

REGULAR ARTICLE

Comparative proteomic analysis of peripheral blood eosinophils from healthy donors and atopic dermatitis patients with eosinophilia

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Atopic dermatitis (AD) is an allergic disease that has recently shown a dramatic increase of incidence in developed countries. Eosinophilia, the accumulation of eosinophils, occurs in AD patients through an anti-apoptotic mechanism. To understand the target proteins involved in the anti-apoptotic signaling of eosinophilia, we used a proteomic approach to analyze eosinophil proteins from AD patients with eosinophilia and healthy donors. Protein spots in two-dimensional electrophoresis (2-DE) gels were identified with peptide mass fingerprinting (PMF) based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and database searching. More spots were observed in the 2-DE proteome map from AD patient samples (1310 ± 58 spots) than in those from healthy donors (1121 ± 40 spots). We identified 51 proteins affected by eosinophilia: 19 related to signaling, 8 involved in regulation of metabolism, 4 related to apoptosis, and 3 involved in inflammation. The other identified proteins were associated with transcription, RNA processing, translation, the cytoskeleton, and unknown functions. Among the identified proteins, we observed prominent increases in the expressions of cyclinA2, voltage-dependent anion channel protein 2, and 38 kDa FK506 binding protein 8 in eosinophils from AD patients in comparison to healthy donors. PMF and immunoblotting of a single spot that was expressed in eosinophils from healthy individuals but not in AD patients identified the protein as phosphorylated growth receptor binding 7 (Grb7) adaptor protein. Increased phosphorylation of Grb7 and its upstream signaling protein, focal adhesion kinase (FAK), was detected in low viability eosinophils such as those from healthy donors or in cultured eosinophils (AML14.3D10 cells) treated with dexamethasone. These results suggest that phosphorylation of Grb7 and the expressions of cyclinA2, voltage-dependent anion channel protein 2, and 38 kDa FK506 binding protein 8 may be related with the anti-apoptosis mechanism of eosinophilia.

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Abbreviations: AD, atopic dermatitis; Grb7, growth receptor binding 7

1 Introduction

Eosinophils act as effectors in the inflammatory reactions of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (AD), and in the chronic development of allergic inflammation [1–3]. Activated eosinophils induce tissue injury and inflammation by secreting many inflammatory and pro-inflammatory mediators including

basic proteins, sulfidopeptide leukotrienes, toxic oxygen products, and cytokines [3–8]. AD, a chronic skin disorder characterized by eosinophilic inflammation, occurs in people of all ages but has recently become more common in children residing in developed countries [9]. AD patients and other sufferers of allergic disorders can suffer from eosinophilia, an accumulation of eosinophils due to increased survival or decreased apoptosis of eosinophils [10]. Mature eosinophils do not divide and regulation of their survival or apoptosis is a critical determinant for the duration and severity of eosinophilic inflammation [7, 8]. Thus, a clear understanding of the eosinophil pro- or anti-apoptotic factors involved in the pathogenesis of AD may lead to the development of novel therapeutic approaches.

Proteomic analysis involves using a combination of techniques, including 2-DE, image analysis, and MS to separate, quantify, and identify proteins, respectively [10]. Proteins in a complex sample mixture can be separated by 2-DE to produce a high-resolution 2-DE map in which stained proteins are visualized as spots of various sizes and intensities depending on the amount of protein in the sample. Computer-assisted comparison of the spot patterns in different 2-DE gels is then performed to assess changes in protein levels that relate to the disease or process of interest [11]. Differentially expressed protein spots selected and isolated from the 2-DE proteome maps can then be identified by MALDI-TOF MS analysis [12–16]. Here, we sought to identify proteins involved in the anti-apoptotic signaling of eosinophilia, using proteomic profiling and comparative analysis of eosinophil proteins from eosinophilia patients and healthy donors.

Because of the rarity of eosinophils in peripheral leukocytes of healthy individuals, and the short life span of these cells, few previous studies have investigated cytosolic eosinophil proteins and their abilities to regulate signal transduction. Differential display, real time RT-PCR and microarray analyses have been used to examine eosinophil genes regulated by IL-5 and to identify genes related with AD and eosinophilia [17, 18]. However, a comparative analysis of human eosinophil proteins in eosinophilic patients and normal donors has not been reported yet.

We herein report the comparative proteomic analysis of protein expression in eosinophils isolated from AD patients with eosinophilia and in healthy individuals. We utilized proteomic approaches to analyze the expression of proteins related to AD eosinophilia, established a proteomic database, and characterized the proteins related with anti-apoptotic signaling of eosinophils. As eosinophilia is a common feature of allergic disorders, it is likely that our identification of proteins involved in eosinophilia may facilitate a better understanding of the anti-apoptotic mechanism of eosinophils and perhaps lead to the future development of new therapeutic targets for allergic diseases.

2 Materials and methods

2.1 Reagents and materials

Dextran, the Accustain® Wright-Giemsa stain kit and Histopaque (density = 1.077) were purchased from Sigma (St. Louis, MO, USA) and the rabbit polyclonal Grb7 and FAK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trypsin and Protein A/G-Agarose were from Promega (Madison, WI, USA) and the anti-phosphotyrosine monoclonal 4G10 antibody was from Upstate Biotechnology (Lake Placid, NY, USA). The horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody was obtained from Bio-Rad (Hercules, CA, USA), and recombinant human IL-5 was from R&D Systems (Minneapolis, MN, USA). Dexamethasone was purchased from Calbiochem (La Jolla, CA, USA) and dissolved (1 mM) in DMSO.

