronchial inflammation of AIA. Global gene expression of the nasal polyps of AIA patients was examined using microarray technology for comparison with nasal polyps of eosinophilic sinusitis (ES) patients: ES is typically characterized by a nasal polyp with an inflammatory cell infiltration similar to that in an AIA polyp but without aspirin sensitivity, thus being an appropriate reference for the selection of AIA-specific genes.

Materials and methods

Nasal polyp tissues and Aspirin-Intolerant Asthma Subjects

Nasal polyp tissues for microarray analysis were obtained from nine Japanese patients (aged from 35 to 76 years, five males/four females) with AIA, five (aged from 34 to 73 years, three males/two females) with ES, and two (aged 61 and 71 years, both males) with only chronic sinusitis (CS) (Table 1). These patients had not been exposed to preoperative treatment with steroids for at least 1 year before surgery. According to the definition of rhinosinusitis, CS with nasal polyps with eosinophilic inflammatory features without fungal hyphae includes aspirin-sensitive and aspirin-tolerant types [11]. Thus, three groups of patients with nasal polyps were sequentially defined as follows: first, CS with nasal polyps was diagnosed based on clinical symptoms, such as nasal discharge, postnasal drip, headache, hyposmia, and nasal obstruction, and endonasal findings of muco-purulent secretion and nasal polyps with a paranasal shadow observed by CT examination [12]. Among CS patients with nasal polyps, ES patients were identified histologically by counting the number of eosinophils at ×200 magnification under light microscopy. Five fields were examined for each section,

Table 1. Clinical characteristics of patients with nasal polyps for microarray analysis

ID	Age/ gender	Parameters in peripheral blood: WBC (/mm^3)	Parameters in peripheral blood: Eosinophil (%)	Allergic rhinitis	Asthma	AIA episode
AIA#1	76/M	8000	3	–	+	+
AIA#2	48/M	5500	13	–	+	+
AIA#3	73/M	6500	3	–	+	+
AIA#4	59/F	9500	28	–	+	+
AIA#5	50/F	5720	14	–	+	+
AIA#6	40/M	9100	4	–	+	+
AIA#7	35/M	8800	6	–	+	+
AIA#8	50/F	6000	9	+	+	+
AIA#9	66/F	7000	8	–	+	+
ES#1	73/F	7200	2	–	+	–
ES#2	64/F	6400	23	–	+	–
ES#3	69/M	7700	4	+	–	–
ES#4	61/M	4900	5	–	+	–
ES#5	34/M	6300	3	+	+	–
CS#1	61/M	7400	10	–	+	–
CS#2	67/M	9700	10	–	–	–

M, male; F, female; WBC, white blood cell; –, no allergic rhinitis, no asthma, or no AIA episode; AIA, aspirin-intolerant asthma; CS, chronic sinusitis; ES, eosinophilic sinusitis.

and the average was considered to be the number of eosinophils infiltrating the sample. Nasal polyps having more than 100 eosinophils were classified as ES [12]. Among ES patients, those who had had apparent episodes of asthma attacks in response to aspirin and other NSAIDs were classified as AIA patients (AIA#1–9). The remaining five ES patients without AIA episodes (ES#1–5) had no troubles even after taking NSAIDs in postoperative courses during hospitalization. The oral provocation test for AIA patients was not performed in most of the patients due to potential risk, although severe reactions against the provocation were improbable [13], and only verbal history has yielded some false positives [14]. The ethics committees of Kagoshima University approved the study protocols, and each participant gave written informed consent.

DNA samples from 219 unrelated individuals with AIA (age: 55.7±13.5 years; 70 males/149 females) and 374 non-asthmatic controls (CTR) (age: 44.5±23.2 years; 181 males/193 females) were obtained as described previously [7]. For AIA-associated SNPs, 282 unrelated individuals with aspirin-tolerant asthma (ATA) (age: 56.0±16.1 years; 132 males/150 females) [7] were also genotyped, and used as asthmatic controls. The subjects were recruited at Niigata University Hospital, University of Tokyo Hospital, Nagoya University Hospital, Doai Memorial Hospital, and Kyushu University Hospital, with Institutional Review Board approvals. The diagnosis of AIA was based on a self-reported history due to the potential risk of a provocation test. ATA was defined as adult asthma diagnosed by expert physicians according to the American Thoracic Society criteria [15] and no history of aspirin or NSAID-induced asthmatic attack, and comprised of 154 atopic asthmatic (age: 48.0±15.6 years; 80 male/74 female) and 128 non-atopic asthmatic (age: 65.9±10.0 years; 52 male/76 female) subjects. CTR were outpatients with diseases (e.g., hypertension) other than respiratory diseases including asthma, and who self-reported no history of aspirin sensitivity. The patients and controls were all of Japanese ethnicity. Although the Japanese population is thought to be genetically homogenous, nearly identical numbers of patients and controls from the various locations were recruited to avoid geographical differences in allelic frequencies.

