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Comparative proteomic analysis of peripheral blood mononuclear cells from atopic dermatitis patients and healthy donors

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that induces changes in various inflammatory skin cells. The prevalence of AD is as high as 18% in some regions of the world, and is steadily rising. However, the pathophysiology of AD is poorly understood. To identify the proteins involved in AD pathogenesis, a comparative proteomic analysis of protein expression in peripheral blood mononuclear cells isolated from AD patients and healthy donors was conducted. Significant changes were observed in the expressions of fourteen proteins, including the vinculin, PITPNB, and Filamin A proteins. Among the proteins, α-SNAP and FLNA decreased significantly, and PITPNB increased significantly in AD patients compared with control subjects; these findings were further confirmed by real-time PCR and Western blot analysis. The comparative proteome data may provide a valuable clue to further understand AD pathogenesis, and several differentially regulated proteins may be used as biomarkers for diagnosis and as target proteins for the development of novel drugs. [BMB reports 2008; 41(8): 597-603]

INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic, chronic inflammatory skin disease that affects children and adults worldwide. Most manifestations of AD result from a complex interplay of susceptible genes, environmental factors, pharmacological abnormalities, skin barrier defects, and immunological responses (1, 2). The skin lesions of AD patients are characterized by increased numbers of IgE-bearing Langerhans cells, inflammatory dendritic cells, macrophages, eosinophils, activated T lymphocytes, and mast cells (3-5). In particular, peripheral blood mononuclear cells (PBMCs) act as the main effectors via the functional regulation of cytokines such as interleukins and interferon-γ. This important role of PBMCs is well established, and has been demonstrated in experimental models. Specifically, the PBMCs in AD patients have a decreased capacity to produce interferon-γ, which is inversely correlated with serum IgE concentrations (1, 2, 6).

Proteomics can provide a global, systemic, and comprehensive approach to the identification and description of the biochemical processes, pathways, and networks involved in both normal and abnormal physiological states at the protein level (7-10). As a typical proteomic analysis, two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) has been used widely in research (11-13). 2-DE can be used to detect differences in protein expression levels in cell states between healthy and diseased cells, and is also a promising tool for use in identifying disease markers and candidates for therapeutic intervention (8, 14).

The number of AD patients has increased with modernization and industrialization. Therefore, further insight into the complex pathogenesis of AD, determining its detailed mechanisms and its regulatory proteins, is needed (10, 15). Although proteomic studies can be employed to identify new target proteins of interest in AD, proteomic approaches have been used less often in the study of AD than have genomic approaches (11, 15-17). Moreover, a comparative analysis of human PBMCs between AD patients and healthy donors has not yet been reported. In this study, 2-DE combined with MS has been used to investigate the protein expression profiles in PBMCs isolated from both AD patients and healthy donors.

RESULTS AND DISCUSSION

PBMCs are a heterologous cell population composed of approx-

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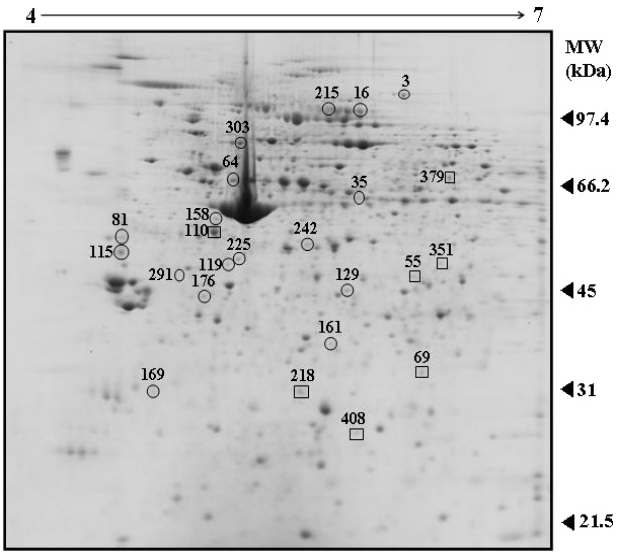