2.2 Isolation of human peripheral blood eosinophils

Peripheral blood samples were obtained from 10 healthy volunteers without history of AD and 5 AD patients with moderate eosinophilia (prior to treatment) whose eosinophil counts ranged from 12% to 15%. The eosinophil count of 1 to 3% was observed in healthy volunteers. Informed consent was obtained from all volunteers, according to the guidelines established by the Catholic University Hospital of Korea. Eosinophils were purified using Histopaque-1077 (Sigma) and MACS (magnetic activated cell sorting) [19]. Briefly, heparinized venous bloods (180 mL) were sedimented in 6% w/v dextran (Sigma), layered over Histopaque-1077 solution (Sigma) and centrifuged at $500 \times g$ for 30 min at 20°C. The polymorphonuclear leukocytes (PMNs) were collected from Histopaque phase and resuspended in PBS. The red blood cells were lysed by hypotonic shock with water for 20 s followed by 8 mL of 3.5% w/v NaCl, washed with PBS, and suspended at 5×10^6 cells/mL in PBS. PMNs were incubated with anti-CD16 antibody-conjugated micro magnetic beads (Miltenyi Biotechnology, Auburn, CA, USA) for 30 min at 4°C and passed through a magnetic column; eosinophils were obtained by negative selection. The purity of the obtained eosinophils was assessed by Wright-Giemsa staining [8].

2.3 Sample preparation for 2-DE

Purified eosinophils were lysed in 1 mL of RIPA buffer (PBS, pH 7.4, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 1.0% w/v SDS, 1 mM EDTA, 1 mM EGTA) supplemented with 2 mM sodium orthovanadate (Na_3VO_4), 1 mM sodium pyrophosphate, 1 mM β -glycerophosphate (ppi) and 1 mM sodium fluoride. Following incubation at 37°C for 30 min, an equal volume of acetic acid was added to the cell lysate and incubated for 1 h at –20°C. The cell lysate was then centrifuged at $12\,000 \times g$ for 30 min at 4°C, and the supernatant was removed and the pellet dried in a SpeedVac Plus SC110A

(Savant, Holbrook, NY, USA) vacuum concentrator for 30 min. The resulting pellet was resuspended in rehydration buffer (7 M urea, 4% w/v CHAPS, 0.5% w/v IPG buffer, pH 3–10 NL 2% w/v DTT and a trace of bromophenol blue) and the protein concentration was measured using the Bio-Rad protein assay reagent.

2.4 2-DE

IEF was performed with an IPGphor unit (Amersham Biosciences, Uppsala, Sweden) using precast 18 cm pH 3–10 nonlinear IPG gel strips (Amersham Biosciences). Equal amounts (100 µg) of total proteins were mixed with rehydration solution to a total volume of 350 µL and allowed to sit for 2 h with no applied voltage. Then, IEF was performed at 500 V for 1 h, 1000 V for 1 h and 8000 V for 5 h. The current was limited to 50 µA *per* gel strip. After IEF separation, the gel strips were immediately equilibrated in equilibrium buffer including 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, and 2% w/v SDS. The second dimension separation was carried out on non-stacked 12%T, 2.7%C SDS-PAGE gel (23.5 × 18 cm). Electrophoresis was performed using a Protean II xi 2-D cell (Bio-Rad), with 10 mA *per* gel applied for 20 min and 20 mA *per* gel applied until the front line of bromophenol blue reached the bottom of the gel.

2.5 Silver staining

Silver staining of 2-DE gels was modified to permit the MALDI-TOF MS analysis of proteins as described [14]. Briefly, gels were fixed with 50% v/v methanol/5% v/v acetic acid/0.5% v/v formaldehyde for 30 min. After several washes in 50% v/v methanol and distilled water, the gels were sensitized by incubating in 0.2% w/v sodium thiosulfate for 30 min with vigorous rotation, washed several times with distilled water, incubated in 0.2% w/v silver nitrate for 30 min, and then thoroughly washed with distilled water. The gels were then developed in 0.5% v/v formaldehyde/6% w/v sodium carbonate for 10 min and the reaction was stopped with 50% v/v methanol/12% v/v acetic acid. The resulting silver stained 2-DE gels were scanned with an ImageScanner (Amersham Biosciences) in transmission mode.

2.6 Protein identification

2.6.1 In-gel digestion

Spots were excised as 1–2 mm² slices, destained with freshly prepared 20 mM potassium ferricyanide/100 mM sodium thiosulfate, washed with 200 mM ammonium bicarbonate/100% ACN, and dried in a SpeedVac Plus SC110A (Savant, Holbrook, NY, USA) vacuum concentrator. The dried gel pieces were rehydrated with 5–15 µL of 10 ng/µL trypsin solution, and digestion was allowed to continue at 37°C for 16 h. The in-gel tryptic peptides were extracted with 50 mM

ammonium bicarbonate for 1 h followed by 100% ACN for 1 h. The extracted solutions were mixed and then dried in a vacuum concentrator. The resulting peptides were solubilized with 0.5% v/v TFA for MS analysis.