RNA extraction

The nasal polyp tissue was removed during endoscopic sinus surgery, submerged in RNA*later* regent (Ambion Inc., Austin, TX, USA) to avoid RNA degradation, and used for RNA extraction within 48 h after resection. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were analysed using the Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) with an RNA6000 Nano LabChip Kit (Agilent Technologies). RNAs from two CS patients were equally pooled, and used as a common reference in the two-colour microarray experiments, where a single microarray was used to compare each test sample from an AIA or an ES patient with the reference sample.

cRNA synthesis, labelling, hybridization, and expression profiling

For fluorescent cRNA synthesis, high-quality total RNA (150 ng) was labelled with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In this procedure, cyanine 5-CTP (Cy5) and cyanine 3-CTP (Cy3) (PerkinElmer, Boston, MA, USA) were used to generate labelled cRNA from the individual AIA or ES RNA and the pooled CS RNA as a reference, respectively. Labelled cRNAs (0.75 μg each) from the AIA, ES, or CS patients were fragmented in a hybridization mixture with the In Situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's instructions. The mixture was hybridized for 17 h at 65 °C to an Agilent Human 1A(v2) Oligo Microarray. After hybridization, the microarray was washed with SSC buffer, and then scanned in Cy3 and Cy5 channels with the Agilent DNA Microarray Scanner, model G2565AA. Signal intensity per spot was generated from the scanned image with Feature Extraction Software ver7.5 (Agilent Technologies) in default settings. Spots that did not pass quality control procedures with the software were flagged and removed for further analysis.

GeneSpring software GX 7.3 (Agilent Technologies) was used for the Lowess (locally weighted linear regression curve fit) normalization of the ratio (Cy5/Cy3) of the signal intensities generated in each microarray and the subsequent data analysis. To determine the AIA-specific expression profile of nasal polyps, ES transcripts with ratios ranging from 0.5 to 2 were extracted, and the AIA transcripts with expression undergoing a twofold change or more were extracted as decreased or elevated genes. Of the transcripts overlapping the two groups, only those with statistically significant differences in expression between the AIA and CS nasal polyps (Benjamini and Hochberg false discovery rate (FDR)<0.01; [16]) were counted as AIA-specific genes. To identify novel expression patterns in nasal polyps from AIA patients, the *k*-means method [17], a well-known unsupervised partitional clustering approach, was applied to the AIA-specific genes. For functional subclassification of the AIA-specific genes, we applied the Gene Ontology (GO) classification for biological processes with DAVID 2.1 (http://david.abcc.ncifcrf.gov/), a web-accessible program [18]. A permutation test with 10 000 iterations was used for multiple test correction when nasal polyps from AIA

and ES patients were compared at the transcriptome level [19]. $P<0.05$ was considered significant in every statistical analysis.

Quantitative real-time reverse transcription polymerase chain reaction analysis

Two transcripts, *INDO* and *IL1R2*, that were differentially expressed between AIA and CS nasal polyps were subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for verification of the microarray data, using a validation set of total RNAs from AIA ($n=10$) and CS ($n=4$) nasal polyps including nine AIA and two CS samples for the present microarray experiment. Total RNA from each nasal polyp was used as a template in first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Tokyo, Japan) with TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The relative quantification method [20] was used to measure the amounts of the respective genes in nasal polyps, normalized to *GAPDH* as an endogenous control. The statistical significance in gene expression between the AIA and the CS samples was determined by the Welch t-test; $P<0.05$ was considered significant.

Single nucleotide polymorphism genotyping

For gene-based association analysis, SNPs of AIA candidate genes were obtained from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) using SNPbrowser Software (Applied Biosystems), to cover the entire regions of the genes positionally and genetically. SNPs were genotyped using the TaqMan SNP Genotyping assay (Applied Biosystems) with the allelic discrimination software SDS version 2.1 (Applied Biosystems) on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis of association study

Differences in allelic frequencies were evaluated by a case–control design with a χ^2 test. Haplotype frequencies for multiple loci were estimated using the expectation-maximization method with SNPAlyze v6.0 software (DYNACOM, Mobara, Japan). Bonferroni's correction was adopted for each gene and haplotype for multiple test correction.

Pairwise LD was estimated as $D=x_{11}-p_1q_1$ where x_{11} is the frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A1 and B1 at loci A and B, respectively. A standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where p_2 and q_2 are the frequencies of the other alleles at loci A and B, respectively [21]. Lewontin's coefficient, D', is given by $D'=D/D_{max}$, where $D_{max}=\min(p_1q_2, p_2q_1)$ when $D>0$ or $D_{max}=\min(p_1q_1, p_2q_2)$ when $D<0$ [22].

The power of the present association analysis was calculated using 'Genetic Power Calculator [23] (http://pngu.mgh.harvard.edu/~purcell/gpc/)'. Using our sample sizes in the AIA–CTR comparison, the study has had 80% power to detect common alleles (risk allele frequency = 0.1) with a relative risk of 1.65, and 50% power to detect the alleles with a relative risk of 1.44 at a threshold of nominal P-value = 0.05 under an additive model in the log-odds scale.

Results and discussion

Microarray analysis of nasal polyp tissues of Aspirin-Intolerant Asthma patients

Bronchial biopsy specimens from AIA patients exhibit a fourfold increase in eosinophils compared with those from ATA patients [24]. The increased influx of eosinophils into the airway mucosa of AIA patients is likely a result of an inflammatory rather than an atopic mechanism. It is noteworthy that the nasal polyps of AIA patients show very similar pathological characteristics such as infiltration of eosinophils into the bronchial mucosa [12, 13]. These observations led us to postulate a common molecular mechanism in the development of a polyp and AIA. In such a case, genes related to nasal polyp development in AIA patients might suggest both potential susceptibility genes and pathways involved in aspirin hypersensitivity and the development of AIA. Because it is not practical to apply bronchial tissues for microarray analysis, we used nasal polyp tissues from AIA patients that were under resection for therapeutic purpose and monitored global gene expressions to demonstrate AIA-specific gene expression profiles. ES is known to be a related disorder of AIA; ES is typically characterized by a nasal polyp with inflammatory cell infiltration similar to that in an AIA polyp but without aspirin sensitivity, thus being an appropriate reference for the selection of AIA-specific genes.

