Quantitative Proteomic Analysis of Stratum Corneum Dysfunction in Adult Chronic Atopic Dermatitis

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TO THE EDITOR

Atopic dermatitis (AD)/eczema is a common condition in children that is also suffered by approximately 2–3% of adults (Eichenfield et al., 2014). Despite intense scientific investigation into the genetics of AD, disease-linked loci are associated with a minority of disease cases (Baurecht et al., 2015), leaving the etiology of spontaneous AD in question.

To better understand the biology of AD and to identify protein markers of disease, we applied quantitative techniques to identify differential stratum corneum (SC) proteins from adult AD patients and normal individuals. Using discovery proteomics, we identified over 1,000 proteins in the SC, with over 200 differentially identified by condition. Selected proteins were quantified with precise targeted methods. Technical details are described in the Supplementary Materials and Methods online.

Specifically, we profiled skin surface tape-strip samples from 11 AD subjects at lesional and nonlesional sites. These were compared with similar samples from 17 healthy subjects (see Supplementary Table S1 online). Combining all spectral evidence, we identified 1,102 proteins at a 1% false positive error rate. To our knowledge, this represents the most comprehensive proteomic study of AD SC to date (Broccardo et al., 2011; Sakabe et al., 2014). The full identification list can be found in Supplementary Table S2 online.

A poorly characterized aspect of skin biology is the role of protein post-translational modifications, which are abundant in the outer layers of the epidermis. One such is modification to citrulline, also called deimination (Gyorgy et al., 2006). We included deimination in our search parameters and filtered the results to avoid false positives. We detected at least one citrullination site on 1,005 proteins (91% of those identified) (see Supplementary Table S3 online). The most citrullinated proteins were trichohyalin and filaggrin, which are known to be heavily modified in vivo. For filaggrin, healthy and nonlesional atopic skin were citrullinated on 78% and 75% of arginine residues, respectively. This decreased to 62% in lesional skin, corresponding to a loss of modification on 56 residues. Of the 386 proteins that were citrullinated in all conditions, 257 exhibited decreased fractional citrullination in lesional sites compared with other conditions (Figure 1). Notably, trichohyalin, an abundant hair protein not considered related to AD, showed a negligible difference by the same comparison.

We next analyzed differential proteins by two methods: Fisher exact test on identification counts by condition and Student t test on label-free quantification results for proteins observed in greater than 10 samples (Figure 2a, b, respectively). There were 252 proteins differentially observed between samples from healthy and AD subjects (Figure 2a, and see Supplementary Table S2). Many were general plasma-related markers of inflammation. Those preferentially observed in AD included proteins related to inflammation and barrier function such as serpins A3, B4, and B5, IL-1 receptor antagonist, IL-36 receptor antagonist, protein disulfide isomerases, and S100s A4 and A6. In addition, 36 proteins were found as differentially abundant via t test (Figure 2b). These included inflammation-related proteins such as serpins A1, B3, and B12 and S100s A8, A9, and A11. Proteins down-regulated in AD skin were generally structural, including several keratins and filaggrin-2. Filagrin-2 was annotated only in 2009 (Wu et al., 2009), and mutations have been recently linked to persistent AD (Margolis et al., 2014). When nonlesional and lesional AD samples were compared, 45 proteins were differentially observed (Figure 2a and see Supplementary Table S2). Sixteen were preferentially found at the lesional site, and 29 were observed more frequently in nonlesional samples. The protein most preferentially identified at the lesional site was PYCARD, a key regulator of inflammasome and activation and apoptosis (Martinon et al., 2002). One protein found at most nonlesional sites but only at 9% of lesional sites was SPINK5, for which the gene with mutations was linked to AD in Japanese patients (Kato et al., 2003).