2.6.2 MALDI-TOF MS analysis and database searching

MS was performed using an Applied Biosystems Voyager-DE™ MALDI-TOF (Foster, CA) and an ETTAN™ MALDI-TOF Pro (Amersham Biosciences) mass spectrometer, using saturated CHCA solution in 0.1% v/v TFA/50% v/v ACN as the matrix. The mass spectrometer was externally calibrated using the matrix and tryptic autodigestion ion peaks as the internal standards. For protein identification, we performed database searching against the Swiss-Prot database using the MS-FIT program (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>) and the *pI* acquired from our image analysis. We used a protein molecular mass range of 6–200 kDa and a mass tolerance of 50 ppm for the internal calibration. Proteins matching more than four peptides were considered significant matches.

2.7 Cell viability assay

The leukemic eosinophil cell line, AML14.3D10, was kindly provided by Drs. C. Paul and M. Baumann (Wright State University, Dayton, OH, USA). AML14.3D10 cells were grown in RPMI-1640 containing 10% v/v FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. Cells were seeded into 96-well plates (1 × 10⁴ cells/200 µL/well) in RPMI medium with 10% v/v FBS and incubated for two days. For experiments, cells were incubated for 24 h in fresh medium, treated with IL-5 (10 ng/mL) for 1 h followed by treatment with 0, 1, 5 or 10 µM dexamethasone for two days. After dexamethasone treatment, the viability of cells was measured by MTS assay (CellTiter 96® AQueous Assay; Promega, Madison, WI, USA). Briefly, MTS dye solution (25 µL) was added into each well and plates were incubated at 37°C for 4 h. Then the absorbance at 490 nm was measured with an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8 Immunoprecipitation and Western blotting

Total cell lysates were prepared using lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% w/v Triton X-100, 2.5 mM α sodium pyrophosphate, protease inhibitors (1 mM leupeptin, 1 mM pepstatin A, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride), and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄). Total cell lysates were incubated with the anti-Grb7 antibody, and immune complexes were collected using Protein A/G-agarose beads. The collected immune complexes were mixed with SDS-PAGE sample buffer (0.5 M Tris-HCl, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 10% w/v SDS, 0.5% w/v bromophenol blue), separated on a 12%T, 2.7%C

SDS polyacrylamide gel, and transferred to a nitrocellulose membrane (Millipore, Bedford, USA). The membrane was blocked with 5% v/v skim milk in TBS-T buffer (TBS with 0.05% w/v Tween-20) and reacted with the 4G10 anti-phosphotyrosine antibody (diluted 1:2000 in 5% v/v skim milk in TBS-T buffer) for 12 h on a rocking platform at 4°C. The membrane was then washed three times with TBS-T buffer for 15 min and incubated with 5% v/v skim milk in TBS-T buffer containing horseradish peroxidase-conjugated goat anti-mouse antibody (diluted 1:3000) for 1 h. The hybridized membrane was washed in TBS-T buffer and visualized using a chemiluminescent ECL detection kit (Amersham Biosciences).

3 Results

3.1 Isolation of human eosinophils and 2-DE

Human eosinophils were isolated from the peripheral blood of healthy volunteers and atopic dermatitis patients and purified by negative selection using anti-CD16 immunomagnetic beads and magnetic separation (MACS). The purity of the isolated eosinophils were checked by Wright-Giemsa staining [8] and found to be greater than 98%. The purified eosinophil counts of healthy donors and AD patients with moderate eosinophilia ranged from 80–240 and 850–1780 *per* mL of whole blood, respectively. Because eosinophils are relatively rare in healthy individuals, we were unable to extract sufficient eosinophil proteins from any one healthy volunteer for 2-DE. Accordingly, purified eosinophils from three healthy donors were pooled for control 2-DE analysis. For 2-DE separation, the first dimension electrophoresis was performed *via* IEF with an IPGphor unit (Amersham Biosciences) using IPGphor™ strips (pH 3–10 NL) with equal amounts of solubilized proteins *per* sample incubated in equilibration solution for 30 min. Then, second dimension electrophoresis were carried *via* 12%T, 2.7%C SDS-PAGE, and proteins were visualized by silver staining. Spot counting revealed that eosinophils from atopic dermatitis patients expressed more proteins (1310 ± 58 spots) than those from healthy donors (1121 ± 40 spots). A 2-DE gel loaded with proteins from healthy donor eosinophils was used for comparative analysis, which revealed that 85.4% of the spots matched with those from a representative gel from AD patient eosinophils (Fig. 1).

3.2 Identification of eosinophil proteins affected by eosinophilia

Seventy differentially expressed spots were selected and analyzed with MALDI-TOF MS (Table 1) and PMF with database searching. Of these, 51 were identified as being affected by eosinophilia. Expression of 46 of these proteins was up-regulated and five were down-regulated in eosinophilia patients. The AD eosinophil-up-regulated proteins (Table 1)

included HLA class II histocompatibility antigen (spot#21) and Integrin β -7 (spot#44) which were previously reported as being expressed in IL-5 or GM-CSF treated eosinophils [20–22]. In addition we observed that expression of cyclinA2 (spot#14), voltage dependent anion channel 2 (spot#32), and 38 kDa FK506 binding protein 8 (spot#51) was increased in eosinophils from AD patients in relation to those of healthy donors (Fig. 1 and Table 1). We divided the identified proteins according to their functions (Table 2), and found that 19 were related to signaling, 8 to regulation of metabolism, 4 to apoptosis, and 3 to inflammatory processes. Of these, catalase (spot#9), cyclin A2 (spot#14) and voltage dependent anion channel 2 (spot#32), all involved in regulation of apoptosis, were expressed at higher levels in eosinophils of AD patients than in healthy donors (Table 2). In addition, we identified differential expression of two transcriptional proteins, three RNA processing proteins, two translational proteins, two cytoskeletal proteins, and ten proteins of unknown function in eosinophils of AD patients in comparison to those of healthy controls.