The global gene expression profiles of AIA nasal polyps and those of ES nasal polyps were then compared. Similar expression profiles were expected in polyps of AIA and ES patients due to the similar histological and biochemical characteristics such as extensive infiltration of eosinophils. Figure 1a shows a hierarchical clustering (HC) dendrogram for the profiles of nasal polyps from nine AIA and five ES patients. Unexpectedly, two discrete clusters appeared, representing AIA and ES nasal polyps, respectively, with the exception of one (ES#5) of the ES tissues, from a patient who was aspirin tolerant and had clinical characteristics similar to those of other ES patients

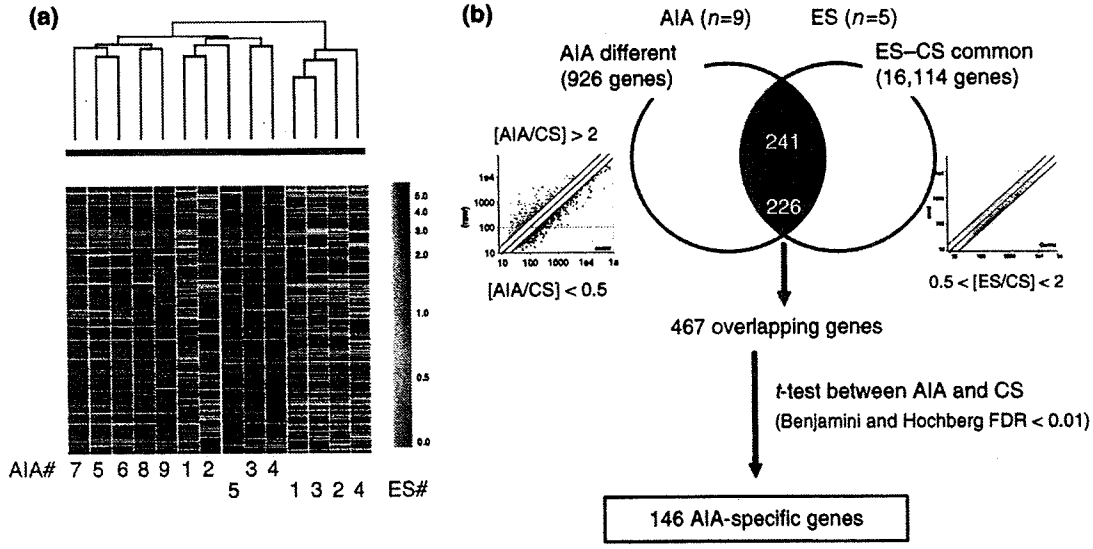

Fig. 1. Experimental design to extract aspirin-intolerant asthma (AIA)-specific genes with microarray analysis. (a) Hierarchical clustering (HC)-based classification of nasal polyps from AIA and eosinophilic sinusitis (ES) patients. Based on the gene expression in nasal polyps, HC clustering shows distinct expression profiles in AIA and ES patients. The clinical characteristics of the patients for the expression analysis are summarized in Table 1. (b) Strategy for discovering AIA-specific gene expression profiles, referred to as 'AIA-specific genes'. From 16,114 genes representing no change (less than twofold) in gene expression between ES and chronic sinusitis (CS) polyps, 146 AIA candidate genes were extracted at the threshold of twofold differences in expression with statistical significance (FDR < 0.01) between AIA and CS polyps.

(Table 1). Thus, AIA and ES nasal polyps appear to exhibit distinct expression profiles. The HC analysis was supported statistically in that 4012 of 18716 transcripts surveyed by microarray displayed significant differences in expression between the AIA and the ES polyps using a permutation test, followed by Student's *t*-test at a significance level of 0.05. While the expression differences between the two groups could be due to an inter-group variation in cell composition within the nasal polyp tissues, they could not have been due to aspirin sensitivity, and so a two-step selection process was used to extract an AIA-specific expression profile (Fig. 1b). We first obtained genes (16114 genes) common to polyp formation, i.e., genes showing no difference (less than twofold) between ES and chronic sinusitis (CS) polyps, in which a difference in the pathological state of the polyps such as infiltration of inflammatory cells would be minimized. We then selected 926 genes differentially expressed between AIA and CS polyps showing twofold differences, which could be related to the pathophysiology of aspirin sensitivity. From the overlapping genes (467 genes) between the two gene lists, 146 genes were statistically extracted including 143 elevated and three decreased transcripts that were defined as AIA-specific genes.

To examine the biological features of these AIA-specific genes, we assigned 146 genes to the GO classification using the web-accessible DAVID program. As shown in Table 2, nine GO terms were highly associated with the AIA-specific genes. It is notable that the genes involved in cell proliferation and immune response were enriched in the AIA candidate genes, indicating successful extraction of the genes related to nasal polyp formation, because both nasal cell growth and acute inflammation in the respiratory tract are clinical characteristics during the development of nasal polyp in AIA patients.

