ORIGINAL COMMUNICATION

A Pilot Study to Explore the Usefulness of Antibody Array in Childhood Atopic Dermatitis

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Background: The pathophysiology of childhood atopic dermatitis (AD) involves complex interactions among cellular, humoral, cytokine and chemokine systems.

Objective: To evaluate protein expressions using antibody microarray.

Methods: Severity-nine proteins were assayed using antibody microarray on AD patients age ≤18 years. Disease severity was assessed with the SCORing Atopic Dermatitis (SCORAD) and Nottingham Eczema Severity Score (NESS), and quality of life with the Children Dermatology Life Quality Index (CDLQI). Serum IgE levels were also assessed. Normal subjects without atopy were used as controls. Cytokines, chemokines and a wide array of proteins were assayed with RayBio® Human Cytokine Antibody Array V (RayBiotech, Norcross, GA).

Results: Nine Chinese children with AD and four normal subjects were recruited. The median SCORAD was 60.7. Among the 79 proteins, the levels of BDNF, Fit-3 ligand, IL-8, IL-16, LIGHT, MIP-1β, MIP-3α, NAP-2, PARC, TGF-β2 and TIMP-2 were significantly different from the controls. Nevertheless, no significance was found when adjusted for multiple comparisons using p=0.0006. Some of these markers showed significant correlations with various components of SCORAD, NESS and CDLQI. The serum IgE level as a marker of atopy correlates significantly with BDNF, LIGHT, PARC and TIMP-2.

Conclusions: The serum levels of BDNF, LIGHT, PARC and TIMP-2 correlate to IgE as a marker of atopy. Although targeting chemokines and chemokine receptors may offer new opportunities for therapeutic interventions in AD, protein assay with cytokine antibody array was generally not helpful in identifying specific molecules pertinent to AD activity.

Key words: dermatology

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INTRODUCTION

The pathophysiology of childhood atopic dermatitis (AD) is complex and involves interactions among immune cells, antibodies, cytokines and chemokines. AD-associated chemokines have been extensively studied in both adults and children and include cutaneous T-cell-attracting cytokine (CTACK), macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC) and leukotriene E4.[1-4] Neuroimmune mediators have also played a role in the pathogenesis of itch. Brain-derived neurotrophic factor (BDNF) is a growth factor initially found in the nervous system.[5] Increased blood levels of BDNF has been documented in AD patients and correlated with disease severity.[6-8] It is also found to be produced, stored and released by human peripheral eosinophils.[9] In addition, eosinophil apoptosis was inhibited by BDNF.[6] Substance P (SP) is another neuropeptide probably involved in the pathogenesis of AD via the neuroimmunocutaneous system and significantly correlated with disease activity.[10] There are possibly many more biochemical markers that have not been well studied in this complex disease. In the past, it was difficult if not impossible to analyze large numbers of potentially important or relevant proteins. Modern technology has made the study of protein expressions possible. An antibody array is a specific form of protein array that a collection of capture antibodies are spotted and fixed on a silicon chip for the purpose of detecting antigens and detecting protein expression from cell lysates. The purpose of this study was to explore if antibody array is helpful in the evaluation of protein expressions in AD comparing with control.

PATIENTS AND METHODS

AD patients age <18 years of age were randomly recruited from the pediatric dermatology clinic of a university teaching hospital in Hong Kong. AD was diagnosed according to criteria proposed by Hanifin and Rajka,[11] and severity and symptomatology (pruritus and

sleep loss in the preceding three days) of AD patients was evaluated with SCORAD.[12] Patients were excluded from the study if they suffered from other inflammatory dermatitides such as psoriasis, seborrheic dermatitis, ichthyosis or overt asthmatic symptoms such as cough, wheeze, shortness of breath or exercise-induced bronchospasm within four weeks before recruitment. Further, patients were not treated with systemic corticosteroids or immunomodulating drugs within four weeks before they entered this study. They were only allowed to use a topical corticosteroid or oral antihistamine on an as-needed basis during the study period. Informed written consent was obtained from subjects' parents, and the clinical research ethics committee of our university approved the study.