Fig. 1. Representative 2-DE image of PBMCs from an AD patient. The protein samples (500 μg) were applied to a first dimension of pH 4-7 nonlinear IPG strips and a second dimension of 12.5% SDS-PAGE visualized by CBB-G250 staining. The rectangles and circles mark spots that show significant changes in expression levels. The indicated spot numbers in Table 1 correspond to the spots shown in Fig. 1.

imately 80-85% lymphocytes, which are known to be key players in the innate and adaptive immune response, including the pathogenesis of AD via functional regulatory cytokines (3-6). In this study, to identify the proteins in the PBMCs that are involved in AD pathogenesis, the protein profiles of the PBMCs from eight pairs of patient-matched healthy donors were analyzed using 2-DE. The section of the proteome that was investigated covers proteins with an isoelectric point from pH 4 to 7 and that range in size from 10 to 100 kDa. After image analysis and visual confirmation, approximately 800 spots were detected in the PBMC samples from AD patients (898 $\pm$77) and healthy donors (826 $\pm$ 68 spots). More than 80% of the protein spots were matched between the two groups. The protein spots showing significant change, greater than 1.5-fold in magnitude, between the healthy donors and AD patients were scored. For each selected spot, a one-way analysis of variance (ANOVA) was performed. Statistical significance was accepted at a level of $P < 0.05$. A total of twenty-four spots showed significant changes between the two groups. Compared with the healthy donors, seven spots were up-regulated and seventeen spots were down-regulated in the AD patients (Fig. 1). Among these, a total of fourteen proteins were identified via mass spectrometry (Fig. 2 and Table 1). These proteins were classified into distinct functional groups of cell motility, regulation of signal transduction, transport, organismal physiological processes, and proteins of unknown function.

