

Towards a proteomic analysis of atopic dermatitis: A two-dimensional-polyacrylamide gel electrophoresis/mass spectrometric analysis of cultured patient-derived fibroblasts

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Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease typically characterized by a distribution of eczematous skin lesions with lichenification, pruritic excoriations, and dry skin with wide varieties of pathophysiologic aspects. Recently, AD was divided into extrinsic and intrinsic forms according to the presence or absence of an allergy. We investigated alterations in protein expression in primary cultured AD cells from the patient biopsy samples by two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight. In the primary cultured fibroblasts, we obtained 31 candidate proteins from the two-dimensional gel image analysis in which 18 proteins were up-regulated, eight proteins were down-regulated and five proteins were post-translationally modified. From these 2-DE results, we found several candidate genes matched proteomic expression patterns by semi-quantitative reverse transcription PCR. Since the exact mechanism of atopic alterations in fibroblasts remains unknown, our results may provide new clues to aid in understanding AD.

Keywords: Atopic dermatitis / Primary cultured fibroblasts / Reverse transcriptase PCR / Two-dimensional gel electrophoresis

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1 Introduction

Proteomic approaches coupled with genomics have been recently applied to analyze many diseases and to probe disease-specific biomarkers for diagnosis usage as well as for therapeutic purposes [1–3]. More now than ever, rapid developments of techniques in both proteomics and genomics have enabled researchers to understand dis-

eases and diseases-associated factors. Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease typically characterized by the distribution of eczematous skin lesions with lichenification, pruritic excoriations, and dry skin with wide varieties of pathophysiologic aspects. Although most patients with AD have high concentrations of total and allergen-specific serum IgE levels, and positive skin prick test reactions to common environmental allergens, a subgroup of AD patients suffers from skin lesions without sensitization to aero- or food allergens. An analogy to the extrinsic and intrinsic types of asthma has been proposed. This analogy uses the term intrinsic type AD (ADi) synonymously with nonallergic AD, nonatopic eczema, or nonatopic AD and as a counterpart to the term extrinsic type AD (ADe). ADi is found in a relevant proportion of all AD patients at a frequency of 15–45%.

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Abbreviations: AD, atopic dermatitis; ADe, extrinsic type AD; ADi, intrinsic type AD; PTM, post-translational modification

Even though patients with ADi tend to have a late onset of the disease, there are neither molecular markers nor clinical diagnostic tools available for distinguishing between ADi and ADe.

cDNA microarrays have been applied to uncover gene expression patterns in asthma subjects [4, 5]. The results showed that atopic nonasthmatic and atopic asthmatic individuals had activated proinflammatory pathways connected with IgE production, and an activated tumor necrosis factor pathway. Gene-array technology aids diagnosis by providing a gene-expression score for atopy [6]. On the genome level, single nucleotide polymorphism associations in atopy have been reported [7, 8]. Polymorphisms in the interleukin (IL)-3 promoter, IL-4 and IL-4 receptor alpha chain might be associated with modulating severity of atopy. Although genomic and mRNA levels in atopic diseases have been reported, expression patterns at the protein level have not been well elucidated. Therefore, in this study, we investigated alterations in protein expressions in primary cultured AD fibroblasts from patient biopsy samples compared to normal primary fibroblasts that were analyzed by 2-DE and MALDI-TOF. From a comparison of proteomic and semiquantitative RT-PCR results, several candidate genes were well matched with proteomic expression patterns. Since the exact role of fibroblasts in atopic alterations has not been well elucidated, our results may provide information to understand the pathogenesis of AD as well as insight into the roles of fibroblasts in AD.

2 Materials and methods

2.1 Subjects

The atopic samples used in this study were classified into extrinsic and intrinsic types according to our previous report [9]. All AD samples were collected from nonasthmatic atopic patients. Nonatopic control subjects, who had no personal/family history or sign of atopic diseases, were selected and samples were obtained from foreskins. Punch biopsies of 5 mm diameter were obtained from lesional skin in both types of AD patients. The ages of both control and AD patients were between 21 and 24 years old. The sex of all samples was male. Informed consent was obtained from all patients and control individuals. The study was approved by the Samsung Biomedical Research Institute Medical Ethics Committee.

2.2 Cell culture

Specimens were briefly sterilized in 70% ethanol, minced, and then treated with dispase overnight at 4°C. The dermis was separated and placed in a solution containing

0.05% trypsin and 0.025% EDTA for 15 min at 37°C. After vigorous vortexing, cells were pelleted and resuspended in DMEM containing 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco, Rockville, MD, USA). Cells were grown at 37°C in a 5% CO₂ incubator.

