

Increased expression of osteopontin is associated with long-term bee venom immunotherapy

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Background: Venom allergen immunotherapy (VIT) is proven to be highly effective for insect allergy, but the mechanisms and the biomarkers associated with clinical efficacy remain elusive.

Objective: The aim of this study was to identify candidate biomarkers associated with successful VIT.

Methods: Gene chip array and clustering analyses of PBMCs from subjects with or without VIT were performed.

Results: From gene chip array and clustering analyses, an increased expression of osteopontin was found in patients who completed 5 to 6 years of VIT and discontinued therapy for 3 to 6 years (completed treatment group) compared with the untreated group. A significantly higher level of serum osteopontin was found in the completed treatment group compared with the untreated group ($n = 16$ in each group; $P < .001$).

Conclusion: The upregulation of osteopontin after VIT suggests a role of osteopontin as a candidate biomarker for VIT. (J Allergy Clin Immunol 2005;115:1063-7.)

Key words: Venom immunotherapy, gene chip microarray, clustering, osteopontin, biomarker

Venom immunotherapy (VIT) reduces the likelihood of a systemic response in patients with systemic allergic reactions to insect venom. VIT is generally effective with less than 3 months of treatment, but persistent protection usually develops after more than 4 years of treatment.^{1,2} Criteria to precisely identify the point in time when protection becomes persistent are unknown. In addition, despite the remote possibility of severe reaction, the supervised sting challenge test is the only available means to evaluate the true efficacy of VIT. The identification of markers that can be used to predict protection is thus needed.

During maintenance VIT, a modest reduction in serum allergen-specific IgE, concomitant with an increase in

Abbreviations used

IFNGR1: IFN- γ receptor 1

MRC1: Mannose receptor, C type 1

PLAU: Urokinase type plasminogen activator

VIT: Venom immunotherapy

allergen-specific IgG,³ particularly of the IgG4 isotype,⁴ has been reported. However, the level of venom-specific IgG antibodies is not an absolute predictor of the outcome of a sting challenge, because patients who are treated for more than 4 years, even those with low venom-IgG levels, do not react to stings.⁵ This observation suggests that immune tolerance to *Hymenoptera* venom becomes established through a non-IgG mechanism after extended maintenance therapy.

Although the evidence accumulated to date has revealed that therapy is associated with many changes in immune cells and cytokines, the mechanism of action has so far remained elusive (see review⁶). The focus has been on the cytokines and mechanisms associated with protection from sting reactions in the early stage of immunotherapy. This approach may have missed and/or obscured the identification of relevant targets that are predictive of long-term clinical outcome. Although the role of IL-10 in the induction of immune tolerance has been demonstrated, its role in rush and conventional VIT is not established. In this report, an increased expression of osteopontin in patients with successful VIT was identified, suggesting that osteopontin is a potential candidate as a biomarker for VIT.

METHODS

Study subjects

Subjects were 18 years of age or older and had been consenting participants in the ongoing insect allergy research project, which included live sting challenge procedures to determine the individual's susceptibility to allergic reactions and to evaluate the efficacy of VIT. All had a history of allergic reactions to insect stings and had evidence of venom-specific IgE antibodies in the skin and/or serum. For gene chip array and validation analysis, a total of 14 individuals allergic to yellow jacket with a positive history of insect sting-induced systemic allergic reactions were divided into 3 different groups (Table I): (1) 5 subjects not on VIT (untreated); (2) 5 subjects on VIT for less than 1

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TABLE I. Study subjects for gene chip analysis

Group	Patient	Age	Sex	Tx# insect	Years on VIT	Years off VIT
Untreated	1*	45	F	—	—	—
	2*	60	M	—	—	—
	3*	36	M	—	—	—
	4	49	M	—	—	—
	5	47	M	—	—	—
Early treatment	1*	42	F	MV	<1 (8 m)	—
	2*	45	F	MV	<1 (11 m)	—
	3*	44	M	MV	<1 (10 m)	—
	4	59	M	MV	<1 (11 m)	—
	5	33	M	MV	<1 (11 m)	—
Untreated	1*	49	F	MV	5	4
	2*	21	M	MV	5	6
	3*	18	M	MV	6	3
	4	42	F	MV	5	3

MV, Mixed vespid.