In order to verify the results from the 2-DE analysis, Western

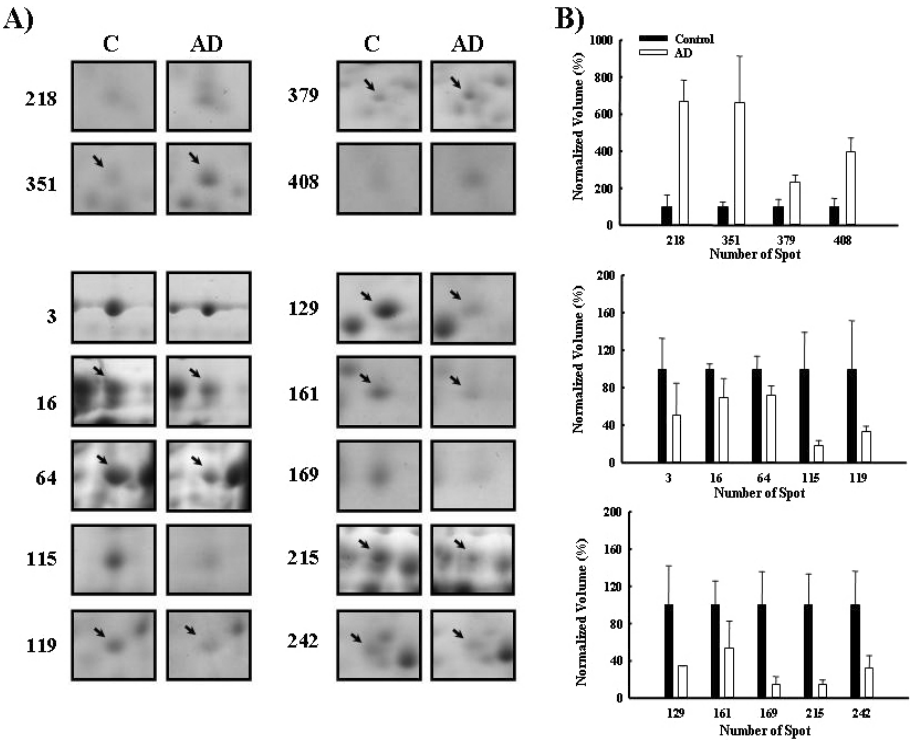

Fig. 2. Comparative analysis of differentially expressed protein spots in normal healthy donors and AD patients. (A) Zoom of the 2-DE gel on up- and down-regulated spots. (B) Quantification of differentially expressed protein spots in AD patients compared to healthy donors. In (B), spot volumes were normalized to those of healthy donor samples. Values are the means $\pm$ SE (n = 4 for AD and healthy donor samples). The indicated spot numbers correspond to the spot shown in Fig. 1.

Table 1. Fourteen differentially expressed proteins in AD patients

Spot No.	Accession No.	Protein name	Expectation value	Mascot score[a]	Coverage (%)	Alteration
Cell motility						
3	gi|24657579	Vinculin	8.40E-22	270	63	↓
Regulation of signal transduction						
16	gi|15779184	FLNA protein	6.70E-22	271	61	↓
115	gi|63252906	Tropomyosin 1α chain isoform 7	4.20E-11	163	66	↓
129	gi|55957302	Glutathione S-transferase omega 1	1.70E-03	79	34	↓
215	gi|15779184	FLNA protein	4.10E-05	95	30	↓
Transport						
64	gi|13162226	Tubulin β1	5.40E-08	124	37	↓
119	gi|3929617	α-SNAP	1.70E-08	129	46	↓
161	gi|49168476	RAB11B	4.00E-02	73	54	↓
351	gi|21594294	PITPNB protein	2.50E-02	75	39	↑
379	gi|62896685	TATA binding protein interacting protein 49 kDa variant	7.40E-03	63	40	↑
Organismal physiological process						
169	gi|48145803	Myosin light chain 9	3.20E-05	96	56	↓
Unknown						
218	gi|42543203	Chain B, X-ray structure of the small G protein RAB11A in complex with GDH	3.10E-02	66	50	↑
242	gi|13786848	Chain B, human heart L-Lactate dehydrogenase H chain, ternary complex with NADH and oxamate	6.70E-10	151	57	↓
408	gi|34811513	Chain A, solution structure of the C-terminal domain of the human elf1b γ subunit	2.70E-08	128	83	↑

[a] The score greater than 61 are significant ($P < 0.05$).
↑ increased spot in PBMCs of AD patients compared to healthy donors.
↓ decreased spot in PBMCs of AD patients compared to healthy donors.

blot and real-time PCR analysis with selected proteins, including α-SNAP, FLNA, and PITPNB, were performed. As expected, the Western blot analysis showed a decreased level of α-SNAP expression in the AD patients, which is consistent with the 2-DE result. In addition, the real-time PCR analysis exhibited a down-regulation of the FLNA and up-regulation of the PITPNB mRNA in the AD PBMCs, confirming the results from the 2-DE experiments (Fig. 3B).

Several soluble proteins play important roles in cellular physiological processes by mediating the intracellular mechanisms through the regulated transport of granule contents (18, 19). SNAP proteins have three isoforms, α-, β-, and γ-SNAP, and they contribute to the disassembly of membrane-specific SNAP receptor complexes to facilitate intracellular membrane fusion events (20). In particular, α-SNAP is a ubiquitous and indispensable component of the membrane fusion function. Alterations in the expression levels of α-SNAP may be associated with a number of specific pathological conditions, such as neurological disorders, Type 2 diabetes, and aggressive neuroendocrine tumors (18, 19). Recently, it was found that α-SNAP has potential applicability as a therapeutic target protein in many human diseases (21). In the present work, α-SNAP is identified as a candidate protein that is associated with AD. α-SNAP was down-regulated more than two-fold in the AD PBMCs compared with that in the healthy donors (Fig. 3). This suggests that a decrease of α-SNAP may be an important factor in the pathogenesis of AD diseases. Moreover, it is tempting to speculate that, similar to the known functional effects of SNAP