2.3 Sample preparation and 2-DE

Primary cultured fibroblasts (less than 4 passages) were harvested in 50 mM Tris buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 1 × protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). The lysates were homogenized and centrifuged at 12 000 × *g* for 15 min. Endonuclease (700 units/mL; Sigma, St. Louis, MO, USA) was added to the mixture and suitably stored at –70°C after quantitation by the Bradford method. For 2-DE analysis, pH 4–7 IPG strips (Amersham Biosciences, Uppsala, Sweden) were rehydrated in swelling buffer containing 7 M urea, 2 M thiourea, 0.4% DTT, and 4% CHAPS. The lysates (500 µg) were cup-loaded into the rehydrated IPG strips using a Multiphor II apparatus (Amersham Biosciences) for a total of 57 kVh. The 2-D separation was performed on 8–16% linear gradient SDS-polyacrylamide gels. Following fixation of gels for 1 h in a solution of 40% methanol containing 5% phosphoric acid, the gels were stained with colloidal Coomassie Blue G-250 (Sigma) for 5 h. The gels were destained in 1% acetic acid for 12 h and then imaged using a GS-710 imaging calibrated densitometer (Bio-Rad, Hercules, CA, USA). Protein spot detection and 2-D pattern matching were carried out using Melanie III software (Genebio, Geneva, Switzerland). To compare spot densities of normal and AD samples, more than 30 spots throughout all gels were correspondingly landmarked and normalized. We comparatively quantified spot densities of candidate proteins with the aid of histograms. To obtain the suitable gel images, we repeated the above procedures and conducted two-pairs of 2-DE gels individually from two different donors for control, extrinsic and intrinsic types.

2.4 MS

Protein spots excised from the gels were washed in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% v/v ACN for 1 h at room temperature. The protein spots were then dehydrated in a SpeedVac for 10 min. Gel pieces were rehydrated in 10 µL (10 ng/µL) of sequencing grade trypsin solution (Promega, WI, USA) in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight. After removing the trypsin solution, the resulting peptides were extracted with 5 µL of 0.5% TFA containing 50% ACN by sonication. Peptide extraction was done at

room temperature for 40 min. The tryptic digested peptide samples (1 μ L) were mixed with equal volumes of CHCA (5 mg/mL in 50% v/v ACN/ 0.1% TFA) and loaded onto a target plate. MALDI-TOF mass spectra were obtained on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, MA, USA). Peptide matching and protein searches against the Swiss-Prot and NCBI databases were performed using the ProFound program (<http://prowl.rockefeller.edu/>).

2.5 Semiquantitative RT-PCR

Total RNA was isolated with easy-BLUE (Intron, Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis was carried out under the following conditions: (1) total RNA (200 ng) from each primary fibroblast population was mixed with 1 μ L of oligo(dT)_{12–18} primer and 1 μ L of 10 mM dNTP mix, (2) the final volume was brought to 13 μ L with nuclease-free water, (3) samples were heated at 65°C for 5 min then quickly chilled on ice, (4) an aliquoted RT mixture (4 μ L of 5 $\times$ First-Strand buffer, 2 μ L of 0.1 M DTT, and 1 μ L of RNaseOUT Recombinant RNase Inhibitor) and 1 μ L of SuperScript III Reverse Transcriptase (Invitrogen, Leek, The Netherlands) were then added to the individual tubes, (5) the reaction was incubated at 50°C for 60 min, and finally (6) inactivated at 70°C for 15 min, then chilled on ice. Primer sequences (Table 1) for target genes were designed on the basis of GENBANK accession using the Primer 3 program (<http://frodo.wi.mit.edu/>). The reaction mixture for PCR of each gene contained a 1 μ L aliquot of the RT reaction, 1 μ L of

each primer, and 17 μ L of *i*-Master mix PCR Solution (Intron). The final reaction volume was 20 μ L. PCR was conducted under the following conditions: (1) initial denaturation for all samples was at 94°C for 2 min, (2) denaturation of optineurin, transitional endoplasmic reticulum ATPase, FK506-binding protein 4, annexin A5, chloride intracellular channel protein 1, and vimentin was at 94°C for 30 s, annealing was at 60°C for 20 s, and extension was at 72°C for 60 s for 30 cycles, followed by a final extension at 72°C for 5 min; for phosphoglucosyltransferase 3 and coatomer delta subunit (Archain), denaturation was at 94°C for 30 s, annealing was at 65°C for 20 s, and extension was at 72°C for 60 s for 30 cycles, followed by a final extension at 72°C for 5 min.

3 Results

3.1 2-DE

2-D protein expression patterns of primary cultured normal and AD fibroblasts were suitably visualized (Fig. 1) and were analyzed by MS and peptide matching as well as protein searches. We obtained 31 candidate proteins from the 2-D gel image analyses in which 18 proteins were up-regulated, eight proteins were down-regulated, and five proteins were post-translationally modified. The eighteen up-regulated proteins are listed in Table 2 and some of their gel images as well as expression ratio changes are shown in Fig. 2. Three types of up-regulation patterns were observed as follows: (1) proteins up-regulated both in extrinsic and intrinsic types such as FK506-