*Samples used for cDNA microarray.

year (early treatment); and (3) 4 subjects who completed 5 to 6 years of VIT, discontinued VIT for at least 3 years, and remained nonreactive to stings (completed treatment). For measurement of serum osteopontin levels, an additional group consisting of 32 subjects with 16 untreated patients and 16 subjects who completed treatment was included in this study (Table II). Conventional VIT was performed for the patients who had systemic reactions after stings according to current practice parameters and consistent with the product package insert as previously reported.^{7,8} All subjects gave informed consent under a protocol approved by the Institutional Review Board of the Johns Hopkins Medical Institutions.

cDNA microarray analysis

Total RNAs were isolated from the PBMCs and purified by using the RNeasy midi kit (Qiagen, Chatsworth, Calif). For gene chip array analysis, 100 ng of total RNAs from 3 subjects randomly selected from each group were pooled for T7-based RNA amplification (Ambion, Austin, Tex). Gene chip array hybridizations were performed by using human 20K cDNA chips (Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Md). Cy3 and Cy5 derivatized cDNAs were prepared by using random primers and reverse transcriptase according to the manufacturer's instructions (Ambion). Fluorescent cDNAs were competitively hybridized to the gene chips. Raw scanned images of Cy3 and Cy5 fluorescence were processed by using GenePix Pro4.0 microarray image analysis software (Molecular Devices Co, Sunnyvale, Calif), and local background was subtracted from the fluorescent value of each spot to obtain a net value. The log ratio of measured Cy3 and Cy5 values obtained from the image analysis software was computed. Global normalization of expression values was performed to obtain an identical distribution of overall gene expression. For each sample, an independent hybridization was performed, and the mean intensity was calculated and used for subsequent analysis. The hybridization and analysis were performed according to the Minimum Information About a Microarray Experiment guideline (www.mged.org/Workgroups/MIAME/miame.html). A statistical cluster program (GeneSpring 6.2; Silicon Genetics, Redwood City, Calif) was used to analyze the 1751 differentially expressed genes with ≥ 2.0 -fold change between untreated versus early treatment or completed treatment groups.

TABLE II. Demographic characteristics of study participants for analysis of serum osteopontin level*

	Untreated	Completed treatment
Number of subjects	16	16
Age (y)	46.9 $\pm$ 13.0	56.6 $\pm$ 16.8
Sex, F/M	8/8	10/6
Tx insect		
Years on VIT (range)	—	5.88 $\pm$ 1.96 (4-11)
Years off VIT (range)	—	9.62 $\pm$ 5.22 (2-16)

*Data are mean $\pm$ SD. Number of patients: mixed vespid, 5; mixed vespid/polistes, 3; mixed vespid/honey bee/polistes, 3; yellow jacket, 2; honey bee, 1; yellow jacket/honey bee, 1; yellow jacket/yellow hornet, 1.

Validation

To confirm differential expression of the osteopontin gene, total RNAs (250 ng) from each sample were reverse-transcribed to cDNAs by using oligo dT primer, followed by quantitative detection of osteopontin transcripts using TaqMan probes and primers purchased from PE Applied Biosystems (Assays-on-Demand, assay ID Hs00167093; Foster City, Calif) on an ABI PRISM 7700 sequence detector (Applied Biosystems) with the following program: the initial step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The mRNA expression levels for all samples were normalized to the levels for a housekeeping gene, β -actin. Each measurement was set up in duplicate. Monocyte-depleted PBMCs were purified with a magnetic cell separation system (MACS Miltenyl Biotec, Bergisch Gladbach, Germany), and the expression of osteopontin was examined by the use of RT-PCR and osteopontin-specific primers (forward, 5'-CCAAGTAAGTCCAACGAAAG-3'; reverse, 5'-GGTGATGTCCTCGTCTGTA-3'). Monocyte-depleted cell suspensions contained less than 1.0% contaminating CD14⁺ cells. PCR was performed for 5 minutes at 95°C for initial denaturing, followed by 35 cycles of PCR at 95°C for 60 seconds, 52°C for 60 seconds, and 72°C for 60 seconds. The PCR products were visualized under UV light after ethidium bromide staining and the intensity measured and normalized to the intensity for glyceraldehyde 3-phosphate dehydrogenase by using National Institutes of Health image 1.63 (<http://rsb.info.nih.nih-image/>).