3.3 Characterization of a protein spot uniquely expressed in eosinophils from healthy donors

Among five proteins down-regulated in eosinophils of eosinophilia patients, one (spot#17) was almost completely absent in the eosinophils of AD patients. We identified this as the Grb7 adaptor protein (Fig. 2A and Table 1). To further identify the protein and test its possible tyrosine-phosphorylation, we performed immunoblotting assays on the nitrocellulose membrane transferred from partial 2-DE gel using anti-Grb7 antibody and anti-phosphotyrosine antibodies. The results indicated that spot#17 was the tyrosine phosphorylated form of Grb7 (Fig. 2B). To further check the expression levels of phosphorylated Grb7, we performed immunoprecipitation and immunoblotting assays on total extracts from eosinophils of healthy donors and AD patients. Total cell lysates were immunoprecipitated with anti-Grb7 antibody, the immune complexes were collected on protein A/G-agarose beads, separated by SDS-PAGE, transferred onto a nitrocellulose membrane and immunoblotted with the anti-phosphotyrosine antibody. We found that the phosphorylation level of Grb7 was significantly higher in healthy donor eosinophils than in those from AD patients (Fig. 2C). These results suggest that phosphorylation of Grb7 may be related with the anti-apoptotic mechanism of eosinophilia in AD patients.

3.4 The phosphorylation level of Grb7 is related with apoptosis in the eosinophil cell line, AML14.3D10

To further confirm the relationship between Grb7 phosphorylation and apoptotic signaling in eosinophils, we studied the effect of an apoptosis-inducing agent, dexamethasone, on the human leukemic eosinophil cell line, AML14.3D10. It has been reported that dexamethasone induces apoptosis of

Table 1. Fifty-one differentially expressed proteins identified by comparative analysis of 2-DE maps of eosinophils from healthy donors and AD patient by PMF searching in Swiss-Prot database