We then applied the *k*-means algorithm [17], an unsupervised partitional clustering approach, to organize AIA-specific genes into functionally meaningful groups. The *k*-means method has been efficient in showing a significant enrichment of genes belonging to given functional categories in the *k*-means-based clusters [25]. In this analysis, we selected an optimal number of clusters (*k*) in which the number of unclassified genes was minimized. As shown in Table 2, four distinct clusters, subsets 1, 2, 3, and 4, in gene expression were generated for the AIA-specific genes using the *k*-means method (figure not shown). Thus, four types of distinct expression patterns across samples were observed using the dataset of 146 AIA-specific genes. The three genes with decreased expression in AIA nasal polyps were categorized into subset 4, and the 143 elevated genes were classified into three subsets, 1, 2, and 3 (Table 2). Interestingly, genes involved in immune response (18/21 genes) and response to external signal (11/15 genes) were highly enriched in subset 2, while another enrichment of cell proliferation-related genes (17/24 genes) was observed in subset 1 (Table 2). These features of the gene enrichments indicate the biological significance of the present *k*-means-based clusters for AIA candidate genes. According to the cluster–GO correlation, the most notable functional patterning occurred for genes relevant to an immune response owing to the highest concentration (85.7% of the genes

Table 2. Enrichements of genes involved in GO-functional categories within *k*-means-based clusters

GO TERM (biological process; level 3)	Count	*P*-value	*k*-means clusters (no. of genes assigned) Subset 1 ($n = 57$)	Subset 2 ($n = 57$)	Subset 3 ($n = 29$)	Subset 4 ($n = 3$)
Cell proliferation	24	0.0000028	17	7		
Immune response	21	0.068		18	3	
Biopolymer metabolism	20	0.034	9	7	3	
Response to stress	17	0.00097	4	11	2	
Response to external stimulus	15	0.083		11	4	
Catabolism	14	0.017	5	5	4	
Cell organization and biogenesis	12	0.031	2	8		1
Cell motility	6	0.012	2	4		
Cellular defense response	4	0.024		3	1	

DAVID v2.1 (http://david.abcc.ncifcrf.gov/) was used to classify 146 AIA-specific genes functionally according to Gene ontology (GO) classification for biological process. Genes in the respective GO categories were mapped to four *k*-means-based clusters for gene expression.

extracted) in one subset (subset 2) of clusters, indicating that their expression might be highly coordinated in nasal polyps.

Association study with candidate genes for AIA

Based on functional clustering of the AIA-specific genes in the *k*-means clustering, immune response-related genes might serve as candidate genes for susceptibility underlying AIA because the AIA-specific changes in gene expression reflect elevated immune and inflammatory reactions in the nasal polyps of AIA patients. Table 3 shows 21 immune response-related genes in descending order based on the expression ratios in the microarray analysis. We focused on the three top-ranked genes, *INDO*, *IL1R2*, and *CLECSF6*, and screened 17 SNPs of these three genes (three SNPs for *INDO*, 11 SNPs for *IL1R2*, and three SNPs for *CLECSF6*) for an allelic association study between 219 AIA patients and 178 non-asthmatic controls (CTR) in the first screening. One SNP of *INDO* and four SNPs of *IL1R2* were significantly associated with AIA evaluated by a simple χ^2 test (data not shown) based on nominal *P*-values. Differential expressions of the two genes, *INDO* and *IL1R2*, in AIA nasal polyps were confirmed by real-time RT-PCR (Fig. 2). Because the three SNPs of *CLECSF6* examined were not associated with AIA, the gene was not pursued, and other ranked genes were also not screened further.

After increasing the sample size of CTR to 374 subjects, we further examined the allelic associations of a total of 14 SNPs (three SNPs for *INDO* and 11 SNPs for *IL1R2*) with AIA in a second screening. As shown in Tables 4 and 5, one SNP of *INDO* (INDO-SNP2: rs7820268) and one SNP of *IL1R2* (IL1R2-SNP10: rs11688145) showed significant associations with AIA after a multiple test correction using Bonferroni's correction (corrected $P = 0.011$ for the INDO-SNP2 and corrected $P = 0.026$ for the IL1R2-SNP10). None of the SNPs in the controls showed deviation from Hardy–Weinberg's equilibrium (data not shown). Both the significant SNPs observed were located in non-coding regions of the respective genes, and so the functional impacts of the SNPs were not demonstrated. In order to examine whether *INDO* and *IL1R2* were genetic

Table 3. Twenty-one genes involved in *immune response*

Expression ratio (normalized)	Gene Symbol	Name
1 3.70	INDO	Indoleamine-pyrrole 2,3 dioxygenase
2 3.31	IL1R2	Interleukin 1 receptor, type II
3 2.75	CLECSF6	C-type lectin, superfamily member 6
4 2.68	CCL11	Chemokine (C-C motif) ligand 11
5 2.65	CD163	CD163 antigen
6 2.63	TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10
7 2.53	AIF1	Allograft inflammatory factor 1
8 2.46	NCF2	Neutrophil cytosolic factor 2
9 2.32	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
10 2.31	FPR1	Formyl peptide receptor 1
11 2.21	TYROBP	TYRO protein tyrosine kinase-binding protein
12 2.21	CTSC	Cathepsin C
13 2.11	IFI30	Interferon, gamma-inducible protein 30
14 2.03	MICB	MHC class I polypeptide-related sequence B
15 1.90	LCP2	Lymphocyte cytosolic protein 2
16 1.86	NCK1	NCK adaptor protein 1
17 1.84	LST1	Leukocyte-specific transcript 1
18 1.83	TLR2	Toll-like receptor 2
19 1.76	PTAFR	Platelet-activating factor receptor
20 1.71	CKLF	Chemokine-like factor
21 1.65	EDG6	Endothelial differentiation, G-protein-coupled receptor 6

susceptibility genes underlying aspirin hypersensitivity, we further genotyped the two significant SNPs in 282 ATA patients for comparison. In the AIA–ATA association study, the INDO-SNP2 also showed a statistically significant association with AIA ($P = 0.038$) (Table 4), whereas an association of the IL1R2-SNP10 with AIA was marginal ($P = 0.073$) with the same direction of genetic effect of the associated allele on AIA susceptibility (Table 5). In contrast, no significant differences in allele frequencies at the two SNPs were observed between CTR and ATA groups (statistical data not shown). These SNP-based association results indicate that the two SNPs in *INDO* and *IL1R2* are associated with the risk of aspirin hypersensitivity rather than an asthmatic reaction in Japanese population.