Serum was collected from the AD patients, centrifuged at 4º C and 2041×*g* for 10 minutes, and then stored at -70º C until analysis. RayBio® Human Cytokine Antibody Arrays V & 5.1 (RayBiotech, Norcross, GA) were preformed in one batch according to the manufacturer's instructions. This set of arrays was chosen as it covers a large number of molecules, many of them have previously been found to be involved in the T-helper 2 pathway and correlate with AD activity. The membrane arrays were first blocked with blocking buffer and then incubated with two-fold diluted serum samples for 1.5

Table 1. Comparison of 79 proteins between AD patients and controls by antibody array

	Control (N=4)	Atopic Dermatitis (N=9)	P Value
	Median (IQR)	Median (IQR)	
Angiogenin	0.693 (0.555, 0.892)	1.030 (0.877, 1.151)	0.090
BDNF	**0.856 (0.716, 0.982)**	**0.356 (0.284, 0.474)**	**0.011**
BLC	0.171 (0.117, 0.202)	0.067 (0.054, 0.086)	0.123
Ck b8-1	0.096 (0.063, 0.132)	0.073 (0.056, 0.131)	0.877
EGF	0.879 (0.520, 1.105)	0.421 (0.306, 0.591)	0.440
ENA-78	0.090 (0.045, 0.136)	0.121 (0.088, 0.140)	0.440
Eotaxin	0.065 (0.053, 0.084)	0.082 (0.055, 0.124)	0.440
Eotaxin-2	0.130 (0.088, 0.161)	0.104 (0.070, 0.175)	0.817
Eotaxin-3	0.051 (0.039, 0.063)	0.075 (0.066, 0.085)	0.189
FGF-4	0.163 (0.109, 0.216)	0.099 (0.072, 0.112)	0.090
FGF-6	0.085 (0.068, 0.106)	0.115 (0.099, 0.118)	0.280
FGF-7	0.122 (0.103, 0.128)	0.107 (0.065, 0.118)	0.355
FGF-9	0.213 (0.172, 0.238)	0.128 (0.080, 0.131)	0.064
Fit-3 Ligand	**0.169 (0.148, 0.178)**	**0.084 (0.062, 0.118)**	**0.025**
Fractalkine	0.127 (0.090, 0.161)	0.098 (0.067, 0.112)	0.217
GCP-2	0.118 (0.089, 0.135)	0.078 (0.068, 0.103)	0.486
GCSF	0.056 (0.047, 0.062)	0.001 (-0.003, 0.090)	0.440
GDNF	0.206 (0.137, 0.253)	0.126 (0.106, 0.162)	0.217
GM-CSF	0.049 (0.035, 0.055)	-0.002 (-0.006, 0.085)	0.440
GRO	0.046 (0.024, 0.063)	0.103 (0.067, 0.168)	0.053
GRO-α	0.034 (0.024, 0.048)	0.006 (-0.008, 0.087)	0.537
HGF	0.351 (0.282, 0.384)	0.206 (0.164, 0.264)	0.165
I-309	0.091 (0.080, 0.105)	0.096 (0.081, 0.110)	0.817
IFN-γ	0.102 (0.089, 0.117)	0.110 (0.090, 0.127)	0.537
IGF-1	0.125 (0.086, 0.153)	0.091 (0.056, 0.100)	0.280
IGFBP-1	0.177 (0.146, 0.196)	0.121 (0.106, 0.196)	0.877
IGFBP-2	0.690 (0.616, 0.709)	0.444 (0.368, 0.527)	0.064
IGFBP-3	0.147 (0.111, 0.178)	0.145 (0.133, 0.149)	0.643
IGFBP-4	0.071 (0.063, 0.076)	0.101 (0.084, 0.123)	0.121
IL-10	0.042 (0.028, 0.060)	0.094 (0.038, 0.116)	0.217
IL-12 p40 p70	0.094 (0.085, 0.117)	0.056 (0.036, 0.102)	0.280
IL-13	0.115 (0.079, 0.190)	0.069 (0.048, 0.126)	0.279
IL-15	0.200 (0.155, 0.217)	0.093 (0.062, 0.167)	0.215
IL-16	**0.081 (0.071, 0.086)**	**0.131 (0.118, 0.156)**	**0.021**
IL-1A	0.243 (0.181, 0.332)	0.114 (0.101, 0.131)	0.064
IL-1B	0.108 (0.094, 0.135)	0.109 (0.097, 0.133)	0.877
IL-2	0.061 (0.039, 0.092)	0.050 (0.044, 0.072)	0.877
IL-3	0.097 (0.074, 0.111)	0.071 (0.049, 0.124)	0.758
IL-4	0.091 (0.077, 0.092)	0.110 (0.078, 0.143)	0.440
IL-5	0.037 (0.029, 0.043)	0.040 (0.029, 0.061)	0.588
IL-6	0.104 (0.074, 0.159)	0.040 (0.017, 0.101)	0.217

IQR: Interquartile range; Proteins with p<0.05 are bold.