Spot no.	Name of protein identified	Accession no.	Mass / pI		Peptide matched	Score	Alteration
			Theoretical	Experimental			
1	Nitric oxide synthase (NOS, type III)	P29474	133.2 / 6.9	135.2 / 7.1	22 / 40	0.54	↑↑
2	VJ recombination activating protein 1	P15918	119.1 / 9.0	105.1 / 7.1	36 / 69	0.52	↑↑
3	Glutamate receptor 2	P42262	98.8 / 7.5	105.2 / 7.2	20 / 74	0.27	↑↑
4	RAC- α -serine/threonine kinase	P31749	55.7 / 5.8	98.4 / 6.2	26 / 70	0.37	↑↑
5	Ariadne-1 protein homologue (ARI-1)	Q9Y4X5	64.1 / 5.0	76.6 / 5.7	24 / 75	0.32	↑↑
6	Rab proteins geranyltransferase component A 1	P24386	73.4 / 4.7	75.1 / 6.4	18 / 56	0.32	↑↑
7	Prominin-like protein 1 precursor	O43490	97.2 / 7.0	88.0 / 7.7	15 / 62	0.24	↑↑
8	G protein-coupled receptor kinase 2	P50148	79.6 / 6.9	81.2 / 7.8	26 / 65	0.4	↑↑
9	Catalase	P04040	59.7 / 6.9	82.0 / 7.9	19 / 31	0.61	↑↑
10	Tyrosine protein kinase TXK	P42681	61.2 / 8.1	78.3 / 7.7	23 / 65	0.35	↑↑
11	Coilin (p80)	P38432	62.6 / 9.2	75.5 / 7.7	27 / 75	0.36	↑↑
12	Mitogen-activated protein kinase phosphatase 5	Q9Y6W6	52.6 / 7.6	80.8 / 7.7	24 / 55	0.43	↑↑
13	COUP transcription factor 1	P10589	46.1 / 8.6	50.5 / 7.8	22 / 50	0.44	↑↑
14	Cyclin A2	P20248	48.5 / 6.1	46.2 / 5.8	13 / 40	0.32	↑↑
15	Synaptotagmin IX	Q86SS6	56.1 / 6.8	49.3 / 6.3	38 / 76	0.5	↑↑
16	Hypothetical protein	Q9UH06	12.4 / 8.8	17.4 / 8.1	11 / 75	0.14	↑↑
17	Grb7 adaptor protein	Q14451	59.7 / 8.9	48.2 / 6.4	27 / 55	0.49	↑
18	Mitochondrial folate transporter/carrier	P13760	35.3 / 7.5	32.7 / 8.4	8 / 15	0.53	↑
19	Insulin-degrading enzyme	P14735	118.0 / 6.3	107.3 / 7.7	8 / 24	0.33	↑↑
20	Acyl-CoA dehydrogenase	P49748	70.3 / 8.9	83.2 / 6.2	4 / 7	0.57	↑↑
21	HLA class II histocompatibility antigen	P20039	30.1 / 6.7	37.5 / 6.0	18 / 75	0.24	↑↑
22	Kell blood group glycoprotein	P23276	82.8 / 8.1	62.0 / 7.3	25 / 67	0.37	↑↑
23	M-phase inducer phosphatase 1 (Cdc25A)	P30304	58.7 / 6.3	46.1 / 8.5	15 / 47	0.32	↑↑
24	Integrin α -6 precursor	P23229	126.6 / 6.4	140.2 / 6.4	15 / 37	0.4	↑↑
25	ADP-ribosylation factor GTPase-activating protein 3	Q9NP61	34.3 / 7.8	65.1 / 5.7	15 / 37	0.4	↑↑
26	Actin	P02570	41.7 / 5.3	44.7 / 6.4	16 / 66	0.24	↑↑
27	Copine VII	Q9UBL6	70.2 / 5.9	76.4 / 7.8	17 / 45	0.53	↑↑
28	Calponin 3	Q15417	36.4 / 5.6	42.1 / 5.9	13 / 35	0.37	↑↑
29	tRNA-methyltransferase	P48549	47.7 / 8.2	37.7 / 5.6	23 / 100	0.23	↑↑
30	G-protein beta-subunit like protein 1	O75841	35.6 / 8.3	39.1 / 8.7	16 / 80	0.2	↑↑
31	Low affinity immunoglobulin epsilon FC receptor	P06734	36.4 / 5.4	32.5 / 6.8	25 / 75	0.33	↑↑
32	Voltage-dependent anion-selective channel protein 2 (VDAC-2)	P45880	38.1 / 6.3	36.5 / 8.0	14 / 73	0.19	↑↑
33	Plasma membrane adaptor AP-2 protein	P20172	49.6 / 9.6	41.5 / 8.8	4 / 9	0.44	↑↑
34	Stac protein	Q99469	44.5 / 8.4	38.9 / 8.6	5 / 15	0.33	↑↑
35	Laminin α -5 chain precursor	O15230	39.9 / 6.6	36.5 / 7.8	8 / 31	0.25	↑↑
36	Ras-related protein	Q9BZG1	29.5 / 8.1	28.0 / 8.3	8 / 31	0.25	↑↑
37	Translation initiation factor elf-3e	Q64252	52.2 / 5.7	44.7 / 6.9	11 / 31	0.35	↑↑
38	F-actin capping protein beta subunit	P47756	31.3 / 5.4	29.5 / 6.3	6 / 15	0.4	↑↑
39	Alanine aminotransferase	P24298	54.5 / 6.7	41.8 / 7.0	7 / 11	0.63	↑↑
40	G-rich sequence factor-1	Q12849	48.0 / 5.5	43.0 / 6.3	13 / 39	0.33	↑↑
41	Heterogeneous nuclear ribonucleoprotein R	O43390	70.9 / 8.2	76.1 / 8.1	5 / 11	0.45	↑↑
42	Collagen α -1	Q14993	115.4 / 8.5	130.0 / 8.4	13 / 43	0.3	↑↑
43	Flotillin-1	O75955	47.3 / 7.1	48.2 / 7.3	13 / 36	0.36	↑
44	Integrin β -7	P26010	86.9 / 5.7	32.5 / 5.8	20 / 91	0.21	↑↑
45	Hypothetical protein	Q9H4I3	42.3 / 8.2	46.1 / 8.2	4 / 23	0.17	↑
46	Hypothetical protein	O94819	65.7 / 5.8	65.5 / 6.2	11 / 33	0.33	↑↑
47	Serine-protein kinase ATM (Ataxia telangiectasia mutated)	Q13315	350.6 / 6.3	310.0 / 6.5	27 / 46	0.56	↑↑
48	Signal transducer and activator of transcription 1- α/β	P42224	87.3 / 5.7	85.5 / 5.8	21 / 40	0.52	↑
49	Mitotic spindle assembly checkpoint protein MAD2B	Q9UI95	24.3 / 6.0	20.5 / 5.9	5 / 33	0.15	↑↑
50	Biglycan precursor	P21810	41.6 / 7.2	42.5 / 7.4	7 / 27	0.25	↑↑
51	38 kDa FK506 binding protein8	Q14318	38.5 / 7.8	39.0 / 7.8	8 / 33	0.24	↑↑