Validation of selected genes was also performed on samples originally used for array analysis. Amplified RNAs (1 μ g) were reverse-transcribed to cDNA by using random N18 primer. Amplification of each cDNA was performed with a Taq DNA polymerase by using gene specific primers for *MRC1* (forward, 5'-TGATGGCTACTGGAAGACAGC-3'; reverse, 5'-GAT-CACAATGATGACTACTCC-3'; 604 bp), *IFN- γ receptor 1* (*IFNGRI*; forward, 5'-CTGAGAGTGATCACTCCAGA-3'; reverse, 5'-AGTTGGTGCAACTTAGCTCA-3'; 284 bp), *CD44* (forward, 5'-GAGACCAAGACACATTCCAC-3'; reverse, 5'-TGC-CACTGTTGATCACTAGC-3'; 251 bp), urokinase type plasminogen activator (*PLAU*; forward, 5'-TATCTCTATCCGGAGCAGCT-3'; reverse, 5'-CCAGGGTAAGAAGTGTGAGA-3'; 278 bp), and *G3PDH* (forward, 5'-ACCACAGTCCATGCCATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3'; 432 bp). Each PCR was performed for 5 minutes at 95°C for initial denaturing, followed by 28 to 35 cycles of 95°C for 60 seconds, 52°C to 60°C for 60 seconds, and 72°C for 60 seconds. The PCR products were analyzed with electrophoresis through 2% agarose gel and visualized under UV light after ethidium bromide staining. The intensity was measured and normalized to the intensity for G3PDH by using National Institutes of Health image 1.63.

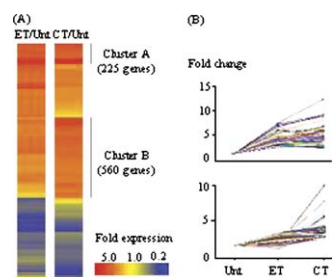


FIG 1. Cluster analysis of 1751 genes with ≥ 2.0 -fold changes; early treatment (ET) versus untreated (Unt) or completed treatment (CT) versus untreated (Unt). **A**, Cluster image of cDNA microarray expression profiles of 1751 genes. The ratio of gene expression levels is represented according to the color scale shown (red, upregulation; blue, downregulation). **B**, Expression profiles of individual genes in cluster A and B. X-axis indicates the fold change of upregulation or downregulation compared with the intensity of the untreated group.

Measurement of serum osteopontin levels

Serum samples from a total of 32 subjects (16 from the untreated group and 16 from the completed treatment group) were collected and subjected to measurement of serum osteopontin levels. Serum osteopontin levels were measured in duplicate by ELISA (IBL Japan, Gunma, Japan).

Statistical analysis

All statistical analyses were performed by using the Stat View 5.0 (1998; SAS Institute Inc, Cary, NC). The serum osteopontin levels were compared by using the Mann-Whitney *U* test or Wilcoxon rank test. A *P* value of $<.05$ was considered statistically significant.

RESULTS

Gene chip array analysis

To identify differentially expressed genes and potential biomarkers associated with VIT, a 2-tiered analytical strategy was used. First, the primary target genes in a core group of patients with and without VIT using gene chip array and clustering approaches were identified. Second, candidate genes were probed and validated in additional samples to increase the validity of the analysis. The microarray hybridization experiments were considered successful when a correlation coefficient of Cy3:Cy5 ratio greater than 0.95 was obtained between 2 independent hybridizations. The gene expression profiles from the untreated group served as the baseline for comparisons with the early and completed treatment groups.

A total of 1751 genes with ≥ 2.0 -fold changes were selected (Fig 1) and subsequently used for hierarchical clustering. Candidate clusters were selected by querying the gene expression data set, derived from the study groups for genes that demonstrate an overall pattern of progressively increasing (or decreasing) expression concordant with different stages of VIT. Among those genes differentially expressed, osteopontin emerged as the leading candidate with higher level of differential expression after VIT, particularly in subjects after 5 to 6 years of successful VIT (1.6-fold, early treatment versus

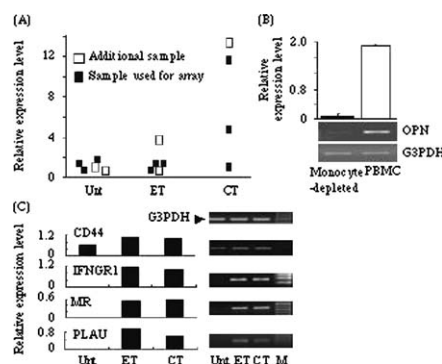


FIG 2. **A**, Osteopontin mRNA expression for 14 individuals from 3 groups by real-time RT-PCR. **B**, Monocytes are the major source of osteopontin expression in PBMC. Osteopontin gene expression of monocyte-depleted cells was compared with PBMC. This shows the representative data from 3 different individuals. **C**, Validation by semiquantitative RT-PCR for selected genes. Semiquantitative RT-PCR was performed for several genes as indicated by using pooled samples.

untreated; 7.0-fold, completed treatment versus untreated), which was validated by real-time RT-PCR assays (Table I; Fig 2, A). Furthermore, monocytes (CD14⁺) were the dominant cell population in the PBMCs expressing osteopontin, because depletion of monocytes significantly reduced the level of osteopontin expression (Fig 2, B).