hours. They were then washed and incubated with biotinylated secondary antibodies for another 1.5 hours, and subsequently with cy3 streptavidin for 1 hour. The signals were captured on a film and scanned to JPEG format. The density of image was quantified by a densitometer (Bio-Rad Laboratories, Hercules, CA), and intensity was normalized with positive controls from the same membrane array. Data were compared between nonatopic controls and patients with AD by Mann Whitney-U test. Besides serum levels of BDNF, CTACK and TARC were also measured by enzyme-linked immunosorbent test (ELISA) as previously described.[1,2,6]

Data were expressed as median and IQR unless otherwise specified. Chi-squared test was used to compare proportions, and Mann-Whitney U test was used to analyze numerical variables. The correlations between different SCORAD components and biochemical markers were analyzed by Spearman's correlation coefficients. All comparisons were made two tailed, and p values <0.05 were considered to be statistically significant. The significance was also set at 0.0006 to adjust for multiple statistical comparisons.

RESULTS

Nine Chinese children with AD [median age (interquartile range) of 11.6 (10.7–12.0) years] and four normal subjects 13.7 (13.3–14.0) years were recruited. The median SCORAD was 60.7. Among the 79 proteins, the levels of BDNF, Fit-3 ligand, interleukin (IL)-8, IL-16, LIGHT, macrophage inflammatory protein (MIP)-1β, MIP-3α,

Table 1. continued

	Control (N=4) Median (IQR)	Atopic Dermatitis (N=9) Median (IQR)	P Value
IL-7	0.046 (0.023, 0.067)	0.011 (0.006, 0.026)	0.247
IL-8	**0.064 (0.045, 0.080)**	**0.139 (0.104, 0.157)**	**0.014**
IP-10	0.214 (0.178, 0.231)	0.182 (0.148, 0.212)	0.279
Leptin	0.145 (0.112, 0.194)	0.277 (0.171, 0.318)	0.089
LIF	0.086 (0.074, 0.097)	0.149 (0.120, 0.154)	0.064
LIGHT	**0.183 (0.151, 0.214)**	**0.112 (0.104, 0.128)**	**0.045**
MCP-1	0.316 (0.264, 0.383)	0.238 (0.216, 0.277)	0.217
MCP-2	0.064 (0.054, 0.070)	0.097 (0.073, 0.128)	0.090
MCP-3	0.079 (0.074, 0.085)	0.067 (0.059, 0.125)	0.758
MCP-4	0.111 (0.082, 0.136)	0.114 (0.109, 0.154)	0.355
MCSF	0.180 (0.135, 0.219)	0.120 (0.100, 0.161)	0.355
MDC	0.124 (0.101, 0.142)	0.198 (0.133, 0.230)	0.123
MIF	0.161 (0.126, 0.175)	0.132 (0.102, 0.145)	0.440
MIG	0.090 (0.054, 0.124)	0.062 (0.052, 0.127)	0.877
MIP-1β	**0.056 (0.046, 0.079)**	**0.127 (0.114, 0.143)**	**0.045**
MIP-1s	0.461 (0.295, 0.619)	0.320 (0.290, 0.367)	0.355
MIP-3α	**0.079 (0.047, 0.116)**	**0.167 (0.136, 0.171)**	**0.031**
NAP-2	**0.167 (0.131, 0.189)**	**0.407 (0.380, 0.486)**	**0.021**
NT-3	0.085 (0.068, 0.116)	0.115 (0.097, 0.134)	0.280
NT-4	0.166 (0.120, 0.207)	0.149 (0.113, 0.159)	0.355
Oncostain M	0.115 (0.095, 0.135)	0.109 (0.071, 0.167)	0.758
Osteoprotegerin	0.221 (0.148, 0.303)	0.144 (0.133, 0.172)	0.280
CCL-18/PARC	**0.451 (0.426, 0.477)**	**0.928 (0.859, 1.033)**	**0.005**
PDGF-BB	0.549 (0.483, 0.612)	0.498 (0.448, 0.565)	0.537
PIGF	0.178 (0.139, 0.217)	0.161 (0.150, 0.186)	1.000
RANTES	0.298 (0.174, 0.457)	0.498 (0.494, 0.542)	0.165
SCF	0.109 (0.092, 0.129)	0.091 (0.085, 0.121)	0.486
SDF-1	0.078 (0.071, 0.097)	0.088 (0.073, 0.123)	0.536
TARC	0.080 (0.069, 0.091)	0.134 (0.085, 0.145)	0.105
TFG-β1	0.090 (0.077, 0.106)	0.117 (0.086, 0.132)	0.216
TGF-β2	**0.382 (0.362, 0.406)**	**0.272 (0.218, 0.301)**	**0.021**
TGF-β3	0.146 (0.109, 0.179)	0.106 (0.091, 0.126)	0.280
Thrombopoietin	0.049 (0.040, 0.060)	0.090 (0.053, 0.114)	0.142
TIMP-1	0.389 (0.323, 0.405)	0.446 (0.437, 0.487)	0.064
TIMP-2	**1.568 (1.235, 1.859)**	**0.663 (0.565, 0.785)**	**0.009**
TNF-α	0.102 (0.079, 0.113)	0.106 (0.063, 0.135)	0.643
TNF-β	0.140 (0.096, 0.178)	0.143 (0.128, 0.161)	0.877
VEGF	0.126 (0.081, 0.165)	0.122 (0.087, 0.136)	0.877