↑ increased spot in healthy donors' eosinophils

↑↑ increased spot in atopic dermatitis patients eosinophils

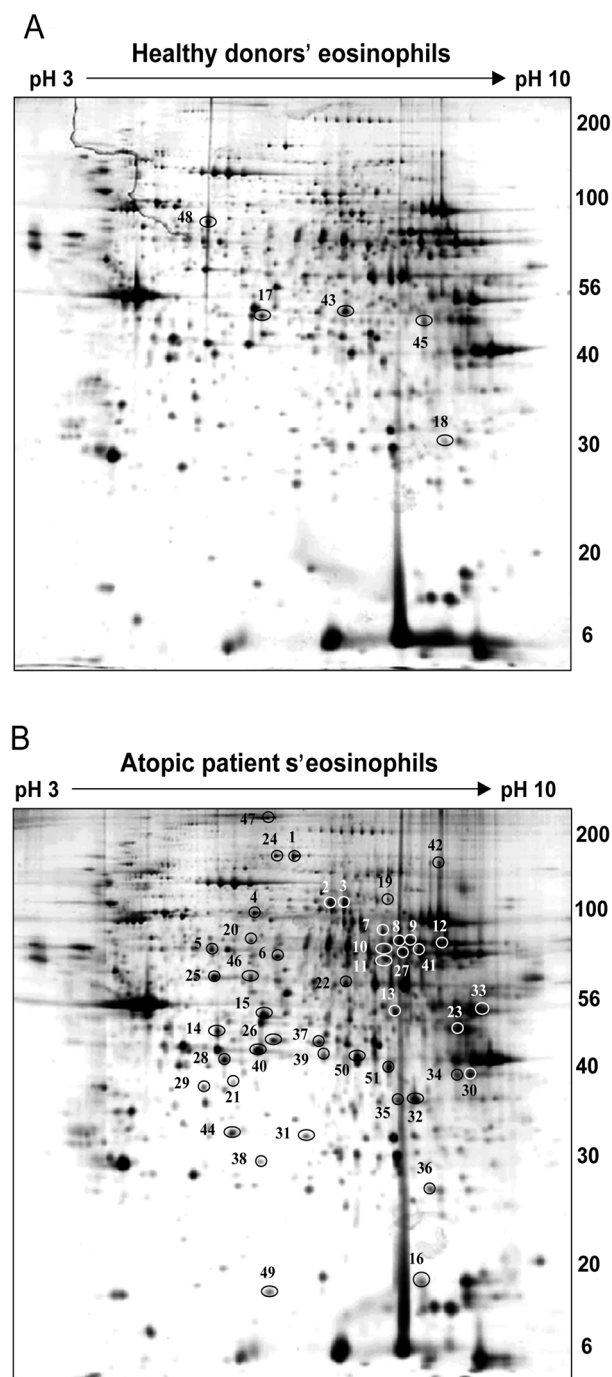


Figure 1. Comparison of 2-DE maps of eosinophils from healthy donors and AD patients with eosinophilia. Eosinophils purified from healthy donors or AD patients were solubilized, and cell lysates (100 μ g) were applied to a first dimension of pH 3–10 nonlinear IPG strips (18 cm) and a second dimension of 12%T, 2.7%C SDS-PAGE visualized by silver staining. (A) Eosinophils from healthy donors. Circled spots indicate proteins that were expressed more in eosinophils from healthy donors than in those from AD patients. (B) Eosinophils purified from AD patients with eosinophilia. Circled spots indicate proteins that were expressed more highly in eosinophils from AD patients than in those from healthy donors.

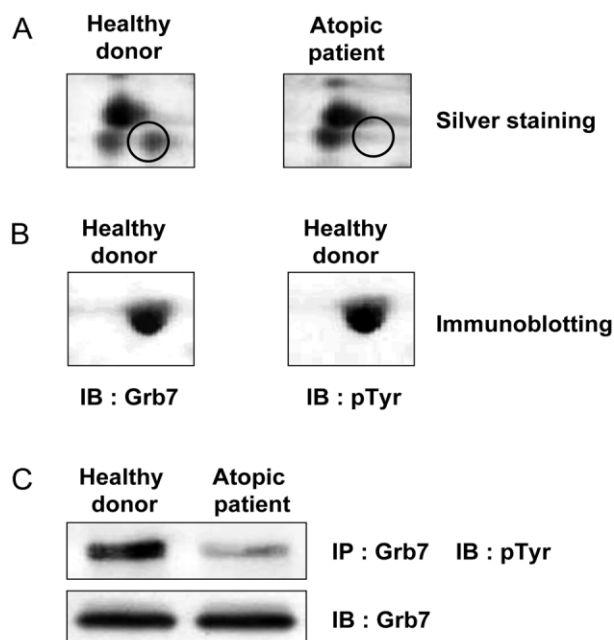


Figure 2. A protein spot uniquely expressed in healthy donors was identified as Grb7, and further shown to be the phosphorylated form. (A) Localization of a protein spot (spot#17, circled) uniquely expressed in the 2-DE map of healthy donors. (B) Characterization of spot#17 by immunoblotting. Partial 2-DE gels were destained, transferred to a nitrocellulose membrane and then probed with anti-Grb7 antibody. The membrane was subsequently stripped and reprobed with an anti-pTyr antibody. (C) Phosphorylated Grb7 was observed by 1-DE immunodetection in eosinophil lysates from healthy donors and AD patients. Lysates of purified eosinophils were immunoprecipitated with an anti-Grb7 antibody and analyzed by immunoblotting with an anti-phospho-Tyr antibody. The membrane was subsequently stripped and reprobed with an anti-phospho-Tyr antibody. The data shown are representative of three independent experiments.