Figure 3 shows D'- and r^2-based LD block structures in the genomic regions around *INDO* and *IL1R2*, respectively. We observed a strong LD ($|D'| = 0.98$) between INDO-SNP1 and -SNP2 in the *INDO* region. A highly structured LD pattern, a major LD block structure ($|D'| > 0.7$) covered by IL1R2-SNP6 to -SNP11, was observed in *IL1R2* (Fig. 3). Next, we conducted a haplotype-based association study within the respective LD blocks (Tables 6 and 7). We found that one haplotype of *INDO*, m/m (double minor haplotype) at INDO-SNP1 and -SNP2, was underrepresented in AIA with statistical significance after multiple test correction with Bonferroni's correction (Table 6; $\chi^2 = 6.74$, df = 1, corrected $P = 0.038$), indicating a protective effect of the m/m haplotype of *INDO*. One haplotype of *IL1R2*, M/M/M (triple major haplotype) at IL1R2-SNP6, -SNP10, and -SNP11, showed a highly significant difference between AIA and CTR (Table 7; $\chi^2 = 8.94$, df = 1, corrected $P = 0.011$), indicating that the M/M/M haplotype represented a risk for AIA.

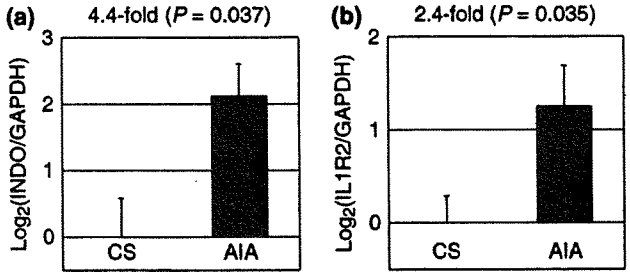

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis validates expression differences for two genes, *INDO* and *IL1R2*, in nasal polyps from aspirin-intolerant asthma (AIA) and chronic sinusitis (CS) patients. Relative amounts of the respective genes in nasal polyps (AIA, $n = 10$; CS, $n = 4$) were measured by real-time RT-RCR using TaqMan Gene Expression Assays. *Y*-axes indicate the $\log_2$ relative expression levels, normalized to the amount of *GAPDH* and relative to the averaged expression levels in CS groups. The expression levels of *INDO* and *IL1R2* were significantly higher in AIA than CS nasal polyps by the Welch *t*-test.

INDO encodes indoleamine 2, 3-dioxygenase, which is a rate-limiting enzyme of tryptophan catabolism and is expressed in various cell types such as fibroblasts, macrophages, and dendritic cells [26]. INDO activity is induced by interferons (IFNs) and further enhanced by inflammatory cytokines such as IL-1 [27] but suppressed by anti-inflammatory cytokines such as TGF-β and IL-4 [28, 29]. INDO-induced tryptophan degradation in macrophages results in inhibition of T cell proliferation [30], suggesting that INDO plays an important role in the regulation of T cell-mediated immune responses. Aspirin inhibits INDO activity in stimulated peripheral blood mononuclear cells indirectly, via its inhibitory effect on the production of IFN-γ [31]. Therefore, the functional disturbance of INDO activity due to the INDO-SNP2 might play a role in the pathogenesis of aspirin sensitivity or AIA. A possibility remains that an unknown SNP in tight LD with the INDO-SNP2 or on the m/m haplotype could serve as a *bona fide* causality, which could prevent AIA induction by keeping INDO activity normal. Further studies are needed to resolve the functional significance of the INDO-SNP2 and the m/m haplotype in the genetic aetiology of AIA.

Interleukin 1 receptor type II (IL1R2) acts as a soluble decoy receptor that inhibits IL-1 signalling [32]. The inhibition of IL-1 binding to the receptor in human monocytes results in a reduction of COX-2 activity but not COX-1 activity [33]. As an imbalance in arachidonate

Table 4. Allelic association of *INDO* SNPs with AIA in Japanese population

					MAF		AIA vs. CTR				MAF	AIA vs. ATA	
SNP no.	Position*	Localization	dbSNP ID	Alleles (M/m)†	AIA $n = 219$	CTR $n = 374$	χ^2	Odds ratio (95% CI)	P	Corrected P‡	ATA $n = 282$	χ^2	P
INDO-SNP1	−1953	5′-upstream	rs3808606	T/C	0.414	0.472	3.65	0.79 (0.62–1.01)	0.056	0.17	ND		
INDO-SNP2	6202	intron4	rs7820268	C/T	0.101	0.163	8.47	0.58 (0.40–0.84)	0.0036	0.011	0.145	4.29	0.038
INDO-SNP3	13994	intron9	rs3739319	A/G	0.474	0.436	1.63	1.17 (0.92–1.49)	0.20	0.60	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.

†M and m denote major and minor alleles, respectively, at each SNP site.

‡Corrected *P* values were obtained using Bonferroni's correction.

MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined; SNP, single nucleotide polymorphism.