IQR: Interquartile range; Proteins with p<0.05 are bold.

neutrophil activating protein (NAP)-2, pulmonary and activation-regulated chemokine (PARC), transforming growth factor (TGF)-β2 and tissue inhibitor of metalloproteinases (TIMP)-2 were significantly different from the controls (Table 1). No significance was found when adjusted for multiple comparisons using $p=0.0006$. Some of these markers showed significant correlations with various components of SCORAD, NESS and CDLQI. The serum IgE level as a marker of atopy correlated significantly with BDNF, LIGHT, PARC and TIMP-2 (Table 2).

Quality of life as measured with CDLQI correlated with Fit-3 ligand, IL-8 and MIP-3α. Both Fit-3 ligand and IL-8 also correlated with intensity of AD lesions. There were no significant correlations between SCORAD and the above 11 markers or BDNF, CTACK and TARC levels measured by ELISA ($\rho=0.217$, $p=0.576$; $\rho=0.217$, $p=0.576$; and $\rho=0.600$, $p=0.088$, respectively).

DISCUSSION

Antibody array is an expensive but potentially useful method to screen a large number of biological markers and molecules to identify relevant underlying pathophysiological mechanism. In this assay of 79 proteins between AD patients and controls with corrections for multiple comparisons, however, we were unable to identify relevant molecules. Chemokines are important mediators of immune-mediated skin diseases.[13] In these inflammatory diseases, recruitment of T lymphocytes is driven by chemokines expressed on the surface of endothelial cells or released by activated resident skin cells such as mast cells, fibroblasts and keratinocytes. Chemokines are produced in a coordinated and sequential manner, with IL-8 and RANTES induced by TNF-α during early stage; and MCP-1, IP-10, Mig, I-TAC, I-309 and MDC induced by IFN-γ during later stage. Infiltrating monocytes, dendritic cells and T cells are additional sources of chemokines that drive further leukocyte accumulation. Distinct T-cell subsets express different chemokine receptors, with type-2 T-helper (Th2) lymphocytes mostly attracted by eotaxin, MDC, TARC and I-309; and type-1 T-helper (Th1) lymphocytes sensitive to IP-10, Mig, I-TAC, RANTES and MIP-1β. MCP-1 is effective on both T-cell subsets. T regulatory cells inhibit dendritic cell function and are probably involved in the termination of AD. They are sensitive to MCP-1, MIPs and TARC, express high levels of CCR8 and are more specifically attracted by I-309.[13] This study demonstrates that protein expressions of both CXC (NAP-2, IL-8) and CC (MIP-1β, PARC, MIP-3α) chemokines may be associated with childhood AD. Nevertheless, no such significance was found after correction for multiple comparisons. Despite this, IL-8 (which is a prototype chemokine of CXC family for neutrophils and lymphocytes)[14,15] significantly correlated with disease intensity and quality of life, and may be a vital inflammatory mediator in the cascade of interacting cytokines in immunologically induced inflammation.[14] In addition, PARC of CC family (which is chemotactic for activated T cells and nonactivated lymphocytes) was found to be significantly correlated with serum IgE levels.

The levels of two other important CC chemokines—CTACK and TARC—were not elevated in this antibody array study. We previously demonstrated that they were elevated and correlated with disease activity in AD.[1,2] CTACK functions by providing a skin-specific signal involved in localization of cutaneous lymphocyte-associated antigen (CLA) memory T cells to skin and provides a potential target to regulate cutaneous T-cell trafficking.[1] TARC is a Th2 chemokine that specifically acts on chemokine receptor CCR4 to attract Th2 cells into sites of allergic inflammation. TARC could be detected in keratinocytes and to a lesser extent in endothelial cells and dermal infiltrating cells, including CD3 lymphocytes and CD1a dendritic cells.[1] There were no correlations between SCORAD and plasma levels of these two chemokines as determined by array study or ELISA in this study. This might be due to the small number of subjects and high individual variance of serum levels of these chemokines.