peripheral blood eosinophils through a loss of mitochondrial membrane potential [23, 24]. AML14.3D10 cells were pretreated with IL-5 to increase their viability by blocking apoptosis [25] and then stimulated with various concentrations (1–10 μ M) of dexamethasone. As shown in Fig. 3A, the viability of AML14.3D10 cells was significantly and dose-dependently reduced by treatment with dexamethasone. Cell viability decreased by 24–32% and 76–80% in samples treated with 5 and 10 μ M dexamethasone, respectively (Fig. 3A). This result confirms that pro-apoptotic signaling in AML14.3D10 cells is induced by dexamethasone. Next, we analyzed Grb7 phosphorylation in AML14.3D10 cells treated with dexamethasone, and found that AML14.3D10 cells treated with dexamethasone showed increased phosphorylation of Grb7 and FAK, which is an upstream kinase of Grb7 (Fig. 3B). The intracellular pro-apoptotic signaling mediated by kinases and transcription factors usually precedes the actual cell death. In eosinophil apoptosis induced by dexamethasone, the loss of viability is detected after 24 h to 72 h

Table 2. Functional groups of identified proteins by comparison of 2-DE maps for eosinophils from healthy donors and AD patients

Functional group	Protein name and spot number (#)
Signaling	Glutamate receptor-2 (3), RAC- α -serine/threonine kinase (4), Rab proteins geranyltransferase component A1 (6), G protein-coupled receptor kinase-2 (8), Tyrosine protein kinase TXK (10), Mitogen-activated protein kinase phosphatase-5 (12), COUP transcription factor 1 (14), Synaptotagmin IX (15), Grb7 adaptor protein (17), Integrin α -6 precursor (24), ADP-ribosylation factor GTPase-activating protein-3 (25), G-protein beta-subunit like protein-1 (30), Plasma membrane adaptor AP-2 protein (33), Stac protein (34), Laminin α -5 chain precursor (35), Ras-related protein (36), Integrin β -7 (44), Signal transducer and activator of transcription 1- α/β (48), FK506 binding protein 8 (51)
Metabolism	Ariadne-1 protein homolog (5), Prominin-like protein-1 precursor (7), Mitochondrial folate transporter/carrier (18), Insulin-degrading enzyme (19), Acyl-CoA dehydrogenase (20), Kell blood group glycoprotein (22), Alanine aminotransferase (39)
Apoptosis	Catalase (9), Cyclin A2 (14), M-phase inducer phosphatase-1 (23), Voltage-dependent anion channel protein 2 (32)
Inflammation	Nitric oxide synthase type III (1), HLA class II histocompatibility antigen (21), Low affinity immunoglobulin epsilon FC receptor (31)
Transcription	VJ recombination activating protein-1 (2), COUP transcription factor-1 (13)
RNA processing	Coilin (11), G-rich sequence factor-1 (40), Heterogeneous nuclear ribonucleoprotein R (41)
Translation	tRNA-methyltransferase (29), Translation initiation factor elf-3e (37)
Cytoskeleton	Actin (26), F-actin capping protein beta subunit (38)
Unknown	Copine VII (27), Calponin-3 (28), Collagen α -1 (42), Flotillin-1 (43), Hypothetical protein (16, 45, 46), Serine-protein kinase ATM (47), Mitotic spindle assembly checkpoint protein MAD2B (49), Biglycan precursor (50)

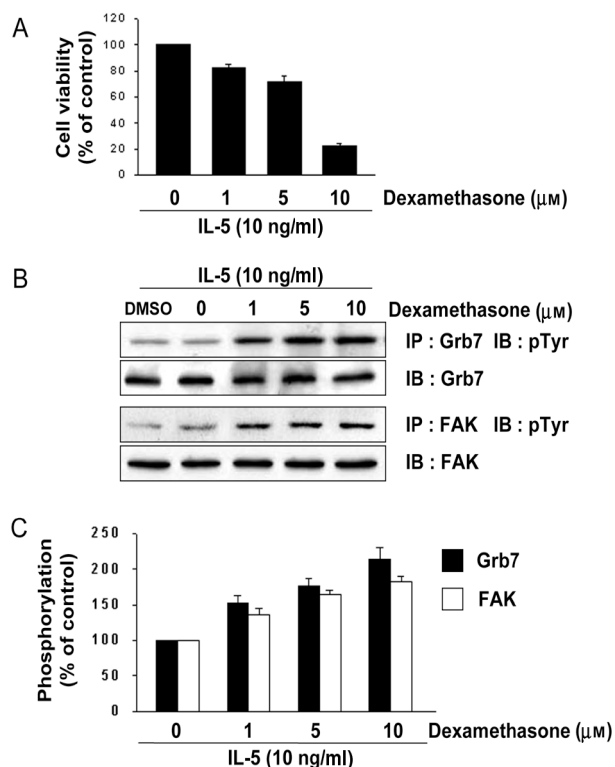


Figure 3. Increased phosphorylated Grb7 is detected in the human eosinophilic cell line, AML14.3D10, following treatment with the pro-apoptotic agent, dexamethasone. (A) Viability of AML14.3D10 cells is decreased by dexamethasone. Cells were pretreated with IL-5 (10 ng/mL) for 1 h and the indicated concentrations of dexamethasone for 48 h, and then analyzed by MTS assay. The control was considered 100% viability. Samples were analyzed in triplicate; the error bars indicate the standard error. The data shown are representative of live independent experiments. (B) Phosphorylation of Grb7 and FAK was increased in dexamethasone-treated AML14.3D10 cells. The cells were pretreated with IL-5 (10 ng/mL for 30 min and then treated with the indicated concentrations of dexamethasone for 30 min. Vehicle control of DMSO (1:500) was used to check the DMSO effect on the phosphorylation of FAK and Grb7. Cell lysates were immunoprecipitated with anti-Grb7 or anti-FAK antibody and analyzed by immunoblotting with the anti-phospho-Tyr antibody to check the level of tyrosine-phosphorylated Grb7 or tyrosine-phosphorylated FAK. The membrane was then stripped and reprobed with anti-Grb7 or anti-FAK antibodies for checking the expression level of total Grb7 or FAK protein. The data shown are representative of three independent experiments. (C) The density of immunoblotting band of FAK and Grb7 was measured by densitometric analysis using an image analysis software, ImageQuant™v5.2 (Amersham Biosciences). The phosphorylation level of Grb7 and FAK proteins are expressed as the percentage of that of the control (0 μ M of dexamethasone). The data shown are representative of three independent experiments.