Table 1. Primers used for RT-PCR

Primer		Sequence	Expected size (bp)
Phosphoglucosyltransferase 3 (PGM 3)	Forward	5'-AGTCATGGTAACAGCGTCCC-3'	194
	Reverse	5'-CAAAGGCATCTTGTTGCAGA-3'	
Optineurin (OPTN)	Forward	5'-GATGGCTGAAGGAGAAGCAG-3'	225
	Reverse	5'-GCCTCCTTGAGTGCAACTTC-3'	
FK506-binding protein 4	Forward	5'-GCGGTGAAGGCTATGCTAAG-3'	252
	Reverse	5'-CATTTGGTGGGATTTGGAAC-3'	
Transitional endoplasmic reticulum ATPase	Forward	5'-ATCCGTGAATCCATCGAGAG-3'	226
	Reverse	5'-GGAATCTGAAGCTGCCAAAG-3'	
Coatomer delta subunit (Archain)	Forward	5'-CAGTATCTGGAGGCAGCACA-3'	202
	Reverse	5'-CGCTTGCCCATAGAGGA-3'	
Annexin A5	Forward	5'-AGACCCTGATGCTGGAATTG-3'	279
	Reverse	5'-GTCTCTGCAAGGTAGGCAGG-3'	
Chloride intracellular channel protein 1 (NCC27)	Forward	5'-CAGCTCTGAACCCTGAGTCC-3'	222
	Reverse	5'-TGCCATCCAAAACTTCCTC-3'	
Vimentin	Forward	5'-CCCTCACCTGTGAAGTGGAT-3'	241
	Reverse	5'-TCCAGCAGCTTCTGTAGGT-3'	

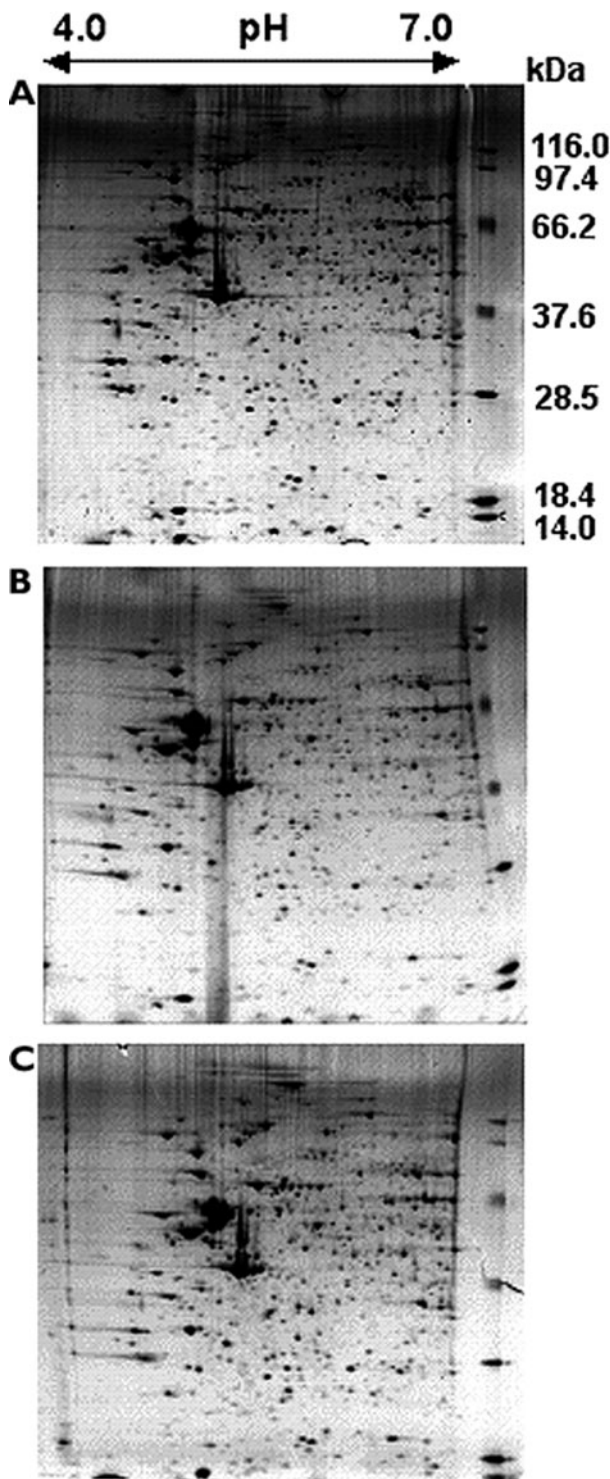


Figure 1. 2-DE maps of primary cultured fibroblasts stained with Coomassie Blue G-250. (A) normal fibroblasts; (B) fibroblasts from extrinsic type AD; (C) fibroblasts from intrinsic type AD. To obtain the suitable gel images, we conducted two-pairs of 2-DE gels individually from two different donors for control, extrinsic and intrinsic types.