Table 2. Spearman's correlation coefficients (ρ) between clinical parameters and 11 laboratory markers in our nine AD patients

	Laboratory Markers										
Clinical Parameters	BDNF	Fit-3 Ligand	IL-16	IL-8	LIGHT	MIP-1β	MIP-3α	NAP-2	PARC	TGF-β2	TIMP-2
Total SCORAD	0.483	0.650	0.500	0.533	0.300	0.192	-0.333	-0.583	0.183	-0.050	0.400
Objective SCORAD	0.467	0.600	0.433	0.600	0.317	0.100	-0.367	-0.567	0.200	-0.017	0.417
SCORAD Components											
Extent	0.350	0.600	0.367	0.633	0.233	0.092	-0.383	-0.533	0.217	-0.117	0.350
Intensity	0.563	0.698*	0.563	0.698*	0.529	-0.042	-0.412	-0.479	0.328	0.059	0.529
Pruritus	0.071	0.293	0.071	0.497	-0.080	0.374	0.018	-0.310	-0.053	-0.293	0.142
Sleep loss	0.319	0.891**	0.597	0.210	0.160	0.101	-0.496	-0.538	0.193	-0.210	0.034
NESS	-0.445	0.094	-0.265	0.342	-0.539	0.253	0.009	-0.060	-0.359	-0.778*	-0.308
IgE	0.917**	0.567	0.317	0.333	0.683*	-0.176	-0.067	0.267	0.833**	0.017	0.683*
CDLQI	0.460	0.817**	0.655	0.826**	0.562	-0.338	-0.672*	-0.358	0.358	-0.247	0.528

* Correlation is significant at the 0.05 level (two tailed); ** Correlation is significant at the 0.01 level (two tailed)

Serum chemokine profiles have also been studied in patients with alopecia areata and bullous pemphigoid. When comparing our findings with those of two immune-mediated skin diseases, we shared few similarities but some differences. AD is associated with much itching and scratching, whereas the other two diseases are generally not associated with significant scratching. Using a sandwich immunoassay-based multiplex protein array system, the serum chemokines MCP-1 and IP-10 were found to be elevated in patients with bullous pemphigoid when compared with patients with pemphigus vulgaris and normal controls.[16] Using cytometric bead array assay, serum Mig, RANTES, IL-8 and eotaxin levels were selectively increased in patients with alopecia areata, compared with normal controls. Apart from IL-8, the 10 proteins in our patients with AD were all different, suggesting that the underlying pathophysiological mechanisms of these immune diseases were all different. Also, some of these chemokines were found to be elevated in allergic contact dermatitis (ACD), suggesting that AD and ACD may share similar pathways in their pathophysiology.[13] Of the 11 protein markers, four were found to correlate with serum IgE levels. IgE is not only a marker of atopy but also a maker disease severity.[17] In the present study, BDNF level correlated best with serum IgE level ($r>0.9$, $p=0.001$). We recently demonstrated that BDNF is an extremely important factor in the mediation of itch and its level correlated with AD severity.[6-8] BDNF has also been found to be produced, stored and released by human circulating eosinophils.[9] In addition, eosinophil apoptosis was inhibited by BDNF.[6] Nevertheless, no correlation with SCORAD was demonstrated in this study with Raybio array or ELISA. Possible explanations for the lack of correlation include small sample size, high individual variance and factors other than disease severity affecting the level of BDNF.

The association between Fit-3 ligand and inflammatory skin condition is unknown. Fit-3 ligand belongs to the platelet-derived growth factor family, and its expression appears to be most pronounced in stem cells and progenitor cells within the hematopoietic systems. The levels of this ligand are unexpectedly found to be correlated with disease intensity, sleep loss and quality of life. More in-depth research should be undertaken to explore the association between AD and mediators involved in the maintenance of hematopoiesis.[18-20]

Furthermore, quality of life is an important parameter in the assessment of disease impact on patients,[21,22] and this antibody array study also documented that some of the proteins (Fit-3 ligand, IL-8 and MIP-3α) were also correlated with CDLQI.

In conclusion, antibody array helps to identify 11 markers which are different in AD patients at $p<0.05$. Nevertheless, no significance was found when adjusted for multiple comparisons using $p=0.0006$. Despite this, the serum levels of some of these markers correlate with IgE and various clinical components of SCORAD. Further characterization of the kinetics and persistence of their secretion should be undertaken with a larger sample size of patients. Although targeting chemokines and chemokine receptors may offer new opportunities for therapeutic interventions in AD, protein assay with cytokine antibody array was generally not helpful in identifying specific molecules pertinent to AD activity.

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