Table 5. Allelic association of *IL1R2* SNPs with AIA in Japanese population

SNP No.	Position*	Localization	dbSNP ID	Alleles (M/m)†	MAF AIA $n=219$	MAF CTR $n=374$	AIA vs. CTR χ^2	Odds ratio	P	MAF Corrected P‡	AIA vs. ATA ATA $n=282$	χ^2	P
IL1R2-SNP1	−6913	5′-upstream	rs4851519	C/T	0.272	0.219	4.27	1.34 (1.01-1.76)	0.039	0.43	ND		
IL1R2-SNP2	−4381	5′-upstream	rs35789178	T/G	0.368	0.358	0.12	1.05 (0.81-1.34)	0.73	1	ND		
IL1R2-SNP3	−3657	5′-upstream	rs12467316	A/C	0.299	0.376	7.21	0.71 (0.55-0.91)	0.0072	0.080	ND		
IL1R2-SNP4	−3145	5′-upstream	rs12468239	C/T	0.090	0.093	0.02	0.97 (0.64-1.47)	0.89	1	ND		
IL1R2-SNP5	9147	intron1	rs11691240	C/T	0.439	0.479	1.73	0.85 (0.67-1.08)	0.19	1	ND		
IL1R2-SNP6	14513	intron1	rs3755482	A/G	0.273	0.340	5.58	0.73 (0.56-0.95)	0.018	0.20	ND		
IL1R2-SNP7	15413	intron1	rs719250	G/A	0.381	0.318	4.73	1.32 (1.02-1.69)	0.030	0.33	ND		
IL1R2-SNP8	21335	intron3	rs2110562	C/T	0.179	0.193	0.32	0.91 (0.67-1.25)	0.57	1	ND		
IL1R2-SNP9	40304	3′-downstream	rs4851531	T/C	0.462	0.408	3.16	1.25 (0.98-1.59)	0.075	0.83	ND		
IL1R2-SNP10	42202	3′-downstream	rs11688145	C/A	0.244	0.330	9.23	0.66 (0.50-0.86)	0.0024	0.026	0.296	3.22	0.073
IL1R2-SNP11	54346	3′-downstream	rs7588933	A/G	0.186	0.206	0.70	0.88 (0.65-1.19)	0.40	1	ND		

*Numbers indicate the nucleotide position from the first nucleotide of exon 1.

†M and m denote major and minor alleles, respectively, at each SNP site.

‡Corrected P values were obtained using Bonferroni's correction.

MAF, minor allele frequency; AIA, aspirin intolerant asthma; CTR, non-asthmatic control; ATA, aspirin tolerant asthma; ND, not determined

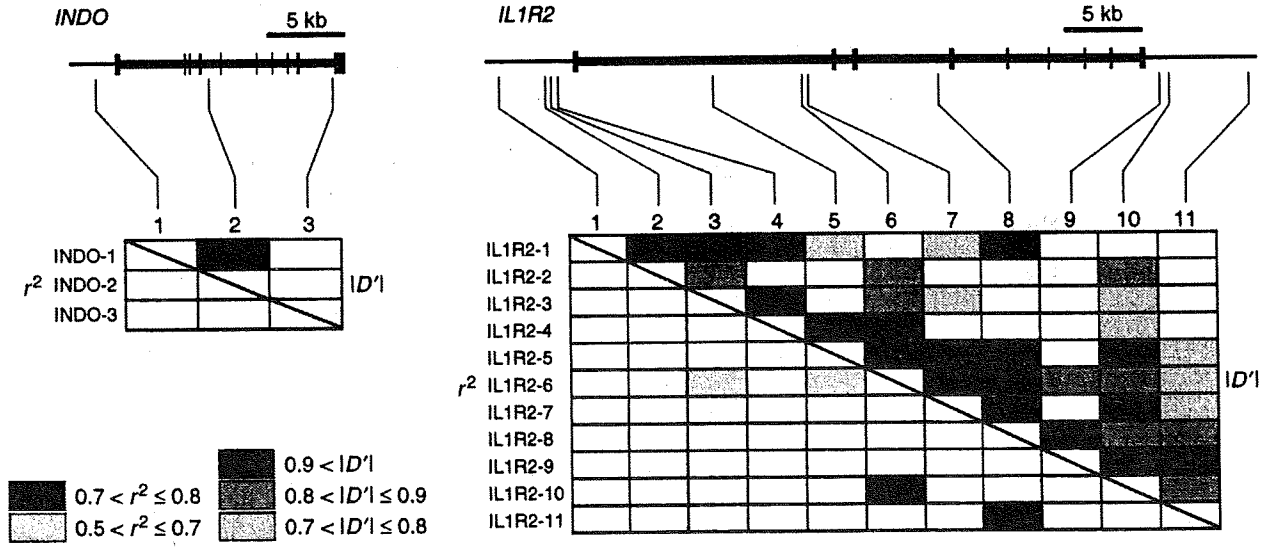

Fig. 3. Linkage disequilibrium pattern of *INDO* and *IL1R2*. The gene structures of *INDO* (left) and *IL1R2* (right), together with positions of the 14 single nucleotide polymorphisms (SNPs) examined, are shown. Pairwise LD coefficients, D' and r^2, were determined and expressed as a block structure. In the schematic block, red boxes indicate a pairwise LD of $|D'|>0.9$, pink $0.9\geq|D'|>0.8$, and orange $0.8\geq|D'|>0.7$; blue boxes indicate a pairwise LD of $0.8\geq r^2>0.7$ and light blue $0.7\geq r^2>0.5$. Blank boxes represent $|D'|\leq 0.7$ or $r^2\leq 0.5$.

metabolism is the usual pathogenesis proposed for AIA, the elevated level of IL1R2 that inhibits the inflammatory effects of IL-1α in the respiratory tract might well be involved in the pathogenesis of AIA and the formation of nasal polyps. Although IL1R2-SNP10 showed the strongest allelic association with AIA (Table 5), the functional impact of an SNP locating 3′-downstream of the gene is currently unclear despite its up-regulated expression in the AIA polyp (Table 3). There is also the possibility that an unidentified functional SNP in LD with the IL1R2-SNP10 could be a causality. Further genetic fine mapping in *IL1R2* will be required to fully understand which genetic variant contributes to the risk of AIA. The functional impacts of the SNP and haplotype also require further investigation.