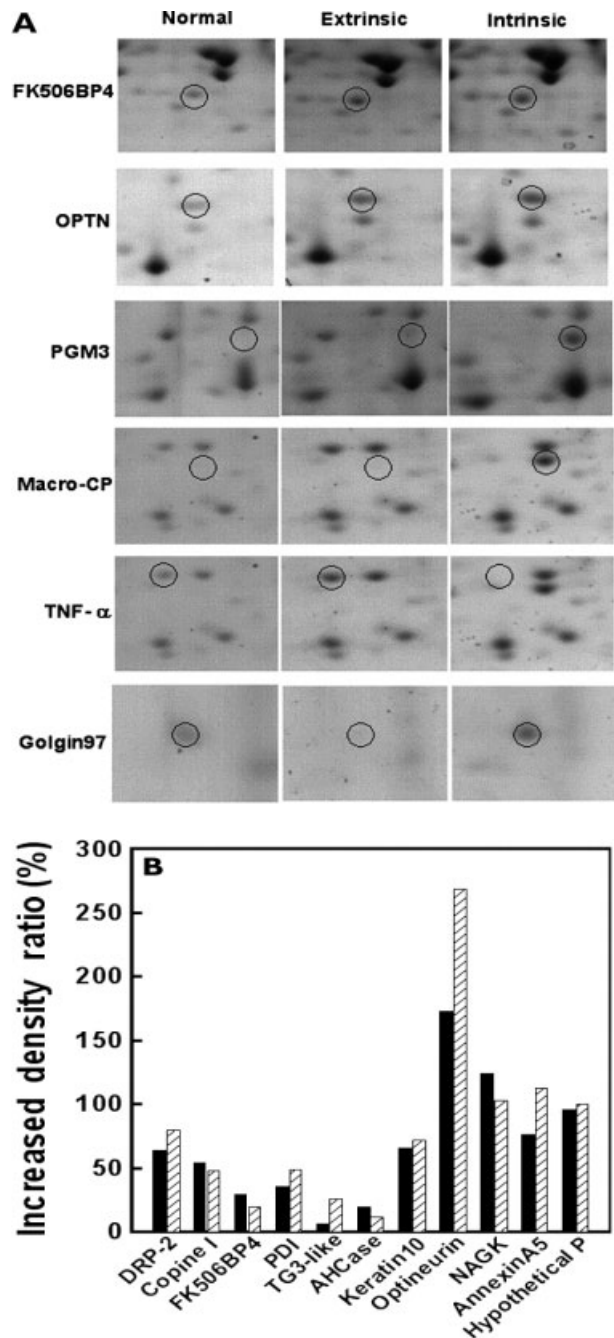


Figure 2. (A) 2-DE images of up-regulated proteins in extrinsic and intrinsic compared to normal fibroblasts. The labels on the left side of the figure show the corresponding proteins. FK506BP4 = FK506-binding protein 4; OPTN = optineurin; PGM3 = phosphoacetyl glucosamine mutase; Macro-CP = macrophage capping protein; TNF- α = tumor necrosis factor-alpha; Golgin97 = golgi autoantigen. (B) Increased density ratio relative to the normal spot. Data were calculated as increased density ratio (%) = (normalized target spot density)/(normalized control spot density) (%)–100 (%). Black bars show the corresponding extrinsic sample spots. Striped bars show the corresponding intrinsic sample spots.

Table 2. Up-regulated proteins in atopic fibroblasts comparative to normal fibroblasts

Protein	Accession no. /database	Measured M_r (kDa)/pI	Seq. cover- age (%)	MOWSE score	No. of matched peptides
Dihydropyrimidinase related protein-2 (DRP-2)	Q16555/Swiss-Prot	67/6.2	30	1.02e + 08	10
Copine I	Q99829/Swiss-Prot	66/5.6	15	3250	8
FK506-binding protein 4 (FK506BP4)	Q02790/Swiss-Prot	60/5.5	25	3.50e + 06	10
Protein disulfide-isomerase (PDI)	S55507/Swiss-Prot	60/5.8	52		26
Protein-glutamine γ -glutamyltransferase 3-like (Transglutaminase 3-like)	O95932/Swiss-Prot	53/6.9	10	6.70e + 06	7
Adenosylhomocysteinase	P23526/Swiss-Prot	45/6.3	30	1.00e + 00	16
Keratin 10	21961605/NCBI	37/6.55	30	1.64e + 08	15
Optineurin	20149572/NCBI	36/5.9	10	612	7
N-acetylglucosamine kinase (NAGK)	Q9UJ70/Swiss-Prot	35/5.9	48	2.21e + 11	12
Annexin A5	P08758/Swiss-Prot	31/4.95	42	2.39e + 10	13
Hypothetical protein	CAB63881/NCBI	27/5.6	42	1.00e + 00	8
TRIO and F-actin binding protein (Protein Tara)	Q9H2D6/Swiss-Prot	60/6.4	19	7.72e + 04	12
Prolyl 4-hydroxylase alpha-2 subunit	O15460/Swiss-Prot	60/5.9	22	1.46e + 06	8
Zyxin 2	Q15942/Swiss-Prot	78/6.5	31	7.52e + 05	11
Macrophage capping protein (Actin-regulatory protein CAP-G)	P40121/Swiss-Prot	35/6.0	18	2.08e + 04	5
Tumor necrosis factor ligand superfamily member 2 (TNF-alpha)	Q8MKG8/Swiss-Prot	35/6.0	32	1.15e + 04	4
Golgi autoantigen (Golgin 97)	Q92805/Swiss-Prot	30/7.0	12	1.02e + 04	10
Phosphoacetyl-glucosamine mutase (PGM 3)	O95394/Swiss-Prot	68/6.1	23	6.40e + 05	10

binding protein 4, optineurin, dihydropyrimidinase related protein-2, copine I, PDI, transglutaminase 3-like protein, adenosylhomocysteinase, keratin 10, NAGK, annexin A5, and hypothetical protein, (2) proteins mostly up-regulated in the extrinsic type such as TNF- α , TRIO and F-actin binding protein, (3) proteins mostly up-regulated in the intrinsic type such as PGM 3, macrophage capping protein, Golgin 97, prolyl 4-hydroxylase alpha-2 subunit, and zyxin 2.