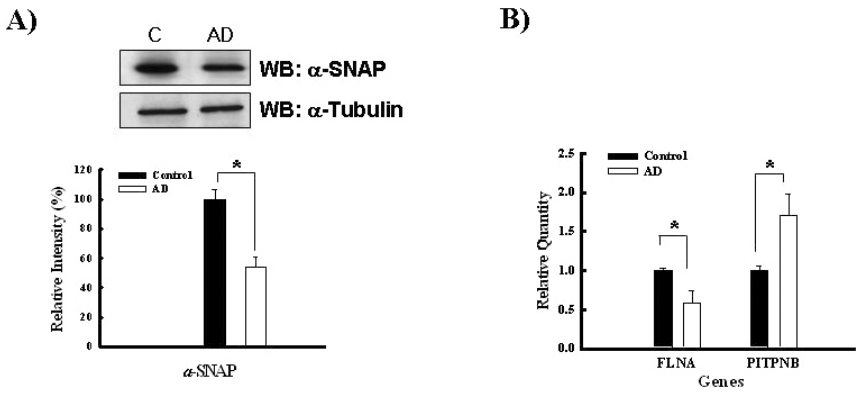

Fig. 3. Validation of α-SNAP and FLNA. (A) Western blot result of α-SNAP from healthy donor and AD PBMCs. Quantification of the α-SNAP level in AD patients compared to healthy donors is also shown (lower graph). (B) Quantitative real-time PCR of mRNA expression of FLNA and PITPNB in AD patients compared to healthy donors. Quantitative data were normalized to the intensity for α-tubulin (Western blot) or β-actin (RT-PCR). The values are means ± SD (n=3 for AD and healthy donor samples) (*P < 0.05).

proteins on the regulation of exocytosis in inflammatory cells (22), the down-regulation of α-SNAP may inhibit the exocytic activity of the PBMCs in the pathogenesis of AD.

Rab proteins are small Ras-like GTPases that function in membrane traffic. Among them, Rab11b is known to be involved in regulated and constitutive exocytosis (23). The down-regulation of Rab11b, as well as α-SNAP, strongly suggests the possibility of an interconnection between the regulation of exocytosis and AD pathogenesis.

The actin cytoskeleton is essential not only for the maintenance of cell shape and motility, but also for the integration of cell signals that initiate and propagate alterations in these cellular properties (24, 25). A recent report showing that the cellular scaffold dynamically regulates various cellular functions led to the suggestion that components of the cytoskeleton may be directly involved in human developmental disorders (25). In this study, several proteins related to the cytoskeleton, including vinculin, FLNA protein, tropomyosin 1α chain isoform 7, and tubulin β1, were identified as differentially expressed proteins in the PBMCs from AD patients.

Vinculin was decreased in the PBMCs from AD patients (Table 1). Vinculin is a ubiquitously expressed actin-binding protein that is known to be involved in cell adhesion, cell spreading, cell migration, and resistance to apoptosis. Notably, vinculin interacts with a number of proteins, including β-catenin, vinexin, PKCα, F-actin, talin, and Arp2/3 (26). However, until now, it was unclear whether vinculin played a role in atopic dermatitis.

FLNA, a cytoskeleton protein, cross-links actin into either networks or stress fibers in a regulated fashion (25, 27). A deficiency of FLNA at a cellular level results in a loss of the ability to generate cytoplasmic extensions and to migrate in response to environmental cues (25, 28). The mediation of some cellular functions through signaling and second messenger pathways has recently been shown to be dependent on FLNA (25, 29-31). In the present study, decreased protein and mRNA expression levels of FLNA in isolated PBMCs from AD patients were clearly observed. This finding suggests that FLNA may be a defective factor that occurs in AD patients. Furthermore, from the reports that FLNA regulates inflammatory signal transduction pathways via kinase activation (32, 33), it may be speculated that the decreased FNLA expression, as noted in the present study, may be caused by abnormal kinase activation in AD patients.