To confirm results from discovery liquid chromatography tandem mass spectrometry (LC-MS/MS), we targeted several AD-related proteins using more sensitive and precise selected reaction monitoring (SRM) mass spectrometry-based assays (see Supplementary Table S4 online). These results support our shotgun LC-MS/MS label-free quantification but with increased sensitivity, as expected (see Supplementary Figure S1 online). Caspase 14, beta-defensin, desmoglein-1, desmplakin, filaggrin, kallikrein-8, keratin 10, S100-A7, and S100-A9 all increased when comparing healthy with atopic nonlesional skin. In addition, beta-defensin, filaggrin, S100-A7, and S100-A9 further increased from nonlesional to lesional atopic skin. The only targeted protein to show a significant decline was filaggrin-2, which decreased substantially from nonlesional compared with lesional epidermis, in agreement with label-free

Abbreviations: AD, atopic dermatitis; LC-MS/MS, liquid chromatography tandem mass spectrometry; SC, stratum corneum; SRM, selected reaction monitoring

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quantification described. Given the evidence for reduced abundance of filaggrin in atopic dermatitis from several studies (Pellerin et al., 2013; Suarez-Farinas et al., 2011), our observation of increased SRM signal in atopic skin may seem counterintuitive. Our label-free quantification found filaggrin to be similar between healthy and AD samples ($P = 0.2$) (see Supplementary Table S2). In the targeted SRM assay, we were monitoring SRM signal from the fully tryptic unmodified peptide, $^{3976}$HGSYGSADY-DYGESGFR$^{3992}$. In addition to the mass shift introduced by citrullination, we observed that this modification typically results in a missed cleavage at arginine, rendering the peptide invisible to the SRM assay used here. Thus, our observation is consistent with decreased citrullination on the endogenous peptide as disease progresses. Indeed, most evidence for filaggrin protein loss comes from immunohistochemistry experiments, in which antibody epitopes are not always characterized. It is possible that these epitopes are citrullinated, similar to those observed in vivo for rheumatoid arthritis (Girbal-Neuhauser et al., 1999).

Although genetic factors tied to AD have been investigated in some detail, these account for a minority of disease incidence (Baurecht and Hotze, 2015). Much focus has been directed toward filaggrin loss-of-function mutations; however, transcriptomic profiles stratified by filaggrin mutation status show an up-regulation of this protein in wild-type AD patients (Cole et al., 2014). We found a modest overlap between transcript and proteomic differential markers (see Supplementary Materials). Nongenetic potential disease factors, including microbiome diversity and epigenetic factors like DNA methylation, have only recently begun to be investigated (Kong et al., 2012; Rodriguez et al., 2014). The data presented here show alterations in the protein posttranslational modification profile of patients with AD lesions, which ultimately adds another dimension to the rich and complex etiology of AD.

**STATEMENT OF INFORMED CONSENT**

All research was carried out under the Declaration of Helsinki protocols, written informed consent was obtained from all individuals, and experiments were approved by the Western Institutional Review Board (study number 1121688).

**CONFLICT OF INTEREST**

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**Figure 1. Fractional citrullination (modifications to arginine) for selected proteins.** Many proteins related to epidermal differentiation and homeostasis decrease in citrullination with perturbation. The unrelated but highly citrullinated protein trichohyalin shows little change with condition. DCD, dermcidin; FABP5, fatty acid-binding protein, epidermal; FLG, filaggrin; FLG2, filaggrin-2; KLK5, kallikrein-5; KRTDAP, keratinocyte differentiation-associated protein; LY6D, lymphocyte antigen 6D; SERPINB13, serpin B13; TCHH, trichohyalin.
REFERENCES


SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.03.037.

Supplementary Table S2 online.