As shown in Fig. 2B, optineurin, NAGK, annexin A5, and hypothetical protein were significantly up-regulated compared to normal samples (more than 2-fold in atopy samples). DRP-2, copine I, PDI, and keratin 10 were up-regulated 50–80% and FK506-binding protein 4, transglutaminase 3-like protein, and adenosylhomocysteinase were up-regulated 20–30% relative to the normal samples. Spots representing TNF- α , TRIO and F-actin binding protein were newly appeared in extrinsic samples, showing

significant up-regulation in the extrinsic type. Similarly, PGM 3, macrophage capping protein, Golgin 97, prolyl 4-hydroxylase alpha-2 subunit, and zyxin 2, spots were newly appeared in intrinsic samples, showing significant up-regulation in the intrinsic type. Table 3 summarizes the eight down-regulated proteins in AD fibroblasts. Similar to the up-regulation patterns observed, three types of down-regulation patterns were observed as follows: (1) spots which disappeared both in extrinsic and intrinsic types such as Ku antigen 70, hypothetical protein FLJ32204, nesprin 2, bile acid receptor, and moesin, (2) spots that disappeared only in the intrinsic type such as DEAD-box protein 41, and (3) spots which not disappeared but were down-regulated both in extrinsic and intrinsic types such as 3-phosphoglycerate dehydrogenase and tropomyosin alpha 3 chain. Some of their gel images as well as expression ratio changes are shown in Fig. 3.

Table 3. Down-regulated proteins in atopic fibroblasts compared to normal fibroblasts

Protein	Accession no. /database	Measured M_r (kDa)/pI	Seq. cover- age (%)	MOWSE score	No. of matched peptides
3-Phosphoglycerate dehydrogenase	2674062M/NCBI	57/6.8	33	2.23e + 10	16
Tropomyosin alpha 3 chain	24119203M/NCBI	31/4.5	33	4.01e + 04	13
Thyroid autoantigen 70kD (Ku antigen 70)	NP_001460.1/NCBI	75/6.6	36	1.00e + 00	–
Bile acid receptor (Retinoid X receptor- interacting protein 14)	Q96RI1/Swiss-Prot	60/5.8	20	1.43e + 04	8
DEAD-box protein 41	Q9UJV9/Swiss-Prot	68/6.0	10	2877	6
Hypothetical protein FLJ32204	Q96ML2/Swiss-Prot	50/4.5	31	1.00e + 00	14
Moesin (Membraneorganizing extension spike protein)	P26038/Swiss-Prot	80/6.6	42	7.27e + 06	27
Synaptic nuclei expressed gene 2 (Nesprin 2)	NP_055995.2/NCBI	80/6.2	9	1.00e + 00	–