The phosphatidylinositol transfer protein (PITP) family is one of the families of transfer proteins that can bind and exchange one molecule of phosphatidylinositol (PI) or phosphatidylcholine (PC), and can facilitate the transfer of these lipids between different membrane compartments. In mammals, three soluble isoforms of PITP (α, β, and RdgBβ) have been identified. Phosphatidylinositol transfer protein β (PITPNB) was originally purified as a sphingomyelin (SM) transfer protein via an assay that monitored the transfer of pyrenylacyl-labelled SM from donor to acceptor vesicles (34). The ability to transfer SM is unique to PITPNB. Recently, it was also reported that PITPNB was up-regulated in AML14.3D10 cells, a human eosinophil cell line, after treatment with IL-5 (10). In the present study, the expression levels of PITPNB were found to be increased in isolated PBMCs from AD patients, thus implying the possibility of an interconnection between the regulation of SM metabolism and atopic dermatitis through PITPNB.

In conclusion, a comparative proteomic study was performed to analyze the differential protein expression in the human PBMCs of AD patients and healthy donors. Several proteins were differentially expressed in the PBMCs from AD patients. Among these, it is suggested that α-SNAP, FLNA, and PITPNB may be used as candidate biomarker proteins or target proteins to further investigate the pathogenesis of AD. Taken together, these proteomic results provide clues for understanding the molecular mechanisms of AD, as well as for identifying the proteins that are implicated in the immune response and physiological changes that are correlated with AD disease.

MATERIALS AND METHODS

Isolation of human PBMCs

Peripheral blood samples were obtained from ten healthy volunteers without a history of AD and from six AD patients. The

diagnosis of AD was based on the criteria provided by Hanifin and Rajka (35). All volunteer information in the present study conformed to the guidelines given by Sun General Hospital (Daejeon, Republic of Korea), and was also approved by the Ethics Committee in Sun General Hospital. The PBMCs were isolated from the blood samples by the Ficoll gradient using Ficoll-Plaque Plus (GE HealthCare, Uppsala, Sweden) according to the instructions of the manufacturer.

Sample preparation for 2-DE

Protein samples were extracted from the isolated PBMCs via sonication after gentle pipetting in a lysis solution [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and protease inhibitors]. The supernatant was then separated by centrifugation at 12,000 × *g* for 20 min at 4°C. The protein concentration in the supernatant was determined using a Bradford assay. A total of 500 μg of protein samples was precipitated by 75% acetone at 4°C for 2 h. After precipitation, the samples were centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was then removed, and the pellet was dried in a SpeedVac Plus SC110A (Savant, Holbrook, NY). The dried samples were redissolved in 250 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 2% pH 4-7 ampholyte, and 10 μg/ml protease inhibitors).

2-DE

The protein samples were directly applied to IPG strips (pH 4-7, 18 cm) and rehydrated for 12 h at room temperature. After rehydration, the protocol for isoelectric focusing was performed using a Multiphor II (GE HealthCare, Uppsala, Sweden). The initial voltage was maintained at 300 V for 15 min. In the second step, the voltage was increased linearly from 300 V to 3,500 V over 5 h. In the final step, the voltage was maintained at a constant level of 3,500 V for 10 h. The plate temperature was maintained at 20°C during isoelectric focusing. After focusing, the focused IPG strips were briefly equilibrated for 20 min with an equilibration solution [50 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, and 30% glycerol] containing 1% DTT, and were then equilibrated again with the same solution containing 5% iodoacetamide instead of DTT for 10 min. The equilibrated strips were loaded directly onto 12.5% SDS-PAGE gels (150 × 150 × 1.5 mm). The gels were run constantly at 30 mA per gel using an SE-600 apparatus (GE HealthCare, Uppsala, Sweden). The gels were stained using a modified Coomassie brilliant blue-G250 method as described previously in (36). The stained gels were scanned using a UMAX PowerLook 21ooxl scanner in the film-scanning mode in order to acquire stained images.