SYNOPSIS

Figure 2. Differentially observed proteins in atopic dermatitis. (a) Differentially identified proteins (P < 0.05) from Fisher exact test. Bar lengths indicate the observations by clinical condition. Proteins with the five lowest P-values are shown for each condition. The full data table is in Supplementary Table S2 online. (b) Differentially abundant proteins (adjusted P < 0.05) for healthy versus atopic (nonlesional and lesional) samples. Bars represent the difference in average Normalized Spectral Index by clinical condition. Positive values indicate higher abundance in atopic samples and negative values indicate higher abundance in healthy samples. CARD, C-terminal caspase-recruitment domain; SINV normalized spectral index.
Urocanic Acid: An Endogenous Regulator of Langerhans Cells

TO THE EDITOR

One of the most intriguing biological effects of UVR is the suppression of adaptive immunity. The molecular mechanisms underlying UVR-induced immunosuppression are complex. One essential molecular mediator is DNA damage, because reduction of DNA lesions by sunscreens or DNA repair enzymes is associated with a mitigation or even prevention of immunosuppression (Ullrich and Byrne, 2012). In addition, cis-urocanic acid (UCA), the photoisomer of trans-UCA, is involved, because upon removal of UCA immunosuppression by UVR was not any longer observed. This was further confirmed by the close fit of the absorption spectrum of UCA to the action spectrum for UV-induced immunosuppression and later on by the demonstration that injection of cis-UCA exerts immunosuppressive features (De Fabo and Noonan, 1983; Ross et al., 1987).

On the other hand, UCA acts as a natural UV filter in the corneal layer and thus should mitigate UVR-induced immunosuppression.

To assess which of these opposite activities of UCA dominates in vivo, we used histidinemic (HIS) mice, which have a strongly decreased amount of UCA (Barresi et al., 2011; Taylor et al., 1993; Wright et al., 1982). Because of a mutation in the Hal gene, HIS mice are deficient in histidase, which converts histidine into UCA (Kacser et al., 1973).

Compared with about 320 nmol/mg UCA in tape-stripped stratum corneum from the shaved back skin of wild-type (WT) mice, HIS mice contain only about 5 nmol/mg of UCA, and the amount of histidine is about 180 nmol/mg in HIS mice compared with about 15 nmol/mg in WT mice (Barresi et al., 2011). If the filtering effect of UCA is predominant, HIS mice should be more susceptible to UVR-induced immunosuppression. If in turn the effect of cis-UCA, which is formed after UV exposure, outweighs the filtering effect of UCA, HIS mice should be resistant to UVR-induced immunosuppression, which would be in accordance with previous preliminary observations (De Fabo et al., 1983).

Barresi et al. (2011) reported that HIS mice display an increased sensitivity to UVR-induced DNA damage. We confirmed these results by immunohistological staining of skin biopsy samples of UVR-exposed HIS and WT mice with an antibody directed against cyclobutane pyrimidine dimers, the major type of UVR-induced DNA damage. Nuclear staining for cyclobutane pyrimidine dimers in keratinocytes of HIS mice was more pronounced than that of WT mice (data not shown), indicating that the same amount of UVR induces a higher degree of DNA damage most likely attributable to the lack of the filtering effect by UCA.

UVR-induced immunosuppression was evaluated in the murine model of contact hypersensitivity (CHS), the induction of which is suppressed by UVR (Schwarz et al., 2011). All animal experiments were in accordance with institutional and state guidelines and were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas of Schleswig-Holstein. The shaved backs of HIS (C57BL/6J background) and C57BL/6J mice were exposed to UVR (UVB, 1.5 kJ/m² daily for 4 consecutive days); 24 hours after the last exposure, mice were sensitized with 2,4,6-trinitrochlorobenzene (TNCB) on the backs. On day 5, challenge with TNCB was performed on the ears, and ear swelling was quantified 24 hours later. For experimental details refer to Supplementary Materials (online). UVR-exposed WT mice were significantly suppressed in their ear swelling response. Suppression was less pronounced in HIS mice, suggesting that HIS mice are less susceptible to UVR-induced immunosuppression (Figure 1a).

To evaluate the development of regulatory T cells (Treg), adoptive transfer experiments were performed. Splenocytes and lymph node cells obtained from the UVR-exposed mice were injected intravenously into naïve WT mice. Recipients were sensitized against TNCB 24 hours after cell transfer. Ear swelling upon challenge was significantly suppressed in recipients, which had received cells obtained from irradiated WT donors, indicating the