It is noted that a group of genes encoding products known to be involved in the osteopontin regulatory pathway was also identified in the same clusters (Table III). For example, this includes *CD44* (a receptor for osteopontin), *IFNGR1*, *MRC1* (mannose receptor, C type 1), and *PLAU* (inducible by osteopontin). Higher levels of gene expression for these genes in the completed treatment group were also validated by RT-PCR analyses (Table III; Fig 2). Further, the genes encoding cytokine receptors, such as TNF- α receptor, GM-CSF receptor, and IL-1 receptor, and their associated signaling molecules were also identified in these clusters (Table III).

Serum osteopontin level is increased after VIT

To confirm differential expression of osteopontin further, serum levels of osteopontin were analyzed in a panel of 16 subjects randomly chosen from the completed treatment and 16 from the untreated group. As seen in Fig 3, significantly higher levels of osteopontin were found in the completed treatment group compared with those seen in the untreated group ($P < .001$). This finding is consistent with the results from gene chip array and quantitative RT-PCR analyses. In these individuals, there was no difference in sex and age distribution, and the type of insect allergen used for therapy did not influence the level of osteopontin.

DISCUSSION

In this report, a significant upregulation of osteopontin expression associated with successful VIT was discovered

TABLE III. Upregulated genes known to be associated with osteopontin

Gene bank		Fold change			Cluster	Reference
Accession ID	Gene name	Symbol	ET/Unt	CT/Unt		
AA775616	Osteopontin	<i>OPN</i>	1.6	7	B	
T72877	IL-1 receptor antagonist	<i>IL1RA</i>	2	4.6	B	9*
AA284669	Urokinase type plasminogen activator	<i>PLAU</i>	3.8	4.3	A	10
AA279147	GM-CSF receptor β	<i>CSF2RB</i>	4.3	3.6	A	11
BC016671	Integrin $\alpha 4$	<i>ITGA4</i>	2.7	2.5	A	12
AA464526	IL-1 receptor type 1	<i>IL1R1</i>	2.4	2.5	B	9
AA488075	STAT1	<i>STAT1</i>	3.1	2.4	A	13*
AA169355	IL-1 receptor-associated kinase	<i>IRAK</i>	1.1	2.4	B	9*
AA495922	IFN- γ receptor 2	<i>IFNGR2</i>	1.6	2.3	B	10
AA478043	IFN regulatory factor 1	<i>IRF1</i>	1.6	2.1	B	13*
H11482	IFN- γ receptor 1	<i>IFNGR1</i>	2.5	2.1	A	13
T69168	CD44	<i>CD44</i>	3	2.1	A	14
AA150416	TNF receptor 2	<i>TNFR2</i>	1.5	2	B	15
H16389	Mannose receptor, C type 1	<i>MRC1</i>	1.4	2	B	11
H78386	IL-1 receptor type 2	<i>IL1R2</i>	1.6	2	B	9

ET, Early treatment; Unt, untreated; CT, completed treatment.

*These are not reported for a direct association but rather an indirect association with osteopontin.

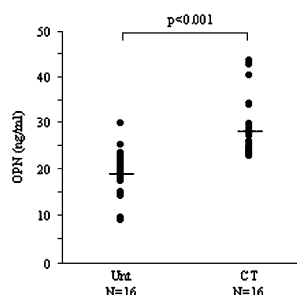


FIG 3. Serum osteopontin levels are higher in patients from the completed treatment group than in the untreated group. Patients characteristics used for this study are shown in Table II. The line represents the median value of each patient group.

level of osteopontin seen in patients after the completion of VIT for 3 to 6 years is currently unclear. Further studies are needed to clarify the pathophysiological mechanisms by which osteopontin affects allergic responses, and studies with large numbers of patients are required to evaluate the utility of serum osteopontin as a functional marker of VIT.

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through the use of gene chip microarray and clustering analyses. These results suggest, therefore, a potential role of osteopontin as a biomarker in VIT. In addition to its known regulatory role in bone metabolism,¹⁶ osteopontin has been postulated to be a T_H1 cytokine and is involved in the T_H1-associated immune responses.¹⁷⁻²³ Although osteopontin's role in directly modulating the T_H2 response is unknown, the known T_H1-inducing activity of osteopontin may potentially play a role in modulating the allergic responses. In a study of patients with large local allergic reactions to venom, increased levels of osteopontin associated with clinical improvement were also noted after beginning VIT (data not shown). Also, *in vivo* administration of osteopontin attenuated IgE but not IgG2a production in antigen-sensitized mice (data not shown), further suggesting a potential link between osteopontin and allergic response. Of interest, a significantly reduced level of osteopontin is found in association with hyper-IgE syndrome.²⁴ Further studies are required to investigate a potential role of osteopontin in regulating IgE response. The reason for an increased and sustained

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