In conclusion, DNA microarray technology was used to monitor global gene expression patterns specific to AIA nasal polyp tissues to clarify the pathophysiology of AIA. From the gene expression profile, candidate genes underlying AIA were selected and subjected to an association study. We identified SNPs in *INDO* and *IL1R2* that may represent genetic susceptibility to AIA. This genetic study

Table 6. Haplotype-based association of *INDO* with AIA

Haplotype (INDO-SNP1/2)*	Haplotype frequency			χ^2	*P*	Corrected $P^\dagger$
	Total	AIA	CTR			
M/M (T/C)	0.552	0.591	0.531	3.75	0.053	0.21
m/M (C/C)	0.306	0.304	0.307	0.01	0.94	1
m/m (C/T)	0.141	0.105	0.161	6.74	0.0094	0.038
			Global comparison	8.05 (df = 3)	0.045	0.18

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

Table 7. Haplotype-based association of *IL1R2* with AIA

Haplotype (IL1R2-SNP6/SNP10/SNP11)*	Haplotype frequency			χ^2	*P*	Corrected $P^\dagger$
	Total	AIA	CTR			
M/M/M (A/C/A)	0.494	0.552	0.458	8.94	0.0028	0.011
m/m/M (G/A/A)	0.277	0.243	0.299	4.03	0.045	0.18
M/M/m (A/C/G)	0.182	0.170	0.189	0.59	0.44	1
			Global comparison	8.72 (df = 4)	0.069	0.28

*M and m denote major and minor alleles, respectively, at each SNP site. The corresponding nucleotides at the respective sites are shown in parentheses.
†Corrected *P* values were obtained using Bonferroni's correction.
AIA, aspirin intolerant asthma; CTR, non-asthmatic control; SNP, single nucleotide polymorphism.

represents only first-stage evidence of the association because only Japanese individuals were included, and so further replication in independent case–control samples is required to confirm the role of *INDO* and *IL1R2* genotypes in the genetic risk for AIA. A pathophysiological link between the two gene products is unclear and further investigation is evidently needed. In addition, further studies including functional analyses of the SNPs with respect to how genetic variants are responsible for the risk of AIA are also required for a full understanding of the pathogenesis of AIA.

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

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ABSTRACT

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Patients

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

CRS was diagnosed based on clinical symptoms, such as

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

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

CRS = chronic rhinosinusitis.

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

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

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

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

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

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

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

Immunohistochemistry of VEGF in Nasal Glands

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

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

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

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

Light Cycler–PCR Conditions

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

Reverse Transcriptase-Polymerase Chain Reaction

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

Statistics

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

RESULTS

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

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

Immunohistochemistry of VEGF in Nasal Glands

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

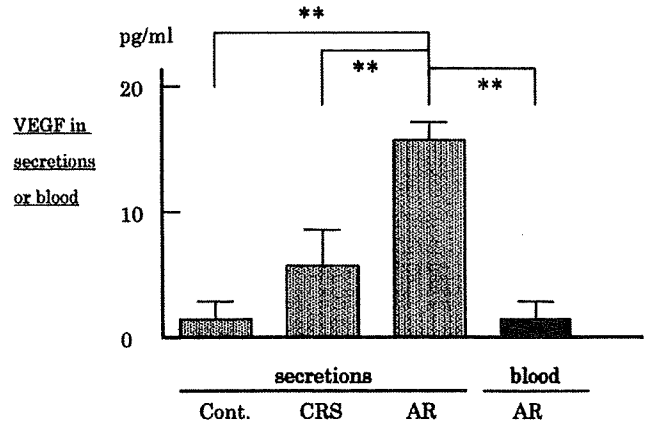

*Figure 1. Vascular endothelial growth factor (VEGF) levels are significantly higher in nasal secretions than peripheral blood in allergic rhinitis patients. Additionally, VEGF levels in nasal secretions are significantly higher in allergic rhinitis than in control or chronic rhinosinusitis patients.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

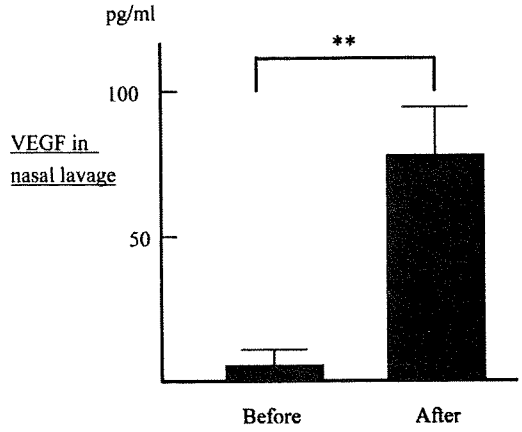

*Figure 2. Vascular endothelial growth factor (VEGF) levels are significantly higher in nasal lavage after the provocation test with house dust. Results represent the average ± SD. **$p < 0.01$. After, after provocation test; Before, before provocation test.*