Image analysis

The total proteins were analyzed using the Phoretix™ 2D expression software (Nonlinear Dynamics, UK). The protein spot volume was evaluated based on the lowest boundary mode of the background selection. To compare the spot densities of the normal and AD samples, more than 10 spots throughout all of the gels were landmarked and normalized. The computer analysis allowed automatic detection and quantification of the protein spots, as well as matching among gels. The significance of the differences of protein spots was evaluated using a Student's *t*-test with a significance of $P < 0.05$.

In-gel digestion and peptide mass fingerprint (PMF)

The spots of interest were either manually excised or automatically detected and excised using Xcise™ (Shimadzu Biotech, Japan). The excised protein spots were digested by trypsin (Promega) for 16 h to 24 h at 37°C. The digested peptides were extracted via sonication for 25 min at room temperature with an extraction solution [50% acetonitrile (ACN) and 2.5% trifluoroacetic acid (TFA)], and the extracted peptides were then dried using a vacuum drier. After the peptide extraction, a Zip-Tip microcolumn (Millipore) was applied according to the instructions of the manufacturer. For the peptide mass fingerprinting, desalted peptides were dissolved by gentle pipetting with 1 μl of 50% ACN and 0.1% TFA for complete dissolution, and a 0.5 μl drop of peptide was applied to a target plate and allowed to air-dry. The mass spectra were acquired via an Axima CFR+ matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS, Shimadzu Biotech, Japan). The sequences of MS/MS spectra were identified using the correlations with peptide sequences present in the NCBI non-redundant protein database via the MASCOT software (www.matrixscience.com).

Western blot analysis

The protein (20 μg) was loaded and separated on a 12.5% SDS-PAGE gel, and was then transferred to an NC membrane according to standard procedures. The membrane was blocked with 5% v/v skim milk in a TBS-T buffer (TBS with 0.05% w/v Tween-20) and reacted with the anti-α-SNAP antibody (diluted 1:2000 in 5% v/v skim milk in the TBS-T buffer) for 12 h on a rocking platform at 4°C. The membrane was then washed three times with the TBS-T buffer for 15 min and incubated for 1 h with 5% skim milk in the TBS-T buffer containing horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted to 1:3000). The hybridized membrane was washed in the TBS-T buffer and visualized using a chemiluminescent ECL detection kit (GE HealthCare, Uppsala, Sweden).

RNA isolation and quantitative real-time PCR

The total RNAs were isolated from the atopic and normal PBMCs using an RNeasy Mini Kit (Qiagen) according to the instructions of the manufacturer. Two micrograms of the total cellular RNA were denatured at 65°C for 5 min, after which the first-strand cDNA was synthesized using M-MLV reverse transcriptase at 37°C for 60 min in the presence of 0.5 μg oligo (dT), 10 mM dNTP, and 0.1 M DTT (all from Invitrogen) in a total volume of 40 μl. After terminating the RT reaction by

heating at 75°C for 15 min, the double-stranded cDNA fragments of the target candidate gene were obtained. For the real-time PCR, SYYBR Premix Ex Tag (TaKaRa) was employed to detect the FLNA and PITPNB expressions with a Dice™ TP 800 Thermal Cycler (TaKaRa). The primers used were as follows: FLNA, 5'-CCCAGGCTTGGTGTCTGCTTA-3' and 5'-CAC TCCTGGCAATCCATCTTCA -3'; PITPNB, 5'-TGTGGGAACAC ACACTTCAAGGA-3' and 5'-CAGCACTCTCATGATGCCTATT CAG-3'; β-actin, 5'-AGGCCCAGAGCAAGAGAGG-3' and 5'-T ACATGGCTGGGGTGTTGAA-3'. Statistical analysis was performed using an independent student's *t*-test, and a P-value of < 0.05 was considered to be statistically significant (37).

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