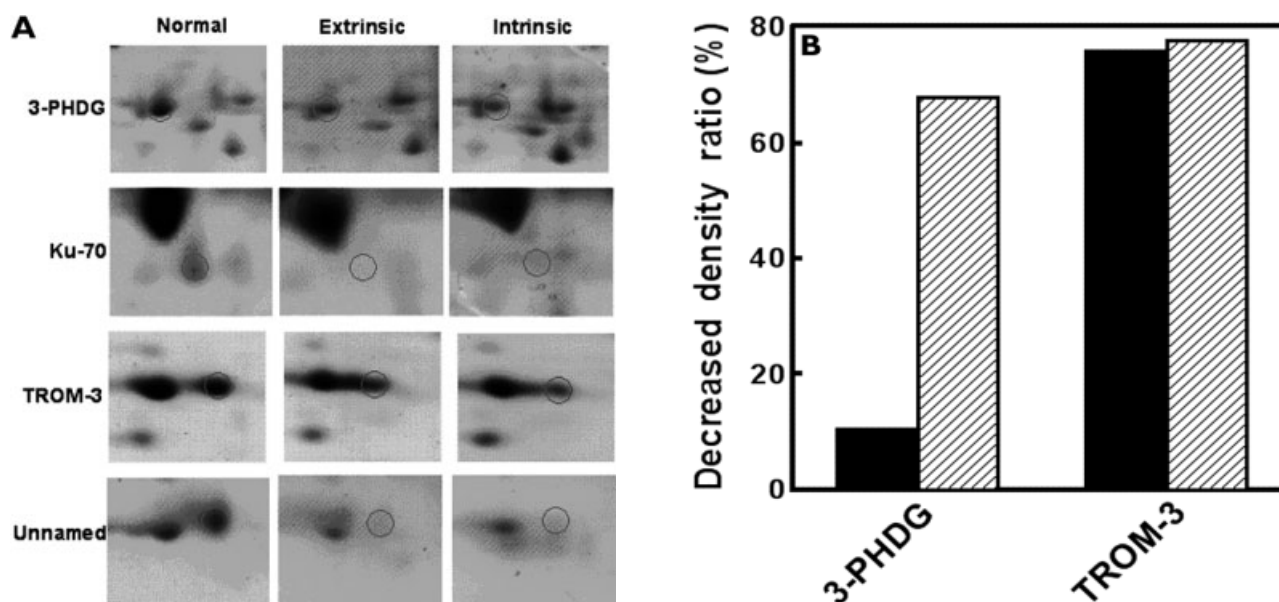


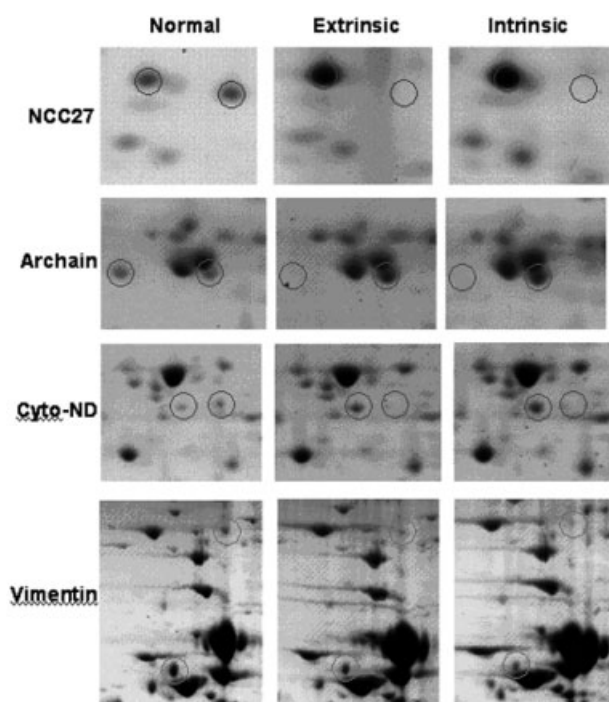
Figure 3. (A) 2-DE images of down-regulated proteins in extrinsic and intrinsic types compared to normal fibroblasts. The labels on the left side of the figure list the corresponding proteins. 3-PHDG = 3-phosphoglycerate dehydrogenase; Ku-70 = ku antigen 70; TROM-3 = tropomyosin alpha 3 chain; Unnamed, hypothetical protein FLJ32204. (B) Decreased density ratio relative to the normal spot. Data were calculated as decreased density ratio (%) = 100 (%) – (normalized target spot density)/(normalized control spot density) (%). Black bars show the corresponding extrinsic sample spots. Striped bars show the corresponding intrinsic sample spots.

Both 3-phosphoglycerate dehydrogenase and tropomyosin alpha 3 chain were down-regulated more than 60% compared to the normal samples (Fig 3B). In the case of Ku antigen 70, hypothetical protein FLJ32204, nesprin 2, bile acid receptor, and moesin, spots were significantly down-regulated both in extrinsic and intrinsic types with expression being below the level of detection.

DEAD-box protein was down-regulated in the intrinsic type (seen as spot disappearance). Interestingly, five proteins listed in Table 4 were detected as multisites, suggesting that PTM had occurred and these modifications were somehow involved in the atopic disease. PTM were subdivided into two types: (1) mostly modified pI values such as NCC27, archaic, and cytosolic non-

Table 4. Post-translationally modified proteins in normal fibroblasts compared to atopic fibroblasts

Protein	Accession no. /database	Measured M_r (kDa)/ pI	Seq. cover- age (%)	MOWSE score	No. of matched peptides
Vimentin	P08670/Swiss-Prot	97/5.0	35	1.00e + 00	19
	20031127/MSDB	60/4.8	49	1.70e + 08	21
Transitional endoplasmic reticulum ATPase	P55072/Swiss-Prot	120/6.3	16	3.77e + 06	9
	P55072/Swiss-Prot	95/5.3	33	2.23e + 12	19
Coatomer delta subunit (Archain)	P48444/Swiss-Prot	66/6.5	23	8.34e + 05	12
	P48444/Swiss-Prot	66/6.3	19	3.45e + 05	10
Cytosolic nonspecific dipeptidase	Q96KP4/Swiss-Prot	55/5.8	54	6.92e + 13	24
	23396498/NCBI	55/5.9	29	3.39e + 05	9
Chloride intracellular channel protein 1 (NCC27)	O00299/Swiss-Prot	30/5.2	63	1.93e + 08	12
	NP_001279.2/NCBI	30/5.3	55	1.00e + 00	9

**Figure 4.** 2-DE images of post-translationally modified proteins in normal, extrinsic, and intrinsic fibroblasts. The labels on the left side of the figure list the corresponding proteins. NCC27 = chloride intracellular channel protein 1; Archain = coatomer delta subunit; Cyto-ND = cytosolic nonspecific dipeptidase.

specific dipeptidase and (2) both pI and M_r were significantly changed such as vimentin and transitional-ER ATPase (Fig. 4).

3.2 Semiquantitative RT-PCR

We performed semiquantitative RT-PCR on eight genes; four from up-regulated proteins (FK506-binding protein 4, optineurin, annexin A5, and PGM 3) and four from post-translationally modified proteins (vimentin, transitional ER-ATPase, archain and NCC27). The primers we used are summarized in Table 1. Of the four up-regulated proteins, optineurin and PGM 3 showed increased mRNA levels in AD. The other two genes did not show any differences between AD and the control. In post-translationally modified proteins, transitional ER-ATPase and archain showed increased expressions at the mRNA level. Vimentin and NCC27 did not (Fig. 5).