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

LMD and PCR

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

DISCUSSION

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

Although there are previous reports on VEGF production in seasonal allergic rhinitis,[15,16] this is the first showing more VEGF hyperproduction in allergic rhinitis versus nonallergic rhinosinusitis. Nasal lavage VEGF levels significantly increased after provocation in pollinosis cases, in a prior study

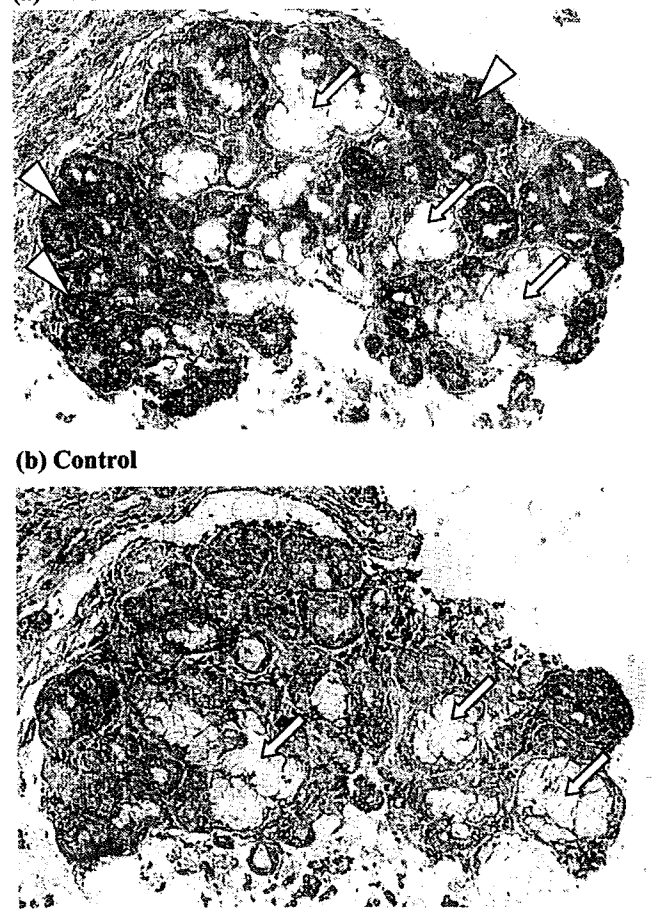

Figure 3. (a) Representative immunohistological vascular endothelial growth factor (VEGF) section is shown. VEGF is positively stained in serous (arrow head) but negative in mucous (arrow) acini of mucosal glands in nasal inferior turbinate mucosa of an allergic rhinitis patient. (b) Control without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

by Benson *et al.*[15] In the present study, such an increase of VEGF was confirmed not only in pollinosis but also in perennial allergic rhinitis. VEGF is thought to be hypersecreted in the acute phase of type 1 allergic reaction irrespective of seasonal or perennial allergic rhinitis. Before analyzing VEGF production in serous and mucous acini at the messenger level, including the isoform patterns, it was imperative to confirm the increase in nasal lavage VEGF after provocation in perennial allergic rhinitis.

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

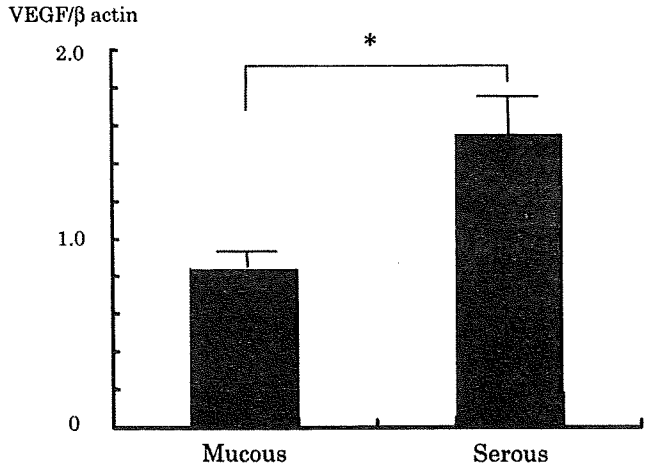

*Figure 4. Light Cycler-PCR confirms total vascular endothelial growth factor (VEGF) mRNA levels (expressed as VEGF/β-actin) is significantly higher in serous versus mucous glands in nasal inferior turbinate mucosa of allergic rhinitis patients. Results represent the average ± SD, *p < 0.05.*

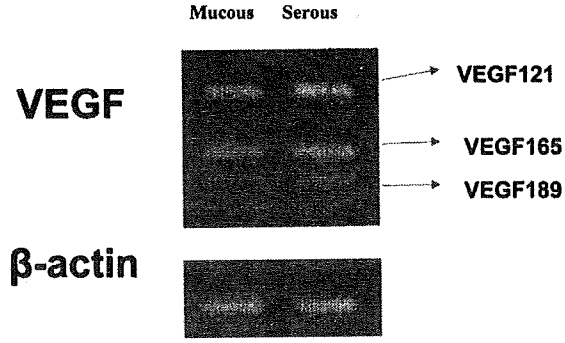

Figure 5. Vascular endothelial growth factor (VEGF) isoform distribution is similar in serous and mucous acini of mucosal glands. The major VEGF subtypes are VEGF121 and VEGF165.

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

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

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

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

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

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

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