of stimulation and early intracellular signaling mediated by kinase activation such as p38 MAPK and JNK was observed during 3–30 min and 15–40 min, respectively [26]. Thus we observed the viability of an eosinophil cell line, AML14.3D10 at 48 h and intracellular activation of FAK at 30 min after dexamethasone treatment. It was reported that IL-5 induced the increased expression but not the activation of adhesion molecules [27]. In order to show that the increased tyrosine phosphorylation of FAK and Grb7 (Fig. 3B) is dose dependent, we also analyzed the tyrosine phosphorylation levels of FAK and Grb7 by densitometric analysis. The treatment of dexamethasone (1–10 μ M) dose-dependently enhanced the phosphorylation levels of FAK and Grb7 (Fig. 3C). Total protein levels of Grb7 and FAK did not show significant increase after dexamethasone treatment. These results suggest that the tyrosine phosphorylation of Grb7 and FAK may be involved in dexamethasone-induced pro-apoptotic signaling in AML14.3D10 cells. Thus, our data indicate that tyrosine phosphorylation of Grb7 is related with apoptotic signaling not only in eosinophils isolated from human peripheral blood, but also in the human eosinophil cell line, AML14.3D10.

4 Discussion

In order to identify proteins related with eosinophila in AD, we performed comparative proteomic analyses in eosinophils from AD patients with eosinophilia and healthy donors. We successfully identified 51 proteins affected by eosinophilia. Among the identified proteins, we observed AD-associated up-regulation of cyclin A2 (spot#14), voltage dependent anion channel 2 (spot#32) and 38 kDa FK506 binding protein 8 (spot#51). Cyclin A2 and FK506 binding protein are known to be associated with cell cycle and cell proliferation, and their enhanced expression may be related with the anti-apoptotic mechanism of eosinophilia [28, 29]. Recently, voltage dependent anion channel family proteins have been reported to play a key role in the apoptotic changes of mitochondria. In particular, voltage dependent anion channel 2 inhibits mitochondrial apoptosis by its physical association with the pro-apoptotic protein, BAK [30, 31]. Thus, increased cellular expression of voltage dependent anion channel 2 may also contribute to the anti-apoptotic mechanism of eosinophilia in AD patients.

To validate the proteins identified by 2-DE and PMF, we selected one protein spot for further analysis. Tyrosine phosphorylated Grb7 was uniquely expressed in eosinophils of healthy volunteers but not in those of AD patients. To further examine its relevance in terms of eosinophilia, we tested the effect of dexamethasone (an apoptosis-inducing agent) on Grb7 expression in the eosinophil cell line, AML14.3D10. Growth factor receptors are important for cell proliferation, and growth receptor adaptor proteins such as Grb7 are known to mediate the coupling of multiple cell surface receptors to the FAK downstream signaling pathways, which

are pivotal in the regulation of cell proliferation [32–34]. Concordant with our observation of phosphorylated Grb7 enhancement in apoptotic eosinophils isolated from healthy volunteers (Fig. 2), we noted increased expression of phosphorylated Grb7 and its upstream kinase, phosphorylated FAK, in dexamethasone-treated (apoptosing) AML14.3D10 cells (Fig. 3). In Fig. 2A and 2B, spot#17 in 2-DE gel was identified as tyrosine-phosphorylated Grb7. The amount of tyrosine-phosphorylated Grb7 was down-regulated in eosinophils from AD patients compared to those from healthy individuals. As shown in Fig. 2C, the amount of tyrosine phosphorylated Grb7 was significantly higher in the eosinophils of healthy individuals, even though the total amount of Grb7 was comparable. There appears to be less tyrosine phosphorylated Grb7 (spot#17) in the eosinophils of AD patients than in those of healthy individuals. Thus, in eosinophils from AD patients there is more Grb7 which was not tyrosine phosphorylated and it may be located in the unidentified spot(s) other than spot#17 in 2-DE gel (Fig. 2A). Because Grb7 family members regulate the Ras signaling pathways, which are important for cell proliferation [32], we speculate that phosphorylation of Grb7 may regulate the apoptosis of eosinophils by inhibition of Ras signaling.

5 Concluding remarks

In conclusion, we herein performed a comparative proteomic study to analyze differential protein expression in eosinophils of AD patients compared to those from healthy donors. We established a protein database related with anti-apoptotic signaling eosinophilia, and further examined one differentially expressed protein, Grb7. Together, our results may facilitate further studies aimed at determining the anti-apoptotic mechanisms of eosinophilia in AD, and our work will hopefully contribute to the clinical study of allergic diseases.

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