4 Discussion

In general, AD is closely associated with type I allergy. The skin lesions of AD patients are infiltrated by activated T cells, eosinophils, and antigen-presenting Langerhans cells that present IgE-complexed allergens on their surfaces. The majority of allergen-specific T cells derived from AD skin lesions showed a polarized Th2 cytokine pattern, which was previously regarded as a specific feature reflecting immune dysregulation. This may be correct for ADe patients who have high IgE and positive food or aeroallergens. However, ADi patients have specific IgG antibodies instead of IgE [10]. However, this finding can not explain the presence of dry skin and pruritus in AD. An allergy may induce the first event of AD pathogenesis, but how can we explain the next step? To solve this question, we need much more information and gene analysis.

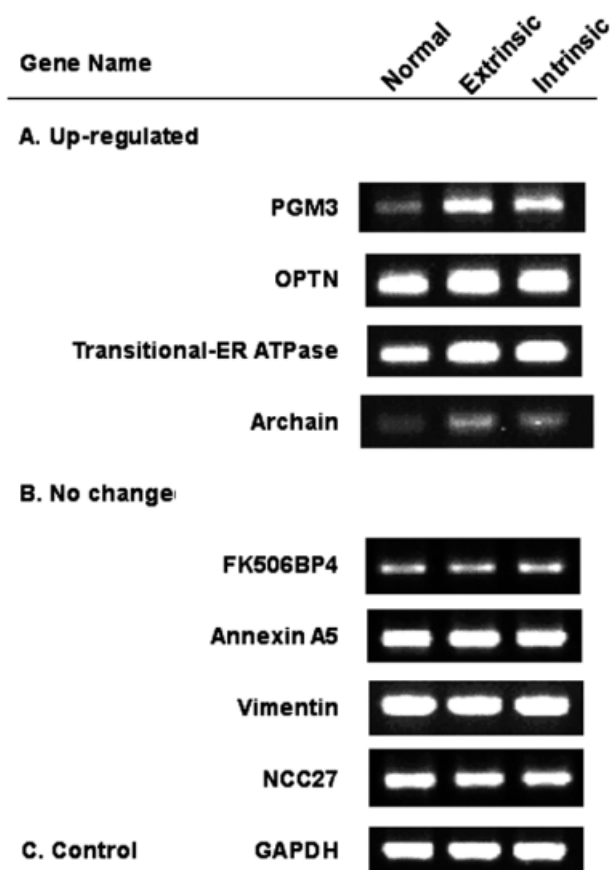


Figure 5. Semiquantitative RT-PCR of mRNAs from normal, extrinsic, and intrinsic fibroblasts. Candidate genes were collected from proteomic results and were classified into two groups (up-regulated (A) and no change (B)) according to the results. GAPDH was used for control quantification (C). To minimize donor-to-donor variations in the samples used in Fig. 1, RNAs were newly prepared from the different donors. PCR products were confirmed by sequencing with the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primers used in the sequencing reaction were the corresponded forward primers for each gene in Table 1.

There may be several ways to get candidate genes for disease analysis, such as cDNA microarrays, subtractive suppression hybridization (SSH) or proteomics approaches including 2-DE and MALDI-TOF. Each method has its own merits and drawbacks. With cDNA microarrays we can obtain lots of genes which are overexpressed or down-regulated in the disease. However, it is hard to choose the really important genes from the list. In our previous SSH experiment of AD [11] we identified many genes that were overexpressed in disease tissues but did not acquire the genes whose actions are very short such as cytokine genes. Using proteomics, we detected several up-regulated, down-

regulated or post-translationally modified proteins. However, there are limitations in identifying the desired proteins because of the different pI levels of the expressed proteins. Furthermore, it is hard to explain the meaning of the identified proteins. Although semiquantitative RT-PCR did not precisely quantify the amount of mRNA, we performed 2-DE and MALDI-TOF with the cultured fibroblasts of AD samples and compared the results with those of the semiquantitative RT-PCRs. Semiquantitative RT-PCRs of candidate proteins listed in Tables 2–4 were conducted. We show the results from eight genes in this study which had good data reproducibility in our experimental conditions. Although there were some differences in the expression levels of proteins between ADe and ADi in the 2-DE experiment, we could not detect the differences in RT-PCR.

We investigated protein expression patterns analyzed in normal *versus* atopic fibroblasts, and classified them into three groups: (1) up-regulated, (2) down-regulated, (3) multi-spotted. We then divided the groups into several subtypes. Among the protein candidates, several molecules such as archain, NCC27, autoantigens, TNF- α , and dihydropyrimidinase related protein 2 (DRP-2) may play a role in AD. Archain is an intracellular protein and has sequence similarity to heat shock proteins and clathrin-associated proteins. It may be involved in vesicle structure or trafficking [12, 13]. It is located on chromosome 11q23.3 where the mixed lineage leukemia and Friend leukemia virus integration 1 genes exist, and where multiple disease-associated chromosome translocations occur. In this study, archain was observed in a post-translationally modified form and up-regulated both at the protein and mRNA levels. Regardless of the up-regulation of archain at the total expression level, it was not post-translationally modified in AD fibroblasts. Interestingly, this phenomena of loss of translational modifications in AD fibroblasts was commonly observed in all multispot proteins such as NCC27, cytosolic nonspecific dipeptidase, and vimentin. The multispot proteins appeared as monospots in AD fibroblasts. Recently, the associations of PTM in human diseases have been gradually elucidated. It has been revealed that multiple sulfatase deficiency (MSD) is due to a defect in the PTM of the C α -formylglycine generating enzyme (FGE). Mutations in *SUMF1* which encodes FGE directly cause MSD by causing a lack of cysteine-to-formylglycine conversion in patients [14, 15]. Transduction of the FGE encoding cDNA into MSD-cell lines can restore the sulfatase activity and the authors suggested the possibility of using enzyme replacement therapy in patients. Defects in PTM of proteins such as archain, NCC27, cytosolic nonspecific dipeptidase, and vimentin may be important factors in AD.

NCC27 is a member of the p64 family and localizes to the cell nucleus. It exhibits both nuclear and plasma membrane chloride ion channel activity [16], however its exact role *in vivo* has not been well elucidated. In general, chloride channels regulate fundamental cellular processes including stabilization of cell membrane potential, maintenance of intracellular pH, regulation of cell volume, and chloride transport. We detected NCC27 as multiple forms due to PTM in normal fibroblasts. However, this modification does not occur in AD fibroblasts. Our unpublished data also shows that NCC27 can be detected in multiple forms in primary keratinocytes similar to AD fibroblasts. Two kinds of autoantigens, Ku antigen 70 and Golgin 97 were differently regulated in AD fibroblasts. Golgin 97, localized to the golgi area, is known to be associated with Sjogren's syndrome [17]. The Ku antigen 70 is the p70 subunit of the p70/p80 autoantigen which consists of two proteins whose M_r are approximately 70 and 80 kDa. Both p70 and p80 contain phosphoserine residues. The roles of Ku antigen 70 in DNA repair or tumor suppression have been suggested [18, 19]. The roles of Golgin 97 and Ku antigen 70 in AD have not been elucidated. Further studies regarding autoantigens may provide information to aid our insights into AD.

It has been reported that tumor necrosis factor pathways are activated in atopic nonasthmatic subjects with increased cytokine and receptor levels [4]. This report supports our proteomic result that TNF- α was up-regulated in extrinsic type fibroblasts. However, our previous RT-PCR result on tissue from AD patients showed that TNF- α mRNA expression decreased in both types of AD patients [9]. The drawback of our previous experiment was that we asked patients not to take oral steroids for 2 weeks before experimentation. We did not realize that histamines were known to inhibit TNF- α via H2 receptors expressed on immune cells. Because of these conflicting results, the role of TNF- α as a major proinflammatory cytokine in AD has to be probed in further studies. DRP-2 has been suggested to play a role in the impairment mechanism of neural network formation in Alzheimer's disease [20]. DRP-2 was up-regulated in AD fibroblasts. Data is lacking to interpret its exact role in AD.

AD is a T-cell mediated disease. T cells from patient's skin lesions produce predominantly Th2 cytokines, and lots of candidate genes for AD have been identified such as cytokines, autoantigens like Hom s 1 [21], natural antimicrobial peptides like defensin [22], and lipid associated genes like sphingomyelinase [23]. It is conceivable that autoantigen and cytokine genes can induce the first event of AD, antimicrobial peptide can develop

frequent infections in AD and lipid genes can cause the dry skin in AD. However, it is not possible to explain the entire pathogenesis of AD or to know how many genes or proteins are involved in the development of AD. According to our proteomics data, we can choose several interesting proteins such as FK506 binding protein, TNF- α , autoantigens like Ku antigen 70 and Golgin 97. The topical immunomodulator, tacrolimus, binds to the intracellular FK506 binding protein and inhibits calcineurin resulting in activation of the promoter region of inflammatory cytokine genes [24]. Although TNF- α is very important for the development of AD, there have been conflicting reports as to the role of this gene as mentioned previously. We think autoantigens are very important in the induction of pathogenesis of AD. This will need verification by more experiments. Consequently, the functions of most of the candidate proteins detected in this study have not been reported in AD or other pathogenesis. We provide new information regarding differently regulated and expressed proteins in AD patients as well as the involvement of PTM. Although we do not suggest the direct role of candidate proteins important in AD, we provide candidate proteins to be targeted in further trials of AD therapy and some insights to determine the response of fibroblasts, the most abundant cells in dermis, in AD.

5 Concluding remarks

The 2-DE approaches used to profile the expression patterns in AD *versus* normal fibroblasts revealed that 18 proteins were up-regulated, eight proteins were down-regulated and five proteins were post-translationally modified. Using semiquantitative RT-PCR four candidate genes (PGM 3, OPTN, Transitional-ER ATPase, archain) were detected that matched proteomic results in the present study. We identified some proteins, such as FK506 binding protein, TNF- α , and autoantigens, know to be involved in the pathogenesis of AD in this experiment. We suggest that defects in PTM of candidate proteins such as NCC27, archain, cytosolic nonspecific dipeptidase, vimentin and three matched up-regulated proteins (PGM 3, OPTN, and Transitional-ER ATPase) might play important roles in AD.

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6 References

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